

**ABSTRACTS FROM THE
2024 ISSX/JSSX MEETING**

**THE 26TH NORTH AMERICAN ISSX MEETING AND
39TH JSSX MEETING**

**September 15-18, 2024
Honolulu, Hawaii, USA**



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SC1.1 - WHY AND WHEN DO I USE PBPK MODELING**Kunal Taskar***GSK, Stevenage, United Kingdom*

This first session of the workshop will endeavour to lay the foundation for the workshop with basics of PBPK modeling. This session will stress on the application oriented strategies to drive the model informed drug discovery and development using PBPK modeling. The workshop session will focus on the applications of the PBPK modeling strategies to appropriately guide the DDIs and other appropriate factors related to CYP3A4 induction. The session will help attendees to discover the true worth of applying PBPK modeling in not just interpreting observed data but even to prospectively use the pBPK modeling simulations to design effective clinical studies.

SC1.2 - HOW DO I BUILD AN 'IDEAL' PBPK MODEL IF MY DRUG IS EITHER A SUBSTRATE OR INDUCER**Ryuta Asaumi***Astellas Pharma, Chuou-ku, Japan*

To predict drug-drug interactions (DDIs) mediated by CYP3A induction using a physiologically based pharmacokinetic (PBPK) model, it is essential to accurately set drug parameters of both the CYP3A substrate (victim drug) and the CYP3A inducer (perpetrator drug) as well as physiological parameters in detail. In this process, it is crucial not only that the plasma concentration-time profiles of both drugs match clinical data, but also that the contribution of CYP3A-mediated metabolism (fractional metabolism: fm) and first-pass metabolism in the intestine for the substrate, and the CYP3A induction parameters (Emax, EC50) for the inducer are appropriately set. Additionally, PBPK models are constructed using a bottom-up approach with *in vitro* data or allometric scaling with animal data in the preclinical stage, but some parameters need to be updated through a top-down approach after obtaining clinical plasma concentrations. This presentation will introduce prediction methods for CYP3A induction effects appropriate to different stages of development, along with their pros and cons. Specifically, it will showcase examples of models developed using midazolam as a typical CYP3A substrate and rifampicin as a strong inducer. These modeling approaches can also be applied to compounds at various stages of development. By predicting DDIs with a typical substrate or a strong inducer and comparing and validating these predictions against clinically observed DDI results, *in vivo* parameters that reflect clinical outcomes, such as fm or induction parameters of the compound, can be obtained. Consequently, it is believed that an ideal model capable of accurately predicting untested DDIs with other substrates or inducers can be established.

SC1.3 - HOW DO I USE PBPK MODELING TO DISCERN COMPLEX DRUG-DRUG INTERACTIONS?**Jayaprakasam Bolleddula***EMD Serono, Boston, MA, United States*

Pharmacokinetic drug-drug interactions (DDIs) often occur due to the inhibition and induction of drug-metabolizing enzymes and/or transporters that are involved in the absorption, distribution, metabolism, and excretion of co-administered drugs. DDIs may increase or decrease the exposure of investigational drugs, which can lead to either reduced efficacy or increased adverse events in patient population. Therefore, understanding the magnitude of DDI is critical and has traditionally been investigated in clinical studies using index inhibitors, inducers, or substrates. Physiologically based pharmacokinetic (PBPK) modeling has proven to be a powerful tool to simulate the magnitude of DDI by integrating system- and drug-specific parameters. In oncology, PBPK model simulated DDI results have been increasingly used to inform inclusion/exclusion criteria in first-in-human trials, clinical pharmacology study designs, dosing guidance, and drug labeling to support DDI management. However, it is extremely challenging to precisely assess the magnitude of drug interaction when multiple pathways are affected by the perpetrator or there are competing DDI mechanisms, such as inhibition (reversible and time-dependent) and induction of cytochrome P450 (CYP) enzymes or transporters or both. This session will discuss the utility of PBPK modeling to discern complex DDIs when other drug metabolizing enzymes and transporters are involved in DDIs along with CYP3A induction.

SC1.4 - HOW WILL CYP INDUCTION RELATED PBPK APPLICATIONS BE EVALUATED DURING THE NDA REVIEWS?**Ying-Hong Wang***FDA, Silver Spring, MD, United States*

Characterization of CYP induction potential of a new drug entity (NME) is important for formulating drug interaction management strategies and ensuring the effective use of concomitantly administered drugs. In this section of the short course, the attendees will learn how CYP induction related PBPK applications are evaluated during the NDA reviews. Case examples of NMEs as a victim and a perpetrator of CYP induction will be presented to discuss common issues and potential solutions.

SC2.1 - INDUSTRIAL PERSPECTIVE OF REACTIVE METABOLITES: WHEN TO BE AFRAID AND WHEN TO BE VERY AFRAID**Jonathan Bauman***Pfizer, Groton, CT, United States*

Reactive metabolites have been a concern in drug discovery and development since the mid-20th century. Initially, their role was not well understood, and they were often seen as benign byproducts of drug metabolism. However, in the 1960s and 1970s, researchers began to realize that some reactive metabolites could bind covalently to proteins and DNA, leading to toxicity and adverse drug reactions (ADRs). This realization prompted the development of methods to detect and characterize reactive metabolites, such as trapping techniques and mass spectrometry. In the 1980s and 1990s, the pharmaceutical industry started to incorporate screening assays for reactive metabolites into drug development pipelines. This was driven by several high-profile cases of drugs being withdrawn from the market due to toxicity issues related to reactive metabolites. While reactive metabolites have been identified for dozens of compounds in various *in vitro* systems, and it has been further demonstrated that they adduct to biological macromolecules *in vitro*, a direct mechanistic link between reactive metabolites and *in vivo* toxicities has been difficult to unequivocally prove. With the tenuous link between metabolic bioactivation and toxicity, there have been many assays described to measure bioactivation. These range from simple and inexpensive assays to qualitatively detect adducts between model nucleophiles and electrophiles generated in drug metabolism incubations to greater investments in quantitative assessments of macromolecule adducts using radiolabeled drugs. Additional advances were made when *in vitro* covalent binding data were combined with overall dose and the relative contribution of bioactivation to the total metabolism to estimate the total covalent body burden, which we have demonstrated can be correlated with clinical drug toxicity, especially in extreme cases. In recent years, there has been a shift towards earlier detection and mitigation of reactive metabolites in drug discovery. This session will cover the challenges of dealing with reactive metabolites from an industry perspective in the past, present, and future.

SC2.2 - MITIGATING STRATEGY FOR DRUG INDUCED LIVER INJURY LED BY REACTIVE METABOLITES**Sean Zhu***Takeda, Cambridge, MA, United States*

The pharmaceutical industry loses billions of dollars annually due to the failure of drug candidates. More important than the loss of money, failure of drug candidates results in the loss of potential treatment opportunities for the patients, despite having compounds with promising preclinical efficacy. One of the major causes of termination of drug development programs and removal of drugs from the market is drug-induced liver injury (DILI). There are two types of DILI, intrinsic and idiosyncratic. Intrinsic DILI results in dose dependent hepatotoxicity and can usually be detected in either *in vivo* or *in vitro* preclinical studies. Because of the nature of intrinsic DILI, the risks associated with a potential drug candidate can often be assessed in earlier stages of drug discovery and avoided during clinical trials. Idiosyncratic DILI (iDILI), on the other hand, is very difficult to predict and occurs at a low frequency in patient populations. Actually, iDILI is defined at having a less than 0.1% frequency of occurrence that does not involve the pharmacological properties of the drug, it occurs in small patient populations at any dose of the drug, and typically has a delayed onset ranging from weeks to months (Uetrecht, 1999). Because of this, iDILI is difficult to predict preclinically. For example, one potential hypothesis for the cause of iDILI is immune mediated DILI, the mechanism of which is thought to be the drug candidate is metabolized to a reactive metabolite which can covalently bind to proteins within the hepatocytes forming neoantigens eliciting an immune response causing hepatotoxicity and resulting in the drug candidate being labelled with a warning or withdrawn. Many drugs involved in idiosyncratic drug reactions (IDR's) have the potential to form reactive metabolites. It is hypothesized that reactive metabolite formation might be the first step in the generation of IDR's. The earlier within a drug discovery/development project a potential risk is identified the easier it is to implement a mitigation strategy within the project and the higher likelihood of success of the project. It is therefore essential to identify potential risks, assess the probability/potential for the risk, and place it within the context of the project as early as possible. Because of this multiple *in vitro* assays in cells and/or subcellular fractions have been developed (for example, different trapping assays (glutathione for soft electrophiles, cyanide for hard electrophiles, semicarbazide for aldehydes, etc.), time dependent inhibition, covalent binding, cellular glutathione consumption, etc.) to evaluate the potential reactive metabolite risk. Each of these assays have their own advantages and disadvantages and taken together can help a project team mitigate the risk for reactive metabolites.

SC2.3 - NOVEL MECHANISMS OF DRUG-METABOLIZING HYDROLASES REGULATING THE TOXICITY CAUSED BY REACTIVE METABOLITES**Tatsuki Fukami***Kanazawa University, Kanazawa, Japan*

In most cases, drug-induced liver injury is initiated by the bioactivation of drugs to chemically reactive metabolites. Arylacetamide deacetylase (AADAC), which has been characterized by our group as one of the major drug-metabolizing

hydrolases responsible for drug detoxification or pharmacological activation, is also sometimes involved in the production of reactive metabolites. For example, AADAC hydrolyzes an anticancer drug trametinib to a metabolite that is hydroxylated by CYP3A4 to potential reactive metabolites, quinoneimine or N-hydroxylamine forms. Recently, we found that plasma alanine and aspartate transaminase levels, biomarkers of liver injury, were more highly increased in knockout (KO) mice of Aadam than in wild-type (WT) mice after administration of carbon tetrachloride (CCl₄) or acetaminophen (APAP). CCl₄-induced liver injury involves the production of a highly toxic trichloromethyl radical by CYP2E1. APAP-induced liver injury involves the production of a reactive metabolite, N-acetyl-p-quinoneimine, by CYP2E1 and CYP3A4. Thus, the hydrolysis reaction is not associated with the development of liver injury of these compounds. Recent studies have reported that ferroptosis, an iron-dependent form of regulated cell death caused by lipid peroxidation and subsequent membrane damage, is related to liver injury induced by CCl₄ and APAP. Pretreatment of ferroptosis inhibitors such as ferrostatin-1 and deferoxamine could suppress the liver injury caused by CCl₄ or APAP in Aadam KO and WT mice, suggesting that the more severe liver injury observed in Aadam KO mice by administration of CCl₄ and APAP was due to the exacerbation of ferroptosis. Co-immunoprecipitation followed by proteomics showed that AADAC interacts with ceruloplasmin, which has ferroxidase activity (oxidation of ferrous iron to ferric iron). Aadam KO mice had significantly lower ceruloplasmin expression level and activity than WT mice, resulting in higher hepatic ferrous iron level in Aadam KO mice than in WT mice. Thus, it was demonstrated that Aadam has a role in maintaining ceruloplasmin protein level, thereby decreasing hepatic ferrous iron and attenuating susceptibility to drug-induced ferroptosis in the liver. In this presentation, I will introduce the novel functions of a drug hydrolase as described above in detail.

SC2.4 - IN VIVO MODELS FOR ASSESSING THE TOXICITY OF REACTIVE METABOLITES

Xinxin Ding

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The toxicity of many drugs or other xenobiotic compounds may depend on how efficiently they are bioactivated in the body by biotransformation enzymes, such as the cytochrome P450 monooxygenases. For most chemicals that require bioactivation to cause toxicity, it is unclear whether this bioactivation process needs to occur in the target tissue for adverse effects, which enzymes or metabolic pathways are most important, or whether variations in the bioactivation enzymes can dictate risks for toxicity. Dr. Ding will describe the efforts of his laboratory to develop novel approaches and genetically engineered mouse models to address these questions.

SC3.1 - QUANTITATIVE ANALYSIS OF GASTROINTESTINAL DRUG ABSORPTION AND DISPOSITION USING STATE-OF-THE-ART TECHNOLOGY

Yoshiyuki Shirasaka

Kanazawa University, Kanazawa, Japan

Oral drugs, which are superior in terms of convenience and safety, are the most preferred dosage form from the perspective of a patient's quality of life. Indeed, oral drugs account for 80 % of the world drug market, clearly indicating their high value and demand. An important aspect of handling oral drugs, both in drug development and in clinical practice, is to understand gastrointestinal (GI) absorption. However, it is not necessarily easy to develop, because the drug absorption profile after oral administration may be easily influenced by the dosing conditions and the physiological state of the gastrointestinal (GI) tract. For example, drug absorption from the GI tract includes disintegration of the drug product, dissolution of the drug substance, and membrane permeation through the epithelial cells of the GI tract. All of these processes are potentially affected by various factors that can change the physiological environment in the GI tract, such as disease/pathology, food/beverage, concomitant medications, age, and gender. Our research group has recently found that variability in the GI fluid volume may influence drug absorption characteristics by altering drug concentration. Indeed, the contributions of passive diffusion, transporters, and metabolic enzymes all depend upon the GI drug concentration levels, which are influenced by changes in GI fluid volume. However, this important variable has been neglected in many prediction methods for oral drug absorption and drug interactions, and for convenience, it is often assumed that the GI fluid volume is fixed at a constant value. However, the actual volume of fluid in the GI tract should be both time- and site-dependent as a result of water intake, absorption, secretion, and GI transit. Regarding fluid dynamics in the GI tract, it may affect not only the above-mentioned "amount (volume)" but also "the exposed area (effective surface area)". In this case, for poorly soluble drugs, changes in the exposed area depending on the fluid volume would be an important factor in the absorption kinetics. On the other hand, for poorly absorbable drugs and controlled-release formulations, it is necessary to understand the absorption kinetics in the lower part of the GI tract, where the fluid volume and the exposure area are small. Because the large intestine contains less luminal fluid than the small intestine, it is expected to be exposed to higher concentrations of drugs, and therefore, theoretically, the large intestine should be a good drug absorption site. Interestingly, however, it is generally considered unimportant as a site of drug absorption. To efficiently develop oral drugs, including formulation development, it is important to understand the complexity of drug absorption after oral administration, considering the fluid dynamics in the GI tract in terms of the volume, area, and site of exposure. This short course will provide an overview on the predictive approach of oral drug absorption and disposition

based on quantitative analysis of GI physiology using a combination of PBPK modeling and various cutting-edge technologies such as quantum dot imaging, tissue transparency, and 3D organoid culture.

SC3.2 - GASTROINTESTINAL IMAGING WITH MRI: INSIGHTS INTO THE BEHAVIOR OF DOSAGE FORMS AND THE CONDITIONS AT THE SITE OF DRUG RELEASE

Werner Weitschies

University of Griefswald, Griefswald, Germany

Magnetic resonance imaging (MRI) provides unique insights into the physiology of the gastrointestinal tract and the performance and fate of drug delivery systems in humans. The continuing development of fast MR imaging techniques and sequences is making it increasingly possible to visualize the dynamics of gastrointestinal processes. Possibilities for identification of drug delivery systems in MRI will be discussed. Examples of the situation in the stomach and small intestine and the effects on the transport of drugs and delivery systems will be shown. The impact of gastrointestinal physiology on pharmacokinetics will also be discussed. Particular attention will be paid to the phenomena of fluid transport in both the stomach ("stomach road") and the small intestine ("small intestinal road") under postprandial conditions.

SC3.3 - HUMAN ENTEROID MONOLAYERS – A FIT-FOR PURPOSE MODEL TO ELUCIDATE INTESTINAL NATURAL PRODUCT-DRUG INTERACTIONS

Kenneth E. Thummel, Eimear O'Mahony, Christopher Arian, and Edward J. Kelly

University of Washington, Seattle, WA, United States

Preclinical screening drug candidates for human intestinal first-pass metabolism and oral bioavailability has been challenged in the past by the lack of an *in vitro* model that fully recapitulates the unique biochemical and physiological properties of the proximal intestinal mucosa. Much has changed following the discovery of intestinal crypt stem cells and procedures for isolation, propagation and differentiation of those cells into the various mature cell types of the mucosal villus. In this vein, we found that following stem cell propagation to increase cell count, reductions in key growth factors resulted in differentiated cells that when plated as a Transwell monolayer, develop tight-junction barriers to drug translocation (evidenced by immunohistochemical staining of occludin, ZO-1 and ZO-3, increased TEER and low lucifer yellow permeability) and CYP3A4 activity comparable to that expected for villus enterocytes. RNA-Seq analysis illuminated the stem cell to enteroid monolayer transition, with decreases in expression of cell proliferation genes such as PCNA, LGR5 and MKI67 and increases in isomaltase and MUC13 expression associated with enterocyte and goblet cells, as well as many of the drug metabolizing enzymes and transporters (notably, CYP3A4 and ABCB1). Selective protein staining demonstrated polarized localization of MRP3 on the basolateral monolayer surface and ZO-3 towards the apical surface. Although the cultured monolayer does not form prominent villi, it does show a pattern of undulations indicative of an increased apical surface area. Of significant importance for studies of gene regulation and long-term DMET/Tox studies is the observation that all of the above cell monolayer properties are fully developed by 14d in culture and can be maintained for up to 42d and possibly beyond. Indeed, the presence of Ki67-positive nuclei in enteroid monolayers suggests the retention of some self-replicating capability.

Focusing on CYP3A4 function, we observed first-pass, apical – basolateral metabolic extraction values for enteroid monolayers derived from different duodenal crypt donor cell lines that were comparable to that observed *in vivo* (18-52% vs 19-59%), and reduced by 6'7'-dihydroxybergamottin and induced by 1 α ,25-dihydroxy vitamin D3 pretreatments. Interestingly, the addition of human serum albumin to the basolateral Transwell media, reducing compartmental unbound midazolam concentrations, did not alter the intracellular rate of midazolam metabolism after an apical dose, but did increase transcellular midazolam permeability and, as a consequence, reduced the first-pass metabolic extraction efficiency by 25%. With regard to drug transport functions, we demonstrated organic cation transporter mediated A>B uptake of metformin and P-glycoprotein mediated efflux of digoxin (B>A/A>B = 3.4). Ongoing experiments are examining an inhibitory interaction between constituents of the natural product goldenseal and metformin absorption, and PXR- and AhR-mediated induction of CYP3A4 and CYP1A1, respectively, by constituents of the natural product oregano. Collectively, these experiments highlight the significant value of our organoid cell-based model for investigating acute and chronic xenobiotic treatment effects on oral drug disposition. It should also be useful for studying the impact of chronic pathological conditions such as inflammation or inherited genetic variation (with a large enough donor stem cell pool) on mucosal epithelial functions.

Supported in part by a grant from NCCIH (U54 AT008909) and funding from the University of Washington Drug Metabolism, Transport and Systems Pharmacology Research (DMTSPR) consortium of pharmaceutical companies.

SC3.4 - TOWARDS A PHARMA[E]COLOGICAL VIEW OF PRECISION MEDICINE**Peter Turnbaugh***University of California, San Francisco, San Francisco, CA, United States*

Although the importance of human genetic polymorphisms and drug-drug interactions in precision medicine is increasingly understood, the role of specific genotypic variants and products elaborated by our “second genome” (the microbiome) has been largely overlooked. In this presentation I will describe a microbiome-informed approach to pharmacology, working to elucidate the direct and indirect mechanisms through which the human microbiome shapes the efficacy and toxicity of small molecule and biologic therapies. For example, research in my laboratory has provided mechanistic insights into the reciprocal interactions between drugs and gut bacteria, leading to the discovery of the genes and enzymes responsible for drug metabolism as well as the genetic determinants of drug sensitivity. These effects extend beyond metabolism to impact other key components of ADME, including the expression and function of drug transporters. Collectively, attendees will learn about exciting new research at the interface between pharmacology and the human microbiome, providing suggestions for incorporating the microbiome into both basic and applied research and even drug development.

SC3.5 - INTEGRATING INTESTINAL PHYSIOLOGY AND DRUG METABOLISM, DISPOSITION, AND INTERACTIONS THROUGH PBPK MODELING**Bhagwat Prasad***Washington State University, Spokane, WA, United States*

The presentation will delve into the intricate dynamics of intestinal drug absorption. Prediction of drug absorption requires the integration of drug properties—such as solubility, permeability, metabolism, and transport—with physiological systems properties, including intestinal enzyme and transporter levels, gastric emptying time, food effects, and residence time. By leveraging physiologically based pharmacokinetic (PBPK) modeling, we can systematically address these critical factors to enhance our understanding and prediction of drug behavior within the gastrointestinal tract. The presentation will highlight key determinants affecting drug absorption that must be incorporated into PBPK models. Specific examples will include the impact of intestinal UGT2B17, BCRP and gastric emptying time on drug absorption. Through this discussion, attendees will gain insights into the importance of integrating physiological and pharmacological parameters for accurate PBPK modeling, ultimately leading to better prediction of drug interactions and interindividual variability in drug absorption.

SC4.1 - DOWNSTREAM EFFECTS OF PROTEIN DEGRADERS**Katherine Donovan***Dana-Farber Cancer Institute, Boston, MA, United States*

The therapeutic potential of targeted protein degradation (TPD) offers a revolutionary pathway for drug development, with degraders such as PROTACs (PROteolysis Targeting Chimeras) leading the forefront. These bifunctional molecules orchestrate the selective degradation of pathological proteins, offering a novel modality for treating a broad spectrum of diseases. However, the complexity of their mechanism of action, encompassing binary binding, ternary complex formation, ubiquitylation, and eventual protein degradation, presents unique analytical challenges. This workshop aims to elucidate a spectrum of cutting-edge analytical methodologies, with a special focus on quantitative proteomics, to unravel the intricacies of the degrader-mediated degradation process.

SC4.2 - BIOANALYSIS, BIOTRANSFORMATION AND PHARMACOKINETICS OF ANTIBODY-DRUG CONJUGATE**Yuki Kishino***Daiichi Sanyko, Tokyo, Japan*

Antibody-drug conjugate (ADC) comprising monoclonal antibodies conjugated to small molecule drugs (payloads) via chemical linkers is one of the fastest growing drug modalities, providing wider therapeutic index by selectively delivering payloads to target tissues and cells.

In vivo behavior of ADCs is evaluated by several bioanalytical platforms in preclinical and clinical stages, and the bioanalytical data are essential for pharmacokinetics/ pharmacodynamics (PK/PD) modeling. The total antibody and ADC concentrations in plasma are generally measured by ligand binding assay with capture/detection reagents such as antigen, anti-idiotypic antibody and anti-payload antibody. The concentrations of released payload are determined by sensitive LC/MS/MS method. The behavior of ADC in the body is complex primarily due to biotransformation including drug-to-antibody ratio (DAR) change, which can be caused by intended enzymatic linker cleavage and/or other linker degradation mechanisms. The transition of DAR after administration can be evaluated by hybrid affinity capture LC-MS/MS, where samples are treated by immunoprecipitation with antibody-conjugated beads and papain digestion to release payloads, followed by determination of payload concentrations. The importance of DMPK research in ADC drug discovery and development will be discussed from the viewpoint of bioanalysis and biotransformation.

SC4.3 - TIPS FOR BIOANALYTICAL METHOD DEVELOPMENT OF PEPTIDES, AND THEIR DDI RISK EVALUATION**Kosuke Saito***National Institute of Health Sciences, Kawasaki, Japan*

In recent years, the development of peptide drugs has significantly expanded the range of molecules that can be targeted and improved their pharmacokinetics through complete chemical synthesis, control of steric structures (such as stapling or cyclization), and conjugation. However, along with this progress, several-related issues have emerged in the field of pharmacokinetics research. Specifically, in terms of analytical aspects, the applicability of conventional ligand binding assay, for analyzing drug concentrations in biological samples is unclear. This uncertainty arises due to the complexity of the structure as well as the demand of discrimination from impurities and metabolites. LC-MS, a method usually used for quantification of small chemical drug concentrations, can alternatively be applicable for peptide drugs with capability of discrimination from impurities and metabolites, although its sensitivity is not compatible to ligand binding assay. To advance its application, there remains several concerns, including but not limited to carryover, selection of quantitative ion, extraction efficacy, has been addressed. On the other hand, there are currently no regulatory guidelines outlining DDI assessments for peptide drugs, which poses significant uncertainty and challenges during drug development for this heterogenous class of molecules. Although there are limited number of approved peptide drugs, few of them with less than molecular weight of 2000 Da have reported the risk of DDI based on *in vitro* study. In addition, we recently found that ATSP-7041 (molecular weight 1745 Da), a stapled alpha-helical peptide, exhibited strong inhibition of transporters, P-gp, BCRP, OATP1B1, and OATP1B3, *in vitro*. These observations raise the importance in assessment of peptides for transporter inhibition.

Based on these situations, I will present our approaches to standardize LC-MS based peptide analysis. A peptide drug conjugate (BT1718) and two cyclic peptides (Cyclorasin 9A5 and PD-1/PD-L1 inhibitory peptide) was selected model peptides and develop analytical methods in multi-laboratory. We are under evaluating the inter-laboratory compatibilities to extract the challenges that were encountered during the development processes, and the effective solutions to these challenges. In addition, I will also present our recent study evaluating *in vitro* inhibitory potency to transporters (P-gp, BCRP, OATP1B1, and OATP1B3) by peptides, including 15 peptides composed of all natural amino acids and 16 peptides including non-natural structured amino acids with over 1000 Da molecular weight. Further, we conducted comprehensive evaluation of their inhibitory potency to physicochemical properties, including charged and hydrophobic amino acid numbers and molecular weights.

SC4.4 - OLIGONUCLEOTIDE TARGET ENGAGEMENT**Michael McCoy***Takeda, Cambridge, MA, United States*

Oligodeoxynucleotides (oligos), short single-stranded or double-stranded DNA/RNA molecules, are emerging as a promising class of therapeutics. Similar to conventional drugs, successful oligo therapeutics rely on efficient, specific target engagement in the target tissue site or cell of interest. In this context, target engagement refers to the specific binding of an oligo to its intended molecular target, which can be RNA, DNA, or protein.

Measuring target engagement for oligos presents unique challenges compared to small molecule drugs. Traditional methods may not be readily applicable due to the specific properties of oligos. However, innovative approaches are being developed to assess oligo target engagement at both macro (tissue) and micro (cellular and subcellular) scales, including:

- Biophysical techniques: Techniques like surface plasmon resonance (SPR) and biolayer interferometry (BLI) can quantify oligo-target binding affinity and kinetics.
- Chemical modifications: Oligos can be chemically modified to incorporate tags or probes that facilitate target engagement assessment through techniques like PCR or fluorescence microscopy. However, probe attachment may alter target engagement capabilities and promiscuity.
- Target cell isolation: Separate a specific population of cells from a heterogeneous mixture to evaluate oligo distribution and the function of specific cell types using Immunomagnetic separation, fluorescence-activated cell sorting (FACS), density gradient centrifugation, laser capture microdissection (LCM).
- Immunohistochemistry (IHC): If the oligo is chemically conjugated to an antibody or other detectable tag or chemical structure, IHC can be used to visualize the oligo within tissues.
- Fluorescence in situ hybridization (FISH): This technique utilizes fluorescently labeled probes complementary to the oligo sequence to detect its location within cells.
- Cellular assays: These assays utilize reporter genes or protein fusions to measure target occupancy or activity modulation by the oligo.
- Sub-cellular assays: RISC-loading assay and RNAseq are techniques used to measure the efficiency and accuracy of siRNA loading into RISC complex which impacts target gene knockdown efficacy.

Effective target engagement assays are crucial for oligo drug development. They allow researchers to:

- Validate target selection: Confirm if the chosen target is accessible and effectively engaged by the oligo.
- Optimize oligo design: Guide modifications to the oligo sequence or chemical structure to enhance target specificity and affinity.
- Predict efficacy and safety: Correlate target engagement with biological response to assess potential therapeutic effects and off-target interactions.

By employing these target engagement strategies, researchers can efficiently develop potent and specific oligo therapeutics.

K1 - AI PLATFORM TO ACCELERATE DRUG DISCOVERY

Yasushi Okuno

Kyoto University, Kyoto, Japan

Facing COVID-19, it was probably the first time for not only researchers and healthcare professionals but also the general public to be so acutely aware of the importance of vaccines and pharmaceuticals in a pandemic. Particularly shocking for us researchers was the swift response by Western pharmaceutical companies and regulatory authorities. Remdesivir was approved in less than six months after COVID-19 became a global concern, and vaccines hit the market within a year—an unprecedented speed that far exceeded the conventional pace of drug development. Typically, it is said that developing a drug requires over ten years and costs over 100 billion yen. However, this extraordinary case of COVID-19 has overturned the conventional wisdom of drug development and is expected to become the new normal for future drug discovery.

On the other hand, just as the third AI boom appeared to be losing momentum, the advent of ChatGPT caused AI to break through from just a trend to becoming a pioneering force for the future. This shift can be equally said for drug discovery, where various AI technologies are being developed worldwide, and expectations are mounting significantly. Nevertheless, many reports, particularly those from the industry appearing in newspapers and other media, may contain information of uncertain veracity. As the academic community, we must soberly assess the current capabilities and limitations of AI applications in drug discovery and strive to develop new technologies.

Against this backdrop, we are working on the research and development of a drug discovery digital transformation (DX) platform aimed at ultra-smartening the drug discovery process through AI and simulation, with the fundamental premise of small teams and low costs. This initiative seeks to enable the Japanese pharmaceutical industry to catch up with and confront global speeds in drug innovation. Specifically, we have been developing AI and simulation technologies for each step of the drug discovery process, from upstream to downstream. These technologies include target discovery, activity prediction (such as efficacy and toxicity), mechanism elucidation, compound design optimized based on these conditions, and the extraction of adverse effect factors from clinical data.

For instance, in a domestic project, we are developing a Graph Convolutional Network (GCN) AI that predicts activity and ADMET properties from chemical structures, by sharing compound data among several pharmaceutical companies. Additionally, we are advancing AI technologies utilizing diverse datasets like multi-omics and clinical data to achieve patient stratification, sample classification, molecular mechanism elucidation, biomarker estimation, and the exploration of drug target molecules. In this lecture, I would like to introduce specific examples of my work and discuss the current state and future prospects of AI in drug discovery.

PL1 - ENDOGENOUS BIOMARKER-INFORMED APPROACHES FOR EVALUATION OF TRANSPORTER-MEDIATED DRUG-DRUG AND DRUG-DISEASE INTERACTIONS

Aleksandra Galetin

University of Manchester, Manchester, United Kingdom

Recent years have seen a discovery of novel biomarkers for hepatic/ renal transporters and advances in their application for evaluation of transporter-mediated drug-drug interactions (DDI) in early drug development. This session will discuss the proposed tier classification of endogenous biomarkers and recommendations for application of biomarkers to improve de-risking of transporter DDIs and management strategies. The presentation will provide examples of leveraging biomarker data in combination with different modelling approaches to predict transporter DDIs and to facilitate the design of prospective clinical interaction studies. The extension of mechanistic biomarker models to patient populations will demonstrate the role of modelling to differentiate the effect of disease on biomarker synthesis and transporter activity. In addition, challenges to delineate the effect of disease and drug-mediated inhibition of transporters in patient populations will be discussed.

S1.1- REVIEW OF FINALIZED ICH M12 GUIDELINE ON DRUG INTERACTION STUDIES**Akihiro Ishiguro***PMDA, Toyko, Japan*

Evaluation of characteristics of drug interactions (DDI) in drug development is essential to minimize potential DDI risks of adverse drug reactions or attenuation of the therapeutic effect of drug. A risk-based approaches are generally used to assess DDI during drug development, but there are some differences in the approaches presented in guidelines published by regulatory agencies in each region. In 2019, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) launched an initiative as an M12 expert working group (EWG) to develop the first international guideline on DDI. The harmonized DDI guideline provides recommendations to promote a consistent approach in designing, conducting, and interpreting enzyme- or transporter-mediated *in vitro* and clinical DDI studies during drug development. A consistent approach will reduce uncertainty for the pharmaceutical industry to meet the requirements of multiple regulatory agencies and lead to more efficient utilization of resources. In addition, this approach will lead to the effective and safe treatment for patients who take multiple medications.

Public consultation on the draft DDI guidelines was conducted in 2022, and approximately 1,000 comments were raised from 30 stakeholders in each region [1]. The EWG ensured evidence-based discussions to address the comments related to topics that impact regulatory decisions or where scientific knowledges are accumulating (e.g., optimization of *in vitro* cut-off values, availability of endogenous biomarker, assessment of drugs with high protein binding). The session will share the EWG's activities of harmonizing differences in the regional guidelines. The emerging approaches on DDI evaluation adopted in the M12 and perspectives by implementing the harmonized guideline will also be introduced.

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S1.2 - PRECLINICAL DDI RISK ASSESSMENT IN DRUG DISCOVERY AND DEVELOPMENT**Niresh Hariparsad***AstraZeneca, Waltham, MA, United States*

Given that polytherapy is commonplace in the treatment and management of most diseases, it is important to understand and quantitate the potential of new chemical entities (NCEs) as perpetrators and victims of drug-drug interactions (DDIs). DDIs are especially problematic when they result in sub-efficacious exposures or conversely drug levels into the toxic range. While tools to assess and quantify DDI risk have improved, continued evolution in this field is required as more complex DDIs are observed with multiple enzymes and/or transporters involved. Added to this challenge are the development of compounds with very high intrinsic stability, very poor physicochemical properties, compounds with non-traditional clearance pathways, beyond the rule of 5 compounds and the emergence of novel modalities such biologics. During this talk the speaker will introduce the current status with regards to *in vitro* tools to assess DDI risk for induction/inhibition of metabolic enzymes and transporters and practices to quantify DDI risk (using static and PBPK modeling). Thereafter, the speaker will focus on emerging challenges and how to assess and quantify complex DDIs e.g. where the perpetrator is an inducer/inhibitor of multiple enzymes and transporters. In addition the speaker will discuss challenges quantifying fraction metabolized for low clearance compounds and the related implications for victim DDI in the clinic. This will extend to victim and perpetrator DDI challenges for protacs and antibody drug conjugates. Finally, the speaker will delve into emerging technologies which will include next generation *in vitro* tools and also novel clinical tools such as biomarkers and extracellular vesicles.

S1.3 - COMPLEX DRUG-DRUG INTERACTIONS: STUDY DESIGN AND CLINICAL INTERPRETATION**Manoli Vourvahis***Pfizer, New York City, NY, United States*

Drug-drug interactions (DDIs) involving drug metabolizing enzymes and drug transporters can lead to changes in drug exposure, which can potentially impact both efficacy and safety. In drug development, evaluation of DDI potential is essential to optimize therapeutic strategies. While successful approaches have been developed to assess/predict DDIs involving cytochrome P450 enzyme inhibition or induction in isolation, the optimal strategy to evaluate and interpret complex DDIs such as mixed enzyme inhibition and induction, combined competitive and time-dependent inhibition (TDI), or simultaneous transporter-enzyme interactions continues to evolve. Complex DDIs can be difficult to deconvolute and can pose a challenge during drug development. Understanding these interactions is essential to predict the potential risks of drug coadministration and minimizing adverse effects. Several novel approaches to facilitate the mechanistic understanding of complex DDIs are emerging. This presentation will explore some examples of complex DDIs and highlight study design, data analysis, and extracellular vesicle (EV)/biomarker strategies that can be utilized to assist with mechanistic interpretation.

S1.4 - UTILIZING PBPK MODELING AND IN VITRO DATA FOR PREDICTING COMPLEX DDIs OF OATP1B AND CYP SUBSTRATES CAUSED BY VARIOUS PERPETRATORS**Yuichi Sugiyama***Josai International University, Tokyo, Japan*

Many drugs that undergo extensive metabolism by CYPs and /or excreted into bile/urine are also substrates of uptake transporters, OATPs and OATs, OCTs in the liver and kidney. According to extended clearance concept, a change in the uptake clearance directly affects the organ clearance of these drugs, whereas a change in the metabolic/excretion clearance sometimes has little influence on its organ clearance^{1,2}). It is thus essential to know the rate-determining process of organ elimination of a drug. In particular, cassette (cocktail) sub-therapeutic dosing of probe drugs is a powerful approach for the simultaneous phenotyping of multiple metabolic enzymes and transporters in the same person without producing side effects caused by a large increase in the plasma concentration of substrate drugs. In this presentation, I will share with you our recent clinical studies using several OATP substrates and CYPs substrates with/without the co-administration of OATP inhibitor and CYPs inhibitors³⁻⁷).

Rifampicin (RIF), an anti-tuberculosis drug, is widely recognized as an inducer and inhibitor of drug transporters and metabolic enzymes, causing clinically significant drug-drug interactions (DDIs).

Rifampicin causes drug-drug interactions (DDIs) with various concomitant medications through inhibition and induction of hepatic uptake transporter OATP1B, P-glycoprotein (P-gp), and drug-metabolizing enzymes. Therefore, when co-administered drugs are multi-substrates of both OATP1B or P-gp and drug-metabolizing enzymes, complex DDIs can occur depending on the timing and duration of rifampicin administration. In this presentation, we predict the DDIs of rifampicin with multi-substrate drugs such as glyburide (OATP, CYP2C9, and CYP3A substrate) and repaglinide (OATP, CYP2C8, and CYP3A substrate) using the PBPK model and compare the results with past reports. Additionally, we introduce the validation process of the constructed PBPK model.³⁻⁷)

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S1.5 - UTILITY OF MECHANISTIC STATIC MODELS FOR PREDICTING CYP- AND TRANSPORTER-MEDIATED DDI**Sheila Peters***Boehringer-Ingelheim, Mainz, Germany*

Mechanistic static and dynamic physiologically based pharmacokinetic (PBPK) models are used in clinical drug development to assess the risk of drug–drug interactions (DDIs). Currently, the use of mechanistic static models (MSM) is restricted to screening DDI risk for an investigational drug, while dynamic PBPK models are used for quantitative predictions of DDIs to support regulatory filing. However, PBPK model development by sponsors as well as a review of models by regulators require considerable resources. There is growing evidence to suggest that MSM can provide quantitative predictions that are comparable with those from PBPK when appropriate inhibitor concentrations. A pragmatic approach would be to use of MSM or PBPK depending on the intended purpose, limiting the use of PBPK models to those applications that leverage its unique strengths, such as what-if scenario testing to understand the effect of dose staggering, evaluating the role of uptake and efflux transporters, and extrapolating DDI effects from studied to unstudied populations.

S2.1 - ASSESSMENT OF ADME AND DDI FOR LNP-MRNA THERAPEUTICS**Lei Ci***Moderna Therapeutics, Cambridge, MA, United States*

Lipid nanoparticle (LNP)-encapsulated mRNA is a novel and expanding modality of human therapeutics. The global deployment of mRNA COVID-19 vaccines was a prominent milestone for this modality, building confidence in mRNA-based therapeutics. The success of these mRNA therapies depends on their encapsulation in lipid nanoparticles, assisting in the transport of exogenous nucleic acids to tissues while simultaneously preventing degradation by endo- and exonucleases. Lipid nanoparticles are non-covalent assemblies of multiple molecular components and are typically composed of ionizable lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-functionalized lipids. Ionizable lipids are a critical LNP component that drive biodistribution, cellular uptake, endosomal escape, and tolerability of mRNA therapeutics. We evaluated a novel amino lipid for its ADME properties and DDI potential. This talk will review the current understanding of LNP-mRNA technologies and the evaluation of ADME and DDI properties for our LNP-mRNA therapeutics.

S2.2 - TARGETED PROTEIN DEGRADERS (TPD): PK/PD TRANSLATION FROM PRECLINICAL TO CLINICAL**Dapeng Chen***Kymera Therapeutics, Watertown, MA, United States*

Targeted Protein Degradation (TPD) is a novel therapeutic modality that uses heterobifunctional small molecules to redirect the cellular machinery responsible for protein degradation and cellular homeostasis, the ubiquitin-proteasome system (UPS), toward specific disease-causing proteins through spatial proximity with the use of affinity rather than occupancy-based catalysis, delivering a potentially transformative new way to treat disease. To realize on the promise of this disease-agnostic technology, Kymera has taken a unique approach to target selection, where we focus on targets that are either undrugged or inadequately drugged within key signaling pathways with clear clinical validation and validation through human genetics/caUnited StatesI biology, and where TPD is the best or the only solution. This can be applied to targets that have both kinase and scaffolding functions that require removal of the protein such as Interleukin-1 receptor–associated kinase 4 (IRAK4). KT-474 (also known as SAR444656) is a potent, highly selective, orally bioavailable IRAK4 degrader, in clinical development for the treatment of IL-1R/TLR-driven autoimmune and autoinflammatory diseases. We have generated and validated predictive models that reflect our deep knowledge and understanding of the interplay between targets and drug properties, driving optimization of drug disposition and *in vitro* and *in vivo* pharmacokinetic (PK) /pharmacodynamic (PD) relationships of our degraders across different tissue and cell types. We have demonstrated high fidelity of translation in the clinic for all the programs that we have advanced in clinical stage to date. In this presentation, we will highlight key ADME and PK characteristics of KT-474, the preclinical PK/PD relationship in pharmacologically relevant models, strategies and approaches in prediction of human PK and pharmacologically active dose, and successful preclinical to clinical translation of IRAK4 degradation.

S2.3 - IMPROVING ADME PROPERTIES OF THERAPEUTIC ANTIBODIES BY NOVEL ENGINEERING TECHNOLOGIES**Kenta Haraya***Chugai Pharmaceutical, Yokohama, Japan*

Therapeutic monoclonal antibodies (mAbs) have become an important option for the treatment of several diseases. Since many mAbs have shown promising efficacy and safety in clinic, the competition to develop mAbs has become severe.

Although mAbs have several advantages over other therapeutic modalities, there are still some issues in many cases such as target mediated drug disposition (TMDD) mediated short half-life, required high therapeutic dosage due to abundant target amount, narrow therapeutic window due to systemic target expression etc. To overcome these issues, several antibody technologies have been developed. I will present the various antibody engineering technologies through improving ADME properties of mAbs that can provide significant benefits for patients.

S2.4 - DEFINING THE THERAPEUTIC WINDOW OF BI-SPECIFIC T-CELL ENGAGERS

Kip Conner

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The therapeutic potential of bispecific T cell engagers (TCEs) has been demonstrated through significant clinical milestones, beginning with the approval of blinatumomab in 2014 for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL). More recently, the approval of tarlatamab for extensive-stage small cell lung cancer (SCLC) has further expanded the application of TCEs to a major solid tumor indication, underscoring their tremendous utility in oncology to treat even the most highly refractory cancers. Despite these successes, the broad development of new TCE therapies continues to encounter significant clinical challenges. Chief among these is a narrow therapeutic window, which frequently leads to dose-limiting toxicities such as cytokine release syndrome (CRS) and off-tumor, on-target tissue toxicity.

This presentation will delve into the mechanisms underlying the narrow therapeutic window of TCEs, focusing on the pharmacokinetic drivers of treatment-related adverse events (AEs) and the dosing strategies employed to control them. An emphasis will be placed on the unique considerations associated with TCE therapy for solid tumors, namely the challenge of selective tumor targeting, as well as the complexities of the tumor microenvironment that can limit TCE biodistribution and control access to critical tumor-infiltrating lymphocytes (TILs). Finally, the discussion will explore molecule engineering strategies aimed at maximizing the therapeutic window of TCEs. This outlook and perspective will provide an overview of the current landscape and future directions in TCE therapy that are paving the way for safer and more effective cancer treatments.

S3.1 - AI-BASED FEATURE GENERATION FOR MOLECULAR DESIGN

Tadahaya Mizuno

University of Tokyo, Tokyo, Japan

While humans can recognize and handle chemical structures in various forms, such as molecular formulas, 2D images, and even 3D models, computers traditionally process them only as numerical data. Therefore, the numerical encoding of chemical structures, known as feature generation, has been a cornerstone of cheminformatics. However, recent advancements in neural networks have enabled computers to recognize and manipulate chemical structures in multiple forms, offering researchers the flexibility to create both task-specific and generalizable features. Deep learning models for chemical structures are primarily categorized into two types: those designed for graph-based representations and those tailored for text-based data. This study focuses on chemical language models, which treat chemical structures as textual data, similar to natural language.

Chemical language models analyze string representations of chemical structures, such as the Simplified Molecular Input Line Entry System (SMILES), by leveraging natural language processing techniques—one of the most advanced areas of neural networks. Since Gomez-Bombarelli's groundbreaking work in 2016, these models have been widely applied to tasks such as structure generation, retrosynthesis prediction, and feature generation. However, the mechanisms through which these models recognize diverse chemical structures remain largely unknown. To address this black-box, we investigated the learning processes of chemical language models and uncovered insights into their recognition capabilities, particularly in relation to partial structures and chirality. Despite its intended purpose, canonical SMILES representations often exhibit non-canonical and inconsistent notations. Therefore, we also explored the impact of dialects within string representations of chemical structures. Our findings indicate that these notational inconsistencies affect the recognition capabilities of chemical language models, although they do not significantly impact downstream tasks such as predicting kinetic properties and compound toxicities.

When applying machine learning models, including AI, it is essential to thoroughly understand their characteristics and limitations. We hope that our presentation provides valuable insights for those either new to or already engaged in AI-based feature generation, helping them better grasp the complexities involved.

S3.2 - MECHANISTIC MULTIPLE PARAMETER OPTIMIZATION IN DRUG DISCOVERY**Fabio Broccatelli***Altos Labs, San Diego, CA, United States*

Computational chemistry and machine learning are used in drug discovery to predict target-specific and pharmacokinetic properties of molecules. Multiparameter optimization (MPO) functions are used to summarize multiple properties into a single score, aiding compound prioritization. However, over-reliance on subjective MPO functions risks reinforcing human bias. Mechanistic modeling approaches based on physiological relevance can be adapted to meet different potential key objectives of the project (e.g. minimizing dose, maximizing safety margins and/or minimized drug-drug interaction risk) while retaining the same underlying model structure. The current work incorporates recent approaches to predict *in vivo* PK properties and validates *in vitro* to *in vivo* correlation analysis to support mechanistic PK MPO. Examples of use and impact in small molecule drug discovery projects are provided.

S3.3 - DRIVING COMPOUND OPTIMIZATION IN EARLY DISCOVERY BY IMPLEMENTING MECHANISTIC PHARMACOKINETIC AND MACHINE LEARNING MODELS**Wenyi Wang***Genentech, Foster City, CA, United States*

Physiology-based pharmacokinetic (PBPK) models are broadly applied in late phase drug discovery/ development. Traditionally, compounds are prioritized based on hierarchical filtering with predefined cut-offs for desirable range of various physical chemical properties and *in vitro* measurements. PBPK models, when combined with related pharmacodynamic assumptions, offer a valuable platform to integrate multiple parameters driving the *in vivo* PK profile required for target engagement. They also provide mechanistic interpretation of key drivers for the predicted profile to further help with compound design strategies. As such, these results enable compound prioritization in a holistic manner, focusing on multi-property optimization (MPO). Machine learning (ML) models for various *in vitro* end points have been well-established in the industry, enriched by a large amount of internal data across a broad chemical space. As such, ML models can be used in lieu of *in vitro* in out parameters, especially for those with lower throughput. Thus, combination of these two types of models can enable early implementation of MPO generation using the mechanistic platform. This presentation will provide a brief overview of the structure and application of an internal PBPK model. Examples of successfully combining the ML and PBPK model on a small molecule drug discovery program to drive compound optimization will be shared.

S3.4 - COMPUTER AIDED METID FOR SMALL MOLECULES AND PEPTIDE DRUGS**Ismael Zamora***Mass Analytica SL, Sant Cugat del Vallés, Spain*

The study of compound degradation in the context of Drug metabolism or/and Chemical degradation studies have an important role in drug discovery with an impact in the development of a new chemical entities. These studies have 2 components one related to the quantification and the calculation of compound clearance or degradation constants and another aspect that is related to the elucidation of the products (degradants or metabolites) formed during the experiment. We are presenting in here a single workflow that can be used in molecules of any size for metabolite identification. A collection of algorithms was developed to perform the two major tasks that are needed peak detection and structure assignment. The peak detection algorithms consider the specific needs for each compound class, i.e. it can use the Monoisotopic mass for molecules smaller than 4000 amu, the most abundant isotope for molecules until 20.000 amu or the average mass for bigger ones. Also, an AI system to reproduce the expert behavior during peak selection will be shown for both small and macro molecules. The structure assignment algorithm has been developed to be able to work with a set of atoms and bonds like the ones described in small molecule or monomers like in oligonucleotides, peptides or proteins or a combination of both molecule representation.

We have analyzed a collection of experimental data for a set of small molecules, protacs peptides, oligonucleotides, or proteins where the data was collected on a Waters Synapt/ Xevo/Vion/ Bioaccord, Thermo Q-Exactive, Agilent QToF, Sciex TripleToF/Zeno, Bruker TimmToF instrument. The degradation studies presented where done on in-vitro metabolite systems like human liver microsome, human hepatocyte, protease incubation or forced degradation analysis.

Results will be presented for each of the compound classes: a) small molecular metabolism in Human liver microsomal for nefazodone measured in 4 different instrument/acquisition modes (Waters Synapt MSe, Sciex Tripe ToF SWATH, Bruker QToF and Aglient All Ions); b) force degradation studies for lanzoprazol measures in Agilent QToF and Thermo Q-Exactive; c) Protac Hepatocyte degradation study on a Thermo DDS d) Somatostatine metabolism study using a Waters acquisition, e) Semiclutide degradation from a Waters Vion; f) Oligonucleotide (sense and antisense chain analysis) from a Thermo DDS analysis

In all cases the computation of the clearance and/or degradation contact will be presented to gether with a collection of

the metabolites found. The analysis of the fragmentation data will be shown for one case of the Nefazone and the oligonucleotide studies.

S4.1 - MEMBRANE TRANSPORTERS IN DRUG DEVELOPMENT AND AS DETERMINANTS OF PRECISION MEDICINE; AN UPDATE FROM THE INTERNATIONAL TRANSPORTER CONSORTIUM

Kim Brouwer

University of North Carolina Eshelman School of Pharmacy, Chapel Hill, NC, United States

Major progress has been made in understanding the roles of membrane transporters in drug disposition and response since the initial Nature Review Drug Discovery publication by the International Transporter Consortium (ITC) in 2010. Scientists in the academic, regulatory, and pharmaceutical industry sectors, with ongoing collaborations and dialogue facilitated by the ITC, have accelerated scientific discoveries regarding membrane transporters and applications in drug development and patient care. Recent research has revealed that intrinsic factors (e.g., genetics, physiologic states, organ-based diseases) and extrinsic factors (e.g., herbals and food, medications, environment) can modulate transporter abundance and/or function. More precise tools and approaches are being developed to assess transporter-mediated activity, toxicity, drug–drug interactions (DDIs), and to predict transporter-mediated drug disposition and pharmacokinetic changes in diseases and in specific populations. Physiologically based pharmacokinetic (PBPK) modeling has emerged as a key translational tool in drug development and has gained broad acceptance in regulatory submissions for drugs that are substrates or inhibitors of transporters. Notable advances have been made in the discovery and validation of endogenous substrates as biomarkers of transporter function, and in the assessment of transporter-mediated DDIs. Key technological advances in transporter research include the determination of the cryogenic electron microscopy structures of SLC and ABC transporters, and modulation of transporter function via genome editing. This presentation will provide an overview of the current status of transporters in drug development with a focus on recent advances. In addition, regulatory science considerations will be discussed. Unanswered questions and future directions in the application of transporter research to achieve the promise of precision medicine will be highlighted, as detailed in the ITC's 2024 Nature Review Drug Discovery publication.

S4.2 - DISCOVERY, VALIDATION, AND APPLICATION OF TRANSPORTER ENDOGENOUS BIOMARKERS: A WORK OF TRANSLATION

Hong Shen

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The ability to predict pharmacokinetic changes due to drug-drug interactions (DDIs) is essential to managing the associated adverse drug reactions in drug development. With the rise of pharmacogenomics and metabolomics, and other advances in bioanalytical sciences, transporter endogenous biomarker studies have emerged in a whole new era and holds promise in monitoring transporter activities *in vivo* and supporting transporter-mediated DDIs in early drug development. ICH M12 globally harmonized regulatory guidance provides recommendations on when and how to evaluate DDI potential using transporter endogenous biomarkers. The last decade has witnessed considerable efforts towards the identification, characterization, and validation of endogenous biomarkers of hepatic and renal uptake drug transporters (i.e., OATP1B1/1B3, OAT1/3, and OCT2). On the other hand, biomarkers of intestinal efflux transporters BCRP and P-gp are not available. This presentation will focus on discussion of 1) the current status, challenges, and knowledge gaps of transporter biomarker investigation, 2) application of coproporphyrin I as a biomarker to evaluate OATP1B-mediated DDIs in real-life examples at BMS, and 3) discovery and validation of riboflavin as a surrogate marker of BCRP.

S4.3 - FOOD-DERIVED NEUROTROPHIC AMINO ACID ERGOTHIONEINE AND ITS TRANSPORTER SLC22A4: FROM BENCH TO HEALTHY FOOD

Yukio Kato

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Solute carrier SLC22A4 was originally identified as a transporter involved in the uptake of various organic cations and thus named carnitine/organic cation transporter 1 (OCTN1). We have constructed octn1 gene knockout (KO) mice and performed metabolome analysis using the mice to clarify its endogenous substrates, revealing that ergothioneine (ERGO) is present in blood and all organs of wild-type mice at μM to mM levels whereas octn1 KO mice do not have it in the body. ERGO is synthesized in fungi and mycobacteria, but not in mammals which ingest ERGO from dietary food. Thus, ERGO has its specific uptake transporter OCTN1 in the body, implying the important roles of this combination of transporter and substrate. So far, octn1 KO mice have been demonstrated to exhibit more severe phenotypes compared with wild-type mice in disease models including liver fibrosis, chronic kidney disease, and intestinal inflammation, and expression of OCTN1 protein is up-regulated in those diseased organs, suggesting that this transporter and possibly its substrate may

play protective roles in organ disease conditions. We also noticed that this highly water-soluble and hydrophilic amino acid ERGO permeates the blood-brain barrier. In addition, ERGO is taken up by neurons and neural stem cells (NSCs) by OCTN1 in the brain. In particular, gene expression of OCTN1 in NSCs is much higher than that of other organic cation transporters. NSCs can be proliferated and differentiated into neurons and glial cells in the brain. An addition of ERGO into primary cultured NSCs promotes their differentiation into neurons, but not glial cells, implying that ERGO and OCTN1 may be involved in neurogenesis including differentiation and/or maturation of neurons. In NSCs ERGO induces expression of brain-derived neurotrophic factor and neurotrophin 5 through amino acid signaling, promoting their receptor TrkB phosphorylation. These findings may indicate that ERGO may be a food-derived neurotrophic factor, promoting further *in vivo* studies to examine its effect on brain functions. Oral administration of ERGO for 2 weeks improves learning and memory ability in mice. Notably, this effect was observed at systemic ERGO concentration relevant to that in humans, promoting the clinical application of this compound. So far, at least two human studies have been performed, showing that ingestion of food-derived and pure ERGO improves verbal memory and sleep difficulties in healthy volunteers. ERGO-containing food components are currently used as healthy food. Interestingly, the stimulation of TrkB phosphorylation is also observed in extracellular vesicles in humans ingested with ERGO-containing tablets, implying that neurogenesis may be promoted in the brain. In addition, oral administration of ERGO also prevented cognitive decline at least partially via the promotion of neurogenesis in Alzheimer's disease model mice. Thus, ERGO may not only be applicable to healthy foods but also to the potential prevention of neurodegenerative disease.

S4.4 - ROLE OF INTRACELLULAR SLC46A3 IN THE CYTOSOLIC EXPOSURE AND PHARMACOLOGICAL EFFECT OF THE PAYLOAD OF TRASTUZUMAB EMTANSINE (ADC)

Katsuhisa Inoue

Tokyo University of Pharmacy and Life Sciences, Hachioji, Japan

The translocation of bioactive compounds across biological membranes is critical to the efficacy of any intracellularly targeted drugs including antibody-drug conjugates (ADCs). Since ADC payloads such as tubulin inhibitors and DNA-modifying agents are released in the lysosomes via endocytosis, drug permeation process through the lysosomal membrane may be important for ADC therapy. Trastuzumab emtansine (T-DM1) is an ADC used to treat HER2-positive metastatic breast cancer, which consists of an antibody against HER2 (trastuzumab) and the microtubule polymerization inhibitor maytansine derivative (DM1) with a non-cleavable linker (SMCC), and thus converted into a cytotoxic catabolite (Lys-SMCC-DM1) in the lysosomes when taken up by endocytosis in HER2-positive cancer cells. Hamblett KJ et al. have identified SLC46A3 as a gene involved in the efficacy of ADC with DM1 attached to a non-cleavable linker (Cancer Res, 75, 5329-40, 2015). Recently, we explored the function of SLC46A3 mutants lacking the C-terminus sequence with lysosomal targeting motif (SLC46A3 dC) and demonstrated the capability of SLC46A3 to transport Lys-SMCC-DM1 in a proton-dependent manner. Our functional transport study showed that SLC46A3 is a lysosomal proton-dependent organic anion transporter, which can mediate the transports of steroid conjugates and bile acids as endogenous substrates. T-DM1 cytotoxicity in HER2-positive breast cancer cells were significantly reduced in the presence of lysosomotropic SLC46A3 inhibitors such as macrolide and ketolide antibiotics, implying a potential drug-ADC interaction. To assess SLC46A3-mediated DDI risk *in vitro*, we established the fluorescence-based assay system for evaluating SLC46A3 function using 5-carboxyfluorescein, which is known as a fluorescent probe for organic anion transporters. This assay system allows to screen promising SLC46A3 substrates, as well as potential SLC46A3 inhibitors. This presentation will highlight the characterization of SLC46A3 inhibitors. To gain the information on SLC46A3-mediated transport and inhibition may be useful to develop effective ADCs and other pharmaceuticals targeting SLC46A3. We will discuss strategies for these purposes.

K2 - IN VITRO - IN VIVO EXTRAPOLATION APPROACHES FOR GLUCURONIDATED DRUGS: PROMISES AND PITFALLS

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After cytochrome P450 mediated drug biotransformation, glucuronidation is the most important metabolic pathway for small molecule drugs. Indeed, glucuronidation contributes to the elimination of drugs from almost all therapeutic classes. The glucuronidation of drugs and other compounds (non-drug xenobiotics and endogenous chemicals) is facilitated by enzymes of the UDP-glucuronosyltransferase (UGT) superfamily, which catalyse the transfer of glucuronic acid from the cofactor UDP-glucuronic acid to a suitable acceptor functional group on the substrate (or aglycone). Available evidence indicates that only enzymes of the UGT1A and 2B subfamilies (viz., 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B10, 2B15 and 2B17) significantly contribute to drug glucuronidation in humans. Of these, UGT1A1, UGT1A9 and UGT2B7 appear to be of most importance in terms of the number of drugs metabolised. The individual UGT enzymes exhibit distinct, but sometimes overlapping substrate and inhibitor selectivities. In the latter respect, drug-drug interactions (DDIs) arising from modulation of UGT enzyme activities are relatively common, although their clinical relevance is often overlooked [1]. The increased recognition of the importance of glucuronidation as a drug elimination mechanism that has occurred in

recent years has stimulated the development of *in vitro* approaches for the characterisation of human drug glucuronidation, including measurement of: metabolic stability (assessed as intrinsic clearance); reaction phenotyping (the fractional contribution of individual UGT enzymes that contribute to the glucuronidation of any given drug); and the selectivity and extent of UGT enzyme inhibition and induction by drugs. These data provide the foundation for quantitative *in vitro* – *in vivo* extrapolation (IV-IVE) strategies that predict metabolic clearance via glucuronidation and DDI liability, which are particularly relevant to the pre-clinical development of new chemical entities. Notwithstanding acceptance of the value of *in vitro* approaches for the measurement of UGT enzyme activities and kinetic parameters (K_m , V_{max} , CL_{int}), experimental conditions often vary between laboratories and consequently the interpretation of results can be problematic. However, evidence-based strategies have been published recently [2], and it is anticipated that these will inform practice and guide ongoing research. Advances in the experimental approaches adopted for UGT reaction phenotyping and the measurement of the inhibitor constant (K_i) determined from *in vitro* inhibition kinetic experiments are similarly proving valuable for predicting the likelihood of a DDI arising from UGT enzyme inhibition, although data may again be confounded by the use of non-optimised experimental conditions.

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S5.1 - REGULATORY ACCEPTANCE OF MPS - CONSIDERATION FROM CONTEXT OF USE

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The U.S. Food and Drug Administration (FDA) Modernization Act 2.0, approved in 2022, now allows the use of cell-based assays, and will accelerate the United States' use of alternatives to animal models. One of such models is microphysiological systems (MPS). According to FDA, MPS model "functional features of a specific tissue or organ of human or animal origin by exposing cells to a microenvironment that mimics the physiological aspects important for their function or pathophysiological condition." In recent years, examples of their applications in Investigational New Drug Applications (IND applications) have been reported. In accepting MPS data into the regulatory situations, it is important to establish the Context of Use (CoU) of the test method using MPS. "Context" of Use does not merely mean the intended use but "the situation, events, or information that are related to something and that help you to understand it," according to the Longman Dictionary. To the better understanding of CoU of MPS, it has become clear that there are "points to consider" that differ from conventional culture methods due to the unique nature of the culture environment. In the MPS-RS project being conducted by the AMED program: Research on Regulatory Science of Pharmaceuticals and Medical Devices (AMED-MPS2 project), the "first-pass effect" in the small intestine and liver is one example of such CoUs. AMED-MPS2 project is developing a first-pass model utilizing gut-liver co-culture MPS developed by Japanese research laboratories. The "points to consider" for the implementation of this model to industry and regulation sections, which are discussing in another AMED project: MPS-RS (regulatory science) project, will be presented. MPS-RS project is preparing to propose a test guideline to the OECD. I will introduce the road-map to the OECD test guideline proposal. I would appreciate your interest in the development of this test guideline.

S5.2 - PREDICTION OF DRUG INTESTINAL ABSORPTION AND TOXICITY WITH THE USE OF HUMAN/ANIMAL CRYPT-DERIVED CULTURED INTESTINAL CELLS

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The quantitative prediction of intestinal absorption of drugs is one of the critical components in the process of the development of orally-administered drugs. However, at the moment, no *in vitro* experimental systems suitable for the characterization of intestinal absorption and drug-induced intestinal toxicity in humans have been established because of the large species difference and the discrepancy of the expression/function of a set of enzymes/transporters between intact intestinal tissue and various model cells such as Caco-2 cells. Thanks to the rapid advance of regenerative medicine, we could establish the 3D spheroid culture of animal/human crypt-derived intestinal stem cells with the use of L-WRN cell conditioned media, which contain Wnt3a, R-spondin 3 and Noggin (WRN). Then, after single cell isolation and seeding cells onto normal dish or culture insert, only by replacing with media without WRN, differentiated intestinal epithelial cells can be efficiently obtained in a week (Michiba et al., DMD, 50, 204-213 (2022)). We confirmed the expression/function of various metabolizing enzymes and transporters in our cells and observed PXR-mediated induction of enzymes/transporters with rifampicin. We also showed that the fraction of drugs escaped from intestinal metabolism (F_g) of CYP3A substrate drugs in humans could be well predicted from their apparent permeability across differentiated cell monolayer in the presence or absence of CYP3A inhibitor with a simple mathematical model. PCFT and ASBT are exclusively expressed in the jejunum- and ileum-derived differentiated cells, respectively, suggesting that their gene expression profile at the origin of crypts is maintained as reported previously. Regarding the screening of drug-induced

intestinal toxicity, we first evaluated the intracellular ATP level and LDH leakage after incubation of a series of EGFR-tyrosine kinase inhibitors (EGFR-TKIs) with spheroids of intestinal stem cells because frequency of severe diarrhea induced by EGFR-TKIs in the clinical trials varies largely (Hashimoto et al., *Toxicol In Vitro*, 93, 105691 (2023)). As a result, the potency of ATP decrease for each drug is related to its relative risk of diarrhea in the clinical settings and the reversibility of ATP level after removing drugs from the media matched the binding mode of drug to EGFR-TK. On the contrary, ATP decrease by incubation with EGFR-TKIs could not be clearly observed in Caco-2 cells. One of the triggers of drug-induced emesis is thought to be the release of serotonin (5-HT) from enterochromaffin (EC) cells. Since the fraction of EC cells in the whole intestinal cells is very small (~1%), we established EC cell-rich spheroids after incubation with DAPT and measured 5-HT level in the media with LC-MS/MS. The risk of emesis of ALK/ROS1 inhibitors could be roughly classified into high-risk and low-risk groups. As a result, whereas 5-HT release was clearly observed at lower concentration of high-risk ALK/ROS1 inhibitors, it was not observed or observed only at the highest concentration of low-risk drugs in our assay. Thus, we consider that human/animal crypt-derived cells can be useful to evaluate the characteristics of the intestinal absorption and drug-induced intestinal toxicity of new chemical entities.

S5.3 - RAT AND DOG QUAD-CULTURE LIVER CHIP MODELS: CHARACTERIZATION AND USE TO INTERROGATE A POTENTIAL FLAVIN-CONTAINING MONOOXYGENASE-MEDIATED, SPECIES-SPECIFIC TOXICITY OF A HISTAMINE RECEPTOR ANTAGONIST

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Microphysiological systems (MPS) are *in vitro* experimental models typically comprised of multiple cell types in three-dimensional architecture often incorporating simulated blood flow. There is growing interest in MPS for use in disease modeling, safety and disposition studies. Animal cell-based MPS are scarce yet needed to bridge *in vivo* findings thereby providing confidence in the translation of findings in human cell-based MPS. We developed rat and dog quad-culture liver MPS incorporating primary hepatocytes, sinusoidal endothelial cells, Kupffer cells and stellate cells in a polydimethylsiloxane chip-based system. After identifying reliable sources of cryopreserved cells, we established a stepwise protocol for co-culturing cells under physiologically relevant flow conditions. Cells in the model were evaluated for viability, morphology and function using microscopy (bright-field and fluorescence) and biochemical endpoints (e.g., albumin production, cytochrome P450 and flavin-containing mono-oxygenase activity). Results demonstrated that the protocol enabled construction and maintenance of high-quality rat and dog chips for up to 7 days. We then evaluated model performance with ABT-288, a histamine-3 receptor antagonist that caused elevated levels of serum transaminases in dogs but not rats. This finding was at least partially attributed to high levels of N-oxide metabolite in the dog. Key findings in our study were 1) dog chips exhibited modestly higher sensitivity to ABT-288 toxicity endpoints (albumin, aspartate transaminase, alanine transaminase and lactate dehydrogenase) compared to rat, and 2) dog chips showed much higher FMO activity compared to rat. The latter was not reproduced in rat and dog hepatocytes cultured in suspension, suggesting that properties of the quad culture MPS were necessary to model higher FMO activity observed in dogs *in vivo*. The data suggest that this preclinical species liver chip model can provide novel understanding of *vitro/in vivo* translation of ABT-288 dog liver toxicity.

S5.4 - IN VITRO EVALUATION METHODS FOR HEPATIC AND RENAL DRUG DISPOSITION AND TOXICITY

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Hepatic and renal disposition affect the systemic exposure of drugs and metabolites as well as their respective tissue concentrations. Since transporters play critical roles in regulating drug disposition in these tissues, it is essential to evaluate tissue concentrations and efficacy/toxicity of drugs in the experimental system with maintained transporter activities. In this presentation, new experimental methods for hepatic and renal drug disposition which we are now developing are presented. For an *in vitro* evaluation of drug biliary disposition, sandwich-cultured hepatocyte (SCH) is widely used. However, SCH does not allow time-dependent and quantitative evaluation because drugs accumulate in the closed bile canaliculi lumen formed between hepatocytes and their amounts cannot be measured directly. This structural problem of SCH can be overcome by inducing a formation of an open canaliculus lumen face to a culture support such as a Transwell insert, which enables a permeation assay for quantitative evaluation of drug biliary excretion. When primary human hepatocytes and PXB-cells, derived from liver-humanized mice, were cultured on a permeable support coated with claudins, a family of tight junction proteins, opened hemi-luminal morphology as indicated by the canalicular membrane marker MRP2 was newly formed on the surface of the permeable support. Therefore, it is considered that the surface of support coated with claudin proteins functioned as a pseudo-hepatocyte membrane for bile canaliculus formation and this culture system is termed as an induced open-form bile canaliculus hepatocyte (icHep (1)). A continuous monolayer of hepatocytes with tight cell-to-cell contact is formed using PXB-cells. In addition, the cells maintained the functions of a wide range of liver-specific drug transporters and metabolizing enzymes. The permeation assay in icHep showed unidirectional transport of taurocholate (BSEP), E217 β G (MRP2), and digoxin (P-gp) from blood to bile side. Each

transport was suppressed upon treatment with the respective inhibitor. Furthermore, the estimated biliary drug excretion from the permeability obtained in icHep was well-correlated with the reported human *in vivo* values. Accordingly, icHep permeation assay system overcomes the problems of the conventional SCH method and enables easy and rapid collection of biliary excreted drugs and their metabolites to estimate their biliary excretion clearance. This approach is useful for assessing hepatic drug disposition as well as drug-induced liver toxicity during the preclinical stage of drug discovery and development. We are also developing an *in vitro* evaluation method for renal drug disposition and drug-induced kidney injury. Primary cultured renal proximal tubular epithelial cells (2D-RPTEC) showed lowered or negligible expression of transporters such as organic anion transporter OAT1. In this study, we propose a simple and reproducible 3-dimensional cultured RPTEC spheroids (3D-RPTEC, (2)) with maintained expression of major drug transporters including OAT1 and OCT2 protein at physiological levels. Cisplatin and adefovir exhibited transporter-dependent ATP decreases in 3D RPTEC spheroids, demonstrating a usefulness of 3D-RPTEC in evaluating human renal proximal tubular toxicity and drug disposition. Furthermore, we evaluated kidney injuries induced by 32 nephrotoxic drugs for up to 28 days using 3D-RPTECs (3). The predictive performance of 3D-RPTECs for drug-induced kidney injury was better than 2D-RPTECs and HK-2 cells. Our results suggested that 3D-RPTECs are useful for *in vitro* evaluation of RPTEC injury.

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S6.1 - UPDATE FROM THE IQ TALG QUANTITATIVE FRACTION METABOLIZED WORKING GROUP

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The proportion of a drug's metabolism mediated by particular enzyme or metabolic pathway is a key determinant of pharmacokinetic variability and susceptibility to victim DDI. Early in the drug development process, a quantitative prediction of fraction metabolized is conducted using hepatic cell-based assays, subcellular fractions, recombinant expression systems, and enzyme selective inhibitors. Although assays for selective inhibition of CYP enzymes, the primary drug metabolizing enzymes of human liver, have been established for several decades, experimental challenges persist, and practices vary widely across the industry. Of particular interest is the universal challenge of characterizing drugs with low metabolic turnover with the current tools. The objective of the Quantitative Fraction Metabolized IQ Working Group is to define an industry baseline for quantitative *in vitro* assessment of fraction cleared by metabolic pathways using current and novel tools and practices. First, irreversible enzyme inhibitors were chosen based on selectivity for individual CYPs and to reduce the effective dose to limit the risk of PXR activation/CYP induction during long term incubation. Next, inhibition studies were conducted in three model assays to assess the utility for conducting reaction phenotyping with low turnover compounds: Hurel, Hepatopac and Hepatocyte Suspension Relay. The results of these efforts and plans for further expansion will be discussed.

S6.2 - PHENOTYPING ASSAY USING GENETICALLY MANIPULATED HUMAN IPS CELL-DERIVED HEPATOCYTE-LIKE CELLS

Hiroyuki Mizuguchi

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The liver plays an essential role in drug metabolism and detoxification. Drugs are metabolized in the liver, and transformed into active (or inactive) metabolites. Some drugs and their metabolites can cause hepatocellular toxicity, compelling pharmaceutical companies to discontinue development of these drugs or withdraw them from the market. Therefore, it is essential for pharmaceutical research to accurately evaluate the kinetic characteristics and toxicity of drugs in the liver. Primary (cryopreserved) human hepatocytes (PHHs) are the main cell source used for preclinical *in vitro* studies of drug metabolism and disposition. However, PHHs have some issues, such as lot-to-lot variability, limited supply, and loss of hepatic functions in culture. Human induced pluripotent stem (iPS) cell-derived hepatocyte-like cells (HLCs) are expected to replace PHHs as a new stable source of functional hepatocytes. One of the advantage of the use of HLC is that HLCs differentiated from each individual-derived cell can be prepared. Human iPS cells derived from individuals with single nucleotide polymorphisms (SNPs) in cytochrome P450 (CYP) 2D6 recapitulated the poor metabolizer phenotype by differentiation into HLCs [1]. In addition, genome editing technology successfully recapitulated the phenotypes of poor metabolizers of CYP2C19 and CYP3A4 in HLCs and revealed the contribution of specific CYP enzymes in pharmacokinetics [2,3]. Furthermore, UGT1A1-KO model enabled evaluation of UGT1A1-specific kinetics and toxicity [4]. Although HLCs are expected to be utilized for pharmaceutical applications, several issues remain; HLCs have lower hepatic functions than PHHs, require a long time for differentiation and cannot be prepared in large quantities because they do not proliferate after their terminal differentiation. To overcome these problems, we established hepatic organoids (iHOs) from HLCs. The iHOs could proliferate approximately 1020-fold by more than 10 passages and

expressed most hepatic genes more highly than HLCs. In addition, to enable their widespread use for *in vitro* drug discovery research, we developed a two-dimensional culture protocol for iHOs. Two-dimensionally cultured iHOs expressed most of the major hepatocyte marker genes and proteins at much higher levels than HLCs, iHOs, and even PHHs [5]. The combination of human iPS cell-derived hepatocyte models, through organoid culture, and genome editing technology, by which drug-metabolizing enzymes' genes are modified, would be a powerful tool to evaluate the contributions of specific drug-metabolizing enzymes to ADMETox properties of the drug candidates in the early phase of pharmaceutical development. I will introduce our study to achieve this goal.

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S6.3 - ADVANCES IN ENABLING IVIVE AND ENZYME PHENOTYPING OF LOW CLEARANCE COMPOUNDS

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The Extended Clearance Concept Classification System (EC3S) was established as a discovery-stage tool to enable compound classification based on cutoff values for MDCK permeability (5×10^{-6} cm/s) and unbound HLM intrinsic clearance (25 μ L/min/mg). IVIVE analysis shows that for compounds with unbound intrinsic clearance values above this cutoff, systemic clearance is generally predicted within three-fold, whereas for those below this value, human clearance cannot be predicted. This observation motivated the search for alternate in-vitro models to enable IVIVE for compounds with low metabolic turnover. As alternatives, hepatic models including spheroids, Hurel® and hepatocyte/fibroblast co-cultures were tested, since low turnover compounds can be incubated for days to weeks. Our data demonstrate that, of the systems tested, hepatocyte spheroids predicted within 3-fold (>70% of compounds) the systemic clearance observed clinically. A further analysis of the major metabolic pathway(s) revealed that most of these compounds were predominately glucuronidated in humans. Current *in vitro* approaches have limited ability to predict tissue specific fraction of glucuronidation (f_{gluc}) and IVIVE clearance of UGT substrates. To address this, recombinant systems expressing clinically relevant UGTs were employed to measure glucuronide formation of substrates (marketed and internal), and activity scaled using either hepatic UGT relative activity or expression to predict clearance and f_{gluc}. Our findings suggest that scaling of UGT glucuronidation in recombinant systems with the relative hepatic UGT activity scalar approach has potential to better predict human clearance and f_{gluc}.

S6.4 - EVALUATION OF LOW CL COMPOUNDS USING CHIMERIC MICE WITH HUMANIZED LIVER

Hiroshi Suemizu

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Information on drug metabolites is essential for the development of safe and effective drugs, but it is difficult to predict drug metabolites produced in humans at the nonclinical stage due to species differences in drug metabolism and the limitations of *in vitro* assays using hepatocytes and liver microsomes. In this study, we would like to present the usefulness of humanized-liver chimeric mice for predicting major or unique metabolites in humans.

PL2 - SPECIES SPECIFICITY AND SELECTION OF ANIMAL MODELS FOR DRUG OXYGENATIONS DIFFERENTLY CONTRIBUTED BY POLYMORPHIC HUMAN ENZYMES

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Drug oxygenation is primarily mediated by polymorphic cytochromes P450 (P450) and flavin-containing monooxygenases (FMO). Species differences exist in terms of drug metabolism in rodents and humans, and such differences can be important in determining the contributions of individual enzymes. Thalidomide is teratogenic in non-human primates and rabbits, but not in rodents. Thalidomide causes human P450 3A enzyme induction in the placenta and liver, and is also

activated by P450 3A through the primary human metabolite 5-hydroxythalidomide (leading to conjugation with glutathione/nonspecific proteins). Differences in species susceptibility to thalidomide teratogenicity may result from differences in the primary biotransformation of compounds by drug-metabolizing enzymes. Approaches used to investigate the roles of human P450/FMO enzymes in drug oxidation and clinical therapy have not yet reached maturity and require further development. Evaluation of hepatic P450-mediated fractions metabolized of drugs is important for evaluating drug interactions and effect modifiers in pharmacogenetics. In an *in vitro* system, ~80% inhibition of single-on-target P450 activity in human liver microsomes pretreated with time-dependent inhibitors was observed under limited off-target effects. Humanized-liver mouse systems pretreated with furafylline, tienilic acid, paroxetine, and azamulin would be useful for creating a standard *in vivo* animal model to investigate the contributions of specific P450s. The *in vivo* fractions of the victim drugs metabolized by a polymorphic human P450 isoform need to be investigated extensively for pharmacology and toxicology. Unexpectedly, high hepatic exposure modeled possibly in poor and intermediate metabolizer patients harboring P450 2C19*2 or *3 and 2D6*10 might contribute to the adverse effects recorded for medicines prescribed alone. The findings summarized here provide a foundation for understanding individual pharmacokinetic and toxicological results in preclinical models and will help further support the understanding of the molecular mechanisms of human P450/FMO functionality.

S7.1 - DISCOVERY OF PF-07817883, A SECOND-GENERATION ORAL SARS-COV2 MAIN PROTEASE INHIBITOR

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Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has become firmly established as a global pandemic. Despite the record-breaking discovery and development of vaccines and antiviral therapeutics, COVID-19 remained the fourth leading cause of death in the world and third highest in the United States in 2022. The lecture will feature SAR highlights leading to the discovery of PF-07817883, a second generation, orally bioavailable, SARS-CoV-2 main protease inhibitor with improved metabolic stability versus nirmatrelvir, the antiviral component of PAXLOVIDTM. PF-07817883 has attractive *in vitro* panhuman coronavirus antiviral activity and off target selectivity profile and has demonstrated oral efficacy in a mouse-adapted SARS-CoV-2 model. Relative to nirmatrelvir, the preclinical disposition studies in animals and human reagents suggest improved oral pharmacokinetics for PF-07817883 in humans, and *in vitro* inhibition/induction studies against major human drug metabolizing enzymes/transporters suggest a low potential for perpetrator drug-drug interactions upon use of PF-07817883 as a single agent. Both of these preclinical hypotheses have been confirmed in first-in-human pharmacokinetics and drug-drug interaction studies.

S7.2 - UNCOVERING TARGET-MEDIATED DRUG DISPOSITION (TMDD) OF SMALL MOLECULE BRAIN PENETRANT EBP INHIBITORS

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Target-mediated drug disposition (TMDD), which leads to nonlinear pharmacokinetics, has been well-characterized and is prevalent for protein drugs. However, while it was initially proposed with small molecules(1), TMDD is not as commonly observed and reported for such drugs, most likely because *in vivo* studies conducted in preclinical development are not designed to detect or investigate this phenomenon.

The pharmacokinetic features of TMDD were observed with brain penetrant EBP (Emopamil Binding Protein) inhibitors in rats and mice. The compounds tested showed time- and dose-dependent nonlinear pharmacokinetics, with greater than dose-proportional increase in exposure at low doses, following single oral administration, which was attenuated after repeated dosing.

In tissues (e.g., brain), administration of varying dose levels resulted in profiles converging towards the same concentration, and displacement studies confirmed the impact of target binding on the pharmacokinetic profiles observed. This presentation will describe various degrees of TMDD observed with EBP inhibitors, the potential impacts on further drug development and approaches to mitigate them.

Reference:

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S7.3 - A TALE OF PRECLINICAL INSIGHTS INTO BILE SALT HOMEOSTASIS DISRUPTION IN DILI

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Drug-induced liver injury (DILI) is a common and serious side effect that can lead to regulatory action or halt drug development. Notable examples include the withdrawal of troglitazone and benoxaprofen from the market due to DILI.

The mechanisms behind DILI are complex, involving factors like drug properties, oxidative stress, mitochondrial susceptibility, immune responses, and bile salt (BA) homeostasis disruption. BAs are produced in the liver and transported into bile by various transporters, such as BSEP, which is essential for BA homeostasis and normal liver function. Thus, inhibiting BSEP can lead to hepatocyte damage due to intracellular BA accumulation. While *in vitro* assays can flag the risk of BSEP inhibition, BA homeostasis involves a complex network of synthesis, metabolism, and transport processes regulated by numerous enzymes and transporters. The intricate balance of these mechanisms is not fully understood, complicating the prediction and prevention of DILI. The presentation will include case examples and discuss the mechanisms associated with DILI, as well as de-risk strategies in drug discovery.

S7.4 - BEST PRACTICE OF MITIGATING P-GLYCOPROTEIN LIABILITIES IN DRUG DISCOVERY - ASSESSMENT AND PREDICTION OF P-GP MEDIATED NONLINEAR PK, BRAIN PENETRATION, AND INTERACTIONS

Hideki Hirabayashi

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Since P-glycoprotein [(P-gp)/multidrug resistance protein 1 (MDR1)] is well known to be involved in various pharmacokinetic processes of administered drugs, it is important to design drug molecules that are free from the significant liabilities associated with P-gp. Although P-gp has been extensively studied for many years, the development of strategies to reduce P-gp liabilities remains a major challenge, especially in drug discovery for drugs acting on the central nervous system (CNS). The key to success lies in how to utilize and combine various *in vitro*, *in vivo*, and *in silico* tools to establish an optimal P-gp evaluation platform. In this talk, the speaker will first review the past 15 years of P-gp research at Takeda and propose best practices for mitigation strategies against P-gp liabilities by presenting internal case studies. Advances in digital technology over the past decade have brought a new dimension to P-gp research. The next generation of P-gp research using cutting-edge technologies such as AI/machine learning and CryoEM will also be touched upon.

S8.1 - UNRAVELING CANNABINOIDS-DRUG INTERACTIONS IN AGING: INSIGHTS FROM PBPK MODELING

Zhu Zhou

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Cannabis has received interest from older adults suffering from chronic pain, cancer-related symptoms, mood disorders, and sleep disturbances that are poorly managed with current treatments. As cannabis use for myriad conditions continues to grow, understanding potential pharmacokinetic interactions between cannabinoids and other drugs remains an urgent public health need. In this session, Dr. Zhou will share recent findings from her lab's work on this topic based on modeling outcomes.

S8.2 - MODEL-BASED META-ANALYSIS OF ETHNIC DIFFERENCES - GENERAL CHANGES IN CLEARANCE BY PATHWAYS AND AGING

Akihiro Hisaka

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Ethnic differences in the oral clearance (CL_{oral}) of drugs are very important for pharmaceutical industries because they may require dose adjustment for each country. To confirm this potential risk, the Japanese regulatory authority has been requiring phase I clinical studies to be conducted in Japanese ethnicity for decades; thus, many comparative pharmacokinetic data of Japanese and Western ethnicities are now accumulated to study the ethnic differences in detail. In fact, the ethnic differences have arisen not only in Japan but also in other non-Western countries. Furthermore, there are cases where the Western regulatory authority required clinical trials conducted in Asia to be validated in the West. We evaluated the ethnic ratios (ERs) of CL_{oral} between Japanese and Western populations by model-based meta-analysis (MBMA) for 81 drugs evaluated in 673 clinical studies, enrolling 16,996 subjects. The drugs were classified into eight groups of clearance mechanisms such as substrates of major CYPs or transporters. The ER for each group was inferred by the Markov chain Monte Carlo method which discriminates inter-individual and inter-study variability from ethnic variability. The goal is not to precisely identify the ER for each drug, but rather to propose a rational and practical strategy to evaluate the ethnic differences for each country with limited resources.

The most evident finding was that ethnic differences in CL_{oral} and the extent of their variability were strongly dependent on the clearance mechanisms. The analysis showed that drugs with a distinct clearance mechanism and little risk for genetic polymorphism had inherently small ethnic differences. Small ethnic differences were suggested also for drugs extensively excreted in urine as unchanged. Nevertheless, drugs with extremely low bioavailability were shown to be potentially at higher risk of ethnic differences. For all drug groups of clearance mechanism, inter-study differences of about half of the inter-individual differences were detected, and if the contribution of these inter-study differences was not taken into account when estimating ethnic differences from the results of different studies, it could lead to erroneous judgements.

To properly assess ethnic differences in CLoral without false positives, the mechanisms of CLoral should be adequately taken into account in the design of phase I studies. If the risk of ethnic differences is small based on the mechanism of CLoral, the risk of false positives of ethnic differences is high and an ethnicity-specific pharmacokinetic study might not be useful.

Changes in hepatic clearance in the elderly analyzed by MBMA based on reported population pharmacokinetic analyses of 18 drugs will also be presented. The analysis showed that hepatic clearance decreased at a rate of 0.8% per year after the age of 40 years, with no gender or ethnic differences in the extent of the change. This change was consistent with the change in liver weight. Compared with changes in renal clearance of 0.97% per year, those in hepatic clearance was somewhat smaller, but 24% for 30 years and 32% for 40 years, suggesting that a dose adjustment of approximately 30% should be considered even for hepatic cleared drugs in the elderly.

S8.3 - MECHANISMS UNDERLYING ALTERED DRUG CLEARANCE IN PREGNANCY

Muluneh Fashe

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Pregnancy can impact how drugs are absorbed, distributed, metabolized, and excreted (ADME) in the body leading to altered clearance and exposure in maternal circulation. As a result, the dose requirements, safety, and effectiveness of many drugs during pregnancy are uncertain. Studies suggest that the changes in maternal physiology and biochemistry during pregnancy can affect the function of organs and tissues involved in drug disposition. The liver, for instance, is a crucial organ that affects the disposition of several drugs, and during pregnancy, hepatic drug metabolism and clearance can be significantly altered. However, the mechanisms behind these changes are not fully understood and vary from drug to drug. These have been attributed, at least in part, to changes in the expression and function of drug-metabolizing enzymes (DMEs) and transport proteins. These changes have been linked to the increased secretion of pregnancy-related hormones (PRHs) and have attracted significant research interest. In *in vitro* models using qualified human hepatocytes, our laboratory, and other groups have demonstrated that the expression and function of certain DMEs and transporters change in response to the PRHs and correlate with observed and predicted changes in hepatic clearance and systemic exposure in pregnant people. Similarly, pregnancy-associated increases in glomerular filtration and expression and function of renal transporters appeared to be associated with altered pharmacokinetics of certain drugs and their metabolites primarily excreted via urine. We are utilizing various experimental methods such as voluntary pregnant and non-pregnant individuals, humanized animal models, and sandwich-cultured human hepatocytes to study the impact of pregnancy on the expression and function of key hepatic and renal drug-metabolizing enzymes and transporters.

S8.4 - ALTERATION OF BLOOD BRAIN-BARRIER PROTEOME IN VARIOUS CONDITIONS

Sumio Ohtsuki

Kumamoto University, Kumamoto, Japan

The blood-brain barrier (BBB) limits the brain distribution of drugs and also expresses various transporters to regulate drug permeability to the brain. The expressions of transporters at the BBB were variable depending on the conditions, and proteomic analysis is a useful methodology to understand the changes of the BBB function. We have developed a novel isolation method for brain microvessels and determined the BBB proteome profile in conditions such as diabetes and Alzheimer's. We also analyzed the differences of BBB proteome profile in neonatal mice from the adults. Brain microvascular endothelial cells (BMECs) also express various membrane receptors, such as insulin receptor (INSR) and transferrin receptor. Although many studies have focused on the receptor-mediated transcytosis ability to enable efficient drug delivery to the brain, those membrane receptors are expected to play important roles in the BBB regulations. Furthermore, dysregulation of membrane receptors can be considered to contribute to the changes of the BBB functions in disease conditions. We are interested in two membrane receptors, INSR and podocalyxin (PODXL) in relation to diabetes. We have analyzed the knockdown effects of those membrane receptors in immortalized human BMECs (hCMEC/D3 cells) by quantitative proteomics, and revealed that those membrane receptors regulate the expression and function of the BBB. I will introduce our recent progress in BBB proteomics in the blood-brain barrier to understand drug distribution to the brain.

PS1.1 - AUTHENTICITY AND ADULTERATION OF BOTANICAL NATURAL PRODUCTS: LESSONS FROM A DECADE OF SOURCING MATERIALS FOR SCIENTIFIC INVESTIGATION

Nadja Cech

University of North Carolina Greensboro, Greensboro, NC, United States

As part of the National Institutes of Health funded Natural Product Drug Interaction Center, our laboratory is responsible for sourcing commercial dietary supplement materials to be evaluated in *in vitro*, pre-clinical, and clinical studies. Using both untargeted mass spectrometry metabolomics and targeted quantitative methods, we have devoted a decade to

analyzing hundreds of commercial samples of plant-based dietary supplements, including preparations of green tea (*Camellia sinensis*), goldenseal (*Hydrastis canadensis*), cinnamon (*Cinnamomum verum*), oregano (*Origanum vulgare*), kratom (*Mitragyna speciosa*) and hemp (*Cannabis sativa*). These studies have provided us a unique insight into the chemical landscape of the botanical natural products being sold to the public in the United States. In some cases (i.e. green tea and hemp), the industry appears to be doing an excellent job of quality control. Other botanicals (i.e. goldenseal and cinnamon) are of variable quality, with some suppliers providing authentic materials and others selling adulterated or inauthentic products. We also identified at least one botanical (kratom), where more than one “chemotype” was present among commercially available materials. This presentation will review some of the pitfalls and challenges in sourcing botanical natural products and provide recommendations for how to rigorously select appropriate material for scientific investigations.

PS1.2 - SCHISANDRA SPHENANTHERA : ITS HEPATOPROTECTION AND BEYOND

Huichang Bi

Southern Medical University, Guangzhou, China

The use of botanical and other plant-based natural products continue to thrive worldwide in recent years. This presentation will discuss *Schisandra sphenanthera*, a famous herb historically listed among China's most important herbs to improve the function of the liver and indexed in the Pharmacopoeia of China. The hepatoprotective effects of *Schisandra sphenanthera* and its active ingredients against drug-induced liver injury such as cholestasis, its effect on liver regeneration and even depression, and the involved underlying mechanisms are summarized and discussed.

PS1.3 - BOTANICAL-DRUG INTERACTIONS: BLAZING THE TRAIL USING TRANSLATIONAL TOOLS

Mary Paine

College of Pharmacy & Pharmaceutical Sciences, Washington State University, Spokane, WA, United States

Consumers are turning increasingly to botanical and other natural products for myriad health and wellness benefits. Based on the common belief that ‘natural’ implies ‘safe’, patients often supplement their prescribed pharmacotherapeutic regimens with these ubiquitous, readily available, and widely marketed products. This practice raises concerns for unwanted natural product-drug interactions. Like drug-drug interactions, pharmacokinetic mechanisms underlying natural product-drug interactions include inhibition and induction of drug metabolizing enzymes and transporters by the natural product, producing alterations in systemic and tissue drug exposure and potentially, toxicity or reduced efficacy. Two textbook example precipitants include grapefruit juice and St. John's wort. Grapefruit juice inhibits cytochrome P450 (CYP) 3A4 and organic anion transporting polypeptides in the intestine, whereas St. John's wort induces CYP3A4 and P-glycoprotein in both the intestine and liver. Given the large repertoire of substrates for these proteins, numerous drug labels carry cautionary language about potential drug interactions with these natural products. Despite these well-characterized precipitants, unlike for drug-drug interactions, harmonized approaches about how best to study potential natural product-drug interactions remain lacking. Because natural products are inherently complex and variable chemical mixtures, a multidisciplinary approach is required to rigorously evaluate and disseminate the drug interaction risk of these products. A center composed of clinical pharmacologists, natural products chemists, bioinformaticists, and health communications experts was established by the United States National Institutes of Health to address these critical knowledge gaps. Results from a series of interaction projects involving methodically selected natural products (e.g., cannabis, cinnamon, goldenseal, and kratom) and translational research tools, including mechanistic *in vitro* studies, physiologically based pharmacokinetic modeling and simulation, and proof-of-concept clinical studies, led to a set of recommended approaches about how best to assess these complex interactions. This multi-disciplinary effort is expected to result in improved design of future studies and ultimately, evidenced-based information for health care providers, educators, and patients about the safe use of certain natural products when taken concomitantly with drugs.

PS1.4 - CHALLENGES AND BREAKTHROUGHS IN THE CONDUCT OF CLINICAL CANNABIS STUDIES

Ryan Vandrey

Johns Hopkins University, Baltimore, MD, United States

This talk will provide an overview of research methods utilized in the conduct of controlled human laboratory studies involving acute administration of cannabis/cannabinoids. Dr. Vandrey will review regulatory requirements, drug preparation and storage considerations, dosing methodology for smoked, vaporized, oral, and topical dosing. He will also provide a brief summary of key findings from recent studies conducted at the Johns Hopkins Cannabis Science Laboratory.

PS1.5 - DEVELOPING NATURAL PRODUCTS AS POTENTIAL THERAPEUTIC AGENTS**Amala Soumyanath***Oregon Health & Science University, Portland, OR, United States*

Natural products, principally herbal or botanical materials, have been used for centuries to preserve or improve health and treat disease. The knowledge held within traditional medicine practices represents a valuable source of potential new treatments for some of the world's most challenging diseases. Indeed, compounds occurring in nature are the source or template for an extensive range of single chemical drugs used in modern medicine. At the same time, complex extracts of natural products, containing multiple compounds, can be used to improve human health particularly where a multi-target approach may be beneficial. Many botanical powders and their complex extracts find use as popular health products available for self-selection from retail outlets. In the United States, these products are regulated by the Food and Drug Administration (FDA) as "dietary supplements" which are distinct from drugs and have associated restrictions on the claims that can be made for their health benefits. Concurrently, the US FDA allows complex, multi-component natural product extracts to be developed as "botanical drugs" which can be marketed for the treatment of disease. The development and registration of these botanical drugs follows the general sequence of preclinical and clinical research studies applied for the development of conventional drugs, yet the complex nature of natural products presents unique challenges for this process.

This presentation will describe our experience with botanical drug development of the herb *Centella asiatica* as a potential treatment for Alzheimer's disease, based on its traditional use to improve nerve function and memory. Studies using standard preclinical cell culture animal models have demonstrated that a water extract of the herb (CAW) has biological activities and mechanisms associated with improving neuronal health and cognitive function. We have also established a role for two groups of compounds, triterpenes and caffeoylquinic acids, in these biological effects supporting the use of a complex mixture for optimum bioactivity. In translational studies, we have examined the possible interaction of CAW with drug metabolizing enzymes, developed a CAW product for human consumption, and explored the plasma pharmacokinetics of triterpenes and caffeoylquinic acids in humans. Based on these studies, a clinical trial is currently underway examining safety and target engagement of CAW in humans with mild cognitive impairment or mild Alzheimer's Disease (NCT05591027).

PRE-DOCTORAL/GRADUATE POSTER FINALISTS (A1 – A6)**A1 - THE BLOOD BRAIN BARRIER DEMONSTRATES CAPABILITY TO INFLUENCE HETEROGENOUS TENOFOVIR AND EMTRICITABINE METABOLISM AND TRANSPORT IN THE BRAIN**

Hannah Wilkins, The Johns Hopkins University School of Medicine, Baltimore, MD, United States

A2 - PHARMACOKINETIC MODELING LEVERAGING *IN VITRO* TO *IN VIVO* EXTRAPOLATION OF THE GUT MICROBIOME'S ROLE IN SULFASALAZINE METABOLISM INTO SULFAPYRIDINE AND MESALAMINE

Lisa Cheng, The University of British Columbia, Vancouver, BC, Canada

A3 - DRUG-INDUCED GASTROINTESTINAL TOXICITY DUE TO DISRUPTION OF THE INTERINFLUENCE BETWEEN SEROTONIN AND GUT MICROBIOTA

Suguru Asaji, Kanazawa University, Ishikawa, Japan

A4 - IMPACT OF TYROSINE KINASES AND INHIBITORS ON SGLT1 ACTIVITY

Kshitee Sandeep Kharve, SUNY BUFFALO, Buffalo, NY, United States

A5 - DEVELOPMENT OF AN *IN VITRO* METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS (MASH) MODEL TO STUDY HEPATIC TRANSPORTERS

Sarina Kyburz, UNC Eshelman School of Pharmacy, Chapel Hill, NC, United States

A6 - CONSTRUCTION OF FUNCTIONAL EVALUATION SYSTEM FOR HUMAN BCRP-MEDIATED BILIARY EXCRETION USING ADENO-ASSOCIATED VIRUS

Shotaro Yoshino, Kanazawa University, Kanazawa City, Japan

POSTDOCTORAL POSTER FINALISTS (A7 – A12)**A7 - PEGYLATED TESTOSTERONE WITH ENHANCED PHARMACOKINETIC PROPERTIES IN MICE**

Chae Bin Lee, Johns Hopkins University, Baltimore, MD, United States

A8 - DOES CANNABIDIOL (CBD) OR DELTA-9-TETRAHYDROCANNABINOL (THC) INDUCE DRUG METABOLIZING ENZYMES IN HUMAN HEPATOCYTES?

Ankit Balhara, University of Washington, Seattle, WA, United States

A9 - THE ONTOGENY AND INTER-INDIVIDUAL VARIABILITY OF HYDROLYTIC DRUG-METABOLIZING ENZYMES IN HUMAN LIVER

Dilip Singh, Washington State University, Spokane, WA, United States

A10 - IDENTIFICATION OF PAZOPANIB AS AN OATP1B1 SUBSTRATE BY COMPETITIVE COUNTERFLOW SCREEN

Kevin Huang, The Ohio State University, Columbus, OH, United States

A11 - DEVELOPMENT OF A STRATEGY TO PREVENT CISPLATIN-INDUCED KIDNEY INJURY BY TARGETING MATE-TYPE TRANSPORTERS

Masaya Kanda, Department of Pharmacy, Tokushima University Hospital, Tokushima, Japan

A12 - INVESTIGATION OF THE MECHANISM OF METRONIDAZOLE-INDUCED ENCEPHALOPATHY FOCUSING ON THE POSSIBLE EFFECT ON THIAMINE TRANSPORT

Yoshiaki Yamagishi, Musashino university, Nishitokyo-shi, Tokyo, Japan

ADC (P1 – P2)**P1 - QUANTITATIVE ANALYSIS OF ANTIBODY CONJUGATE PAYLOAD BY LC MS/MS WITHOUT IMMUNOCAPTURE**

Koppara Samuel, Frontage Laboratory, Exton, PA, United States

P2 - *IN VITRO* STUDY OF PAYLOAD RELEASE OF AN ANTI-TNF GLUCOCORTICOID RECEPTOR MODULATOR IMMUNOLOGY ANTIBODY-DRUG CONJUGATE (ADC-T) AND PAYLOAD METABOLISM

Mingshe zhu, MassDefect Technologies, Princeton, NJ, United States

ANTIBODY (P3 – P4)**P3 - RAPID PRODUCTION OF RABBIT MONOCLONAL ANTI-IDIOTYPE ANTIBODY FOR ANTIBODY DRUGS BY ECOBODY TECHNOLOGY**

Shin Kawano, iBody Inc., Nagoya, Aichi, Japan

P4 - DEVELOPMENT AND QUALIFICATION OF AN *IN VITRO* ASSAY TO DETERMINE TARGET MEDIATED INTERNALIZATION OF TREM2 RECEPTOR SPECIFIC ANTIBODIES FOR TMDD ASSESSMENT

Thomas Kraft, Roche, Penzberg, Germany

BIOANALYSIS (P5 – P6)**P5 - EVALUATION FOR BIOTRANSFORMATION OF ANTIBODY-PEPTIDE CONJUGATES USING NATIVE MASS SPECTROMETRY**

Hiraku Hagita, Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan

P6 - DEVELOPMENT OF A PAC-LC-MS/MS METHOD FOR SIMULTANEOUS QUANTIFICATION OF MIPOMERSEN, ITS METABOLITES, AND ANALOGUES IN TISSUE SAMPLES

Yuchen Sun, National Institute of Health Sciences, Kawasaki, Kanagawa, Japan

BIOAVAILABILITY (P7 – P12)**P7 - ENHANCED EFFECT OF IONIC LIQUID ON INTESTINAL ABSORPTION OF MACROMOLECULES (< 10 KDA)**

Shoichiro Fukuda, Tokushima University, Tokushima, Tokushima, Japan

P8 - MUCOADHESIVE HEMI-POROUS JANUS MICROPARTICLES: POTENTIAL FORMULATION TO IMPROVE INTESTINAL ABSORPTION FOR BCS CLASS 3 DRUGS

Akihiro Matsumoto, Osaka Ohtani University,
Tondabayashi-shi, Osaka, Japan

P9 - A HYBRID TRIAL APPROACH BY COMBINING 13C8-LABELED AND RADIOLABELED BRILAROXAZINE TO ASSESS ABSOLUTE BIOAVAILABILITY AND HAME IN A SINGLE CLINICAL TRIAL

Li Shen, Frontage lab, Exton, Pennsylvania, United States

P10 - DEVELOPMENT OF DISSOLUTION EVALUATION SYSTEM FOR DRUG ABSORPTION (V): ADDITIONAL INTESTINAL ABSORPTION MODULE OF IN VITRO 3D GASTRIC EMPTYING MMODEL FOR ORALLY DISINTEGRATING TABLET

Kozo Tagawa, Doshisya Women's College of Liberal Arts,
Kyotanabe-shi, Kyoto, Japan

P11 - IDENTIFICATION OF PHYSIOLOGICAL FACTORS SUPPRESSING COLONIC DRUG ABSORPTION USING QUANTUM IMAGING TECHNOLOGY

Yugo Yasugi, Kanazawa University, Kanazawa, Ishikawa,
Japan

P12 - EXCEEDING 100% BIOAVAILABILITY IN DOG PK: IMPLICATION OF CYP2D15 POLYMORPHISM

Richard Zang, IDEAYA, South San Francisco, CA, United States

BIODISTRIBUTION (P13)**P13 - VALIDATION OF QPCR METHOD TO DETECT HUMAN CELLS IN MOUSE AND RAT TISSUES**

Kuniyoshi Abe, SHIN NIPPON BIOMEDICAL LABORATORIES, LTD. (SNBL), Kagoshima, Japan

BIOLOGICAL SAMPLE (P14)**P14 - EVALUATION OF DIFFERENT TISSUE PROCESSING METHODS IN BAMA PIG SKIN AS AN ANIMAL MODEL FOR TOPICAL DELIVERY SYSTEMS**

Lijuan Hou, WuXi AppTec, Suzhou, Jiangsu, China

BIOMARKERS (P15)**P15 - IMPLEMENTATION OF BLOOD COLLECTION USING THE TASSO MICROSAMPLING DEVICE TO MEASURE THYROID HORMONE BIOMARKERS**

Chad Christianson, Alturas Analytics, Moscow, ID, United States

CLEARANCE PREDICTION (P16 – P19)**P16 - ADDRESSING THE CHALLENGES OF DETERMINING FRACTION METABOLIZED FOR LOW TURNOVER DRUGS – AN UPDATE FROM THE QUANTITATIVE FRACTION METABOLIZED WORKING GROUP OF THE IQ CONSORTIUM**

Lawrence Cohen, Takeda Pharmaceuticals, Cambridge, MA, United States

P17 - INCORPORATION OF WELL-STIRRED, DISPERSION AND PARALLEL TUBE LIVER MODELS INTO SIMPLE AND PERMEABILITY LIMITED PBPK FRAMEWORKS FOR CLEARANCE PREDICTION

Swati Nagar, Temple University School of Pharmacy,
Philadelphia, PA, United States

P18 - SELECTION OF LIVER MODELS OF HEPATIC DRUG CLEARANCE: IMPROVEMENTS AND IMPLICATIONS FOR IVIVE AND PBPK MODELING

K. Sandy PANG, Leslie Dan Faculty of Pharmacy,
University of Toronto, Toronto, ON, Canada

P19 - INTESTINAL SECRETION IS POTENTIALLY AN IMPORTANT CLEARANCE MECHANISM FOR HIGHLY PLASMA-BOUND AND LOW METABOLIC CLEARANCE EFFLUX SUBSTRATES

Murali Subramanian, Gilead Sciences, Foster City, CA,
United States

CONJUGATION REACTIONS AND ENZYMES (P20 – P22)**P20 - CONFOUNDING EFFECTS OF B-GLUCURONIDASE ON UDP-GLUCURONOSYLTRANSFERASE STABILITY: DEVELOPMENT OF A MODIFIED METABOLIC STABILITY ASSAY TO ENABLE SAR FOR A DISCOVERY PROGRAM**

Sarah Glass, Johnson & Johnson, San Diego, CA, United States

P21 - EFFECTS OF MEFENAMIC ACID ON THE CONJUGATIVE METABOLISM OF INDOLE AND P-CRESOL IN RELATION TO CELLULAR NECROSIS IN A METABOLICALLY COMPETENT HUMAN HEPATOMA CELL LINE

Tony Kiang, Faculty of Pharmacy and Pharmaceutical Sciences / University of Alberta, Edmonton, AB, Canada

P22 - SDS-STABLE HOMO-OLIGOMERS OF UDP-GLUCURONOSYLTRANSFERASE 2B7: INVOLVEMENT OF DISULFIDE BOND FORMATION VIA A SPECIFIC CYSTEIN RESIDUE

Yuu Miyauchi, Sojo-University, Kumamoto, Japan

CYTOCHROME P450 (P23 – P39)**P23 - THE REGULATORY ROLES OF THE LNCRNA HNF1AOS1 IN HEPATIC DRUG METABOLISM IN MICE**

Beshoy Armanios, University of Connecticut, Storrs, CT, United States

P24 - A SEVEN CYTOCHROME P450 INDEX SUBSTRATE COCKTAIL FOR CLINICAL TRIALS – PROFILING THE EFFECTS OF CLOPIDOGREL AND GEMFIBROZIL ON CYP ENZYMES AND OATP1B1 BIOMARKERS

Janne Backman, University of Helsinki, Helsinki, Finland

P25 - SCREENING TOWARDS A CYP3A7-SPECIFIC MARKER REACTION AGAINST CYP3A4 AND CYP3A5

Elias Carvalho Padilha, NCATS, Rockville, MD, United States

P26 - PROTEIN EXPRESSION AS ENDPOINT FOR ASSESSMENT OF CYP2B6 INDUCTION: APPLICATION OF IMMUNO-AFFINITY CAPTURE METHOD REVEALS IMPROVED DYNAMIC RESPONSE RANGE

Julius Enoru, AbbVie, South San Francisco, CA, United States

P27 - A COMPREHENSIVE PLATFORM FOR EVALUATING THE *IN VITRO* CYP REACTION PHENOTYPING OF NEW MOLECULAR ENTITIES (NME)

Lifang Jiang, WuXi AppTec Lab Testing Division - Shanghai, Shanghai, Pudong, China

P28 - INFLUENCE OF THE GENETIC POLYMORPHISM OF CYP2D1 ON METABOLISM AND PHARMACOKINETICS OF E2027 IN RATS

Nana Kasamori, Eisai Co., Ltd., Tsukuba, Ibaraki, Japan

P29 - INTRA-PATIENT VARIATION IN CYP2D6 ACTIVITY OVER TIME ASSESSED USING THE DIETARY BIOMARKER SOLANIDINE IN BREAST CANCER PATIENTS ON TAMOXIFEN

Denise Keller, London Health Sciences Centre, London, ON, Canada

P30 - A RAPID LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS) METHOD FOR QUANTIFYING THE METABOLITES OF SEVEN MAJOR PROBE SUBSTRATES IN HUMAN LIVER MICROSOMES

Xiaotong Li, WuXi AppTec, SHANGHAI, China

P31 - THE GUT MICROBIOME REGULATES HEPATIC DRUG PROCESSING GENES IN LIVERS OF HUMANIZED PXR-CAR-CYP3A4/3A7 MICE

Jennifer Liem, University of Washington, Seattle, WA, United States

P32 - SOLANIDINE METABOLITES AS DIETARY CYP2D6 BIOMARKERS OF TAMOXIFEN METABOLISM

Samantha Medwid, University of Western Ontario, London, ON, Canada

P33 - AN EVALUATION OF CYP AND NON-CYP REACTION PHENOTYPING STUDY TRENDS FROM 2000-2023: A CRO PERSPECTIVE

Tina Mueller, BioIVT, Hicksville, NY, United States

P34 - CYTOCHROME P450 (CYP) PHENOTYPING OF AFICAMTEN, A NEXT-IN-CLASS INHIBITOR OF CARDIAC MYOSIN, USING HUMAN LIVER MICROSOMES AND HUMAN RECOMBINANT CYP ENZYMES

Rajaa Sukhun, Cytokinetics, South San Francisco, CA, United States

P35 - IMPACT OF DNA G-QUADRUPLEX FORMATION ON CYP3A4 GENE TRANSCRIPTION

Seiya Takemoto, Kanazawa University, Kanazawa, Japan

P36 - CYTOCHROME P450 3A CHEMICAL INHIBITORS: UNDERSTANDING SELECTIVITY IN HUMAN LIVER MICROSOMES FOR USE IN REACTION PHENOTYPING

Elaine Tseng, Pfizer Inc, Groton, CT, United States

P37 - METABOLISM OF PIROCARPINE IN CHIMERIC MICE WITH HUMANIZED LIVER

Shotaro Uehara, Central Institute for Experimental Medicine and Life Science, Kawasaki, Kanagawa, Japan

P38 - GUT MICROBIAL METABOLITES ACTIVATE PREGNANE X RECEPTOR TO INCREASE CYP3A4 EXPRESSION IN THE LIVER

Kyoung-Jae Won, Purdue University, West Lafayette, IN, United States

P39 - ASSESS CYP450 INDUCTION BY QRT-PCR QUANTIFICATION OF CYP450 MRNAS FROM SMALL EXTRACELLULAR VESICLES (SEVS) DERIVED FROM PLASMA

Shengjie Xu, Merck & Co., Inc, West Point, PA, United States

DATABASE ANALYSIS (P40)**P40 - ELUCIDATING SYNERGISTIC RISK FACTORS AMONG COMORBIDITY COMBINATIONS FOR NEPHROTIC SYNDROME BASED ON TIME SERIES STATISTICAL EVALUATION**

Katsuyuki Chida, Chiba University, Chuo-ku, Chiba-shi, Chiba, Japan

DIFFERENCES IN METABOLISM (SPECIES, GENDER, AGE, DISEASES) (P41 – P50)**P41 - HEPATIC EXPRESSION OF DRUG METABOLIZING ENZYMES IS ALTERED BY CONCOMITANT OBESOGENIC LIPIDS AND ALCOHOL IN MICE AND HUMAN HEPATIC ORGANIDS**

Joseph Dempsey, University of Washington, Seattle, WA, United States

P42 - CHANGES IN THE EXPRESSION OF DRUG-METABOLIZING ENZYMES AND TRANSPORTERS ASSOCIATED WITH DECREASED EXPRESSION OF CYP3A AND MRP2

Chieri Fujino, Ritsumeikan University, Kusatsu, Shiga, Japan

P43 - ASSESSMENT OF UNINTENTIONAL EFFECTS ON ENDANGERED SPECIES ENDEMIC TO THE BONIN ISLANDS CAUSED BY DEPLOYMENT OF ANTICOAGULANT RODENTICIDE

MASASHI KOIDE, Hokkaido University, Japan, Sapporo, Hokkaido, Japan

P44 - INVESTIGATING THE INFLUENCE OF CYP3A AND CYP2C8 PHENOTYPES ON TUCATINIB CLEARANCE IN A DIVERSE COHORT OF HUMAN HEPATOCYTE DONORS

Bethany Latham, UNC Chapel Hill Eshelman School of Pharmacy, Chapel Hill, NC, United States

P45 - COVID-19 SEVERITY DYSREGULATES THE EXPRESSION OF CLINICALLY RELEVANT DRUG PROCESSING GENES

Chukwunonso Nwabufu, University of Toronto, Toronto, ON, Canada

P46 - USING INTESTINAL MICROSOMES TO ASSESS THE IMPACT OF GUT METABOLISM ON THE INTESTINAL ABSORPTION OF BIDAC DEGRADERS.

Ritu Singh, C4 Therapeutics, Watertown, MA, United States

P47 - THE INFLUENCE OF ETHNIC DIFFERENCE ON SIMVASTATIN AND ITS METABOLITE PHARMACOKINETICS

THANATE SRIMATIMANON, Chulalongkorn University, Bangkok, Thailand

P48 - THE INFLUENCE OF ALPHA-1 ACID GLYCOPROTEIN BINDING ON TOTAL PLASMA FREE FRACTION AND THE EFFECT ON CLINICAL PHARMACOKINETICS

Kelly Wang, Gilead Sciences, Inc, Foster City, CA, United States

P49 - ALTERATION OF RENAL-LIVER-CARDIAC INTERACTIONS LINKED BY CIRCADIAN MACHINERY UNDERLIES CHRONIC KIDNEY DISEASE- ASSOCIATED CARDIAC INFLAMMATION AND FIBROSIS

Yuya Yoshida, Kyushu university, Fukuoka, Fukuoka, Japan

P50 - AGE AND SEX INFLUENCE THE PHARMACOKINETICS AND BRAIN DISTRIBUTION OF REC-3599, A SELECTIVE INHIBITOR OF PROTEIN KINASE C, IN RATS

Vamshi Manda, Recursion Pharmaceuticals, Salt Lake City, UT, United States

DILI (P51)**P51 - GUT BACTERIAL METABOLITE 3-PHENYLPROPIONIC ACID INHIBITS CYTOCHROME P450 2E1 EXPRESSION AND ATTENUATES ACETAMINOPHEN-INDUCED CYTOTOXICITY IN PRIMARY MOUSE HEPATOCYTES**

Pei-Ru Jin, Purdue University, West Lafayette, IN, United States

DISPOSITION (P52 – P66)**P52 - CFT8634, A POTENT, SELECTIVE AND ORALLY BIOAVAILABLE BIDACTM DEGRADER OF BRD9, HIGHLIGHTS THE POTENTIAL OF C4T'S TORPEDO® PLATFORM TO DEVELOP DEGRADERS WITH EXCELLENT DRUG-LIKE PROPERTIES AGAINST CURRENTLY UNDRUGGABLE TARGETS**

Prasoon Chaturvedi, C4 Therapeutics, Watertown, MA, United States

P53 - THE INVESTIGATION OF THE METABOLISM AND DISPOSITION MECHANISM OF BUPRENORPHINE AND ITS ANALOGS

Jie Chen, University of Houston, Houston, TX, United States

P54 - METABOLITE PROFILING OF [14C]BMS-986166 AND ITS ACTIVE METABOLITE (BMS-986166-P) FOLLOWING A SINGLE ORAL DOSE TO INTACT/BILE DUCT CANNULATED RATS

Weiqi Chen, Bristol Myers Squibb, Princeton, NJ, United States

P55 - CONTRIBUTION OF MCT6 IN REGULATING CELLULAR ACCUMULATION OF THE N-ACETYL CYSTEINE S-CONJUGATE METABOLITE OF CISPLATIN

Arthur Germakovski, University at Buffalo, Buffalo, NY, United States

P56 - PRIMARY CULTURE OF RAT KIDNEY TISSUE SLICE FOR EVALUATION OF DRUG-INDUCED KIDNEY INJURY USING GAS-PERMEABLE PLATES

Moeno Kadoguchi, Kanazawa University, Kanazawa, Ishikawa, Japan

P57 - PHARMACOKINETICS OF MONOCLONAL ANTIBODIES LOCALLY-APPLIED INTO THE MIDDLE EAR OF GUINEA PIGS

Tomoko Kita, Kyoto University, Kyoto, Japan

P58 - ALTERATION IN INTERCELLULAR LIPID COMPOSITION OF STRATUM CORNEUM IN GOTO-KAKIZAKI RATS: A MODEL OF TYPE 2 DIABETES WITH AN INCREASED FENTANYL RELEASE RATIO FROM THE TRANSDERMAL FORMULATION

Satoshi Mizuno, Kanazawa University, Kanazawa, Ishikawa, Japan

P59 - MASS BALANCE RECOVERY, ABSORPTION, METABOLISM, AND EXCRETION OF [14C]-ACOZIBOROLE IN HEALTHY MALE SUBJECTS AFTER SINGLE MICROTRACER ORAL DOSING – I – PREPARATION OF GMP [14C]-ACOZIBOROLE DRUG SUBSTANCE

Iain Shaw, Quotient Sciences, Nottingham, United Kingdom

P60 - MASS BALANCE RECOVERY, ABSORPTION, METABOLISM AND EXCRETION OF [14C]-ACOZIBOROLE IN HEALTHY MALE SUBJECTS AFTER SINGLE ORAL DOSING - II - MASS BALANCE AND CLINICAL SAFETY ASSESSMENTS

Iain Shaw, Quotient Sciences, Nottingham, United Kingdom

P61 - MASS BALANCE RECOVERY, ABSORPTION, METABOLISM, AND EXCRETION OF [14C]-ACOZIBOROLE IN HEALTHY MALE SUBJECTS AFTER SINGLE MICROTRACER ORAL DOSING – III – INVESTIGATION OF METABOLITE PROFILE IN PLASMA, URINE AND FECAL HOMOGENATE SAMPLES USING UPLC-MS FRACTI

Iain Shaw, Quotient Sciences, Nottingham, United Kingdom

P62 - PROTAC TISSUE DISTRIBUTION

Stefanus Steyn, Pfizer, Cambridge, MA, United States

P63 - SCREENING OF RENAL PLASMA FLOW BIOMARKER BASED ON UNTARGETED METABOLOMICS ANALYSIS

Tomoko Suzuki, Kanazawa university, Kanazawa city, Japan

P64 - MASS BALANCE, PHARMACOKINETICS, AND TISSUE DISTRIBUTION OF [14C] ZETOMIPZOMIB IN RATS BY QUANTITATIVE WHOLE-BODY AUTORADIOGRAPHY

Jinhai Wang, Kezar Life Sciences, South San Francisco, CA, United States

P65 - OPTIMIZATION OF LINKER SEQUENCE OF AMINOPEPTIDASE A-FC FUSION PROTEINS IN PHARMACOKINETICS AND ANTIHYPERTENSIVE EFFECT

Yukiko Yamaguchi, Kanazawa University, Kanazawa City, Japan

P66 - RADIOKINETICS, PHARMACOKINETICS, MASS BALANCE AND EXCRETION, AND METABOLISM OF RADIOLABELED PROTAC® ANDROGEN RECEPTOR DEGRADER BAVDEGALUTAMIDE (ARV-110) FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION IN RATS

George Zhang, Arvinas, New Haven, CT, United States

DRUG DELIVERY (P68)**P68 - TRIGGERING OF PACLITAXEL RELEASE FROM PEG MONOGLYCERIDE NIOSOMES BY EMPTY SPAN85 NIOSOMES**

Ken-ichi Ogawara, Kobe Pharmaceutical University, Kobe, Hyogo, Japan

DRUG DELIVERY SYSTEM (P69)**P69 - BLOOD GLUCOSE CONTROL VIA ORAL ZN-INSULIN ADMINISTRATION TOGETHER WITH SMALL INTESTINE-PERMEABLE CYCLIC PEPTIDES IN MOUSE MODELS OF DIABETES MELLITUS**

Shoma Chikamatsu, Kumamoto university, Kumamoto, Kumamoto, Japan

DRUG DISCOVERY AND DEVELOPMENT (P70 – P99, P339 – P340)**P70 - A CASE STUDY FOR VISUALIZING OLIGONUCLEOTIDE THERAPEUTICS DISTRIBUTION IN RAT ORGANS USING MALDI MASS SPECTROMETRY IMAGING**

Hiroaki Aikawa, Mediford Corporation, Tokyo, Japan

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Medical Institute, Mahidol university, Samut Prakan,
Thailand*

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*Tianran Hao, Washington State University, Spokane, WA,
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*Lifang Jiang, WuXi AppTec Lab Testing Division -
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Terry Podoll, IV-PO, LLC, Seattle, WA, United States

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Terry Podoll, IV-PO, LLC, Seattle, WA, United States

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Christiane Fæste, Norwegian Veterinary Institute, Ås, Norway

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Christiane Fæste, Norwegian Veterinary Institute, Ås, Norway

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Jie Wang, WuXi AppTec (Shanghai) Co Ltd, Shanghai, China

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Kunihiro Yoshisue, *Taiho Pharmaceutical Co., Ltd., Tsukuba, Ibaraki, Japan*

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Qing Zhang, *University of the Pacific, Stockton, CA, United States*

P336 - INVESTIGATION IN THE PREDICTIVE ACCURACY OF BRAIN KPUU ACROSS SPECIES USING *IN VITRO* AND *IN VIVO* METHODS

Ling Zou, *Amgen, South San Francisco, CA, United States*

P337 - THE IMPACT OF UPTAKE AND EFFLUX TRANSPORTERS ON THE DISPOSITION AND CHOLESTEROL LOWING EFFECT OF EZETIMIBE

Ting Du, *Texas Southern University, Houston, TX, United States*

A1 - THE BLOOD BRAIN BARRIER DEMONSTRATES CAPABILITY TO INFLUENCE HETEROGENOUS TENOFOVIR AND EMTRICITABINE METABOLISM AND TRANSPORT IN THE BRAIN**Hannah Wilkins¹**, Ben Orsburn¹, and Dionna Williams²¹The Johns Hopkins University School of Medicine, Baltimore, MD, United States, ²The Emory University School of Medicine, Atlanta, GA, United States

Neurologic deficits associated with human immunodeficiency virus (HIV) infection demonstrate the need to eliminate viral reservoirs in the brain. Nucleotide reverse transcriptase inhibitors tenofovir (TFV) and emtricitabine (FTC) are antiretroviral therapeutics (ART) prescribed for the treatment and prevention of HIV. Recently, we demonstrated that the distribution of TFV, FTC, and their active metabolites, tenofovir-diphosphate (TFV-DP) and emtricitabine-triphosphate (FTC-TP), localized heterogeneously across brain regions. However, the mechanisms that contribute to this distribution pattern remain unknown. We hypothesize that the blood brain barrier (BBB), which is dynamic in brain microvascular endothelial cell (BMVEC), pericyte, and astrocyte composition, contain differential expression and activity of nucleotide metabolizing kinases and transporters that may lead to heterogeneity in drug localization. To assess the capability of BBB cells to metabolize TFV and FTC, primary human BMVEC, pericytes, and astrocytes were exposed to clinically relevant concentrations of ART for 24 hours. We determined that TFV-DP and FTC-TP were quantifiable in all BBB cells (n=3-4) by liquid chromatography-mass spectrometry (LC-MS/MS). However, pericyte FTC-TP concentrations were 80% higher than in BMVEC and astrocytes (p<0.05). TFV-DP and FTC-TP concentrations were comparable among BMVEC and astrocytes. Next, we performed proteomics by LC-MS/MS and western blot to quantify the kinases and transporters responsible for TFV and FTC metabolism and transport. TFV and FTC transporters MRP4, MRP1, ENT1, BCRP, and P-gp were significantly differentially expressed across the three BBB cell types (n=4-8). Several TFV and FTC nucleotide metabolizing kinases were also significantly differentially expressed among BBB cells (n=8), including creatine kinase brain-type (CKB), pyruvate kinase muscle-type (PKM), and phosphoglycerate kinase 1 (PGK1). Further, MRP4, BCRP, and P-gp activities were assessed by a flow cytometric efflux transporter activity assay. Interestingly, BCRP activity was comparable across BBB cells, whereas P-gp activity in pericytes had 81% and 64% higher efflux than in astrocytes and BMVEC, respectively (n=4 each, p<0.01). In contrast, pericytes displayed 69% and 75% less MRP4 efflux than BMVEC and astrocytes, respectively (n=4 each, p<0.01). Next, we performed a CKB-mediated TFV activation assay, which demonstrated that astrocytes had 5-fold higher TFV activation compared to BMVEC (n=5, p<0.01). Overall, our findings indicate that the cells that comprise the BBB contain specific and heterogenous activities in the metabolism and transport of TFV and FTC. These data suggest that TFV and FTC brain disposition may be influenced by the nucleotide metabolizing kinases and transporters at the BBB, impacting their central nervous system disposition and efficacy.

A2 - PHARMACOKINETIC MODELING LEVERAGING *IN VITRO* TO *IN VIVO* EXTRAPOLATION OF THE GUT MICROBIOME'S ROLE IN SULFASALAZINE METABOLISM INTO SULFAPYRIDINE AND MESALAMINE**Lisa Cheng¹**, Jingwei Cai², Alexis Auster², Sungjoon Cho², and Miaoran Ning²¹The University of British Columbia, Vancouver, BC, Canada, ²Genentech, South San Francisco, CA, United States

The human gastrointestinal tract hosts the gut microbiome, which contributes to the metabolism of orally administered drugs. For example, sulfasalazine can be cleaved by colonic bacterial azoreductases to yield two metabolites: sulfapyridine and mesalamine. Previous studies suggested that the microbiome residing in the anaerobic colon plays a critical role in its biotransformation.^{1,2} Currently, quantitative assessment of the gut microbiome's influence on *in vivo* outcomes is lacking. This highlights the necessity to establish a physiologically-based pharmacokinetic (PBPK) modeling framework for gut microbiome-mediated metabolism through *in vitro* to *in vivo* extrapolation. To address this knowledge gap, we used sulfasalazine as a model drug to predict its colonic metabolism and the generated metabolites' pharmacokinetics. The Simcyp® Simulator V22 (Sheffield, UK) was employed for PBPK simulations. Three PBPK models (sulfasalazine, sulfapyridine, and mesalamine) were built using a middle-out approach where model parameters were collected from literature or estimated from intravenous clinical data. The models' performances were verified with oral pharmacokinetic data from individual dosing of each compound through a visual check and fold-error analysis of exposure parameters. *In vitro* gut microbiome-mediated metabolism parameters (the maximum reaction velocity, V_{max}; the substrate concentration at which half V_{max} is reached, K_m) were obtained from pooled human fecal slurries incubated with a range of sulfasalazine concentrations under anaerobic conditions. The difference in bacterial abundance in the colon and feces were addressed by scaling the experimental V_{max} by a factor of 0.66. Scaled colon luminal metabolic kinetics (V_{max} of 651 pmol/mg of luminal content/min and K_m of 3.65 mM) were integrated into the sulfasalazine model and linked to the sulfapyridine and mesalamine models. After integrating colon luminal metabolism parameters into the sulfasalazine model, concentration-time profiles for sulfasalazine, sulfapyridine, and mesalamine following a sulfasalazine dose were simulated and compared against clinical data. Our results suggested that this IVIVE coupled with PBPK modeling approach successfully captured clearance of sulfasalazine by the gut microbiome and its metabolites' colonic absorption, distribution, clearance, and elimination within two-fold. Future research will explore additional compounds where gut microbiome-mediated metabolism plays a significant role, thereby enhancing the robustness of the current translational framework. In summary, the IVIVE of gut microbiome-mediated metabolism and PBPK modeling strategy can

provide a better understanding of the gut microbiome's contribution in drug metabolism and moreover, it underscores a novel approach to evaluate gut microbiome-mediated metabolism for targeted colonic delivery by prodrugs.

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A3 - DRUG-INDUCED GASTROINTESTINAL TOXICITY DUE TO DISRUPTION OF THE INTERINFLUENCE BETWEEN SEROTONIN AND GUT MICROBIOTA

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Gastrointestinal (GI) toxicity (e.g., diarrhea and constipation) is one of the most common adverse events leading to discontinuation of oral medications. Therefore, predicting GI toxicity as well as bioavailability is important to develop oral drugs having higher safety and effectiveness. However, drug-induced GI toxicity is not necessarily easy to assess and predict, because its mechanism is complicated by multiple factors. For example, serotonin (5-HT), a major GI hormone, is associated with gut microbiota, which plays a role in various GI physiology, and therefore drug effect on interinfluence between 5-HT and gut microbiota dynamics may lead to drug-induced GI toxicity. In the present study, we examined our hypothesis that changes in the transporter-mediated GI disposition of 5-HT due to orally administered drugs could influence gut microbiota dynamics, leading to disorders of the GI fluid regulation, using metformin which clinically causes diarrhea as a side effect. Studies using transporter-expressed *Xenopus laevis* oocytes and Caco-2 cells suggested that multiple transporters (SERT, PMAT, THTR2 and OCT3) are involved in the intestinal uptake of 5-HT, and metformin inhibits 5-HT uptake mediated by these transporters. The influence of metformin on luminal fluid and luminal 5-HT levels were estimated by employing rat in situ closed-loop method. Administration of metformin into the intestinal lumen significantly increased the GI fluid volume, demonstrating diarrhea symptoms as an adverse effect of metformin. At the same time, elevated levels of 5-HT in the GI lumen were also observed. A similar tendency was observed after long-term *in vivo* oral administration of metformin. Interestingly, a significant positive correlation was identified between colonic 5-HT levels and fecal moisture content. Thus, it is suggested that colonic 5-HT levels may be a major determinant of the onset of metformin-induced diarrhea symptoms. Based on these findings, it is considered that metformin may increase luminal 5-HT levels by inhibiting transporter-mediated intestinal uptake of 5-HT, resulting in diarrheal symptoms. Further analysis for fecal microbiota indicated that metformin influences gut microbiota dynamics (mainly *Turicibacter*) as well as GI 5-HT levels, implying that gut microbiota dynamics are linked with luminal 5-HT. When transferring the feces of these rats into the intestinal lumen of another untreated rat, the recipient rat showed the same diarrheal symptoms as the donor rat. Thus, it was speculated that diarrhea symptoms derived from changes in luminal 5-HT levels may be mediated by effects on the gut microbiota. Taken together, our findings indicate that drug-induced GI toxicity is caused by the changes in gut microbiota via an increase of the luminal 5-HT levels by inhibition of transporter-mediated GI 5-HT uptake.

A4 - IMPACT OF TYROSINE KINASES AND INHIBITORS ON SGLT1 ACTIVITY

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Tyrosine kinase inhibitors (TKIs) are widely used for cancer treatment due to their ability of suppressing oncogenic cellular signaling events. However, TKIs are associated with adverse events, such as gastrointestinal distress or hypoglycemia,¹ that can impact treatment outcome or quality of life. The mechanism responsible for these adverse events are unclear, however similar gastrointestinal and glycemic complications are seen in the absence of sodium-glucose-linked transporter 1 (SGLT1) activity. We hypothesized that SGLT1 is sensitive to inhibition by TKI exposure resulting in reduced glucose absorption. To address this hypothesis, we measured 14C- α -methyl-D-glucopyranoside (14C-AMG) uptake in SGLT1 overexpressing HEK293 cells in presence and absence of ten different TKIs (20 μ M). We observed that seven TKIs reduced SGLT1 activity by at least 50% ($p < 0.05$), with regorafenib and sorafenib being the most potent inhibitors that reduced transport activity to ~10% ($p < 0.05$). Exposure of SGLT1 overexpressing HEK293 cells to regorafenib was also associated with loss of transporter phosphorylation at Y144 as determined by mass spectrometry analysis. This phosphorylation event was deemed important for SGLT1 function, as when we transiently transfected HEK293 cells with a Y144F mutant variant of SGLT1, transport activity, as determined by 14C-AMG uptake, was lost ($p < 0.05$), despite the SGLT1 being localized to the plasma membrane. Due to BRAF being a common target of SGLT1-inhibiting TKIs, we analyzed the impact of BRAF-targeting siRNA on 14C-AMG uptake in SGLT1-overexpressing HEK293 cells, in the presence or absence of sorafenib. This study revealed that siRNA mediated knockdown of BRAF resulted in a 40% reduction in SGLT1 function ($p < 0.05$). Overall, the collected data indicates that SGLT1 is sensitive to several TKIs, including regorafenib and sorafenib, and that TKI exposure can reduce phosphorylation of tyrosine at position 144, which

is an important amino acid for SGLT1 function. Moreover, BRAF, which is a common target among SGLT1 inhibiting TKIs, is a major regulator of SGLT1 function. Further study is warranted to assess the influence of SGLT1 inhibition in contributing to the onset or severity of TKI-induced adverse events.

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A5 - DEVELOPMENT OF AN *IN VITRO* METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS (MASH) MODEL TO STUDY HEPATIC TRANSPORTERS

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Metabolic dysfunction-associated steatotic liver disease (MASLD) is a highly prevalent condition that can progress to MASH. Understanding the impact of MASLD/MASH on hepatic drug transporter function is critical for predicting potential alterations in drug disposition in patients¹. This study aimed to develop an *in vitro* MASH model to predict changes in hepatobiliary drug disposition. MASH-inducing lipid-cytokine treatments (fatty acids, lysophospholipids, free cholesterol, and pro inflammatory cytokines) were optimized using a differentiated human hepatoma (HuH-7) cell model². The impact of optimized MASH-inducing treatments on select transporters was subsequently investigated in sandwich-cultured human hepatocytes (SCHH). On Day 0, HuH-7 cells were seeded at 75,000 and 350,000 cells/well for toxicity and MASH phenotype assessment (96-well plates) and transporter function (24-well plates), respectively. Differentiation of HuH-7 cells started on Day 2 by supplementing culture media with 1 μ M dexamethasone and 0.5% dimethylsulfoxide. On Day 7, cells were overlaid with 0.25 mg/mL Matrigel. For SCHH studies, transporter certified human hepatocytes (n=3 donors) were seeded at 450,000 cells/well in 24-well plates and overlaid with Matrigel after 16–20 hours. HuH-7 cells and SCHH were cultured with lipid-cytokine treatments for 72 hours beginning on Days 14 and 2, respectively; endpoint assays were performed on Days 17 and 5, respectively. HuH-7 cell viability was measured using the ATP assay. Lipid accumulation (BODIPYTM 493/503) and lipid peroxidation (BODIPYTM 665/676) were measured using high content imaging for MASH phenotype assessment. Taurocholic acid (TCA) accumulation and biliary excretion index (BEI) were determined using B-CLEAR[®] technology. Protein abundance of select transporters in SCHH was determined using LC-MS/MS quantitative targeted absolute proteomics. Increasing cholesterol concentrations (0.05–0.1 mM) in lipid-cytokine treatments resulted in reduced cell viability and higher lipid accumulation in differentiated HuH-7 cells. To reduce cellular toxicity risk, a lower cholesterol concentration (0.01 mM) was selected for subsequent treatments. Lipid-cytokine treatments increased TCA accumulation in differentiated HuH-7 cells from 42.9 $\pm$ 2.7 pmol/mg protein (control) to 64.8 $\pm$ 1.6–73.4 $\pm$ 4.5 pmol/mg protein, and decreased TCA BEI from 45.1% (control) to 7.4–13.9% (n=3/treatment). In SCHH, three of the optimized lipid-cytokine treatments significantly (p<0.05) decreased protein abundance of basolateral uptake transporters (OATP1B1, OATP1B3, OATP2B1, NTCP and OAT2) 0.33–0.9-fold, and decreased apical efflux transporters (MRP2 and BSEP) 0.48–0.71-fold (n=3/donor). Furthermore, MRP3 was significantly increased 1.22–1.27-fold for all treatments. Decreased abundance of uptake transporters and increased abundance of efflux transporters aligns with published data in liver tissue from patients with MASH³. Ongoing studies involve measurement of functional changes in transporters following lipid-cytokine treatment in SCHH.

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A6 - CONSTRUCTION OF FUNCTIONAL EVALUATION SYSTEM FOR HUMAN BCRP-MEDIATED BILIARY EXCRETION USING ADENO-ASSOCIATED VIRUS

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Purpose: Breast cancer resistance protein (BCRP/ABCG2) is an efflux transporter expressed on the luminal side of intestinal epithelial cells and the canalicular side of hepatocytes. BCRP in the small intestine may limit exposure to its substrates in the body whereas information on potential involvement of hepatic BCRP in its substrate drug disposition is

limited. The tissue-selective gene knock-down/knock-in approach is needed to assess the pharmacokinetic roles of hepatic BCRP *in vivo*. Adeno-associated virus (AAV) vector is a useful gene delivery modality for exogenous protein expression. Therefore, the purpose of the present study is to construct the functional evaluation system of human BCRP-mediated biliary excretion *in vivo* by using AAV-mediated gene delivery in the liver.

Methods: AAV vectors carrying mouse and human BCRP gene, and that with a genetic variant (AAV-mBcrp, AAV-hBCRP, and AAV-hBCRP(Q141K)), and tdTomato as a control (AAV-tdTomato) were produced in AAV293 cells, and AAVs were purified by ultracentrifugation. AAV was intravenously administered to Bcrp^{-/-} mice to knock-in BCRP gene in the liver. A typical BCRP substrate sulfasalazine was orally or intravenously administered to Bcrp^{-/-} mice treated with AAV-mBcrp, AAV-hBCRP, AAV-hBCRP(Q141K), and AAV-tdTomato, and plasma, bile, and liver were collected. Concentrations of drugs and endogenous compounds in those samples were measured by LC-MS/MS or LC-qTOFMS.

Results and Discussion: Both western blotting and immunostaining detected BCRP gene product in the liver of AAV-mBcrp, AAV-hBCRP, and AAV-hBCRP(Q141K) treated Bcrp^{-/-} mice, indicating successful knock-in of BCRP by AAV-mediated gene delivery. AUC of sulfasalazine after oral administration in AAV-mBcrp and AAV-hBcrp treated Bcrp^{-/-} mice was 10-fold lower than that in AAV-tdTomato treated ones, suggesting functional expression of mBcrp and hBCRP in the liver. In addition, AAV-hBCRP(Q141K) treated Bcrp^{-/-} mice showed 3-fold higher AUC compared with that in AAV-hBCRP treated ones, reflecting reduced function and/or expression of BCRP by this SNP. This was further supported by the finding that biliary clearance of sulfasalazine in Bcrp^{-/-} mice treated with AAV-hBCRP and AAV-hBCRP(Q141K) was 20 and 4-fold higher than that in AAV-tdTomato treated Bcrp^{-/-} mice. AUC and biliary clearance of sulfasalazine showed a slight difference between AAV-mBcrp and AAV-hBCRP treated Bcrp^{-/-} mice, suggesting possible species differences in function and/or expression between mouse and human. Biliary excretion of food-derived BCRP substrates isoflavone sulfates in AAV-mBcrp treated Bcrp^{-/-} mice was higher than that in AAV-tdTomato treated ones, whereas their plasma concentration in AAV-mBcrp treated Bcrp^{-/-} mice tended to be only slightly lower than that in AAV-tdTomato treated ones, suggesting that the systemic exposure to these food-derived sulfates may be primarily determined by BCRP in the small intestine, but not the liver.

Conclusion: AAV-mediated gene delivery is useful for evaluating the role of hepatic BCRP in the biliary excretion of its substrates. Further characterization is needed to apply this approach for the quantitative evaluation of biliary excretion of BCRP substrate drugs in humans.

A7 - PEGYLATED TESTOSTERONE WITH ENHANCED PHARMACOKINETIC PROPERTIES IN MICE

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Testosterone, an androgenic steroid hormone, plays a crucial role in regulating primary sexual characteristics. Beyond its impact on physical attributes, testosterone levels also influence overall mood, cognition, social behavior, and sexual function. Various factors such as medical conditions, aging, or inheritance are responsible for testosterone deficiency, which is mainly treated by exogenous testosterone replacement. In addition, low testosterone levels have been linked to metastatic breast cancer, osteoporosis, Alzheimer's disease, frailty, obesity, diabetes, and cardiac disorder[1,2]. Current testosterone therapies have not always been feasible due to extensive metabolism, low solubility, and unwanted side effects [2,3]. In attempt to improve the physicochemical and pharmacokinetic properties, we synthesized a conjugate of testosterone with a 4-Arm PEG-OH (a multi-armed polyethylene glycol with hydroxyl groups at each terminal of the four arms). Pharmacokinetic comparison of the PEG-testosterone conjugate versus testosterone were conducted using IP route at a 10 mg/kg equivalent testosterone dose. Blood samples were collected at 0.25-12 hr post administration and analyzed using LC/MS-MS. Following IP administration PEG-testosterone exhibited a ~6-fold improvement in half-life versus equimolar testosterone (0.21 hr versus 1.18 hr); and a substantial 54-fold improvement in exposures with an AUC_{0-t} = 36.0 $\mu\text{M}\cdot\text{hr}$ versus 0.67 $\mu\text{M}\cdot\text{hr}$ for testosterone respectively. Moreover, it has been reported that the use of testosterone is associated with severe hepatotoxicity. In fact, testosterone and its derivatives have been shown to induce a specific form of cholestasis, peliosis hepatis, and hepatic benign and malignant tumors [4]. Given this we also measured the tissue exposures and identified that PEG testosterone had significantly (10-fold) lower tissue/plasma ratio (based on AUC) compared to testosterone in the liver i.e. 0.03 versus 0.46 suggesting that PEG-testosterone may potentially help avoid toxicities related to liver [5]. Other tissue analysis is underway. Overall, the synthesis and evaluation of a testosterone-4-Arm PEG-OH conjugate represents a promising approach to advancing testosterone replacement therapy, with the potential to improve patient outcomes and quality of life across a range of medical conditions by minimal toxicity. Further preclinical and clinical studies will be necessary to fully assess its efficacy, safety, and therapeutic utility in diverse patient populations.

A8 - DOES CANNABIDIOL (CBD) OR DELTA-9-TETRAHYDROCANNABINOL (THC) INDUCE DRUG METABOLIZING ENZYMES IN HUMAN HEPATOCYTES?**Ankit Balhara**, Yik Pui Tsang, and Jashvant D. Unadkat*University of Washington, Seattle, WA, United States*

The *in vitro* and *in vivo* potential of cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC) to cause multiple CYP and UGT inhibitory drug interactions has been well documented by us and others [1-4]. CBD (but not THC) is a time-dependent inhibitor of CYP1A2, 2C19 and 3A4 [2]. While we found that single oral dose CBD (640 mg) inhibited oral midazolam clearance by 51% [5], others have reported no such inhibition after chronic oral CBD administration (Epidiolex NDA application) suggesting that chronic CBD administration can induce CYP3A activity. CBD product labels indicate that CBD can induce CYP1A2, 2B6 and 3A4 enzymes in human hepatocytes. Therefore, we investigated the potential of CBD and THC to induce drug metabolizing enzymes (DMEs) in 4 lots of plated human hepatocytes (PHH, 1 male and 3 females). PHH were seeded in 24-well plates and treated, in triplicate, for 72 hours with CBD or THC (medium was refreshed daily at 8 and 16 hr). The CBD concentrations were 420nM and 42nM, corresponding to the average maximum CBD plasma concentrations (C_{max}) after a single high (700 mg) and low (25 mg) oral dose of the drug, respectively. The THC concentrations were 700nM and 250nM, corresponding to the average THC C_{max} after 70 mg and 25 mg single inhalational dose, respectively. At 72 hr, after washing with the incubation media, the PHH were incubated for 1 hr at 37°C with a cocktail of CYP probes, namely 100 µM phenacetin (CYP1A2), 100 µM bupropion (CYP2B6), 90 µM diclofenac (CYP2C9), 150 µM testosterone (CYP3A) and 20 µM dextromethorphan (CYP2D6). Then, cells were lysed and mRNA expression of DMEs was determined using qPCR (using beta-actin as the housekeeping gene). CYP activity was quantified by measuring, in the medium (using LC-MS/MS), the peak area ratio of the probe metabolite relative to the internal standard. Induction of CYP3A mRNA by rifampin (10 µM) was used as a positive control. Significant inter-lot variability was observed in the induction of CYP mRNA expression or activity by the cannabinoids. Of the DMEs, the mRNA of CYP3A4 was induced the most (>3-fold in three of four lots) by CBD (420nM) or THC (700nM). CBD (420nM) induced the mRNA of CYP1A2 by >2-fold in only two lots (ZKF and ADR). CBD (42nM and 420nM) significantly induced CYP3A activity (>2-fold) in two lots (YTW and ZKF) while THC (700nM) significantly induced CYP3A activity (>2-fold) in only one lot (YTW). CBD (420nM) significantly induced CYP1A2 activity (>1.7-fold) in two lots (YTW and ADR). CBD or THC did not significantly induce the activity of any other CYPs. Rifampin significantly induced both mRNA expression and activity of CYP3A enzymes in all four lots. A cannabinoid concentration-dependent study needs to be conducted to estimate the E_{max} and EC₅₀ of the induction of CYP3A and 1A2 activity prior to populating our THC and CBD PBPK models to predict the potential of these cannabinoids to cause inductive drug interactions.

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A9 - THE ONTOGENY AND INTER-INDIVIDUAL VARIABILITY OF HYDROLYTIC DRUG-METABOLIZING ENZYMES IN HUMAN LIVER

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Drug dosing for children is frequently suboptimal when relying on the traditional approach of normalizing doses based on body weight or surface area. Thus, the ontogeny profiling of drug-metabolizing enzymes (DMEs) is important to help develop physiologically-based pharmacokinetic (PBPK) models for predicting safer pediatric dosing. In particular interest are hydrolases are one of the most diverse classes of DMEs and catalyze reactions containing esters, amides, phosphates, etc. in both drugs and prodrugs. Hydrolases such as carboxyl esterases, cathepsins, and arylacetamide deacetylase have been studied for their involvement in drug metabolism. However, a significant number of hydrolases have not yet been appropriately characterized, and as such their influence on drug metabolism remains unknown. Moreover, there is a lack of comprehensive data on the ontogeny and inter-individual variability in expression of these enzymes. Here a characterization of the age-dependent protein abundance of hydrolases in viable primary human hepatocytes isolated from pediatric (n=50) and adult (n=8) donors (Female=28; Male=30) using quantitative global proteomics-based total protein approach was performed. An optimized trypsin digestion protocol was deployed for sample preparation, and the proteomics data were acquired using nano-liquid chromatography-high-resolution mass spectrometer and analyzed using DIA-NN software. A total of 251 hydrolases were detected and quantified in human hepatocytes, which were classified based on their hydrolytic activities towards different chemical bonds: ester (n=108), peptide (n=82), non-peptide carbon-nitrogen (n=33), glycosidic (n=11), ether (n=5), acid anhydride (n=5), carbon-carbon (n=4), and carbon-halide (n=3). The majority of these proteins (n=165) had an abundance of less than 1 pmol/million cells. Additionally, 64 hydrolases fell within the range of 1 to 10 pmol/million cells, while 12 hydrolases were measured between 10 to 100 pmol/million cells. Notably, the protein abundance of one hydrolase, specifically 2-iminopropanoate deaminase, exceeded 100 pmol/million cells. Seventy-six of the studied hydrolases exhibited a notable increase in abundance with age. When comparing neonates aged 0-12 days (n=7) to adults (>18 years, n=8), 20 hydrolases, including GDA, CES1, and SAMHD1, exhibited a more than 10-fold increase with age. Thirteen hydrolases exhibited a 3- to 10-fold increase, including GGT5, G6PC, and CES2 and 42 hydrolases showed a 1.5- to 3-fold increase, such as GUSB, PON1, and EPHX1. Further, 24 hydrolases showed a significant decrease in abundance with age (e.g., ARSA, CTSC, AFMID). The majority of these changes in expression were observed during the first year of life. For samples from the donors older than one year, 31 hydrolases (moderate to high abundance, >0.1 pmol/million cells) exhibited high inter-individual variability (%CV > 50). This variability may potentially be attributed to genetic polymorphism or other population factors. No significant sex-dependent differences were observed for most of the hydrolases, except PPT1, ARSD, FUCA1, PREP, LONP1 (20-30% lower in males), and PON3 and GCHFR (20-25% higher in males) in samples >1 year. These data will be useful in deriving ontogeny equations for PBPK models to predict age-dependent hepatic clearance of drugs metabolized by hydrolases in pediatric population.

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A10 - IDENTIFICATION OF PAZOPANIB AS AN OATP1B1 SUBSTRATE BY COMPETITIVE COUNTERFLOW SCREEN

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Tyrosine kinase inhibitors (TKIs) belong to a drug class of small molecules that inhibit the function of key protein kinases involved in the pathogenesis of a broad range of human diseases. Although the primary elimination pathway for most TKIs involves hepatic CYP3A metabolism, the mechanism by which these agents are taken up into hepatocytes remains unclear. Furthermore, the presence of high levels of extracellular binding creates experimental challenges in identifying a transporter-mediated process. Here, we optimized and validated a competitive counterflow (CCF) approach to examine TKIs as substrates of the hepatic uptake transporter OATP1B1. The CCF method was based on the stimulated efflux of radiolabeled estradiol-17 β -glucuronide after steady state in HEK293 cells engineered to overexpress OATP1B1. A panel of FDA-approved TKIs was examined for transport by OATP1B1, in which Pazopanib was identified as a representative hit through CCF for further verification studies. The transport of Pazopanib was demonstrated by decreased kinase activity of vascular endothelial growth factor receptor 2 (VEGFR2), its target protein kinase, in OATP1B1 overexpressing cells but not cells lacking OATP1B1. Similarly, the activity of VEGFR2 in hepatocytes isolated from wild-type mice harboring functional OATP1B1 was also decreased compared to mice with OATP1A/1B-deficiency. Furthermore, the

molecular docking of Pazopanib exhibited a binding pattern that is similar to estrone-3-sulfate, a well-established OATP1B1 substrate. Taken together, these findings further support the notion that OATP1B1 mediates the transport of Pazopanib. The clinical utility of Pazopanib is limited by severe and potentially fatal hepatotoxicity. Thus, we further hypothesized that this toxicity phenotype is dependent on OATP1B1. In a series of *in vivo* approaches, higher liver concentrations of Pazopanib that is accompanied by elevated liver transaminases (ALT and AST) was observed in wild-type mice compared to OATP1A/1B-deficient mice. Moreover, these findings occurred without pharmacokinetic differences between the two genotypes. Taken together, our study provides the utility of the CCF method in identifying substrates of OATP1B1. Our additional verification studies shed light into a previously unknown initiating mechanism of Pazopanib hepatotoxicity and provides translational relevance of OATP1B1 as a key mediator of this toxicity.

A11 - DEVELOPMENT OF A STRATEGY TO PREVENT CISPLATIN-INDUCED KIDNEY INJURY BY TARGETING MATE-TYPE TRANSPORTERS

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Cisplatin-induced nephrotoxicity, a currently incurable health issue, is a critical clinical challenge, as it negatively affects the treatment of diseases and the quality of life of patients. Cisplatin-induced kidney damage is hypothesized to be caused by cisplatin accumulation in the proximal tubular cells of the kidneys, which is associated with multidrug and toxin extrusion (MATE) transporters. We hypothesized that SGLT2 inhibition would increase MATE excretory activity, reducing cisplatin accumulation in proximal tubular cells and mitigating renal damage. We aimed to investigate the effects of SGLT2 inhibitors on cisplatin-induced kidney injury in a mouse model generated using 10-week-old male C57BL/6 mice intraperitoneally administered 15 mg/kg cisplatin. Impairment of renal function and the impact of cisplatin accumulation in the kidneys were evaluated. Renal injury was assessed 72 h after cisplatin administration using blood urea nitrogen, creatinine clearance, and histological evaluation. SGLT2 inhibitors were administered once daily for four days, starting one day before cisplatin injection. The accumulation of platinum in the kidneys, blood, and urine was measured 8 h after cisplatin administration using ICP mass spectrometry. SGLT2 inhibitors and various other inhibitors were administered 24 h and 30 min before cisplatin administration, respectively. Renal function parameters that were significantly worsened by CDDP were significantly improved by the concomitant use of SGLT2 inhibitors. Concomitant use of ondansetron, a MATE inhibitor, further exacerbated renal impairment compared with that achieved with CDDP alone. Cisplatin administration resulted in significant accumulation of the drug in the kidneys. Pretreatment with the MATE inhibitor ondansetron significantly increased cisplatin accumulation in the renal tissue. Conversely, administration of the SGLT2 inhibitor dapagliflozin significantly reduced cisplatin accumulation in the kidneys. The results suggest that co-administration of cisplatin with SGLT2 inhibitors may reduce cisplatin accumulation in the kidneys and prevent the occurrence of cisplatin-induced nephrotoxicity. This represents a promising strategy to alleviate an important effect of cisplatin chemotherapy and may benefit patients' quality of life and cancer treatment.

A12 - INVESTIGATION OF THE MECHANISM OF METRONIDAZOLE-INDUCED ENCEPHALOPATHY FOCUSING ON THE POSSIBLE EFFECT ON THIAMINE TRANSPORT

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Aims: Metronidazole (MTZ)-induced encephalopathy has been suggested to be associated with thiamine deficiency based on pathological similarities with Wernicke's encephalopathy, but the mechanism of MTZ-induced encephalopathy is unknown. Based on the structural similarity between thiamine and MTZ, we hypothesized that MTZ interferes with the transport of thiamine at gastrointestinal tract and blood-brain barrier via inhibition of transporters for thiamine such as thiamine transporter (THTR) 1, THTR2 and organic cation transporter (OCT) 1. The objective of this study was to test this hypothesis using human-derived intestinal epithelial cell line Caco-2, which has been demonstrated to have functional transporters for thiamine, for the purpose of elucidating the mechanism of MTZ-induced encephalopathy.

Methods: 1) Effect of MTZ on the expression level of the thiamine transporters: Caco-2 cells were cultured in 24-well plates to form a monolayer and thiamine-d3 was added with or without MTZ (0.08-10 mM). After incubation at 37°C for 72 hours, the mRNA levels of THTR1, THTR2 and OCT1 in the cell lysate were quantified using real-time RT-PCR. 2) Effect of MTZ on cellular uptake of thiamine-d3: Caco-2 cells were cultured in 24-well plates to form a monolayer and thiamine-d3 was added with or without MTZ (0.01-50 mM). After incubation at 37°C for 7 minutes, the intracellular concentration of thiamine-d3 was measured using LC-MS/MS. 3) Effect of MTZ on transcellular transport of thiamine-d3: Caco-2 cells were cultured in Transwell® and confluency was confirmed by the transepithelial electrical resistance values of more than 300 $\Omega \cdot \text{cm}^2$. Thiamine-d3 was added to the apical side with or without MTZ (0.3-50 mM) and, after incubation at 37°C for 120 minutes, concentrations of thiamine-d3 in the basal side were measured using LC-MS/MS.

Results: No significant change in the mRNA levels of THTR1, THTR2 and OCT1 were observed in the MTZ-treated cells compared with the control cells. Unexpectedly, in the uptake study, the intracellular concentrations of thiamine-d3 were significantly higher (about 1.4- and 2.1-fold) in the cells treated with 10 and 50 mM MTZ, respectively, compared with the control cells. However, MTZ (0.3-50 mM) did not exhibit significant changes in the transcellular transport of thiamine-d3 up to 120 minutes.

Conclusions: Clinically relevant concentrations of MTZ did not affect the expression of thiamine transporters or the transcellular transport of thiamine-d3, suggesting that MTZ-induced encephalopathy may not be due to inhibition of gastrointestinal absorption of thiamine or its transport into central nervous system. Based on the findings of the present study, the possibility of MTZ affecting thiamine bioactivation is under investigation in order to elucidate the mechanisms of encephalopathy.

P1 - QUANTITATIVE ANALYSIS OF ANTIBODY CONJUGATE PAYLOAD BY LC MS/MS WITHOUT IMMUNOCAPTURESuhong Zhang and **Samuel Koppara**

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Antibody-drug conjugates (ADCs) have attracted extensive attention in recent years due to precise targeting of monoclonal antibodies and high potency of payloads. Quantitative analysis of total and conjugated antibody, total, free, conjugated payload and its metabolites are critical to evaluate efficacy, on and off-target toxicity. Liquid chromatography-mass spectrometry (LC-MS) has become a complementary tool to ligand binding assay for ADC analysis owing to its high specificity, sensitivity, and dynamic range. In early discovery, measurement of payload and its metabolites is a valuable way to determine its efficacy and toxicity. LC-MS based quantitative analysis of conjugated payload is usually coupled with immunocapture of ADC and cleavage of linker. However, antibodies used to capture ADCs may not be easily available at an early stage and immunocapture procedure can be lengthy with high reagent cost. In this study, an alternative sample extraction procedure for conjugated payload analysis was proposed and investigated.

One of our bile duct cannulation (BDC) study, only small molecules including free payload and metabolites, total and conjugated payload are of interest to be analyzed. Generally, immunocapture method is used to process samples for the analysis of conjugated payload and protein precipitation extraction is applied for the analysis of free payload/metabolites and total payload. Immunocapture is a powerful tool to enrich and clean up samples. In this study, an alternative method is utilized for the analysis of conjugated payload. Aliquots of samples such as rat plasma, urine, bile and feces were precipitated by ACN, the supernatants were used for free payload/metabolites analysis and the protein precipitates were washed 2x with methanol. The washed protein residues were redissolved and hydrolyzed with 1N NaOH. The mixtures were precipitated with ACN again and the supernatants were used for conjugated payload analysis. To prove the feasibility of this method, ADC was used to prepare for standards in each matrix, ADC and free payload were mixed at equal concentration to prepare QCs in each matrix. As a result, QCs in all matrices were passed with over 90% accuracy on average. To cross validate the results, the same sets of QCs were analyzed by immunocapture method. The values of QCs from both methods had no significant difference for all matrices. The test results suggest that an alternative way to immunocapture to analyze conjugated payload is possible.

P2 - IN VITRO STUDY OF PAYLOAD RELEASE OF AN ANTI-TNF GLUCOCORTICOID RECEPTOR MODULATOR IMMUNOLOGY ANTIBODY-DRUG CONJUGATE (ADC-T) AND PAYLOAD METABOLISM**Mingshe Zhu**¹, Zhiqiang Li², Chongzhuang Tang², Yang Gao², Lijuan Chen², and Xingxing Diao²¹MassDefect Technologies, Princeton, NJ, United States, ²XenoFinder, Suzhou, China

Study of the payload release from an ADC is very important in ADC lead optimization and nonclinical development. Release of the payload or payload-containing components from ADC in targeted and non-targeted tissues are directly associated with ADC efficacy and toxicity, respectively. The payload or major payload-containing component(s) in plasma of Tox species and human are subjected to bioanalysis in ADC safety evaluation and clinical studies. Furthermore, metabolism of a new payload-related compound should be conducted across species *in vitro* and in Tox species. Glucocorticoid receptor modulators (GRMs) have been developed as anti-inflammatory drugs for treating or preventing diseases related to glucocorticoid receptor signaling via affecting the immune system function. However, existing GRMs often have many undesirable side effects. ADC-T is an immunology antibody-drug conjugate that delivers a GRM payload via a cleavable linker and designed to reduce unwanted side effects of GRMs. The primary objective of this study was to study the release of the payload and payload-containing components from ADC-T in acidified liver lysosomes and S9 of rat, monkey and human using untargeted LC-HRMS method developed at XenoFinder. In addition, metabolite profiles of the GRM payload in hepatocytes across species were determined. Results showed that 23 payload-related components were detected and structurally characterized in the incubations with liver lysosomes and S9 for 24 h. The GRM payload was the single major payload-containing component released from ADC-T. Metabolite profiling of the GRM payload revealed that mono-oxidations were the major metabolic pathways, while glucuronidation was minor metabolic pathway. Currently, CYP phenotyping of the GRM payload metabolism was on-going. In summary, this study confirmed the design of ADC-T and selection of Tox species. In addition, it provided guidance for bioanalysis and *in vitro* DDI evaluation of the payload containing component.

P3 - RAPID PRODUCTION OF RABBIT MONOCLONAL ANTI-IDIOTYPE ANTIBODY FOR ANTIBODY DRUGS BY ECOBODY TECHNOLOGY**Shin Kawano**, Tomoe Yamauchi, Tomiko Yamada, Risumi Isogai, Satoko Yamamoto, and Shoji Ohuchi

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Anti-idiotypic (aID) antibody specifically recognizes a target antibody as an antigen. One of the most important applications of aID antibodies is pharmacokinetic (PK) assays, particularly the quantitation of administered therapeutic antibodies in serum. The generation of aID antibodies on their needs is expected to shorten the periods of therapeutics development.

However, since therapeutic antibodies are usually made from human or humanized IgG, it is difficult to obtain aID antibodies that are specific to the target antibody and do not react with surrounding human IgG. In general, antibodies derived from rabbits exhibit higher affinity and specificity than those derived from mice. Indeed, our previous studies have shown that rabbit monoclonal antibodies can distinguish slight amino acid differences between target and non-target proteins. However, rabbit is known as a difficult resource for generating monoclonal antibodies because of the inefficiency of conventional antibody generation technologies. Here, we report the rapid development of high performance aID monoclonal antibodies from rabbit using our proprietary technology, Ecobody, the combination of single-cell and *in vitro* translation technologies. Ecobody has enabled rapid, comprehensive, and effective antibody screening from a variety of resources including rabbit, alpaca, and human. By utilizing Ecobody technology in a fine source, rabbit, we are able to obtain antibodies with performance that is difficult to obtain using conventional methods. The lymphocytes of rabbits immunized with trastuzumab (Tra) and ipilimumab (Ipi) were applied for generating aID antibodies against these therapeutic antibodies. The single B cells with a desired reactivity were isolated by fluorescence-activated cell sorting (FACS), followed by the single-cell RT-PCR for the amplification of monoclonal antibody genes. The amplified DNAs were subjected to *in vitro* Fab expression, and the following ELISA evaluation identified varieties of aID clones against Tra and Ipi. The kinetical analyses by bio-layer interferometry showed KD values ranging from 10⁻⁹ to 10⁻¹⁰ M for full-bodies of the selected clones expressed in mammalian cells. These affinities are sufficient for PK assay applications, demonstrating that rapid generation of high performance aID antibodies from rabbit lymphocytes was successfully achieved employing Ecobody technology.

P4 - DEVELOPMENT AND QUALIFICATION OF AN *IN VITRO* ASSAY TO DETERMINE TARGET MEDIATED INTERNALIZATION OF TREM2 RECEPTOR SPECIFIC ANTIBODIES FOR TMDD ASSESSMENT

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Target mediated drug disposition (TMDD) is a key process, important for many targets that are relevant for drug development of therapeutic monoclonal antibodies (mAbs) as it can have a substantial impact on exposure and efficacy. It is affected by several factors like target expression and its turnover, exposure of the target site to the mAb, mAb-target binding kinetics forming complexes, internalization of these complexes, and finally recycling of the target to the cell surface.

Here, we present the development and qualification of a quantitative, flow cytometry based method to determine internalization parameters *in vitro* which we use for Michaelis–Menten modeling of TMDD parameters such as Km and Vmax for mAbs targeting the triggering receptor expressed on myeloid cells 2 (TREM2). The TREM2 receptor is selectively expressed, mainly on macrophages in various tissues throughout the human body, including microglia cells in the brain. Developing therapeutic antibodies that achieve a durable, pharmacologically relevant concentration in patients is essential for their success as it is tightly linked to efficacy, patient convenience (e.g. through long dosing intervals and options for subcutaneous dosing), and cost.

For this method, the amount of fluorescently labeled mAb is quantified in target expressing cells using flow cytometry via a calibration curve while the contribution of mAbs bound to the cell surface to the total signal is accounted for by using an antibody directed against the fluorophore, quenching its fluorescence selectively at the cell surface. The labeled mAb is titrated and an absolute cellular uptake rate (molecules per cell per time) is determined which is fitted to a Michaelis–Menten model for TMDD.

We first qualified the method by using a molecule, cross reactive to murine Trem2. For this, the mouse *in vivo* TMDD parameters obtained from a PK study were compared to the corresponding *in vitro* parameters obtained using primary cells (macrophages) from the same mouse model. We found that the TMDD relevant parameters which we obtained using the new *in vitro* method were in agreement with the *in vivo* parameters when using approximations from the literature for TREM2 expressing cell types and cell numbers in the mouse.

We then used the method to characterize development antibodies, i.e. bispecific mAbs targeted against human TREM2 and a second non-TREM2 target (not affected by TMDD in systemic circulation). We determined TREM2-specific cellular internalization kinetics of TREM2-bispecific mAbs in order to aid in the assessment of TMDD properties of and to support translation from preclinical studies to humans. We therefore used M1 and M2 type macrophages, differentiated from monocytes of human donors as tissue agnostic model systems to study the TMDD parameters of anti-TREM2 antibodies. Our results revealed significant differences in the uptake kinetics of mono- vs bivalent human TREM2 binders in human monocyte derived macrophages. Bivalent TREM2 binders show significantly higher Vmax and lower Km compared to the monovalent binder. This finding is a crucial insight for the evaluation of drug candidates regarding their potential PK properties in humans which needs to be balanced with other factors like potency, safety and developability.

P5 - EVALUATION FOR BIOTRANSFORMATION OF ANTIBODY-PEPTIDE CONJUGATES USING NATIVE MASS SPECTROMETRY

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Purpose: As modalities have become more complex in recent years, bioanalyses of them have also become more difficult, and there is an increased risk of misinterpreting the pharmacokinetic behavior of compounds when drug concentrations are measured using analysis methods that focus only on part of their structure. A compound was prepared by chemically conjugating a peptide, which is a ligand for a specific receptor, to a monoclonal antibody using the cysteine rebridging technique, and it was administered intravenously to mice. Upon measuring the total antibody concentrations in the plasma, it was observed that while the LC/MS method showed similar concentration profiles for both the naked antibody and the conjugated antibody, the LBA method showed a time-dependent increase in the concentrations of the conjugated antibody. Here, we hypothesized that biotransformation was the cause of this phenomenon and evaluated biological samples using native mass spectrometry.

Methods: The conjugated antibody in plasma was extracted as an intact protein by affinity purification. This sample was analyzed by native mass spectrometry using size exclusion chromatography/mass spectrometry (SEC/MS), and the obtained data was deconvoluted to estimate the molecular weight of the compound in the samples 4 and 24 hours after administration. We also considered the quantification method by LBA using anti-human IgG antibodies that had different specificities.

Results and Discussion: The conjugated antibody has four peptides, and the deconvolution from intact protein mass-to-charge spectra to mass spectra showed a molecular weight corresponding to a single peptide cleavage midway from the sample 4 hours after administration and corresponding to 2 to 4 peptide cleavages in the same region from the sample 24 hours after administration. Similar products were not observed in the *in vitro* plasma stability evaluation and were unique to the *in vivo* samples. Moreover, development of the LBA method, which gave results similar to the LC/MS assay, was not possible even when using anti-human IgG antibodies that had different specificities.

Conclusions: It is difficult to apply subunit analysis, which is a middle-up approach method, to compounds chemically conjugated by cysteine rebridging. Among the top-down approaches to evaluate intact proteins, native mass spectrometry, which does not involve protein denaturation, was shown to be one of the most useful tools for evaluating complex modalities in pharmacokinetic studies. The LC/MS assay was also useful for determining the total antibody concentrations.

P6 - DEVELOPMENT OF A PAC-LC-MS/MS METHOD FOR SIMULTANEOUS QUANTIFICATION OF MIPOMERSEN, ITS METABOLITES, AND ANALOGUES IN TISSUE SAMPLES

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Antisense oligonucleotides (ASOs) are single-stranded therapeutic oligonucleotides used for various intractable diseases. To date, it has been reported that usually, ASOs accumulate in the liver and kidneys and are metabolized by endonucleases and exonucleases. Therefore, the measurement of ASOs along with their metabolites is considered important in both non-clinical and clinical pharmacokinetic studies. Recently, liquid chromatography coupled with mass spectrometry (LC-MS) has been used as a major bioanalytical tool in the drug development of ASOs. In our previous research, we developed an LC-MS-based bioanalytical method for the quantification of plasma ASOs, and the developed method has been validated by multicenter validation studies. However, LC-MS-based bioanalytical methods for the simultaneous quantification of ASOs and their metabolites in tissue samples are sparsely available and have yet to be standardized. Besides, the applicability of acceptance criteria outlined in the International Council for Harmonisation (ICH) M10 guideline to such methods remains unclear. To address these challenges, we aimed to develop LC-MS-based bioanalytical methods capable of simultaneous quantification of ASOs and their metabolites in tissues. In this study, mipomersen and its reported metabolites (5'-N-6, 5'-N-7, 5'-N-9, 5'-N-10, 5'-N-10-PS, 5'-N-12, 3'-N-10, 3'-N-11, 3'-N-12, 3'-N-13) along with two analogues (5'-N-1, 3'-N-1) were used as model compounds. Extraction of mipomersen and its metabolites/analogues from Sprague-Dawley rat liver and kidney lysate (50 mg/mL, dissolved in Clarity OTX Lysis-Loading buffer) was performed using a Clarity OTX solid phase extraction plate. We used peptide adsorption-controlled

(PAC)-LC-MS/MS system capable of measuring samples with high concentrations of organic solvents for the analysis of the extract. LC separation was performed using mobile phases supplemented with triethylamine, hexafluoroisopropanol, and acetylacetone. Nitrogen bubbling was applied during the measurement to prevent their deterioration. The phosphorothioate-derived ion (m/z 95.0) was primarily used as a product ion in the measurement, while nucleotide-derived ions (m/z 402.1 for 5'-N-10, m/z 328.0 for 3'-N-10) were employed for the measurement of metabolites with similar molecular weight. The full validation for the developed bioanalytical method was conducted by referring to the ICH M10 guideline. As a result of method development, we established a bioanalytical method for quantifying the concentrations of mipomersen and its metabolites/analogues in liver and kidney tissues, covering a quantification range of 0.1 – 50 $\mu\text{g/g}$ tissue. The validation tests for the developed method demonstrated excellent linearity ($r^2 > 0.9930$) and achieved good intra-/inter-day accuracy (relative errors: -9.0% to 18.0%) and precision ($< 16.8\%$) across quality control samples, including lower limit of quantification samples, for all analytical targets. Additionally, the other validation tests, including selectivity, carryover, dilution integrity, stability, reinjection reproducibility, also satisfied the acceptance criteria specified in the ICH M10 guideline. However, during the specificity evaluation, we noted interference signals from impurities of reference standards. Nevertheless, we have confirmed that these interference signals do not affect the measured values when using the reference standard mixture of mipomersen and its metabolites/analogues at the same concentration. In conclusion, we have developed a bioanalytical method for the simultaneous quantification of mipomersen and its metabolites/analogues in the liver and kidneys. This method holds promise for accurately quantifying ASOs and their metabolites in tissues, thus offering valuable insights for the development of ASO therapeutics.

P7 - ENHANCED EFFECT OF IONIC LIQUID ON INTESTINAL ABSORPTION OF MACROMOLECULES (< 10 kDa)

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Introduction: Ionic liquids are defined as organic salts composed of an organic cation and an organic anion. We recently showed that ionic liquids have a potential to increase intestinal absorption of large molecules up to 20 kDa. However, the mechanism behind the increased intestinal absorption is not clear yet. In this study, therefore, we studied the effect of ionic liquids on tight junction (TJ) in the intestinal epithelium and on the intestinal translocation of orally administered compounds, which may contribute to the increased absorption.

Methods: To study whether the treatment with ionic liquids affects intestinal cellular adhesion between the intestinal epithelium cells, ionic liquids were orally administered and then the change of TJ protein Claudin 3 expression was studied. To study the effect ionic liquids on translocation of orally administered compounds, indocyanine green (ICG) in the ionic liquids were orally administered to mice and ICG localization in the small intestine were recorded at 1 h by the *in vivo* imaging system (IVIS). To study the effect of ionic liquids on small intestinal or colon absorption of macromolecules, FITC-dextran 10 (FD 10) (MW: 10 kDa), with/without ionic liquids was directly administered into the small intestine or colon of rats. Blood samples were collected over time and measured for fluorescence intensity (FI) of FITC of FD.

Results: The expression of the TJ protein Claudin 3 clearly decreased in ionic liquids concentration-dependent manner. This finding demonstrates that ionic liquids may decrease cell-to-cell interactions in the small intestine presumably by decreasing the expression of Claudin 3. Consequently, the enhancement of macromolecules absorption by ionic liquids, previously observed, was probably mediated by the paracellular route. In addition, the dynamics of ICG in the small intestine after oral administration to mice was observed by IVIS. The ICG solution showed that ICG moved rapidly through the intestine, whereas the ionic liquid group relatively moved through slowly. We observed higher FD absorption when FD 10 with ionic liquids was directly administered into the small intestine, suggesting that ionic liquids improve the absorption of macromolecules from the small intestine. These findings suggest that the enhanced absorption of macromolecules by ionic liquids probably occurs due to prolonged residence time in the small intestine.

Conclusion: In this study, we showed that the macromolecules-intestine absorption enhancement effect of ionic liquids may be caused by the prolonged residence time of administered compounds in the small intestine and increased permeability to macromolecules via opening TJ between intestinal epithelial cells.

P8 - MUCOADHESIVE HEMI-POROUS JANUS MICROPARTICLES: POTENTIAL FORMULATION TO IMPROVE INTESTINAL ABSORPTION FOR BCS CLASS 3 DRUGS

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Introduction: Recently, therapeutic biological products have addressed unmet medical needs. The most active ingredients of these products often fall under Class 3 of the Biopharmaceutics Classification System (BCS). For Class 3 drugs, absorption enhancers play an important role in improving their intestinal permeability. However, these enhancers exhibit a dose-dependent effect, and dilution in intestinal fluid can often lead to critical issues. Typically, conventional formulations

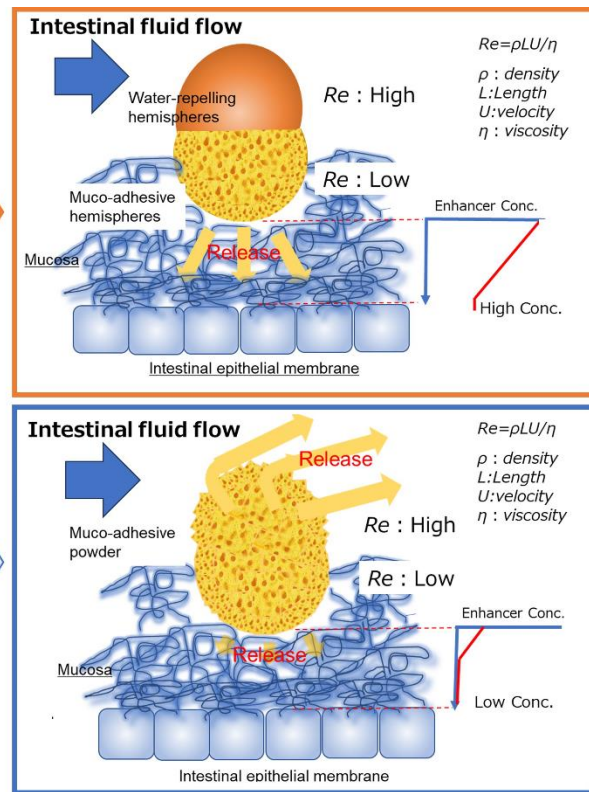
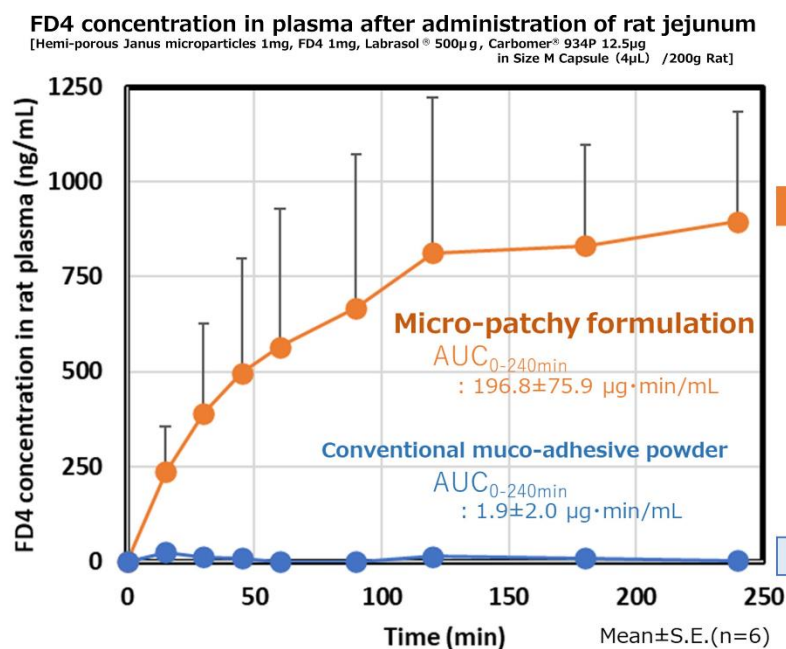
release drugs omnidirectionally. However, absorption enhancers released into the luminal side are susceptible to dilution by intestinal fluid. Janus particles, characterized by their heterogeneous nature and distinct hemispheres each serving different functions, offer a promising solution. We designed a novel “hemi-porous Janus microparticle,” comprising both a rigid and porous hemisphere to enable uni-directional drug release. These hemi-porous Janus microparticles equipped with mucoadhesive properties impregnate drugs, absorption enhancers, and mucoadhesive polymers into their porous hemispheres, releasing these substances only on the mucus side. In this study, we investigated the jejunum and ileum absorption of Class 3 drugs in rats using mucoadhesive hemi-porous Janus microparticles. We also explored the possibility of improving intestinal drug absorption by preventing the dilution of absorption enhancers in the micro-order area.

Methods: A hemi-porous Janus microparticle was fabricated with the water-in-oil emulsion-type solvent evaporation method using ethyl cellulose, hypromellose phthalate, and hard fat. Fluorescein isothiocyanate-dextran (FD4) with a molecular weight of 3000–5000 Da was used as a model Class 3 drug. To prepare the hemi-porous Janus microparticles, an aqueous solution of FD4, Labrasol®, and Carbomer® was impregnated into the microparticles by decompression and allowed to dry overnight. FD4 release was evaluated via a dissolution test using 0.05% polysorbate 80-JP 2nd fluid. The formulations were administered into rat jejunum or ileum, and blood samples were collected from the jugular vein at predetermined intervals. FD4 concentrations in the plasma were measured using fluorometry. The drug release from the formulations in the mucus model was simulated using the lattice Boltzmann method.

Results: Mucoadhesive hemi-porous microparticles achieved complete drug release within 30 min and demonstrated a similar *in vitro* drug release profile to that of conventional mucoadhesive formulations (mixture of FD4, Labrasol®, and Carbomer® 934P). Based on the drug release simulation from the microparticles between the two layers of low (mucus) and high (lumen) Reynolds numbers, drug release from a conventional formulation on mucus was estimated to occur preferentially towards the luminal side, whereas drug release from the microparticles to the luminal side was effectively suppressed. Compared to the conventional formulation, the AUC of FD4 after the administration of the mucoadhesive Janus microparticles in the jejunum or ileum of the rats was significantly elevated by up to 100 times, varying according to the installation site and intestinal fluid flow.

Conclusions: This study suggests that mucoadhesive hemi-porous microparticles have the potential to improve the intestinal absorption of Class 3 drugs by preventing the dilution of absorption enhancers in the micro-order area.

Additionally, these mucoadhesive hemi-porous microparticles can be used for the effective enteral delivery of Class 3 drugs, such as polypeptides and nucleic acids.



P9 - A HYBRID TRIAL APPROACH BY COMBINING ¹³C8-LABELED AND RADIOLABELED BRILAROXAZINE TO ASSESS ABSOLUTE BIOAVAILABILITY AND H₁ IN A SINGLE CLINICAL TRIAL

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Brilaroxazine (RP5063) is an investigational drug in development by Reviva Pharmaceuticals for the treatment of neuropsychiatric and inflammatory disorders. It is a serotonin-dopamine modulator displaying partial agonist activity at D₂/3/4 and 5-HT_{1A}/2A receptors and antagonist activity at 5-HT_{2B}/7 receptors with binding affinity for 5-HT_{2B} > D₂. Both comprehensive absolute bioavailability (ABA) and ¹⁴C-human studies of brilaroxazine have been conducted in the same healthy human subjects at Frontage Laboratories, Inc. to explore the ABA, pharmacokinetics, metabolism, and excretion of brilaroxazine. Eight healthy male human subjects were administered a single oral dose of brilaroxazine (15 mg) followed 4 hours later (~C_{max}) by an IV microdose of ¹³C₈-labeled brilaroxazine at 100 µg. Blood/plasma were collected at pre-determined time points up to 192 hr post-oral dose. Concentrations of ¹³C₈-labeled and non-labeled brilaroxazine were determined in human plasma using high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) using the validated methods. After 5 half-lives washout period, six of the eight human subjects were continually administered a single oral dose of [¹⁴C]brilaroxazine at 15 mg (~163 µCi). Whole blood, plasma, urine, and feces were collected at pre-determined time points up to 456 hr post-dose. Total radioactivity in whole blood, plasma, urine, and feces were determined by liquid scintillation counting (LSC) or combination of combustion and LSC. The radioactive parent/metabolite profiles in pooled plasma, urine, and feces of each subject were obtained by HPLC with radioactive detection, and major radioactive metabolites were identified by LC/HRMS and/or by comparisons with synthesized reference standards. High absolute bioavailability (~73%) were obtained for brilaroxazine in healthy humans. Approximate 86% of the dose was recovered in feces (~53%) and in urine (~33%). More than 90% of the radioactive components have been characterized in plasma, urine, and feces. Oxidation, N- or O-dealkylation with subsequent sulfation and glucuronidation were the metabolic pathways of brilaroxazine in humans. The hybrid trial approach by combining stable isotopically labeled and radiolabeled drug candidates have been successfully applied to brilaroxazine to improve quality and efficiency for both absolute bioavailability and absorption, distribution, metabolism, and excretion in a single clinical trial study under the same healthy human subjects.

P10 - DEVELOPMENT OF DISSOLUTION EVALUATION SYSTEM FOR DRUG ABSORPTION (V): ADDITIONAL INTESTINAL ABSORPTION MODULE OF *IN VITRO* 3D GASTRIC EMPTYING MODEL FOR ORALLY DISINTEGRATING TABLET

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Purpose: We developed a new system for the 38th annual JSSX poster presentation that mimics the disintegration and swallowing of OD tablets (BCS1, 2, 3) in the human oral cavity after oral administration without water intake, and evaluates *in vivo* gastric content emptying, dissolution, precipitation, and redissolution. In this study, we conducted an additional research on the IVIVC when applying a new intestinal absorption module (50 mL Rapid FlowTM, Disposable Filter Unit: 0.22 µm) of this system to OD tablets with enteric coating granule of lansoprazole.

Methods: (1) We investigated the solubility of lansoprazole in water, artificial intestinal fluids in the fasting condition (FaSSIF) and the fed condition (FeSSIF). The disintegration of the OD tablets (artificial saliva volume/tongue pressure) was compared under various conditions. (2) Using a combination of an oral surgery module and a 3D stomach model, OD tablets were orally administered in the oral cavity without water with artificial saliva and a constant tongue pressure. On the other hand, each OD tablet was administered to the stomach with 150 mL/250 mL of water. By using a rolling ball-type massage device in simulated gastric fluid supplied at a predetermined ratio, gastric emptying fluid was collected over time. The drug was dissolved/precipitated and redissolved in the duodenal pH shift medium (pH 6.5) of the new intestinal absorption module.

Results and Discussion: (1) The disintegration behavior of OD tablets was confirmed to vary depending on the artificial saliva volume and tongue pressure. We were able to set conditions that adequately reflect the *in vivo* disintegration behavior in this device. (2) The disintegration and dissolution behavior of each OD tablet when orally administered with 150 mL of water and without water intake varied depending on various artificial saliva, gastric content emptying rate, and water content / pH shift / surfactant concentration of the intestinal absorption module.

Conclusions: These results indicate that this new system, which includes an oral absorption module to mimic disintegration and dissolution in the human gastrointestinal tract and its effects on OD tablets, is a useful system for estimating the *in vivo* performance of test formulations.

P11 - IDENTIFICATION OF PHYSIOLOGICAL FACTORS SUPPRESSING COLONIC DRUG ABSORPTION USING QUANTUM IMAGING TECHNOLOGY

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The large intestine has typically been considered an unimportant site for drug absorption due to its smaller surface area and tight junctions. However, the longer residence time and lower luminal fluid volume in the large intestine suggest that drugs are exposed to higher concentrations for longer periods. This implies that, theoretically, the large intestine could be a good absorption site for drugs. Therefore, understanding the differences in absorption sites after oral administration of drugs is important for the efficient development of oral drugs, including formulation development. In the present study, we aim to quantitatively characterize drug absorption in the small and large intestines of rats and identify the factors that suppress drug absorption in the large intestine. First, the absorption of various drugs (antipyrene, theophylline, metoprolol, metformin, amoxicillin, and atenolol) in the jejunum and colon was evaluated using an *in situ* closed-loop method. The results revealed that the intestinal permeability of antipyrene and metoprolol was slightly lower in the colon than in the jejunum, whereas theophylline, metformin, amoxicillin, and atenolol showed similar permeability in both jejunum and colon. Since the intestinal permeability obtained from these *in situ* experiments is an apparent value when the intestinal tract is regarded as a simple tube, the theoretical permeability was calculated taking into account the difference in the surface area caused by the circular folds and villi. In this case, significantly higher permeability was estimated in the colon than in the jejunum for all drugs. Next, the contribution of the colon to the oral absorption of each drug was evaluated by *in vivo* oral administration studies in rats undergoing cannulation surgery to prevent drug entry into the colon. No significant differences in the plasma concentration of each drug were observed between cannulated and control rats. These findings suggest that the contribution of the large intestine to the oral absorption of drugs is very small, even though the large intestine has the potential ability to absorb drugs. To investigate the factors that suppress drug absorption in the colon, *in vivo* imaging using quantum dots was applied to visualize drug disposition in the gastrointestinal tract. The result showed that drugs were accumulating in the feces 30 minutes after oral administration. These findings suggest that fecal uptake of the drugs significantly limits gastrointestinal absorption, especially colonic absorption, after oral administration.

P12 - EXCEEDING 100% BIOAVAILABILITY IN DOG PK: IMPLICATION OF CYP2D15 POLYMORPHISM

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The extensive investigation into Cytochrome P450 (CYP) polymorphism underscores its significant role in the variability of drug pharmacokinetics (PK) across both nonclinical and clinical settings. While polymorphic CYPs like CYP2D6/CYP2C8 have received considerable attention in human studies, research into the strategy, implications, and mechanistic understanding of polymorphic CYPs (e.g., CYP2D15) in PK variability from nonclinical species such as dogs remains relatively limited. The aim of this study was to elucidate the abnormal bioavailability (>100%) observed in dog PK and to develop *in vitro* and *in vivo* DMPK strategies to mitigate risks associated with polymorphic CYPs during nonclinical development.

IDY-1, a small molecule, exhibited bioavailability (%F) surpassing 100% and significant inter-subject exposure variability in dog PK studies. To decipher the anomaly in oral bioavailability and exposure variability, *in vitro* and *in vivo* reaction phenotyping studies were conducted, including the use of the reference drug celecoxib. The results indicated that IDY-1 is metabolized by CYP2D15 in dogs and that the abnormal %F was attributable to a mixed utilization of poor metabolizers (PM) and extensive metabolizers (EM) in the dog IV/PO PK study. A cross-over dog PK study was undertaken to obtain more precise %F values and to further validate the involvement of CYP2D15 in drug metabolism.

Considering that dogs are the primary nonrodent species utilized for drug developability assessment, understanding the impact of CYP2D15 polymorphism on drug metabolism from early stages becomes imperative. Thus, we propose integrating a parallel study design into initial dog PK studies, encompassing both CYP2D15 EM and PM, facilitating early detection of CYP2D15 polymorphism's influence on the drug candidate. In instances where notable exposure variability is observed in dog PK studies that included EM and PM dogs, additional *in vitro* phenotyping studies may be conducted to further evaluate CYP2D15's contribution to drug metabolism. Phenotyping of the dog colony is essential to identify EMs and PMs when transitioning to toxicity studies with the drug candidate.

P13 - VALIDATION OF QPCR METHOD TO DETECT HUMAN CELLS IN MOUSE AND RAT TISSUES

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Introduction: In preclinical safety assessment of human cell products, understanding their biodistribution in animal models is important for the evaluation of safety and efficacy. qPCR assays targeting Alu repeat elements (Alu-qPCR) have high sensitivity and are therefore commonly used for evaluating the biodistribution of human cells. However, because of their very high sensitivity, Alu-qPCR have also a higher risk of getting false positive results caused by the contamination with human DNA during the sample handling. We previously showed that DUF1220, a human lineage-specific multi-copy gene, could be used as a potential target with high sensitivity and low background signals to assess the biodistribution of human cells. In the study presented here, we validated Alu-qPCR and DUF1220-qPCR considering the needed amount of template DNA and matrix effects using DNA from NOG mice and nude rat rodent models. **Materials and Methods:** Naïve NOG mice or nude rats are typical immunodeficient animals used in the biodistribution study of cell products. Genomic DNA was extracted from liver and spleen, and mixed for each animal species for use as matrix DNA. Calibration curves, specificity, accuracy and precision (intra- and inter-assay), matrix effect (extracted DNA from blood, liver, lung, spleen, and testis), limit of detection, and recovery were tested as validation items for qPCR. For recovery, A549 cells, a cell line derived from human lung epithelial cells, were added to the collected biological samples from NOG mice (liver) and nude rats (blood and liver), and genomic DNA was extracted from the samples and analyzed by both qPCR assays.

Results: For Alu-qPCR, the template amount was 10 ng/reaction with no interference by background signals and no matrix inhibition. For DUF1220-qPCR, matrix inhibition was observed at 1000 ng/reaction during validation, and the template amount was set to 100 ng/reaction. The quantitative range of the Alu-qPCR and DUF1220-qPCR was confirmed at 103-108 and 50-107 copies/reaction, with detection limits of 500 and 10 copies/reaction. Accuracy for the Alu-qPCR and DUF1220-qPCR was $\pm 40\%$ and $\pm 50\%$, and precision was 30% and 25%. In specificity, no Cq values were detected in the DUF1220-qPCR; however, background signals were detected in the Alu-qPCR. For recovery, the Alu and DUF1220 sequences were detected in biological samples containing 100 to 10,000 cells, and for the liver, there were no clear differences in the copy numbers for DUF1220 and Alu sequences determined in both species. The results of all items of the above validation in the Alu-qPCR and DUF1220-qPCR, respectively, were similar in the presence of both mouse and rat matrices.

Conclusion: The appropriate amount of template DNA in the DUF1220-qPCR was 10 times more than that in Alu-qPCR. Both assays were reliable for the detection of human cells in biological samples from NOG mouse and nude rat. No differences were observed in the validation results between the matrices from the two different rodent species. In this poster, we will discuss the pros and cons of both assays.

P14 - EVALUATION OF DIFFERENT TISSUE PROCESSING METHODS IN BAMA PIG SKIN AS AN ANIMAL MODEL FOR TOPICAL DELIVERY SYSTEMS

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WuXi AppTec, Suzhou, Jiangsu, China

Bama pig skin is commonly used as an animal model for TDS due to its close match for human skin. Literature reports suggest that pig skin exhibits similar histology and biological structure to human skin, making it the optimal animal model for assessing TDS. Based on the measured skin layer thickness, we can isolate the stratum corneum, epidermis, dermis, and subcutaneous tissue for further analysis. To ensure accurate compound content analysis in the skin tissues, a homogenization process was required.

In this poster, we compared the bead milling method with the traditional homogenization method to determine their impact on sample processing efficiency and cross-contamination avoidance.

The bead milling method demonstrated significant advantages over the traditional homogenization method. It improved sample processing efficiency by several orders of magnitude and effectively avoided the risk of cross-contamination. Microscopic observations of the skin samples processed using these methods revealed that the bead milling method resulted in smaller and more uniform particle sizes compared to the traditional homogenization method. Furthermore, the bead milling method proved suitable for handling unstable compounds. Its low-temperature operation provided a stable environment for processing thermally sensitive substances.

In conclusion, the bead milling method offers substantial improvements in sample processing efficiency and contamination control compared to the traditional homogenization method. Its ability to generate smaller and more uniform particles makes it an attractive choice for TDS assessment using Bama pig skin. Additionally, the bead milling method is versatile and can accommodate the processing of unstable substances.

P15 - IMPLEMENTATION OF BLOOD COLLECTION USING THE TASSO MICROSAMPLING DEVICE TO MEASURE THYROID HORMONE BIOMARKERS

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Collection of samples for biomarker analysis is typically conducted by a trained phlebotomist at a clinic or hospital and requires a painful blood draw and several milliliters of blood. The large volume of blood required makes pediatric blood sampling difficult and the travel requirement for the collection can be burdensome for the elderly or critically ill. The Tasso-M20 device enables at-home, automated, self-collection of sample aliquots. The device automatically collects four 17.5 microliter samples in minutes with only a few easy steps and very little pain or blood loss. The accurate quantitation of two thyroid biomarkers extracted from the Tasso-M20 device and analyzed using HPLC-MS/MS was performed in this study. Thyroid hormones Thyroxine (T4) and 3,3',5-triiodothyronine (T3) are regulators of metabolism, growth, and development. The levels of T3 and T4 are useful biomarkers of overall thyroid function. Hyperthyroidism or hypothyroidism can result from autoimmune disorders, such as Graves' disease, certain medications, thyroid cancer, and can often occur during pregnancy. Individuals suffering from thyroid dysfunction suffer from a broad range of symptoms including weakness and fatigue. A method was developed to extract T3/T4 from blood collected using a Tasso device followed by HPLC-MS/MS analysis. For standard preparation T4 and T3 were spiked into a surrogate matrix and 17.5 microliters of this solution was pipetted onto exposed blank Tasso-M20 tips. The tips were dried at ambient and placed into a DWP 96 and spiked with stable label internal standards and 500 microliters of methanol containing 1% formic acid. After 60 minutes of shaking, 400 microliters of the extract was transferred, evaporated to dryness, and reconstituted with 100 microliters of 80:20 Methanol:H₂O containing 1% formic acid. The extract was analyzed on a SCIEX triple quad mass spectrometer. The binary HPLC method utilized an Agilent Pursuit Diphenyl column and Water/Acetonitrile mobile phases containing formic acid. TASSO samples were collected from 17 individual donors. Sixteen of the donors had no diagnosed thyroid dysfunction and one donor was diagnosed with hyperthyroidism prior to testing. The samples were analyzed in duplicate and T3/T4 was quantified from an eight point calibration curve (0.100-500 ng/mL dynamic range) prepared in surrogate matrix and analyzed in duplicate. The data collected from the 16 healthy volunteers had an average T3 value of 0.380 ng/mL and an average T4 value of 24.5 ng/mL. The subject with diagnosed hyperthyroidism was found to have a T3 value of 1.28 ng/mL (over 3X the pool average) and a T4 value of 237 ng/mL (over 9X the pool average). This data suggests that the TASSO collection device couple with HPLC-MS/MS analysis can be used as a tool to help diagnose T3/T4 thyroid conditions.

P16 - ADDRESSING THE CHALLENGES OF DETERMINING FRACTION METABOLIZED FOR LOW TURNOVER DRUGS – AN UPDATE FROM THE QUANTITATIVE FRACTION METABOLIZED WORKING GROUP OF THE IQ CONSORTIUM

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The proportion of a drug's metabolism mediated by a particular enzyme or metabolic pathway is a key determinant of pharmacokinetic variability and susceptibility to victim DDI. Early in the drug development process a quantitative prediction of fraction metabolized is conducted using hepatic cell-based assays, subcellular fractions, recombinant expression systems, and selective enzyme inhibitors. Although assays for selective inhibition of CYP enzymes, the primary drug metabolizing enzymes of human liver, have been established for several decades, experimental challenges persist, and practices vary widely across the industry. Of particular interest is the universal challenge of characterizing drugs with low metabolic turnover with the current tools. The objective of the Quantitative Fraction Metabolized IQ Working Group is to define an industry baseline for quantitative *in vitro* assessment of fraction cleared by metabolic pathways using current and novel tools and practices. First, the team identified substrates for phenotyping studies based on the availability of human ADME data. Mechanism-based enzyme inhibitors were then chosen based on selectivity for individual CYPs and to limit the risk of PXR activation and cytotoxicity in long term culture systems. Next, inhibition studies were conducted in three model assays to assess the utility for conducting reaction phenotyping with low turnover compounds: Hurel, Hepatopac and hepatocyte suspension relay.

Initial experiments conducted in the 3 models selected for reaction phenotyping evaluation (Hurel, Hepatopac and Hepatocyte Relay) showed that most of the probe substrates' intrinsic clearance values were low, with values of less than 3 uL/min/million cells. The results were similar across assays (within ~3-fold) and agreed well with current literature findings. The inhibitor concentrations selected demonstrated a reduction in both substrate depletion and metabolite formation of up to 95% which was maintained for a minimum of 4 days in long term cultures. Inhibitor concentration and metabolite formation were monitored throughout the incubation and used to establish timing of inhibitor replenishment.

These data suggest that the approach of using mechanism-based inhibitors was advantageous for conducting reaction phenotyping assessments in suspension and long-term hepatocyte cultures. The results of these efforts and plans for further expansion will be discussed.

P17 - INCORPORATION OF WELL-STIRRED, DISPERSION AND PARALLEL TUBE LIVER MODELS INTO SIMPLE AND PERMEABILITY LIMITED PBPK FRAMEWORKS FOR CLEARANCE PREDICTION

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Hypothesis: Well-stirred (WSM), parallel tube (PTM), and dispersion (DM) models modeled as one-compartment (1C) and permeability-limited livers can be incorporated into whole-body physiologically based pharmacokinetic (PBPK) frameworks, to improve clearance predictions.

Methods: 1C and permeability-limited model were to evaluate the ability of microsomal and hepatocyte intrinsic clearances to predict hepatic clearance. WSM, PTM, and DM models were evaluated within the liver as well as within whole-body PBPK frameworks. Curated datasets^{1,2} including *in vitro* microsome and hepatocyte clearance (CL) an *in vivo* CL were used for model comparison. The curated Tess dataset¹ included n=32 drugs (16 acids, 11 bases, 5 neutrals), and the PQ dataset² included n=16 drugs (4 acids, 9 bases, 3 neutrals). A scaling factor that reduced interstitial fluid (ISF) plasma protein binding of acids was used, and compared with a published scalar³ that reduces drug plasma protein binding.

Results: A linear combination of well-stirred and parallel-tube average liver blood concentrations to approximate dispersion model blood concentrations was derived. Using a flow/permeability-limited model, a very large systematic error was observed for acids and no systematic error for bases. A scaling factor that reduced interstitial fluid (ISF) plasma protein binding could greatly decrease the AAFE for acids. Using a 1C model, a scalar to reduce plasma protein binding decreased the microsomal clearance AAFE for both acids and bases. We also show that for hepatocyte intrinsic clearance *in vitro-in vivo* correlations (IVIVCs), a 1C model is mechanistically appropriate since hepatocyte clearance should represent the net clearance from ISF to elimination. A relationship was derived that uses microsomal and hepatocyte intrinsic clearance to solve for an active hepatic uptake clearance, but the results were inconclusive.

Conclusions: A linear concentration of PTM and WSM average liver blood concentrations accurately reproduces the average liver blood concentration for the DM. Therefore, the WSM, PTM, and DM liver can be evaluated and incorporated into ODE-based PBPK models. A scalar to reduce plasma protein binding decreased microsomal 1C model clearance AAFE for both acids and bases. A possible explanation for the errors observed with the permeability-limited model is that pH partitioning into hepatocytes increases the intracellular concentration of bases and the scalar for plasma protein binding corrects for the lack of pH partitioning in 1C models. The mechanism of the apparent increased cytosolic concentrations for acids remains unknown. Finally, the PTM model generally performed better than the WSM or DM models, suggesting the presence of additional systematic IVIVC errors. Additional studies will be necessary to better model the intricacies of hepatic clearance. Funding: Grants NIGMS 2R01GM104178 and 3R01GM114369.

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P18 - SELECTION OF LIVER MODELS OF HEPATIC DRUG CLEARANCE: IMPROVEMENTS AND IMPLICATIONS FOR IVIVE AND PBPK MODELING

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There is an overwhelming preference for application of the unphysiologic, well-stirred model (WSM) over the parallel tube (PTM) and dispersion (DM) models to predict hepatic drug clearance, CL_H, despite that liver blood flow is dispersive in nature¹. The reasoning is due to ease in computation in relating the hepatic intrinsic clearance (CL_{int} or V_{max}/K_m), total hepatic blood flow (QH), unbound fraction in blood (f_{ub}) and the transmembrane clearances (CL_{in} and CL_{ef}) to CL_H. To demonstrate that the miniPTM of 3 subcompartments best mimic DM properties in terms of CL_{int} and CL_H, we simulated data based on the same drug extraction ratio (EH) to compare properties of the models. The WSM was found to be the least physiologic model that required a higher CL_{int} over those for the miniPTM, PTM and DM, models that are similar, to yield the same EH: CL_{int},WSM > CL_{int},DM > CL_{int},PTM. Trends for the unbound substrate concentration in liver [S] and the liver to outflow blood concentration ratio, KL/B, were opposite: PTM > DM > WSM. Because CL_{int}, [S] and KL/B are model-dependent, we also examined given V_{max}s and K_{ms} on sulfation and glucuronidation of a flow-limited substrate, harmol, into rat perfused liver and *in vivo* studies. Liver models nested within PBPK models were used to describe whole body kinetics of the harmol and conjugates, using the WSM (one single compartment), a 3-compartment tank-in-series model of uniform enzymes (miniPTM), and zonal liver models (ZLM) of unevenly-distributed glucuronidation and sulfation

activities to predicted CLH. The WSM furnished the lowest extraction ratio (EH,WSM = 0.5) compared to EH,miniPTM and EH,ZLM (>0.68), and the Vmaxs for sulfation and glucuronidation of the WSM were raised 5.7 to 1.15-fold to enable EH,WSM to approach EH,miniPTM and EH,ZLM, confirming the need for scaling factors. The strategy of using the CLint,miniPTM to approximate the CLint,DM yielded sound values for CLH and improved IVIVE, and provided a sound approach towards selection of the miniPTM as the CLH model. However, newer definitions for KL/B for the miniPTM must be used for PBPK modeling. The higher KL/B values for the miniPTM, PTM, (DM), and ZLMs will increase the effective volume of distribution of the liver, and ultimately, the total volume of distribution of drug at steady-state².

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P19 - INTESTINAL SECRETION IS POTENTIALLY AN IMPORTANT CLEARANCE MECHANISM FOR HIGHLY PLASMA-BOUND AND LOW METABOLIC CLEARANCE EFFLUX SUBSTRATES

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Intestinal secretion/excretion (IE) of drug-related material from the systemic circulation via the enterocytes into the intestinal lumen has traditionally been considered a minor clearance (CL) pathway. Other clearance routes such as metabolism, biliary and renal elimination have rightfully garnered more attention, and have been systematically studied in humans and animals. Current drug discovery paradigms have been able to significantly minimize metabolic and renal clearance. Hence, clearance routes such as biliary elimination and intestinal excretion (IE) have come into greater prominence. In particular, for highly metabolically stable molecules that are not eliminated via the bile, IE can present as an important clearance mechanism. In the present work, we have analyzed IE observed in animal studies from the Gilead database.

PK studies from SD rats, beagle dogs and cynomolgus monkeys were included in this analysis. In total 91 studies were analyzed of which 55, 7, and 29 were from rat, dog, and monkey, respectively. Fourteen of the molecules had more than 20% of IV dosed parent in feces while 27 of the molecules had more than 10% of IV dosed parent in feces. The chemicals represented in this analysis cover a wide range of targets and physiological-chemical spaces. The cLogD for these molecules ranged from -1 to 8, while the *in vivo* hepatic extraction ratios ranged from 0.0008 to 2.7. The % of parent in the feces also varied widely from 0 to 50%, while the plasma protein binding ranged from 0.01 to 98% unbound. IE clearance (IE CL) was calculated by multiplying the systemic IV CL with the % of parent found in feces from an IV Bile-Duct-Cannulated (BDC) study. IE extraction ratio (IE ER) was calculated by dividing IE CL by the hepatic blood flow in that species in order to compare IE ER with hepatic CL extraction ratio.

Several correlations were discernable from the data. It was seen that lower-clearance molecules had a higher propensity for showing intestinal clearance. This is intuitively reasonable since compounds with low clearance have excellent metabolic stability and minimal renal clearance and need other routes of elimination such as IE. It was also observed that compounds with low plasma binding are less susceptible to IE. It was also verified that there was no correlation between IV clearance and plasma free fraction in this dataset- hence the correlation between IE and plasma protein binding is independent of clearance considerations. It was also observed that being an efflux substrate in the Caco-2 assay was a necessary precondition for significant IE. It was found that if the Caco-2 efflux ratio was less than 3.5, then the IE ER was < 4% and hence of minor consideration. In conclusion, this is the first systematic review of IE that we are aware of, and several important correlations and criteria that govern IE have been identified. Highly bound, low systemic clearance molecules that are an efflux transporter substrate are more susceptible to IE, and in general, the IE ER doesn't exceed 6% with some exceptions.

P20 - CONFOUNDING EFFECTS OF B-GLUCURONIDASE ON UDP-GLUCURONOSYLTRANSFERASE STABILITY: DEVELOPMENT OF A MODIFIED METABOLIC STABILITY ASSAY TO ENABLE SAR FOR A DISCOVERY PROGRAM

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Initial PK profiling for a discovery project illustrated that compounds possessed low oral bioavailability in mouse (<1 %F). Identification of factors leading to poor oral bioavailability in mouse was essential to enable compound optimization and progression of the series in PK/PD and safety studies. Compounds from the program contained carboxylic acids, making them susceptible to glucuronidation by UDP-glucuronosyltransferases (UGTs) along with cytochrome P450-mediated oxidation. Glucuronidation was identified as the major metabolic route in mouse, with very rapid metabolism to the acyl

glucuronide (AG) observed in hepatocytes *in vitro*. Despite significant AG formation, compounds often appeared stable in standard hepatocyte intrinsic clearance assays when measuring parent compound depletion. These conflicting results made it challenging to understand and profile compound metabolic stability, precluding necessary optimization to improve bioavailability. We hypothesized that AGs were efficient substrates for mouse β -glucuronidase which allowed for rapid regeneration of aglycone parent compound from the AG metabolite. To test this hypothesis, a UGT stability assay in mouse liver microsomes was conducted in the presence and absence of a β -glucuronidase inhibitor, D-saccharic acid 1,4-lactone (saccharolactone). In the absence of saccharolactone, parent depletion was linear for only a portion of the time course and often plateaued with a high concentration of compound remaining. With inclusion of the inhibitor, AG formation continued to increase throughout the incubation and linear parent compound depletion was observed, illustrating that β -glucuronidase-mediated futile cycling was confounding interpretation of UGT stability in standard assays. Over ~100 compounds from the program were profiled in a modified assay in the presence of saccharolactone to enable direct assessment of UGT stability. A large proportion of the compounds had very low UGT stability ($t_{1/2} < 4$ min) on initial profiling, but utilization of the assay enabled SAR and ultimately resulted in identification of metabolically stable (UGT $t_{1/2} > 180$ min) compounds, which significantly improved oral bioavailability in mouse. While saccharolactone is often not recommended for routine incubations, this work highlights that in cases where non-linear compound metabolism is observed, inclusion of a β -glucuronidase inhibitor can be beneficial to enable assessment of UGT stability.

P21 - EFFECTS OF MEFENAMIC ACID ON THE CONJUGATIVE METABOLISM OF INDOLE AND P-CRESOL IN RELATION TO CELLULAR NECROSIS IN A METABOLICALLY COMPETENT HUMAN HEPATOMA CELL LINE

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Background: Dietary p-cresol (4-methylphenol) and indole (1H-indole) are conjugated in the liver to produce p-cresol sulfate (pCS), p-cresol glucuronide (pCG), indoxyl sulfate (IS), and indoxyl glucuronide (IG), respectively. These are protein-bound uremic compounds that are accumulated in patients with renal disease. The two sulfonated metabolites (pCS and IS) have been associated with organ pathologies, but the formation of pCG is detoxifying in a metabolically competent liver cell line (HepaRG) as demonstrated in our lab. Recently, we reported the potent, non-competitive inhibitory effects of mefenamic acid (2-[(2,3-dimethylphenyl)amino]benzoic acid) on the formation of pCS (inhibitory constant, $K_i=2.4$ nM) in human liver cytosols [1]. We hypothesize that mefenamic acid can reduce the in-situ formations of pCS and IS (without affecting the glucuronidation pathways) and attenuate the cellular toxicities generated by p-cresol or indole in HepaRG cells. **Methods:** Cells were cultured per protocol (Biopredic International) in 24-well plates with a density of 0.4×10^6 cells/well (N=6 replicates). Cells were exposed to p-cresol or indole (0–1 mM) for 24 hours to determine the kinetics of pCS, pCG, IS, and IG formation. Subsequently, cells were treated with p-cresol or indole (0.5 mM, linear kinetic conditions) with or without mefenamic acid (3–30,000 nM) for 24 hours. Concentrations of pCS, pCG, IS, and IG in the culture supernatant were quantified using liquid chromatography-tandem mass spectrometry in our lab. Cellular necrosis was quantified by lactate dehydrogenase (LDH) activities. **Supporting Data and Results:** HepaRG cells exposed to 0–1 mM p-cresol or indole for 24 hours generated metabolites following substrate inhibition kinetics (maximum concentrations obtained for pCS was 29.98 ± 5.60 μ M at 0.5 mM p-cresol and 8.43 ± 3.00 μ M of IS at 0.5 mM indole) and Michaelis-Menten kinetics (maximum concentrations obtained for pCG was 479.95 ± 24.73 μ M at 1.0 mM p-cresol and 10.23 ± 6.08 μ M IG at 1.0 mM indole). In cells exposed to 0.5 mM p-cresol, mefenamic acid (0–30,000 nM) decreased the formation of pCS in a concentration-dependent manner. No evident LDH release was observed for mefenamic acid alone up to 30 nM, which reduced pCS formation by 41.53 ± 17.2 % ($p < 0.05$). On the other hand, mefenamic acid generated a trend toward concentration-dependent reductions in IS formation in cells exposed to 0.5 mM indole ($p > 0.05$). In all scenarios, mefenamic acid did not affect pCG or IG formation. Co-treatments of p-cresol or indole with 30 nM mefenamic acid reduced LDH release by 50.00 ± 16.07 % and 56.25 ± 9.80 %, respectively ($p < 0.001$ vs. p-cresol or indole alone) (Figure 1). **Conclusion:** Our novel findings indicate mefenamic acid could be utilized to reduce cellular necrosis generated by p-cresol or indole in HepaRG cells, but different mechanisms might be involved (i.e., inhibition of pCS formation with p-cresol but other un-identified pathways with indole). Experiments are ongoing with additional toxicity markers in this cellular model. **Reference:** [1] Rong, Y & Kiang, TK. Characterization of human sulfotransferases catalyzing the formation of p-cresol sulfate and identification of mefenamic acid as a potent metabolism inhibitor and potential therapeutic agent for detoxification. *Toxicol Appl Pharmacol* 425, 115553 (2021).

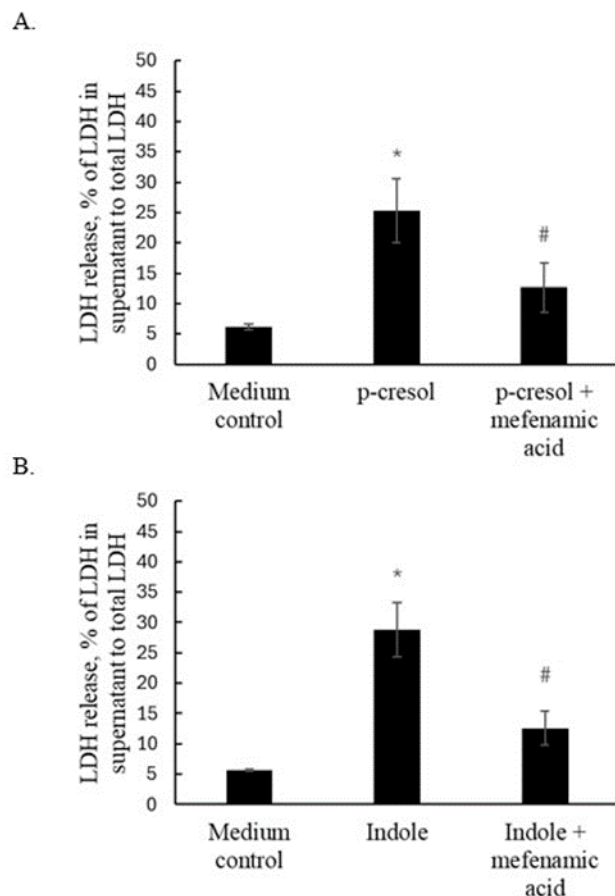


Figure 1. Effects of 30 nM mefenamic acid on LDH release in HepaRG cells treated with (A) 0.5 mM *p*-cresol or (B) indole for 24 hours (N=6; mean $\pm$ standard deviation) * p < 0.001 for *p*C or indole treatment vs. medium control; # p < 0.001 for *p*-cresol or indole combined with mefenamic acid vs. *p*-cresol or indole alone. Mefenamic acid alone was not toxic vs. the medium control.

P22 - SDS-STABLE HOMO-OLIGOMERS OF UDP-GLUCURONOSYLTRANSFERASE 2B7: INVOLVEMENT OF DISULFIDE BOND FORMATION VIA A SPECIFIC CYSTEINE RESIDUE

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UDP-Glucuronosyltransferase (UGT) plays an important role in drug metabolism by catalyzing the glucuronidation of hydrophobic chemicals. UGT is a type-I membrane protein localized in the endoplasmic reticulum. It has been reported that UGT forms a dimer, tetramer, and higher-order oligomers of the same or different isoforms. Interestingly, this oligomerization can alter UGT function by enhancing/suppressing conjugation ability, altering regio-selectivity, and acquiring new substrate-specificities. However, the molecular mechanism underlying such oligomer formation remains unclear. To address this issue, we transiently expressed UGT2B7 fused with a C-terminal HA-tag in COS-1 cells and analyzed homo-oligomer formation.

First, intracellular oligomers were cross-linked with two types of chemical cross-linker, disuccinimidyl suberate (DSS) or dithiobis(succinimidyl propionate) (DSP). UGT2B7 monomer and higher-order oligomers were detected by Western blotting using anti-HA and anti-UGT2B7 antibodies as the primary antibody. Both cross-linkers form covalent bonds targeting primary amines on the lysine side chain. DSS forms non-cleavable cross-links, whereas DSP forms cross-links that can be cleaved by reducing agents like thiols. When lysates from cells treated with these reagents were analyzed under nonreducing conditions, only higher molecular mass UGT2B7 forms were detected. In contrast, in the presence of reducing agents, monomers were detectable in the DSP-treated sample but not in the DSS-treated sample, suggesting

that the majority of UGT2B7 forms higher-order oligomers in cells. Some UGT2B7 oligomers could be detected by nonreducing SDS-PAGE without cross-linker. Such SDS-stable oligomers disappeared in the presence of a reducing reagent, which indicated that disulfide bonds are involved in the formation of UGT oligomers.

To identify the cysteine residue(s) important for the formation of inter-molecular disulfide bonds, systematic deletion mutants of UGT2B7 were generated and expressed in COS-1 cells. Although most of the mutants generated exhibited the ability to form SDS-stable oligomers, the mutant lacking amino acids 253-310 failed to form higher-order oligomers and was only detected as a monomer in nonreducing conditions by Western blot. Given that UGT2B7 has only one cysteine, Cys282, in this region, this cysteine was considered as a candidate for disulfide bond formation. This was confirmed when substitution of Cys282 by alanine markedly decreased formation of SDS-stable oligomers of full length UGT2B7 as well as of a mutant lacking amino acids 24-200 and 493-529. These results indicate that UGT2B7 has several oligomer-formation sites and that Cys282 plays an important role in forming inter-molecular disulfide bonds in UGT2B7.

P23 - THE REGULATORY ROLES OF THE LNCRNA HNF1AOS1 IN HEPATIC DRUG METABOLISM IN MICE

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The cytochrome P450 (CYP) enzymes, predominantly found in the liver, intestine, and kidney, metabolize over 60% of prescription drugs. They are considered as the primary drug- metabolizing enzymes in the liver. Various factors including genetic, epigenetic, and physiological influences modulate the expression of CYP enzymes, contributing to variations in metabolizing capabilities among the population. *in vitro* studies have shown that HNF1A-AS1, a long non-coding RNA (lncRNA) transcribed from the HNF1A-AS1 gene on the negative strand in Human Chromosome 12, can regulate expression of these enzymes under a basal or induced state by drugs. It is reported that many lncRNAs exhibit diverse expression patterns across cells, tissues, organs, and species, resulting in functions that vary based on the model types used in research. Certain mechanistic studies of HNF1A-AS1 have been conducted in human *in vitro* experiments. However, it is a challenge to conduct *in vivo* experiments in the human body. Thus, an animal model is essential to compensate for this disadvantage and gain comprehensive insights *in vivo*. There is little work regarding the regulation of lncRNA HNF1AOS1, a HNF1A- AS1 homolog in mice, in a complex biological model. To address this, the loss-of-function *in vitro* and *in vivo* mice models were established in this study to elucidate the regulation of HNF1AOS1 on hepatic drug metabolism. By designing siRNAs targeting HNF1AOS1 and selecting the most efficient one, initial *in vitro* experiments in Hepa1C1C7 cells demonstrated that silencing HNF1AOS1 significantly reduced CYP3A4 levels without impacting other CYP enzymes. Meanwhile, the *in vivo* studies utilized an AAV-shRNA with the same target site as the siRNA, confirming the successful transduction of the AAV-shRNA via *in vivo* imaging system (IVIS). Subsequent RT-qPCR analysis of CYP mRNA expression levels revealed diminished HNF1AOS1 expression leading to a decrease in CYP3A4 and an increase in CYP1A2 and 2E1 levels. These findings illuminate the distinct regulatory effects of HNF1AOS1 on drug metabolism in both *in vitro* and *in vivo* settings in mice. Ongoing RNA-sequencing, Proteomics studies, and enzyme activity experiments will be conducted to further determine the potential effects of HNF1AOS1 on various phase I, II, and III enzymes that play an important role in hepatic drug metabolism. Our study offers valuable insights into interspecies differences, demonstrating the feasibility and effectiveness of using animal models to investigate the role of human lncRNAs in drug metabolism research.

P24 - A SEVEN CYTOCHROME P450 INDEX SUBSTRATE COCKTAIL FOR CLINICAL TRIALS – PROFILING THE EFFECTS OF CLOPIDOGREL AND GEMFIBROZIL ON CYP ENZYMES AND OATP1B1 BIOMARKERS

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Introduction: Clinical cocktails based on administration of cytochrome P450 (CYP) index drugs simultaneously can be used for profiling of drug metabolism, for example in drug-drug interaction trials. However, none of the validated cocktails includes an index drug for CYP2C8, which plays an important role in the metabolism of multiple drugs. Moreover, the clinical effects of the CYP2C8 inhibitors clopidogrel and gemfibrozil on other CYP enzymes and OATP1B1 activity have not been studied sufficiently.

Aims: Our aim was to add the CYP2C8 index drug repaglinide to the Geneva cocktail (caffeine/CYP1A2, bupropion/CYP2B6, flurbiprofen/CYP2C9, omeprazole/CYP2C19, dextromethorphan/CYP2D6 and midazolam/CYP3A4) and investigate if its addition leads to any mutual interactions between the index drugs. To validate the cocktail approach and to improve understanding of the effects of clopidogrel and gemfibrozil on CYP enzymes and OATP1B1, we also aimed to investigate their effects on the full 7 CYP enzyme cocktail and organic anion transporting polypeptide 1B1 (OATP1B1) biomarkers.

Methods: Sixteen healthy volunteers were recruited to a randomized controlled trial with five phases. The following drugs were administered in the different phases: 1) 0.05 mg repaglinide, 2) the Geneva cocktail (50 mg caffeine, 20 mg bupropion, 10 mg flurbiprofen, 10 mg omeprazole, 10 mg dextromethorphan and 2 mg midazolam), 3) repaglinide with the

Geneva cocktail (full cocktail), 4) clopidogrel (300 mg dose) followed by the full cocktail, and 5) gemfibrozil pretreatment (600 mg twice daily for three days) followed the full cocktail. Venous blood samples were collected for 23 hours after index drug administration. Concentrations of the drugs and their metabolites were quantified with LC-MS/MS.

Results: The AUC of repaglinide was increased 1.2-fold by the Geneva cocktail, compared with repaglinide alone. Repaglinide slightly increased the 1-hydroxymidazolam/midazolam AUC0-23h ratio but did not affect the pharmacokinetics of the other index drugs. According to expectations, clopidogrel and gemfibrozil increased the AUC0-23h of repaglinide 4.5-fold (90% CI 3.7-5.5) and 7.5-fold (90% CI 5.9-9.5), respectively ($p < 0.01$). Similarly, the previously documented moderate CYP2B6 inhibition and weak CYP2C19 inhibition by clopidogrel were detected with the respective metabolite to index drug AUC0-12h ratios ($p < 0.01$). As novel findings, gemfibrozil slightly increased caffeine AUC0-12h and decreased the paraxanthine to caffeine AUC0-12h ratio by 23% (90%CI 14-31%, $p < 0.05$). Additionally, gemfibrozil increased the metabolite to index drug AUC0-12h ratios for CYP2C9, CYP2C19, CYP2D6 and CYP3A4 1.6-fold (90%CI 1.3-1.9), 1.5-fold (90% CI 1.3-1.6), 1.8-fold (90%CI 1.2-2.6), and 2.1-fold (90%CI 1.9-2.4), respectively ($p < 0.01$), without affecting index drug AUCs. Additionally, glycochenodeoxycholate 3-O-glucuronide plasma levels 0-4 hours after gemfibrozil intake were increased by 33% by gemfibrozil in subjects with normal or decreased function SLCO1B1 genotype, confirming that gemfibrozil is a weak inhibitor of OATP1B1. No adverse effects or hypoglycemia were observed.

Conclusions: The CYP2C8 index drug repaglinide can be safely administered with the Geneva cocktail to produce a seven-in-one CYP enzyme cocktail with applicability in drug-drug interaction and drug metabolism phenotyping studies. The results suggest that apart from strong CYP2C8 inhibition, gemfibrozil slightly inhibits CYP1A2 and can interfere with the elimination of the CYP index metabolites of flurbiprofen, omeprazole, dextromethorphan and midazolam.

P25 - SCREENING TOWARDS A CYP3A7-SPECIFIC MARKER REACTION AGAINST CYP3A4 AND CYP3A5

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CYP3A4 is widely recognized as the primary CYP enzyme involved in xenobiotic metabolism. However, in human embryonic, fetal, and newborn liver, CYP3A7 predominates as the most abundant CYP enzyme, and is still relatively underexplored. While substrates such as testosterone and dehydroepiandrosterone sulfate (DHEAS) are commonly used to study CYP3A7 activity, they are also metabolized by CYP3A4 to varying extents, making the search for a CYP3A7 selective substrate or marker reaction a challenging endeavor, similar to the challenge in differentiating CYP3A4 and CYP3A5 activities, as these 3 CYP3A enzymes present a significant substrate overlap. Hence, there is a pressing need to identify a specific marker substrate for CYP3A7.

In pursuit of this, we conducted a metabolic stability assay on approximately 3300 compounds, including FDA-approved drugs and drug-like molecules, using CYP3A4 and CYP3A7 supersomes. From this screening, we selected 64 compounds with a half-life ($t_{1/2}$) of less than 30 minutes for CYP3A7 and more than 30 minutes for CYP3A4.

Subsequently, these compounds underwent further metabolic stability testing against CYP3A4, CYP3A5, and CYP3A7 supersomes to identify candidates with a higher likelihood of being CYP3A7-specific among the major CYP3A enzymes.

Of the 64 compounds, 8 exhibited minimal depletion in CYP3A4 and CYP3A5 but significant depletion in CYP3A7. Metabolite identification (MetID) studies on these 8 compounds revealed that NCGC00179658 produced a unique metabolite with CYP3A7, indicating its potential as a marker substrate candidate. Subsequent incubation of NCGC00179658 with human liver microsomes, including those from 8 infant donors under 1 year old with presumably elevated CYP3A7 activity, failed to generate the unique CYP3A7 metabolite, even when the primary metabolic pathway of NCGC00179658 was inhibited by a CYP2C9 inhibitor.

This study outlines our efforts in the pursuit of a specific CYP3A7 marker substrate, particularly against CYP3A4 and CYP3A5, and its applicability in liver microsomal incubations. Additionally, we present metabolite identification data for all 8 compounds that had shorter $t_{1/2}$ in 3A7 compared to 3A4 in the supersome incubations. Additional research is being conducted on increasing the number of compounds of the screening. That will increase the likelihood of finding a CYP3A7 specific marker substrate that can be useful in a practical scenario such as with human liver microsomes setting.

P26 - PROTEIN EXPRESSION AS ENDPOINT FOR ASSESSMENT OF CYP2B6 INDUCTION: APPLICATION OF IMMUNO-AFFINITY CAPTURE METHOD REVEALS IMPROVED DYNAMIC RESPONSE RANGE

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Introduction: Regulatory guidance for CYP450 Induction DDI assessments generally recommends fold change in mRNA expression, with enzyme activity as an acceptable additional endpoint. Due to potential limitations of these methods, there's increasing interest in exploring the use of protein expression as an endpoint (Maclean et al., 2017, Savaryn et al., 2020, Savaryn et al., 2022). While these initiatives have successfully measured and showed correlation for mRNA, enzyme activity and protein expression for two of the major inducible CYP450 enzymes (CYP1A2 and CYP3A4), there was a significant disconnect between protein expression vs mRNA expression/enzyme activity for CYP2B6. This

disconnect triggered further investigations for methods to improve analysis of CYP2B6 protein expression.

Purpose: In this study, our goals were: a) investigate the use of immuno-affinity capture (IAC) enrichment method for measuring CYP2B6 protein expression; b) derive CYP2B6 induction kinetic parameters (EC₅₀ and E_{max}) using protein expression; c) compare results across three endpoints.

Methods: The CYP2B6 mRNA, enzyme activity and protein levels were measured following treatment of two lots of human hepatocytes with CYP2B6 prototypical inducers carbamazepine, CITCO, phenytoin, and rifampin.

Results: Using the IAC method for sample preparation, the EC₅₀ and E_{max} values for the CYP2B6 protein expression endpoint across the two lots were 6.8-28 μ M and 4.5-5.9-fold for carbamazepine, 1.1-6.9 μ M and 5.7-fold for phenytoin, 0.071-0.19 μ M and 6.8-7.4-fold for CITCO and 0.22-1.7 μ M and 5.3-5.9-fold, for rifampin, respectively. With the plate to peak (P2P) method, the CYP2B6 protein expression kinetic parameters could not be calculated due to poor dynamic response range. E_{max} values were generally $\leq$ 2.2-fold for all compounds tested in the two hepatocyte lots.

Conclusion: IAC shows improved CYP2B6 induction dynamic response range than P2P. The CYP2B6 kinetic values (EC₅₀ and E_{max}) derived from protein expression using IAC were generally comparable with those obtained from mRNA and enzyme activity endpoints. Therefore, when using the P2P method, it is recommended to supplement with IAC to determine the induction kinetic parameters for CYP2B6 protein induction response. The results further facilitate the use of protein as an additional or alternative endpoint for CYP2B6 induction assessment. Incorporating protein expression as an endpoint may enhance confidence in clinical DDI modeling predictions.

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P27 - A COMPREHENSIVE PLATFORM FOR EVALUATING THE *IN VITRO* CYP REACTION PHENOTYPING OF NEW MOLECULAR ENTITIES (NME)

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Purpose: Although the non-CYP enzymes are frequently involved in the elimination of many drugs, CYP is still the major enzyme involved in metabolic clearance. Careful assessment of the contribution of individual CYP toward the overall metabolism of a new molecular entity (NME) is therefore crucial to accurately assess the victim DDI liability of an NME in the clinic.

Method: Validated method will be used to monitor substrate loss or metabolite formation in two recognized test systems. Pooled human liver microsomes with specific chemical inhibitors and recombinant individually expressed human enzymes with required cofactors are both validated in our lab. Major CYPs (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A) are used as the standard design. If a discrepancy is found in these two systems for the identification of the major metabolic enzyme(s), additional efforts would include the following: (1) Addition of a time-dependent inhibitor for a CYP to ensure the specificity of the inhibitory effects on that specific CYP (2) Additional CYPs (CYP1A1, CYP2A6, CYP2E1, CYP2J2, CYP 3A5, CYP4F2) are all validated in our lab for the comprehensive assessment of the enzyme(s) involved.

Results & Discussion: Application 1: Results from the chemical inhibition study showed that CYP3A was the major metabolic enzyme and CYP2C8 played a secondary role in the metabolism of the test compound. However, CYP2C8 was identified as the major metabolic enzyme by recombinant CYP metabolism study. Additional CYP enzymes that might be inhibited by Ketoconazole, the chemical inhibitor of CYP3A used in the chemical inhibition study, were included in this study. CYP3A5 was identified as the major metabolic enzyme in reaction phenotyping study. Application 2: Results from the chemical inhibition study showed that CYP1A2 was the major metabolic enzyme. However, CYP2C19 was identified as the metabolic enzyme that contributed most to the clearance of the test compound by a recombinant CYP metabolism study. Results from a separate CYP inhibition study showed that the test compound might be an inhibitor for all the major CYPs (CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A). The role of CYP1A and CYP3A in the metabolism of the test compound was not different between the two concentrations, namely 0.1 and 0.5 μ M. CYP1A1 was included in the recombinant CYP metabolism study due to the off-target potential of the CYP1A2 inhibitor (α -Naphthoflavone), and CYP1A1 was identified as playing a role in the metabolism of the test compound.

Conclusions: A comprehensive platform to accurately assess the CYPs contributing to the metabolism of the compound of interest was set up in our lab. The knowledge/experience acquired from this can also be extended to other enzymes.

P28 - INFLUENCE OF THE GENETIC POLYMORPHISM OF CYP2D1 ON METABOLISM AND PHARMACOKINETICS OF E2027 IN RATS

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E2027 is a novel, highly selective and potent inhibitor of phosphodiesterase 9 under development for the treatment of dementia with Lewy bodies. In the nonclinical pharmacokinetic (PK) studies, large inter-individual PK variability was observed in Sprague-Dawley (SD) rats, in which E2027 PK was apparently classified into high and low clearance groups. The plasma AUC(0-inf) value in low clearance group was 11-fold larger than that in high clearance group after single oral administration. This study was designed to clarify the underlying mechanism for the inter-individual PK variability of E2027 observed in rats. *in vitro* studies using recombinant rat P450 systems (rCYP) revealed that E2027 was predominantly metabolized by rCYP2D1 and rCYP3A1. Genetic polymorphism has been reported for CYP2D1 in SD rats with 418A/421C (ATCC) and 418G/421T (GTCT), corresponding to V123/F124 and I123/L124, respectively¹. After single intravenous administration of E2027 to CYP2D1 genotyped rats with ATCC/ATCC (ATCC homo), GTCT/GTCT (GTCT homo), and ATCC/GTCT (hetero), mean total clearance of E2027 in male and female rats with GTCT homo was 7.7- and 8.1-fold lower than those with ATCC homo, respectively. The terminal elimination phase half-lives in male and female rats with GTCT homo were 8.4- and 10- fold longer than those with ATCC homo. There were no remarkable differences in the PK parameters between ATCC homo and hetero. In the liver microsomes prepared from each genotype, the metabolic clearance of E2027 in GTCT homo was lower than those in ATCC homo and hetero. We quantified protein abundance of CYP2D1 in the rat liver microsomes by liquid chromatography with mass spectrometry. Using a peptide sequence specific to ATCC genotype, the expression level of CYP2D1 protein was approximately half in the hetero compared with ATCC homo, but not detected in the GTCT homo. Using a peptide sequence commonly shared between the genotypes, the expression level of CYP2D1 protein was similar regardless of genotypes, suggesting that 418G/421T mutation affected enzyme activity without alteration of protein expression levels. These results demonstrated that hepatic metabolism of E2027 was mainly mediated by genetically polymorphic CYP2D1, contributing to the PK variability in SD rats.

Reference:

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P29 - INTRA-PATIENT VARIATION IN CYP2D6 ACTIVITY OVER TIME ASSESSED USING THE DIETARY BIOMARKER SOLANIDINE IN BREAST CANCER PATIENTS ON TAMOXIFEN

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Cytochrome P450 (CYP) 2D6 is a highly polymorphic enzyme involved in the metabolism of nearly 25% of currently prescribed medications, including structurally divergent drugs such as certain opioid analgesics, beta blockers, antidepressants, antipsychotics, and antiemetic medications. Moreover, a number of widely prescribed antidepressants are known to inhibit CYP2D6. Recent studies suggest solanidine, a phytochemical present in potatoes, after consumption undergoes CYP2D6-mediated metabolism to 4-OH-solanidine and 3,4-seco-solanidine-3,4-dioic acid (SSDA) and the metabolite:parent ratios predict CYP2D6 phenotype. Tamoxifen is a selective estrogen receptor modulator used for the treatment of hormone-sensitive breast cancer. Tamoxifen undergoes bioactivation primarily via CYP2D6 to its active metabolite endoxifen and CYP2D6 genetic variation has shown to be linked to tamoxifen metabolism. The goal of this retrospective cohort study was to better define the extent of intra-patient variation of solanidine and its metabolites over an extended period of time in the presence or absence of CYP2D6 interacting medications among female breast cancer patients on tamoxifen therapy. We compared CYP2D6 normal metabolizers (n=40) who were on mild (escitalopram, citalopram, venlafaxine and desvenlafaxine (n=11)), moderate (duloxetine and sertraline, (n=5)) or strong (bupropion, fluoxetine and paroxetine, (n=4)) CYP2D6 inhibitors to those with no interacting medications (n=20). Three single time point plasma samples were assessed over an average of 2.14 years (range 0.67-5.14). Plasma concentrations were measured by mass spectrometry, and all data was natural log (ln) transformed to normalize before statistical analysis. A mixed-effect model was used to analyze the effect of time and CYP2D6 inhibitor status (none, mild, moderate and strong) on metabolite to solanidine ratios. Solanidine metabolite:parent ratios did not differ over time (4-OH-solanidine/solanidine p=0.829, SSDA/solanidine p=0.196). However, a significant effect was observed for the use of CYP2D6 inhibitors for 4-OH-solanidine/solanidine (p<0.001) and SSDA/solanidine ratios (p<0.001), with a trend to reduced ratios with CYP2D6 inhibitor use compared to no inhibitor for each time point (pairwise comparison). In conclusion, solanidine metabolite:parent ratios do not exhibit significant intra-patient variation over prolonged periods of time in breast cancer

patients, while the use of interacting medication appears to be reflected as a reduced ratio. Accordingly, diet-derived solanidine metabolites have the potential to serve as a reliable measure of CYP2D6 activity *in vivo*.

P30 - A RAPID LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS) METHOD FOR QUANTIFYING THE METABOLITES OF SEVEN MAJOR PROBE SUBSTRATES IN HUMAN LIVER MICROSOMES

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Background: Cytochrome P450 (CYP) family of enzymes are commonly involved in important drug-drug interactions (DDIs). The FDA recommends an *in vitro* CYP inhibition assay for the 7 major human hepatic CYP isoforms: CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A. Compared to the way individual metabolites are investigated separately with a single probe, cassette incubation and analysis, known as the "cocktail" approach, can simultaneously detect multiple metabolites to improve efficiency and throughput, which involves simultaneous determination of acetaminophen (CYP1A2), hydroxyl-bupropion (CYP2B6), n-desethyl-amodiaquine (CYP2C8), 4'-hydroxy-diclofenac (CYP2C9), 4'-hydroxymephenytoin (CYP2C19), dextrophan (CYP2D6) and 1'-hydroxy-midazolam (CYP3A4/5). This cassette analysis method faces many challenges such as interference separation issues, solvent effect of acetaminophen, sensitivity of 4'-hydroxymephenytoin, etc. Therefore, a novel 7-in-1 LC-MS/MS analysis method has been developed, which can simultaneously detect these metabolites in nM and only requires 2.5 minutes per injection. This new method greatly facilitates the assessment of potential compound inhibition of the seven major CYPs during the drug discovery phase. Methods: Seven metabolites were extracted from human liver microsomes using acetonitrile including 7 stable isotope labeled internal standards. They were detected in MRM mode by SCIEX Triple Quad™ 6500+ coupled with Shimadzu Nexera UHPLC-40 and a CTC autosampler. The transitions were m/z 152.1 → 110.1, 256.4 → 238.0, 328.2 → 283.2, 312.0 → 231.0, 235.1 → 150.2, 258.2 → 157.2, and 342.1 → 203.1 for acetaminophen, hydroxyl-bupropion, n-desethyl-amodiaquine, 4'-hydroxy-diclofenac, 4'-hydroxymephenytoin, dextrophan, and 1'-hydroxy-midazolam, respectively. The analysis was carried out using an ACQUITY HSS T3 column (2.1 x 50 mm) with mobile phases of 2% formic acid in water and 2% formic acid in acetonitrile. All metabolites were eluted using a gradient method with a flow rate of 0.7mL/min. Result: The method's selectivity was evaluated, and the results demonstrated no interference in the analysis of the seven metabolites by any potential substances, including the seven substrates and the metabolites themselves. All seven metabolites, acetaminophen, hydroxyl-bupropion, n-desethyl-amodiaquine, 4'-hydroxy-diclofenac, 4'-hydroxymephenytoin, dextrophan, and 1'-hydroxy-midazolam, exhibited good linearity in the ranges of 15-4000 nM, 1.5-400 nM, 6-1600 nM, 3-800 nM, 30-8000 nM, 1.2-320 nM, and 3-800 nM, respectively. The calibration curve was linear with $1/x^2$ as the weighting factor, and the regression coefficient (r) values were higher than 0.99. The accuracy and precision results were within ± 25% of their nominal values. This analytical method has been optimized to 2.5 minutes, which is 3 times faster than the method reported in the literature. Conclusion: A novel and rapid LC-MS/MS method was developed for simultaneous quantification of 7 metabolites of major CYPs in human liver microsomes within 2.5 minutes.

P31 - THE GUT MICROBIOME REGULATES HEPATIC DRUG PROCESSING GENES IN LIVERS OF HUMANIZED PXR-CAR-CYP3A4/3A7 MICE

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Pharmacomicrobiomics is an emerging field that investigates the contribution of the microbiome on drug disposition, action, and toxicity. Previous work has shown that the gut microbiome can regulate the expression of certain drug processing genes (DPGs) in germ-free (GF) wild-type mice. However, inter-species differences exist between mice and humans in nuclear receptors (e.g., PXR and CAR), which adds complexity to investigating the regulatory effect of the microbiome on downstream genes of human nuclear receptors. To facilitate the investigation of these issues, we used a humanized mouse model (hPXR-hCAR-CYP3A4/CYP3A7) to study the modulatory effects of microbiome on the expression of human PXR and downstream DPGs, such as CYP3A4. Specifically, we studied three groups (4 male and 4 female 12-14-week-old mice per group): conventional (CV) mice with a mouse microbiome, GF mice, and conventionalized mice (GFCV) which were GF mice that were inoculated with pooled human feces. We quantified the mRNA expression of 408 DPGs in the liver using RNA-seq. In addition, we performed metagenomics shotgun sequencing on extracted microbial DNA of the colon contents of CV and GFCV mice, and the pooled human feces used to inoculate mice in the GFCV group. We found that male CV and GFCV mice had 176 and 21 differentially expressed DPGs, respectively, compared to male GF mice. For many of the differentially expressed DPGs in male mice, the CV mice had an opposite expression pattern compared to the GF and GFCV mice. We found that female CV and GFCV mice had 46 and 51 differentially expressed DPGs, respectively, compared to female GF mice. Unlike the male mice, the lack of microbiome had less impact on the hepatic DPG expression in female mice. The mRNA expression of human genes was verified by RT-qPCR. Based on the metagenomics shotgun sequencing data, the beta diversity (Bray-Curtis Index) differed between the CV and GFCV groups and certain bacteria were enriched in one group over the other. For example, the genus *Parasutterella* were present in male CV and female CV mice but absent in male GFCV and female GFCV mice.

We hypothesized that the microbiome diversity may contribute to the regulation of CYP3A4 expression as well as other DPGs, and absence or presence of certain bacteria may suppress or facilitate CYP3A4 expression in liver. Further work will be done to test this hypothesis and elucidate the mechanism of CYP3A4 regulation by the microbiome.

P32 - SOLANIDINE METABOLITES AS DIETARY CYP2D6 BIOMARKERS OF TAMOXIFEN METABOLISM

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Tamoxifen is a selective estrogen receptor modulator prescribed to reduce the risk of breast cancer recurrence. However, response to tamoxifen therapy can vary substantially, which is thought to be in part due to altered activity of the drug-metabolizing enzyme cytochrome P450 (CYP) 2D6. Tamoxifen is a prodrug, with the majority being metabolized to N-desmethyltamoxifen (NDM-tamoxifen) by CYP3A4/5 and then further transformed by CYP2D6 to its primary active metabolite, endoxifen. Previous studies have used CYP2D6 genotype-based phenotyping and endoxifen therapeutic drug monitoring to optimize tamoxifen therapy. Recently, solanidine, a glycoalkaloid found in potatoes, has been suggested as a dietary biomarker of CYP2D6 metabolism. Therefore, we investigated the correlation between solanidine and tamoxifen metabolism in a large cohort of breast cancer patients on tamoxifen therapy. Ultra-high performance tandem mass spectrometry was used to measure tamoxifen, NDM-tamoxifen, endoxifen, solanidine and solanidine metabolites (m/z 414, 416, 432, and 444) in plasma of 538 breast cancer patients. There was a strong correlation between endoxifen to tamoxifen ratio with m/z 414 ($p = 0.2990$), 416 ($p = 0.3986$), 432 ($p = 0.3986$) and 444 ($p = 0.4911$) to solanidine ratios ($P < 0.0001$). Furthermore, this effect was stronger when examining endoxifen to NDM-tamoxifen ratio compared to m/z 414 ($p = 0.3504$), 416 ($p = 0.4415$), 432 ($p = 0.4389$) and 444 ($p = 0.5226$) to solanidine ratios ($P < 0.0001$), which is the CYP2D6-specific step in tamoxifen biotransformation to endoxifen. Lastly, linear regression models demonstrated m/z 414 ($R^2 = 0.3477$), 416 ($R^2 = 0.3758$), 432 ($R^2 = 0.3846$), and 444 ($R^2 = 0.4146$) to solanidine ratios were predictive of endoxifen to tamoxifen ratio when including the covariates, sex, age and CYP2D6 phenotype ($P < 0.0001$). Similar results were seen with models of m/z 414 ($R^2 = 0.3885$), 416 ($R^2 = 0.4273$), 432 ($R^2 = 0.4351$), and 444 ($R^2 = 0.4467$) to solanidine ratios for predicting endoxifen to NDM-tamoxifen ratio ($P < 0.0001$). Accordingly, solanidine metabolites in plasma, particularly m/z 444, can predict endoxifen exposure, and may be useful as CYP2D6 biomarkers in determining the clinical efficacy of tamoxifen in breast cancer patients.

P33 - AN EVALUATION OF CYP AND NON-CYP REACTION PHENOTYPING STUDY TRENDS FROM 2000-2023: A CRO PERSPECTIVE

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In preclinical drug development, it is critical to understand the enzyme-mediated metabolism of drug candidates. Drug metabolizing enzymes are often divided into the following two groups: Cytochrome P450 (CYP) enzymes and non-cytochrome P450 (non-CYP) enzymes such as UDP-glucuronosyltransferases (UGT), flavin monooxygenase (FMO) or sulfotransferases (SULT) among others. It has been well established that CYP enzymes are primarily involved in the metabolism of small molecule drugs and contribute to the potential risk of drug-drug interactions (DDIs). To reduce DDI liability, drug developers sometimes target non-CYP-mediated metabolism over CYP-mediated metabolism. Consequently, if drug developers attempt to design molecules that are metabolized by non-CYP enzymes, an increase of non-CYP enzyme evaluations in preclinical studies over time is expected. We analyzed sponsored reaction phenotyping studies implemented by XenoTech, now a BioIVT company, to evaluate whether there is a trend towards the development of drug candidates that are metabolized by non-CYP enzymes. We analyzed a total of 494 reaction phenotyping studies conducted from 2000-2023. The data was divided into three time periods with period 1 being 2000-2007, period 2 from 2008-2015, and period 3 spanning 2016-2023. The percentage of non-CYP enzyme monitoring over these three periods varied only slightly from 27.8 to 29.4% with no observable trend. Of note, there was a shift in the spectrum and prevalence of the different non-CYP enzymes that were assessed. From 2000–2007, FMO was evaluated >50% and UGT in 36% of the studies, while UGT was the most studied non-CYP enzyme after 2007 (>60%). We further evaluated the results and conclusions of these reaction phenotyping studies to gain insights into the prevalence of CYP and non-CYP enzyme-mediated *in vitro* metabolism of drug candidates. From 2000-2007, >68% of studies concluded that CYP enzymes were involved in the *in vitro* metabolism of these drug candidates whereas 11% non-CYP enzyme metabolism was observed. From 2008-2015, 55% of all studies observed CYP enzyme-mediated *in vitro* metabolism and approximately 15% implicated non-CYP enzymes and the prevalence stayed similar for the period from 2016-2023. Last, we recorded the studies when these enzyme classes were shown to not contribute to the *in vitro* metabolism of the drug candidates. From 2000-2007, <2% of all studies showed no involvement of CYP enzymes, while this increased to >17% in the other two time periods. Taken together, our data showed no clear increase in non-CYP enzyme evaluations over the last two decades, but the results and conclusions of the evaluated studies give insights about possible trends towards non-CYP mediated drug metabolism in early drug development to potentially reduce DDI liability.

P34 - CYTOCHROME P450 (CYP) PHENOTYPING OF AFICAMTEN, A NEXT-IN-CLASS INHIBITOR OF CARDIAC MYOSIN, USING HUMAN LIVER MICROSOMES AND HUMAN RECOMBINANT CYP ENZYMES**Rajaa Sukhun***Cytokinetics, South San Francisco, CA, United States*

Aficamten, also known as CK-3773274, is a next-in-class small molecule cardiac myosin inhibitor being developed for the chronic oral treatment of patients with symptomatic hypertrophic cardiomyopathy (HCM). The objective of the present study was to determine which cytochrome P450 (CYP) enzymes are involved in the *in vitro* metabolism of aficamten using two different approaches: 1) heterologous expressed (recombinant) human CYP enzymes (rCYP) and 2) human liver microsomes (HLM) in the presence or absence of a CYP enzyme-selective inhibitor. Aficamten is known to be metabolized in HLM and human hepatocytes predominantly by hydroxylation to ω -1-hydroxylated metabolite of the ethyl group of the ethyl-1,2,4-oxadiazol-3-yl moiety forming metabolite M1. Due the high metabolic stability of aficamten, the formation rate metabolite M1 was measured and used for intrinsic clearance (CL_{int}) determinations. For these CYP phenotyping methods, the focus was on the eight common CYP isoforms (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5). In the human rCYP phenotyping test system, the relative activity factor (RAF) approach was employed using RAF-characterized Supersomes™ prepared from insect cells engineered to over express cDNA for human CYPs. Incubation conditions were chosen based on preliminary time- and aficamten-concentration studies to ensure that M1 formation in RAF studies was performed under first-order kinetic conditions. CL_{int} in recombinant P450s (CL_{int}, rP450) was calculated for aficamten and extrapolated to human liver microsomal (HLM) clearance for the P450 of interest for an estimate of CYP fraction metabolized (percent fm,CYP). In the second approach using HLM incubations with CYP-selective chemical inhibitors (HLM CI), aficamten (1000 nM) was incubated from 0- to 120 minutes with HLM (0.25 mg/mL) and a panel of seven CYP-selective chemical inhibitors, namely: α naphthoflavone (CYP1A2), 2 phenyl-2-(1-piperidinyl)propane (CYP2B6), montelukast (CYP2C8), sulfaphenazole (CYP2C9), (-)-N-3-benzylphenobarbital (CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A) for the measurement of M1 formation and estimation aficamten CL_{int},P450. The percent fm,CYP was determined by the calculated HLM CL_{int},P450 in the presence and absence of each CYP-selective inhibitor using the equation: fm,CYP = ((CL_{int}, no inhibitor - CL_{int},CYP-selective inhibitor)/CL_{int}, no inhibitor) * 100. Results from *in vitro* CYP phenotyping studies indicate that aficamten undergoes metabolism contributed by several CYP enzymes, including CYP2D6, CYP2C8, CYP2C9, CYP2C19, and CYP3A4. Importantly, none of these individual CYP isoforms were found to be responsible for more than 50% of aficamten's metabolism.

P35 - IMPACT OF DNA G-QUADRUPLEX FORMATION ON CYP3A4 GENE TRANSCRIPTION**Seiya Takemoto**, Masataka Nakano, Tatsuki Fukami, and Miki Nakajima*Kanazawa University, Kanazawa, Japan*

Guanine-rich sequence in genomic DNA can form tetra-stranded structure known as G-quadruplex (G4), which is linked by Hoogsteen base pairing. G4s on the upstream region of oncogenes such as MYC, BCL2, or KRAS have been reported to play a critical role in the transcriptional regulation of their expressions via the modulation of chromatin structure or binding of transcriptional factors. However, it is unknown whether G4 modulates the expression of drug-metabolizing enzymes. We noticed that there are three potential G4-forming sequences in the 5'-flanking region of the CYP3A4 gene, which is the most abundant drug-metabolizing P450 isoform in the human liver. In the present study, we examined whether G4s modulate CYP3A4 transcription and sought to explore drugs that stabilize G4s, affecting CYP3A4 expression. By qPCR stop assay to assess the G4 formation or chromatin immunoprecipitation (ChIP) assay using BG4, a peptide that binds to G4, we found that three guanine-rich sequences on the 5'-flanking region of the CYP3A4 gene can form G4s in HepG2 cells. CYP3A4 expression levels in HepG2 cells and primary human hepatocytes were increased by treatment with pyridostatin (PDS), a small molecule that stabilizes G4s. The reporter activity of a plasmid containing three G4-forming sequences on the CYP3A4 gene was increased by PDS treatment, suggesting that G4 formation would be critical for the transcriptional regulation of CYP3A4. Formaldehyde-assisted isolation of regulatory elements (FAIRE) analysis and ChIP assay showed that the stabilization of G4s on the 5'-flanking region of CYP3A4 relaxes chromatin structure to recruit pregnane X receptor, a transcriptional factor regulating CYP3A4. Finally, we performed screening using FDA-approved drug library to identify small molecules that stabilize the G4 on the 5'-flanking region of CYP3A4 by qPCR stop assay. We found that digoxin can promote G4 formation of one of the three G4-forming sequences on CYP3A4. Interestingly, the treatment with digoxin (not a ligand of PXR) significantly increased CYP3A4 expression in HepG2 cells and primary human hepatocytes. Collectively, we found that G4s on the 5'-flanking region of CYP3A4 enhance its transcription via the relaxation of chromatin structure. We propose a novel concept: drugs that can stabilize the G4s, such as digoxin, would affect pharmacokinetics and drug response of CYP3A4 substrates.

P36 - CYTOCHROME P450 3A CHEMICAL INHIBITORS: UNDERSTANDING SELECTIVITY IN HUMAN LIVER MICROSOMES FOR USE IN REACTION PHENOTYPING

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Cytochrome P450s are major drug metabolizing enzymes contributing towards Phase I metabolism and clearance pathways of most marketed drugs. Cytochrome P450 3A (CYP3A) is the predominant isoform responsible for the majority of these biotransformations. As such, understanding the extent of contribution by CYP3A during phenotyping experiments is imperative during drug discovery and development to appropriately assess potential for drug-drug interactions (DDI). Assays are well established in the literature and recommendations of commonly used CYP3A inhibitors are outlined in regulatory documents. However, selectivity of these inhibitors against other CYP isoforms may not be well defined, leading to over predictions of CYP3A contribution and unnecessary conduct of clinical DDI studies. Ketoconazole is a CYP3A inhibitor which is widely used to assess CYP3A contribution. However, Doran et al. (1) have recently reported on the CYP selectivity profile of the inhibitor as rather promiscuous, limiting the selection of a single optimal concentration to accurately determine contributions by CYP3A. These findings led to a comprehensive evaluation of ten CYP3A time-dependent inhibitors (6-methyl-7-ethoxy-dihydroxybergomottin, atazanavir, azamulin, clarithromycin, CYP3A4 inhibitor, diltiazem, itraconazole, ritonavir, troleandomycin, and verapamil) to define their potency for CYP3A and selectivity against other CYP isoforms in human liver microsomes. Preliminary results confirm previous reports indicating that azamulin possesses a highly potent and selective profile that is suitable to replace ketoconazole in phenotyping experiments. Conversely, 6-methyl-7-ethoxy-dihydroxybergomottin's non-selective profile may serve as a better pan-CYP inhibitor over the commonly used 1-aminobenzotriazole. Understanding the selectivity profiles of the inhibitors will aid in choosing the most appropriate CYP3A inhibitor to use in phenotyping studies. Additionally, the selectivity profiles of the inhibitors can assist in data interpretation when off target contributions by other isoforms are observed.

Reference:

1. Doran AC, et al. Defining the Selectivity of Chemical Inhibitors Used for Cytochrome P450 Reaction Phenotyping: Overcoming Selectivity Limitations with a Six-Parameter Inhibition Curve-Fitting Approach. *Drug Metabolism and Disposition* September 2022, 50 (9) 1259-1271

P37 - METABOLISM OF PIROCARPINE IN CHIMERIC MICE WITH HUMANIZED LIVER

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Pilocarpine is a cholinergic muscarinic receptor agonist used to treat glaucoma and dry mouth. In humans, pilocarpine is converted into 3-hydroxypilocarpine and pilocarpic acid by hepatic cytochrome P450 (CYP)2A6 and plasma paraoxonase 1 (PON1). CYP2A6- and PON1-mediated drug metabolism in humanized-liver mice has not yet been well-characterized. We hypothesized that pilocarpine would be a suitable substrate for evaluating the characteristics of hepatic CYP2A6 and plasma PON1 activity in humanized-liver mice. Specifically, we studied the conversion of pilocarpine to 3-hydroxypilocarpine and pilocarpic acid via *in vitro* and *in vivo* metabolic assays using liver microsomes/plasma and humanized-liver mice, respectively. Pilocarpine was administered orally at a single dose of 10 mg/kg to humanized-liver mice and non-humanized mice, and plasma and urine samples were obtained. The concentrations of pilocarpine and its metabolites in the samples were measured using liquid chromatography-tandem mass spectrometry. The area under the curve and maximum plasma concentration of 3-hydroxypilocarpine in humanized-liver mice were significantly greater than those in non-humanized mice, whereas there was no difference in pilocarpic acid levels. 3-Hydroxypilocarpine was the more abundant metabolite in humanized-liver mouse urine, as similarly reported for humans. The *in vitro* metabolic assay showed that the 3-hydroxylation and hydrolysis of pilocarpine in liver microsomes and plasma, respectively, were remarkably different between humans and laboratory animal species. However, both activities in humanized-liver mice were similar to those in humans. Kinetic analyses suggested that liver microsomes and plasma from humans and humanized-liver mice catalyzed the 3-hydroxylation and hydrolysis of pilocarpine with similar affinities for the substrate. The 3-hydroxypilocarpine formation rate in liver microsomes from chimeric mice harboring different donor hepatocytes correlated with the CYP2A6 content. These results suggest that pilocarpine metabolism in humanized-liver mice mimics that in humans based on hepatic CYP2A6 and plasma PON1 functions. Thus, humanized-liver mice are potentially useful *in vivo* models for drug metabolism studies.

P38 - GUT MICROBIAL METABOLITES ACTIVATE PREGNANE X RECEPTOR TO INCREASE CYP3A4 EXPRESSION IN THE LIVER**Kyoung-Jae Won¹**, Pei-Ru Jin¹, Lydia Davis², Jimmy Orjala², Hyunwoo Lee³, and Hyunyoung Jeong¹*Department of Industrial and Molecular Pharmaceutics, College of Pharmacy, Purdue University, West Lafayette, IN, United States, ²Department of Biopharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago, Chicago, IL, United States, ³Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University, West Lafayette, IN, United States*

CYP3A4 is a key enzyme that metabolizes about half of clinically used drugs. Pregnane X receptor (PXR) is a ligand-activated transcription factor that primarily governs the expression of CYP3A4. The large interindividual variations in hepatic expression and activity of CYP3A are well known, but the responsible factors remain unclear. To investigate the potential roles of the gut microbiota on hepatic CYP3A4 regulation, humanized CYP3A4/PXR mice were treated with an antibiotic cocktail, and hepatic CYP3A4 expression was examined. Depleting the gut microbiota with antibiotics led to a significant decrease in hepatic CYP3A4 expression in the mice. Organic extracts from the cecum contents from the antibiotic-treated mice exhibited a lower PXR activation in the reporter assays as compared to those from the control mice, suggesting the production of PXR-activating compounds by the gut microbiota. To identify potential microbial metabolites that activate PXR, we screened organic extracts from 113 gut bacterial cultures. Approximately 10% of the tested bacteria activated PXR more than two-fold. We selected *Fusobacterium nucleatum* (Fn) as a model bacterium, which showed the highest PXR activation. Through activity-guided fractionation and spectroscopic methods, we identified new PXR-activating metabolites (FTIN and TIN) from Fn culture. The half-maximal effective concentration (EC₅₀) of FTIN and TIN for PXR activation in HepG2 cells were 298 and 454 nM, respectively. We measured the concentrations of FTIN and TIN in extracts from various *Fusobacterium* species and observed a correlation between FTIN level and PXR activity, suggesting that FTIN is a major metabolite that activates PXR. Collectively, our results provide mechanistic insights into the role of gut microbiota in influencing the variability in human CYP3A4 expression.

P39 - ASSESS CYP450 INDUCTION BY QRT-PCR QUANTIFICATION OF CYP450 MRNAS FROM SMALL EXTRACELLULAR VESICLES (SEVS) DERIVED FROM PLASMA**Shengjie Xu¹**, Brahim Achour², Christopher Gibson¹, Weixun Wang¹, Daniel Spellman¹, and Rena Zhang¹*¹Merck & Co., Inc, West Point, PA, United States, ²University of Rhode Island, Kingston, RI, United States*

Cytochrome P450s catalyze the biotransformation of drugs. Accurate assessment of CYP450s induction is critical to drive dose selection and adjustment for concomitant medications and evaluate the drug-drug interaction (DDI) potential of new drug candidates, especially as potential inducers. The proof-of-concept validation of CYP450s expressions in blood-derived sEVs for enzyme induction has been published on relatively small selection of subjects [1]. Thorough evaluation of the analytical methods on many aspects, such as sensitivity and reproducibility, remains essential to achieve confidence before supporting ongoing clinical studies. Initially, we found unsatisfying mRNAs detection when we followed vendor-suggested sEV isolation methods, mRNA extraction protocols, and regular qRT-PCR methods. In this study, we aim to develop a reliable method of quantifying mRNAs of CYP450s in blood-derived sEVs with qRT-PCR and validate its utility as a biomarker of CYP450 induction by Dexamethasone (a strong inducer) in rats and Modafinil (a moderate inducer) in human.

We first evaluated and optimized six sEV isolation methods (Ultracentrifugation, ExoEasy kit, FUJIFILM MagCapture kit, Izon qEV2 Gen 2 kit, ExoQuick kit, and Invitrogen Total Exosome Isolation kit) from 1ml human plasma and serum based on the physical characteristics of exosome samples with Nanoparticle Tracking Analysis (NTA). ExoQuick kit showed the higher yield with moderate purity of isolated sEVs, which was used to quantify mRNA expression of CYP450s in sEVs. Our results showed successful mRNA quantification of CYP enzymes (e.g., CYP3A4, CYP3A5, CYP2C9, CYP2E1, CYP1A2, CYP2B6, CYP2D6), exosome markers (e.g., TSG101, CD63), liver markers (e.g., APOA2, AHSG1), and housekeeping genes (e.g., 18S, GAPDH) by qRT-PCR in plasma-derived exosomes. Next, we sought to evaluate our optimized protocols through analytical method validation. First, the detectability and expression levels of genes increased in a volume-dependent manner in sEV RNA samples isolated from 0.5-, 1-, or 2-ml plasma, suggesting good linearity of the method. Also, our method demonstrated great precision of with intra CV% < 7% and reproducibility with inter CV% < 8% (n = 5) based on the Ct values of multiple genes. To further validate its utility as a CYP450 induction biomarker, we isolated sEV RNA from 2ml Sprague-Dawley rat plasma, and Cyp3a23/3a1 showed significant induction at mRNA level following dexamethasone treatment (50 mg/kg/day * 7 days, p.o.) (p = 0.002, unpaired t test, n = 5), while housekeeping genes and exosome marker genes remain unaltered. Additional efforts are underway to validate the assay using samples from a recent modafinil clinical DDI study. Taken together, we developed and optimized a novel method to isolate and quantify sEV mRNAs from plasma samples with excellent sensitivity and reproducibility, which showed promises as a non-invasive tool for evaluating DDI potential and gene expression patterns among populations, offering valuable insights for drug development.

Reference:

1. Rodrigues, A.D., et al., Exploring the Use of Serum-Derived Small Extracellular Vesicles as Liquid Biopsy to Study the Induction of Hepatic Cytochromes P450 and Organic Anion Transporting Polypeptides. Clin Pharmacol Ther, 2021. 110(1): p. 248-258.

P40 - ELUCIDATING SYNERGISTIC RISK FACTORS AMONG COMORBIDITY COMBINATIONS FOR NEPHROTIC SYNDROME BASED ON TIME SERIES STATISTICAL EVALUATION

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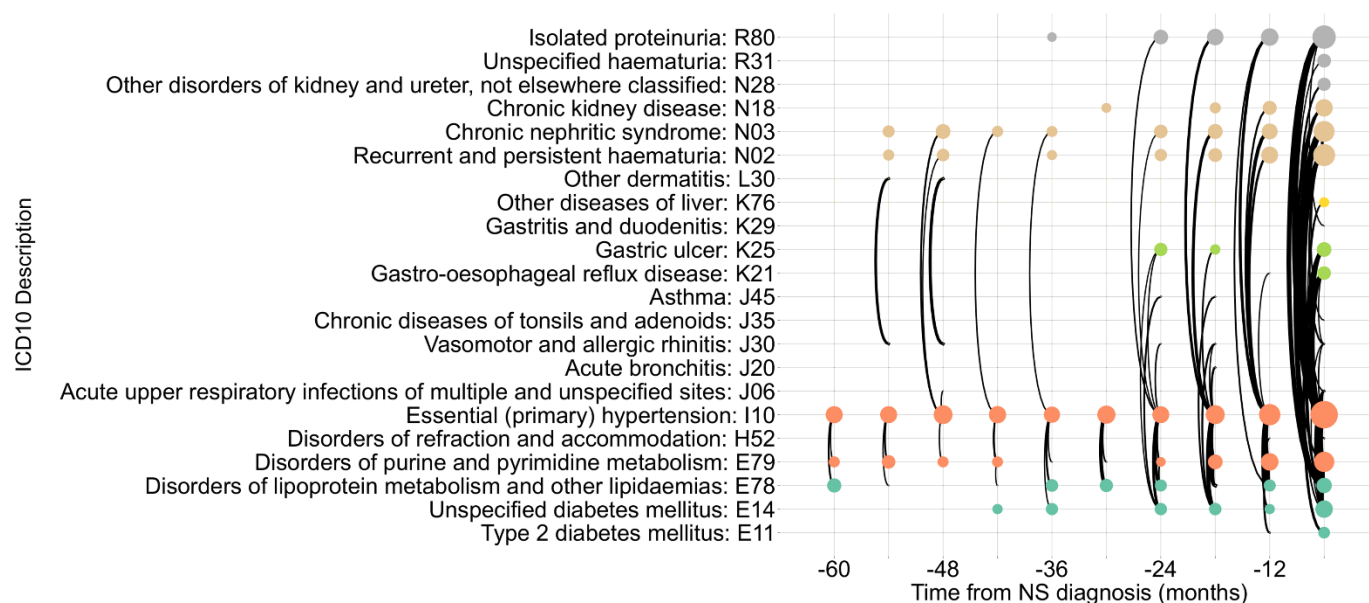
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Hypothesis: Nephrotic syndrome (NS) is characterized by massive urinary protein and accompanying hypoalbuminemia. NS causes various symptoms due to a disruption of homeostasis. Predicting NS enables us to prepare for a renal biopsy to obtain definitive diagnosis. However, the risk factors for NS are poorly understood. We hypothesized that patients with NS exhibit comorbidities that reflect various symptoms from an early stage, and these combinations distinguish a population at high risk of developing NS.

Methods: We used data consisting of receipts and health checkup data obtained from several health insurance associations, collected in the JMDC Claims Database. This data includes people ($n = 329,500$) who underwent at least five physical checkups between April 2008 and March 2019 and had all 17 major items included in data collected from January 2005 to March 2019. Patients with a diagnosis of N04 of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10) code were analyzed as a case group ($n = 411$). A control group involved subjects who had never been diagnosed with NS, which matched 1:1 to the case group for age at the start of the observation, sex, and length of the observation periods using nearest neighbor methods. The NS diagnosis date of individuals in the control group was assumed to be the same as in the matched NS individuals. We applied Fisher's exact test and correction for multiple testing by the Limitless-Arity Multiple-testing Procedure (LAMP) to analyze combinations of comorbidities within 1-year intervals. These time frames were adjusted in six-month increments leading up to NS diagnoses.

Data and Results: In the temporal network, nodes represented individual diseases with significant differences, while edges represented significantly different combinations of two diseases (Figure). We did not draw the combination of three or more diseases on the temporal network. We focused on diseases that occurred more than 24 months before the onset of NS to identify early indicators. The combination of primary hypertension (I10), and hyperuricemia (E79) was more frequently diagnosed within the time frames of 48 months prior to onset (adjusted $p = 0.034$) in the NS group compared to the control group. In addition, Agents acting on the renin-angiotensin system prescribed for hypertension (I10), and antigout preparations for hyperuricemia (E78) were prescribed more often in the NS group at 48 before the onset of NS (odds ratio = 9.69).

Conclusion: This study is a case-control study; further investigation is needed to translate these findings into clinical practice. This study implies that our method can find the difference among comorbidity combinations and prescription combinations.



P41 - HEPATIC EXPRESSION OF DRUG METABOLIZING ENZYMES IS ALTERED BY CONCOMITANT OBESOGENIC LIPIDS AND ALCOHOL IN MICE AND HUMAN HEPATIC ORGANIDS

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MetALD is the subtype of steatotic liver disease that develops from metabolic dysfunction-associated steatotic liver disease and ethanol. Previous studies in mice demonstrate that diet or ethanol alter the RNA expression of genes that metabolize xenobiotics, including TZDs that manage insulin resistance in MetALD patients. However, how the concomitant exposure of obesogenic lipids and ethanol (MetALD) alter the expression of xenobiotic metabolizing genes for *in vitro* and *in vivo* model systems is not fully known. We hypothesize that concomitant obesogenic lipids and ethanol exposure will have a synergistic effect on the altered RNA expression of xenobiotic metabolizing genes for *in vivo* and *in vitro* mouse models of MetALD. For our MetALD mouse model, we fed C57BL6/J male mice a chow (Chow) or a high-fat, fructose, cholesterol (FFC) diet and co-administered either saline or EtOH for four experimental groups: Chow, EtOH, FFC, and FFC-EtOH for 12 weeks, followed by measuring biomarkers for MetALD and sequencing the hepatic transcriptome. For our *in vitro* models, we exposed primary human hepatic organoids and VL-17A cells to EtOH (100 mM), oleate (1 mM), or EtOH and oleate, followed by isolating RNA for performing RT-qPCR. In mice, FFC-EtOH induced more body weight gain, glucose intolerance, and steatosis, compared to Chow, EtOH, or FFC. Gene set enrichment analysis showed that FFC-EtOH, compared to EtOH alone, led to dysregulated biological pathways related to xenobiotic metabolism and oxidative stress. Among 330 Phase I (oxidation) enzymes, the expression of 114 genes was decreased by either FFC or FFC-EtOH. Among 97 Phase II (conjugation) enzymes, expression of 37 genes, including glutathione-S-transferases, were altered by at least one diet. In human hepatic organoids, EtOH increased the expression of the major drug metabolizing enzyme CYP2B6, whereas oleate and oleate-EtOH decreased the expression of CYP2C8 and CYP3A4, which both metabolize the TZD pioglitazone. Expression of the xenobiotic and lipid metabolism regulating nuclear receptor CAR was decreased by oleate and oleate-EtOH, which may be responsible for the decreased expression of CYP2C8 and CYP3A4. However, VL-17A cells had no changes in RNA expression of xenobiotic metabolizing genes, indicating human hepatic organoids provides a superior model that better reflects MetALD pathology *in vivo* and further determining molecular mechanisms leading to the pathology. This study provides information towards the potential effects of obesogenic diet, EtOH, and MetALD on xenobiotic metabolism in mouse liver and human hepatocytes. Overall, obesogenic lipids alter the expression of xenobiotic metabolizing genes more than EtOH. Identifying the changes to enzymes that metabolize therapeutics may help improve and guide drug treatment of metabolic diseases.

P42 - CHANGES IN THE EXPRESSION OF DRUG-METABOLIZING ENZYMES AND TRANSPORTERS ASSOCIATED WITH DECREASED EXPRESSION OF CYP3A AND MRP2

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Changes in the expression of drug-metabolizing enzymes and transporters can alter the pharmacokinetics of certain drugs and may affect drug efficacy and safety. The mechanisms of transcriptional regulation of drug-metabolizing enzymes are similar to those of transporters. It has been reported that knockout animals of certain drug-metabolizing enzymes or transporters showed collaborative changes in gene expression of other drug-metabolizing enzymes and transporters in the liver. Therefore, an interplay of transcriptional regulation between drug-metabolizing enzymes and transporters is suggested, although the significance and mechanisms of this phenomenon have not been fully elucidated. In this study, we focused on cytochrome P450 (CYP) 3A and multidrug resistance-associated protein (MRP) 2 and investigated the effects of their decreased expression on the gene expression of other drug-metabolizing enzymes and transporters. First, we obtained the microarray data of Cyp3a knockout mice from Gene Expression Omnibus, a public genomics database provided by National Center for Biotechnology Information. In the liver of Cyp3a knockout mice, the expression of Cyp2b10, Cyp2c55 and multidrug resistance 1a was higher than that of the control mice. The activation of transcription factors that are involved in the regulation of drug-metabolizing enzymes and transporters expression was evaluated based on changes observed in downstream gene expression. The gene expression of insulin induced gene 2, transcobalamin 2, and cytochrome P450 oxidoreductase was also higher in the liver of Cyp3a knockout mice than that of the control mice, suggesting the activation of pregnane X receptor (PXR). Second, MRP2 siRNA was transfected into human hepatocellular carcinoma HepG2 cells. Knockdown of MRP2 expression decreased the sulfotransferase (SULT) 1E1 mRNA and protein expressions, which were evaluated by RT-qPCR and Western blotting, respectively. In the liver of MRP2-deficient Eisai hyperbilirubinemic rats (EHBR), mRNA and protein expressions of SULT1E1 were also lower than that of the control rats. The enzymatic activity of SULT1E1 was also decreased, as evaluated using liver cytosol and β -estradiol as a specific substrate. The changes observed in downstream gene expression in the liver of EHBR suggested the activation of farnesoid X receptor (FXR) and liver X receptor (LXR). Bile acids and cholesterol, known as endogenous agonists of FXR and LXR, respectively, were higher levels in the plasma of EHBR than that of the control rats. These findings suggest that a decrease in CYP3A or MRP2 expression caused a collaborative variation in the gene expression

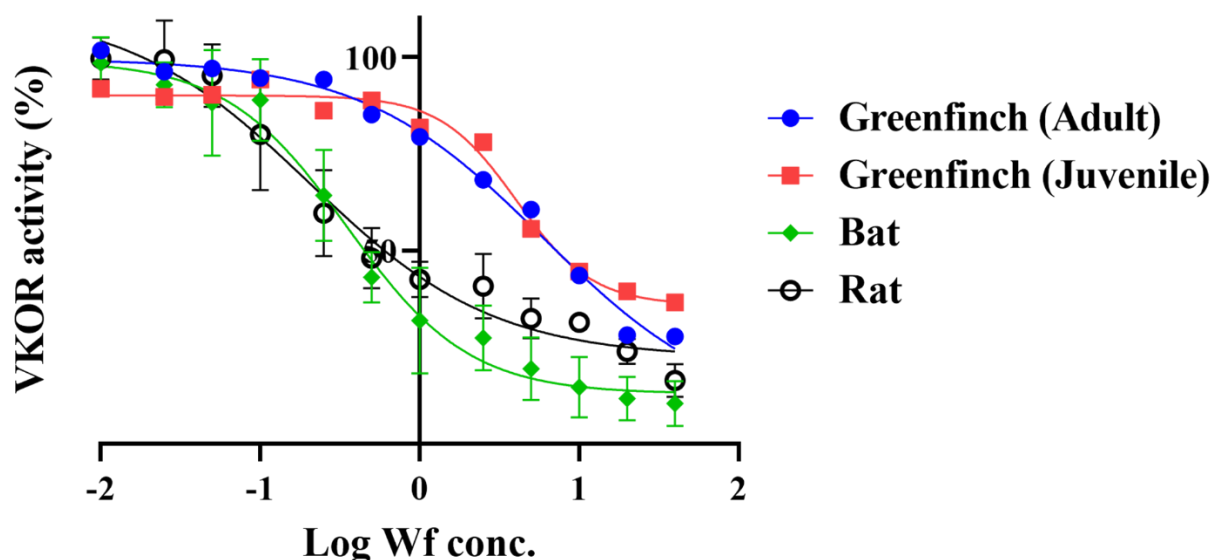
of other drug-metabolizing enzymes and transporters through the activation of transcription factors by endogenous molecules.

P43 - ASSESSMENT OF UNINTENTIONAL EFFECTS ON ENDANGERED SPECIES ENDEMIC TO THE BONIN ISLANDS CAUSED BY DEPLOYMENT OF ANTICOAGULANT RODENTICIDE

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The Bonin Islands are Japanese island group with a unique ecosystem comprising many endemic plants and animals. Some animals in the islands such as Bonin fruit bat (*Pteropus pselaphon*) and Bonin greenfinch (*Chloris sinica kittlitzii*) are listed as endangered species by the IUCN. One of the factors threatening the survival of these species is ecosystem disturbance caused by non-native rodents represented by Black rats (*Rattus rattus*). In order to control these rodents in the islands, Diphacinone, one of the anticoagulant rodenticides (ARs), has been applied. However, the potential effects of the use of rodenticides on non-target animals in this area are still unclear. ARs inhibit the blood coagulation system by blocking vitamin K epoxide reductase (VKOR), ultimately causing fatal hemorrhage. When absorbed into the body, ARs are metabolized by Cytochrome P450 (CYP) and excreted. There are differences between species in sensitivity to ARs, of which the binding affinity to the target molecule VKOR and the ability of CYP to metabolize ARs are important determinants. In this study, microsomal fractions were purified from the livers of Oriental greenfinches (*Chloris sinica minor*), as an alternative to Bonin greenfinch, and of Bonin fruit bats. With use of these microsomal fractions, inhibition tests of VKOR activity using Warfarin and Diphacinone were conducted. In order to assess *in vitro* susceptibility to rodenticides, Warfarin metabolic tests were also performed. In addition, the metabolic pathway of rodenticides in greater detail was analyzed by next-generation sequencing using RNA derived from the liver of Bonin fruit bats. The IC₅₀ values obtained from the activity inhibition tests using Warfarin were 0.204, 0.351, and 5.95 (μ M), respectively for SD rats, Bonin fruit bats, and Oriental greenfinches, showing higher values for greenfinches compared to rats, while that of bats was comparable to that of rats. The equivalent values from the Diphacinone tests were 0.131, 0.133, and 0.648 (μ M), respectively, suggesting a higher susceptibility in bats than in rats. V_{max}/K_m was calculated as an indicator of metabolic capacity for each metabolite found in the Warfarin metabolic tests, yielding totals of 4.28, 3.23, and 4.40 (mL/min/nmol CYP), respectively, rats, bats, and greenfinches. These results indicate that both of bats and greenfinches could have similar Warfarin metabolic capacity as that of rats. Next-generation sequencing results showed a high level of expression of different types of CYPs, indicating that these may contribute significantly to metabolism of rodenticides in bats. Thus, the metabolic pathway of bats differs from that of rats. Better estimation of the susceptibility requires further studies such like metabolic assay using Diphacinone, *in vivo* administration tests, functional analysis of each CYP type by cloning, risk assessment of misfeeding, or the same kind of tests using samples from the close related species to Bonin fruit bats.



P44 - INVESTIGATING THE INFLUENCE OF CYP3A AND CYP2C8 PHENOTYPES ON TUCATINIB CLEARANCE IN A DIVERSE COHORT OF HUMAN HEPATOCYTE DONORS**Bethany Latham**, Klarissa Jackson, and Pegah Montazeri*UNC Chapel Hill Eshelman School of Pharmacy, Chapel Hill, NC, United States*

Tucatinib (6-N-(4,4-dimethyl-5H-1,3-oxazol-2-yl)-4-N-[3-methyl-4-([1,2,4]triazolo[1,5-a]pyridin-7-yloxy)phenyl]quinazoline-4,6-diamine) is a tyrosine kinase inhibitor (TKI), that is metabolized by the enzymes cytochrome P450 (CYP) 3A and CYP2C8. The activity of these enzymes can determine the rate at which the drug is metabolized. Tucatinib has been associated with hepatotoxicity in patients; therefore, elucidating its metabolic pathway is vital for patient safety. Interindividual variability in clearance of TKIs has been seen, however it is unknown which factors contribute to this variability. Genetic variability in CYP enzymes has been shown to influence drug metabolism phenotypes. This study aims to understand what factors or combination of factors influence tucatinib metabolism and interindividual variability in clearance rates. The purpose of this study is to determine the influence of genetics and CYP activity on the clearance of tucatinib in primary human hepatocytes (n = 19). Donor demographics include: n = 11 African American/Black, n = 5 White, and n = 3 Hispanic. Donors include n = 10 male and n = 9 female donors. Commercially available cryopreserved human hepatocytes from individual donors and 5-donor pooled lots were purchased from BioIVT. To determine the clearance of each donor, substrate depletion experiments were performed with tucatinib. Primary human hepatocytes in suspension were seeded at a density of 0.5×10^6 cells/mL and incubated with 1 μ M tucatinib over the time points 0, 15, 30, and 60 minutes. Tucatinib levels were quantified using liquid chromatography - tandem mass spectrometry (LC-MS/MS), and the percentage remaining tucatinib was calculated in reference to time 0 minutes. CYP3A and CYP2C8 activities were obtained from the vendor using the rate of 1'-hydroxymidazolam formation and N-desethylamodiaquine formation respectively. The tucatinib clearance rates varied 24-fold between individual donors; 0.75 to 18.46 (μ L/million cells/min). The 5-donor pooled human hepatocyte lot had a clearance of 32.34 (μ L/million cells/min). There was a significant correlation between tucatinib depletion and CYP2C8 activity (Pearson correlation coefficient $r = 0.74$, $p = 0.0059$). There was also a significant correlation between tucatinib clearance and CYP3A activity (Pearson correlation coefficient $r = 0.65$, $p = 0.023$). The findings of this study indicate high interindividual variability in tucatinib clearance *in vitro* in primary human hepatocytes. Genetic variants of CYP3A and CYP2C8 can have a major impact on drug clearance and efficacy. In the future, the impacts of CYP protein concentration, CYP genotypes, and genetic ancestry on tucatinib clearance will be evaluated in this diverse set of donors.

P45 - COVID-19 SEVERITY DYSREGULATES THE EXPRESSION OF CLINICALLY RELEVANT DRUG PROCESSING GENES**Chukwunonso Nwabufor**¹, Jessica Luc², Allison McGeer³, Jeremy Hirota⁴, Samira Mubareka⁵, Andrew Doxey², and Theo Moraes⁶¹University of Toronto, Toronto, ON, Canada, ²University of Waterloo, Waterloo, ON, Canada, ³Mount Sinai Hospital, Toronto, ON, Canada, ⁴McMaster University, Hamilton, ON, Canada, ⁵Sunnybrook Research Institute, Toronto, ON, Canada, ⁶Hospital for Sick Children, Toronto, ON, Canada

Recently, we reported that SARS-CoV-2 infection dysregulates the expression of clinically relevant drug metabolizing enzymes and membrane transporters (DMETs) in both monkey kidney epithelial cells and postmortem COVID-19 human lung tissues¹ and may underpin the altered systemic drug concentrations observed in hospitalized COVID-19 patients^{2,3}. However, the impact of COVID-19 severity on the expression of DMETs is still unknown and there are no putative biomarkers to identify patients at risk of COVID-19 – drug interactions. The overall goal of this study was to investigate the impact of COVID-19 severity on the expression of clinically relevant DMETs and identify potential biomarkers of COVID-19 – drug interactions. Transcriptomic analysis was carried out on nasopharyngeal swabs obtained from 50 SARS-CoV-2 positive patients with varying clinical severity including 17 outpatients, 16 non-ICU patients, and 17 ICU patients, as well as 13 SARS-CoV-2 negative individuals in a clinical study cohort within the Greater Toronto Area. We investigated the changes in the expression of 102 DMETs, 10 inflammatory markers, and 12 xenosensing regulatory genes following SARS-CoV-2 infection and varying ranges of disease severity as indicated by hospitalization status. We detected statistically significant differences in gene expression, including 41 DMETs, 5 inflammatory markers, and 9 xenosensing regulatory genes. COVID-19 severity correlated with increased expression of AKR1C1, MGST1, SULT1E1, FMO5, and decreased expression of ABCC10, CYP3A43, GSTM3, SLC29A4. Notably, CYP3A4 exhibited upregulation specifically in non-ICU patients, aligning with our earlier research that identified CYP3A4 upregulation in SARS-CoV-2-infected Vero E6 cells. The observed changes in DMETs expression were linked to significant alterations in inflammatory and xenosensing regulatory genes. GSTM3, PPARA, and AKR1C1 emerged as potential biomarkers for the observed DMET dysregulation pattern in nasopharyngeal swabs of outpatients, non-ICU, and ICU patients, respectively. COVID-19 severity is associated with the dysregulation of the gene expression of DMETs involved in processing commonly prescribed drugs. This dysregulation may be driven by an imbalance in SARS-CoV-2-induced inflammatory and anti-inflammatory processes. Our studies and that of others suggest that SARS-CoV-2-mediated interactions with DMETs may be tissue

specific^{1,4}, indicating the urgent need to further investigate its impact on drug concentrations at different peripheral tissues^{5,6}.

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P46 - USING INTESTINAL MICROSOMES TO ASSESS THE IMPACT OF GUT METABOLISM ON THE INTESTINAL ABSORPTION OF BIDAC DEGRADERS.

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Accurate projection of human pharmacokinetic (PK) parameters, including oral absorption, is an important component of drug candidate selection. *in vivo* PK studies in pre-clinical species such as mouse, rat, dog, and monkey are conducted to guide the prediction of PK parameters, including intestinal absorption and oral bioavailability; however, occasional variability in oral bioavailability across pre-clinical species is observed and can lead to challenges with making accurate projections. While many factors may contribute to this issue, understanding the contributions of the different mechanisms to overall clearance for a given molecule can play an important role in projecting accurate oral bioavailability. In this study, we focused on factors determining metabolic stability beyond the hepatic contribution. We evaluated the use of intestinal microsomes as an approach to correlate drug metabolism in the gut with observed oral bioavailability and the extent of intestinal absorption following oral dosing. Representative BIDAC™ degrader compounds selected across multiple programs were tested to assess the extent of both Phase I and Phase II metabolism using intestinal microsomes obtained from rat, dog, monkey, and human. The percentage of compound remaining following incubation was correlated with extent of absorption *in vivo* ($f_a \times f_g$ or fraction of administered drug entering the portal vein). These data showed relatively high metabolic instability of compounds in cynomolgus monkey intestinal microsomes, which correlated well with the lower extent of absorption observed *in vivo* in this species. The metabolic stability in human intestinal microsomes was closer to that observed in rat and dog intestinal microsomes. The higher metabolic activity of cytochrome P450 enzymes in the cynomolgus monkey intestine compared to the human intestine is a probable explanation for the differences in stability of compounds in monkey versus human intestine (1). *in vitro* intestinal metabolic stability data using intestinal microsomes from pre-clinical species and humans may provide valuable insights into the contribution of gut metabolism to the extent of intestinal absorption and oral bioavailability across species.

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P47 - THE INFLUENCE OF ETHNIC DIFFERENCE ON SIMVASTATIN AND ITS METABOLITE PHARMACOKINETICS

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Ethnicity is a well-established determinant of the pharmacokinetics of various drugs. Comparison between clinical pharmacokinetic studies using a microdose cocktail revealed the relative difference in midazolam pharmacokinetics, suggesting a potential ethnic effect for altered CYP3A4/5 metabolism between Thai and Caucasian populations. However,

the difference in OATP1B1/3 function was inconclusive. Simvastatin, a commonly prescribed medication in Thailand for hypercholesterolemia, is a substrate of CYP3A4/5, and its active metabolite, simvastatin acid, is a substrate of CYP3A4/5 and OATP1B1/3. Comparison between clinical simvastatin pharmacokinetic studies demonstrated a relatively high simvastatin acid-to-simvastatin AUC ratio in Thais compared to Caucasians. To confirm the observed high AUC ratio in Thais and determine the potential cause, this study was performed using a larger sample size and incorporated genetic analysis. Fifty healthy Thai participants aged 23-77 years were orally administered a single dose of 20-mg simvastatin. Validated liquid chromatography with tandem mass spectrometry was used to quantify plasma concentrations of both simvastatin and simvastatin acid. The pharmacokinetic parameters were calculated using non-compartmental analysis. The AUC₀₋₁₂ of simvastatin was 15.38 ng·h/mL (95% CI: 13.28-17.82) while the AUC₀₋₁₂ of simvastatin acid was 33.32 ng·h/mL (95% CI: 27.80-39.94). The AUC ratio in Thais was approximately 3.6-fold higher than that in Caucasians, concordant with the previous study in Thais. Interestingly, the 949 Thai Genome Reference Database revealed that several SNPs in PON1 had significantly higher frequency of the alternative alleles in Thais compared to those in Caucasians retrieved from gnomAD, including, but not limited to, rs3917490, rs3917529, and rs2237582 (adjusted $P < 0.01$). The expression quantitative trait locus (eQTL) analysis suggested candidate functional SNPs relating to the increased mRNA expression of PON1 in liver ($P < 0.05$). Therefore, the relatively high AUC ratio in the Thai population might be caused by the genetic polymorphisms of PON1 that may affect the PON1 expression in liver, leading to an increase in simvastatin acid formation in the body. Further study using the physiologically-based pharmacokinetic model in the Thai population appeared supportive of these results. Nevertheless, investigations into candidate functional SNPs of pharmacokinetic-related genes of simvastatin and simvastatin acid are ongoing. Determining the effect of these SNPs on enzyme activity is necessary to confirm these results. The study was funded by a grant from the Health Systems Research Institute (HSRI), Thailand, and conducted with support from the Maha Chakri Sirindhorn Clinical Research Center Under the Royal Patronage, Thailand.

P48 - THE INFLUENCE OF ALPHA-1 ACID GLYCOPROTEIN BINDING ON TOTAL PLASMA FREE FRACTION AND THE EFFECT ON CLINICAL PHARMACOKINETICS

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Plasma binding has the potential to modulate efficacy, toxicity, clearance (CL) and drug-drug interactions of pharmaceutical compounds. An assessment of the extent of plasma binding is thus important in the prediction of pharmacokinetics (PK) and understanding biological effects. The two main drug binding proteins in human plasma are human serum albumin (HSA) and α 1-acid glycoprotein (AAG, AGP or ORM), and other plasma components such as lipoproteins can also play a role. HSA concentrations display relatively little interindividual variability and are typically ~40 mg/mL in children and adults, except in subjects with hepatic impairment. AAG concentrations show greater variability with a typical value of 0.8 - 0.9 mg/mL, but increasing 3 – >5 fold in some pathological states. Understanding the preferred site(s) of binding of a ligand can assist in predicting its plasma free fraction in subjects with hepatic impairment or other pathological conditions. The proposed method described here facilitates understanding of the preferred plasma binding partner of test compounds. Equilibrium dialysis is widely accepted as a reliable technique to assess binding. The semi-micro (1 mL half-cell) sized apparatus maximizes the potential for reaching equilibrium, and therefore determining free fractions accurately, even for highly bound compounds, but the method could be adapted for other formats (e.g. HTD96 or RED device). Binding is assessed using; 1) pooled human plasma with known concentrations of HSA and AAG, 2) physiological (40 mg/mL) purified HSA, 3) physiological (e.g. 0.8 mg/mL) purified AAG, 4) "pathological" (5x) purified AAG (e.g. 4.0 mg/mL) and 5) pooled human plasma overspiked with purified AAG to 5x. We find that condition (5) provides a more reliable understanding of the influence of AAG as it is tested within the plasma matrix (so, at physiological pH and ionic strength etc.). We also note that isolated proteins can exhibit artificially high binding, likely due to the lack of endogenous competing ligands, such as fatty acids and bilirubin. We routinely use bictegravir as an HSA-selective ligand (insensitive to AAG concentration) and disopyramide as a ligand for AAG. Using the protocol described above we found that alobresib is a ligand for AAG and this likely explains the high binding observed in plasma from late-stage cancer patients, the variability in PK in those subjects and the low apparent volume of distribution and CL.

P49 - ALTERATION OF RENAL-LIVER-CARDIAC INTERACTIONS LINKED BY CIRCADIAN MACHINERY UNDERLIES CHRONIC KIDNEY DISEASE-ASSOCIATED CARDIAC INFLAMMATION AND FIBROSIS

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Dysfunction of the circadian clock has been implicated in the pathogenesis of cardiovascular disease. The CLOCK protein is a core molecular component of the circadian oscillator, so that mice with a mutated Clock gene (Clk/Clk) exhibit abnormal rhythms in numerous physiological processes, including metabolism. However, here we report that chronic kidney disease (CKD)-induced cardiac inflammation and fibrosis are attenuated in Clk/Clk mice even though they have high blood pressure and increased serum angiotensin II levels. A search for the underlying cause of the attenuation of

heart disorder in Clk/Clk mice with 5/6 nephrectomy (5/6Nx) led to identification of the monocytic expression of G protein-coupled receptor 68 (GPR68) as a risk factor of CKD-induced inflammation and fibrosis of heart [1]. 5/6Nx induces the expression of GPR68 in circulating monocytes via altered CLOCK activation by increasing serum levels of retinol and its binding protein (RBP4). The high-GPR68-expressing monocytes have increased potential for producing inflammatory cytokines, and their cardiac infiltration under CKD conditions exacerbates inflammation and fibrosis of heart. The increase in retinol levels in 5/6Nx mice is caused by the reduced expression of CYP3A11 in the liver. The release of TGF β due to renal inflammation causes retinol to accumulate in the blood by suppressing transcription of Cyp3a11 and Cyp26a1, the encoding key proteins in retinol metabolism in the liver[2]. In addition, serum retinol and RBP4 levels in CKD patients are also sufficient to induce the expression of GPR68 in human monocytes [1,3]. Our present study reveals an uncovered role of monocytic clock genes via hepatic dysfunction in CKD-induced heart failure.

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P50 - AGE AND SEX INFLUENCE THE PHARMACOKINETICS AND BRAIN DISTRIBUTION OF REC-3599, A SELECTIVE INHIBITOR OF PROTEIN KINASE C, IN RATS

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This study focuses on REC-3599, a potent, selective, orally bioavailable small molecule inhibitor of protein kinase C, which has been demonstrated in preclinical disease models to potentially treat GM2 gangliosidosis - a rare, early childhood genetic disorder. We examined whether age or sex impacts the pharmacokinetics and brain distribution of REC-3599 and its primary metabolite, REC-65250. This is determined following both single and repeat (5 days) oral administration of 30 mg/kg of REC-3599 to either juvenile or adult Fischer 344 rats. Results indicate adult female rats had significant higher REC-3599 C_{max} (adult vs juvenile: 483 vs 62 ng/mL and 577 vs 49 ng/mL on Days 1 and 5, respectively) and AUC_{0-24hr} (adult vs juvenile: 4892 vs 506 ng*h/mL and 6434 vs 333 ng*h/mL) than juvenile female rats. Conversely, age had less influence on REC-3599 C_{max} (adult vs juvenile: 134 vs 69.8 ng/mL and 116 vs 715 ng/mL on Days 1 and 5, respectively) and AUC_{0-24hr} (adult vs juvenile: 1034 vs 494 ng/mL and 1327 vs 1922 ng/mL on Days 1 and 5, respectively) in male rats compared to females. In the brain, both male and female juvenile rats had comparable REC-3599 concentrations (female vs male: 204 vs 193 ng/mL and 187 vs 143 ng/mL on Days 1 and 5, respectively). However, adult female rats displayed higher brain concentrations of REC-3599 than adult male rats (female vs male: 43.5 vs 6.75 ng/mL and 53.1 vs 6.95 ng/mL on Days 1 and 5, respectively). Notably, juvenile rats had a higher concentration of REC-3599 than adult rats, possibly pointing to age-dependent rat blood-brain barrier (BBB) permeability for this compound. REC-65250 displayed similar profiles and slightly higher plasma exposures across groups, along with longer T_{max} and t_{1/2} than seen for REC-3599. These findings can inform dose selections in disease-relevant animal models and future interpretations of REC-3599 pharmacokinetics and pharmacodynamics in humans.

P51 - GUT BACTERIAL METABOLITE 3-PHENYLPROPIONIC ACID INHIBITS CYTOCHROME P450 2E1 EXPRESSION AND ATTENUATES ACETAMINOPHEN-INDUCED CYTOTOXICITY IN PRIMARY MOUSE HEPATOCYTES

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Acetaminophen (APAP)-induced hepatotoxicity has been a major cause of acute liver failure worldwide and is caused by APAP bioactivation into a toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI), by hepatic drug-metabolizing enzymes. The gut microbiota has emerged as a modulator of the hepatic expression of drug-metabolizing enzymes and may contribute to inter-individual variability in APAP-induced hepatotoxicity. Our group previously reported that a gut bacterial metabolite phenylpropionic acid (PPA) alleviates APAP-induced hepatotoxicity in mice. This was accompanied by decreased hepatic protein (but not mRNA) levels of cytochrome P450 2E1 (CYP2E1), the major enzyme responsible for APAP bioactivation. In this study, to elucidate the mechanisms of PPA action, PPA effects on APAP toxicity and CYP2E1 expression were investigated in primary mouse hepatocyte culture and AML-12 mouse hepatocyte cell line. PPA, at physiological concentrations, decreased APAP-induced cytotoxicity (determined by the lactic dehydrogenase, LDH, release) in both primary mouse hepatocytes and AML-12 cells. Furthermore, we observed a decreased CYP2E1 protein expression in the PPA-treated hepatocytes, suggesting that the previous *in vivo* findings in PPA-supplemented mice are attributable to the direct effects of PPA on CYP2E1 expression. Unlike in mice, however, PPA treatment

decreased the mRNA levels of CYP2E1 in primary mouse hepatocytes. Blocking protein synthesis through co-treatment with cycloheximide minimally impacted the decrease in CYP2E1 protein levels induced by PPA, supporting the minimal effects of PPA on the degradation rate of CYP2E1 protein. Overall, these results suggest the role of PPA in the transcriptional regulation of CYP2E1 in primary mouse hepatocytes. Our future study will investigate the detailed molecular mechanisms underlying PPA action in primary mouse hepatocytes and AML-12 cells.

P52 - CFT8634, A POTENT, SELECTIVE AND ORALLY BIOAVAILABLE BiDACTM DEGRADER OF BRD9, HIGHLIGHTS THE POTENTIAL OF C4T'S TORPEDO® PLATFORM TO DEVELOP DEGRADERS WITH EXCELLENT DRUG-LIKE PROPERTIES AGAINST CURRENTLY UNDRUGGABLE TARGETS

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Close to 85% of proteins are presently undruggable or poorly drugged. Targeted Protein Degradation (TPD) is a powerful modality that offers the potential to expand the landscape of druggable targets. C4T's TORPEDO® platform can be used to design protein degrading therapeutic candidates that have the potential to improve patient care. Bromodomain-containing protein 9 (BRD9) is a component of the non-canonical BRG1/BRM-associated factor (ncBAF) chromatin remodeling complex involved in regulating gene expression and DNA damage repair. Synovial sarcoma (SS) is a rare and aggressive soft tissue malignancy that harbors a SS18-SSX fusion gene, resulting in a synthetic lethal dependence on BRD9. Inhibition of the BRD9 bromodomain is insufficient to manifest this synthetic lethality; however, genetic ablation or small-molecule mediated degradation of BRD9 blocks tumor cell proliferation in SS preclinical models. CFT8634, (S)-3-((4-(4-((S)-1-(2,6-dimethoxy-4-(1,4,5-trimethyl-6-oxo-1,6-dihydropyridin-3-yl)benzyl)-3,3-difluoropiperidin-4-yl)piperazin-1-yl)-3-fluorophenyl)amino)piperidine-2,6-dione, is a potent, selective and orally bioavailable BRD9 degrader with excellent drug-like ADME properties. CFT8634 is comprised of a BRD9-binding ligand moiety, a chemical linker, and a cereblon E3 ligase-binding ligand moiety. This presentation will focus on the preclinical *in vitro* and *in vivo* ADME profile of CFT8634 along with some early clinical pharmacokinetic (PK)/pharmacodynamic (PD) results. The *in vitro* cellular potency and *in vivo* PK/PD/efficacy of CFT8634 in patient-derived xenograft (PDX) models and global proteomic evaluation data will also be shared. In preclinical studies, CFT8634 demonstrated favorable drug-like properties in both *in vitro* and *in vivo* systems. CFT8634 was modestly metabolized in mouse, rat, dog, and human cryopreserved hepatocytes and the qualitative metabolite profiles were comparable across hepatocytes from preclinical species and human. The plasma protein binding of CFT8634 to mouse, rat, dog, and cynomolgus monkey was high, while it was moderate to high for human plasma proteins. The PK properties of CFT8634 were consistent across preclinical species. The in-vitro drug-drug interaction profiles in combination with the expected unbound systemic concentrations in the predicted efficacious dose range, suggest a low likelihood of CFT8634 being a perpetrator of CYP450 enzymes and/or causing transporter mediated drug-drug interactions in humans. The early clinical data of CFT8634 showed a dose-proportional linear PK profile in humans with concomitant robust degradation of BRD9 in tumor samples. These data highlight the potential of using C4T's TORPEDO® platform to design drug-like degrader molecules effective against targets that are beyond the reach of conventional inhibitors.

P53 - THE INVESTIGATION OF THE METABOLISM AND DISPOSITION MECHANISM OF BUPRENORPHINE AND ITS ANALOGS

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The misuse of opioid-related substances has become a significant public health concern in recent years. Understanding the metabolism and distribution of buprenorphine and its analogs is crucial as it can greatly impact their analgesic effects and potential adverse reactions. The major metabolism pathway for buprenorphine and its analogs is phase 2 glucuronidation with morphine-6-glucuronide has been identified to be biologically active. Despite this importance, the study of glucuronidation of buprenorphine and its analogs has often been overlooked.

In this study, we conducted a comprehensive investigation into the metabolism and disposition mechanisms of buprenorphine and its analogs, including morphine (MOR), nalorphine (NLP), nalbuphine (NBP), naloxone (NLX), naltrexone (NTX), nalmeferene (NMF), and buprenorphine (BPN). Firstly, a highly sensitive LC-MS/MS method was established and validated for the bioanalysis of both the aglycone and glucuronide, including sensitivity (limit of detection, LOD and limit of quantification, LOQ), the lower limit of quantification (LLOQ), calibration curve, selectivity and specificity, accuracy, and precision (both interday and intraday), matrix effect, recovery and carry over.

Secondly, we conducted *in vitro* comparisons of glucuronidation rates in liver and intestine microsomes to identify the primary organ for buprenorphine and analogs' metabolism. Our results revealed that compared to the intestine, the liver exhibited significantly higher glucuronidation rates for BUP-G (6960 nmol/ (g*h)), NLX-G (2458 nmol/ (g*h)), MOR-3-G (431 nmol/ (g*h)) and MOR-6-G (1754 nmol/ (g*h)). For NTX, NBP, and NLP, the liver exhibited the highest glucuronidation formation rate, followed by duodenum, jejunum, and ileum microsomes.

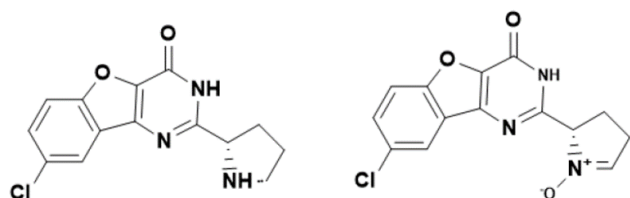
Lastly, we performed an in-situ rat perfusion study to further validate the glucuronidation mechanism. We found bile had exceptionally high concentrations of glucuronide metabolites compared with blood and perfusate. Our findings indicated that orally administered buprenorphine and its analogs are likely to undergo enterohepatic circulation, leading to prolonged drug exposure over time.

Overall, our study sheds light on the metabolism and disposition of buprenorphine and its analogs, providing valuable insights into their pharmacokinetic profiles and potential clinical implications in the future.

P54 - METABOLITE PROFILING OF [14C]BMS-986166 AND ITS ACTIVE METABOLITE (BMS-986166-P) FOLLOWING A SINGLE ORAL DOSE TO INTACT/BILE DUCT CANNULATED RATS

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Sphingosine 1 phosphate receptor (S1PR) 1 controls the egress of lymphocytes from peripheral lymphoid organs and their subsequent peripheral localization, and has been targeted as a way to inhibit unwanted immune responses and thus treat immune-mediated diseases. BMS 986166 is a novel prodrug that is phosphorylated to its pharmacologically active moiety, BMS 986166 P. BMS 986166 P is an S1P receptor modulator, a biased agonist of S1PR1, with agonist activity for S1PR4 and S1PR5 as well. BMS 986166-P induces dose-dependent lymphopenia with maximal effect, and efficacy in animal models of MS, inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and atopic dermatitis (AD). In an *in vitro* kinase assay, BMS-986166 was shown to be a substrate for both sphingosine kinase 1 (SphK1) and sphingosine kinase 2 (SphK2) with SphK2 being more efficient. BMS-986166-induced lymphopenia required SphK2 as no lymphopenia was observed in SphK2 KO mice *in vivo*. Disposition of BMS-986166 and fate of BMS-986166-P were investigated in rats following a single oral dose of [14C]BMS-986166. Metabolism was the major clearance pathway of BMS-986166 in rats, while direct biliary/fecal excretion (6-8% of the dose) and renal excretion (~5% of the dose) were the minor pathways. BMS-986166 was extensively metabolized via multiple biotransformation pathways including oxidation, N-demethylation, glucuronidation, and phosphorylation to form the active metabolite, BMS 986166 P. BMS 986166 P was a major drug-related component in blood (~45% of radioactivity AUC). Urinary and biliary excretion of BMS 986166 P was low (3% of the dose). However, notable amounts of BMS 986166 P were recovered in feces in both intact rats (15.2% dose) and bile duct cannulated rats (11.4% dose), suggesting BMS 986166 P underwent intestinal excretion in rats, possibly via an active transport process.



P55 - CONTRIBUTION OF MCT6 IN REGULATING CELLULAR ACCUMULATION OF THE N-ACETYL-CYSTEINE S-CONJUGATE METABOLITE OF CISPLATIN

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Up to 75% of patients who receive cisplatin experience dose-limiting and permanent hearing loss that can devastate patient quality of life. Unfortunately, the factors that regulate cisplatin ototoxicity are unclear and there are no known clinical interventions. Genetic variation of the monocarboxylate transporter MCT6 has been associated with protection from cisplatin-induced hearing loss, however, its role in mediating this outcome is unknown. We hypothesize that MCT6 regulates cellular uptake of the toxic N-acetylcysteine S-conjugate metabolite of cisplatin (NAC-1). To address this hypothesis, we generated MCT6 expressing *Xenopus laevis* oocytes, as well as a doxycycline mediated inducible MCT6 overexpressing Madin-Darby Canine Kidney (MDCK) cell model, that can be used to measure NAC-1 uptake. Platinum concentrations were measured using inductively coupled plasma mass spectrometry. We found that platinum accumulation in MCT6 or mock-transfected oocytes was no different after exposure to cisplatin for 1 hour at 37°C, but intracellular concentration of platinum increased by approximately 2-fold in MCT6 overexpressing oocytes compared to control cells after exposure to NAC-1 ($p < 0.05$). Function of MCT6 activity in our MDCK model was confirmed by exposing cells to 5 μM of the MCT6 substrate 5-carboxyfluorescein (5-CF) for 15 minutes at 37°C, which resulted in approximately 4-fold increased 5-CF fluorescence (excitation and emission of 492 and 518 nm, respectively) in cells with induced MCT6 expression compared to cells without MCT6 ($p < 0.01$). MDCK cells with induced expression of MCT6 also

showed 30% increased intracellular platinum concentration compared to cells without MCT6 when exposed to NAC-1 for 1 hour at 37°C ($p < 0.05$). Collectively, our findings support NAC-1 as an MCT6 substrate and indicate that future endeavors to assess the role of MCT6 in regulating cisplatin-induced toxicity are required.

P56 - PRIMARY CULTURE OF RAT KIDNEY TISSUE SLICE FOR EVALUATION OF DRUG-INDUCED KIDNEY INJURY USING GAS-PERMEABLE PLATES

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Introduction: Drug-induced kidney injury (DIKI) is a major cause of clinical trial failure in drug discovery and development. However, it has been still a challenge to predict DIKI by *in vitro* studies using experimental animals. DIKI is observed in the various cells composing the kidney, it should be evaluated in a culture system where tissue structure is maintained. Since rat kidney tissue slices maintain physiological kidney tissue structure and function, it is expected to be possible to evaluate various types of DIKI using primary cultured kidney tissue slices. However, tissue culture of kidneys remains challenging since high oxygen supply is required for maintaining tissue survival. In this study, we aimed to construct a primary culture method for rat kidney tissue slices using the high oxygen-permeable InnoCell™ T plate for evaluation of DIKI.

Methods: Kidney tissue slices were prepared from rats were cultured in a CO₂ incubator using InnoCell™ T plates (Mitsui Chemicals). The ATP levels and histopathology in rat kidney slices were examined up to day 3 of culture in the absence or presence of drug exposure. To evaluate drug adsorption to the plates, drug concentration in the medium was measured 24 hours after the addition of drugs to InnoCell™ T plates.

Results: The ATP level in rat kidney tissue slices cultured by InnoCell™ T plates was maintained for 7 days and was higher than that by gas-impermeable polystyrene plates. Moreover, the InnoCell™ T plates exhibited lower adsorption rate of lipophilic drugs than that of poly-dimethylsiloxane (PDMS) plates and exposure to nephrotoxic lipophilic drug reduced the ATP content in the slices compared to that in the untreated slices. By immunohistochemical study, Megalin, a receptor for protein reabsorption in proximal tubular epithelial cells, was observed in cultured rat kidney slices. Moreover, uptake of metformin, a substrate of organic cation transporter 2, and para-aminohippuric acid, a substrate of organic anion transporter 1, was decreased by each transporter inhibitor cimetidine and probenecid, suggesting that transporter-mediated DIKI can be evaluated by primary cultured rat kidney slices.

Conclusion: The rat kidney tissue slice culture system using the highly oxygen-permeable and low-adsorption InnoCell™ T plate is a promising tool for DIKI evaluation.

P57 - PHARMACOKINETICS OF MONOCLONAL ANTIBODIES LOCALLY-APPLIED INTO THE MIDDLE EAR OF GUINEA PIGS

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Sensorineural hearing loss (SNHL) has been a global disease burden for decades. Approximately 466 million people worldwide have hearing disabilities. However, the available pharmacotherapies for SNHL are currently limited, and the main treatments for stable SNHL are medical devices, hearing aids and cochlear implants.

Countless antibodies are currently being used to treat a wide range of diseases. Some of the targets of therapeutic antibodies have been implicated in the pathogenesis of SNHL, suggesting that SNHL may be a new target for monoclonal antibody (mAb) therapy. mAbs can be administered by injection or topical, and the most common site of administration for SNHL drug therapy is local application in the middle ear, which provides the best access to the therapeutic target, cochlea. However, the pharmacokinetics of macromolecular drugs such as mAbs when administered topically in the middle ear are largely unknown. Therefore, we decided to clarify the basic characteristics of mAb pharmacokinetics after topical application to the middle ear using guinea pigs with a large inner ear volume. We used mouse monoclonal antibodies against the FLAG tag (FLAG-mAbs), which do not have specific binding sites in the middle and inner ear, as model antibodies and analyzed their pharmacokinetics. The results showed that FLAG-mAbs rapidly migrated from the middle ear into the cochlear fluid, indicating high permeability of the round window membrane to mAbs. FLAG-mAbs, like small molecules, disappeared from the cochlear fluid 3 h after application. Whole body autoradiography and quantitative evaluation of cerebrospinal fluid and serum indicated that the biodistribution of FLAG-mAbs was restricted to the middle and inner ear. In conclusion, the pharmacokinetics of mAbs during local administration to the middle ear are similar to those of small molecules, suggesting the need for a drug delivery system to ensure adequate delivery of mAbs to the cochlear fluid after local administration to the middle ear.

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P58 - ALTERATION IN INTERCELLULAR LIPID COMPOSITION OF STRATUM CORNEUM IN GOTO-KAKIZAKI RATS: A MODEL OF TYPE 2 DIABETES WITH AN INCREASED FENTANYL RELEASE RATIO FROM THE TRANSDERMAL FORMULATION

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Fentanyl, an opioid, is used to relieve cancer-related pain. However, it has a large inter-individual variability in the release ratio from its transdermal formulation, together with a narrow therapeutic window of blood concentration, causing severe toxicity at higher doses. Therefore, it is important to clarify intrinsic factors that contribute to the variability of the fentanyl release ratio from the transdermal formulation. Previous studies have shown an approximately 10% increase in the fentanyl release ratio from the transdermal formulation in Goto-Kakizaki (GK) rats, a model of type 2 diabetes, at 0-24 hours. This study aimed to clarify changes of the stratum corneum (SC), the outermost layer of the skin where drugs are first distributed, in male GK rats, with Wistar rats as a control. We first confirmed that GK rats show approximately 2-fold higher non-fasting blood glucose level than Wistar rats. In the lateral abdominal skin (the same site as in the previous study), transepidermal water loss (TEWL), skin hydration and surface pH were measured as indicators of the skin properties. In addition, cholesterol, free fatty acid (FFA), and ceramide (CER), the major components of intercellular lipids in the SC, were measured by HPLC-UV, GC-MS, and LC-MS/MS, respectively. There were no apparent differences in the skin appearance or in the indicators of the skin properties (TEWL, skin hydration, and surface pH) between the two groups. However, in intercellular lipids analysis, although the contents of cholesterol, saturated FFA and unsaturated FFA in GK rats did not differ from those in Wistar rats, CER content (total of 12 subclasses) was decreased by approximately 30% in GK rats. It is possible that this alteration in the composition of intercellular lipids in GK rats could have affected the lipid packing structure in the SC, the main permeation pathway for drugs. Our results suggest that a decrease of the CER content in the SC is one of factors contributing to the increased fentanyl release ratio from the transdermal formulation in GK rats.

P59 - MASS BALANCE RECOVERY, ABSORPTION, METABOLISM, AND EXCRETION OF [14C]-ACIZIBOROLE IN HEALTHY MALE SUBJECTS AFTER SINGLE MICROTRACER ORAL DOSING – I – PREPARATION OF GMP [14C]-ACIZIBOROLE DRUG SUBSTANCE

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¹Selcia, Ongar, United Kingdom, ²Quotient Sciences, Nottingham, United Kingdom, ³DNDi, Geneva, Switzerland

Acoziborole (Figure 1), an oxaborole-6 carboxamide, is being developed as an oral single dose treatment for Human African trypanosomiasis (also called "sleeping sickness"). As part of the overall development programme the mass balance, pharmacokinetics, metabolism and excretion of acoziborole were studied in an open-label, clinical phase 1 study in 6 healthy male subjects.

This poster will describe the radiolabelling and purification of [14C]-acoziborole as a GMP radiolabelled Drug Substance to support the human metabolism investigation. The mass balance and clinical safety assessments and the metabolite characterisation investigations are described in separate posters.

A typical process for GMP [14C]-Drug Substance purification is described in Figure 2.

The synthesis of non-GMP [oxaborol-3-14C]-acoziborole, the designated radiolabelled starting material supplied by the Sponsor, is described starting from barium [14C]-carbonate. A risk assessment of the TSE/BSE status of the supplied [oxaborole-14C]-acoziborole was conducted based on information relating to its original manufacture and provenance. [14C]-Acoziborole drug substance was repurified at high specific activity (HSA) from the radiolabelled precursor [14C]-acoziborole using preparative-HPLC. An aliquot was held in storage at -20 °C for use in the GMP [14C]-Drug Substance preparation. Radiochemical dilution using non-radiolabelled Drug Substance provided [14C]-acoziborole at medium specific activity (MSA) for use in stability studies. The MSA batch was subjected to three further sequential dilutions to provide a low specific activity (LSA3) technical batch for a trial Drug Product (IMP) manufacture at Quotient Sciences. Recrystallisation was confirmed to give the desired physical form (Form B).

The validated HPLC chemical purity and assay method used was formally transferred from DNDi and adapted for GMP [14C]-Drug Substance release and radio-stability testing. MSA [14C]-acoziborole showed that the product was stable for at least 4 weeks, both at -20°C and at -80°C.

Repurification was performed in a dedicated GMP radiosynthesis laboratory starting from retained [14C]-acoiziborole to provide GMP [14C]-acoiziborole. QC analysis and QA review of the batch record confirmed the required specifications had been met.

This radiosynthesis and GMP repurification process provided the necessary supply of GMP [14C]-Drug Substance required to support Drug Product manufacture and regulatory submission for a human AME study with acoiziborole in a time and material efficient manner.

Figure 1: [Oxaborol-3-¹⁴C]-acoiziborole

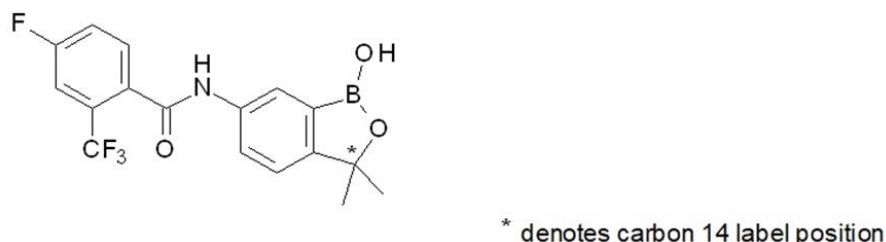
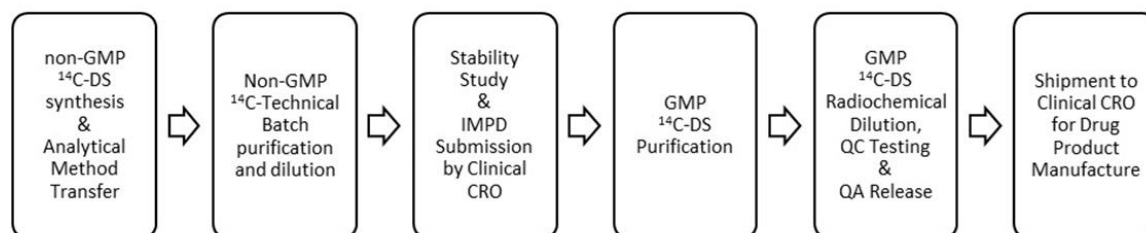


Figure 2: GMP [¹⁴C]-Drug Substance (DS) Purification Process



P60 - MASS BALANCE RECOVERY, ABSORPTION, METABOLISM AND EXCRETION OF [14C]-ACOIZIBOROLE IN HEALTHY MALE SUBJECTS AFTER SINGLE ORAL DOSING - II - MASS BALANCE AND CLINICAL SAFETY ASSESSMENTS

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Acoiziborole (Figure 1), an oxaborole-6 carboxamide, is being developed as a novel oral treatment for Human African trypanosomiasis (also called "sleeping sickness"). Thanks to its long half-life, acoiziborole is active as a single dose treatment. As part of the overall development programme the mass balance, pharmacokinetics, metabolism and excretion of acoiziborole were studied in an open-label, clinical phase 1 study in 6 healthy male subjects.

This poster (2 of 3) will describe from the human ADME study. The radiolabelling of acoiziborole and the metabolite characterisation investigations are described in separate posters.

Six healthy male subjects were dosed as a single cohort in this study, which outline is described in schematic form in Figure 2. Each subject received a single oral dose of 960mg [14C]-acoiziborole (containing approximately 37kBq 14C). Radiolabelled drug product manufacturing was performed in a GMP manufacturing suite that is co-located with the clinical pharmacology unit where the study was performed.

Blood, plasma and faecal samples were collected periodically up to 120 days post dose. Urine collection was impacted by the COVID-19 pandemic and collection was restricted to 360 hours post dose.

All samples were assayed for total radioactivity. Concentrations of acoiziborole and of its metabolite SCYX-3109 were determined over time in plasma and urine. Pharmacokinetics parameters were determined from plasma concentration-time profiles for both total radioactivity and acoiziborole.

Mass balance data showed that an approximately 87% of the administered radioactivity was recovered in the urine and faeces by the end of the sampling period. Elimination was predominantly through the bile and faeces with the majority of radioactivity (>76%) recovered in the faeces and 14% recovered from the urine.

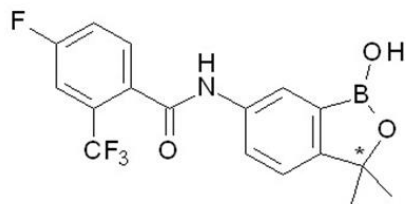
The plasma concentration-time profiles for total radioactivity and acoiziborole were similar up to 1392 h (58 days) and acoiziborole represented 106% and 111% of total radioactivity in plasma, considering AUC_{0-240h} and AUC_{0-inf}, respectively. The metabolite SCYX-3109 accounted for 0.17%. Pharmacokinetics of acoiziborole and SCYX-3109 were consistent with previously observed kinetics in other clinical trials.

There were 13 treatment-emergent adverse events (TEAEs) in total. The majority were mild and/or considered not related

to the IMP. Overall, acoziborole was well tolerated.

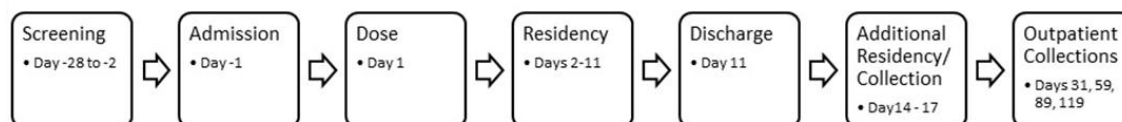
This study and the associated sample analysis provided a thorough understanding of the mass balance of acoziborole. The co-location of GMP manufacturing for radiolabelled drug product with the clinical facility for dosing of study subjects creates an efficient and effective means of study conduct that enables the investigation and understanding of human drug disposition in pharmaceutical development.

Figure 1: [Oxaborol-3-¹⁴C]-acoziborole



* denotes carbon 14 label position

Figure 2: Study outline



P61 - MASS BALANCE RECOVERY, ABSORPTION, METABOLISM, AND EXCRETION OF [14C]-ACOZIBOROLE IN HEALTHY MALE SUBJECTS AFTER SINGLE MICROTRACER ORAL DOSING – III – INVESTIGATION OF METABOLITE PROFILE IN PLASMA, URINE AND FECAL HOMOGENATE SAMPLES USING UPLC-MS FRACTI

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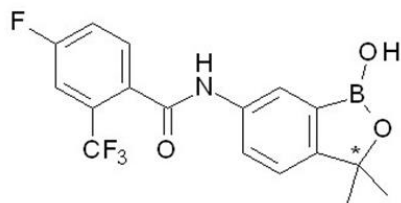
Acoziborole (SCYX-7158, Figure 1), an oxaborole-6 carboxamide, is being developed as an oral single dose treatment for Human African trypanosomiasis (also called "sleeping sickness"). As part of the overall development programme the mass balance, pharmacokinetics, metabolism and excretion of acoziborole were studied in an open-label, phase 1 study in 6 healthy male subjects. This poster (3 of 3) will describe the metabolite characterisation investigations following the human ADME study.

The radiolabelling of Acoziborole and the mass balance and clinical safety assessments from the human ADME study are described in separate posters.

In this ADME study, the metabolite profile of acoziborole was investigated in plasma, urine and faeces homogenate samples. For plasma (0-336h), urine (0-240h) and faeces homogenates (0-240h) individual pools were prepared first, after which a pool per matrix was prepared across subjects. Analytes were extracted from the matrix using acetonitrile (plasma and faeces homogenate samples only) and extracts were injected onto a UPLC (Acquity UPLC system, Waters) equipped with an Acquity HSS C18 100 mm x 2.1 mm, 1.8 µm d.p. (Waters) column, coupled to a Q-Exactive (Thermo) MS system. Separation of analytes was accomplished using gradient elution (0.1% formic acid in water and 0.1% formic acid in acetonitrile). Fractions (120) were collected and analysed off-line on an Accelerator Mass Spectrometer (AMS) system, consisting of an Elemental Analyzer, Double Gas Interface and a 1MV multi-element AMS (model 4110 Bo, High Voltage Engineering). MS data was acquired using data dependent acquisition using an MS2 inclusion list of to-be expected metabolites. Metabolites were tentatively assigned to fractions in which activity greater than the baseline was observed. Acoziborole and metabolite SCYX-3109 (oxidative deboronation of acoziborole) were identified based on the retention time of the reference standard. All other identities were tentatively assigned based on the observed and calculated molecular mass. The vast majority of the radioactivity in plasma and faeces was accounted for by the parent compound (95.1% and 66.0% of the radioactivity in the profile, respectively); it accounted for only 7.6% in urine. The only other entity detected and identified in plasma was mono-oxidized acoziborole (2.3%). In faeces also SCYX-3109 was found (24.2%). Other minor metabolites in faeces included oxidative deboronation + oxidation (2.2%) and mono-oxidized acoziborole (2.4%). More extensive metabolism was observed in urine. Besides acoziborole (7.6%), the following metabolites were identified: oxidative deboronation + glucuronidation, desaturation + urea (together accounting for 27.7% as peaks were not completely resolved), oxidative deboronation + oxidation (5.6%), mono-oxidation (in two different places; 2.2% and 27.2%), desaturated metabolite + di-oxidation (3.5%) and desaturation (11.3%). However, most peaks

account for less than 1% of the administered dose, as only 7.7% of the administered dose was recovered in urine, and its identities were therefore not further elucidated.

Figure 1: [Oxaborol-3-¹⁴C]-acoziborole



* denotes carbon 14 label position

P62 - PROTAC TISSUE DISTRIBUTION

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PROTACs or protein degraders (Prodegs) are large heterobifunctional molecules. The objective of degraders is to degrade the protein of interest instead of classical inhibition of activity. Degradation involves the proteasome via ubiquitination of the protein of interest. This is achieved through the structure of a degrader which involves a target binder, an E3-ligase, and a linker, linking the two mentioned moieties at the correct orientation and distance apart to ensure ubiquitination of a lysine amino acid at the surface of the protein. Hence, Prodegs are complex molecules with likely high molecular weights and lipophilicities resulting in the majority of Prodegs to fall outside the Rule of 5 (i.e., beyond rule of 5, bRo5). To add to the challenge, most therapeutic targets of interest are intracellular requiring these bRo5 molecules to permeate cell membranes. Passive permeability assessments using classical approaches such as Caco-2, MDCK II or RRCK cells suggest - as expected - passive permeability is low, if at all measurable. This makes it challenging to rank order Prodegs during the discovery phase in terms of their ability to permeate cell membranes. Yet, many reports indicate that Prodegs amongst others, display potent antitumor efficacy using xenograft mouse models[1]. This suggests that at least *in vivo* cell permeation is possible. Intrigued by this apparent dichotomy between *in vitro* and apparent *in vivo* permeability, we investigated tissue distribution of Prodegs in rodents. We included both VHL- as well as cereblon containing degraders. We explored if Prodegs have different distributions to organs such as spleen, kidney, heart, and liver. In addition, these distributions were contrasted to distribution into xenografts. As part of our investigations, we also studied the time required to reach equilibrium between various tissues and plasma. We also compared acute versus steady state distributions. Finally, we categorized observed tissue distribution properties using classical physiochemical properties. Our data suggest that Prodegs do indeed distribute effectively into tissues. In addition, Prodegs were detectable in xenografts from about 1 hour post dose. Acute dosing distributions were not different from steady state exposures. Interestingly though, our data indicated a tissue distribution differentiated based on the E3-ligase used. Our analysis, data and conclusions will be shared with this poster.

Reference:

1. Chen, Z., et al., Discovery of CBPD-268 as an Exceptionally Potent and Orally Efficacious CBP/p300 PROTAC Degradable Capable of Achieving Tumor Regression. *Journal of Medicinal Chemistry*, 2024.

P63 - SCREENING OF RENAL PLASMA FLOW BIOMARKER BASED ON UNTARGETED METABOLOMICS ANALYSIS

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Purpose: Effective renal plasma flow (RPF) is an index for quantitatively evaluating renal function. RPF is clinically estimated from the urinary excretion rate and plasma concentration of para-aminohippuric acid (PAH) following intravenous administration. However, application of this method is limited due to its high invasiveness and low throughput. Conversely, creatinine is commonly used in clinical settings but does not accurately represent RPF. According to pharmacokinetic theory, the blood concentration of endogenous compounds highly eliminated/extracted in the kidney with a constant biosynthesis rate may be useful as biomarkers reflecting RPF. However, the systematic approach to identifying such compounds is quite limited. In the present study, simultaneous collection of arterial and renal venous blood was performed for comprehensive measurement of endogenous substances (arteriovenous metabolomics) to identify compounds with high renal extraction ratios to explore candidates of biomarkers for RPF.

Methods: Blood was simultaneously collected from the femoral artery and renal vein in rats. Plasma samples were

injected into LC-qTOFMS, and MS scanning was performed. Picked ions (features) were integrated and analyzed by KNIME software. Features with a vein-to-artery (V-A) peak intensity ratio of less than 0.3 were picked up to identify highly renal extracted compounds. Chronic kidney disease (CKD) model rats and rats with partial ligation of the renal artery and vein were created to alter RPF gradually. Subsequently, PAH was continuously infused, and plasma concentrations of both PAH and biomarkers candidate compounds were quantified using LC-qTOFMS or LC-MS/MS to focus on blood-flow sensitive ones.

Results and Discussion: Features with a V-A peak intensity ratio of less than 0.3 were compared between renal vein ligated rats and sham-operated ones, leading to the identification of a putative amino acid metabolite as an RPF biomarker candidate. In rats with the gradual change in RPF resulting from the partial ligation of the renal artery and vein, the change in plasma concentration of compound X was similar to that of PAH among other amino acid metabolites. The uptake of compound X into hOAT1 cRNA-injected *Xenopus laevis* oocytes was significantly higher than that in vehicle-injected ones. Thus, the plasma concentration of compound X could be a biomarker reflecting RPF probably because it is subjected to OAT1-mediated renal secretion. The conventional assessment of RPF by PAH requires intravenous injection in renal failure patients, whereas the utilization of such endogenous compounds for this purpose could significantly alleviate inconvenience in the patients.

Conclusion: The plasma concentration of compound X may be the potential biomarker that could reflect RPF. It is necessary to evaluate its utility and robustness through correlation with actual measured values of RPF in humans.

P64 - MASS BALANCE, PHARMACOKINETICS, AND TISSUE DISTRIBUTION OF [14C] ZETOMIPZOMIB IN RATS BY QUANTITATIVE WHOLE-BODY AUTORADIOGRAPHY

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Zetomipzomib is a tripeptide ketoepoxide-based selective inhibitor of the human immunoproteasome. Inhibition of the immunoproteasome results in a reduction in cytokine production across multiple immune cell types *in vitro*, reduced activity of inflammatory T-helper cell subsets, increased numbers of regulatory T-cells, and a reduction of plasma cell numbers and autoantibody production. Zetomipzomib is being evaluated in phase 2 studies of LN, and Autoimmune Hepatitis (AIH).

The study utilized 4 groups of male SD rats. Groups 1 (intact) and 2 (bile duct cannulated [BDC]) were used for evaluation of excretion mass balance (MB). Group 3 provided serial plasma samples for evaluation of plasma total radioactivity PK. Group 4 was used for comparison of blood and plasma radioactivity profiles, as well as for evaluation of tissue distribution of total radioactivity using quantitative whole-body autoradiography (QWBA). All rats received a single SC dose of [14C] zetomipzomib at 3 mg/kg as free base and a target radioactive dose of ~100 μ Ci/kg.

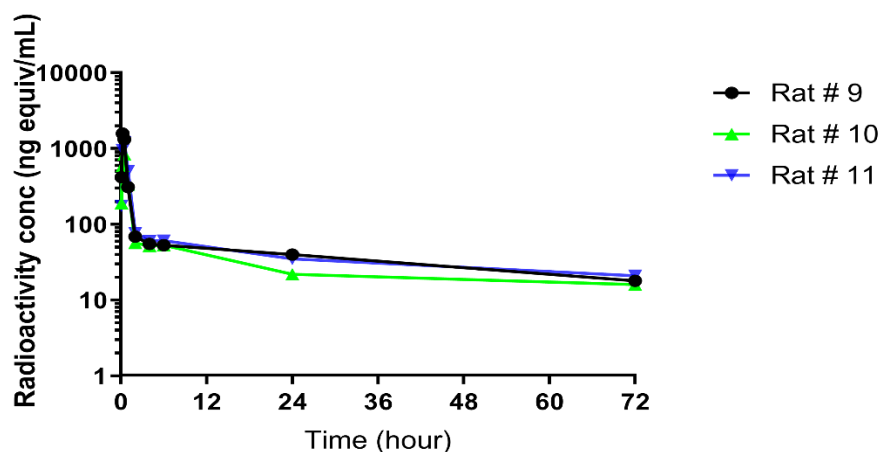
The primary route of elimination of radioactivity in intact rats (Group 1) after a single SC dose of [14C] zetomipzomib was in the feces (65.3%) with elimination also in the urine (26.1%) through 168 h with the total recovery of radioactivity in Group 1 rats of 93.3% of the administered dose. The primary route of elimination of radioactivity in BDC rats (Group 2) after a single SC dose of [14C] zetomipzomib was in the bile (66.6%). Urinary excretion in BDC rats was 20.3%, and 5.8% of the administered dose was recovered in the feces. The total recovery of radioactivity in Group 2 rats was 93.9% of the administered dose. Excretion in bile in the 0-4 h period was 61.3%. This rapid initial elimination indicates that absorption of the SC dose was rapid. Since biliary excretion was the major route of elimination in Group 2 rats, it is likely that a majority of the recovery of radioactivity in feces of Group 1 rats was due to biliary excretion.

In Group 3 rats, the quantifiable concentrations were observed at all collection time points, Radioactivity was rapidly absorbed after SC dosing of [14C] zetomipzomib. After achieving C_{max} , plasma total radioactivity concentrations decreased biphasically (Figure 1).

Drug-derived radioactivity was widely distributed throughout the body of Group 4 rats, with quantifiable concentrations present in nearly all tissues through 168 h (35 of 41 sampled tissues).

Tissues with the greatest overall exposure to radioactivity, based on AUClast values, were lymph node, pituitary gland, spleen, thyroid, and liver. Central nervous system tissues (brain and spinal cord) had very low exposure to radioactivity. The results were consistent with an autoradiogram from a group 4 rat at 0.5 h.

Following a single subcutaneous (SC) dose of [14C] zetomipzomib to male Sprague Dawley (SD) rats, zetomipzomib was found to be excreted in feces, urine and bile. The radio activity of [14C] zetomipzomib was rapidly absorbed and widely distributed to tissues with little in brain.



P65 - OPTIMIZATION OF LINKER SEQUENCE OF AMINOPEPTIDASE A-FC FUSION PROTEINS IN PHARMACOKINETICS AND ANTIHYPERTENSIVE EFFECT

Yukiko Yamaguchi¹, Yusuke Masuo¹, Ayano Tsumoto¹, Katsuya Sakai¹, Ryu Imamura¹, Hidesuke Mizutani², Takafumi Ushida², Tomomi Kotani², Hiroaki Kajiyama², Kunio Matsumoto¹, Shigehiko Mizutani³, and Yukio Kato¹

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Purpose: Hypertensive disorders of pregnancy (HDP) occurs in 3~5% of pregnant women and may cause serious complications for both mother and child. Many antihypertensive drugs are contraindicated for administration during pregnancy, and no therapeutic agent with satisfactory efficacy is available for HDP. Aminopeptidase A (APA) is responsible for the hydrolysis of a vasoconstrictive peptide angiotensin II (Ang II). It was reported that the activity of APA in the blood of HDP patients was reduced, leading to the accumulation of Ang II in the blood, and this may be one of potential mechanism causing HDP. Therefore, administration of recombinant APA protein (rhAPA) can be expected to be a hypotensive modality, and low placental transfer of APA due to its high molecular size may also be preferable to avoid side effect in fetus. The present study aimed to construct a fusion protein between rhAPA and the IgG Fc domain, and its linker sequence was attempted to be optimized to obtain the fusion protein with appropriate pharmacokinetic properties and pharmacological activity. **Methods:** APA-Fc fusion protein (rhAPA-Fc), in which various linkers were introduced between human APA and Fc domain, was expressed in mammalian cells, and rhAPA-Fc was purified from the culture supernatant by affinity chromatography. The enzymatic activity of rhAPA-Fc was evaluated by quantifying the hydrolysis activity of Glu-MCA and the production rate of Ang III during incubation with Ang II which was measured by LC-MS/MS. After intravenous administration of rhAPA-Fc in mice, blood samples were collected, and plasma concentration of rhAPA-Fc was evaluated as Glu-MCA hydrolysis activity. HDP model mice were produced by narrowing down both abdominal aorta and vena cava, and rhAPA-(G4S)3-Fc was administered into the tail vein on days 15.5 and 16.5 of pregnancy, followed by evaluation of systolic blood pressure (SBP) and fetal mortality on day 17.5 of pregnancy. **[Results/Discussion]** Various linkers were introduced into rhAPA-Fc, and the hydrolysis activity (kcat/Km) of Ang II was found to be improved by up to 8 times. The terminal half-life after intravenous administration was 1.4 times compared to rhAPA-Fc without a linker. In HDP model mice, intravenous administration of rhAPA-Fc was shown to suppress the increase in SBP and tend to reduce fetal mortality. These results suggest that linker-introduced rhAPA-Fc may confer benefits to both the mother and the fetuses in the HDP model mice by improving enzymatic activity and blood stability, proposing validity as an antihypertensive therapeutic agent.

P66 - RADIOKINETICS, PHARMACOKINETICS, MASS BALANCE AND EXCRETION, AND METABOLISM OF RADIOLABELED PROTAC® ANDROGEN RECEPTOR DEGRADER BAVDEGALUTAMIDE (ARV-110) FOLLOWING INTRAVENOUS AND ORAL ADMINISTRATION IN RATS

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Bavdegalutamide, also known as ARV-110, is an orally bioavailable PROteolysis Targeting Chimera (PROTAC®) androgen receptor (AR) degrader being evaluated in prostate cancer. An absorption, distribution, metabolism, and excretion study was performed in male and female Sprague Dawley rats to determine radiokinetics, bavdegalutamide pharmacokinetics, mass balance and excretion, and metabolite profiles of bavdegalutamide using two alternatively labeled [¹⁴C]-bavdegalutamide (nitrile and phthalimide carbonyl) molecules which were dosed separately in parallel groups in order to assess overall contribution of metabolic pathways related to PROTAC linker-breakage. Intact and BDC

(bile duct-cannulated) rats received a single IV dose at 1 mg/kg (30 μ Ci/kg); intact rats received an oral dose at 6 mg/kg (100 μ Ci/kg). Blood, plasma, bile, and excreta were collected, and total radioactivity was determined. Plasma concentrations of bavdegalutamide were determined by LC/MS/MS. In addition, the metabolite radio-profiling and identification were performed in plasma, bile, and excreta. Overall, the radiokinetics, bavdegalutamide pharmacokinetics, mass balance, and excretion results from the two labels and sex were similar. The plasma clearance of bavdegalutamide was low at 5.0 – 7.0 mL/min/kg. The volume of distribution was high at 7.9 – 11.2 L/kg. Bavdegalutamide was the major [^{14} C]-derived radioactivity component in plasma, accounting for 50.1 – 59.5% of plasma radioactivity. Bavdegalutamide was moderately absorbed based on blood and/or plasma radioactivity, with the fraction of radioactivity dose absorbed estimated at 25% to 36% and the oral bioavailability at 28% to 33%. The similar value of oral bioavailability to the estimated fraction of radioactive dose absorbed suggests minimum first pass metabolism. There was limited partitioning of [^{14} C]-derived radioactivity into blood cells with blood/plasma concentration ratio of 0.55 to 0.90. The total radioactivity recovery in excreta ranged from 84% to 96% over the 168 h for intact rats and from 67% to 72% over the 72 h for the bile duct cannulated (BDC) rats during the sampling periods. The major route of radioactivity excretion was feces in intact animals and bile in BDC animals. Urine was a minor route of excretion for all groups. Unchanged bavdegalutamide was the most abundant radioactive component in the feces after either IV or PO dosing but was minimal in the bile in BDC rats after IV dosing, suggesting intestinal secretion as a significant clearance pathway along with metabolism. Bavdegalutamide was metabolized in rats and the metabolites were excreted primarily in bile and feces. Up to sixteen metabolites were detected in various matrices. The major metabolic pathways involved hydrolysis, mono-oxidation, oxidative deamination, alone or in combination thereof. Overall, metabolic pathways related to linker-breakage did not contribute meaningfully to the overall metabolic clearance of bavdegalutamide in the rat.

P67 - ABSTRACT WITHDRAWN

P68 - TRIGGERING OF PACLITAXEL RELEASE FROM PEG MONOGLYCERIDE NIOSOMES BY EMPTY SPAN85 NIOSOMES

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Purpose: PEG liposomes are one of the excellent nanoparticle formulations that can efficiently deliver drugs into tumor tissues. However, due to the high drug retention capacity and resultant insufficient drug release from liposomes that have distributed to tumor tissues, there have been some cases where antitumor effects are not as satisfactory as expected from the amount of liposomes distributed in tumors. Our previous studies revealed that adding empty Span85 niosomes to paclitaxel (PTX)-encapsulated PEG liposomes significantly improved the release rate of PTX. It was also suggested that the membrane fusion of PTX-encapsulated PEG liposomes with Span85 niosomes would be at least partly responsible for this result. Niosomes are vesicles possessing an aqueous core enclosed in bilayer structure, which mainly consist of non-ionic surfactant and cholesterol. Similar to liposomes, niosomes are capable of encapsulating not only lipophilic but also hydrophilic drugs. Therefore, in this study, we used three types of monoglycerides (MG), MG-C12, MG-C16, and MG-C18 to prepare PTX-encapsulated PEG niosomes as nonionic surfactants with structures similar to the phospholipids used in liposome preparation. We prepared three different types of PTX-encapsulated PEGylated MG niosomes and evaluated their PTX release behavior by adding empty Span85 niosomes.

Methods: MG niosomes and Span85 niosomes were prepared by conventional thin film hydration method. To evaluate the PTX release behavior, dialysis assay was used where empty Span85 niosomes were added to PTX-encapsulated PEG MG niosomes at various mixing ratios. PTX concentrations in different samples were measured by HPLC.

Results and Discussion: The release of PTX from the PTX-encapsulated MG niosomes showed sustained release pattern, and the released PTX at 48 hours after the start of dialysis was approximately 10% of total PTX amount. On the other hand, the addition of empty Span85 niosomes significantly improved the PTX release rate in all PTX-encapsulated MG niosomes, and the released amount of PTX exceeded 40% even with the combination that showed the lowest value. Furthermore, it was suggested that the optimal mixing molar ratio of PTX-encapsulated MG niosomes and empty Span85 niosomes was 1:3 within the range studied. We also investigated the mechanisms underlying these above-mentioned PTX release triggering from MG niosomes by empty Span85 niosomes, and it was suggested that the mechanism would not be the same with the one involved when empty Span85 niosomes are added to PTX-encapsulated PEG liposomes.

P69 - BLOOD GLUCOSE CONTROL VIA ORAL ZN-INSULIN ADMINISTRATION TOGETHER WITH SMALL INTESTINE-PERMEABLE CYCLIC PEPTIDES IN MOUSE MODELS OF DIABETES MELLITUS

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Oral insulin can potentially improve insulin therapy in patients with Diabetes Mellitus (DM). In a previous study, we demonstrated that oral co-administration of an insulin hexamer stabilized by zinc ions (Zn-insulin) with a small intestine-permeable cyclic peptide synthesized from D-amino acids (D-DNP peptide) effectively lowered blood glucose levels in

mice. However, the hypoglycemic effect of co-administering oral insulin with this D-DNP peptide is insufficient for therapeutic applications, highlighting the need for novel strategies to enhance its efficacy. To address this need, we synthesized a D-DNP-V peptide by linking a D-DNP peptide with a modified insulin-binding peptide (IBP) via a G3S linker. We aimed to investigate the hypoglycemic potency of orally administered Zn-insulin in combination with the D-DNP-V peptide in wild-type and DM mouse models. Co-administration of Zn-insulin with the D-DNP-V peptide into the mouse small intestine resulted in a 3.3-fold enhancement of hypoglycemic potency compared to co-administration of Zn-insulin with the D-DNP peptide. To improve oral absorption of Zn-insulin with D-DNP-V peptide (referred to as oral D-DNP-V/Zn-insulin), we included a trypsin inhibitor, increased the ZnCl₂ concentration, and optimized the ratio of insulin to D-DNP-V peptide. Following oral administration of D-DNP-V/Zn-insulin, blood glucose levels decreased to approximately 50% of their baseline levels after 90 min and returned to normal after 360 min. To maintain the hypoglycemic effect, we administered omeprazole (a gastric acid secretion inhibitor) and pepstatin (a pepsin inhibitor) two hours prior to D-DNP-V/Zn-insulin. This pretreatment (oral pre/D-DNP-V/Zn-insulin) sustained the hypoglycemic effect of D-DNP-V/Zn-insulin for up to 300 min. Furthermore, oral pre-(+) D-DNP-V/Zn-insulin normalized blood glucose levels in a streptozotocin (STZ)-induced DM mouse model. In a genetically induced DM mouse model (Kuma mice), which exhibits higher blood glucose levels than STZ-induced DM mice, oral D-DNP-V/Zn-insulin demonstrated significant hypoglycemic efficacy. Kuma mice treated with oral pre-(+) D-DNP-V/Zn-insulin daily for three days showed consistent hypoglycemic effects. Lastly, oral pre-(+) D-DNP-V/Zn-insulin showed hypoglycemic effects when administered subcutaneously at the same dose, with a pharmacological availability of 32% in wild-type mice and 41% in STZ-induced DM mice. In summary, oral insulin combined with D-DNP-V and gastrointestinal and gastric acidolysis inhibitors effectively controlled blood glucose levels in DM mice. This study may pave the way for the development of oral insulin therapy, potentially improving the quality of life for patients with DM.

P70 - A CASE STUDY FOR VISUALIZING OLIGONUCLEOTIDE THERAPEUTICS DISTRIBUTION IN RAT ORGANS USING MALDI MASS SPECTROMETRY IMAGING

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Therapeutic oligonucleotides (OGNs), which are part of next-generation pharmaceuticals, have been introduced to the market continuously since around 2016, and have been actively developed in recent years. Various quantitative methods (e.g. ligand binding assays utilizing hybridization, fluorescent-LC of the hybridized sample with the fluorescence probe, LC-MS/MS using ion pair reagents or hydrophilic interaction liquid chromatography (HILIC) columns) have been reported for measuring OGNs present in the body after administration. However, these methods cannot provide information on the localization of administered OGNs within tissues. On the other hand, mass spectrometry imaging (MSI) has attracted attention in recent years in the field of drug discovery research. Additionally, this technique has allowed visualization of the spatial distribution of target compounds in biological samples without labeling. Therefore, the aim of this study is to visualize the distribution of OGNs in tissues using MSI. At first, nusinersen, an antisense OGN, was administered to male rats at a single dose of 10 mg/kg (i.v.). Kidneys, livers, and other tissues were collected at 1, 8, 24, and 48 hours after administration (n=1). The organs were rapidly frozen using dry ice and stored in a deep freezer (-80°C) until tissue-sectioning. Secondly, we developed an MSI method to visualize nusinersen and its two metabolites (3'N-1, 3'N-2) using MALDI-FT-ICR mass spectrometer (solariX 2xR, Bruker, MA). Subsequently, the distribution of nusinersen and its two metabolites in kidneys and livers was evaluated. The results showed that nusinersen tended to be distributed to the cortex of the kidneys across all administration groups. Similarly, the two metabolites of nusinersen also showed a tendency to be distributed to the cortex. In contrast, it was confirmed that nusinersen was distributed spreading into the entire liver tissues of all the administration periods. In the livers, the signal intensity of nusinersen was lower compared to that in the kidneys, and its two metabolites were not detected due to insufficient sensitivity. Finally, quantitative mass spectrometry imaging (qMSI) analysis of nusinersen was performed using mimetic tissue models with blank homogenates of the kidneys and livers for calibration curve samples. The calibration curves for the kidneys (5 to 300 µg/g tissue) and livers (5 to 100 µg/g tissue) showed good linearity and accuracy of the back-calculated values. Quantitative calculations were performed by selecting the entire tissue section as the region of interest. Measurements by qMSI were performed twice using serial sections for each tissue, and the average values were used as the quantitative values. The tissue concentrations of nusinersen were 65.6-107 µg/g tissue in the kidneys and 7.63-15.0 µg/g tissue in the livers. Furthermore, the concentrations of nusinersen in the tissue sections were also quantified using LC-MS/MS. Comparison of the quantitative values obtained from qMSI and LC-MS/MS methods showed that the qMSI quantitative values were 68.2% to 108.0% of the LC-MS/MS, indicating a close correlation between the two methods. Thus, spatial distribution analysis in tissue section using MSI provides new insights into both metabolism and disposition of oligonucleotide therapeutics. Additionally, we are currently working on MSI measurement for other organ tissues.

P71 - INVESTIGATION OF PHARMACOKINETIC PROPERTIES AND BRAIN PENETRATION OF GENIPIN: A POTENTIAL PHARMACOPHORE FOR PARKINSON'S DISEASE TREATMENT

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Parkinson's disease (PD) is characterized by the misfolding of α -synuclein, which aggregates into toxic clusters known as Lewy bodies. Once the Lewy body is formed, it spreads throughout the brain through neurons, leading to onset of PD. It is also characterized by dopamine depletion, mitochondrial dysfunction, and progressive damage and loss of dopaminergic neurons in the substantia nigra of the midbrain, contributing to its development. A number of drug candidates have been discovered and developed to treat PD, and recent research has shown that genipin as a noteworthy pharmacological candidate, revealing its significant efficacy in mitigating the aggregation and toxicity of α -synuclein. Genipin, an iridoid compound abundantly found in Gardenia fruits exhibits numerous biological activities by modulating various cellular signaling pathways. Additionally, it has been found to reduce the generation of various neurotoxic molecules from activated microglia, thereby protecting neurons. Although considerable efforts have been dedicated to elucidating the pharmacological mechanisms of genipin, the comprehension of its pharmacokinetic properties remains unknown. In this study, we investigated the plasma and brain pharmacokinetics of genipin in mouse using liquid chromatographic-electrospray ionization-time-of-flight/mass spectrometric (LC-ESI-TOF/MS) method. In plasma, Genipin exhibited high clearance in mouse with IV 2 mg/kg at 231.44 ± 72.29 mL/min/kg, and the AUClast was 8126.46 ± 2345.42 min*ng/mL with IV 2 mg/kg and 4597.75 ± 1535.98 min*ng/mL with PO 10 mg/kg. The observed Vss was 1575.00 ± 408.03 mL/kg for IV administration. In the brain, brain-to-plasma ratio of genipin was 123.32% after IV 10 mg/kg administration and 103.82% after PO 10 mg/kg administration. Despite the partition coefficient (Kp) value for genipin falling within the acceptable range for central nervous system (CNS) drugs, the high clearance rate in plasma made us doubt the existence of active brain metabolites. So, we executed a brain metabolite identification experiment using high-resolution mass spectrometry. However, no metabolites of genipin were detected within the brain. Through this series of experimental investigations employing genipin, we have elucidated the pharmacokinetic characteristics of genipin. Furthermore, the synthesis of metabolically stable derivatives of genipin is currently underway. Subsequent efforts will focus on identifying genipin derivatives optimized for the treatment of Parkinson's disease, leveraging drug efficacy assessments and *in vivo* pharmacokinetic evaluations utilizing these derivatives.

P72 - FORMULATION EFFECTS ON ORAL APE1/REF1 INHIBITORS PHARMACOKINETICS IN MICE

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Apurinic/aprimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) is a multifunctional protein known for its pivotal roles in DNA repair and redox signaling. Its involvement in these critical cellular processes makes it an attractive therapeutic target for a range of diseases, including a variety of cancer types, retinal and inflammatory bowel disease 1-4. The APE1/Ref-1 inhibitor APX3330 has recently concluded Phase II clinical trials for diabetic retinopathy (NCT03375086), underscoring its therapeutic potential. Building upon the success of APX3330, second-generation analogs, including APX2009 and APX2014, have been developed to further disrupt APE1's redox signaling activity⁷. In the realm of drug development, selecting the optimal formulation is a critical step, particularly in the early stages. This process is essential for generating robust pharmacokinetic (PK) and safety data, which are crucial for informing decision-making and mitigating potential failures in later phases of development^{8,9}. Therefore, in this study, we aimed to assess the optimal formulation conditions for APX2009 and APX2014 through *in vivo* PK studies conducted in mice. Male C57BL/6J mice were administered single doses orally (25 mg/kg) and intravenously (5 mg/Kg) of APX2009 and APX2014 formulated in various vehicles (Cremophor, Tween 80, and PEG 400). Blood samples were collected via the saphenous vein at different time points to assess plasma drug concentrations. Subsequently, plasma samples were subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to quantify APX2009 and APX2014 by the IUSCCC Clinical Pharmacology Analytical Core. PK data were then analyzed using non-compartmental analysis (NCA) with Lixoft® PKanalix 2023R1 software. Our results revealed distinct differences in the pharmacokinetic profiles of APX2009 and APX2014 across different formulations. Specifically, formulations containing 4% Cremophor in ethanol demonstrated superior PK parameters compared to alternative formulations. These formulations exhibited higher exposures (AUC_{0-inf} = 317.27 and 16619.42 h.ng/mL, respectively) and favorable clearance rates (CL/F), indicating enhanced bioavailability (F = 13.8% and 77.2%, respectively) and systemic exposure. Overall, our study highlights the importance of formulation optimization in maximizing the pharmacokinetic performance of novel compounds like APX2009 and APX2014. By identifying formulations that offer improved PK profiles, we provide valuable insights to guide further drug development efforts. This enhanced understanding of the pharmacokinetic behavior of APX2009 and APX2014 contributes to the advancement of potential therapeutic options for various diseases, including cancer. Acknowledgments: We acknowledge the IUSCCC Preclinical Modeling and Therapeutics Core for supporting the animal studies. This work was supported by

grants from the National Institute of Health, National Cancer Institute R01CA167291; R01CA254110; R01CA264471; R01CA231267 and R01CA205166; U01CA274304 and by the IU Simon Comprehensive Cancer Center, P30CA082709. COI: Mark R. Kelley has licensed APX2009 and APX2014 through Indiana University Research and Technology Corporation to Apexian Pharmaceuticals LLC and Ocuphire Pharma. Neither Apexian Pharmaceuticals nor Ocuphire Pharma had either control or oversight of the studies, interpretation, or presentation of the data.

P73 - COMPARISON OF TITER AND SIGNAL-TO-NOISE RATIO FOR DETERMINATION OF ANTI-DRUG ANTIBODY MAGNITUDE IN IMMUNOGENICITY ASSESSMENT OF BIOPHARMACEUTICALS BY USING ANTI-DRUG ANTIBODY REFERENCE PANEL

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Immunogenicity assessment is one of the critical issues in clinical evaluation of biopharmaceuticals. The emergence of anti-drug antibody (ADA) may lead to reduce efficacy of the drug by shortening blood half-life or inhibiting pharmacological effects, or to adverse reactions by activating complement and/or Fc receptors. In the immunogenicity assessment, measurement of ADA in serum samples by tiered approach is performed. In this approach, i) ADA positive samples are screened by drug-binding assay (ADA screening assay), ii) ADA positivity is confirmed by a competitive assay (ADA confirmatory assay), and iii) the positive samples are characterized in terms of titer, neutralizing activity, immunoglobulin subclass and epitopes. For determining titer, titer assay (i.e., measurement of ADA in serially diluted samples) is a standard approach. Titer is a relative value calculated as dilution factor that intersects a cut point and the assay is semi-quantitative. Compared with the information obtained by the titer assay, the assay procedures are time and resource consuming. Recent reports suggested that the signal-to-noise (S/N) ratio obtained by ADA screening assay correlates well with titer, although there are some discrepancies due to the non-linear response of ADA. In this study, we compared the ADA magnitude obtained by S/N ratio and titer by using the ADA reference panel against infliximab and adalimumab. The reference panel consists of a series of monoclonal ADAs established by NIHS, and also international reference monoclonal ADA reference standard established by NIBSC. Because the sensitivity of ADA assay varies depending on the assay protocol, the data using international reference panel can provide the reference information for ADA assay. The ADA assay methods used were electrochemiluminescent (ECL) assay and enzyme-linked immunosorbent assay (ELISA) with bridging format. As a threshold value to determine the ADA positivity, four types of cut point used in the literatures were examined. The cut points consist of the screening cut point (i.e., 95th percentile of individual response of drug-untreated healthy subjects) and three types of titer cut point (i.e., 99.9th percentile of the drug-untreated healthy subjects, mean + 6 x SD from the response of drug-untreated healthy subjects, and mean of negative control in the plate + (specific multiplier x (within-plate SD)) (Reference: E. Wakshull et.al., *Bioanalysis* 3, 2627, 2011). As a result, S/N ratio correlated well with titer, although the samples with hook effect in ADA screening assay showed lower correlation. Regarding the cut point, we demonstrated that using higher titer cut point may be useful to avoid bias between titer and S/N ratio due to the flat region of concentration-response curve in very low ADA concentration range. Because titer is relative value, this approach can be acceptable. Further case study and discussion for regulatory acceptability of this approach is desirable.

P74 - STABILITY TESTING AND METABOLITE PROFILING TO SUPPORT OPTIMIZATION OF CYCLIC PEPTIDES FOR ORAL DELIVERY

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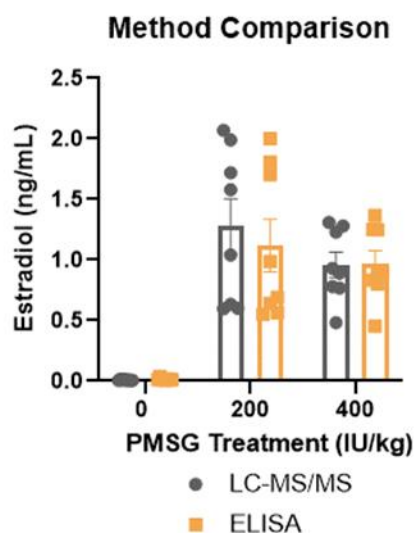
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Delivery of peptides through the oral route is challenging. They are inherently susceptible to degradation and enzyme mediated hydrolysis in the gastric and intestinal environment. Even if they survive the gastro-intestinal environment they need to have reasonable stability in the liver. Chemists have used several approaches to generate stable peptides which include modification of the amide bond, structural modification of the natural amino acid and introducing D-amino acids into the structure. Even with these modifications, the peptides need to be tested for stability in the gastro-intestinal environment. We use a tiered approach to study the stability of the peptides. Initially the peptides are tested for stability in simulated gastric fluid (SGF), simulated intestinal fluid (SIF) and plasma. Once they are identified as stable in these matrices we advance the peptides through additional testing. One of the key pieces of information in supporting the SAR is identifying the primary sites of modification. For this we use a medium throughput assay where we incubate the peptides in 96-well format for up to 4 hrs using a liquid handler. The samples are then processed for HRMS analysis and analyzed on a vendor independent software system, enabling future machine learning. These data get uploaded into Janssen proprietary database for review by multiple groups. This poster will highlight the approaches and challenges that the group faced in establishing the peptide workflow and the lessons learned in applying it to support internal programs.

P75 - AN IMPROVED SAMPLE PREPARATION AND ANALYSIS PROCEDURE FOR ESTRADIOL LC-MS/MS QUANTIFICATION

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The measurement of steroid hormone levels in biological samples as endogenous biomarkers can be important and even crucial for some drug discovery and development programs. However, the sample preparation and analysis requirements to quantify these biomarkers at endogenous levels or lower can be challenging due to factors such as large sample volume requirements (200-500 μ L) as well as time-consuming and/or labor-intensive multi-step preparation techniques such as liquid-liquid and solid phase extractions. We have successfully developed a method that requires a small sample volume (50 μ L) and utilizes a simpler and more commonly used protein precipitation sample clean up and extraction procedure followed by a simple derivatization prior to sample analysis by LC-MS/MS. With this improved sample preparation and analysis methodology, we have successfully been able to detect estradiol concentrations in rat plasma samples down to 0.001 ng/mL that are linear up to 2.5 ng/mL (10-2500 pg/mL). The results obtained with this method correlated very well ($r = .95$) with a commercially available enzyme-linked immunosorbent assay (ELISA) kit. Using this improved method with smaller sample volume requirements and a simpler sample preparation process has enabled us to measure estradiol concentrations at multiple timepoints in the same animal and has also allowed us to prepare and analyze much larger sample batches with relative ease.

**P76 - USING HIGH-RESOLUTION MASS SPECTROMETRY AND SOFTWARE AIDED METABOLITE ASSESSMENT FOR CYTOCHROME P450 REACTION PHENOTYPING OF LOW CLEARANCE SMALL MOLECULE DRUGS**

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Understanding Cytochrome P450 (CYP) metabolism is an important (and FDA required) aspect of small molecule drug discovery for IND submission. CYP reaction phenotyping is routinely performed via measurement of *in vitro* metabolic clearance of potential substrates with liver microsomes +/-inhibitors or recombinant enzyme suspensions to estimation the percent (%) contribution to the total *in vitro* metabolic clearance of your drug candidate by one or more CYP isoforms. Low clearance compounds pose a major challenge for CYP phenotyping, as well as metabolic clearance determination. In addition, early and late states metabolite identification studies may not necessarily provide sufficient information required to identify responsible enzymes and metabolic pathways for clearance and predicted human metabolite profiles.

With many drug discovery programs aiming to reduce metabolic clearance to obtain longer half-life and lower dose drugs, characterizing metabolite profiles and potential drug-drug interactions (DDI) becomes a challenge. CYP phenotyping can be a labor intensive (and expensive) assay, therefore reliably identifying major enzymes involved in the metabolism of a drug candidate before investing in costly clinical DDI studies during drug development is important. We offer the ability to measure and characterize major metabolites formed during CYP Phenotyping and other clearance related assays, useful for both high clearance and low clearance drug candidates. In this work we used high-resolution mass spectrometry (HRMS) to estimate the contribution of eight human cytochrome P450 enzymes (CYP 1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5 isoforms) to the *in vitro* NADPH-dependent metabolism of various compounds utilizing human liver microsomes, recombinant human CYP enzymes (rhCYPs), and long-term human hepatocyte co-cultures in the presence

of selective CYP inhibitors. In addition to the metabolic clearance, metabolite formation was also monitored in the same sample. Furthermore, LC-MS data was collected with data-dependent MS/MS scans for metabolite structure identification and matching to MetID data.

P77 - COMPARATIVE PHARMACOKINETICS OF ANDROGRAPHOLIDE AFTER REPEATED ORAL ADMINISTRATION OF *Andrographis paniculata* FORMULATION CAPSULES IN BEAGLES

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Andrographis paniculata is a medicinal herb recommended by the World Health Organization for the treatment of the common cold. Our previous studies showed *in vitro* anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) activity of andrographolide, and *A. paniculata* extract could prevent death in hamsters infected with the delta variant of SARS-CoV-2 *in vivo*. According to Lipinski's rule of five, andrographolide complies with all of the criteria for potential development as an oral drug candidate. However, this compound is classified as BCS class II as high permeability and low solubility. Therefore, we developed *A. paniculata* formulation capsules by adding a solubilizer (sodium dodecyl sulfate [SDS]) and/or a bioenhancer (Piperine). We administered these capsules orally to Beagles equivalent to andrographolide 5–10 mg/kg/day for 7 consecutive days. We collected blood and excreta 24 h after dosing on days 1 and 7. We used a validated liquid chromatography–mass spectrometry method to determine the andrographolide levels in the biological samples. All of the beagles tolerated the *A. paniculata* formulation capsules and showed no signs of toxicity. The addition of a solubilizer and/or bioenhancer improved the oral bioavailability of andrographolide by approximately 150%–200%. Increasing the solubilizer (SDS) concentration from 0.5% to 1.0% (w/w) improved systemic exposure of andrographolide after a single administration and multiple oral administrations. Increasing the dose of the *A. paniculata* formulation from 5 to 10 mg/kg/day increased the systemic exposure of andrographolide by approximately 2–3-fold. In conclusion, the addition of a solubilizer, bioenhancer and an increase in the dose of *A. paniculata* formulation capsules could enhance systemic exposure of andrographolide in Beagles. This information might be useful for the further development of *A. paniculata* formulations for clinical use against future coronavirus outbreaks.

P78 - STUDY ON ENHANCED GLUCOSE EXCRETION IN CACO2 CELLS BY METFORMIN USING ASYMMETRIC LIPID NANOPARTICLES

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The antidiabetic drug metformin (Met) is thought to decrease blood glucose levels by suppressing gluconeogenesis in the liver, improving insulin resistance in muscle and fat, and inhibiting intestinal absorption of glucose. In addition, in recent years, PET-MRI studies using [18F] Fluorodeoxyglucose have reported that Met may promote glucose excretion into the intestinal lumen [1]. Facilitative glucose transporter 2 (GLUT2), which is normally expressed on the basolateral membrane (B) of small intestinal epithelial cells and transports glucose bidirectionally, is also expressed on the luminal membrane (A) of small intestinal epithelial cells by Met treatment [2]. However, whether the promotion of glucose excretion into the intestinal lumen by Met can be a target for new treatments for diabetes depends on the magnitude of its impact on total hypoglycemic effect.

First, we seeded Caco-2 cells, which are used as a model of intestinal epithelial cells, on Transwell chambers. After addition of Met, immunostaining experiments for GLUT2 and transcellular transport experiments of 3-O-methyl-D-glucose (3OMG), a non-metabolizable glucose analogue, from A to B (absorption) and from B to A (excretion) were performed. As a result, the addition of Met at a high concentration (10 mM) induced expression of GLUT2 in A. Furthermore, accelerated 3OMG transport was observed in the excretion (B to A) compared with the absorption (A to B). A correlation between increased expression of GLUT2 and the enhanced excretion of 3OMG was suggested.

Furthermore, we performed an experiment of Met uptake into Caco-2 cells seeded on culture dishes, and measured the intracellular Met concentration. As a result, intracellular accumulation of Met was observed. It is suggested that Met may remain intracellularly after being taken up by Caco-2 cells and induce GLUT2 expression to A. Currently, in order to achieve a high concentration of Met in the small intestinal epithelial cells, we have prepared an asymmetric lipid nanoparticle formulation containing Met, and are trying to evaluate its intracellular kinetics in Caco-2 cells.

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P79 - QUANTITATIVE PROTEOMICS-BASED IDENTIFICATION OF NOVEL THERAPEUTIC TARGETS FOR PROGRESSIVE SUPRANUCLEAR PALSY USING POST-MORTEM HUMAN BRAIN

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Purpose: Progressive supranuclear palsy (PSP), which is a neurodegenerative disease, causes astrocyte activation and is considered to be involved in various pathological conditions such as decreased energy production and angiogenesis. However, it is unclear how astrocytes are activated and how reactive astrocytes are involved in the pathogenesis of PSP. Therefore, there is currently no effective treatment for PSP because the exact molecular mechanism is not known. Comprehensive analysis of PSP postmortem brain at proteome level would be a rational approach to clarify the pathological molecular mechanisms and promising therapeutic targets. The purpose of the present study was to quantitatively clarify the pathological molecular mechanisms of PSP and identify the promising therapeutic agents using comprehensive and accurate quantitative proteomics. **Method:** The motor cortex (Motor) and posterior cingulate cortex (PCC) were sampled from frozen autopsy brains of PSP patients and healthy controls (Cont.) and applied to SWATH (sequential window acquisition of all theoretical fragment ion spectra) proteomics. Whether the specimens are PSP or not was diagnosed by immunohistochemical staining for phosphorylated tau, and the age matched specimens were used for Control samples.

Result: 5036 and 5691 proteins were quantified in the Motor and PCC, respectively. In Motor (PSP lesion site), the expression levels of 22 proteins related to mitochondrial energy production were decreased in PSP. The previous single cell RNAseq study has reported that 9 out of 22 proteins are the most abundant in astrocytes among neuron, oligodendrocyte, pericyte and endothelial cells etc. By contrast, in PCC, only 4 out of 12 proteins involved in energy production were reduced. Furthermore, unlike PCC, in Motor of PSP, the expression levels of 29 proteins related to the RAS/ERK/MEK pathway, and 8 proteins related to ubiquitination were increased, while the expression levels of 3 proteins involved in proteasome were decreased. It has been reported that the astrocyte is activated by the mitochondrial dysfunction via the RAS/ERK/MEK pathway in the experiment using primary cultured astrocyte. From this previous report and our data, it is considered that the mitochondrial dysfunction activates the RAS/ERK/MEK pathway leading to the astrocyte activation in PSP lesion site. The abnormal protein expressions of ubiquitin-proteasome pathway may be involved in the phosphorylated tau accumulation. Connectivity map (CMAP) was used to search for agents that could potentially restore these fluctuating proteins. The several candidates were treated with primary cultured astrocytes from rats. Bezafibrate and Resveratrol significantly restored the protein expression levels of 70 and 72 out of 161 proteins significantly changed in PSP, respectively.

Conclusion: The present study was the first to clarify the activation of RAS/ERK/MEK pathway and the abnormalities of ubiquitin-proteasome pathway in PSP lesion site of post-mortem brain at proteome levels, in addition to the decreased energy metabolism previously reported. Furthermore, we identified Bezafibrate and Resveratrol as potential therapeutic agents for PSP based on the protein expression levels affected by drug treatment to astrocyte.

P80 - DEVELOPMENT OF A NOVEL LC-MS METHOD FOR METHYLENE BLUE AND ITS APPLICATION TO THE PHARMACOKINETIC AND BRAIN PENETRATION STUDIES IN MOUSE

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by the accumulation of amyloid beta and tau proteins in the brain. Methylene blue (MB), a blue dye used for textile dyeing, is a phenothiazine derivative compound that has been shown to inhibit tau protein aggregation and is currently in phase 2 clinical trials for AD treatment. For therapeutic agents targeting the central nervous system disease such as AD and Parkinson's disease, to manifest their intended efficacy, it is imperative that they possess the ability to permeate the blood-brain barrier (BBB). Despite evidence indicating that MB can inhibit tau protein aggregation in a range of assays, investigations into its pharmacokinetic (PK) attributes and penetration of the BBB *in vivo* remain relatively scarce. Furthermore, while numerous liquid chromatography-mass spectrometry (LC-MS/MS) analytical techniques exist for MB, the majority are not suited for PK studies that necessitate the analysis of extensive sample sets, owing to their prolonged liquid chromatography separation durations. This study developed a novel quantitative method of MB using LC-MS/MS to investigate its PK properties and brain penetration ratio. The method was developed using difluoroacetic acid (DFA) to achieve a fast and reproducible result. A PolymerXTM RP-1 column was used with mobile phases A and B consisting of 0.5% DFA in distilled water and 0.5% DFA in methanol. A calibration curve was generated using the developed method, with curve ranges of 3.05 or 9.15 ~ 2222.22 ng/mL. Quadratic regression and 1/x² weighting were used for curve fitting. Method qualification was performed to suit the in-house criteria with various conditions. In mouse plasma, MB exhibited high clearance with IV 2 mg/kg at 69.35 mL/min/kg. The bioavailability of MB was around 80% based on the AUClast ratios of IV 2 mg/kg and PO 5 mg/kg, which are 25914.85 min*ng/mL and 54258.55 min*ng/mL, respectively. In this study, a novel

LC-MS/MS method which is especially appropriate for PK studies for MB was developed and successfully applied to mouse PK studies and brain penetration study.

P81 - RAPID ADENOSINE BIOANALYSIS WITH LS-LC-MS/MS

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Introduction: Adenosine, a signaling molecule composed of adenine and nucleoside, plays a significant role in the hypoxic tumor microenvironment (TME). Under hypoxic conditions, overexpressed enzyme CD73 catalyzes the dephosphorylation of AMP, resulting in an abundant production of adenosine. This molecule is known to suppress immune cell activity, enhance immune evasion, and foster tumor progression. Traditional LC-MS methods for adenosine are complicated, so sample derivatization, complex mobile phases (such as HILIC mode), and long analytical conditions are required. These methodological demands often limit their application, especially in high-throughput research or studies that involve large sample sizes. LeadSampler (LS) from Sound Analytic, centered around a high-speed sampling robot with up to eight UHPLC capable valves, provides seamless integration to support a multitude of LC workflows and applications. In this study, we developed a rapid and efficient LS-LC-MS/MS method for adenosine quantification in a biological matrix. This innovative approach equips researchers with a robust tool to unravel the complex role of adenosine in the TME and its implications in cancer pathogenesis. Method: Adenosine was analyzed using two parallel Shimadzu Nexera UHPLC-40 with SCIEX Triple Quad™ 6500+ in MRM mode, equipped with a rapid autosampler, Sound Analytics LS-1 autosampler. Samples were pretreated by protein precipitation and adenosine was then separated on a Waters ACQUITY UPLC BEH C18 column (1.7 μ m, 2.1*50 mm) employing a gradient mode of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was maintained at 0.8 mL/min. Mass spectrometry conditions were optimized to monitor the MRM transitions, m/z 268.1/136.1 for adenosine, and m/z 273.2/136.2 for the internal standard, 13C5-adenosine. Result: In this study, a 5% TCA acid in water was selected as an acid precipitant method, instead of commonly used organic solvents. This approach ensured the retention of Adenosine on the column and improved peak shape and ionization efficiency. Furthermore, when sensitivity was not sufficient, the injection volume could be increased without compromising chromatographic efficiency, thereby improving operational feasibility. The method exhibited linearity ($r \geq 0.987$) for the quantification range of 2.0-1000 nM for adenosine in biological matrix. The precision for three-level QC samples ($n = 6$), as denoted by %CV, remained within 1.5 to 7.7%, while accuracy, expressed as %Bias, was within -15.4 to 12.9%. Each sample was analyzed within 30 s and the throughput was about 48 min for one 96-well plate. Conclusion: In this study, we developed a rapid, direct, efficient LS-LC-MS/MS method for high-throughput detection, Adenosine could be well retained, and showed good peak shape and sufficient sensitivity. Analysis of 37 samples was finished within 18.5 min by LS-LC-MS/MS system. The analytical time was shortened to 0.5 min/sample, which reduced 88% of the analysis time compared to the traditional method.

P82 - COMPARATIVE ANALYSIS OF PHARMACOKINETIC CHARACTERISTICS FOR THE IN-SITU ALBUMIN DRUG CONJUGATE

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The therapeutic efficacy and safety of small molecule drugs, particularly in oncology, are often limited by its poor pharmacokinetic properties and lack of specificity. Various attempts have been made to overcome these pharmacokinetic shortcomings and off-target toxicity. A notable approach is the development of antibody-drug conjugates, which have garnered significant attention in the pharmaceutical industry for their improved clinical efficacy and safety based on their tumor specificity and favorable pharmacokinetic profiles. Despite the clinical achievements of ADCs, the complexities of their manufacturing processes and the associated high production costs remain substantial obstacles for both pharmaceutical companies and patients. In this context, in situ albumin drug conjugate might be a possible answer for these challenges. In situ albumin drug conjugate indicates that administered linker-drug, which belongs to small molecule compounds could be conjugated to the originally existing plasma albumin by chemical reaction. Albumin, the most abundant protein in animal plasma, offers several pharmacokinetic advantages, including a long half-life and preferential accumulation in tumor tissues via the enhanced permeability and retention (EPR) effect. Also, it has free nucleophile thiol at its cysteine 34 regardless of animal species and this could react with electrophile moiety such as maleimide, which is widely used for an antibody-drug conjugate's linker, especially in case of cysteine conjugation. Hence, upon intravenous administration of a compound featuring a maleimide moiety, it promptly forms a covalent bond with plasma albumin, thereby hijacking the pharmacokinetic profiles and tumor-targeting capabilities inherent to albumin. In this study, we conducted a comparative analysis of two linker-drugs in the perspective of pharmacokinetic characteristics, both of them possess maleimide functionalities and have different drug release mechanism. One is the MC-VC-PABC-SN38 which is the most widely used peptidase cleavable linker for the antibody-drug conjugate and another one is the MAC-glucuronide-SN38 which could be cleaved by the β -glucuronidase known to be overexpressed at the tumor. The *in vivo* mouse pharmacokinetic evaluation revealed a significant improvement in the pharmacokinetic profiles of the albumin-hijacking

strategy, where the unconjugated SN-38 exhibited a clearance of 191.49 mL/min/kg. Conversely, upon conjugation with albumin, the clearance rates for MC-VC-PABC-SN38 and MAC-glucuronide-SN38 were markedly reduced to 1.26 mL/min/kg and 0.35 mL/min/kg, respectively. This significant reduction in clearance rates for both conjugated forms highlights their enhanced ability to prolong the systemic retention of SN-38, with the MAC-glucuronide-SN38 conjugate, in particular, demonstrating superior stability. The outcomes of this research facilitated an evaluation, from a pharmacokinetic perspective, of the specific linker-drug characteristics that are more conducive to the development of in-situ albumin drug conjugates

P83 - BUILDING AN AUTOMATED, HIGH THROUGHPUT *IN VITRO* ADME SCREENING PLATFORM

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The potential of artificial intelligence and machine learning (AI/ML) in drug discovery is intrinsically tied to the rapid and reliable generation of data for the construction of global models. While published data can be used for constructing a model, variability in assay execution may limit the reliability of the results. In addition to producing reliable data for global AI models, there is also a need for experimentally determined *in vitro* ADME results for the swift ranking of compounds in pharmaceutical development. Assay automation is essential to meet the growing data demand, to support drug discovery program teams and provide AI modelers with enough data to construct global models that contain sufficient structural diversity. Recursion has addressed this dual need by building RADME-01 (Recursion Absorption Distribution Metabolism Excretion), a fully automated platform capable of screening 768 compounds through six iterations of three assays—microsomal stability (human and rat liver microsomes), protein binding (human and rat plasma, human liver microsomes), and PAMPA—every week. This platform automates the entire process, from compound queueing, experiment creation and design, and assay execution, to data processing, review, and warehousing. Samples from all assays are analyzed on a quadrupole time of flight mass spectrometer being served by a reverse phase liquid chromatography system. A compound cassetting and pooling strategy significantly reduces the mass spectrometer instrument time from over 22 days to less than 7 days. Chromatograms are extracted and integrated and the raw data is processed by a commercially derived software tool. All results are then analyzed through an automated decision tree to class the results as passing and reportable or failing. Control compounds, imipramine, fluoxetine, ketoconazole, diltiazem, erythromycin, tolbutamide, and propranolol, were thoroughly scrutinized during the platform and assay validation. Each control demonstrates precision with a percent coefficient of variation below 25%, alongside excellent chromatography and ionization in positive mode. The build of RADME-01 has resulted in a 90x increase in lab throughput with no increase in lab staffing. This high-throughput, automated, and robust platform supports the rapid screening of large compound sets for our drug discovery program teams and facilitates AI/ML modeling for more efficient ranking of hits derived from our phenomic platform.

P84 - HIGHLY DEEP QUANTITATIVE PROTEOMICS ELUCIDATE THE MECHANISM OF ALZHEIMERS' DISEASE TARGETING REACTIVE ASTROCYTE

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Introduction: In the brain, the number of glia cells are about 9 times more than that of neurons. Astrocytes, which consists of glia cells, are activated at an earlier stage than amyloid- β accumulation. On the other hand, it isn't well known about why astrocytes are activated in patients with Alzheimer's disease at the protein level and whether it is related to abnormal energy metabolism. Many AD studies have been conducted in mice, rats, and cells, but many papers have pointed out that they may not accurately reflect the environment in the human brain. Considering the lack of success in the development of therapeutic drugs for AD, it is very significant to conduct a comprehensive protein expression analysis using AD postmortem brains. The purpose of this study is to elucidate the molecular mechanisms in astrocytes in AD patients by comprehensive and accurate quantitative proteomics.

Methods: The Posterior Cingulate Cortex (PCC) and Motor cortex (Motor) were sampled from frozen autopsy brains of AD patients and healthy controls (Cont.) and applied to SWATH (sequential window acquisition of all theoretical fragment ion spectra) proteomics. In addition, we obtained the mass spectra measured by SWATH-MS in the Olfactory Tract (OT) of MCI (Mild Cognitive Impairment) and AD patients from public database to reanalyze by recent software (DIA-NN).

Results/Discussion: More than 5000 proteins were identified in PCC and motor regions, and about 3000 proteins were identified in OT. Compared to Cont., in the PCC of AD patients, the expression levels of 122 proteins significantly decreased, and the expression levels of 197 proteins including astrocyte cytoskeleton-related proteins [GFAP (Glial fibrillary acidic protein) [3.09-fold], HEPACAM (Hepatic And Glial Cell Adhesion Molecule) [1.54-fold], MAOB (Monoamine

Oxidase B) [1.42-fold], etc] and TGF- β signaling proteins significantly increased. By contrast, no increase in expression of these astrocyte-activating molecules was observed in motor. In motor, 27 ribosomal protein (RPs) expressions were significantly upregulated in AD compared to Cont. Also in OT, many RPs (19RPs) were upregulated in MCI and less so in AD (4RPs). The astrocyte marker VIM(Vimentin) was not significantly upregulated in MCI, but was significantly upregulated in AD. [1.61-fold in AD($p < 0.05$), 1.15-fold in MCI (not significantly different)]. This suggests that the increased expression of RPs occurs prior to astrocyte activation.

Conclusion: In this study, we report for the first time that increased expression of RPs occurs in the Motor and OT. We also demonstrated for the first time how astrocyte activation occurs at the protein level. Furthermore, we propose that preventing astrocyte activation is a novel target for AD.

P85 - IDENTIFICATION OF DRUG TARGET ENGAGEMENT OF REPURPOSING CANDIDATES FOR POLYCYSTIC KIDNEY DISEASE BY THERMAL PROTEOME PROFILING

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There is currently only one drug approved by the FDA, EMA and PhMDA for autosomal dominant polycystic kidney disease (ADPKD), a chronic, progressive hereditary disease characterized primarily by fluid-filled cysts in the kidneys. In the search for additional drugs, drug repurposing represents a promising approach for ADPKD. In this strategy, clinically validated drugs with established pharmacokinetics and safety profiles are used for new indications. Here, 2D-thermal proteome profiling (2DTPP), a powerful mass spectrometry-based tool to study a variety of protein interactions, was applied to validate known and identify novel targets of ADPKD repurposing candidates. The compounds birinapant, bardoxolone methyl and salicylic acid were selected for drug repurposing based on their previously demonstrated attenuation of cyst growth *in vitro*, in clinical trials and in preclinical studies, respectively. To mimic *in vivo* conditions, our experiments were performed under physiological oxygen tension in the renal proximal tubular epithelial cell line RPTEC/TERT1. First, we found the expected modes of action (MoA) of each of these three drug candidates. We also identified shared effects on mRNA translation, monocarboxylate transport, and mitochondrial transport and energy metabolism. The latter is a known pathophysiological process in ADPKD, as shown by our proteomic profiling of human ADPKD 3D cyst cultures and tissues (poster title: Global proteomic profiling of a patient-derived 3D cyst model and reference tissue of polycystic kidney disease). In addition, we discovered focal adhesion kinase, an activator of the proliferative PI3K/Akt/mTOR pathway, as a novel potential target that may contribute to the anti-proliferative effect of salicylic acid. Furthermore, we observed thermal destabilization of the glucosidase GANAB, a disease-causing gene involved in polycystin maturation and trafficking, in response to salicylic acid treatment, highlighting the broad range of mechanisms exerted by salicylic acid on cyst growth. Overall, our findings highlight the utility of 2DTPP for evaluation of drug target engagement of repurposing candidates, enabling complex MoA validation and target discovery. Salicylic acid emerges as a particularly promising repurposing candidate for ADPKD due to its anti-proliferative effects and favorable safety profile.

P86 - PROPOSED WORKFLOW TO WEED OUT POTENTIAL DILI-POSITIVE TEST ARTICLES

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Drug-induced liver injury (DILI) is a major cause of drug attrition during drug discovery and clinical development. No single pre-clinical study is adequate to clearly predict the clinical outcome of DILI. To understand and mitigate the risk of DILI in various research programs, Drug Metabolism and Pharmacokinetics (DMPK) along with Toxicology, employs a suite of *in silico* assessments, *in vitro* assays, and *in vivo* studies to triage and select drug candidates with a reduced potential for clinical DILI for further development. The *in vitro* assays that are routinely conducted as part of screening strategy by DMPK (e.g. formation of reactive metabolites during GSH screening assay, IC50 shift during time dependent inhibition assay, inhibition of liver transporters, such as bile salt export pump) in concert with *in vivo* PK assessments and various toxicology assessments (e.g. QSAR, cytotoxicity, integrated 3D spheroid screening) are used to inform on the potential or probability of DILI for early drug candidates. The objective of the poster is to showcase a framework under active development to screen for potential DILI candidates for various neuroscience projects.

P87 - TLC-2716, A NOVEL LIVER X RECEPTOR (LXR) INVERSE AGONIST WITH HIGH ENTEROHEPATIC BUT LOW SYSTEMIC EXPOSURE

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The Liver X receptors (LXR α and β), are key regulators of hepatic and systemic lipid homeostasis, with high expressions in liver and intestine, but also significant expression in immune cells, brain, kidney and other organs. Activation of LXR by physiological agonists (oxysterols) promotes the transcription of multiple genes involved in de novo lipogenesis and cholesterol homeostasis. Inverse agonists of LXR downregulate LXR target genes, thereby reducing liver and systemic lipid levels and representing potential treatments for metabolic diseases. An optimal LXR inverse agonist should have potent repressive effects in the “metabolic” target tissues but should avoid high exposure in post-enterohepatic system where LXR agonism might be beneficial. TLC-2716, a 1-(phenylsulfonyl)indole derivative, is a small molecule pan-LXR inverse agonist currently being developed as an oral agent for the treatment of severe dyslipidemias and nonalcoholic steatohepatitis. In a Phase 1, first-in-human study, TLC-2716 was safe and well tolerated and led to dose-dependent reductions in atherogenic lipids, including plasma triglycerides, low-density lipoprotein (LDL) cholesterol, LDL particles, and apolipoprotein B (1). In this study, the potency and ADME properties of TLC-2716 were evaluated in both *in vitro* and *in vivo* systems. TLC-2716 demonstrated potent biochemical direct binding, nuclear receptor co-repressor recruitment, and cellular reporter activities of LXR with EC₅₀ of 7 to 15 nM. Pharmacokinetics studies demonstrated low plasma exposures and preferential delivery of TLC-2716 into liver with high liver-to-plasma ratios of >100, 23, and 24 in mouse, rat, and dog, respectively. The hepatic extraction ratio was determined to be >90% in portal vein-cannulated rats. Based on *in vitro* transporter studies, the preferential delivery of TLC-2716 into liver appeared to be facilitated by multiple hepatocyte uptake transporters, including NTCP, OATP1B1, and OATP1B3. Mass balance studies in mouse and monkey using [14C]-TLC-2716 indicated approximately 90% of the radioactivity was recovered in feces during a 48-hour period after oral dosing. Consistent with low systemic exposure, very little radioactivity was recovered in urine (<0.2% of the administered dose). In addition, the PK of the radioactivity in plasma, liver, and bile demonstrated high and prolonged exposures in liver and bile suggesting potential enterohepatic recirculation. A QWBA analysis in mice further indicated that the TLC-2716 derived radioactivity was almost exclusively localized in liver and intestine. Together, these data indicate that following intestinal absorption, TLC-2716 is highly extracted in the liver and TLC-2716 and/or its metabolites are secreted into bile with limited systemic exposures thus sparing blood cells and tissues where LXR inverse agonism should be avoided. The potent lipid-lowering effects of TLC-2716 via LXR transcriptional inhibition, a high on-target liver distribution, and the low off-target systemic exposures, come close to the ideal profile of an LXR inverse agonist for metabolic diseases, thus warranting further clinical development of TLC-2716.

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P88 - DEVELOPMENT OF A SUPER HIGH-PLEX IMMUNOHISTOCHEMISTRY PLATFORM PROVIDING IMPROVED CONFIDENCE IN TRANSLATIONAL RESEARCH

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Efforts over the past decades to improve the reliability of translational science have resulted in increasing demand for simultaneous spatial profiling of multiple proteins in tissues using multiplexed immunohistochemistry (mIHC). To meet this demand, significant effort has been made in developing mIHC platforms by changing the labeling method of Abs used for staining. Examples include fluorescent labeling in the PhenoCycler system and metal labeling in Imaging Mass Cytometry. Recently, a new method has been developed to increase multiplexing capacity by staining a single tissue multiple times and integrating the resulting images. This method has attracted attention as a useful approach to improve plex number in mIHC. However, this approach is not compatible with the tyramide amplification (TSA) method, which is capable of achieving the most sensitive fluorescence imaging among current mIHC platforms. This is primarily because the fluorescent dye removal method used in this approach cannot remove the strong fluorescent dye derived from TSA, and high-sensitivity and high-plex mIHC has accordingly not been achieved. Here, we hypothesized that even the highly sensitive fluorescent dyes produced by TSA can be removed by selecting an appropriate TSA reagent and optimizing the fluorescent dye removal procedure. To investigate this, we first selected appropriate TSA reagents, then optimized the methods for removing fluorescent dyes between each round. We then established a method for removing antibodies that strongly bind to tissue antigens, which arose as a new issue during the study. Finally, we determined the conditions required for digital imaging analysis to integrate the images obtained from each round. These efforts resulted in the successful development of an mIHC platform with both high TSA-based sensitivity and a repetitive staining-based high-

plex number. Our improved mIHC is expected to be a useful tool for unravelling the complexities in translational research that have remained unaddressed mainly due to lack of staining sensitivity and plex number. In this presentation, the details of the trial and error that went into realizing the desired method using commercially available human tonsil specimens will be shared, and the wide range of possibilities for utilizing the established method in drug discovery and development research (e.g. diseases prognosis, evaluation of xenobiotics treatment response) will be discussed. Andrea et al., IBEX: an iterative immunolabeling and chemical bleaching method for high-content imaging of diverse tissues, *Nature Protocols*, 17, 378-401 (2022)

P89 - DEVELOPMENT OF A SYSTEM TO EVALUATE BILIARY EXCRETION OF DRUGS USING HUMAN CHOLANGIOCYTE ORGANOID MONOLAYERS

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Purpose: During new drug development, screening of drug excretion into the bile is required. Several evaluation systems have been proposed to estimate the biliary excretion of drugs, but problems persist such as the inability to directly quantify the drug excreted in bile. Recent studies on human liver-derived organoids have been plentiful, and it has also been reported that cholangiocyte organoids can be established from primary human hepatocytes.¹ In this study, we aimed to develop an evaluation system for drug excretion into bile using cholangiocyte organoids. **Methods:** Cholangiocyte organoids were seeded on the insert membrane and formed a cell monolayer. The expression of efflux transporters (ETPs) involved in biliary excretion of drugs was confirmed by mRNA and its localization by immunostaining. We examined the apparent permeability coefficient (Papp) and calculated the efflux ratio (ER) from a transport study using a typical fluorescence-substrate of ETPs. **Results and Discussion:** The cholangiocyte organoids expressed mRNA for ETP, i.e. P-glycoprotein (P-gp), multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein (BCRP). Subcellular localization of each ETP protein was observed, suggesting that cholangiocyte organoid monolayers are oriented with the upper side being the apical side (A side, bile duct lumen side) and the lower side being the basal side (B side, hepatocyte side), and that each ETP is localized to the apical membrane side. Transport studies using a substrate of each ETP showed that the Papp from the B side to the A side was higher than the reverse, and each ER was above 1.0. In addition, this directionality disappeared in the presence of an inhibitor of each ETP. These results indicate that our evaluation system can quantitatively evaluate biliary excretion of drugs. **Conclusion:** The results of this study constitute an original system using cholangiocyte organoid monolayers, one which may prove to be useful as a model for estimating the contribution of biliary excretion to drug clearance.² **References:** 1) Nuciforo S, Heim MH, *JHEP Rep.* 2020, 3(1), 100198. 2) Mizoi K, Ogihara T, et al. *Biol Pharm Bull.* 2024, 47(2), 427-433.

P90 - PM2.5 ENGAGES IMPAIRMENT OF MUCOSAL IMMUNITY, INVOLVING ALTERATION OF COMPLEMENT ACTIVATION AND CELL JUNCTION ASSEMBLY BY REGULATED IL-17 LEVELS: PATHOLOGICAL AND PROTEOMIC APPROACHES

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Fine particulate matter (PM2.5) exacerbates respiratory morbidity, posing significant health and environmental concerns. Although PM2.5 causes various adverse effects on the respiratory tract, the mechanisms of PM2.5-induced lung toxicity have been incompletely characterized. Thus, this study aims to investigate the mechanisms of PM2.5-induced lung inflammation. BALB/c mice were intratracheally administered with concentrations of PM2.5 daily for 7 days. Histopathological analyses of lung tissue were performed. Bronchoalveolar lavage fluid proteomics was analyzed using QTOF LC-MS/MS, and differentially expressed genes were visualized through volcano plots and heatmaps. Functional protein data were further assessed by GO enrichment analysis, KEGG pathways, and STRING protein networking. Moreover, specific proteins of interest were validated using multiplex ELISA. We found that PM2.5 induced marked neutrophilic inflammation in the lungs, as evidenced by the increase of polymorphonuclear cells and macrophages in a dose-dependent manner. Proteomics analysis identified 147 differentially expressed proteins, with 43 down-regulated and 104 up-regulated. Gene ontology analysis revealed that up-regulated proteins are mainly secretory and implicated in the inflammatory response, complement activation, and assembly of various cell junctions. Additionally, a decrease in serum IL-17A levels, supported by STRING network analysis, suggests its involvement in PM2.5-induced mucosal immunity impairment in the respiratory tract and potentially in non-exposed organs. These findings provide mechanistic insight into PM2.5-induced inflammation, facilitating the development of biomarkers of PM2.5 exposure and targeted therapies for PM2.5-related lung diseases.

P91 - ASSESSMENT OF PLASMA PROTEIN BINDING OF PEGYLATED BILIRUBIN: A COMPARATIVE STUDY OF RAPID EQUILIBRIUM DIALYSIS AND ULTRACENTRIFUGATION METHOD

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In the drug discovery and development, the measurement of plasma protein binding (PPB) values of drug candidates is a pivotal process. It helps to understand the critical pharmacokinetic attributes, including absorption, distribution, metabolism, and excretion, which are integral for comprehending a drug's pharmacodynamics and toxicological properties. Additionally, these measurements play a vital role in prediction of the effective doses for human administration. To this end, methodologies such as rapid equilibrium dialysis (RED), ultrafiltration (UF), and ultracentrifugation (UC) are routinely employed to assess PPB, with the RED method often used as the gold standard due to being easy and requiring no special equipment. The choice among these techniques is typically guided by the physicochemical characteristics of the specific drug being analyzed, as each method has distinct advantages and limitations based on the nature of the compound. Bilirubin is renowned for its substantial antioxidant potential. Despite its promising therapeutic benefits, the clinical application of bilirubin has been substantially impeded by its considerable hydrophobicity. To overcome this limitation, a novel derivative named BX-001N, PEGylated bilirubin, was engineered by covalently bonding a PEG36 chain to a synthetic bilirubin 3 α . This innovative modification markedly improved its water solubility, achieving a solubility of approximately 125 mg/mL, as determined by Real thermodynamic solubility tests. This represents a significant enhancement compared to the native solubility of bilirubin, which stands at about 213.8 μ g/mL when measured by Nephelometry. Furthermore, the amphiphilic nature of BX-001N could make it self-assembled into micelles within aqueous environments, indicating the probability in presence of BX-001N in both monomeric and micellar forms in solution. This research aimed to evaluate the PPB of BX-001N employing both the RED which consists of dialysis membrane with 8 kDa molecular weight cut off and UC techniques under varied centrifugal conditions using male Sprague-Dawley rat plasma. The results unveiled a pronounced discrepancy in PPB values, with the RED method demonstrating a PPB of 99.74%, while the UC method yielded values of 87.23% and 89.83% under centrifugal conditions of 155,000 xg for 4 hours and 480,000 xg for 4.5 hours, respectively. This variance in PPB values accentuates the critical impact of a drug's self-assembly behavior on the selection of a suitable PPB measurement methodology. Given these variances, the study advocates for a more nuanced consideration of a drug's physicochemical properties when selecting a PPB measurement technique, particularly for compounds like BX-001N that possess the ability to self-assemble into micelles. These findings underscore the importance of tailoring PPB measurement approaches to the unique properties of the drug candidate in question. This approach is crucial for enhancing the precision of PPB measurements throughout the drug development process, thereby contributing significantly to the optimization of drug dosing regimen.

P92 - PHARMACOKINETIC EVALUATION OF NEUTRAL-SPHINGOMYELINASE2 (NSMASE2) INHIBITOR PRODRUGS IN DOGS

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Extracellular vesicles (EVs) can carry pathological cargo and play an active role in disease propagation. The enzyme neutral sphingomyelinase 2 (nSMase2) causes the hydrolysis of sphingomyelin (SM) to ceramide and is involved in EVs biogenesis. Chronically activated nSMase2 has been implicated in modulating brain inflammation, amyloid deposition, tau protein propagation, cancer metastasis, and viral spreading. Moreover, genetic and pharmacological inhibition of nSMase2 has been demonstrated to reduce EVs secretion and improve disease pathology [1, 2]. From a human nSMase2 high throughput screen (HTS) of >350,000 compounds, our laboratory previously discovered DPTIP (2,6-Dimethoxy-4-(5-Phenyl-4-Thiophen-2-yl-1H-Imidazol-2-yl)-Phenol), a potent (IC₅₀ = 30 nM) and selective inhibitor of nSMase2. However, DPTIP exhibited poor rodent pharmacokinetic (PK) properties with low oral bioavailability (F = <5%), poor brain penetration (AUC_{brain}/AUC_{plasma} = <0.2) and rapid clearance (t_{1/2} = <0.5h). To overcome DPTIP's PK limitations, we synthesized and reported a series of DPTIP prodrugs by masking its phenolic hydroxyl group. When administered orally in mice, several of these prodrugs showed an improvement in plasma and brain exposures. Moreover, in a mouse model of acute brain injury, the best DPTIP prodrug significantly inhibited IL-1 β -induced EV release into plasma and attenuated nSMase2 activity in the brain [3]. Given that prodrugs often depict species specific metabolic differences, we decided to evaluate selected DPTIP prodrugs in dogs for metabolic stability and oral PK [4, 5]. We selected three prodrugs to profile including P-5 (dimethyl carbamate-based), P-15 (methyl phosphate-based) and P-20 (double valine ester-based). All three were evaluated for *in vitro* stability in dog liver microsomes and dog plasma where the ester-based prodrugs showed moderate stability (25-75% remaining at 60 min) and carbamate based showed high stability (>75% remaining at 60 min). We further conducted metabolite identification studies using high resolution mass spec and confirmed that the major metabolite was DPTIP. Given the three prodrugs showed good stability profile, we initiated PK studies in dogs. Male Beagle dogs (non-naïve colony animals 9 to 11 Kg at dosing) were obtained from the test facility's colony. Animals were fasted overnight prior to dose administration and fed 4 hours after dosing. Animals

were dosed with either i.v. DPTIP (1 mg/kg) or oral DPTIP, P5, P15 or P20 (2 mg/kg DPTIP equivalent dose (N=2 animal/cohort). An intravenous dose of 1 mg/kg DPTIP resulted in an observed C_{max} of 2400 nM and AUC of 3799 nM·h. DPTIP's overall exposure from oral dosing was 0.697 nmol.h/mL, indicating poor oral bioavailability (F = 9%). Of the three prodrugs tested, P20 with the val-val promoiety was the best with an AUC of 1382 nM·h, representing a 2-fold increase in oral bioavailability versus DPTIP (F = 18%). Prodrug P5 and P15 both showed poor DPTIP exposures with F < 5%. Additionally, we conducted an analysis of intact prodrug concentrations in plasma, however the intact prodrug levels were below the limit of quantitation, suggesting successful prodrug conversion and release of the active compound. These studies report the discovery of a DPTIP prodrug with improved oral bioavailability that could potentially aid its clinical translation.

P93 - BIOPHARMACEUTICAL, PHARMACOKINETIC, AND ACTIVITY PROFILING OF A NOVEL LOCALLY BIOAVAILABLE SELECTIVE COX-2 INHIBITOR FOR COLORECTAL CANCER PREVENTION

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Despite evidence of high efficacy in clinical studies, current selective COX-2 inhibitors (COXIBs), such as celecoxib, are currently not used as chemopreventive agents against colorectal cancer (CRC) due to significant ON target OFF organ cardiovascular toxicity upon their long-term use. We designed novel locally bioavailable derivatives of celecoxib with designated phenolic groups in its structure to limit their systemic exposure. The PK-directed structural design of locally bioavailable drugs (LBD) exploits the drug disposition mechanisms of hepatic first pass metabolism and enterohepatic recycling of glucuronides to reduce systemic exposure and improve colon targeting for better toxicity and efficacy profile of COXIBs. The COX-2 inhibition potency and selectivity of the synthesized analogs were determined with celecoxib as positive control. The most active analog, 4-(3-((2-Hydroxyphenoxy)methyl)-5-(p-tolyl)-1H-pyrazol-1-yl)benzenesulfonamide (6a1) was selected for further pharmacokinetic and target engagement studies. The results showed that 6a1 exhibited good potency against COX-2 (IC₅₀ of rCOX-2 = 0.118 μM) and selectivity over COX-1 (rCOX-1/rCOX-2 = 43), though addition of phenolic group caused some loss of activity (IC₅₀ of celecoxib = 0.014 μM). Results were validated using HT-29 cells (colon cancer cells), HCA-7 cells (which constitutively express human COX-2), and Raw264.7 cells (expressing COX-2, UGTs, and SULTs). 6a1 was efficiently and preferentially glucuronidated in rat (~6-fold higher) and human (~11-fold higher) liver than colon microsomes, and rate of sulfation in rat/human S9 fraction was significantly lower than glucuronidation. 6a1 showed rapid diffusion in Caco-2 cells (Papp values = 5x10⁻⁶ cm/sec). In a multiple-dose oral PK in PIRC (polyposis in rat colon) rat, a genetic model of CRC, the steady state average blood concentrations of celecoxib (at both doses) were 100 times higher than 6a1 at 40 mg/Kg b.i.d. for 4 days. Both celecoxib and 6a1 achieved colon concentrations higher than their respective IC₅₀ values, though celecoxib levels (~34.3 μM) were much higher than 6a1 (~1.06 μM). Based on protein binding data (96.7% for 6a1 versus 99.4% for celecoxib), free drug colon concentrations of celecoxib were 100-200 nM (2-5x > IC₅₀), whereas 6a1 was 34 nM (3x < IC₅₀). Tissue distribution of 6a1 showed that the drug was primarily distributed in the colon (461 pg/mg tissue), with moderate levels in the liver and kidney, and low levels in the heart, etc. In conclusion, LBD is a new drug design concept which can be used to synthesize safe and active COXIBs for long-term therapeutic prevention of CRC without undue systemic exposure to prevent ON target OFF organ toxicities. However, further lead optimization is needed to improve the potency of 6a1 for better *in vivo* efficacy potential. Moreover, LBD can be applied to various novel therapeutic targets in colon.

P94 - CHALLENGES OF AN ACTIVE METABOLITE: THE POTENTIAL OF DISPROPORTIONATE HUMAN EXPOSURE AND THE COMPLEX KINETICS UPON DRUG-DRUG INTERACTIONS

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MT-202 was identified as a potent lead compound with favorable ADME properties. During pre-clinical characterization, in-vitro studies revealed that MT-202 formed a pharmacologically active metabolite (M1) with the potential for disproportionate human exposure. To evaluate the feasibility of achieving adequate exposure of M1 in nonclinical toxicology studies, we determined its exposure in proposed safety species (rat and NHP) at the highest feasible dose levels. The low ratios of plasma M1 to MT-202 in rats (0.075%) and NHP (3.7%) following oral administration of MT-202, combined with the *in vitro* disposition kinetics of M1 across species, raised the concern over disproportionate M1 exposure in clinical settings, based on projected clinical therapeutic dose of MT-202 and estimated AUC ratio of M1 to MT-202 in humans.

The developability of MT-202 was potentially further complicated by the complex kinetics of M1 in the presence of rifampin (an inhibitor of OATP1B) and/or 1-aminobenzotriazole (1-ABT, a pan CYP inhibitor). MT-202 clearance in NHP was moderately reduced by rifampin (by ~36%) and minimally by 1-ABT (by ~15%). Dual inhibition by rifampin and 1-ABT gave rise to an inhibitory effect comparable to that of rifampin alone. However, M1 exposure was increased by ~20-fold and ~6-fold by rifampin and 1-ABT, respectively. Even more surprising was the effect of dual inhibition where M1

exposure was increased by ~80-fold. This unusual “amplifying” drug-drug interaction magnitude reflected by M1 exposure changes warrants further investigation of the mechanism(s).

The early identification and assessment of potential development challenges related to the MT-202 metabolite M1 outlined in this presentation has greatly facilitated decision-making in candidate selection.

P95 - MODEL-BASED EXPLORATION OF PARENT-METABOLITE KINETICS OF MT-202: NONCLINICAL CHARACTERIZATION AND FEASIBILITY OF CLINICAL METABOLITE EXPOSURE PREDICTION

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During pre-clinical ADME characterization, a lead compound MT-202 was identified to potentially form a disproportionate metabolite (M1) that is pharmacologically active and has demonstrated complex kinetics in the presence of rifampin (inhibitor of OATP1B) and/or 1-aminobenzotriazole (1-ABT, a pan CYP inhibitor). These findings raised concerns over MT-202 developability and thus triggered the effort of predicting clinical M1 exposure in the presence and absence of extrinsic interferences.

We conducted exploratory studies to assess the feasibility of predicting M1 exposure after MT-202 administration to humans. Different model-based approaches were utilized to characterize the parent-metabolite kinetics of MT-202 observed in nonclinical species and assess the confidence of predicting disproportionate metabolite potential for MT-202. The formation clearance (CL_f) and elimination clearance of M1 (CLM₁) were characterized using rat, monkey (NHP), and human liver microsomes in the presence of NADPH and UDPGA and are expressed as scaled values (mL/min/kg). In rats, the low CL_f and high CLM₁ (CL_f,int, rat = 0.9, CLM₁,int, rat = 361) are consistent with the low ratio of M1 to MT-202 observed *in vivo* (0.075%). However, this is not the case with monkeys where the comparable CL_f and CLM₁ (CL_f,int,NHP = 380, CLM₁,int,NHP = 356) obtained *in vitro* would suggest the M1 exposure similar to that of MT-202 based on metabolism-driven static models. This prediction is not in good agreement with the low M1 to MT-202 exposure ratio observed *in vivo* (3.7%). This apparent *in vitro-in vivo* disconnection in monkeys poses a challenge in predicting the relationship between M1 and MT-202 in humans using the determined human CL_f and CLM₁ values (CL_f,int,human = 33, CLM₁,int,human = 31). This challenge, combined with the complex M1 kinetics we previously observed, prompted additional studies to characterize the disposition of M1 in rats and monkeys. The result was then applied to refine our modeling of MT-202 and M1 pharmacokinetic behavior.

The exploration of MT-202 and M1 parent-metabolite kinetics and the modeling work outlined in this presentation provides a solid foundation to assess disproportionate metabolite potential and clinical metabolite exposure prediction.

P96 - THE SITE-SELECTIVE TRITIUM LABELING OF MODIFIED NUCLEIC ACIDS USING FUNCTIONALITY TRANSFER REACTION FOR PHARMACOKINETIC STUDIES

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In the discovery of nucleic acid drugs, it is essential to understand their pharmacokinetic characteristics. A radiolabeled compound has generally been used for exhaustive and quantitative pharmacokinetic analysis. It is desired for the radiolabeling position of nucleic acid drugs for pharmacokinetic studies to be inside the molecular structure rather than at the terminal position because the terminal position of a nucleic acid is easy to be cleaved metabolically. The radiolabeling technique of nucleic acids are important for pharmacokinetic studies. To date, several radiolabeling techniques for nucleic acids have been developed. However, most of the techniques have disadvantages, such as high-cost, technical difficulty, substrate applicability and stability of the compound. Recently, RINAT Imaging Inc. has developed a unique technology for labeling the inside position of nucleic acids with tritium (FT-labeling) to overcome these disadvantages. 1) This technique is that a pyridinyl vinyl keto group is introduced to the amino group of adenine or cytosine by a functionality transfer reaction with reactive oligodeoxynucleotides, followed by a reduction with NaBT₄. With this method, the targeted internal position of the nucleic acid can be labeled site-selectively and rapidly with tritium. In this study, we collaborated with RINAT Imaging and Luxna Biotech Co, Ltd. to demonstrate the usefulness of the FT-labeling technique for pharmacokinetic studies. The Luxna Biotech's nucleic acids LX-A3945, which is bearing amide-bridged nucleic acid (AmNA)² and spirocyclopropylene-bridged nucleic acid (scpBNA)³, and LX-A8140, which has 2'-O-methoxyethylated nucleic acid moieties, were labeled with tritium by the FT-labeling technique. The labeling reaction of both nucleic acids was completed satisfactorily to obtain the tritiated nucleic acids with above 90% radiochemical purities. Then, quantitative whole body autoradiography (QWBA) of these labeled nucleic acids was performed to evaluate their biodistribution. As the result of the evaluation for urine, the production of tritiated water, which is the representative decomposed compound of radiolabeled nucleic acids, was found to be low, though sometimes large production of tritiated water is found when a conventional tritiated nucleic acid is used. The QWBA of these nucleic acids showed enough

results of their biodistribution. These results suggests that the FT-labeling technique is useful for pharmacokinetic studies aimed at development of novel nucleic acid drugs.

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P97 - TRENDS IN *IN VITRO* POTENCY AND PK PARAMETERS; CAN THERAPEUTIC DOSES BE ESTIMATED FROM *IN VITRO* K_i?

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The *in vitro* potency of a new compound to its target (e.g. inhibitory constant (K_i)) is one of the earliest parameters obtained in drug development. It is important to estimate its therapeutic dose at early stage to determine whether it should be further developed or not. One way is to estimate the ideal exposure and dosage based on the *in vitro* K_i and target receptor occupancy (RO). To assess the effectiveness of such an approach, we first collected and analyzed *in vitro* K_i and human pharmacokinetic (PK) parameters of orally-administered small-molecule drugs approved in Japan during the period 2010-2021 from the drug "Interview Form" provided by the manufacturer. Then, the clinical dose was estimated based on *in vitro* K_i by setting a target RO. Assuming the relationship of RO, average unbound concentration in blood (Cave,u) and K_i to be $RO = 1/(K_i/Cave,u + 1)$, a target RO was set to a certain value to back-calculate the Cave,u and daily doses using the observed oral clearance in humans. In total, 144 drugs were collected. Drugs with lower *in vitro* K_i values tended to have larger molecular weight, higher logD (or logP), protein binding rate, hepatic intrinsic clearance, and a larger Vd/fp (volume of distribution corrected by plasma protein binding). *in vitro* K_i also correlated positively with therapeutic dose and Cave, u. Interestingly, K_i and Cave,u/K_i showed a negative correlation. For therapeutic dose estimation, target RO was set for each drug according to its drug classification. As a result, 33% and 64 % of the dose prediction fell in 3-fold and 10-fold errors, respectively. This approach yielded a better prediction accuracy rather than when the target RO was uniformly fixed at 0.5 or 0.8. Overall, the therapeutic dose can be roughly predicted from *in vitro* K_i, however, it must be kept in mind that there will be PK prediction errors in actual practice. By comparing the *in vitro* K_i collected in our study (modern drugs) and classic low molecule drugs approved before 1990, modern drugs have 10-fold lower K_i, however, only a 4-fold difference in dose. This may be due to an increase in hepatic intrinsic clearance as the drugs become more modern. Our survey highlights a new challenge in recent drug development: Increasing *in vitro* potency may not necessarily yield the benefit in terms of lower therapeutic doses as expected.

P98 - DRUG READ-OUT IN UNTARGETED METABOLOMICS USING A MS/MS LIBRARY OF DRUGS AND METABOLITES PROPAGATED FROM METABOLOMICS DATA REPOSITORY

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To study the relationships between drug exposure and health-related outcomes, clinical research has been relying on patient questionnaires or medication records. However, such information is costly to acquire, often incomplete, and does not reflect drug metabolites and exposure from other routes. Untargeted metabolomics offers the opportunity to accurately read out drug exposure from patient biological samples. We developed a MS/MS spectral library for drugs, metabolites, and related molecules to facilitate drug exposure read-out in untargeted metabolomics. From GNPS Public Spectral Library, we collected reference spectra of drugs and metabolites through a partial name search against four drug knowledge bases. We then created a propagated "analog library" by searching against 1.2 billion public MS/MS spectra to obtain those with high similarity to drug reference spectra. We tested the performance of the library on 115 and 48 plasma samples from patients with human immunodeficiency virus (HIV) or severe SARS-CoV-2, respectively, and 2089 fecal samples representing a general international population to enable stratification of patients based on drugs. In total, we collected reference MS/MS spectra for 4,954 drugs (105,938 spectra) and metabolites for 517 drugs (3,057 unique spectra). By searching the library against the HIV (47 ± 11 yrs) and hospitalized SARS-CoV-2 (62 ± 16 yrs) cohorts, we observed that drugs detected in the two cohorts were significantly different. The top drugs in patients with HIV included antiviral drugs such as protease inhibitors, while antibiotics and corticosteroids were among the most frequently detected

drugs in SARS-CoV-2, demonstrating that the drug read-out from untargeted metabolomics accurately reflect expected drug consumption. In the cohort representing the general population (47 yrs, range 3-93), ≥ 1 drug was detected in 29.7% of the samples. As expected, drug classes such as antihypertensives and antihyperlipidemics were mainly detected in patients >45 yrs, while antihistamines, analgesics, and antibiotics were detected in all ages. Our data demonstrate that the drug library is enabling accurate read-out of drug exposure in untargeted metabolomics studies. A novel, comprehensive spectral library for drugs and metabolites was developed using untargeted metabolomics that will have utility in improving annotation of drugs and providing insight into drug biotransformations.

P99 - CIRCULATING BIOMARKER DISCOVERY FOR MASH

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Circulating biomarkers are extremely useful in the clinic for monitoring disease, patient stratification, and pharmacodynamic assessments without the need for invasive tissue biopsies. Additionally, blood-based biomarkers are amenable to longitudinal and frequent measurement. The challenges with blood-based biomarker quantitation are tissue specificity, proximity, and complexity of blood. Specificity challenges arise because blood levels are the sum of proteins secreted from all tissues and thus the source of the biomarker modulation in plasma is uncertain. In plasma, only tissue secreted factors and proteins released due to cell death are detected and therefore the signature is not proximal to the source of the disease or treatment. Lastly, large complexity and dynamic range of plasma make it difficult to measure low abundant proteins.

We evaluate different proteomics platforms for circulating biomarker discovery that address the above challenges. Our efforts are focused on plasma proteomics and exosomal analysis.

For plasma proteomics, we leverage three different platforms: LCMS, SomaLogic, and Olink. SomaLogic relies on aptamers and OLINK relies on antibodies for detection of approximately 6000 proteins in human plasma. Olink and Somalogic are primarily utilized for analysis of human plasma/ serum with only limited coverage of rodent species. Thus, there are limited opportunities for translational research. LCMS typically has lower throughput than Olink or SomaLogic. Additionally, LCMS of crude plasma can only quantify up to 600 proteins because of large dynamic range of plasma where the top 10 proteins constitute 90% of the total protein mass. Fractionation and antibody-based depletion of abundant proteins can increase the coverage to about 2000 proteins. However, depletion and fractionation require significant time and effort, and can add variability to the results.

We evaluated and leveraged SEER platform to quantify > 6000 proteins in mouse and > 4000 proteins in cyno plasma. This platform was then utilized to identify proteomics changes in plasma after treatment with MASH drugs. We identified over a hundred proteins that were modulated in PXB mice on a high-fat diet after treatment with a siRNA drug.

To supplement our circulating biomarker discovery efforts, we have also evaluated extra-cellular vesicles (EVs). EVs can be isolated using ultracentrifugation or approaches relying on membrane affinity. EVs isolated this way are called global EVs. EVs can also be enriched using tissue specific surface markers. We have leveraged both approaches to isolate EVs from *in vitro* and *in vivo* systems. After isolation, cargo of the EVs can be analyzed using immunoassays, LCMS, or somalogic platform. In this presentation we will present data for EV isolation, characterization, and analysis from both *in vitro* and *in vivo* systems.

P100 - EXPENDING THE APPLICATION OF MECHANISTIC STATIC MODELS FOR REGULATORY USE IN PREDICTING CYP- AND TRANSPORTER-MEDIATED DRUG-DRUG INTERACTIONS

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Mechanistic static models (MSM) and physiologically based pharmacokinetic (PBPK) models are both valuable tools for translating *in vitro* observations into clinical drug-drug interaction (DDI) predictions. While MSM has been primarily recommended by regulatory agencies (FDA, EMA and PMDA) to be used before Phase I, PBPK models have been more widely accepted for regulatory applications. However, there is growing evidence that MSM can also be utilized for regulatory purposes [1], as acknowledged by the ICH M12 guideline when appropriate perpetrator concentrations in gut and liver are considered [2-4].

With an embedded MSM model, an extensive repository of over 400 victim drugs and 500 perpetrators, and a flexible user-friendly interface Drug-Drug Interaction Risk Calculator (DDIRC) from Pharmapendium® is uniquely positioned to investigate the preclinical and clinical assessment of DDIs. Prediction accuracy will be evaluated using different Perpetrator unbound concentrations in the liver inlet and plasma ($C_{max,u}$, $C_{avg,u}$, $[I]_{inlet,max,u}$ $[I]_{inlet,avg,u}$) for enzymes and transporters. When the CYP3A4 will be involved in the metabolism of the substrate the perpetrator gut concentration will be considered to assess DDIs.

Even though focusing on the concentration of the perpetrator is crucial for precise DDI predictions using the MSM method, other factors related to the substrate, such as the liver metabolism fraction (f_m) for enzymes, gut metabolism (F_g), and transported fraction (f_t) for intestinal, renal, and hepatic transporters must be carefully assessed to support accurate DDI

predictions to be utilized for regulatory purposes.

This study aims to use MSM to predict DDIs for selected drugs, cytochromes and transporters against clinical observations and compare it with PBPK models to encourage broader application in internal and regulatory decision-making, ultimately saving time and resources for the industry.

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P101 - IN VITRO ASSESSMENT OF PSILOCIN FOR CYP-DEPENDENT DRUG-DRUG INTERACTION POTENTIAL USING RAPID LUMINESCENT ASSAYS

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Recent clinical studies with the psychedelic prodrug psilocybin ([3-[2-(dimethylamino) ethyl]-1H-indol-4-yl] dihydrogen phosphate) provided promising data on its potential for treating psychiatric disorders such as major depressive disorder with a single administration (1). While psilocybin was well-tolerated, its drug-drug interaction (DDI) potential has not been fully explored. Here we present initial studies addressing the cytochrome p450 (CYP) dependent DDI potential of the psilocybin psychoactive metabolite psilocin (4-hydroxy dimethyl tryptamine), which is found in the circulation after oral administration while the prodrug is not. DDIs may occur when CYP gene induction by one drug accelerates metabolism of a co-administered drug causing diminished efficacy, or when CYP inhibition slows its metabolism and increases exposure to a toxic level. We used *in vitro* assays to detect CYP enzyme inhibition or gene induction by psilocin. Each assay utilized a luminogenic probe substrate with selectivity for a CYP enzyme activity of interest. The probes enabled rapid simple luminescent assay protocols. We first demonstrated that the luminescence-based system produces control inhibitor IC₅₀s that correlate closely to IC₅₀s from conventional assays with recombinant CYPs. We then employed recombinant CYP1A2, -2B6, -2C8, -2C9, -2C19, -2D6, and -3A4 for reversible inhibition mode assays and primary human hepatocytes for induction assays with readouts for CYP1A2, -2B6, and -3A4 enzyme activity. Psilocin inhibited the 7 recombinant CYP enzymes with characteristic K_i values varying from 2.2 to 23.4 micromolar. We considered the DDI risk implications of these K_i values in light of R₁ values ($R_1 = 1 + (C_{max}/K_i)$). FDA guidance only recommends further DDI study when $R_1 \geq 1.02$ (2), inferring lower values carry minimal risk. We used the mean C_{max} of 16 micrograms/L (56.3 nM) total psilocin reported by Brown et al from a clinical study that used a dosing regimen similar to recent positive clinical outcome studies (e.g., 25mg single dose) (1, 3). R₁ values calculated from this C_{max} and our K_i values range from 1.002 -1.013. While this range is less than the 1.02 FDA cutoff suggesting low DDI risk, we also note that the R₁ range from a preferred unbound C_{max} would likely be lower, further suggesting low DDI risk via reversible CYP inhibition. In our test for CYP induction, basal CYP1A2, -2B6, and -3A4 activities were observed from monolayers of primary human hepatocytes but these activities were not significantly induced by psilocin across a concentration range encompassing a hypothetical 56.3 nM C_{max}. As levels of these three CYPs are modulated by the three main CYP gene induction pathways, it is reasonable to conclude that psilocin also does not pose a significant risk of CYP induction dependent DDIs. Nevertheless, additional studies are required to more fully address psilocybin DDI risk. These include repetition of the present study for the additional major psilocybin metabolites, 4-hydroxy indole acetic acid and psilocin glucuronide, and time-dependent CYP inhibition assays for the three metabolites.

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P102 - PBPK MODELING OF DRUG-DRUG INTERACTIONS BETWEEN ANTIANDROGENS AND HIV MEDICINESKuan-Fu Chen¹, Laura Shireman¹, and Lisa Almond²¹Certara, Bellevue, WA, United States, ²Certara, Sheffield, United Kingdom

The antiandrogens finasteride and dutasteride are among the most frequently prescribed with estrogens in feminizing hormone therapy to facilitate male-to-female transitioning for transwomen [1]. HIV prevalence is disproportionately high among transwomen [2], which may lead to increased risk of drug-drug interactions (DDI) between antiandrogens and anti-HIV drugs. No clinical DDI studies assessing how HIV medications affect the exposures of finasteride and dutasteride were found using the Drug Interaction Database (DIDB®) [3]. A qualitative assessment on potential interactions with finasteride and dutasteride using the Liverpool HIV drug interactions database identified CYP3A4-mediated DDI liability for multiple HIV drugs [4]. In this study, we predicted potential DDI effects of various HIV medications on the exposures of finasteride and dutasteride by developing physiologically based pharmacokinetic (PBPK) models for finasteride and dutasteride based on published *in vitro* and clinical data and leveraging existing PBPK models for HIV drugs within the Global Health PBPK Model Repository [5]. In the absence of clinical data, DDI predictions may inform effective dose adjustments for finasteride and dutasteride in transwomen taking HIV medications, potentially making treatment more accessible for this important and understudied HIV patient group.

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P103 - POTENTIAL TRANSPORTER-MEDIATED HERB-DRUG INTERACTIONS OF ZINGIBER OFFICINALE AND UNCARIA TOMENTOSANora Szilvasy¹, Csilla Temesszentandrasi-Ambrus¹, Tamas Varga¹, Judit Hohmann², Istvan Zupko², Emese Kis¹, and Zsuzsanna Gaborik¹¹Charles River Laboratories Hungary Kft, Budapest, Hungary, ²University of Szeged, Szeged, Hungary

Ginger (*Zingiber officinale*, Zo) and cat's claw (*Uncaria tomentosa*, Ut) have been widely used in natural medicine for centuries. Beneficial properties attributed to these herbs include antiarthritic, anti-inflammatory, antibacterial, and anticancer effects. While investigation of transporter-mediated drug-drug interactions (DDIs) is mandatory during preclinical drug development, such studies are rarely done for herbs or other food supplements, albeit the risks associated with the concomitant use of herbal products and therapeutic drugs, i.e., herb-drug interactions (HDI), are amply documented. The most common mechanism of HDI is the inhibition and/or induction of transport proteins and/or drug-metabolizing enzymes by herbal ingredients, leading to altered pharmacokinetic disposition of the victim drug. In this study, we investigated the effect of Zo and Ut extracts and their major phytochemical components on 33 clinically relevant uptake (solute carrier, SLC) and efflux (ATP-binding cassette, ABC) transporters. Transporter-overexpressing cell lines were used in cell-based (for SLCs) and membrane vesicle-based (for ABCs) systems to assess such interactions. Zo and Ut extracts both had significant *in vitro* inhibitory effect on the majority of the investigated transporters. Based on *in vitro* determined IC₅₀ values and standard calculations of interaction risk, Zo extract at a typical single oral dose of 170-250 mg was predicted to inhibit intestinal BCRP, MDR1, OATP2B1, and OCT1 (IC₅₀: 9.6, 75.8, 67.1, and 18.6 µg/mL, respectively). Of the purified active ingredients of Zo, 6-gingerol exhibited potentially relevant inhibition of OAT3 (IC₅₀: 2.7 µM) and, assuming high (2.0 g) oral doses of the extract, also ENT1 (IC₅₀: 25.9 µM). Ut extract at a typical single oral dose of 500 mg was predicted to inhibit intestinal BCRP, MDR1, OATP2B1, and OCT1 (IC₅₀: 10.6, 54.6, 40.9 and 29.7 µg/mL, respectively). Of the major active Ut oxindole alkaloids, pure isopteropodine *in vitro* inhibited BCRP, MDR1, OCTs, OATPs, OAT3, and SGLT2 (IC₅₀s between 2.8 and 132 µM), and pure mitraphylline inhibited MDR1, OCT1, and ENT1 (IC₅₀: 28.3, 7.05, and 49.6 µM, respectively); however, the scarcity of published pharmacokinetic data precludes meaningful extrapolation of these effects to the *in vivo* inhibition of extraintestinal transporters. Nevertheless, our data indicate that both ginger and cat's claw extracts at commonly applied doses may potentially interact with intestinal transporters and therefore cause HDIs by altering the absorption of orally administered drugs.

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P104 - COMPARATIVE PHARMACOKINETIC PROFILES OF FAVIPIRAVIR ALONE AND IN COMBINATION WITH THAI HERBAL EXTRACT IN RATS**Dhirarin Junsang¹**, Arimi Fujita¹, Tsutomu Shimada¹, Phisit Khemawoot², and Yoshimichi Sai¹¹Kanazawa University, Kanazawa, Ishikawa, Japan, ²Mahidol University, Samutprakarn, Thailand

Home use of herbal remedies for treating various diseases is becoming more common, but herb-drug pharmacokinetic interactions may cause changes in drug absorption and distribution to target tissues, potential impacting on clinical outcomes and patient safety. *Andrographis paniculata* (APE) and *Boesenbergia rotunda* (BRE) extract is reported to show an antiviral effect, including against SARS-CoV-2 *in vitro* and *in vivo*, while favipiravir is a selective RNA polymerase inhibitor with broad-spectrum antiviral activity, used to treat COVID-19. So far, however, it is not clear whether coadministration of these herbs and favipiravir might result in a pharmacokinetic interaction. To investigate this issue, we randomly divided male Sprague-Dawley rats into three groups ($n = 6$ in each). Rats were orally administered favipiravir (5 mg/kg), favipiravir (5 mg/kg) plus APE (200 mg/kg), or favipiravir (5 mg/kg) plus BRE (300 mg/kg). Favipiravir concentration in biological samples was determined by HPLC-UV. Lower concentrations of favipiravir were seen in both combination groups compared to favipiravir alone. The area under the concentration-time curve (AUC_{0-t}) and the area under the concentration-time curve from 0 to infinity (AUC_{0-inf}) of favipiravir were significantly reduced in the BRE combination compared to favipiravir alone, indicating altered drug exposure. The apparent total body clearance (CL_{tot}/F) was significantly higher in both combination groups. Furthermore, coadministration with APE resulted in a significant decline in maximum plasma concentration (C_{max}) and AUC_{0-inf} compared to favipiravir alone, along with a marked decrease in favipiravir concentration during the initial absorption phase. These results show that combining BRE or APE with favipiravir significantly changed the drug's pharmacokinetics parameters, especially CL_{tot}/F and AUC_{0-inf}, as well as drug exposure and clearance rates during the absorption and elimination phases. Caution may be necessary in the administration of favipiravir to patients taking herbal remedies containing APE, and BRE.

P105 - POTENTIAL DRUG-DRUG INTERACTIONS OF FREQUENTLY PRESCRIBED MEDICATIONS IN ELDERLY PEOPLE WITH OSTEOPOROSIS AND NON-COMMUNICABLE DISEASES, AND A COMPARISON WITH REAL-WORLD PRACTICE**Werapat Suechanyapong**, Tulaton Sodsri, Noppavut Sirirak, Sorawut Thamyongkit, Sutida Sumrithe, and Dr. Phisit Khemawoot

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Elderly patients with osteoporosis may take several drugs that could cause drug-drug interactions (DDIs) and lead to poor adherence to osteoporosis treatments. We aimed to evaluate the potential DDIs of frequently prescribed medications in elderly people with osteoporosis, as determined by online databases, and compared these findings with real-world practice. We selected 73 frequently prescribed medications for elderly people with osteoporosis from the Thai National List of Essential Medicine 2022 and the CNMI Hospital Drug List 2023. We used two online databases, Micromedex and Medscape, to identify potential DDIs. Micromedex is a subscription database for healthcare professionals and Medscape is a free online database for non-medical personnel. We analyzed the prevalence of potential DDIs, as reported by both databases, for agreement regarding severity, documentation, the mechanisms of interaction, and major problematic drugs. Micromedex and Medscape revealed potential DDIs with approximately 17% of the drug pairs (835/5000). Among these potential DDIs, 20% were classified as moderate to severe by both databases (171/835). The main mechanisms of these serious DDIs were QT interval prolongation (20%) and glycemic imbalance (19%), including hypoglycemia and hyperglycemia. The major culprit drug classes were antidepressants (22%), which were most related to QT interval prolongation. There were negligible potential DDIs for osteoporotic medicines, including calcium carbonate, with potentially decreased oral bioavailability of co-administered drugs such as digoxin, ciprofloxacin, and propranolol. The overall agreement between the two databases based on Fleiss' kappa was 0.2552, which indicates fair agreement. The information obtained from this study will be used to compare the prevalence of DDIs in real-world practice with elderly people with osteoporosis at the CNMI Hospital under ethical approval from the Faculty of Medicine Ramathibodi Hospital, Mahidol University, Thailand.

P106 - EFFECT OF CIMETIDINE AND DOLUTEGRAVIR ON RENAL CLEARANCE OF METFORMIN AND PUTATIVE ENDOGENOUS BIOMARKERS FOR OCT2/MATE1/2K (1-NMN, M1A, AND CREATININE) IN HEALTHY SUBJECTS.**Tomoki Koishikawa¹**, Kaku Fujiwara¹, Kenichi Furihata², Yuichi Sugiyama³, and Hiroyuki Kusuhara¹¹The University of Tokyo, Tokyo, Japan, ²P-one Clinic, Tokyo, Japan, ³Josai International University, Tokyo, Japan

Background: The risk of transporter-mediated drug-drug interactions (DDI) by investigational drugs is routinely assessed during drug development. Recently, endogenous biomarkers that serve as surrogate probes to investigate transporter-mediated DDI in the early phase of clinical development have emerged. There are great concerns regarding the predictive performance of endogenous substrates of drug transporters in humans. This study focused on organic cation transporters in the kidney (OCT2 and MATE1/2K), which mediate the renal elimination of metformin.

Methods: We designed a clinical study to investigate OCT2/MATE1/2K-mediated DDI in the kidney using metformin (500 mg, po) as a probe drug and cimetidine (400 or 800 mg) and dolutegravir (50 mg, bid) as perpetrator drugs in healthy Japanese subjects. Metformin was given at 1st and 5th dose of dolutegravir because dolutegravir was accumulated by repeated doses. Plasma concentrations and cumulative amounts of urinary excretion were quantified using LC-MS/MS (QTRAP5500) as reported previously [1].

Results and Discussion: The endogenous compounds N1-methylnicotinamide (1-NMN), N1-methyladenosine (m1A), and creatine were also analyzed in the same subjects. An *in vitro* inhibition study suggested that cimetidine inhibits MATE1/2K rather than OCT2 and that dolutegravir inhibits OCT2 more potently than MATE1/2K. Both drugs reduced the renal clearance (CL_r) of metformin in an exposure-dependent manner. Geometric mean of CL_r ratio of metformin were 0.85 and 0.57 for cimetidine 400 and 800 mg, and 0.73 and 0.43 for dolutegravir at 1st and 5th doses, respectively. The CL_r of 1-NMN and m1A was significantly decreased by treatment with 800 mg of cimetidine. Notably, impact of transporter inhibition on CL_r of the endogenous biomarkers (1-NMN and m1A) showed calculation time interval dependence for cimetidine, and when using the data collected by 4 hours after administration of metformin, the CL_r of 1-NMN and m1A were significantly decreased by 400 mg of cimetidine (CL_rR 0.68 and 0.78, respectively for 1-NMN and m1A, respectively). Dolutegravir decreased the CL_r of 1-NMN in an exposure-dependent manner, whereas the CL_r of m1A was significantly decreased, but no clear systemic exposure dependence was observed. Although the AUC_r of creatine was above 1 by cimetidine and dolutegravir, a reduction in CL_r could not be detected. In conclusion, the inhibition of OCT2/MATE1/2K accounts for the DDI between metformin and cimetidine or dolutegravir. 1-NMN is a better biomarker for OCT2/MATE1/2K than m1A. For weak inhibitors, the clinical design, particularly the collection time interval of natural urine, must be carefully designed considering the predicted duration of transporter inhibition by investigational drugs.

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P107 - IMPAIRMENT OF HEPATIC CYP2C AND CYP3A ACTIVITIES IN THE MICE TREATED WITH FLUOROPYRIMIDINE ANTICANCER DRUGS

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Purpose: Enhanced anticoagulation and bleeding have been noted by the combined use of warfarin with a fluoropyrimidine (FP) anticancer drug, capecitabine (Cape). This drug-drug interaction is thought to be a result of CYP2C9 inhibition by Cape and/or its active metabolite, 5-fluorouracil (5-FU). In contrast, CYP2C9 inhibition by Cape and 5-FU *in vitro* has been reported to be very weak, and therefore, whole body animal experiments are considered necessary to elucidate the inhibition mechanism. In this study, we developed murine models of impaired hepatic CYP activities by the administration of FPs.

Methods: Cape or 5-FU was intraperitoneally administered to male CD-1 mice at 25 mg or 100 mg/kg/day for 3 days, and the liver was removed 1 day after the final administration. Similarly, a single dose of 100 mg or 200 mg/kg was administered, and the liver was removed 7 days later. Microsomes and total RNA were isolated from the livers, and microsomal enzyme activities, mRNA expression of CYP isoforms and inflammation mediators were determined.

Results and Discussion: Significant decreases in hepatic microsomal CYP2C and CYP3A activities were observed in mice 7 days after a single administration of 5-FU, whereas no significant decrease was observed in these activity 1 day after continuous administration of Cape or 5-FU, or 7 days after single administration of Cape. These results suggest that Cape acts as 5-FU to impair CYP enzymes, and that the time elapsed after administration is more important as a factor in reducing activity than the total exposure amount of 5-FU. In contrast, mRNA expression of CYP2C9 and CYP3A11 was unchanged in the mice treated with 5-FU, indicating that the decrease in CYP activities is not due to down-regulation of gene expression. No liver damage was observed in mice under any treatment conditions, while hepatic mRNA expression of an inflammatory cytokine, interleukin-6, was increased in the 5-FU-treated mice, suggesting its involvement in decreased CYP activities.

Conclusions: We developed a murine model of impaired multiple CYP activities by FP administration rather than that specific for CYP2C enzyme. The impairment of hepatic CYP activities by 5-FU might not be accounted for by direct inhibition or down-regulation, but by modification of CYP enzymes with inflammatory mediators presumably released from tissues damaged by 5-FU.

P108 - SCREENING METHODS COMPARISON OF THE EFFECT OF PHARMACEUTICAL EXCIPIENTS ON DRUG PERMEABILITY**Tokio Morita**, Hiroyuki Yoshida, Naomi Tomita, and Yoji Sato*National Institute of Health Sciences, Kawasaki-shi, Kanagawa, Japan*

Statement of Hypothesis: Recent studies highlight the impact of pharmaceutical excipients on intestinal drug absorption, underscoring the need for careful excipient selection to develop biologically equivalent formulations [1]. This study aimed to describe and compare the characteristics of some *in vitro* screening methods for evaluating the effect of excipients on drug permeability.

Methods: Three *in vitro* models—Corning® BioCoat® Pre-coated PAMPA plate system, PermeaPad® plate, and Caco-2 monolayer—were used to evaluate the effect of 14 common excipients on the permeabilities of propranolol (BCS class I), risperidone (class II), atenolol (class III), and sulpiride (class IV). In screening experiments, excipients were set at clinically maximum or higher concentrations, but the concentrations were adjusted based on the effects on lucifer yellow (LY) permeability and cell viability. Concentration-dependent effects were further analyzed to distinguish specific from non-specific activities.

Results and Supporting Data: Many excipients compromised membrane integrity at maximal clinical concentrations in PAMPA. The permeability of the poorly permeable drug sulpiride was increased more than 5.0-fold by 0.3 mg/mL hydroxypropyl cellulose and 0.06 mg/mL povidone K30. In contrast, the PermeaPad® maintained membrane integrity at high concentrations (0.5 to 3 mg/mL). Some excipients decreased the permeability of the highly permeable drugs propranolol and risperidone in PAMPA. Among them, CMS and SLS showed a concentration-dependent manner. Similarly, CMS and SLS showed a concentration-dependent permeability change (maximal 0.48- and less than 0.01-fold at 3 mg/mL, respectively) in PermeaPad®. Caco-2 experiments showed minimal excipients (set at 0.05 to 1 mg/mL) effect for all drugs in the screening experiment. Sodium lauryl sulfate (SLS) notably reduced propranolol and risperidone permeability concentration-dependent (maximal 0.25-fold at 0.5 mg/mL), although LY permeability was increased. Permeability depression by SLS was observed from 0.2 mg/mL in PermeaPad® and Caco-2, while a much lower concentration of 0.01 mg/mL was observed in PAMPA. It is suggested that the micellization of SLS is the reason for the permeability decrease in the PermeaPad® and Caco-2 experiments, whereas the lipid membrane and SLS interaction occurred in PAMPA.

Conclusions: This study showed that PAMPA is highly sensitive to excipient effects, making it suitable for conservative evaluations. The robustness of the Caco-2 method allows for the evaluation of a wide range of excipient concentrations. The PermeaPad® can accommodate highly cytotoxic excipients due to its high tolerance to excipient concentrations. These results emphasize the importance of method selection based on the evaluation objectives and the characteristics of the drug being evaluated.

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P109 - DRUG-DRUG INTERACTION STUDIES OF CORTISOL IN CYNOMOLGUS MONKEYS BY MEASURING CORTISOL, CORTISOL-D4, AND THEIR 6β-HYDROXYMETABOLITES IN PLASMA**Yasuharu Nakanishi**¹, Yuki Nishimura¹, Taiki Aikawa¹, Fumi Tanaka¹, Takatoshi Nakamura¹, and Masahiro Utoh²Shin Nippon Biomedical Laboratories, Ltd., Kainan, Japan, ²Shin Nippon Biomedical Laboratories, Ltd., Tsukuba, Japan

Purpose: Drug-drug interaction (DDI) studies provide very important information about clinically efficacious and safe usages of newly approved drugs. *in vitro* and *in vivo* clinical DDI studies of new drug candidates are conducted under DDI guidelines and guidances, and are essential for approval of new drugs. Although the DDI studies are mainly conducted under clinical study design, information about DDI in preclinical studies during new drug development offers important information about clinical DDI.

Cynomolgus monkeys (Cyno) are recognized as a useful animal model in preclinical studies in new drug development and information about DDI in Cyno before starting clinical studies are valuable for estimating clinical DDI of new drug candidates. In this study, we investigated effect of cytochrome P450 3A (CYP3A) inhibitor on *in vivo* cortisol (F) metabolism in Cyno. We administered orally clarithromycin (CAM), CYP3A inhibitor, to Cyno and exogenous cortisol-9,11,12,12-d4 (F-d4) was intravenously injected before and after CAM administration. Changes of plasma concentrations of F-d4 and 6β-hydroxycortisol-9,11,12,12-d4 (6β-OH-F-d4) were measured. Moreover, endogenous F and 6β-hydroxycortisol (6β-OH-F) concentrations in plasma were concomitantly determined in the same Cyno. We evaluated effect of CAM administration on metabolism (6β-OH-F-d4/F-d4 and 6β-OH-F/F) of exogenously administered F-d4 and endogenous F.

Methods: F-d4 was intravenously administered (0.5 mg/kg, injected before the first oral (PO) administration of CAM and 5 minutes after the last CAM administration on day 5) to 3 male Cyno (3 years old) and blood samples were collected. CAM at 15 mg/kg was administered orally twice a day on days 1-4 and once a day on day 5. Plasma concentrations of F-d4, 6β-

OH-F-d4, F, and 6 β -OH-F were measured by LC/MS/MS.

Results and Discussion: After PO administration of CAM followed by intravenous (IV) administration of F-d4 to Cyno, there were no significant differences between before and after administration of CAM in the pharmacokinetic parameters of F-d4. In contrast, the plasma concentration of 6 β -OH-F-d4 were significantly decreased by CAM administration, and maximum concentration and area under the plasma concentration-time curve in plasma were approximately 3 to 4 times lower than those after IV administration of F-d4. In addition, endogenous 6 β -OH-F/F in plasma after PO administration of CAM was approximately 2 to 3 times lower than that before administration of CAM. The decreases of endogenous 6 β -OH-F and 6 β -OH-F/F after PO administration of CAM corresponded to the decrease of exogenous 6 β -OH-F-d4 and 6 β -OH-F-d4/F-d4, which suggests that Cyno are a suitable animal model to detect DDIs of CYP3A enzymes. Using the plasma samples of a repeated dose study of Cyno, it may be possible to detect *in vivo* DDI of CYP3A by measuring endogenous F and 6 β -OH-F before commencing clinical studies.

P110 - *IN VITRO* MECHANISTIC INVESTIGATION OF UNUSUAL CLINICAL DRUG-DRUG INTERACTION OF TRAZPIROBEN ON A MAJOR REDUCTIVE METABOLITE UPON RIFAMPIN CO-ADMINISTRATION

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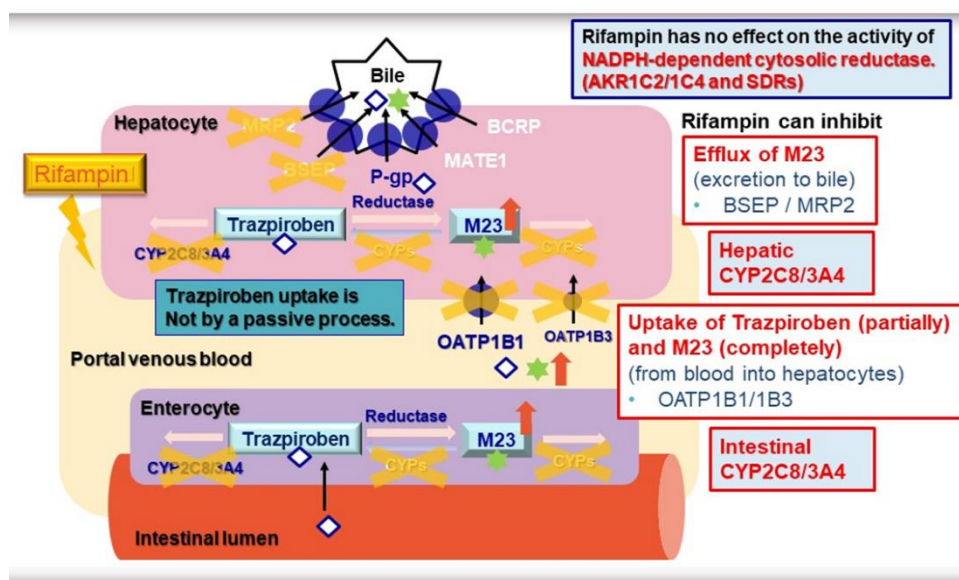
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Trazpiroben (TAK-906) is a peripherally active dopamine D2/D3 receptor antagonist for the treatment of gastroparesis. Trazpiroben is metabolized to M23, a major metabolite in humans, through a non-CYP, cytosolic, NADPH-dependent Aldo-keto reductase and/or Short-chain dehydrogenase/reductases (56.7%), and CYP2C8/3A4 (43.3%) [1]. Trazpiroben is also a substrate of organic anion transporting polypeptide (OATP) 1B1/1B3. Co-administration of rifampin (OATP1B1/1B3 inhibitor) with trazpiroben resulted in approximately five-fold increase in plasma trazpiroben AUC $_{\infty}$ and C $_{max}$, compared with the administration of trazpiroben alone, suggesting that trazpiroben is a sensitive substrate for OATP in the clinic [2]. Interestingly, the M23 plasma exposure was also approximately four- to five-fold higher than the corresponding measurements for plasma trazpiroben and the magnitude of M23 drug-drug interaction (DDI) with rifampin was higher than trazpiroben in the clinic. To elucidate this mechanism for the higher M23 plasma exposure, enzyme- and transporter-based investigations were conducted. Rifampin is known to inhibit uptake from blood into hepatocytes by OATP1B1/1B3 or OATP2B1, and increase efflux excretion to bile by P-gp, BCRP, BSEP and MRP2. Rifampin could also inhibit oxidation of M23 back to trazpiroben by CYP2C8/3A4, which are involved in trazpiroben metabolism. According to the substrate evaluation of transporters, both trazpiroben and M23 were not substrates of the human BSEP, MRP2 (efflux), or OATP2B1 (uptake) transporters, but were substrates of P-gp, BCRP, and OATP1B1/1B3 transporters *in vitro*. For rifampin to increase M23 significantly, it would have to activate the cytosolic reductase or inhibit the oxidative metabolism of M23, including oxidation back to trazpiroben. Trazpiroben underwent both hepatic and extra-hepatic (kidney and intestine) metabolism to M23, while M23 also underwent oxidative metabolism back to trazpiroben in liver, kidney, and intestine. M23 is formed upon first-pass metabolism predominantly in enterocytes followed by transporter mediated uptake into hepatocytes and excretion into bile, and undergoes further metabolism including oxidation back to trazpiroben. Additional *in vitro* assessments revealed that rifampin inhibited the transporter-based uptake clearance of trazpiroben (66% inhibition) and M23 (100% inhibition) in human hepatocytes, but did not inhibit trazpiroben's enzymatic reduction to M23 in human liver, kidney, or intestine S9 fractions. It also facilitated oxidation of M23 back to trazpiroben in human liver S9 fractions. In conclusion, we hypothesize that the elevated M23 plasma exposure in response to rifampin co-administration is most likely due to a combination of its formation in enterocytes and hepatocytes, and inhibition of its hepatic uptake clearance.

Estimated mechanism of the unusual clinical M23 exposure elevation upon rifampin-mediated DDI of trazpiroben



Takeda Pharmaceutical Company Limited

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P111 - QUANTIFICATION AND VISUALIZATION OF OATP1B1 EXPRESSION IN *IN VITRO* UPTAKE SYSTEM FOR RISK ASSESSMENT OF DDI

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Background and Purpose: In predicting transporter-mediated drug-drug interaction (DDI), it is important to construct robust *in vitro* cell system with highly translatable and extrapolatable potential to *in vivo*. In this study, we investigated whether the expression level of OATP1B1 in HEK293 cells used for drug development is consistent to that in liver tissue, and whether the transport activity has a linear relationship with expression level.

Methods: We prepared frozen human liver tissue and HEK293 cells with varying OATP1B1 expression levels by changing the amount of cDNA transfected. OATP1B1 expression level and its subcellular localization in HEK293 cells were compared with that in human liver tissue. The OATP1B1 expression level was determined by quantifying mRNA or proteins from total cell lysate and plasma membrane fraction by qPCR or LC-MS/MS. The localization of OATP1B1 expression was observed with a confocal microscopy after immunostaining. Furthermore, transport activities at each expression levels were determined by uptake of estradiol- β -glucuronide and rosuvastatin into OATP1B1-expressing HEK293 cells and plotted against its expression level determined in respective way.

Results and Discussion: The expression level of OATP1B1 in transiently expressing HEK293 cells was 4.8-times higher than that in liver tissue, and both were found to be on plasma membrane. The obtained images from immunostaining showed that the expression of OATP1B1 was homogeneous through liver tissue, but not in transiently expressing cells with various OATP1B1 expression levels depending on the cell. The expression level of OATP1B1 had a linear relationship with transfected cDNA amount, but the OATP1B1-mediated transport activity was less than proportional to expression levels of OATP1B1. Regarding this non-linear relationship, OATP1B1 expression on plasma membrane was quantified using immunostaining images to demonstrate if the heterogeneity of expression levels in transiently expressing

cells affects transport efficiency in individual cells. As a result, normalized transport activity in individual cells with high OATP1B1 expression group was lower than that in individual cells with low OATP1B1 expression group.

Conclusion: When extrapolating to *in vivo*, attention needs to be paid by considering differences in expression levels and localization with tissues, and the presence of linear relationship between expression levels and transport activity. In this process, additional attention on the expression level per individual cells is also needed for scaling up of transport activity from expression levels in *in vitro* uptake system.

P112 - APPLICATION OF ENTEROID MONOLAYERS TO EVALUATE POTENTIAL PHARMACOKINETIC OREGANO OIL-DRUG INTERACTIONS

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FDA-approved medications undergo rigorous evaluation for potential drug interactions before reaching consumers, but this policy does not apply for most botanical and other natural products. Concomitant use of natural products with prescribed or OTC drugs raises concern for unwanted drug interactions, which can transpire through pharmacokinetic and/or pharmacodynamic mechanisms. Oregano oil, extracted from the plant *Origanum vulgare*, is a popular botanical dietary supplement used for its antioxidant, anti-inflammatory, and antibacterial properties; carvacrol, its primary constituent, is believed to contribute to these effects. *in vitro*, oregano oil was shown to activate the human pregnane X receptor and human aryl hydrocarbon receptor, as well as induce hepatic cytochrome P450 (CYP) 3A4 and CYP1A2 activity. However, oregano oil is taken orally, creating opportunity for drug interactions in the intestine due to transiently high intestinal exposure. Accordingly, we evaluated the potential inductive effects of oregano oil on intestinal CYP3A4 and CYP1A1 expression and activity using human intestinal stem-cell derived enteroid monolayers, a promising microphysiological system, to advance oral drug disposition predictions. Specifically, we tested the effects of oregano oil at different cell differentiation stages and subsequently determined the time course of change in CYP3A4 and CYP1A1 gene expression and probe enzyme catalytic activities. A higher inductive effect was observed in monolayers differentiated for 21 days compared to 0, 1, 7, and 14 days. Following 24-hour exposure to oregano oil (50 µg/mL carvacrol) in monolayers differentiated for 21 days, both CYP1A1 expression and activity (granisetron 7-hydroxylation) increased 25-fold (Figure 1A & C). Additionally, CYP3A4 expression and activity (midazolam 1'-hydroxylation) increased 3-fold and 2-fold, respectively (Figure 1B & D). The time course of oregano oil induction was next evaluated by investigating the transcriptional and functional response for various exposure durations. Monolayers prepared from three different donors displayed a higher CYP1A1 transcriptional response after 72 hours of exposure (4.0- to 6.0-fold) compared to those exposed for 24 hours (1.0- to 5.0-fold) ($p = 0.002$). Preliminary CYP1A1 activity data from one donor showed a 4-fold increase after 24 hours of exposure and a 20-fold increase after 72 hours. Monolayers from these same three donors also displayed a higher CYP3A4 transcriptional response after 72 hours of exposure (3.0-7.0-fold) compared to 24 hours (2.0-3.5-fold) ($p = 0.006$). Preliminary CYP3A4 activity data from one donor showed no oregano-mediated induction after 24 hours of exposure but a 3-fold increase after 72 hours. Future research with oregano oil includes additional *in vitro* experiments to determine the kinetics and mechanism of enzyme induction and a clinical pharmacokinetic drug interaction study.

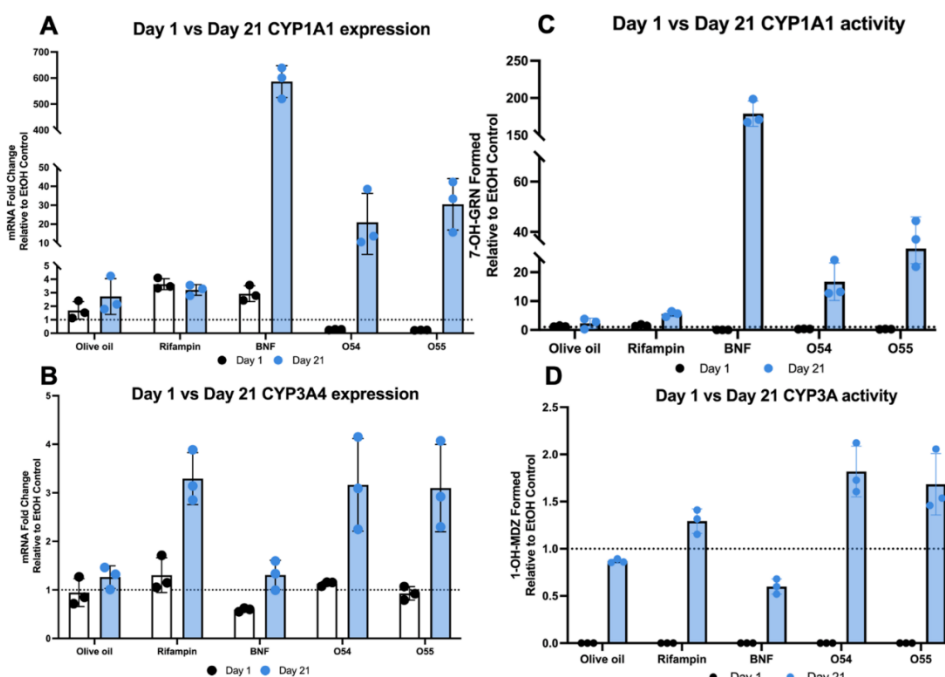
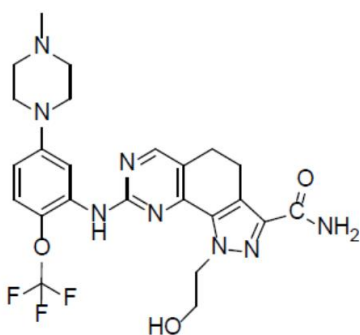


Figure 1. Effects of cell differentiation on inductive response. A more pronounced induction of CYP1A1 [A] and CYP3A4 [B] transcription by oregano oil (O54, O55) and positive controls (rifampin, β -Naphthoflavone) seen in monolayers differentiated for 21 days compared to 1 day. Increased 7-OH-GRN [C] and 1-OH-MDZ formation [D] by oregano oil and positive controls seen in monolayers differentiated for 21 days compared to 1 day.

P113 - IN VITRO ASSESSMENT OF POTENTIAL FOR ONVANSERTIB TO CAUSE CYTOCHROME P450 AND TRANSPORTER-MEDIATED PHARMACOKINETIC DRUG-DRUG INTERACTIONS**Aparna Shinde** and Peter Rix

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Background: Onvansertib, a small molecule inhibitor of PLK-1, is currently under development by Cardiff Oncology, Inc for the treatment of various cancers. *in vitro* studies were conducted to evaluate the potential for clinical drug-drug interactions of onvansertib. Objectives: To determine the role of human CYP enzymes in the *in vitro* metabolism of onvansertib. To evaluate the ability of onvansertib to inhibit CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5 in human liver microsomes. To evaluate onvansertib as an inhibitor of the human transporters P-gp (MDR1), BCRP, OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1 and MATE2-K and as a substrate of the human transporters P-gp and BCRP. Methods: To determine the individual CYP enzymes involved in onvansertib metabolism, reaction phenotyping experiments were performed following both incubation with recombinant CYP enzymes and incubation with human liver microsomes in the presence and absence of specific inhibitors. Both the reversible (competitive) and time-dependent inhibitory potency of onvansertib towards CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/5 (using midazolam and testosterone) were determined *in vitro* by measuring the activity of each enzyme in human liver microsomes in the presence and absence of onvansertib. *in vitro* experiments were designed to measure the concentration of inhibitor that causes 50% direct, time- or metabolism-dependent inhibition (IC₅₀) of each enzyme as measured by probe substrate activity. Transporter P-gp, BCRP, OATP1B1, OATP1B3, OAT1, OAT3, OCT2, MATE1 and MATE2-K inhibition was evaluated by measuring probe substrate transport in the presence and absence of the onvansertib. P-gp and BCRP substrate potential was evaluated by measuring onvansertib transport in the presence and absence of specific inhibitors. A qualified LC-MS/MS method was used to quantify probe substrates in onvansertib incubations with human liver microsomes and recombinant human enzymes and transporters. An LC-MS/MS method to quantify onvansertib was developed and qualified at the testing facility. The range and maximum concentrations of onvansertib tested in each assay were based on published FDA and ICH guidance documents. Results and Conclusions: The results indicated that metabolism of onvansertib in human liver microsomes is predominantly mediated by CYP2C19 and CYP3A4/5. Possible involvement of CYP2C8 and CYP2D6 was observed in one of the two approaches used for CYP reaction phenotyping. No evidence of involvement of CYP1A2, CYP2B6 and CYP2C9 was observed. Onvansertib was not a direct inhibitor of any human CYP enzyme evaluated. At the highest concentration tested (30 μ M), onvansertib directly inhibited CYP3A4/5-mediated midazolam 1'-hydroxylation by 20.9%; therefore, IC₅₀ values were reported as > 30 μ M. There was no evidence of time- or metabolism-dependent inhibition of any of the CYP enzymes evaluated. Onvansertib is an inhibitor of BCRP, OAT3 and MATE1 with IC₅₀ values of 25.1, 6.06 and 0.863 μ M, respectively. Onvansertib inhibited the following transporters by less than 50%: P-gp (26.0%), OATP1B1 (40.9%), OATP1B3 (38.4%) and MATE2-K (34.2%). Onvansertib did not inhibit OAT1 or OCT2. Onvansertib is a substrate for P-gp and BCRP, based on an efflux ratio $\geq$ 2 and a reduction in efflux > 50% in the presence of specific inhibitors. Structure of Onvansertib is uploaded.

**P114 - A NEW INDICATOR FOR DETECTING WEAK INDUCERS BY 4 β -HYDROXYCHOLESTEROL AS A BIOMARKER OF CYP3A4 INDUCTION, AND PROTOCOL FOR HANDLING CLINICAL SAMPLES****Yuki Taya**¹, Mari Mizunaga¹, Shunsuke Nakao¹, Asami Hayashi¹, Soichiro Ito¹, Kiyotaka Nakagawa², and Yukihiro Nomura¹¹JAPAN TOBACCO INC., Takatsuki, Osaka, Japan, ²Tohoku University, Sendai, Miyagi, Japan

Background: 4 β -hydroxycholesterol (4 β -HC) is produced through cholesterol oxidation by CYP3A4. It has been reported that the concentration of 4 β -HC and the 4 β -HC/total cholesterol ratio (4 β -HC/TC ratio) in human plasma increase after administration of a strong inducer of CYP3A4 (e.g., rifampicin) in clinical studies. Furthermore, to utilize 4 β -HC as a practical biomarker, it is important that the 4 β -HC level or 4 β -HC/TC ratio enables assessment of the drug-drug interaction (DDI) risk even with weak CYP3A4 induction, because there are few reports of such cases. In addition, an accurate

measurement of 4 β -HC and assessment of its stability in plasma samples are essential to appropriately evaluate the relationships between CYP3A4 inducers and the 4 β -HC level or 4 β -HC/TC ratio. In this study, we optimized collection and storage protocols of clinical study samples for accurate quantification of 4 β -HC and attempted to find an indicator to detect defective plasma samples. Furthermore, clinical trials using in-house developed new chemical entities (NCEs) with weak CYP3A4 induction were conducted to validate the utility of 4 β -HC as a biomarker.

Methods and Results: To optimize the sample collection and storage protocol for clinical trials, the long-term and short-term storage stability and the freeze-thaw stability of 4 β -HC were evaluated using human plasma with multiple anticoagulants, serum, and acidified plasma. 4 β -HC was stable in samples stored at 4°C or room temperature for 6 hours and also for up to five freeze-thaw cycles. In the long-term stability tests, 4 β -HC was stable in samples stored at -80°C for more than 6 months, while the concentration of 4 β -HC increased in samples stored at -20°C, presumably due to autoxidation. Additionally, an artifact compound presumed to be the cholesterol oxidation product was found to have been generated in samples in which 4 β -HC concentration increased. The correlation between the amount of the cholesterol oxidation product and the rate of increase in 4 β -HC concentration suggests that the amount of the cholesterol oxidation product can be used as an indicator to identify samples stored under inappropriate conditions. Based on the above results, clinical trials of NCEs were conducted using appropriate sample collection and storage protocols. Furthermore, the indicator was monitored in the measurement of 4 β -HC concentrations in clinical trial samples. The clinical DDI studies were conducted using two in-house developed NCEs, both of which were weak inducers of CYP3A4, and midazolam as a probe substrate for CYP3A4 activity in the studies. The 4 β -HC levels and the area under the curve (AUC) of midazolam were collected at pre- and post-administration of NCEs. The weak induction of CYP3A4 by the NCEs was determined based on a slight decrease in midazolam AUC and was detected by a significant increase in 4 β -HC levels.

Conclusion: Our novel approach, relying on the monitoring of an indicator to identify plasma stored under inappropriate conditions, facilitated an accurate measurement of 4 β -HC. As a result, this enabled the evaluation of weak CYP3A4 inducers using 4 β -HC in clinical trials without the use of probe substrates.

P115 - EFFECT OF SUBSTRATES AND INHIBITORS OF BREAST CANCER RESISTANCE PROTEIN ON APIXABAN AND RIVAROXABAN PHARMACOKINETICS IN JAPANESE PATIENTS

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Direct activated coagulation factor X inhibitors such as apixaban and rivaroxaban are orally administered to patients with atrial fibrillation to prevent stroke or systemic embolism (AF). Both apixaban and rivaroxaban are known to be transported by the ATP-binding cassette transporters P-glycoprotein (P-gp, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) in the small intestine, liver, and kidney, and metabolized by the intestinal and hepatic cytochrome P450 (CYP) 3A4/5. Therefore, the pharmacokinetic variabilities of apixaban and rivaroxaban can be explained by changes in the expression and/or function of these proteins. CYP3A4/5 and/or P-gp mediated drug-drug interactions of apixaban and rivaroxaban have been reported, whereas there is little information concerning BCRP mediated their drug-drug interactions. This study aimed to evaluate the effect of BCRP substrates and inhibitors on apixaban and rivaroxaban pharmacokinetics in Japanese patients with AF. In this study, 224 adult patients treated with apixaban and 56 adult patients treated with rivaroxaban were enrolled. Pharmacokinetic profile of apixaban was described using a 1-compartment model with first-order absorption, whereas that of rivaroxaban was described using a 1-compartment model without first-order absorption. Effects of patient characteristics, concomitant use of BCRP substrates (atorvastatin, rosuvastatin, olmesartan and telmisartan) and inhibitor (febuxostat), and ABCG2 421C>A polymorphism on the pharmacokinetics of apixaban and rivaroxaban were analyzed using non-linear mixed effect modeling (NONMEM™) program. The apparent oral clearance (CL/F) of apixaban nonlinearly correlated with creatinine clearance (CL_{Cr}). The population mean of CL/F of apixaban was higher in patients with the ABCG2 421C/C or C/A genotype compared with patients with the ABCG2 421A/A genotype. Febuxostat decreased the CL/F of apixaban in patients with the ABCG2 421C/C or C/A genotype, whereas febuxostat did not affect the CL/F of apixaban in patients with the ABCG2 421A/A genotype. The CL/F of rivaroxaban nonlinearly correlated with CL_{Cr}, although BCRP substrates and inhibitor as well as the ABCG2 421C>A polymorphism did not exhibit the impact on the CL/F of rivaroxaban. These results suggest that concomitant febuxostat, as well as the ABCG2 421A/A genotype and renal function, are considered to be important factors affecting apixaban pharmacokinetics in Japanese patients with AF. In contrast, concomitant use of BCRP substrates and inhibitor as well as ABCG2 421C>A polymorphism are not associated with changes in rivaroxaban pharmacokinetics in these patients. These findings may provide useful information for anticoagulant therapy with apixaban and rivaroxaban to prevent the risk of adverse reactions.

P116 - CLINICAL SIGNIFICANCE OF DRUG-DRUG INTERACTION STUDIES DURING THERAPEUTIC PEPTIDE DRUG DEVELOPMENT**Kai Wang**¹, Carolina Säll², Upendra Argikar³, Constanze Hilgendorf⁴, Hilmar Schiller⁵, Anders Sonesson⁶, and Kenichi Umehara⁷¹Janssen R&D, San Diego, CA, United States, ²Novo Nordisk A/S, Måløv, Denmark, ³Bill & Melinda Gates Medical Research Institute, Cambridge, MA, United States, ⁴AstraZeneca Gothenburg, Mölndal, Sweden, ⁵Novartis Institutes for BioMedical Research, Basel, Switzerland, ⁶Ferring Pharmaceuticals A/S, Kastrup, Denmark, ⁷Roche Innovation Center, Basel, Switzerland

The absence of drug-drug interaction (DDI) guidelines for therapeutic peptides make it difficult to identify when and what DDI studies are most or least informative for this heterogeneous class of molecules. Our cross-industry working group was formed under the sponsorship of the EFPIA to investigate the landscape of peptide DDI activities across the industry. We compiled a database containing DDI data from submission packages for 31 peptide drugs approved between January 2008 and August 2021. We reported our findings in a White Paper published in Clinical Pharmacology and Therapeutics¹, where we describe diverse challenges arising from an absence of DDI guidance during peptide drug development. Our analysis of submission packages from 2008-2021 suggest that DDI likelihood is low for larger peptides (>2 kDa). In line with recommendations from the Editor-in-Chief of Clinical Pharmacology and Therapeutics², we stress that much more peptide DDI data from diverse molecules (with different sizes, structures, and modes of action) will be required to establish clear guidelines for peptide DDI studies that are both feasible and meaningful. Here, we used the same criteria¹ to identify seven additional peptide drugs³ that have been approved between September 2021 and September 2023 for further analysis: trofinetide (0.3 kDa; linear), nirmatrelvir (0.5 kDa; linear), odevixibat (0.7 kDa; linear), rezafungin (1.2 kDa; cyclic), motixafortide (2.2 kDa; cyclic), vosoritide (4.1 kDa; cyclic), and tirzepatide (4.8 kDa; linear). CYP inhibition was investigated for all seven peptides using a standard CYP panel (CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A) in HLMs, while some (odevixibat, rezafungin, and motixafortide) submission packages included additional studies (e.g., hepatocytes or recombinant enzyme investigations). All peptides had investigated CYP induction, including standard CYP panel (CYP1A2, CYP2B6 and CYP3A4) in human hepatocytes, but none were found to be inducers of the relevant CYPs at clinically relevant concentrations. Phenotyping investigations varied from standard studies (e.g., HLMs in the presence of selective CYP inhibitors) to submission packages without phenotyping studies. Drug transporter studies (substrate and inhibition properties) varied substantially from peptide to peptide. Clinical studies investigating metabolism- or transporter-mediated DDIs were performed for some small peptides (odevixibat, rezafungin, and nirmatrelvir; all <2 kDa), but results attributed to the peptide drug showed only minimal changes in PK parameters. Overall, data from these seven newly approved therapeutic peptides was largely consistent with earlier observations from the 2008-2021 approved peptide dataset. Most notably, analyses here are consistent with our published observation that DDI likelihood is low for larger peptides (>2 kDa), while smaller peptides, especially those with xenobiotic structural properties (e.g., nirmatrelvir), demonstrate a DDI risk profile more akin to small molecule drugs. As we continue to benchmark the evolving landscape of peptide DDI activities, we strongly encourage our colleagues to actively publish positive and negative results from peptide DDI studies, as this will be invaluable to inform clear and harmonized guidelines moving forward.

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P117 - INVESTIGATION OF OATP1B INHIBITORY EFFECT BY A CYCLIC PEPTIDE USING AN ENDOGENOUS BIOMARKER COPROPORPHYRIN-I IN MONKEYS**Ryosuke Watari**¹, Hiromi Sawada¹, and Kei Mayumi²¹Shionogi & Co., Ltd., Toyonaka-city, Osaka, Japan, ²Shionogi & Co., Ltd., Osaka-city, Japan

Purpose: Cyclic peptides are an expected modality for undruggable targets, such as protein-protein interaction. European Federation of Pharmaceutical Industries and Associations issued a white paper for industry perspective on therapeutic peptide of drug-drug interaction (DDI) assessments [1], and it is concluded that the DDI likelihood is low for peptides with >2 kDa. Compound A, which is a 3.5 kDa cyclic peptide, was synthesized by Shionogi & Co., Ltd. as an inhibitor of the tropomyosin receptor kinase A and assessed the potential for the treatment of pain. In this study, we investigated the inhibitory effect of Compound A on organic anion transporting polypeptide (OATP) 1B in monkeys using coproporphyrin-I (CP-I), which is an endogenous substrate of OATP1B, and tried to elucidate the utility of CP-I for the assessment of

OATP1B inhibitory effect by large cyclic peptide.

Methods: The *in vitro* OATP1B inhibitory effect and serum protein binding was assessed using OATP1B expressing cells and the equilibrium dialysis method, respectively. In addition, the *in vivo* OATP1B inhibitory effect were evaluated by determination of CP-I in monkeys after single intravenous and subcutaneous administrations of Compound A at 1 and 2 mg/kg, respectively, which are estimated pharmacologically active doses. The *in vivo* inhibition constant (K_i) of Compound A against OATP1B was estimated by fitting of CP-I data after intravenous administration to the simple pharmacokinetics model of CP-I constructed with synthesis and renal/hepatic elimination processes. The accuracy of the estimated *in vivo* K_i value was confirmed using CP-I data after subcutaneous administration.

Results and Discussion: Compound A exhibited potent inhibitory effect on OATP1B and very high serum protein binding in *in vitro* studies. After single intravenous and subcutaneous administration of Compound A to the monkeys, the CP-I concentration in plasma increased by 8-fold and 4-fold, respectively, compared to the baseline. Based on the CP-I data after intravenous administration, the *in vivo* K_i value of Compound A against OATP1B was estimated to be sub-pg/mL as the unbound plasma concentration. Using the estimated *in vivo* K_i value from the intravenous administration study, the CP-I concentration after subcutaneous administration of Compound A were successfully replicated, suggesting that the estimated *in vivo* K_i value is likely to be reliable.

Conclusion: A cyclic peptide of Compound A exhibited significant inhibitory potential on OATP1B. We succeeded to estimate the *in vivo* OATP1B inhibitory potential of Compound A using CP-I in monkeys with the simple pharmacokinetic model.

Reference:

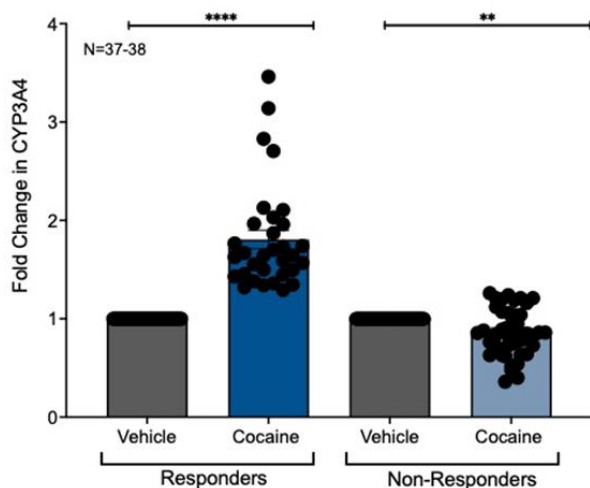
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P118 - COCAINE MODULATES CYP3A4 in PRIMARY HUMAN MACROPHAGES IN A DONOR-SPECIFIC FASHION

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CYP3A4 metabolizes 60% of therapeutic drugs, including antiretroviral therapies (ART). CYP3A4 also contributes to cocaine metabolism. People living with HIV that also consume illicit substances have poor virologic control, despite access to ART. While this is likely attributable to lack of adherence, we hypothesize adverse drug-drug interactions also contribute to loss of virologic suppression. To evaluate this, we obtained peripheral blood mononuclear cells from healthy donors and differentiated them into monocyte-derived macrophages (MDM) that were treated with cocaine 10 μ M cocaine for 24 hours. While CYP3A4 was expressed in primary human MDM, interdonor variability occurred. Interestingly, cocaine caused a dichotomous response wherein it significantly increased CYP3A4 for some donors, whom we termed "responders". However, "non-responders" were resistant to this effect and exhibited no change or even a decrease following cocaine treatment. CYP3A4 enzymatic activity confirmed the dichotomous response. The mechanism behind the dichotomous response was not related to CYP3A4 polymorphisms, plasma cytokines, cytokines in cell supernatant, or sex. However, we determined that CYP3A4's primary transcription factor, PXR, contributed to cocaine's effect as there was a similar dichotomous response in its expression. Cocaine had no effect on CAR, another CYP3A4 regulator. Our data suggest that some individuals are vulnerable to adverse drug-drug interactions with ART and cocaine that may result in higher viral load.



P119 - MECHANISTIC STUDIES SUPPORTING THE EVALUATION OF PHARMACOKINETIC-DRUG INTERACTIONS WITH DRUGS APPROVED BY THE U.S. FOOD AND DRUG ADMINISTRATION IN 2023: A SYSTEMATIC REVIEW OF NEW DRUG APPLICATIONS

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The mechanistic evaluation of enzyme- and transporter-based drug-drug interactions (DDIs) is an integral part of the drug development process and supports the safe and effective clinical use of new therapies. In the present work, DDI data for small molecular drugs approved by the U.S. Food and Drug Administration in 2023 (N = 38) were analyzed using Certara Drug Interaction Database (<https://www.druginteractionsolutions.org/>). The mechanism(s) and clinical magnitude of the observed interactions were characterized based on information available in the new drug application reviews. DDI data from dedicated clinical trials, pharmacogenetics studies, physiologically-based pharmacokinetics (PBPK) modeling and simulations, and population PK analyses were examined. Positive study results defined as mean area under the curve ratios (AUCRs) ≥ 1.25 for inhibition DDIs and ≤ 0.8 for induction DDIs, were then fully analyzed. When new drugs were evaluated as victims of enzyme-based DDIs, a total of 23 drugs (61%) were affected by perpetrator drugs (through inhibition and/or induction). Seven drugs were found to be sensitive substrates of CYP1A2 (fezolinetant), CYP2C8 (daprodustat), and CYP3A (elacestrant, gepirone, nirmatrelvir, nirogacestat, and repotrectinib), with AUCRs of 5.27-18.60 when co-administered with the strong marker inhibitors fluvoxamine, gemfibrozil, itraconazole, or ketoconazole. Four drugs, leniolisib, omaveloxolone, palovarotene, and sparsentan were found to be moderate sensitive substrates of CYP3A (AUCRs 2.12-4.12 with co-administration of itraconazole or ketoconazole). Regarding transporters, only three drugs, iptacopan (P-gp, BCRP, OATP1B1/1B3), momelotinib (OATP1B1/1B3), and zavegepant (OATP1B3, NTCP), were clinical substrates of transporters, with a maximum AUCR of 2.39 predicted for zavegepant using PBPK modeling and simulations following single-dose rifampin administration. As perpetrators, only one combination drug, nirmatrelvir and ritonavir, was considered a strong inhibitor of CYP3A (midazolam AUCR 14.30). No drug exhibited strong inhibition of transporters. The following four drugs were found to be moderate inhibitors (marker substrates AUCRs 2.07-2.73): momelotinib (BCRP), nirogacestat (CYP3A), pirtobrutinib (BCRP, CYP2C8), ritlecitinib (CYP1A2, CYP3A). No strong inducer of enzymes or transporters was identified. Four drugs showed enzyme induction, with repotrectinib showing the maximum induction and considered a moderate inducer of CYP3A (midazolam AUCR 0.31). As expected, almost all DDIs (except one) with AUCRs ≥ 2 or ≤ 0.5 (≥ 2 -fold change) triggered dosing recommendations in the product labels. PBPK modeling and simulations continued to be increasingly used in lieu of clinical trials, with 12 drugs evaluated as victims of enzymes (N = 11) or transporter (N = 1), and 5 drugs as inhibitors of enzymes (N = 4) or transporters (N = 1). In line with the trend observed in recent years, oncology was the most represented therapeutic area, including 24% of all 2023 approvals. However, drugs found to be either sensitive substrates or strong inhibitors of enzymes included treatments for a variety of diseases, e.g., cancer treatments, antianemia preparations, anti-estrogens, antidepressants, and antivirals. This finding highlights the continuous challenge of effectively managing the risk of significant pharmacokinetic interactions in the clinic in patient populations who often receive numerous concomitant medications.

P120 - CELLULAR MECHANISMS OF AFATINIB-INDUCED FLUID SECRETION IN A 3D MOUSE ENTEROID MODEL

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Afatinib is a second-generation epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) and the first-line drug of non-small cell lung cancer with EGFR mutation. Despite its high efficacy, afatinib is associated with life-threatening adverse effects especially diarrhea occurring in 75-95% of patients, which leads to dose reduction or cessation of therapy in over half of patients. The mechanism of afatinib-induced diarrheas is still unclear, particularly under prolonged treatment conditions, which correlate with the onset of diarrhea that requires 1-2 days to occur and typically occurs within a week. Excessive chloride secretion in the intestine is a main driving force for fluid secretion resulting in secretory diarrheas, a type of diarrheas commonly associated with TKI drugs. We hypothesized that excessive chloride-driven fluid secretion may be the underlying pathophysiology of afatinib-induced diarrheas. Therefore, this study aimed to investigate the effect and mechanisms of afatinib on fluid secretion under prolonged treatment conditions using a 3D colonoid model, a model of various epithelial cells derived from colonic stem cells recapitulating complexity and physiology of colon. Herein, we showed that prolonged treatment of afatinib amplified forskolin-induced fluid secretion by up to 2 folds using a swelling assay. For mechanistic studies, inhibitors of cystic fibrosis transmembrane conductance regulator (CFTR), calcium-dependent chloride channels (CaCCs), intermediate-conductance calcium-activated potassium channel (KCa3.1), and Kv7 family of voltage-gated potassium channels (Kv7) were used in swelling assays. We found that CaCCs and Kv7 inhibitors completely blocked the amplification effect of afatinib. On the other hand, the amplification effect of afatinib was not diminished by KCa3.1 inhibitor and was CFTR-independent. Taken together, this study revealed that afatinib enhanced the forskolin-induced fluid secretion via CaCCs and Kv7 channels under prolonged treatment

conditions and these channels may be therapeutic targets for alleviating diarrheal side effects of afatinib in lung cancer patients.

P121 - THE PSYCHOTROPIC BOTANICALS KRATOM AND HEMP INDUCE SELECT CYTOCHROME P450 ENZYMES IN HUMAN HEPATOCYTES: INHIBITION IS NOT THE ONLY GAME IN TOWN

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Kratom (*Mitragyna speciosa*) and hemp (*Cannabis sativa*) are psychotropic botanical products often used as natural alternatives to pharmaceutical medications to self-treat a variety of neurological conditions such as chronic pain, anxiety, and sleep disorders.^{1,2} Extracts prepared from both products have been reported to induce and inhibit the mRNA expression and activity, respectively, of multiple cytochrome P450 (CYP) enzymes *in vitro*.³⁻⁶ As these products are typically taken chronically, evaluating the net effect of induction and time-dependent inhibition on CYP-mediated metabolism is critical to assess drug interaction risk. The time-dependent inhibitory effects of these extracts and major phytoconstituents on various CYP enzymes have been extensively studied,^{3,4} whereas the inductive effects remain largely unknown. The objective of this study was to investigate the impact of a kratom and hemp on the mRNA expression and activity of major CYPs in the liver using plated human hepatocytes. The selected kratom (methanolic extract) and hemp (encapsulated oil) products were chemically characterized using a metabolomics approach to ensure authenticity.⁷ Cryopreserved human hepatocytes (Lot Hu1489, Thermo Fisher Scientific) were seeded onto collagen I-coated 24-well plates at a density of 7×10^5 cells/mL (0.5 mL/well) and kept at 37°C with 5% CO₂. Upon cell attachment (~5 hours after seeding), plating medium was replaced with incubation medium containing kratom extract (<0.1% methanol, v/v) or hemp oil (<0.1% acetone, v/v) at two concentrations (5 and 25 µg/mL). Freshly prepared incubation medium containing the botanical was replaced every 24 hours. After a 48-hour incubation, hepatocytes were washed and incubated with a CYP cocktail consisting of dextromethorphan (CYP2D6, 10 µM), midazolam (CYP3A, 4 µM), phenacetin (CYP1A2, 100 µM), S-mephenytoin (CYP2C19, 150 µM), and tolbutamide (CYP2C9, 100 µM) for 1 hour at 37°C. The medium was then analyzed for the corresponding metabolites (dextrophan, 1'-hydroxymidazolam, acetaminophen, 4'-mephenytoin, and 4'-hydroxytolbutamide) by LC-MS/MS. Total RNA was isolated, and mRNA of each CYP was quantified by real time qPCR. Rifampicin (10 µM) was used as a positive control for CYP3A4 induction. Relative to vehicle control, rifampicin induced CYP3A4 mRNA expression by ~75-fold. Kratom at 25 µg/mL induced CYP3A4 mRNA expression by ~14-fold but had no effect on activity. Conversely, kratom at 5 µg/mL induced CYP1A2 and CYP2C19 activity by ~3- and 6-fold, respectively, but had no effect on mRNA expression. Hemp at 25 µg/mL induced CYP1A2 mRNA expression by ~2-fold but had no effect on activity. Hemp at 5 µg/mL had no effect on neither mRNA expression nor activity of all CYP enzymes tested. These preliminary data suggested a discordance between CYP mRNA expression and activity in human hepatocytes upon exposure to both kratom and hemp. The inductive effects of these two psychotropic botanicals on CYP mRNA expression and activity will be evaluated with additional hepatocyte donors to assess inter-individual variability in response. A range of concentrations will be used to determine the kinetic parameters EC₅₀ and E_{max} for enzymes that show induction (≥ 2 -fold relative to vehicle control). The recovered parameters will be applied to an FDA-recommended mechanistic static model to assess natural product-drug interaction risks.

P122 - INDUCTION OF DRUG METABOLIZING ENZYMES AND TRANSPORTER GENES BY CARBAMAZEPINE, CARBAMAZEPINE-10,11-EPOXIDE, AND CARBAMAZEPINE-10,11-DIOL

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Carbamazepine (CBZ) is used clinically as a strong inducer of cytochrome P450 (CYP) 3A4 despite knowledge gaps regarding its transcriptional profile beyond CYP3A4. Even less is known about induction potential of CBZ metabolites carbamazepine-10,11-epoxide (CBZ-E) and carbamazepine-10,11-diol (CBZ-diol). Previously, the transcriptional profile of genes induced by CBZ were investigated in liver samples obtained from patients treated with CBZ where >2-fold induction was observed for numerous CYPs, SULTs and transporters⁽¹⁾, but it is unclear if the response comes from CBZ and/or metabolites because CBZ-E and CBZ-diol circulate at concentrations 19 to 40% of CBZ, at steady state ⁽²⁾. The aim of this work was to investigate the transcriptional fingerprint of CBZ, CBZ-E, and CBZ-diol at concentrations approximating 1x, 10x, and 30x human C_{avg} in primary human hepatocytes using customized gene expression cards. Moreover, this study evaluated the ligand's effect on multiple enzymes and drug transporters including those not previously investigated *in vitro* (e.g., MATE1, OCT1, OAT2, BCRP, AOX1, CES1, UGT2B4, UGT2B7, etc.). Induction of CYP3A4, CYP2B6, CYP1A2, CYP2C8, CYP2C9, and CYP2C19 were further characterized using a 12-concentration induction response curve spanning 1 – 600 µM for CBZ and 1 – 500 µM for CBZ-E and CBZ-diol. These studies showed potential for CBZ to induce CYP3A4, CYP2B6, CYP1A2, and CYP2C mRNA ≥ 2 -fold. CBZ-E induced CYP3A4, CYP2B6, and CYP2C8, and CBZ-diol induced CYP3A4, CYP2B6, CYP2C8 and CYP2C9. Understanding CBZ and its metabolite's ability to induce beyond CYP3A4 is an important consideration when designing clinical studies and interpreting clinical data, especially for

new chemical entities dependent upon non-CYP3A4 mediated disposition. These studies should contextualize potential for induction beyond CYP3A4, but further work is needed to prospectively predict translation to the clinic.

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P123 - ROLES OF NOLC1 IN NUCLEAR TRANSLOCATION OF PXR

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Pregnane X receptor (PXR) is a nuclear receptor regulating the expression of drug-metabolizing enzymes such as cytochrome P450 (CYP) 3A4, CYP2C9, and UDP-glucuronosyltransferase (UGT) 1A1 in the human liver. PXR is mainly localized in cytoplasm forming a complex with heat shock protein 90 and cytoplasmic CAR retention protein. Upon the activation by binding of ligands such as rifampicin, PXR translocates from cytoplasm to nucleus and upregulates target genes. According to our previous results of co-immunoprecipitation assay using anti-PXR antibody against the lysate of rifampicin-treated ShP51 cells (HepG2 cell line stably expressing human PXR), PXR seems to interact with nuclear and coiled-body phosphoprotein 1 (NOLC1), a protein shuttling between cytoplasm and nucleus and between nucleus and nucleolus. The purpose of this study is to clarify the role of NOLC1 in the nuclear translocation of PXR. The induction of CYP3A4 mRNA by rifampicin in ShP51 or HepaSH (primary hepatocytes from humanized-liver mice) cells was attenuated by the knockdown of NOLC1, although the basal CYP3A4 mRNA level was not affected. The increases in CYP3A4 protein level and activity by rifampicin in HepaSH cells were suppressed by the knockdown of NOLC1. These results suggest that NOLC1 positively regulates CYP3A4 induction by rifampicin treatment. The reporter activity of a plasmid containing the promoter region and the distal enhancer region of the CYP3A4 gene was increased by rifampicin, and the increase was suppressed by the knockdown of NOLC1 in ShP51 cells, suggesting that NOLC1 enhances the ligand-dependent transcriptional activation of PXR. Immunofluorescence staining using anti-PXR antibody in HepaSH cells revealed that the nuclear translocation of PXR was promoted by rifampicin treatment, and the translocation was attenuated by the knockdown of NOLC1. Finally, Western blotting using cytosolic, nuclear soluble, and chromatin fractions in rifampicin-treated ShP51 cells revealed that PXR protein level in chromatin fraction was increased upon rifampicin treatment, and the increase was suppressed by the knockdown of NOLC1, indicating that NOLC1 facilitates the ligand-dependent binding of PXR to chromatin. Taken together, we found that NOLC1 plays a role in enhancing PXR nuclear translocation and PXR binding to chromatin and transactivation of downstream genes such as CYP3A4. This is the first study revealing that NOLC1 functions as a novel drug metabolism regulator in the human liver.

P124 - CONSTRUCTION OF PBPK MODEL FOR MULTIPLE TYROSINE KINASE INHIBITOR TSU-68 BASED ON *IN VITRO* INDUCTION KINETICS OF CYP1A

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Purpose: Enzyme induction sometimes causes drug-drug interaction, because it may reduce systemic exposure to the victim drugs. In particular, the auto-induction of enzymes may lead to decreasing the systemic exposure to the drug itself. In enzyme induction especially auto-induction, understanding the extent of change in drug exposure during/after repeated administration is important, but it is quite difficult to quantitatively estimate the extent of decrease in the exposure in humans based on the *in vitro* studies. TSU-68, an inhibitor of multiple tyrosine kinases involved in tumor angiogenesis, is a substrate of and an inducer of both CYP1A1 and CYP1A2. Clinical studies demonstrate that the plasma concentrations of TSU-68 decreased to one half after the repeated administrations when compared with the single administration probably attributable to auto-induction of CYP1A enzymes. In this study, we constructed the PBPK model of TSU 68 using the *in vitro* data and thereby quantitatively explained the decrease in the plasma concentration of TSU-68 in clinical studies. **Methods:** Pooled cryopreserved human hepatocytes obtained from 10-donors were used in the *in vitro* CYP induction studies. The TSU-68 concentrations in the incubation medium were measured at several time points by an LC/MS/MS method. After incubation with TSU 68 for 24, 48, and 72 hours, CYP1A1/2 mRNA expression was measured by the real-time PCR method, and the metabolic activities of TSU-68 were also measured. A specific inhibitor of CYP1A1 was used to estimate the respective CYP1A1 and CYP1A2 activities. A mathematical induction model was constructed using these *in vitro* data and incorporated into the PBPK model, and its parameters were also estimated by fitting to the plasma concentration-time profile of TSU-68 after a single administration. **Results and Discussion:** TSU-68 concentrations in the incubation medium decreased in a time-dependent manner. CYP1A1/2 mRNA expression and TSU-68 metabolic activity increased, while the time profile of induction of CYP1A1 activity was different from that of CYP1A2 activity. We

developed the mathematical induction model by fitting the observed concentration profiles in the incubation medium and thereby explained these different time profiles of CYP1A inductions. The PBPK model of TSU-68 was then constructed by incorporating this mathematical induction model and well explained plasma concentration-time profiles of TSU-68 after repeated administrations. Notably, the model that did not take into account the decrease in TSU-68 concentration in the medium underestimated the extent of induction; this suggesting the importance of estimation of the inducer concentration in the medium when constructing the induction model. Conclusion: The PBPK model of TSU-68 describing the decrease in systemic exposure to TSU-68 after repeated administration due to CYP1A auto-induction was constructed. In the induction study, taking the decrease of enzyme inducer in the medium into account is essential to precisely estimate the decrease in the exposure in the clinic.

P125 - EFAVIRENZ-INDUCED HEME PROTEIN INDUCTION AND COPROPORPHYRIN LEVELS: INSIGHTS FROM *IN VITRO* EXPERIMENTS AND *IN VIVO* STUDIES

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Organic Anion Transporting Polypeptides (OATPs) are membrane proteins known to impact pharmacokinetics of their substrates. Specifically, members of the OATP1B-subfamily, namely OATP1B1 and OATP1B3, have been studied for their impact on drug efficacy and safety. In recent years, expanding clinical data on drug-drug interactions involving hepatic OATP1B-transporters strengthened the role of coproporphyrin (CP) I and CPIII, which are byproducts of the heme biosynthesis, as appropriate endogenous biomarkers for hepatic OATP1B-transporters (Lai et al. 2016)¹. However, there is an increasing interest to understand whether changes in CP levels can be caused by other mechanisms besides the inhibition of OATP1B1. Considering the origin of coproporphyrins, one possible mechanism could be the induction of cytochrome P450 (CYP) heme proteins leading to a higher demand of heme. This could result in an increase in heme synthesis accompanied by an elevated production of byproducts, including CPI and CPIII.

In this study, we focused on the HIV therapeutic efavirenz. Initially, we verified its function as a CAR-inducer in cell-based reporter gene assays by applying the Dual-Luciferase® Reporter assay system (Promega, Dübendorf, Switzerland). Then, we tested efavirenz for its interaction with OATP1B1 using MDCKII-OATP1B1 cells. In addition, we evaluated its impact on mRNA and protein expression of ALAS1, the key enzyme of heme synthesis, and the heme proteins CYP3A4 and CYP2B6 in differentiated HepaRG cells, treated with efavirenz for 72h, by real-time PCR and Western blot analysis. Moreover, we quantified coproporphyrin as well as 4β-hydroxycholesterol plasma levels in healthy volunteers treated with efavirenz by a validated UHPLC-MS/MS method.

In HepG2-based reporter gene assays, we observed an increased transactivation of the CYP2B6-promoter in presence of CAR and efavirenz. Subsequent transport experiments in MDCKII-OATP1B1 cells excluded an impact of efavirenz on the OATP1B1-mediated cellular accumulation of coproporphyrins. Moreover, investigating CYP enzyme induction, mRNA expression analysis of CYP3A4 and CYP2B6 by real-time PCR revealed increased mRNA levels of both heme proteins in HepaRG cells treated with efavirenz, but no impact on ALAS1. Yet, Western blot analysis showed no change in CYP2B6, CYP3A4, or ALAS1 protein expression. Finally, we report on the coproporphyrin levels analyzed for their association with the CYP3A-activity marker 4β-hydroxycholesterol in plasma of healthy volunteers treated with efavirenz.

In conclusion, we validated efavirenz as an activator of CAR, which induced CYP3A4 and CYP2B6 mRNA expression *in vitro* and we excluded its interaction with the OATP1B1 mediated transport of CPI and CPIII supporting its applicability to test for whether mechanisms other than OATP1B1 inhibition affect the levels of CPs in humans.

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P126 - THINKING BEYOND CYTOCHROME P450 INACTIVATION KINETICS: ENHANCED UNDERSTANDING OF THE MECHANISM OF TUCATINIB INTERACTION WITH CYP3A4

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Tucatinib has demonstrated remarkable efficacy and safety in treating adult patients with advanced, unresectable, or metastatic HER2+ breast cancer, including individuals with brain metastases. *in vitro* cytochrome P450 (CYP) inhibition kinetics studies of tucatinib showed time-dependent inhibition (TDI) of CYP3A4 with a mean kinact value of 0.011 min⁻¹ and a KI value of 0.54 μM, which suggests low TDI potential due to low inactivation efficiency. The purpose of this investigation was to characterize the mechanism of inactivation of CYP3A4 by tucatinib, utilizing a combination of UV

spectral and *in vitro* chemical approaches. To assess the potential for modification or destruction of the heme of CYP3A4 as a mechanism of inactivation, spectral evaluation by carbon monoxide (CO) binding and characterization of P450 heme loss using LC-MS following incubation with tucatinib were both performed. In addition, to assess the potential of quasi-irreversible inhibition via a metabolite-intermediate (MI) complex with the heme of CYP3A4, two distinct approaches were employed, including searching for spectral evidence of an MI complex (e.g. absorbance at 455 nm) and assessing chemical reversibility of inactivation following incubation with potassium ferricyanide.

Results from these studies suggest that no loss of intact heme or formation of a distinct covalent heme adduct was observed following inactivation of CYP3A4, which was confirmed by minimal observed loss of CO binding using UV spectroscopy. Moreover, the observed inactivation by tucatinib was not able to be reversed by treatment with potassium ferricyanide, and no appreciable evidence for a classic MI complex spectrum was observed. These findings suggest that the mechanism of tucatinib inactivation of CYP3A4 cannot be clearly explained by modification of the heme or formation of a metabolite-intermediate (MI) complex. Further studies are currently underway using targeted proteomics and high-resolution mass spectrometry to investigate if inactivation might be the result of covalent modification of the CYP3A4 apoprotein.

P127 - ELUCIDATING MECHANISMS OF PROMISCUOUS AGGREGATION ON CYP INHIBITION ASSAYS

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Many small organic molecule drugs have been shown to form aggregates in buffers used for biochemical assays, which can result in artifacts such as enzyme inhibition false positives. Pan-CYP inhibition and/or atypical inhibition profiles are occasionally observed with such compounds, described as nuisance compounds due to an undesired mechanism (e.g. aggregation) eliciting their biological effect (Reference 1). These types of data can be difficult to interpret, however formation of *in vitro* aggregates may help explain this unusual data. Little is known about the effect of aggregates on routine CYP inhibition assays conducted with human liver microsomes or recombinant CYP enzymes. Putative mechanisms of CYP inhibition by aggregates include sequestration of probe substrate(s), which can be dependent on the physicochemical properties of the aggregate and/or probe, denaturation of the enzyme and/or co-enzymes (Reference 2), disruption of CYP-oxidoreductase interaction, perturbation of membrane structure, or binding to or trapping the enzyme rendering it inactive. To conduct our investigations, a prototypical aggregator (3',3'',5',5''-tetraiodophenolphthalein, TIPT) was selected from a nuisance set of known aggregators and its aggregation in assay buffer was measured using a Lucifer yellow based fluorometric assay. Various *in vitro* studies were conducted to investigate the mechanisms of its CYP inhibition. Equilibrium dialysis and CYP inhibition studies, using a diverse set of CYP2D6 substrates, were designed to investigate the role of substrate sequestration. Spectroscopic data was generated to investigate destruction of P450. CYP inhibition studies were also used to investigate the potential for various additives to reverse *in vitro* aggregation while maintaining enzyme activity. Preliminary data suggests that substrate sequestration by TIPT varies by probe substrate. Ongoing studies will determine if substrate sequestration translates to CYP inhibition artifacts that can be reversed in the presence of an aggregate-preventing additive and if additional mechanisms (e.g. P450 destruction) are involved. This work demonstrates that CYP inhibition assays are vulnerable to artifacts, such as false positives, caused by self-aggregation of the test drug.

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P128 - EVALUATION OF CYTOCHROME P450 INHIBITION IN THE 7-IN-1 COCKTAIL USING PREFERRED SUBSTRATES RECOMMEND BY REGULATORY AGENCIES

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Purpose: An investigational drug's potential to inhibit CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A should be evaluated as recommended by regulatory agencies such as FDA and PMDA. The selection of the probe substrate for the aforementioned CYPs is the first step for *in vitro* experiments. Numerous combinations of substrates have been characterized in previously reported substrate cocktails *in vitro* screening assays to evaluate the inhibitory potency of the compound of interest. However, not all of the substrates in the 7-in-1 cocktail were the preferred substrates¹. We designed and introduced a 7-in-1 cocktail approach with each of the substrates being the preferred ones recommended by the regulatory agencies.

Method: The 7-in-1 substrate cocktail, with each of the preferred substrates, was incubated with human liver microsomes (HLM). The substrates are as follows: Phenacetin (CYP1A2), bupropion (CYP2B6), Amodiaquine (CYP2C8), Diclofenac

(CYP2C9), S-mephenytoin (CYP2C19), dextromethorphan (CYP2D6) and midazolam (CYP3A). Inhibitory profiles of known inhibitors for each of CYPs were studied. The results were compared to those generated in-house using individual substrates.

Results and Discussion: To optimize the reaction conditions, the probe substrates and initial concentrations were selected based on the guidelines from the regulatory agencies, literature values², and K_m values generated in-house. Finally, a cocktail of seven probe substrates with its concentration (25 μ M phenacetin, 5 μ M bupropion, 0.2 μ M amodiaquine, 5 μ M diclofenac, 15 μ M S-mephenytoin, 5 μ M dextromethorphan and 0.2 μ M midazolam) was selected for a 10-min incubation with 0.2 mg protein/mL of HLM as the optimized condition. To validate this method, seventeen known inhibitors, including α -naphthoflavone, ticlopidine, montelukast, sulfaphenazole, (S)-(+)-N-3-Benzylrivanol, quinidine, and ketoconazole were evaluated. The IC_{50} values generated were compared with those generated in-house using an individual substrate approach. The IC_{50} values obtained from these two approaches are summarized. The data from the 7-in-1 substrate cocktail approach were correlated well with the data from the single substrate approach with the R^2 value of 0.995.

Conclusions: We established a 7-in-1 cocktail approach with each of the preferred substrates for CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A for evaluating CYP inhibition potential of compounds of interest in early drug discovery phase.

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P129 - IS THE DILUTION METHOD FOR MEASURING IC_{50} SHIFT VALUES A MORE SENSITIVE APPROACH FOR EVALUATING TIME-DEPENDENT INHIBITION?

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Purpose: In 2022, with the promulgation of ICH M12, the evaluation methods for TDI in drug interactions became elusive. The original regional guidelines, such as the FDA, did not specify the IC_{50} shift method, but the M12 guidelines clearly pointed out that the dilution method should be used for the IC_{50} shift. This has sparked extensive discussions in the industry. Based on the internally validated IC_{50} shift system, we conducted a comparative study of the dilution and non-dilution methods.

Method: We used an internally validated system to ensure the same substrate concentration and reaction time during incubation. The only difference was in the pre-incubation period: the dilution method used higher microsomal concentration (1.0 mg/mL) and compound concentration, then the solution was added to the incubation solution with a 10-fold dilution factor, while the non-dilution method used lower microsomal concentration (0.1 mg/mL) and compound concentration, and directly added to the incubation solution. We performed comparative tests with 3-5 commercially available compounds for each CYP isoform recommended by the ICH M12 guideline.

Results and Discussion: All the IC_{50} shift values generated in both the dilution and non-dilution methods were compared. It was found that most of the compounds yielded the same results regardless of the dilution and non-dilution methods. However, three exceptions were observed: Phenelzine on CYP2C8 and Ticlopidine on CYP3A (using midazolam and testosterone as substrates, respectively). By comparing the reported TDI levels of the compounds in the literature, we found that the dilution method produced one false negative (Phenelzine on CYP2C8) and two false positives (Ticlopidine on CYP3A (using midazolam and testosterone as substrates, respectively)), while the non-dilution method could accurately assess the time-dependent properties of the compounds. To further confirm the properties of the compounds, we conducted Kinact/KI experiments on the two exceptional compounds. The inactivation of CYP2C8 by phenelzine was characterized by a KI value of 199 μ M and kinact value of 0.0474 min⁻¹. However, no inactivation values of Ticlopidine could be generated. It turned out that the properties of the two compounds were correctly classified using the non-dilution method.

Conclusions: In summary, the dilution method for measuring IC_{50} shift values is not a more sensitive approach for evaluating time-dependent inhibition; we recommend using the non-dilution method for its accuracy.

P130 - DEVELOPMENT OF AN *IN VITRO* PLATFORM FOR SCREENING UGT INHIBITORS USING HUMAN LIVER MICROSOMES**Lifang Jiang¹**, Yating Kan², Xi Liu², Hongying Ma², Yi Tao¹, Liang Shen¹, and Genfu Chen¹¹WuXi AppTec Lab Testing Division - Shanghai, Pudong, Shanghai, China, ²WuXi AppTec Lab Testing Division - Nanjing, Jiangsu, China

Purpose: UGT-mediated conjugation with glucuronic acid ('glucuronidation'), derived from the cofactor UDP-glucuronic acid (UDPGA), is a major clearance pathway in phase II metabolism. In particular, after cytochrome P450 (CYP) mediated biotransformation, glucuronidation is the most important metabolic pathway for small molecule drugs¹. With the anticipated release of the new ICH M12 guidelines, screening UGT inhibitors is becoming increasingly important in drug development. Therefore, there is an urgent need to implement accurate and cost-effective high-throughput *in vitro* methods.

Method: The UGT inhibition potential of the compound of interest is evaluated using a human liver microsome (HLM) with relatively selective substrates. The inhibition potential of the compound of interest on UGT1A1 (β -Estradiol or bilirubin as the substrate), UGT1A3 (Telmisartan as the substrate), UGT1A4 (Trifluoperazine as the substrate), UGT1A6 (Serotonin as the substrate), UGT1A9 (Propofol as the substrate), UGT2B7 (Morphine as the substrate), and UGT2B10 (Cotinine as the substrate) was evaluated in pooled human liver microsomes.

Results and Discussion: These newly established methods utilize different concentrations (0.01~0.1mg/mL) of human liver microsomal protein selected based on the results from the initial condition determination. Unlike cytochrome P450, UGT activity remains stable over extended incubation periods. In some instances, we have incorporated incubation periods of up to 40 minutes to enhance metabolite production. This approach improves detection sensitivity while maintaining initial-rate conditions. The substrate concentration selected for each assay was determined after characterizing the enzyme kinetics of each reaction. Finally, the conditions used to evaluate UGT inhibition potential were optimized. Taking UGT1A1 as an example, β -Estradiol or bilirubin were used as the substrates, respectively. Nilotinib was used as a positive inhibitor for UGT1A1, which yielded IC₅₀ values of 0.0396 μ M and 0.122 μ M, respectively. Daidzein, Ethinylestradiol, Ketoconazole, Regorafenib, and Ritonavir were also validated in the system as UGT1A1 inhibitors for inhibiting β -Estradiol glucuronidation in HLM. The inhibition profiles, as well as IC₅₀ values, were presented. The results of the in-house data were found to correlate well with those from the literature.

Conclusions: In conclusion, UGT inhibition assays using human liver microsomes as the test system have been developed. As the assays were designed to reduce costs compared with human recombinant UGTs, they should provide an excellent tool to evaluate new chemical entities' *in vitro* DDI potential in the early discovery stage.

P131 - DEVELOPMENT OF AN *IN VITRO* TEST SYSTEM FOR CYP INHIBITION STUDIES USING CRYOPRESERVED HUMAN HEPATOCYTES AND COMPARISON WITH HUMAN LIVER MICROSOMES**Noémi Szili**

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Inhibition, especially time-dependent inhibition (TDI) of major drug metabolizing enzymes, such as cytochrome P450s (CYPs) may result in clinically significant drug-drug interactions (DDIs). Therefore, accurate prediction of inhibition potential of drug candidates towards these enzymes is crucial for drug development. Human liver microsomes (HLMs) are the standard *in vitro* system for enzyme inhibition studies due to being cost-effective, and simple to use, but they are likely not accurate representations of the complex *in vivo* situation [1-3]. The use of primary human hepatocytes has been proposed by numerous groups to offer a better representation for determining the kinetics of time-dependent inhibition [4,5]. In case of certain biologics, CYP inhibition studies using microsomes resulted in false positive findings which were not occurring when incubations were repeated using hepatocytes; TDI data from HLMs overestimated the magnitude of DDI [6]. As ADME properties of oligonucleotide therapeutics are different from those of small molecule drugs, it is necessary to discuss which approaches for evaluating DDIs are directly applicable, particularly in terms of the choice of materials for *in vitro* experiments, selection of clinically relevant concentrations, and cutoff criteria in the decision tree [7, 8].

The aim of this work was to develop CYP inhibition assays using cryopreserved human hepatocytes, validate the system with known reversible and time-dependent inhibitors, and compare IC₅₀ and IC₅₀-shift results with data acquired using HLMs. Experiments were performed in 96 well plates in triplicate using pooled (n=50) mixed gender human cryopreserved hepatocytes for suspension culture. Cells were thawed, counted, and incubated in Williams E medium containing 2 mM L-glutamine and 50 mM HEPES (pH 7.4) in 96-well plates. All incubations were conducted at 37°C in a thermal plate shaker. Reactions were initiated by addition of the probe substrate and/or inhibitor and stopped after 10 minutes by mixing the samples with ice-cold acetonitrile. Samples were centrifuged, and supernatant was collected for analysis. Formation of enzyme-specific metabolites was quantified by LC/MS/MS. First, time courses and optimal cell number/well were established for the selected probe substrates (CYP1A2, phenacetin; CYP2B6, bupropion; CYP2C8, amodiaquine; CYP2C9, diclofenac; CYP2C19, mephenytoin; CYP2D6, dextromethorphan; CYP3A4, midazolam), followed by determination of kinetic parameters K_m and V_{max} for each probe substrate. Finally, one selective reversible and one

time-dependent inhibitor for each isoform were used to test the setup. Time-dependent inhibitors were preincubated with hepatocytes for 30 minutes before adding probe substrates to the reaction mixture.

P132 - NOVEL BIOENGINEERED RNA MOLECULES FOR THE STUDY OF GENE REGULATION IN DRUG AND NUTRIENT TRANSPORT AND METABOLISM

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Metabolic enzymes and transporters are crucial for the absorption, disposition, metabolism, and excretion (ADME) of drugs and nutrients. Alterations in ADME gene expression and function can significantly influence the pharmacokinetic and pharmacodynamic properties of drugs, as well as nutrient transport, metabolism, and homeostasis that may alter disease progression. MicroRNAs (miRNAs) constitute a superfamily of small noncoding RNAs that govern posttranscriptional gene regulation (PTGR). To study the ADME gene functions and the importance of PTGR, multiple kinds of RNA interference (RNAi) agents such as miRNA mimics, small interfering RNAs (siRNAs), and short hairpin RNAs, have been developed and widely used. However, those RNAi agents are mainly synthesized chemically or biochemically *in vitro*, many of which encompass various artificial modifications that may lead to different physicochemical and pharmacological properties. Therefore, we have developed a one-of-a-kind RNA molecular bioengineering technology to produce RNAs through bacterial fermentation and FPLC purification, using tRNA-fused pre-miRNA as carriers. These novel bioengineered RNAs (BioRNAs) produced and folded in living cells may better recapitulate the structures and functions of natural RNAs. We have produced and applied a number of unique BioRNAs to ADME studies, including miR-27b-3p targeting CYP3A4, miR-328-3p targeting ABCG2, let-7c-5p targeting ABCC5, and miR-22-3p targeting folate and glucose metabolism and transport. Among them, BioRNA/miR-27b-3p significantly reduced the mRNA and protein levels of CYP3A4 in cells, leading to a lower midazolam 1'-hydroxylase activity. BioRNA/miR-328-3p inhibited the expression of ABCG2 and elevated intracellular mitoxantrone accumulation, thereby enhancing drug efficacy. Likewise, BioRNA/let-7c-5p increased the cellular uptake of 5-FU via attenuating the expression of the efflux transporter ABCC5 and thus co-administered BioRNA/let-7c and 5-FU synergistically inhibited the proliferation of human hepatocellular carcinoma cells. In addition, BioRNA/miR-22-3p disrupted folate metabolism and glucose uptake in human non-small cell lung cancer (NSCLC) cells through modulating the expression of respective enzymes and transporters, and BioRNA/miR-22-3p therapy effectively reduced tumor growth in NSCLC patient-derived xenograft mouse models. Our results demonstrate the utility of unparalleled BioRNA molecules produced *in vivo*, which shall improve our understanding of PTGR in ADME and the development of novel therapeutics.

P133 - ASSESSMENT OF *IN VITRO* METHODS FOR UGT1A1 INHIBITION IN RECOMBINANT ISOZYME

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Human UGT1A1 is one of the major enzymes responsible for drug metabolism via phase 2 biotransformation. It is recommended by regulatory agencies to further evaluate the inhibitory potential of the investigational drug on UGT1A1 if direct glucuronidation product(s) are observed as one of the major elimination routes. Despite that SN-38 has been the recommended substrate of UGT1A1 for clinical DDI studies and it has been characterized *in vitro* previously¹, its application for *in vitro* drug-drug interaction evaluation as a substrate for UGT1A1 inhibition remains inconclusive. Therefore, the objectives of this study are 1) To establish initial rate conditions and enzyme kinetics for clinically recommended substrate SN-38 and broad substrate 4-methylumbelliferone (4-MU) in recombinant human UGT1A1 (rhUGT1A1); 2) To evaluate the inhibitory effect of a general inhibitor zafirlukast on rhUGT1A1-mediated SN-38 and 4-MU glucuronidation. To assess the linear incubation condition, rhUGT1A1 isozyme at multiple concentrations were incubated with SN-38 for up to 20 minutes in the presence of cofactor uridine diphosphate glucuronic acid (UDPGA). The formation of SN-38 β -D-glucuronide was quantitatively monitored by LC-MS/MS. To characterize the enzyme kinetics, SN-38 at various concentrations were incubated with rhUGT1A1 under the established linear condition. The results suggested the metabolism of SN-38 under experimental conditions followed allosteric sigmoidal model. Using inhibitor zafirlukast for rhUGT1A1-mediated SN-38 glucuronide and 4-methylumbelliferone- β -D-glucuronide formation, comparable IC₅₀ values were obtained. To conclude, this study suggested the assessment of *in vitro* methods to evaluate inhibitory potential of UGT1A1-mediated metabolism using clinically recommended substrate in recombinant isozyme.

P134 - IN VITRO INVESTIGATION ON INHIBITION POTENTIAL OF DEUCRAVACITINIB TOWARD HUMAN CARBOXYLESTERASE (CES) 2**Ming Yao** and Wenying Li*Bristol Myers Squibb, Princeton, NJ, United States*

Deucravacitinib (DEUC, Sotyktu), a tyrosine kinase (Tyk) 2 inhibitor, has been approved for the treatment of moderate to severe plaque psoriasis in multiple markets including US. Previous data show that DEUC is a substrate of human carboxylesterase (CES), thus inhibition potential of DEUC towards recombinant CES2 (rCES2) was investigated in this study. Mycophenolate mofetil (MMF, a CES2 substrate) is an immunosuppressive agent that is widely used in autoimmune diseases and anticipated to be a concomitant medication of DEUC in the clinics. Thus, MMF was used as probe substrate of CES2 in the assay. Inhibition studies were performed by incubating MMF (25 μ M) with human rCES2 supersomes™ (5 μ g/mL) in the presence of varying concentrations of DEUC (0.01, 0.1, and 1 μ M). Peak area responses of mycophenolate acid (MPA, the active metabolite of MMF formed via CES2-mediated hydrolysis), and an internal standard (stable labeled d3- mycophenolate acid) were determined using a triple quadrupole LC-MS/MS. A known CES inhibitor, phenylmethanesulfonyl fluoride (PMSF, at 0.01, 0.1, and 1 μ M), was also evaluated to demonstrate the functional activity of the test system. At 0.01, 0.1, and 1 μ M, DEUC was not a meaningful inhibitor of rCES2. The positive control inhibitor, PMSF, caused appreciable inhibition of CES2 and produced inhibitory effects that were similar to literature values, indicating acceptable assay performance for the evaluation of inhibition potential of DEUC towards CES2-mediated MMF hydrolysis. These data suggest that coadministration of DEUC at a clinical dose is not likely to affect PK of CES2 substrates like MMF.

P135 - IMPACT OF N-ACETYLTRANSFERASE (NAT) GENETIC POLYMORPHISMS ON NAT CATALYTIC ACTIVITY IN HUMAN COLON MUCOSA**Thomas Massey**¹, Will King¹, and Vikki Ho²¹Queen's University, Kingston, ON, Canada, ²University of Montreal Hospital Research Centre (CRCHUM), Montréal, QC, Canada

Meat consumption is associated with an elevated risk of colorectal cancer, and exposure to heterocyclic aromatic amines (HAAs), carcinogens that are produced when meat is cooked at high temperatures, are hypothesized to be important contributors to this association. HAAs undergo bioactivation by N-acetyltransferases (NAT1 and NAT2) to mutagenic DNA-binding metabolites, and NAT polymorphisms have been associated with biomarkers of cancer as well as colorectal cancer risk. This study assessed the impact of polymorphisms in NAT genes on NAT activities in human colon mucosa, the site of CRC tumorigenesis. Following informed consent, colon tissues from colon resection patients were obtained, and mucosa from healthy appearing tissues (~4 cm²) was collected and stored at -80°C until analysis. NAT1 genotype category was defined by genotyping NAT1*10 (rs1057126 and rs15561); participants with 1 or more variant allele(s) constituted the rapid / intermediate genotype category. NAT2 genotype category was defined by the 2-SNP genotype (rs1041983 and rs1801280); participants with 0 variant alleles constituted the rapid / intermediate category. NAT activities were assessed by acetylation of the selective substrates para-aminobenzoic acid for NAT1 and sulfamethazine for NAT2. Because some NAT alleles have been reported to affect NAT protein stability, we also examined NAT1 and NAT2 protein levels by immunoblotting. For NAT1, genotype did not alter catalytic activity in colon mucosa (2.39 $\pm$ 1.21 nmol/min/mg for rapid / intermediate genotype category vs 2.31 $\pm$ 0.93 nmol/min/mg for slow genotype category; mean $\pm$ SD, n = 14 for each category, p = 0.836, t-test). However, for NAT2, tissues from individuals with genotypes reported in the literature to confer relatively rapid or intermediate activity in recombinant systems, had substantially higher activities (35.9 $\pm$ 16.2 pmol/min/mg) versus those with genotypes reported to be associated with slow acetylation (12.9 $\pm$ 4.7 pmol/min/mg); n = 14 for each category, p < 0.0001). Overall, NAT1 and NAT2 protein levels were consistent with genotype category (i.e. higher in rapid / intermediate versus slow acetylators tissues). These results suggest that, for carcinogens such as HAAs that are metabolized by NAT2, genotype may affect in situ bioactivation in the colon mucosa, and hence colorectal cancer risk.

P136 - EVALUATION OF ALDEHYDE-ODORANT METABOLISMS AND AOX AND ALDH EXPRESSION PROFILES IN HUMAN NASAL MUCOSA AND MUCUS**Naoki Takaoka**¹, Seigo Sanoh¹, Mami Kurosaki¹, Mineko Terao¹, Enrico Garattini², Hideki Sakatani¹, Daichi Murakami¹, Masamitsu Kono¹, Muneki Hotomi¹, and Shigeru Ohta¹¹Wakayama Medical University, Wakayama-shi, Wakayama-ken, Japan, ²Istituto di Ricerche Farmacologiche Mario Negri, Milan, Italy

Introduction: The olfactory epithelium (OE) is a mucosal epithelium that functions as an olfactory sensor. Several reports have implicated that drug-metabolizing enzymes exist in the OE and its secretions, nasal mucus. However, the physiological role of the drug-metabolizing enzymes in the nose has not been fully understood. We previously reported that aldehyde oxidase (AOX) is involved in aldehyde odorant metabolism in mouse OE and nasal mucus¹. However, it

remains unknown about the aldehyde odorant metabolism in human nasal mucosa and mucus, as well as the involvement of aldehyde dehydrogenase (ALDH). In this study, we investigated the AOX and ALDH expression profiles and the metabolism of aldehyde odorants in human nasal mucosa and mucus.

Method: Human nasal mucosa and mucus samples were collected from patients undergoing sinus surgery in accordance with a study protocol approved by the Ethics Committee of Wakayama Medical University. Enzyme expression in the nasal mucosa and mucus was evaluated by proteomic analysis. Metabolite formation after reactions of aldehyde odorants with nasal mucosa 9,000×g supernatant (S9) and nasal mucus were analyzed by LC-MS/MS.

Results and Discussion: The proteomic analysis revealed the expression of a variety of AOX and ALDH isoforms in human nasal mucosa. The most abundant ALDH isoform was found to be ALDH1A3. The AOX and ALDH isoform expression profiles differed significantly from those of C57BL/6J mouse OE. Metabolic studies using human nasal mucosa S9 showed the formation of oxidized metabolites for aliphatic and aromatic aldehydes. Involvement of AOX and ALDH in the aldehyde oxidations was confirmed using by AOX inhibitors and ALDH coenzymes. AOX-mediated aldehyde oxidation activity in human nasal mucosa was lower than in mouse OE, while ALDH-mediated aldehyde oxidation activity was comparable. The aldehyde metabolic activity profile reflects the results of proteomic analysis. In addition, there were interindividual differences in their nasal aldehyde oxidative activities. Furthermore, the presence of various ALDH isoforms and oxidative activity of aldehyde odorants were also observed in human nasal mucus, suggesting the possibility of ALDH isoform secretion from nasal mucosa into the nasal mucus. There were marked interindividual differences in the aldehyde oxidative activities in human nasal mucosa S9 and mucus.

Conclusion: This study has revealed the expression profiles of AOX and ALDH and the metabolic activity of aldehyde odorants in the human nasal mucosa and mucus. In the future, the relationship between odorant metabolism via AOX and ALDH in the human nose and odor perception should be investigated to clarify the physiological significance of extrahepatic metabolism by non-P450 phase I enzymes in the nose.

Reference:

1. Takaoka N et al., (2022). Involvement of aldehyde oxidase in the metabolism of aromatic and aliphatic aldehyde-odorants in the mouse olfactory epithelium. *Arch. Biochem. Biophys.* 715, 109099.

P137 - ASSESSMENT OF INTEGRATED STRATEGY FOR PRODRUG METABOLIC TRANSFORMATION IN PLASMA: STATISTICAL AND ANALYTICAL

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In drug discovery, plasma of different species was applied to evaluate the metabolic stability and activation of prodrugs. Our investigation provided comprehensive data mining of prodrug conversion efficiency and species difference in plasma stability assay for variety of prodrug designs, as well as recommendation of data driven high throughput assay optimization portfolio to improve assay efficiency and guide medicinal chemistry efforts. Further, we proposed integrated strategy with semi-quantitative assessment of parent drug formation kinetics in addition to prodrug stability to fully capture the prodrug biotransformation. In current study, the high frequency distribution ranges of half-lives ≤ 1 hr and ≥ 24 hr agreed with the chemical design rationale of matrix and reaction specific metabolic biotransformation. The statistical profiling of half-lives indicated the species differences for varieties of prodrug designs based on large database, suggesting prodrug metabolic biotransformation in plasma is highly species and prodrug design dependent. In addition, our investigation has shown that the practice of using prodrug MS response to evaluate active drug formation could be misleading. The MS relative response factor (RRF) values obtained in this study ranged from 0.006 to 100, with 62 % of these out of the range between 0.5 (2-fold under-prediction) and 2.0 (2-fold over-prediction). The proposed integrated strategy with RRF corrected semi-quantitation profile of parent drug successfully improved the accuracy of active drug estimation in plasma.

P138 - UNRAVELING THE ROLE OF HNF1A-AS1 IN HEPATOCYTE MATURATION AND SUBNUCLEAR SPACES ORGANIZATION

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The human liver is the major organ responsible for drug metabolism, where hepatocytes play a critical role in processing xenobiotic substances through a variety of metabolic enzymes, including phase I and phase II enzymes. Approximately 50-60% of commonly used drugs are primarily metabolized by cytochrome P450 (CYP) enzymes, a key group in phase I metabolism. Emerging evidence has highlighted the significant role of long non-coding RNAs (lncRNAs) in the regulation of drug metabolism via modulating CYPs. lncRNA HNF1A-AS1 has been found to affect the CYP3A4 enzyme by interacting with transcription factor pregnane X receptor (PXR) and altering histone modification status at the promoter of the CYP3A4 gene. Despite these findings, the dynamic expression of HNF1A-AS1 during hepatocyte maturation and its cellular localization have not been fully understood. To address this, we applied RNAScope technique to visualize

HNF1A-AS1 expression in HepaRG cells, which underwent a transition from bipotential progenitor cells (two weeks for growth) to a mix of hepatocyte-like and biliary cell-like cells (two weeks for differentiation) over a four-week period. Our observations revealed that HNF1A-AS1 expressed in only a small portion of cells during the growth period. However, during the differentiation period, expression levels of HNF1A-AS1 significantly increase. Interestingly, HNF1A-AS1 specifically localized to the nuclei of hepatocyte-like cells, not in biliary-like cells, indicating a crucial role in hepatocyte maturation. To precisely identify its subnuclear position, RNAScope was conducted in HepG2 cells to visualize the co-localization of HNF1A-AS1 and subnuclear organelles. Our results demonstrated that HNF1A-AS1 didn't exist in any known nuclear substructures, such as nucleoli, speckles, paraspeckles, Cajal bodies, and promyelocytic leukemia (PML) bodies. Notably, it displayed an irregular, droplet-like distribution within the nucleus, suggesting its potential involvement in the formation of novel liquid-liquid phase separation (LLPS) spaces. In conclusion, our study revealed the novel function of HNF1A-AS1 in hepatocyte maturation, particularly highlighting its potential involvement in organizing novel intranuclear compartments through LLPS, which may serve as critical functional units for the metabolic functions of hepatocytes.

P139 - CYP7A1 GENE INDUCTION VIA SHP-DEPENDENT OR INDEPENDENT MECHANISMS CAN INCREASE THE RISK OF DRUG-INDUCED LIVER INJURY INDEPENDENT OR IN SYNERGY WITH BSEP INHIBITION

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Objective: Drug-induced liver injury (DILI) is a common adverse effect that often results in regulatory action or causes drug trial failures during drug development. The causative elements behind DILI are complex and can involve various factors such as drug physicochemical properties, pharmacokinetics, mitochondrial susceptibilities, immune responses, reactive metabolites, and disruption of bile salt homeostasis. This study aims to explore potential mechanisms that may contribute to DILI as independent or synergistic risk factors in addition to BSEP inhibition.

Methods: Twenty-eight compounds, including those with discontinued development or use, boxed warnings, and clean labels for DILI, were selected for the regulations of genes associated with BA metabolism and transport. PXR and AhR ligands, rifampicin and omeprazole, and four mitogen-activated protein kinase kinase (MEK1/2) inhibitors were also included. Hepatocytes were cultured in a sandwich format and treated with testing compounds at concentrations ranging from 0.1 to 30 μ M. The gene and protein expressions were measured by the targeted proteomics method and RT-qPCR, respectively. Briefly, the total protein was extracted from the hepatocytes. After denaturation and reduction, the protein was digested using trypsin. The levels of CYP7A1 protein were quantified using a Sciex 7500 triple-quadrupole mass spectrometer coupled to a Shimadzu LC (SLC-30A) system with optimized ionization conditions. Total RNA was isolated from cells using a Qiagen RNeasy kit. The mRNA levels were measured by RT-qPCR. β -actin was used to normalize target gene expression. Bile acid production in the supernatant of SCHH was measured at each collected time point. Individual BA analysis was conducted using a Sciex 7500 mass spectrometer coupling with a SHIMAZU LC system (LC-40). The BSEP inhibition assay was conducted in suspended human hepatocytes using BSEPcyte® methods.

Results and Conclusion: BSEP inhibitors with more severe DILI, notably pazopanib and CP-724714, significantly upregulated the expression of 7 α -hydroxylase (CYP7A1), independent of small heterodimer partner (SHP) expression. CYP7A1 expression was marginally induced by omeprazole. In contrast, its expression was suppressed by mometasone (10-fold), vinblastine (18-fold), hexachlorophene (2-fold), bosentan (2.1-fold), and rifampin (2-fold). All four MEK1/2 inhibitors that show clinical DILI were not potent BSEP inhibitors but significantly induced CYP7A1 expression, accompanied by a significant SHP gene suppression. SULT2A1 and BSEP were marginally upregulated, but no other genes were altered by the drugs tested. Protein levels of CYP7A1 were increased with the treatment of CYP7A1 inducers and decreased with obeticholic acid, an FXR ligand. CYP7A1 inducers significantly increased BA production in hepatocytes, indicating the overall regulatory effects of BA metabolism. This study demonstrates that CYP7A1 induction via various mechanisms can pose a risk for DILI, independently or in synergy with BSEP inhibition, and it should be evaluated early in drug discovery.

P140 - CYP INDUCTION ASSAYS USING 10-DONOR POOLED HUMAN HEPATOCYTES

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Plateable cryopreserved human hepatocytes from individual donors that have been pre-characterized for responsiveness to model inducers represent the “gold standard” experimental system for CYP induction studies, especially in the pharmaceutical industry for the development of drug-drug interaction (DDI) data packages for marketing approval by regulatory agencies. We report here our successful preparation of cryopreserved human hepatocytes pooled from multiple donors (Gentest's 10 Donor Pooled Plateable Human Hepatocytes (PPH)) for CYP induction evaluation. PPH were prepared by thawing, pooling, and re-cryopreservation of plateable cryopreserved human hepatocytes from 10 individual donors using our internationally patented protocol. Results from multiple independent preparations of the 10 donor PPH consistently yielded hepatocytes with optimal post-thaw viability (>80%) as well as their ability to attach onto collagen-coated 96-well plates to form near confluent cultures with polygonal morphology, properties akin to that of cryopreserved

human hepatocytes from individual donors. Upon our success in retention of high viability and plateability of the PPH, we thereby proceeded to apply the PPH in CYP induction studies by evaluating their responsiveness to the prototypical CYP inducers omeprazole, phenobarbital and rifampicin for CYP1A2, CYP2B6, and CYP3A4 induction, respectively. Relative mRNA expression and CYP enzyme activities were determined by LCMS and q-PCR after 48 hours induction. From two independent tests, fold changes for mRNA expression were CYP1A2 (29-fold and 35-fold), CYP2B6 (7.2-fold and 4.7-fold), and CYP3A4 (17-fold and 13-fold) respectively. In agreement, CYP enzyme activity fold changes were at CYP1A2 (10-fold and 21-fold), CYP2B6 (3.0-fold and 2.9-fold), and CYP3A4 (3.0-fold and 3.4-fold). Our results suggest that pooled donor plateable cryopreserved human hepatocytes represent a novel experimental system for CYP induction studies with utilities in both screening of new chemical entities as well as applications in the development of DDI data package for submission to regulatory agencies.

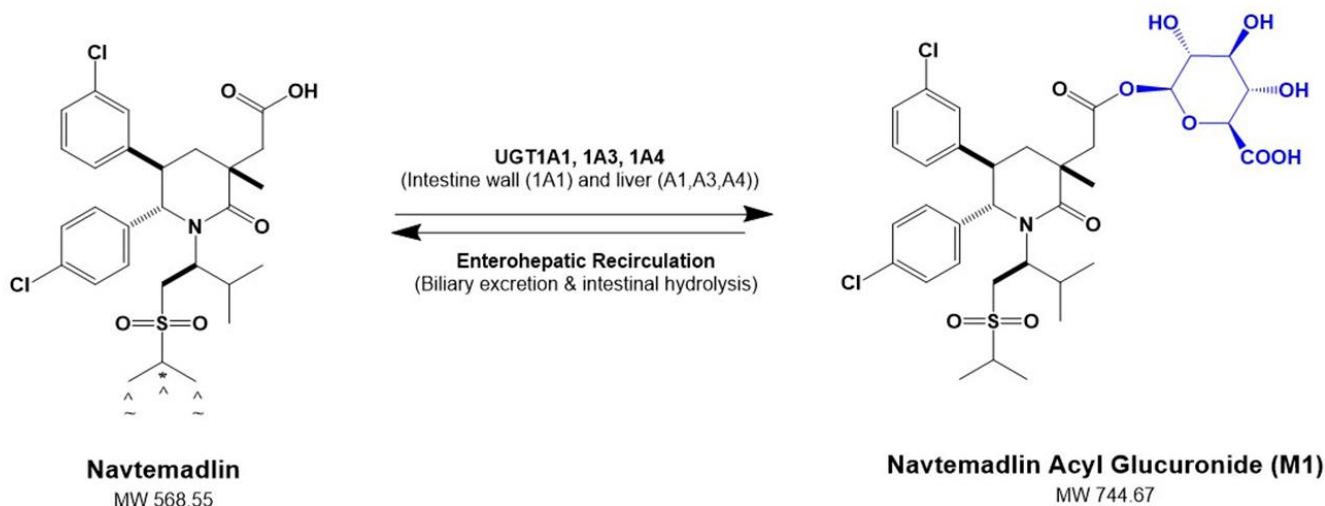
P141 - ABSOLUTE BIOAVAILABILITY, BIOTRANSFORMATION, AND EXCRETION OF THE MOUSE DOUBLE MINUTE 2 INHIBITOR NAVTEMADLIN IN HEALTHY HUMAN SUBJECTS

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Navtemadlin is a potent, selective, Mouse Double Minute 2 (MDM2) inhibitor that restores p53 activity and induces apoptosis in TP53WT tumors. Navtemadlin is under development for treatment of patients with myelofibrosis and endometrial cancer.

Methods: Absolute bioavailability was determined using a 100 µg stable-label [2H613C3]navtemadlin intravenous (IV) microdose, given to 8 healthy male or female subjects at 2 hours (h) after an unlabeled 60 mg navtemadlin oral tablet dose. Navtemadlin pharmacokinetics, biotransformation, and excretion were determined using a 100 µCi/60 mg oral suspension of [14C]navtemadlin, administered to 6 healthy male subjects. Blood, plasma, urine, and feces were collected for high performance liquid chromatography/tandem mass spectrometry and radioanalysis. Plasma concentrations of unlabeled navtemadlin and major circulating acyl glucuronide metabolite (M1) were determined from 0.5-24 h postdose by a validated method with lower limit of quantitation (LLOQ) of 1.00 ng/mL. IV [2H613C3]navtemadlin was determined against laropiprant internal standard using a validated method with a 20 pg/mL LLOQ. Absolute Bioavailability: Navtemadlin total plasma IV clearance was 16.2 L/h, which is <30% of hepatic blood flow, indicating low hepatic extraction. The mean IV navtemadlin $t_{1/2}$ was 30 h, and mean volumes of distribution, V_z , and V_{ss} , were 306 L, and 523 L, respectively. For the 60 mg tablet dose, navtemadlin T_{max} and $t_{1/2}$ were 2.75 h and 27 h, respectively. Geometric mean absolute oral bioavailability, based on AUC_{0-last}, was 67.2% ($\pm$ 23.3%) with a range of 41.9-88.6%. [14C]ADME: Mean $\pm$ SD total recovery of radioactivity was 87.7 $\pm$ 8.25% (range 77.8%-102%). Recovered radioactivity was predominantly in feces (87.1 $\pm$ 8.72%), with a small fraction in urine (0.15 $\pm$ 0.08%). With the 60 mg suspension dose, median plasma T_{max} was 1.25 h, and mean $t_{1/2}$ was 25.9 h. The molar ratio of M1 to parent AUC_{0-last} was 21%. Mean radioactivity blood to plasma ratios were 0.4900 to 0.5913 throughout the postdose period, confirming plasma is a suitable matrix for navtemadlin bioanalysis. Navtemadlin accounted for 56 and 59% of circulating radioactivity based on C_{max} and AUC, respectively, while M1 accounted for 13 and 14%. Typical of an acyl glucuronide, M1 underwent acyl migration and circulated along with three acyl-migrated regioisomer peaks that respectively accounted for 3.73%, 4.34%, and 2.42% of radioactivity in Hamilton-pooled plasma. No other metabolite accounted for more than 10% of drug related material in plasma. Radioactivity in feces was primarily navtemadlin (82.5%), reflecting extensive hydrolysis of biliary M1 in the intestine. Safety: Navtemadlin was safe and well tolerated. There were no serious adverse events reported and all non-serious adverse events were mild to moderate in severity (Grade 1 or 2). Conclusions: Navtemadlin, an orally dosed drug with a low hepatic extraction ratio, has relatively low first pass metabolism, relatively high absolute bioavailability, and a half-life that enables once daily administration. Only one major plasma metabolite was observed, an acyl glucuronide that circulated along with isobaric acyl-migrated forms. Metabolite M1 undergoes intestinal hydrolysis to regenerate navtemadlin, which enables enterohepatic recirculation and excretion of navtemadlin in feces.

Figure: Structures of navtemadlin and its major acyl glucuronide metabolite M1

^Δ ¹³C label, ~ ²H₃ label (absolute bioavailability)
* ¹⁴C label (ADME)

P142 - ABSOLUTE BIOAVAILABILITY, BIOTRANSFORMATION, AND EXCRETION OF THE BRUTON TYROSINE KINASE INHIBITOR TL-895 IN HEALTHY HUMAN SUBJECTS

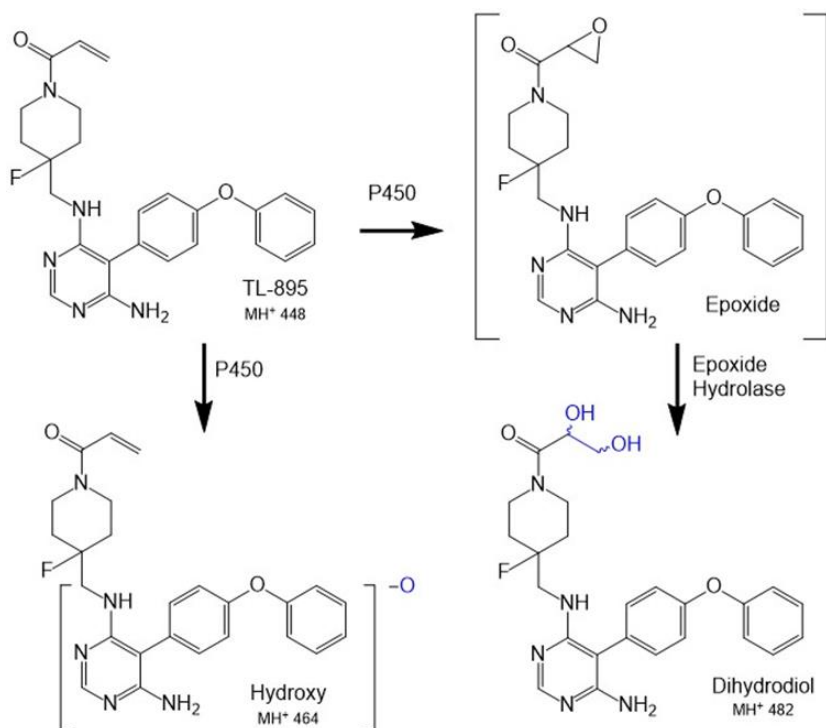
Terry Podoll¹, Martine Allard², Cecile Krejsa², Nick Khorlin², Jonathan Fong², Sue Masten², and J. Greg Slatter²

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TL-895 is a highly potent, selective, covalent Bruton tyrosine kinase (BTK) inhibitor currently under development for treatment of patients with ocular inflammatory disorders and hematologic diseases. Methods: TL-895 absolute bioavailability was determined using a 1 μCi/6 μg [¹⁴C]TL 895 intravenous (IV) microtracer dose, given to 8 fed healthy male or female subjects 2 hours (h) after an unlabeled 300 mg TL-895 oral tablet administration. TL-895 pharmacokinetics, biotransformation and excretion were determined via total radioactivity (TRA) measurement following a 100 μCi/300 mg oral suspension dose of [¹⁴C]TL-895 given to 8 fed healthy male subjects. Blood, plasma, urine and feces were collected for high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and radiometric analysis. Bioanalytical Methods: Plasma concentrations of unlabeled TL-895 were determined using a validated HPLC-MS/MS method, with a lower limit of quantitation (LLOQ) of 500 pg/mL. Plasma concentrations of [¹⁴C]TL-895 were determined using a validated HPLC + Accelerator Mass Spectrometry (AMS) method, with a LLOQ of 0.04 dpm/mL. Metabolite profiling used HPLC + AMS, with identities verified by HPLC-high resolution MS and comparison to synthetic standards. Radiometric kinase assays were performed using kinase-specific substrate peptides and full-length recombinant enzymes. Absolute Bioavailability: TL 895 IV total plasma clearance was 62.5 L/h, near hepatic blood flow, indicating high hepatic extraction. Mean TL-895 IV t_{1/2} was 2.6 h, and mean volumes of distribution, V_z, and V_{ss}, were 245 L, and 76 L, respectively. For the 300 mg tablet dose, TL 895 T_{max} and t_{1/2} were 2.5 and 2.8 h, respectively. Mean absolute oral bioavailability, based on AUC_{0-∞}, was 25% (90%CI, 20-31%) with intrasubject variability of 21%. [¹⁴C]ADME: Mean t_{1/2} of plasma TL-895 was 4.3 h, compared to ~21 h for plasma TRA. The longer TRA t_{1/2} indicated circulating metabolites. TL-895-related radioactivity persisted at low levels in the blood cellular fraction, consistent with binding to BTK and BMX, another covalent target, in blood cells. Mean total recovery of radioactivity was 91.6 ± 3.5% (range 85.7% - 95.7%), with 14.1 ± 2.4% in urine and 77.6 ± 4.2% in feces. TL-895 was the major component in Hamilton-pooled plasma at 20.9% of TRA, while a hydroxy metabolite accounted for 18.3%, and a dihydrodiol metabolite accounted for 8.28% in the AUC_{0-12h} pool (Figure). Other metabolites individually accounted for <7% of circulating radioactivity. Urine contained an unidentified peak (2.8% of dose), plus hydroxy (2.75%) and dihydrodiol (0.30%). Radioactivity in feces was mainly hydroxy (13.5% of dose), along with dihydrodiol (4.04%) and parent TL-895 (0.78%), inferring near-complete absorption and metabolism. All other radioactive peaks individually accounted for < 7% of TRA in feces. The two metabolites were inactive against BTK and BMX in kinase assays.

Safety: TL-895 was generally safe and well tolerated. All adverse events reported were mild in severity (Grade 1) and non-serious. Conclusions: TL-895 has rapid, near-complete absorption, extensive metabolism, and primarily biliary-fecal excretion. The major TL-895 metabolites were inactive against BTK and BMX. This study characterized TL-895 ADME that results in relatively brief exposure, which sustains covalent BTK target inhibition over the clinical dose interval.

Figure: Structures of TL-895 and its major metabolites



P143 - IDENTIFICATION OF A NOVEL OATP2B1 SUBSTRATE BY A DOCKING STUDY USING ALPHAFOLD2 STRUCTURE

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Human organic anion transporting polypeptide (OATP) 2B1 has multiple substrate recognition sites and transports a variety of compounds. It is challenging to predict OATP2B1 substrates by an *in silico* approach since the three-dimensional structure of OATP2B1 has not been solved, but the predicted structure was recently shown by an AI-based approach (AlphaFold2, AF2). The purpose of this study was to identify novel substrates by docking studies using the AF2 structure. The effective amino acid residues involved in OATP2B1 substrate recognition were estimated by *in silico* docking with several known OATP2B1 substrates and non-substrates using a docking software, AutoDock Vina. The substrate recognition model was then developed based on the docking energies. The docking energies for 2999 FDA-approved drugs which were available as a drug library were tested and the drugs were categorized into candidates and non-candidates for OATP2B1 substrates. Uptake studies by tetracycline-inducible OATP2B1/TREx-293 cells were conducted and the amount of drug taken up into the cells was measured by LC-MS/MS. As a result, the predicted amino acid residues of OATP2B1 responsible for the recognition of estrone-3-sulfate, fexofenadine, pravastatin, and prostaglandin E2 were in part consistent with the residues reported in previous uptake studies. The precision rate of the developed substrate recognition model was 74%, defined as the percentage of true positive predictions to the sum of true positive and false positive predictions. Screening based on the docking energy of the developed model using 2999 drugs resulted in 942 drugs being categorized as candidates. As a consequence of uptake studies of 50 compounds each were randomly selected from the candidates and non-candidates, OATP2B1-expressing cells showed a significant uptake activity for acrivastine among the randomly selected 50 candidates, while OATP2B1 did not show significant uptake for any of the randomly selected 50 non-candidates. In conclusion, the substrate recognition model for OATP2B1 was established using known substrates and the AI predicted structure. Through *in silico* screening using the OATP2B1 substrate recognition model, acrivastine, which belongs to BCS class 3, was identified as a novel substrate for OATP2B1.

P144 - BUILDING ROBUST HUMAN LIVER MICROSOMAL STABILITY PREDICTIONS: LEVERAGING INTER-SPECIES CORRELATION WITH RAT DATA

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Hepatic metabolic stability plays a crucial role in drug discovery, impacting both oral bioavailability and compound elimination. The primary enzymatic system responsible for xenobiotic metabolism is the cytochrome P450 (CYP450)

isoenzyme family, accounting for over 75% of known xenobiotic metabolism in humans. At the National Center for Advancing Translational Sciences (NCATS), we utilize a single time-point rat liver microsomal (RLM) stability assay as a Tier I screening assessment, generating RLM stability data for every compound synthesized. Additionally, a high-throughput multi-time point human liver microsomal (HLM) stability assay serves as a Tier II assessment. Over recent years, we've screened >30,000 compounds from >300 projects for RLM stability and ~7,000 compounds from >100 projects for HLM stability. Our study aims to: (1) evaluate the correlation between Tier I RLM data and Tier II HLM data; (2) develop robust quantitative structure-activity relationship models for predicting HLM stability. Since our Tier I RLM and Tier II HLM assays have different extrapolated maximum half-lives; 30 min and 120 min respectively, Tier II HLM half-life was capped at 30 min for correlation analysis. Despite variations in assay and species, our analysis reveals that 81% of the compounds exhibited less than a two-fold difference, and ~ 90% of the compounds showed less than a three-fold difference. This vindicates our rationale for selecting RLM as the matrix for Tier I screening. Utilizing both traditional and advanced machine learning techniques, we identified XGBoost with RDKit descriptors as the best model for predicting HLM stability, achieving balanced accuracies >80%. Notably, incorporating RLM predictions as an additional descriptor for HLM stability prediction resulted in improved model performance. We are currently investigating the reasons behind this enhancement, likely attributable to the significantly larger RLM dataset contributing to increased model sensitivity. The best model along with a subset of our dataset will be made publicly available on the NCATS@ADME website (<https://opendata.ncats.nih.gov/adme/>) to benefit the greater drug discovery community.

P145 - INFLUENCE OF BUFFER COMPOSITION ON PARAMETERIZATION OF LIVER AND KIDNEY SCALING FACTORS IN RATS AND MICE

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Pharmacokinetic modeling can enhance our understanding of efficacy, safety, and risk throughout preclinical and clinical drug development. The extrapolation of *in vitro* metabolic data to *in vivo* clearance informs the underlying predictions of the mathematical models, and requires scaling factors. For microsomal and cytosolic subcellular fractions, scalars that define the amount of protein within the organ fraction per gram of tissue are used. However, methods to generate scalars have arisen ad hoc and understanding the ways in which preparation methodologies impact scalar parameterization is limited. Here, we investigated the impact of buffers, sex, and strain on parameterization of liver and kidney scaling factors for mice (B6C3F1/J, CD-1, C57BL/6, and Swiss Webster) and rats (Sprague Dawley and Wistar), the two most common preclinical species. Organs (previously collected after sacrifice and snap frozen at -80°C) were thawed, dissected, and separated into two different buffers: 250mM sucrose, 5mM HEPES, or 0.1M tris-HCl; then mechanically homogenized. Following centrifugation (10,000 xg, then 100,000 xg), protein content of fractions was quantified using a bicinchoninic acid assay. Additional assays for microsomal intactness using mannose-6-phosphatase activity (quality) and western blotting (purity) were conducted to evaluate fractions. Scaling factors were parameterized using protein content, fraction volume, and wet tissue mass. Liver microsomal intactness (quality) showed no strain or sex differences nor differences due to buffer composition in either mice or rats, except for Wistar rat microsomes where sucrose-HEPES had significantly greater intactness than tris-HCl ($p < 0.05$). Fraction purity evaluated by western blotting showed presence of the microsomal marker cytochrome P450 reductase in microsomes that was absent from the cytosolic fractions, indicating successful separation of fractions without contamination. There were no strain or sex differences in liver or kidney microsomal and cytosolic scaling factors in mice or rats. Our major finding was that that liver cytosolic scaling factors parameterized in tris-HCl buffer were significantly greater on a mg/g basis than the same organ processed in sucrose-HEPES buffer ($p < 0.05$) for all mouse strains except B6C3F1/J. In conclusion, rat and mouse liver and kidney scaling factors are not affected by sex or strain giving confidence in published parameters that historically used male animals and generalized across multiple strains. However, methodologies used to generate scaling factors are likely critical in influencing scaling factor numbers, that could propagate error in subsequent mathematical transformations. Further understanding of methodological approaches such as other buffer compositions, and fresh vs. frozen tissue, can support inter-laboratory standardization of conditions facilitating comparisons across studies and improving accuracy, safety and predictivity of scaling approaches.

P146 - CHALLENGES AND OPPORTUNITIES FOR IMPROVED PREDICTION OF CNS PENETRATION ACROSS SPECIES USING MDR1-OVEREXPRESSING CELLS

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Determining blood-brain barrier penetration of CNS targeting drugs early in drug discovery is crucial but challenging. Recent efforts to implement the 3R initiative to reduce animal experiments urge for more predictive *in vitro* and *in silico*

models. Still, the availability of *in vivo* brain K_{puu} data, used for determining the prediction performance of *in vitro* models, are restricted to preclinical species: predominantly rat, and with a lesser extent mouse and cynomolgus monkey. To help *in vitro-in vivo* extrapolation, preclinical *in vitro* models are required to reveal species differences and provide reliable data. Systems overexpressing the two most important efflux transporters, MDR1 and BCRP, are widely used to predict CNS penetration. We created an *in vitro* assay system, where the parental cell line is endogenous Abcb1 knock-out-MDCKII and the overexpression of the Mdr1 transporter of human, mouse, rat and cynomolgus monkey origin is controlled and comparable. 83 drugs were screened to determine efflux ratios (ER) in all cell lines. ERs were mostly comparable across cell lines, with the best correlation among rat and mouse ($R^2 = 0.96$), but even the weakest correlation is still good ($R^2 = 0.75$), suggesting limited species specificities. *in vivo* K_{puu} data from literature were used to investigate whether *in vitro* ERs determined in screening setup can be used for BBB penetration classification. Using cut-off value of 0.3 for K_{puu,brain} and 2 for ERs, the best sensitivity (85%) was achieved with the hMDR1 cells, which has the largest transporter abundance, therefore its resolution for weaker substrates is the highest. Total ERs derived from hMDR1 and hBCRP cells further increased the predictive power. For quantitative prediction of K_{p,uu,brain} from ERs different published models were used [1-3]. The best correlation ($R^2 = 0.83$) between *in vivo* and predicted K_{p,uu,brain} was achieved with mathematically parametrized calculation, using scaling factors. Inhibitory potency of 21 known MDR1 interactors using both digoxin and quinidine as probe substrate was also tested to identify species differences. We confirmed that inhibitory potency of many drugs is highly substrate-dependent, resulting in generally lower IC₅₀ with quinidine, despite the good overlap in rank order of inhibitors. In some cases, IC₅₀ difference between orthologs was resolved by transporter abundance correction, but this correlation is not always proportional and dependent on substrate-inhibitor interaction, suggesting that correlation between IC₅₀ and MDR1 abundance is dependent on the mode of inhibition. In summary, this is the first report of systematic comparison of inhibitor and substrate interactions of multiple compounds across MDR1 orthologs. Species differences in DDI potential can be investigated in this system, but transporter abundance and mode of inhibition need to be considered. Our *in vitro* assay system is ready to be integrated into CNS drug discovery screening programs to predict BBB penetration. It can be used to reveal potential divergences in transporter susceptibility between preclinical animal and human, improving the translatability of *in vivo* preclinical data.

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2. Sato et al S, Eur J Pharm Sci. 2020 15;142:105119
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P147 - MONKEY BLOOD-BRAIN BARRIER CO-CULTURE MODEL AS AN ALTERNATIVE METHOD FOR PREDICTING BRAIN PENETRATION**Satomi Kanki**

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Selecting a compound that can cross the blood-brain barrier (BBB) is crucial for central nervous system (CNS) drug discovery. Efflux transporters at the BBB can impede drug delivery, necessitating the selection of compounds that can evade transporter recognition in humans. However, species differences in transporter expression and function complicate direct extrapolation of brain distribution results in rodents to humans. There is thus a need for methodologies to predict BBB penetration in humans. This study aimed to establish an *in vitro-in vivo* correlation of BBB permeability in monkeys using the *in vitro* monkey BBB co-culture model comprised of primary cultures of monkey brain capillary endothelial cells, rat brain pericytes and rat astrocytes and monkey *in vivo* studies with eight marketed drugs. The apparent permeability coefficient calculated from the *in vitro* monkey BBB model exhibited a good correlation with those obtained from *in vivo* studies. In our previous presentation at JSSX 2021, we discussed the correlation between the apparent permeability coefficient and *in vivo* total brain concentration. Subsequent analysis revealed an improved correlation when considering the unbound fractions in the brain compared with total brain concentration ($R=0.957$ versus 0.867). This provided us with valuable insights into the parameters that contribute to the predictability of BBB penetration. Additionally, we investigated if cerebrospinal fluid (CSF) concentration can serve as a surrogate of CNS penetration *in vivo*. CSF concentration in monkeys showed a high correlation with unbound brain concentration, supporting the use of CSF as a surrogate for comparing brain penetration among multiple compounds. Utilization of CSF concentration as a surrogate can help us reduce the scale of *in vivo* animal studies, particularly monkey studies, for confirming the CNS disposition profiles of clinical candidates. In summary, our study suggests a novel approach to CNS-targeted drug discovery. The use of the *in vitro* monkey BBB model, given the structural similarity and limited species differences in transporters between monkeys and humans, is helpful for the selection of candidates with high brain penetration in humans in the early discovery stage. Subsequent *in vivo* monkey studies can then project human CNS penetration using CSF concentration as a surrogate before progressing to clinical trials.

P148 - QUANTITATIVE PREDICTION OF P-GP/CYP3A SUBSTRATE DRUG ABSORPTION IN HUMANS USING A MONOLAYER MODEL DERIVED FROM INTESTINAL ORGANOID

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Predicting human absorption of drugs, which are substrates for pharmacokinetic factors such as P-glycoprotein (P-gp) and cytochrome P450 3A (CYP3A), presents a known challenge. To address this, we established a human intestinal organoid-derived monolayer model that retains both P-gp and CYP3A functions. This model was used to evaluate its potential for predicting human absorption of drugs that are substrates for P-gp and/or CYP3A. Permeability tests were conducted from the apical to the basal side of the monolayer model for 28 marketed drugs, referred to as evaluated compounds, for which the fraction of administered compounds that reach the portal vein (FaFg) data could be obtained from literatures. To account for variability in tight junction formation ability and P-gp and CYP3A activity between experiments, and the impact of the evaluated drugs on these factors, we mixed lucifer yellow, digoxin, and midazolam as reference compounds and simultaneously evaluated their permeability in all evaluations. Four conditions were set for each evaluated compound: only reference compounds (Ref condition), a mix of reference compounds and evaluated compound (Ref/Cpd condition), a mix of reference compounds, evaluated compound, and the P-gp specific inhibitor Zosuquidar (Ref/Cpd/ZOS condition), and a mix of reference compounds, evaluated compound, and the pan-CYP inhibitor 1-ABT (Ref/Cpd/ABT condition). In a preliminary study, it was confirmed that the Zosuquidar and 1-ABT treatment in this study did not affect CYP3A and P-gp functions, respectively. As a result of the subsequent main experiments, increased permeability was observed in the Ref/Cpd/ZOS condition for P-gp substrates and in the Ref/Cpd/ABT condition for CYP3A substrates, confirming the validity of our system. From the results of reference compounds' permeabilities under the Ref condition, we compared and verified P-gp and CYP3A functions between experiments. For some evaluated compounds such as Cyclosporine A, the permeability of digoxin and/or midazolam increased under the Ref/Cpd condition compared to the Ref condition, suggesting that the function of P-gp and/or CYP3A was partially or completely saturated with these compounds. To calculate human FaFg from the permeability of reference and evaluated compounds in the four condition experiments, we constructed a novel static model which allows for consideration of the *in vivo* and *in vitro* activity differences of P-gp and CYP3A, and saturation under *in vitro/in vivo* conditions. Although the calculated FaFg values were not determined to be unique for some drugs due to uncertainties of the nonlinearity, we found that this method could effectively predict FaFg for drugs that are substrates for P-gp, CYP3A, or both. Consequently, this study proposes a new methodology that utilizes intestinal organoids for the quantitative prediction of human absorption, leveraging the appropriate setting of references and the novel static model.

P149 - CONFIRMATION OF ALBUMIN-MEDIATED EFFECT ON ORGANIC ANION TRANSPORTER 1/3 AND ITS IMPROVEMENT TO PREDICTION OF RENAL CLEARANCE

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The inclusion of human serum albumin (HSA) or plasma has been reported to affect activity of hepatic OATP1B transporters and improve *in vitro*-to-*in vivo* extrapolation (IVIVE) of hepatic clearance for highly protein-bound substrates[1]. However, limited evidence is available for the relevance of albumin-mediated uptake phenomenon for renal transporters. In this study, transport of eight substrates of renal organic anion transporter 1 or 3 (OAT1/3) was investigated with the inclusion of 1% and 4% HSA in conditionally immortalised proximal tubular epithelial cells transduced with OAT1/3. The unbound substrate concentration was kept constant between albumin-free control and HSA condition by measuring the fraction unbound in the presence of 1% and 4% HSA (f_u, inc). Highly protein-bound substrates showed a greater albumin-mediated uptake effect and larger fold increase in unbound intrinsic active uptake clearance ($CL_{int,u,active}$). In the presence of 4% HSA, the $CL_{int,u,active}$ of six highly protein-bound ($f_u, inc < 0.20$) OAT1/3 substrates increased up to 122-fold (OAT1) and 28-fold (OAT3) relative to the control. No significant effect was observed for weakly protein-bound ($f_u, inc \geq 0.95$) adefovir (OAT1-specific) and oseltamivir carboxylate (OAT3-specific). Relationship between the OAT1/3 $CL_{int,u,active}$ and extent of protein binding in the presence of albumin agreed with the proposed facilitated-dissociation model[2]. Furthermore, a linear relationship was established between the albumin-mediated fold change in $CL_{int,u,active}$ and the f_u, inc for both OAT1 and OAT3. For IVIVE of renal clearance (CLR), relative expression factor (REF) based on global proteomic quantification of *in vitro* OAT1/3 expression, and relative activity factor (RAF) derived from OAT1/3 $CL_{int,u,active}$ of adefovir and oseltamivir carboxylate were applied and compared. Inclusion of albumin significantly improved the bottom-up prediction of OAT1/3-mediated secretion clearance (CL_{sec}) and CLR with both REF or RAF approaches. This study confirms, for the first time, the presence of the albumin-mediated uptake effect with renal OAT1/3 transporters. The inclusion of HSA in routine OAT1/3 *in vitro* assays during drug development is recommended due to significant improvements to the IVIVE of CL_{sec} and CLR.

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P150 - A CASE STUDY: THE EXTRAHEPATIC CLEARANCE CONTRIBUTING TO THE *IN VITRO* - *IN VIVO* EXTRAPOLATION DISCONNECT OF AN INTERNAL NOVEL CHEMICAL ENTITY A1

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Accurate clearance prediction plays a critical role in first-in-human dose predictions for new chemical entities. The use of *in vitro* - *in vivo* extrapolation (IVIVE) for drug clearance prediction is a widely accepted practice in the pharmaceutical industry. However, IVIVE disconnect often observed during drug development, and the cause can be multi-faceted. An internal novel chemical entity (NCE) A1 exhibited a significant IVIVE disconnect in rodent. *in vivo* clearance in mouse was 3 to 5-fold faster than predicted using *in vitro* mouse liver microsomes and hepatocytes, while *in vivo* rat clearance was 22 to 39-fold faster than *in vitro* liver fractions predicted. The current study aimed to identify the cause of the significant underprediction in rodent clearance of A1. To better understand the biotransformation of A1, metabolite identification was conducted in hepatocytes (human/rat/mouse), rodent plasma and tissues (lung and spleen). The most abundant metabolites of A1 included an aldehyde metabolite M1 derived from deamination, and two sequential metabolites of M1: M2 (an alcohol metabolite) and M3 (a carboxylic acid metabolite). Reaction phenotyping was conducted in recombinant human monoamine oxidases (MAO), human liver mitochondria (HLMt) and human liver microsomes (HLM) in the presence and absence of NADPH and different MAO inhibitors. The formation of M1 was observed in HLMt and HLM in the absence of NADPH and the formation was not affected by the addition of NADPH, indicating CYP enzymes are not responsible for A1 deamination. Also, M1 formation was much higher in recombinant MAO-B than in MAO-A, and deprenyl (a MAO-B selective inhibitor) decreased 60-80% of the formation of M1, M2 and M3 in HLMt and HLM. Taken together, it indicates that MAO-B is a critical drug metabolizing enzyme for A1 metabolism. Given that MAO-B expression is reported in multiple extrahepatic tissues and plasma, extrahepatic metabolism was hypothesized to be a major contributing factor to the IVIVE disconnect of A1. However, further *in vivo* rodent PK studies with MAO-B and CYP inhibitors showed that the clearance and AUC of A1 was not affected by the pretreatment of deprenyl (5 mg/kg; IP) or 1-ABT (a non-competitive pan MAO inhibitor and a pan-CYP inhibitor; 100 mg/kg; PO), even though the positive control group showed that the pretreatment of deprenyl caused a 32% increase in AUC and a 30% decrease in clearance of 4-(dimethylamino)benzylamine, a MAO probe substrate, in comparison to the no deprenyl pretreatment group. The data indicated that the IVIVE disconnect is not likely caused by extrahepatic metabolism mediated by MAO-B. Furthermore, a rat bile duct cannulation study was conducted to better understand the clearance pathway of A1. The data showed that ~72% of the total dose was recovered in the rat excreta (bile, feces, and urine) by measuring parent compound A1 and a few metabolites. The parent compound excreted in bile, urine and feces accounted for about 17%, 9% and 5% of the total dose, respectively. Hence, the extrahepatic clearance, including bile excretion and kidney clearance, is likely to partially contribute to the IVIVE disconnect of A1 observed in rodent.

P151 - EVALUATION OF ACUTE KIDNEY INJURY INDUCED BY NEPHROTOXICANTS USING THREE-DIMENSIONAL CULTURED HUMAN PROXIMAL TUBULAR EPITHELIAL CELLS

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Purpose: The onset of acute kidney injury (AKI) affects patient prognosis; therefore, there is a great concern for an appropriate evaluation of DKI induced by drug candidates in drug discovery and development. Renal proximal tubular epithelial cells (RPTEC) are the primary target site for drug-induced kidney injury (DIKI) and express transporters involved in drug disposition in kidney. However, primary cultured human RPTEC has low level of expression of drug transporters compared with human renal cortex, leading to difficulty of evaluation of transporter-mediated DIKI. We previously showed the utility of three-dimensional cultured human RPTEC (3D-RPTEC), whose expression levels of major drug transporters are comparable to those of human renal cortex¹). This study aimed to evaluate the utility of 3D-RPTECs for DIKI including middle molecules.

Methods: 3D-RPTEC and two-dimensional cultured RPTEC (2D-RPTEC) were exposed to 32 kinds of drugs (positive: 17, negative: 15). Cytotoxicity was primarily evaluated by intracellular ATP content. In addition to ATP content, organelle function and morphology were evaluated by quantifying them using images obtained from the cells.

Results and Discussion: The ATP content in 3D-RPTEC was reduced by tenofovir, organic anion transporter (OATs) substrate, and cisplatin, an organic cation transporter 2 (OCT2) substrate in time-dependent manner up to 28 days. For

detection of RPTEC injury, EC20/Cmax≤100 was used as a cut-off values. As a result, the sensitivity in 3D-RPTEC was 82.4% and the specificity is 100% after 7-days of exposure. On the other hand, the sensitivity of 2D-RPTEC for 7 days exposure was 82.4%, but the specificity was 60%. In order to evaluate nephrotoxic drugs other than fluctuating ATP content, confocal imaging parameters were obtained using fluorescent probes for several kinds of organelle such as mitochondria. Several markers were increased by exposure to nephrotoxic drugs within 7 days, indicating that the imaging analysis is a useful approach to evaluate DIKI.

Conclusions: 3D-RPTEC is useful for *in vitro* evaluation of DIKI by the combination with ATP measurement and confocal imaging approach.

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P152 - PRIMARY HUMAN INTESTINAL CELL MODEL FOR ESTIMATING DRUG ABSORPTION, EFFLUX AND METABOLISM

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The intestinal absorption of compounds administered orally can be impacted by the permeability as well as metabolism and uptake/efflux transport. Traditional *in vitro* systems, such as Caco-2 or MDCK cells, which are utilized in the measurement of permeability and prediction of % Fa, do not allow the concomitant assessment of these processes in a physiologically relevant manner. Here we present a novel primary human intestinal cell model (EpiIntestine) developed from duodenum enterocytes and cultured in 24-well membrane bottom plates which can be used to assess drug absorption, transport, metabolism and drug-drug interactions simultaneously in the intestinal tract. The functional activities of intestinal transporters and enzymes expressed in this human relevant model were determined using probe substrates and inhibitors. Efflux ratios (ER) of digoxin (ER = 21 + 9) for P-gp, sulfasalazine (ER = 4.2 + 0.8) for BCRP and vinblastine (ER = 45 + 12) for both MRP2 and P-gp were reduced to approximately 1 in the presence of inhibitors, confirming the activity of key intestinal efflux transporters. The formation of metabolites 1-hydroxy-midazolam, 4-hydroxy-midazolam and raloxifene-6β-glucuronide in the incubations of midazolam and raloxifene indicated the functional activities of CYP3A4 and UGT1A10 enzymes. An extraction ratio of 0.34 was measured for midazolam and could be reduced to 0.07 in the presence of troleandomycin. The permeability of a set of drugs with information on human absorption, ranging from 1 to 100%, also correlated well with the fraction absorbed in human. The functional activity of PEPT1 was also detected in this system with the uptake of cefadroxil and the uptake (1.7×10^{-6} cm/s) was reduced to 0.6×10^{-6} cm/s in the presence of PEPT1 inhibitor Gly-Sar. We applied this human relevant model to study the impact of P-gp efflux and CYP3A4 metabolism on the permeability of nicardipine, a dual substrate of P-gp and CYP3A4, in the intestine. The model showed that the permeability (Papp) of nicardipine in the presence of the P-gp inhibitor elacridar was not dramatically different from the control (Papp = $2.8 + 1.4 \times 10^{-6}$ cm/s versus Papp with elacridar = $3.7 + 1.3 \times 10^{-6}$ cm/s). However, nicardipine permeability increased markedly (Papp = $6.6 + 1.5 \times 10^{-6}$ cm/s) when co-incubated with elacridar and ketoconazole suggesting the inhibition of intestinal CYP3A4 was the main driver of the permeability increase. The results obtained in this primary human intestinal cell model with expression and functional activities of intestinal transporters and metabolizing enzymes suggest it could be suitable for assessing not only drug absorption, transport and metabolism but also intestinal enzyme-transporter interplay and drug interaction in the intestinal tract.

P153 - CLOSING THE TRANSLATIONAL GAP: HUMAN MULTI-TISSUE CHIP PLATFORM FOR PREDICTIVE AND MECHANISTIC PRECLINICAL STUDIES

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Current paradigm of drug development faces significant challenges with translational gap between preclinical findings and human clinical outcomes. Extrapolating pharmacokinetic (PK) parameters from existing preclinical models including *in vitro* cell culture and nonclinical animals, often proves unreliable due to inherent limitations to humans. This challenge is further amplified with the emergence of new modality drugs beyond small and large molecules. Species-specific pathophysiology differences in animals lead to inconsistencies between preclinical and clinical results. Additionally, limited complexity of conventional *in vitro* cultures is not suitable to study complex biological interactions relevant to drug absorption, distribution, metabolism, and excretion (ADME), urging the need for more sophisticated models.

Javelin's advanced Microphysiological Systems (MPS; single-/multi-tissue chip) are purpose built for ADME applications to expand mechanistic knowledge of novel therapeutics towards first in human (FIH) dose estimation. Javelin's tissue chips, *in vivo*-like *in vitro* platform, combine human-specificity, and long-term metabolically functional culture to evaluate human PK parameters. This dynamic system provides continuous oxygenation through media recirculation and MPS – MPS (liver, gut, kidney, muscle) interaction to study multiple ADME processes within a single *in vitro* system. We have

demonstrated that Javelin's single- and multi-tissue chip platform could accurately predict clinical pharmacokinetics parameters (hepatic clearance, renal clearance, absorption, and volume of distribution) from on chip drug profiles. This platform can be utilized for investigating both intra-venous and oral drug ADME profile of 1) low clearance compounds, 2) drug-drug interaction like victim-perpetuator studies, 3) bioavailability studies and finally 4) to evaluate disposition and mechanism of action of new modality drugs that have not extensively studied before.

The Javelin platform can offer an alternative to ADME studies in laboratory animals for the purposes of understanding drug disposition, DDI and exposure-response relationship as a surrogate for human. This translational data can also be incorporated into PBPK modeling to predict human drug exposure and FIH dose estimation.

P154 - EFFECT OF CHITOSAN ON CURCUMIN STABILIZATION

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Curcumin is a natural polyphenolic compound derived from the rhizome of *Curcuma longa*. Curcumin is reported to exhibit broad antimicrobial activity; however, its utilization has been limited by low aqueous solubility and chemical instability in neutral and alkaline aqueous environments. We hypothesized that the addition of a cationic macromolecule such as chitosan could enhance the antimicrobial potential of curcumin by protecting curcumin from alkaline-mediated hydrolysis. This hypothesis was tested by examining the stability of curcumin alone and in combination with chitosan in phosphate-buffered saline (PBS, pH = 7.4) at 25°C and 37°C over 24 hours using UV absorption at 420 nm as an indicator of the stability of the parent compound, as curcumin degradation products do not absorb at this wavelength. We additionally examined the antimicrobial activity of curcumin alone and in combination with chitosan against *Pseudomonas aeruginosa* isolates using a resazurin assay, with absorbance at 570 nm being used as a marker of bacterial growth. Curcumin exhibited a reduced absorbance by 3 hours of incubation, which was especially pronounced at 37°C (~80%). However, at both temperatures, chitosan exhibited a protective effect against degradation when used at a 1:1 mass ratio. Curcumin alone at the highest concentration tested (128 µg/mL) exhibited slight inhibitory activity against a clinical isolate of *P. aeruginosa* (~17%); however, this was not improved with the inclusion of 200 µg/mL of chitosan; indicating that curcumin itself may have little-to-no activity against this isolate. Future studies will examine further the impact of chitosan on the production of curcumin degradation products using LC-UV, and the combination will also be tested with the additional inclusion of an antibiotic with known gram-negative activity to determine if synergy occurs.

P155 - Microfluidic chip for construction of 3D microvascular network of blood-brain barrier

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The blood-brain barrier (BBB) strictly regulates the transport of molecules, including nutrients and ions, between the blood and the central nervous system and maintains homeostasis of the central nervous system. Its basic structure consists of three types of cells (brain microvascular endothelial cells, pericytes, and astrocytes) and a basal membrane. These structural features of the BBB prevent blood components and administered drugs from nonspecifically entering the CNS through the endothelial cell gap. In particular, many biopharmaceuticals such as antibodies and nucleic acid drugs, which are the recent trend in drug discovery, are macromolecules, and thus control of the transport through the BBB is a critical issue for their delivery to the brain.

Micro-physiological systems (MPS) are one of technologies that can potentially replace animal experiments and the BBB-MPS model is expected to be able to assess drug permeability and pharmacokinetics. Many microfluidic BBB models have been proposed so far, but most of them are two-dimensional models using membranes and cannot reflect the three-dimensional (3D) structure of the BBB *in vivo*. The 3D structure of cellular tissue has the potential to bring various biological activities closer to the *in vivo* environment, so the development of models with 3D tissue structures is expected. Here we evaluated several structures of microfluidic chips which can integrate 3D microvascular network of BBB. These chips have three channel structures, namely CNS channel, vascular channel, and BBB channel. Each channel is connected in the central region of the chip. The BBB channel, centered between the CNS and vascular channels, has one or two unique micro-structure on the top surface of the chip to hold the gel solution loaded in the central BBB channel and to provide a constant interface between each channel.

Fibrin gels containing brain microvascular endothelial cells, pericytes, pericytes and astrocytes were filled into the central channel and cultured for 7 days. After culturing, a 3D microvascular network, called BBB-NET [1], was successfully established in the central channel. The 3D microvascular structures in the central channel have a BBB function that can be visualized by fluorescence microscopy and optical coherence tomography (OCT) using previously described image processing techniques [2]. The distribution of the three cell types, transporters and tight junctions in the microvascular network was confirmed by immunostaining. In addition, the transport activity of blood vessels to the brain was investigated using different model compounds. We expect that our microfluidic BBB chip will provide a more convenient platform for screening and evaluation of drugs in the BBB.

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P156 - ESTABLISHMENT OF A NOVEL *IN VITRO* ASSAY SYSTEM WITH CRYPT-DERIVED DIFFERENTIATED INTESTINAL CELLS TO EVALUATE DRUG-INDUCED GASTROINTESTINAL TOXICITY CAUSED BY THE DISRUPTION OF MUCUS LAYER

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Drug-induced gastrointestinal (GI) toxicity is one of the most frequently observed adverse events in the clinical trials of new drug candidates. However, it is difficult to accurately evaluate the GI toxicity in humans at the moment since *in vivo* animal study shows large phenotypic differences. Thus, to cope with the multiple mechanisms of the GI toxicity, we have proposed some *in vitro* assays with the use of crypt-derived intestinal stem cells and their differentiated cells for the evaluation of the risk of serious diarrhea, nausea, and vomiting¹). Cell surface mucus layer mainly composed by mucin (Muc2) secreted from goblet cells works as one of the defense mechanisms of the intestinal tract. Previous studies show that oral administration of drugs which induce GI toxicity reduced the intestinal expression of Muc2²). Hence, we aim to establish a novel *in vitro* assay to evaluate drug-induced mucosal damage using enterocytes derived from mouse small intestinal crypts. In our experiments, nonsteroidal anti-inflammatory drugs (NSAIDs) were exposed to crypt-derived differentiated cells, and Muc2 mRNA expression levels were quantified. Furthermore, for the quantitative evaluation of mucin layer thickness and density, fluorescent beads were added to the cells seeded in 96-well plates, and after the designated period, Z-stack pictures of beads were sequentially collected with fluorescent microscopy. The thickness of the mucus layer was estimated by the histogram of the height of each bead from cell surface. Then, we attempted to optimize the culture conditions to increase the thickness of the cell surface mucin layer. The addition of DAPT (an inhibitor of notch signaling) and FGF-2 to the cell culture media increased mRNA expression of Muc2. In addition, air-liquid interface culture further increased mRNA expression of Muc5AC as well as Muc2. Furthermore, we found that Muc2 mRNA expression in ileal crypt-derived differentiated cells was significantly higher than that in duodenal-derived cells. After two day-exposure to various NSAIDs, Muc2 mRNA expression in both ileal- and duodenal-derived cells was significantly decreased compared to the non-treated group. The estimated mucus layer thickness was also decreased in a drug concentration-dependent manner. These results suggest that this *in vitro* assay system may be useful to evaluate the risk of drug-induced gastrointestinal toxicity caused by the disruption of mucin layer.[1] Hashimoto et al., *Toxicology in Vitro*, 93, 105691 (2023) [2] Federer C et al., *Assay and Drug Development Technologies*, 14, 557-566 (2016)

P157 - Generation of *in vitro* blood-placental barrier (BPB) models using human placental stem cells

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The placenta, an essential organ for fetal development, plays crucial roles, including hormone production, nutrient and waste exchange, and acting as a barrier against xenobiotics such as drugs. Despite its protective function, some drugs can cross the blood-placental barrier (BPB) and reach the fetus, potentially causing adverse effects. Therefore, assessing the transplacental passages of chemicals is important in drug development to ensure fetal safety. Ex vivo perfusion systems utilizing the human term placenta are considered the gold standard for measuring the transplacental passage of drugs. However, they also come with certain limitations, such as the complexity of system setup. Animal experiments are suboptimal due to large species differences in the BPB structure. Therefore, developing BPB models using human placental cells has been extensively studied. In the human placenta, the placental villus serves as a barrier between the maternal and fetal circulations. This villus is encased in a bilayer of trophoblast, consisting of an inner cytotrophoblast (CT) layer and an outer syncytiotrophoblast (ST) layer. The CT cells have the ability to fuse and give rise to multinucleated ST cells, which act as the primary barrier at the interface between the mother and fetus. Various *in vitro* models have been developed to recapitulate the human BPB, including those using choriocarcinoma cell lines cultured on porous membranes. While these models are simple and easy to use, the cancer cells do not fully recapitulate *in vivo* trophoblast functions. To overcome these limitations, we previously established human placental stem cells [1]. The stem cells possess similar functions to CT cells and can differentiate into ST cells in the presence of inducers. In the present study, we show the first placental stem cell-based models for the human BPB [2]. First, we attempted to create a human placental organoid similar to the human placental villus. The placental stem cells were seeded in agarose microwell plates and cultured with three kinds of medium for 8 days, leading to the generation of apical-out spherical organoids. The

spherical placental organoids possess the ST cells on undifferentiated TS cells, and microvilli were observed on the ST cells similarly to those on the ST cells of the *in vivo* placental villus. Subsequently, we constructed a placental barrier model with a column insert and basal collagen membrane, allowing for a quantifiable drug permeability assay. Single nuclei RNA-seq analysis revealed that the BPB model includes several kinds of ST cells and CT cells. Co-culturing with endothelial cells further enhanced the model's resemblance to the human placental villus structure, making it a promising tool for drug development and studying placental physiology.

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P158 - AVAILABILITY OF POLYDIMETHYLSILOXANE CHIP IN EVALUATION OF SMALL INTESTINAL ABSORPTION

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Background: In drug development, conventional *in vitro* evaluation systems have limitations that restrict their utility and cannot completely predict clinical pharmacokinetics. Recently, microphysiological systems (MPS) gained attention in the drug discovery process due to their ability to recapitulate *in vivo* microenvironments *in vitro*, enabling the prediction of human responses. However, the use of polydimethylsiloxane (PDMS) as a widely used material for MPS raises concerns regarding its highly adsorptive properties for drugs, which may negatively affect the extrapolation of *in vitro* results to *in vivo* settings. In this study, we aimed to evaluate the adsorption/sorption properties of five compounds using a Gut model built by Emulate with a PDMS chip. Additionally, we investigated the permeability of these compounds to assess the concerns related to the PDMS chip in evaluating intestinal drug absorption. Throughout the study, we aimed to assess the utility and limitations of the PDMS chips in ADME studies. Method: The Emulate's Human Duodenum Intestine-Chip was utilized as the test system for this study. Human biopsy-derived duodenum organoids from one adult male donor and small intestinal microvascular endothelial cells from one adult male donor were seeded onto Emulate MPS devices and cultured under flow conditions for four days to establish the gut MPS. Metoprolol, talinolol, quinidine, tacrolimus, and felodipine were used as test substances, as they are known transcellular markers, transporters, and/or cytochrome P450 3A substrates. Permeability was evaluated 2, 6, 24, and 48 hours after the addition of the test articles, with a flow rate of 120 μ L/h. Result and discussion: The vehicle control and all compound treated groups indicated a healthy epithelium with characteristic villi-like undulations representative of *in vivo* intestinal morphology and a healthy microvascular endothelium with typical cobblestone morphology at 48 h post-treatment. The permeability studies conducted in the present study demonstrated a correlation between clinical FaFg (product of fraction absorbed and intestinal availability) and *in vitro* apparent permeability coefficient, supporting the kinetic theory. Although a decrease in donor concentrations of the test articles was observed, potentially due to sequestration to PDMS, it was not a significant issue for the compounds tested in this study from a practical viewpoint, as the concentrations remained relatively constant throughout the study. However, it should be noted that for felodipine and tacrolimus, a difference in donor concentration between the inlet and outlet was observed. These findings emphasize the importance of monitoring and ensuring effective drug concentrations on the donor side to accurately assess permeability, especially when the adsorption to the device is a concern. We would like to discuss these results at the poster presentation venue.

P159 - FREE FRACTION COMPARISON METHOD FOR A HIGHLY PROTEIN BINDING COMPOUND BY DYNAMIC ANALYSIS IN BI-DIRECTIONAL DIALYSIS

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Free fraction of a compound (fraction unbound, fu) in plasma is an important parameter for pharmacokinetics study to interpretate drug exposure by free concentration. While it has been technical difficulties for obtaining an accurate fu of a highly protein binding compound, in this research, we focus on obtaining a ratio of fu between different biological materials, by analyzing bi-directional dialysis data with newly developed dynamic analysis method (using Matlab® software). The fu ratio of three test compounds (nifedipine, ibuprofen, and cyclosporin) among different serum samples (mouse, rat, dog, monkey, and human) showed the correlation of the fu ratio between conventional equilibrium dialysis and the dynamic analysis. To evaluate the dynamic analysis model, the compounds that could show highly protein binding feature (asunaprevir, elbasvir, deferasirox, aripiprazole, beclabuvir, lomitapide, and ibrutinib) in rat and/or human plasma were selected, and the predictability of an *in vivo* correlated parameter of fu, steady state distribution volumes (Vss) was evaluated. Using the fu ratio by the dynamic model, 67% of the predictability of human Vss within 1.25-fold compared with

the observed value of human Vss, and the absolute average fold error (AAFE) was 1.61-fold. Using the fu ratio determined by the conventional equilibrium dialysis showed 11% of the predictability of human Vss within 1.25-fold compared with the observed value of the human Vss, and the AAFE was 1.70-fold. The results suggested better predictability of human Vss, by using the fu ratio (rat/human) of dynamic analysis and rat Vss. Estimation of drug-drug interaction of CYP3A inhibitors (aripiprazole, deferiasirox, lapatinib, ticagrelor, ketoconazole, and itraconazole, and RG12525) was also evaluated whether the better predictability could be obtained for AUCR of CYP3A substrate, midazolam, by using the fu ratio of dynamic analysis between serum and microsomes. The fu ratio by the dynamic model showed 1.12-fold error of AAFE, while conventional equilibrium dialysis method showed 1.17-fold error of AAFE, and the fu ratios obtained by human plasma fu value of 0.01 showed and 1.37-fold error of AAFE. The newly developed dynamic analysis method successfully detected a fu ratio before equilibrium state in different matrices, which could be applied to estimate human Vss and AUCR of CYP3A substrate. It will be further discussed its potential usage for pre-clinical research applications.

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P160 - ESTABLISHMENT OF MONKEY JEJUNUM CRYPT-DERIVED CELLS TO INVESTIGATE SPECIES DIFFERENCE BETWEEN MONKEYS AND HUMANS

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Background: Crypt-derived differentiated cells are emerging *in vitro* models for examining the absorption of oral drugs from the lumen. We previously reported that human jejunum crypt-derived differentiated cells are a useful *in vitro* model for assessing the availability of CYP3A4 substrates in the small intestine (Fg)¹. Monkeys are used as experimental animals in drug development. Species differences exist between monkeys and humans. FaFg of CYP3A4 substrate is generally underestimated in monkeys². The purpose of this study was to investigate whether monkey crypt-derived differentiated cells can serve as an *in vitro* model for investigating the impact of drug metabolism on absorption. Methods: Crypts were isolated from cynomolgus monkeys and maintained in Matrigel to establish spheroids as described previously¹. Undifferentiated cells were seeded on the filter of the cell culture inserts and differentiated to form a monolayer. The amounts of drugs and midazolam metabolites (1'-hydroxymidazolam and 4'-hydroxymidazolam) were quantified using LC-MS/MS (QTRAP5500). Results and Discussion: After seeding, switching the differentiated medium from 50% L-WRN-conditioned medium did not induce an increase in TEER. EGF Supplementation improved the TEER of the monolayers from 20 to 60 Ω -cm². Under these conditions, the activities of CYP3A4, P-gp, and BCRP were tested using midazolam, quinidine, and sulfasalazine as the probe substrates. Fg of midazolam was estimated to be 0.75, and efflux ratios (ER) were 6.7 and 1.16 for quinidine and sulfasalazine, respectively. Metabolic activities of CYP3A were unable to account for the *in vivo* Fg. Culture of the differentiated cells for 2 days in the presence of a PXR agonist (rifampicin, 20 μ M) induced the metabolic activity of CYP3A4. Fg was decreased to 0.13, comparable to the *in vivo* Fg (0.098) estimated using data reported previously in monkeys³. These culture conditions will be useful for predicting the Fg of test drugs in monkeys during drug development. Further studies are necessary to elucidate the effects of rifampicin on the ADME activities of other enzymes and transporters.

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P161 - GLOBAL PROTEOMIC PROFILING OF A PATIENT-DERIVED 3D CYST MODEL AND REFERENCE TISSUE OF POLYCYSTIC KIDNEY DISEASE

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Autosomal dominant polycystic kidney disease (ADPKD) manifests as fluid-filled cysts, mainly in the kidneys, which progressively increase in number and size and lead to end-stage renal disease in most patients. Although it is the most common hereditary kidney disease, treatment options are limited as there is currently only one FDA-, EMA- and PhMDA-

approved drug available. While transcriptomic studies have revealed deregulated pathways and potential therapeutic targets, comprehensive proteomic profiling of human patient derived samples remains largely unexplored. Here, we evaluated the utility of 3D cyst cultures used in phenotypic drug screenings as an *in vitro* model for ADPKD research. First, we analyzed and compared 3D cyst cultures derived from healthy human kidney tissue and late-stage ADPKD patient kidney tissues using mass spectrometry-based quantitative global proteomic profiling. We then compared the protein expression profiles with the corresponding reference tissues, mostly from the same patients. In addition, we quantified drug transporting and drug metabolizing enzymes as well as known drug target proteins. Proteomic profiling of human 3D cyst cultures reflected previously reported pathophysiological alterations, including aberrant protein expression in inflammation and metabolic reprogramming with a Warburg-like effect. Moreover, downregulated protein synthesis pathways were observed in both ADPKD cyst cultures and reference tissues. While the 3D cyst cultures partially recapitulated the disease phenotype *in vitro*, transporter expression was notably reduced compared to *in vivo* conditions. *in vitro*, the expression of the cation transport axis OCT/MATE was below the limit of detection, while the anion transport axis OAT/MRP was preserved. Known target proteins for particularly promising drug repurposing candidates such as the AMPK activator salicylate showed similar expression *in vitro* and in tissues, supporting the suitability of the 3D *in vitro* model for drug screenings. Ribosomal proteins and mitochondrial transporters were among our differentially expressed proteins. We have recently shown drug target engagement of several repurposing candidates for ADPKD with these proteins using thermal proteome profiling (poster title: Identification of drug target engagement of repurposing candidates for polycystic kidney disease by thermal proteome profiling). In summary, our findings provide novel insights into the ADPKD proteome and offer a starting point for further research to improve treatment options for affected individuals.

P162 - CHARACTERIZATION OF HUMAN IMMORTALIZED CELL-BASED BLOOD-BRAIN BARRIER SPHEROID MODELS AS A TOOL FOR BRAIN VASCULAR INFLAMMATION STUDIES

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Background and Objective: The blood-brain barrier (BBB), which is primarily formed by brain microvascular endothelial cells (BMEC) together with brain vascular pericytes and astrocytes, strictly limits the entry of various substances from the blood circulation into the brain. Since inflammation associated with attenuation of the barrier functions and immune cell recruitments to the BBB contributes to pathogenesis and progression of various central nervous system (CNS) diseases, regulation of BBB inflammation has gained considerable attention as a good target for new CNS drug discovery. As the first step to create BBB models that can facilitate such drug development, we have created human immortalized cell-based multicellular spheroidal BBB models (hiMCS-BBB) using three types of human immortalized BBB cells (BMEC [HBMEC/ci18], astrocytes, and pericytes). In this study, we aimed to characterize the inflammatory response profiles of the hiMCS-BBB models to clarify their potentials as a tool for CNS drug development studies.

Methods: The hiMCS-BBB models were prepared based on the previously-described method (Kitamura et al. Mol Pharm. 2022;19:2754-2764). Induction of cell adhesion molecule expression in HBMEC/ci18 cells after treatment of pro-inflammatory cytokines (TNF- α 20 ng/mL and IFN- γ 20 ng/mL) were analyzed by qPCR and immunocytochemistry. Recruitment of THP-1 cells (a human acute monocytic leukemia cell line) and Jurkat cells (a human acute T-cell leukemia cell line) to the hiMCS-BBB models were examined by cell adhesion assays.

Results and Conclusion: First, we confirmed that HBMEC/ci18 cells possessed inflammatory response capacity to pro-inflammatory cytokine treatments, where mRNA expression levels of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin were higher in the treated cells compared to those in the untreated cells (1,116, 41,040, and 27,775-fold, respectively). Induction of their protein expression levels were also observed in the treated cells. Then, we performed cell adhesion assays to ask their functions, showing that the numbers of THP-1 and Jurkat cells adhering to the hiMCS-BBB models increased by 4.3- and 3.2-fold under inflammatory conditions, respectively. To summarize, we have demonstrated that cell adhesion molecule expression are induced in the hiMCS-BBB models in response to inflammatory stimuli to promote immune cell recruitment and adhesion. While further characterization is absolutely necessary, it can be expected that the hiMCS-BBB models recapitulate *in vivo* BBB inflammation, thereby becoming a useful research tool for CNS drug development.

P163 - ASSESSING THE FIRST-PASS EFFECTS OF OF FELODIPINE WITH THE SMALL INTESTINE-LIVER COUPLED MICROPHYSIOLOGICAL SYSTEM

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Purpose: In recent years, no system has accurately predicted the *in vitro* pharmacokinetics of orally administered drugs. Microphysiological systems, also known as organ-on-a-chip, have gathered attention as novel pharmacokinetic evaluation platforms capable of replacing animal experiments. However, a key concern arises from its inability to replace dynamic environments such as the blood flow inherent in living organisms. This limitation results in inaccurate assessments of efficacy and safety in humans, contributing to the discontinuation of drug development. To replicate organ interactions and

blood flow in the small intestine and liver, we evaluated the first-pass effects between felodipine and grapefruit juice. This was achieved by perfusing the medium through a small intestine-liver MPS.

Methods: F-hiSIECTM (FUJIFILM) cultured on cell culture inserts were preincubated with bergamottin, a key component of grapefruit juice. The inhibitor was subsequently removed, and Primary Human Hepatocytes (PHH) were mounted on MS plates. The calcium channel blocker felodipine was then added to the apical side of the small intestine and perfused for 3 hours. The samples were taken at 15, 30, 60, 90, 120, 150, and 180 minutes. LC-MS/MS was utilized to assess the kinetics of felodipine and its metabolite, dehydrofelodipine.

Results and Discussion: In the MS plates containing the novel F-hiSIECTM and PHH, felodipine introduced into the small intestine compartment was detected in the portal and systemic circulation compartments after 15-30 minutes, mirroring the behavior of dehydrofelodipine. Moreover, the groups to which the inhibitor was added exhibited a lower production of dehydrofelodipine compared to the control group, indicating inhibition of CYP3A4. The inhibition caused by grapefruit juice is likely to be attributable to the furanocoumarins contained in the fruit. These results indicate the potential application of this system for evaluating first-pass effects and interactions influencing pharmacokinetics.

Conclusion: MS plates containing intestinal and liver cells enable *in vitro* assessment of the pharmacokinetics of orally administered drugs. Furthermore, this system addresses the limitations of static culture systems, which fail to replicate blood flow or organ interactions, and alleviates concerns regarding the animal welfare 3Rs issues by eliminating the need for utilizing laboratory animals for experimentation.

Therefore, we conclude that MS Plates could beserve as a valuable tool for pharmacokinetic studies.

P164 - EXPRESSION AND FUNCTIONAL ANALYSES OF THE PK-RELATED GENES IN DIFFERENTIATED INTESTINAL EPITHELIAL CELLS DERIVED FROM CRYOPRESERVED HUMAN INTESTINAL MUCOSAL EPITHELIUM (CHIM)

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Our previous report suggested that human intestinal stem cell-derived differentiated epithelial cells originated from surgical specimens can be a powerful tool for quantitative prediction of intestinal absorption and toxicity of drugs (Michiba et al., Drug Metabolism and Disposition, 50, 204-13 (2022)). However, it may become an obstacle to utilize these cells because of their ethical issue. Therefore, we attempted to establish spheroids of intestinal stem cells from commercially available cryopreserved human intestinal mucosal epithelium (CHIM) and investigated the expression/function of various drug transporters and metabolizing enzymes as others previously reported (Okada et al., Drug Metabolism and Pharmacokinetics, 54, 100532 (2024)). Intestinal stem cells were isolated and propagated from Caucasian-origin CHIM (Discovery Life Science) in Matrigel with L-WRN cell-conditioned medium. mRNA expression level and function of PK-related genes were evaluated in 2D-cultured differentiated intestinal epithelial cells 5-7 days after seeding and changing to differentiation medium. Differentiated cells showed mRNA expression of a wide variety of PK-related genes such as metabolizing enzymes, transporters, and nuclear transcriptional factors. Functions of selected transporters/enzymes have also been confirmed. In our differentiated cells originated from jejunum, mRNA expression and transport activity of SLC10A2 (ASBT), which is exclusively expressed in ileum, was relatively very small, whereas those of SLC46A1 (PCFT), which is predominantly expressed in jejunum, was clearly observed, indicating that these cells reflected the region-selective expression. The mRNA expression of CYP3A5 and UGT2B17, whose loss of function frequencies showed large ethnic differences (Japanese > Caucasian) was also observed, which suggested that these cells may be useful as an *in vitro* tool that reflects the ethnic differences of intestinal absorption. In addition, it was observed that PXR-mediated induction of expression/function of CYP3A by rifampicin was also clearly observed, which cannot be observed in Caco-2 cells. These results suggested that intestinal stem cells and their differentiated intestinal epithelial cells derived from CHIM maintain the functions of multiple enzymes/transporters as those from surgical specimens and can be useful as a novel *in vitro* system for predicting intestinal absorption and toxicity of drugs.

P165 - MULTICELLULAR SPHEROIDAL HUMAN BLOOD-BRAIN BARRIER MODELS ARE A USEFUL TOOL FOR CHARACTERIZATION OF BRAIN-PENETRATING ANTIBODIES

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Background: A brain-penetrating drug delivery system (DDS) carrier is necessary for CNS-active drugs, which otherwise cannot easily across the blood-brain barrier (BBB), to sufficiently get into the brain. Numerous research efforts have been made to develop such DDS carriers, as exemplified by a brain-penetrating anti-human transferrin receptor monoclonal antibody (hTfRMab). To facilitate these studies, we have developed a multicellular spheroidal human BBB model (hMCS-BBB)¹ that shows application potentials to BBB permeability assays of brain-penetrating DDS carriers. In this study, we aimed to provide further evidence for the utility of the hMCS-BBB models in development of brain-penetrating DDS carriers using several hTfRMABs with different *in vivo* BBB-permeable properties.

Methods: The hMCS-BBB models were developed using three types of immortalized BBB cells as reported previously². The BBB permeability assay of five types of fluorescein-labelled hTfRMabs was performed, and the uptake levels in each spheroid were analyzed semiquantitatively by measuring the fluorescence intensity. The dependency of incubation temperature (4°C or 37°C), time (0.5–6 hours), or concentration (1–40 µg/mL) in their BBB permeabilities were analyzed. The TfR gene knock-out brain microvascular endothelial cells (TfR-KO) were also used for a comparison purpose.

Results: Among the five hTfRMabs, the three showed significant temperature-dependent BBB permeability, which were more than two-fold higher than the others. These BBB permeabilities were clearly abolished when TfR-KO were used in the assays, suggesting involvement of TfR-mediated transcytosis in their uptake process into the spheroids. Next, the time- and concentration-dependency of the BBB permeability of the hTfRMabs were analyzed and found that they were saturable (stating at around 6 hours and 40 µg/mL, respectively).

Conclusion: To summarize, we clearly discriminate and characterize the different BBB permeability properties of the five hTfRMabs using the hMCS-BBB models. Therefore, it can be expected that the hMCS-BBB models are useful for characterizing human BBB permeable properties of various brain-penetrating DDS carriers, including TfR-targeted antibodies.

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P166 - PREDICTING CLINICAL ADME PARAMETERS WITH MULTI-TISSUE CHIP PLATFORM USING PRIMARY HUMAN CELLS

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A crucial step in lead selection during drug development is accurate measure and estimation of adsorption, distribution, metabolism, and excretion (ADME) properties using *in vitro* methods. In the drug metabolism and pharmacokinetics (DMPK) field, *in vitro-in vivo* extrapolation (IVIVE) has become a standard method to predict clinical PK/PD behavior from established in-vitro assays compared to allometric scaling method. However, current invitro models are limited by lack of physiological relevance, and long-term drug exposure leading to poor IVIV translation of drug efficacy and safety. Tissue chips are microphysiological systems (MPS) under dynamic environment that has human relevant pathophysiology enabling improved cellular activities and long-term cultures. Javelin's tissue chip, an *in vivo*-like in-vitro platform, combines human-specificity, and long-term metabolically functional culture to evaluate human PK parameters. Here, we utilized Javelin's multi tissue chip (Entero-Hepatic chip; LTC+) to demonstrate the accurate predict of clinical ADME parameters like hepatic clearance, oral bioavailability, and Drug-Drug interactions (DDI). This platform integrates long-term metabolically functional hepatic culture and polarized and functional intestinal culture (RepliGut® planar – jejunum) under continuous media recirculation providing constant MPS interaction, and nutrient and oxygen supply. Low turnover drug clearance studies on hepatic platform demonstrated high *in vitro in vivo* correlation (IVIVC) and accurate clinical exposure predictions (1.1 average absolute fold error) with physiologically based pharmacokinetic (PBPK) modeling. To demonstrate improved predictivity of oral bioavailability, we investigated intestinal and hepatic clearance of midazolam in LTC+ compared to hepatic only culture. Further, we induced LTC+ using rifampicin and observed decrease in AUC of midazolam depletion and increase in metabolite formation kinetics compared to control. We used advances in bioengineering to develop a novel purpose-built tissue chip platform to address current challenges in preclinical invitro platform. The preliminary data demonstrated high reproducibility and the utility of this platform to study pharmacokinetic and DDI processes to advance mechanistic understanding how drugs will perform with human physiology. This translational data can also be incorporated into PBPK modeling to predict human drug exposure and first in human dose estimation.

P167 - DEVELOPMENT OF A MONOLAYER MODEL DERIVED FROM INTESTINAL ORGANOID TO EVALUATE THE EFFECT OF THE MUCUS LAYER ON DRUG ABSORPTION

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Prediction of bioavailability in humans is essential in the development of drugs that can be orally administered. The small intestine, the site of drug absorption, is an important organ that can limit oral bioavailability. The small intestine actively absorbs nutrients and water; however, it also possesses a selective barrier function that prevents undesired substances including drugs from entering the human body. Mucus layer is one of the compositions of these factors, that can impact

drug absorption. Goblet cells in the intestinal epithelium produce mucus; however, the Caco-2 / MDCK monolayer model, which are gold standard *in vitro* model for analyzing drug absorption, lack these cells and the function of production of mucus, such as MUC2. Intestinal organoids, which include organ specific cell types and can recapitulate several aspects of the *in vivo* organ function. These organoids derived from human biopsy or surgical specimens encompass intestinal stem cells, which can be differentiated into mature intestinal cells such as absorptive enterocytes, goblet cells. In this study, we isolated crypts from the adjacent normal colon of surgical specimens and established colon organoids. To assess the effect of the mucus layer on drug absorption, we have developed the monolayer model using these organoids. Our model produced a mucus layer-like formation which was stained with AB-PAS staining. Penetration assay using fluorescent beads in the size of bacteria (1 μ m) demonstrated that this layer inhibited the sedimentation of fluorescent beads, which is considered to simulate the function of the mucus layer *in vivo* in preventing the invasion of bacteria into the human body. The removal of the mucus layer by pipette aspiration did not notably affect the permeability of some compounds such as lucifer yellow, and Metoprolol, but it significantly enhanced the permeability of Cyclosporine A. Our new model enables to evaluate the impact of the mucus layer on drug absorption, and might be a useful tool to accurately predict drug absorption in humans, and to apply to the development of new formulations that improve drug absorption.

P168 - IMPROVEMENT OF LIVER FUNCTION AND ITS LONG-TERM MAINTENANCE BY APPLYING SYNTHETIC MATRICES, HYDROX, TO PRIMARY HUMAN HEPATOCYTE-DERIVED ORGANOID AND THEIR POSSIBLE APPLICATION IN HEPATOTOXICITY RESEARCH

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Purpose: Primary human hepatocyte (PHH)-derived organoids (PHH-Org) are *in vitro* three dimensionally (3D) cultured proliferative cells that exhibit bipotent stem cell phenotype. Translational research of PHH-Org for drug discovery has been limited by the challenge of their low liver function. Although various attempts have been made to generate functional hepatocyte-like cells from PHH-Org, their liver function is still far from PHH, the gold-standard of hepatocyte in pharmaceutical research. In this study, we aimed to generate a highly functional hepatocyte-like cells from PHH-Org that is applicable to pharmaceutical research. We attempted to establish a novel hepatic differentiation system for PHH-Org, using HYDROX, a chemically defined 3D nanofiber based on an amphiphilic PSar-PLLA polymer (Matsui et al., Mater. Today Commun., 2017).

Methods: Commercially available PHH were embedded in Matrigel to establish PHH-Org. To perform HYDROX-culture, PHH-Org were extracted from Matrigel and re-seeded on a culture-plate coated with HYDROX (HYDROX-plate). The histological characteristics of HYDROX-cultured PHH-Org (PHH-Org-H) were examined under a phase contrast microscope. To further evaluate their characteristics, we examined the individual gene expression level of key hepatocyte markers in PHH-Org-H, followed by a comprehensive gene expression analysis. The applicability of PHH-Org-H to a drug-induced hepatotoxicity test was investigated by treating PHH-Org-H with well-known hepatotoxic drugs. Finally, long-term culture of PHH-Org-H were performed and its ability to predict chronic drug-induced hepatotoxicity caused by fialuridine was examined.

Results: PHH-Org re-seeded on HYDROX-plate formed cell aggregates, indicating that PHH-org were able to culture with HYDROX. PHH-Org-H exhibited improved gene expression levels of hepatocyte markers regarded to drug-metabolizing enzyme capacity, xeno receptors, hepatic transporters, and lipid metabolism, along with culture period up to 21 days. Enzymatic activities of cytochrome P450 3A4 (CYP3A4), CYP2C19, and CYP1A2 in PHH-Org-H approximate those in PHH. When treated with hepatotoxic drugs such as troglitazone, amiodarone and acetaminophen, PHH-Org-H showed similar cell viability to PHH, suggesting that PHH-Org-H could be applied to drug-induced hepatotoxicity tests with the same degree of accuracy as PHH. Furthermore, we demonstrated that PHH-Org-H could maintain its high hepatic functions for up to 35 days and could successfully predict chronic drug-induced hepatotoxicity caused by fialuridine.

Conclusion: In this study, we successfully generated highly functional hepatocyte-like cells from PHH-Org, using HYDROX-culture system, that could possibly be applicable to pharmaceutical research. Further detailed analysis will be performed in the future to clarify the mechanism by which liver function was improved.

P169 - UTILIZING PATIENT-DERIVED TUMORIDS AS A SCREENING PLATFORM FOR EVALUATING CELL-BASED IMMUNOTHERAPIES IN SOLID TUMORS

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With the ongoing expansion of cancer immunotherapy into the treatment of solid tumors, selecting suitable experimental models for developing and validating immunotherapies is a major obstacle. Patient-derived tumoroid models are

increasingly attractive in this area, as they better capture patient-specific genomic, transcriptomic, and proteomic profiles, all in a 3D environment. Here, we demonstrate use of patient-derived tumoroids in a screening platform for effectively evaluating cell-based immunotherapies. Tumoroid models were established *in vitro* from fully consented patient-derived tumor tissue. Chimeric antigen receptor (CAR) T cells recognizing Meso3 or CD19 were knocked-in to donor T cells using CRISPR/Cas9 and a CAR cassette adeno-associated virus (AAV). Alternatively, natural killer (NK) cells were isolated from donor blood and expanded using CTS™ NK-Xpander™ Medium. Immune effector cells (primary NK or CAR T) were co-cultured with tumoroid cells in 96-well plates at varying effector-to-target ratios and monitored via live imaging, where cell killing was directly measured via caspase live staining. CAR T killing was shown to be specific, with killing by Meso3 CAR T cells only observed in mesothelin-expressing tumoroids. CD 19 CAR T cells failed to efficiently kill any tumoroids, which are CD19-negative. NK cell killing efficiency at various effector-to-target ratios was successfully captured in a ratio-dependent manner via live imaging/analysis. Taken together, we show that patient-derived tumoroid models can be utilized in multiplexed killing assays. This platform can predict tissue responses and help to screen solid tumor immunotherapies in real-time, cell-based assays.

P170 - ESTABLISHMENT OF AN AIR-LIQUID INTERFACE INTESTINAL ORGANOID MONOLAYER AND ITS CHARACTERIZATION FOR DRUG ABSORPTION AND METABOLISM

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Investigation and prediction of intestinal absorption and metabolism is a key step in the development of orally administered drugs. Even though the Caco-2 model is extensively used and considered as gold standard *in vitro* model, it has several limitations. A major drawback is the lack of cytochrome P450 3A4 (CYP3A4) and therefore Caco-2 models cannot fully recapture the human *in vivo* first-pass metabolism. Biopsy-derived intestinal organoids can overcome these limitations and have been increasingly used in pharmacokinetic studies in recent years. Organoids provide many advantages including cell type heterogeneity, expression of relevant enzymes and scalability to high-throughput assays. In this study we established an air-liquid interface intestinal organoid monolayer and compared it to a submerged culturing condition. In an in-depth characterization of intestinal organoid monolayers derived from duodenum and jejunum biopsies several advantages of an air-liquid interface culture for intestinal absorption and metabolism studies could be shown. We could demonstrate a reproducible long-term culture and observed physiologically relevant and stable transepithelial electrical resistance values. In comparison to submerged culture conditions we observed distinct differences in cell differentiation, morphology and most importantly higher expression and activity of drug metabolizing enzymes. Furthermore, the apparent permeability (Papp) was evaluated for several compounds from apical-to-basolateral side. This study supports air-liquid interface culture of intestinal organoid monolayers to predict human drug absorption and metabolism.

P171 - DEVELOPMENT OF HUMAN GLUCURONIDE ATLAS AND ITS APPLICATION FOR IDENTIFYING ENDOGENOUS UGT SUBSTRATES BY A NOVEL METABOLOMIC-BASED DMET BIOMARKER DISCOVERY (MDBD) APPROACH

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Identification of endogenous substrates of drug-metabolizing enzymes and transporters (DMETs) is crucial not only for understanding the physiological roles of these proteins but also for discovering biomarkers to predict drug-drug interaction (DDI) potential and inter-individual variability.¹ UDP-glucuronosyltransferases (UGTs) are one of the major and highly variable DMEs involved in the biotransformation of xenobiotics and endobiotics. In this study, we aimed to discover novel endogenous substrates of UGTs by analyzing human urine samples using a state-of-the-art metabolomics-based biomarker discovery (MDBD) approach. For this, we first created a library of 705 potential endogenous glucuronide metabolites from open-access metabolome databases and literature with their predicted high-resolution mass spectrometry data. The analysis of pooled human urine samples (undiluted, 1x) against the glucuronide library detected 103 unique m/z features (195 total signals at different retention times, mass error < 5 ppm) as potential endogenous glucuronides in human urine. Multiple signals for a single unique m/z feature indicate isomers or in-source fragmentation of glucuronides. In the next aim, the pooled urine sample was incubated with beta-glucuronidase enzyme (GUSB) expressed in *E. elegans* and *E. coli*, which confirmed a significant depletion of 51 unique m/z features (83 total signals) belonging to glucuronide and the formation of 31 unique m/z features (88 total signals) belonging to their parent that were significantly higher in the GUSB-treated urine sample. Further, endogenous substrates of UGT2B17 were confirmed by monitoring the formation of glucuronide metabolites in incubations of GUSB-treated urine (undiluted, 1x and 5-fold diluted, 0.2x) with recombinant human UGT2B17 (rUGT2B17) and UDPGA (cofactor). GUSB-urine samples (1x and 0.2x) treated with rUGT2B17 showed a significant increase (fold change > 1.5, p < 0.05) in 10 and 28 unique m/z features (26 and 33 total features), respectively, representing UGT2B17-mediated glucuronide metabolites. Similarly, rUGT2B17-treated 1x and 0.2x GUSB-urine samples showed significant depletion of 3 and 5 unique features (9 and 12 total), respectively. The

pairing of the glucuronide formation and parent depletion further confirmed the identity of most of the metabolites. However, some unique unpaired glucuronide or parent were detected likely due to the low sensitivity of either of these moieties. The top five unique *m/z* values of the endogenous UGT2B17 substrates or metabolites detected in 1x and 0.2x GUSB-urine included the following: i) substrates: *m/z* 109.06, 169.08, 195.06, 201.18, and 293.24; and ii) glucuronides: *m/z* 325.09, 353.13, 377.21, 463.26, and 465.24. The major potential substrates of UGT2B17 were predicted to be *p*-cresol, benzyl alcohol, retinol, lauric acid, trans-cinnamic acid, testosterone, and dihydrotestosterone. Interestingly, testosterone glucuronide and some other metabolites were not detected in the 1x GUSB-urine sample, indicating substrate-dependent inhibition of UGT2B17 activity by other high-affinity substrates present in urine. When validated using synthetic standards, the identified UGT2B17 endogenous substrates can be used as biomarkers for predicting variability in UGT2B17 activity *in-vivo* including DDI for applications in translational pharmacology and precision medicine. This approach can be further extended to identify endogenous substrates of other human UGT isoforms.

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P172 - ADDRESSING COMMON METABOLISM PROBLEMS IN DRUG DISCOVERY WITH *IN SILICO* METHODS

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in silico metabolism prediction can address critical questions to guide lead optimization. Using several case studies, we demonstrate the application of these models to address design challenges involving metabolic (in)stability, the formation of reactive and/or toxic intermediates, and to mitigate the risk of genetic polymorphisms and drug-drug interactions. In addition, we illustrate how these models can inform the selection of *in vitro* and *in vivo* pre-clinical experiments to avoid surprises in late-stage trials. Furthermore, accurate predictions of metabolite profiles early in the discovery process provide essential guidance for drug design. Optibrium's mechanistic metabolism models cover metabolism by P450, AOX, FMO, UGT, and SULT enzymes¹⁻⁵. By combining these models, metabolic pathway analysis proposes the most likely metabolites with greater precision than other methods, assisting in metabolite identification studies and enabling potentially active, reactive, or toxic metabolites to be identified⁶.

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P173 - THE MASS BALANCE, RECOVERY, ABSORPTION, METABOLISM, AND EXCRETION OF [14C]-MIRDAMETINIB IN HEALTHY MALE PARTICIPANTS AFTER ORAL DOSING

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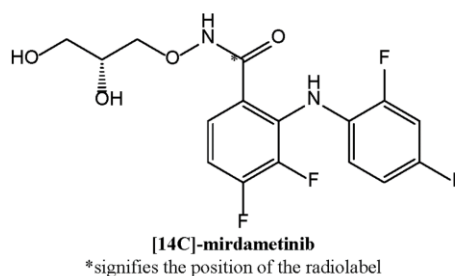
Background: Mirdametinib is an investigational, selective non-adenosine triphosphate (ATP)-competitive inhibitor of mitogen-activated protein kinase MEK-1 and MEK-2. As part of the clinical pharmacology development program, an open-label, single-center, nonrandomized, radiolabeled study was conducted in healthy male participants to assess the mass balance, pharmacokinetics, routes and rates of excretion, and metabolism profile after administration of a 14C-labeled oral dose of mirdametinib.

Methods: Participants received a single 4-mg oral dose of [14C]-mirdametinib (containing ~80.0 µCi 14C; Figure). All 8 participants were dosed as a single cohort in the study. Aliquots of plasma and excreta were prepared from samples collected during the study. Metabolite profiling of total radioactivity was performed using plasma, urine, and feces sample pools collected from all participants. Subsequently, metabolite quantification was achieved by collection of liquid chromatography (LC) fractions followed by construction of radiochemical profiles, which were assessed for the relative and absolute abundance of observed radiochemical components. Structural elucidation was performed by LC/mass

spectrometry on selected samples, and based on the structural elucidation data of metabolites, the likely routes of metabolism of [14C]-mirdametininb were determined.

Results: Overall, 16 metabolites were identified in plasma, urine, and feces. Recovery of total radioactivity averaged 95.0% (0-264 hours post-dose), with urine and feces accounting for 67.7% and 27.3%, respectively. In plasma, unchanged parent drug was the most prominent circulating component at 35.3%. Two metabolites accounted for more than 10% of total drug-related material in plasma. M15 (PD-0315209, via amide hydrolysis) accounted for 24.0%, while M22 (O-β-glucuronide) accounted for 14.7% of total radioactivity in plasma. Elimination of [14C]-mirdametininb in human participants occurred primarily by metabolism with 8.7% of the dose being recovered as intact drug observed in feces (8.0%) or urine (0.7%). The largest drug-related components in urine were found to be M20, M21 and M22 (glucuronides), and M33 (M15 + glucuronide), representing a mean of 7.7%, 19.8% and 14.0%, and 11.9%, respectively, across participants. The largest drug-related components in feces were found to be mirdametininb and M32 (reductive hydrolysis-cleavage of the glycerol), representing a mean of 8.0% and 9.0% across participants, respectively.

Conclusion: This study provided a thorough understanding of the metabolite profile of mirdametininb. The primary metabolic clearance pathways involved were (1) amide hydrolysis to the carboxylic acid (PD-0315209) with glucuronide conjugation (M33); (2) glucuronide conjugation (M20, M21, and M22); and (3) reductive hydrolysis (cleavage of the glycerol) to form the amide (M32).



Funding: SpringWorks Therapeutics.

P174 - THE APPLICATION OF KIRCHHOFF'S LAWS TO SATURABLE, MICHAELIS-MENTEN, PHARMACOKINETICS

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Rates of drug elimination (mass/time) are defined by multiplying either drug concentrations or drug amounts by the relevant coefficient of proportionality, a clearance for measured concentrations and a rate constant for measured amounts. We previously demonstrated that coefficients of proportionality for linear kinetic processes could be derived independent of differential equations using Kirchhoff's Laws, by adding rate-defining parallel processes to give the relevant total clearance or rate constants or by adding the inverse of rate-defining processes in series to give the inverse of the relevant total clearance or rate constant. A rate-defining elimination process is one that by itself limits the rate of loss, i.e., the total clearance or total rate constant experimentally may equal this parameter alone (e.g., liver blood flow). We have shown that it is possible to simply derive the linear hepatic clearance (CL_H) parameter for what was previously regarded as the well-stirred model by considering the rate-defining in series clearance processes, hepatic blood flow (Q_H) and fraction of drug unbound in blood (f_{uB}) multiplied by intrinsic hepatic clearance (CL_{int}), when hepatic basolateral transporters are not relevant.

$$\frac{1}{CL_H} = \frac{1}{f_{uB} \cdot CL_{int}} + \frac{1}{Q_H} \quad (1)$$

Similarly, the clearance parameter may be solved for the in series saturable metabolism relationship

$$\frac{1}{CL_{H,saturable}} = \frac{1}{\frac{V_{max}}{K_M}} + \frac{1}{\frac{V_{max}}{[S]}} + \frac{1}{Q_H} \quad (2)$$

where the units of V_{max} are mass/time, versus the Michaelis-Menten V_{max} units of concentration/time. To our knowledge, the dampening effect of Q_H on $CL_{H,saturable}$ has not been considered previously and can have a significant impact on predicting in vivo nonlinear kinetics. For example, for ethanol at a substrate concentration $[S]$ of 0.1 g/L, where $V_{max} = 10$

g/hr and $K_M = 0.08$ g/L, calculated CL_H is 34.4 vs 55.6 L/hr neglecting Q_H . Even at the drunk-driving limit (0.8 g/L = $10 \times K_M$), the effect of Q_H is not negligible (13% difference)

$$\frac{1}{CL_{H,saturable}} = \frac{1}{\frac{10}{0.08}} + \frac{1}{\frac{10}{0.8}} + \frac{1}{90} = \frac{1}{10.1 \text{ L/h}} \quad (3)$$

When Q_H is very large compared to the other two denominator terms, the usual case for most nonlinear metabolic processes, then solution of Eq. 2 gives the familiar Michaelis-Menten relationship, but with V_{max} units of mass/time,

$$CL_{H,saturable} = \frac{V_{max}}{K_M + [S]} \quad (4)$$

where when $K_M \gg [S]$, then linear elimination is observed and $CL_H = f_{uB} \cdot CL_{int} = \frac{V_{max}}{K_M}$. When $[S] \gg K_M$, then

$CL_{H,saturable} = \frac{V_{max}}{[S]}$. However, we maintain that for saturable kinetic processes following Eqs. 2-4, the *in vitro* V_{max} and K_M values may not be the same as the *in vivo* values; these parameters should be determined experimentally *in vivo* for different steady-state $[S]$ conditions. This is because the *in vitro* Michaelis-Menten parameters are determined in the same volume of fluid, serving as the basis for the Briggs-Haldane derivation, so that the difference between using units of amounts vs concentrations introduces no error. However, *in vivo* certainly the volume of distribution for lipophilic drugs will not be the same as the metabolic enzymes, nor will the volume of distribution for the enzyme-substrate complex be the same as the substrate. We further maintain that because of these volume differences, enzyme-substrate on and off rates *in vitro* may not be predictive *in vivo*. The Kirchhoff's Laws derivation, however, gives the appropriate relationship, Eq. 2, independent of these differences.

P175 - AUTOMATED WORKFLOW FOR TARGETED PROTEIN DEGRADER (TPD) METABOLITE IDENTIFICATION USING COLLISION-INDUCED DISSOCIATION (CID) AND ELECTRON-ACTIVATED DISSOCIATION (EAD)

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Formation of drug metabolites is a critical factor during drug development as it contributes to efficacy, toxicity and drug-drug interactions. The detection and accurate identification of drug metabolites is crucial to ensure the safety and efficacy of a drug. Because LC-MS platforms provide good selectivity and sensitivity, it is the preferred workflow for metabolite identification. Here, confident metabolite identification was performed using unique fragments from EAD spectra and CID spectra. Unique fragments from EAD and CID spectra were processed in a single result file to achieve confident structure assignment of and localization of metabolites.

ARV 771 at 5 μ M starting concentration was incubated in rat hepatocytes at 37°C for 30 minutes. Samples were removed from incubation and quenched with methanol at a ratio of 2:1 (v/v). Samples were vortexed for 30 seconds and centrifuged at 12000 rcf for 12 minutes at room temperature. The supernatant was transferred to a vial. Analytes were separated using a Phenomenex Kinetex Polar C18 column (2.1 x 100 mm, 2.6 μ m, 100 Å) column. The ExionLC AD system was operated at a 0.5 mL/min flow rate. Mobile phase A was 0.1% (v/v) formic acid in water and mobile phase B was 0.1% (v/v) formic acid in methanol. An injection of 5 μ L was subjected to analysis. The samples were analyzed using the data-dependent acquisition (DDA) method with Zeno CID DDA and Zeno EAD DDA on the ZenoTOF 7600 system. Data was processed using Molecule Profiler software.

Several phase 1 metabolites were identified including demethylation to carboxylation at the linker site following the loss of the target protein binding site. Unique EAD fragments at m/z 574.2840, 530.2602, 518.1804, 486.2271, 429.2055 and 357.1622 were used to identify the site of carboxylation. A unique fragment at m/z 113.0229 in the CID spectrum confirmed the site of carboxylation on the proteasomal recognition moiety.

Localization of a carboxylated metabolite of ARV 771 on the linker following the loss of the proteasomal recognition site was observed. EAD fragments at m/z 471.1475, 444.1251, 413.1060 and 382.0643 were used to identify the exact site of carboxylation on the aliphatic chain.

An oxidation metabolite of ARV 771 on the protein targeting site was also observed. A unique fragment at the m/z 613.0744 (EAD) was able to eliminate the sulfur oxidation. Common fragments between CID and EAD eliminated nitrogen oxidation. Therefore, oxidation was localized on the second carbon next to the carboxylate group.

The workflow demonstrates a streamlined path to confident TPD metabolite identification with more the informative spectra provided by EAD.

P176 - DETOXBUG – BIOTRANSFORMATION IN BLACK SOLDIER FLY LARVAELada Ivanova¹, Nada Dhaoui², Eva Byhrø², and **Christiane Fæste**¹¹Norwegian Veterinary Institute, Ås, Norway, ²Pronofa ASA, Gamle Fredrikstad, Norway

Reduced dependence on non-renewable resources is a major aim of bioeconomy strategies. The valorisation of low-value forms of biomass requires the development of innovative approaches enabling transformation into useful products. Raw materials intended for food and feed production are often infected with mycotoxin-producing fungi, presenting a serious risk to human and animal health. About one-third of all produced food- and feedstuffs are lost because they become spoilt with mycotoxin contents exceeding set limits. In this context, the project DetoxBug investigates if mycotoxin-contaminated raw materials can safely enter feed and food chains via detoxification in insect larvae. Rearing insects on inedible resources and using insect proteins and lipids in feed for domestic animals will add value to waste materials and offers environmental advantage, considering that insect farms use less water, chemicals and space than conventional agriculture. The larvae of the Black Soldier Fly (BSFL) (*Hermetia illucens*) have promising properties since they can grow on poor substrates including manure, sludge, straw, offal and spoilt food- and feedstuffs. However, the full utilisation of the BSFL's versatility is currently hindered by safety regulations that restrict the contents of insect feed to conventional ingredients. Before changes can be initiated, evidence for the capability of BSFL to detoxify xenobiotics efficiently has to be provided. The ability of the larvae to resist adverse environmental and dietary contaminants is likely a consequence of extensive biotransformation activity by oxidative and conjugative metabolism enzymes, comparable to what has been found in migratory locusts (*Locusta migratoria*) (Liu et al., 2018). Cytochrome P450 enzymes (CYP) and UDP-glycosyltransferases (UGT) are present in practically all insect tissues, with the highest contents in the Malpighian tubes and fat bodies. Profiling of the BSFL genome (17,770 protein-coding genes) has revealed expanded gene families of biotransformation-relevant enzymes (Zhan et al., 2020), and their involvement in the exceptional xenobiotic resistance of the larvae can be expected. We have therefore prepared S9 fractions of fastened and fed BSFL with and without prior removal of the gut and characterised the activities of important metabolism enzymes using model substrates. Extraction methods and incubation conditions were optimised. Compound depletions and formation of the respective metabolites were analysed by all-in-one liquid chromatography high-resolution mass spectrometry (LC-HRMS), demonstrating substantial biotransformation capacities. Subsequently, the metabolism of typical food-spoiling mycotoxins such as deoxynivalenol (DON) and enniatin B (ENNB) was studied in BSFL S9. Metabolites produced were identified by LC-HRMS and compared to known profiles in vertebrate species. The *in vitro* results were validated by an *in vivo* exposure study in BSFL, confirming high metabolism activity in the larvae.

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P177 - MOXIDECTIN METABOLISM IN REINDEERLada Ivanova¹, Rebecca Davidson², Ingebjørg Nymo², and **Christiane Fæste**¹¹Norwegian Veterinary Institute, Ås, Norway, ²Norwegian Veterinary Institute, Tromsø, Norway

Infections of free-grazing semi-domesticated reindeer (*Rangifer tarandus tarandus*) with the parasitic nematode *Elaphostrongylus rangiferi* (also called brainworm) occur frequently in Northern Norway and result in reduced animal welfare and losses for Sami herders. The parasite has an indirect lifecycle with a highly temperature-dependent development in the intermediate gastropod (slugs and snails) host. With increasing average temperatures in the subarctic regions due to climate change, the brainworm infection risk has increased. The specificities of reindeer herding allow medical treatment only a few times during the year, when the herds are gathered. Successful drugs have thus to provide long-term protection. However, anthelmintic drugs with high stability can be excreted unchanged in the faeces and lead to ecotoxicological concerns. Thus, the optimal drug should be sufficiently stable, but nevertheless, undergo biotransformation to metabolites with reduced biological activity. There are currently no approved treatments for brainworm infection in reindeer. Moxidectin is considered as a potential candidate because it is already used in farm animals, showing effect duration up to 100 days (Pritchard and Geary, 2019). We have established *in vitro* metabolism assays using hepatic S9 and microsomes with the aim of studying moxidectin biotransformation in reindeer. After optimisation of the assay conditions major cytochromes P450 and UDP-glycosyltransferases were characterised by analysing the depletion of model substrates and formation of the respective metabolites by all-in-one liquid chromatography high-resolution mass spectrometry (LC-HRMS). Moxidectin was metabolised by *in vitro* incubation and detected metabolites were compared to those found in other species. Finally, the predictability of the *in vitro* system was evaluated by alignment with the results of an *in vivo* study with subcutaneous application of moxidectin in female reindeer. The newly established *in vitro* method for studying biotransformation in reindeer offers the unique opportunity to determine

the metabolism of veterinary drugs in a species that is usually difficult to access and rarely approachable for *in vivo* experiments.

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P178 - CHARACTERIZATION OF 14C-ELTANEXOR METABOLISM AND DISPOSITION IN RATS

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Eltanexor (KPT-8602; (2E)-3-{3-[3,5-bis(trifluoromethyl)phenyl]-1H-1,2,4-triazol-1-yl}-2-(pyrimidin-5-yl)prop-2-enamide) is an orally bioavailable, investigational and novel exportin-1 (XPO1) inhibitor that is being evaluated in various solid tumors and hematological malignancies. Eltanexor covalently binds to cysteine residue 528 in the cargo binding pocket of XPO1 and inhibits the nuclear export of key tumor suppressor proteins (TSPs) leading to accumulation of these proteins in the nucleus.[1] This results in cell cycle arrest and selective cytotoxicity to cells with genomic alterations (e.g., neoplastic cells). The metabolism of eltanexor was characterized after oral and intravenous administration of 14C-eltanexor to Sprague-Dawley rats. Tissue distribution was evaluated using quantitative whole-body autoradiography (QWBA) in Lister Hooded rat (partially pigmented). 14C-eltanexor was dosed in rats at 4 mg/kg (oral) and 2.5 mg/kg (intravenous) with a radioactive dose level of 4.0 MBq/kg (108 µCi/kg). Radioactivity concentrations were measured in plasma, blood, blood cells, expired air and excreta by liquid scintillation counting. Time-course of tissue concentrations in the carcass were determined using a phosphor imaging technique. In addition, radioactive components in plasma and excreta were quantified by high performance liquid chromatography (HPLC) with radioactivity detection. High resolution mass spectrometry was used to identify the major metabolites in plasma and excreta and correlated with radiochemical peaks of the metabolites. Following a single oral or intravenous dose of eltanexor to male and female rats, majority of radioactivity was eliminated in the feces with minor contribution from urinary elimination. The major metabolic pathway of eltanexor was through glutathione (GSH) conjugation, hydrolysis, and N-dealkylation. The most prominent metabolites >10% dose were M6A (cysteine conjugated metabolite) and KPT 452 (3-(3,5-bis(trifluoromethyl)phenyl)-1H-1,2,4-triazole). The major circulating metabolites that contributed to >10% of total drug related materials was KPT-494 (3,5-bis(trifluoromethyl)benzoic acid) in the AUC0-168 Hamilton pooled plasma sample. The pharmacokinetics (PK) of the total radioactivity after oral administration was characterized by fast absorption peak concentration at 1 hour with gradual decrease by 4 hours; thereafter the concentration gradually increased and reached the second peak at 24 or 48 hours post dosing with significant radioactivity remaining at the last collection timepoint of 72 hours. The long half-life of total radioactivity was attributed to KPT-494, a long-lived metabolite. The maximal concentrations in tissues were generally observed between 1 and 8 hours post dose. As expected, the greatest concentrations were generally observed in the visceral and excretory organs. Concentrations in the majority of tissues were generally approaching or below the limit of quantification by 168 hours post dose. Reversible melanin binding was observed with distribution to pigmented tissues such as the uveal tract and pigmented skin. The metabolic fate of 14C-eltanexor and tissue distribution in rat was well characterized. Preliminary evaluation of circulating human metabolites using pooled steady-state samples was conducted to address questions regarding metabolites in safety testing (MIST) [2] and identified KPT-494 as a major human circulating metabolite.

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P179 - SPECIES-SPECIFIC PLASMA MATRIX MATCHING FOR MIST COVERAGE DETERMINATION

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The FDA requires any disproportionate drug metabolite, defined as greater than 10% of the total drug-related exposure (DRM), to have exposures in pre-clinical species commensurate to the human exposures; major metabolites not sufficiently covered in animals is subject to further safety testing that can be resource intensive and cause late-stage development delays. It is therefore important to understand metabolite coverage as early as possible, often using plasma or blood samples from GLP-toxicology studies and Phase I clinical trials. When measuring parent drug and metabolites via mass spectrometry, the background matrix of the sample can have significant impact on the analyte signal, potentially resulting in either suppression or enhancement of the signal depending on what matrix components are co-eluting at a given retention time. This effect can be normalized away to compare metabolite profiles between matrices using authentic

standards, radiolabeled dose preparations, or ex vivo matrix-matching. At early stages of development, matrix-matching approach is the most commonly used method. While it does not require advanced planning for radiolabeling drug or synthesizing standards; however, it still requires more effort in-lab and dilutes the signal-to-noise of the analyte peaks by mixing preclinical species and human matrices together. For low dose drugs, and ADC warheads, maximizing limits of detection can be critical for characterizing metabolites at the 10% DRM cut-off. Because these metabolite coverage analyses are comparing plasma to plasma, we would like to determine variability in analyte signal due to species-specific matrix effect and whether it is significant enough to require mitigation. To do this, we have generated metabolite mixtures for 15 commercially-available compounds with an approximate molecular weight range of 300-900 Da and including a variety of phase I and phase II metabolites; these metabolite pools are spiked with extracted plasma matrix backgrounds of human and relevant pre-clinical species (individually) and measured via identical mass spectrometry methods to determine any signal variance due to matrix effects. Minimal differences peak intensity would suggest that matrix matching is not necessary to predict major metabolite coverage, while significant variation would support continuing to matrix match even when comparing plasma to plasma between species.

P180 - SIMULTANEOUS DETERMINATION OF LANOSTEROL AND ITS MAJOR METABOLITE FF-MAS IN BIOLOGICAL SAMPLES BY LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Lanosterol, also known as lanosterin, is a tetracyclic triterpenoid, involving in the initial step of steroids biosynthesis in animals. 14-Demethylation of Lanosterol yields follicular fluid meiosis activating factor (FF-MAF), a sterol intermediate that can induce oocyte resumption of meiosis, catalyzed by lanosterol 14 α -demethylase (CYP51, a member of the cytochrome P450 family) [1]. The previously reported analysis methods for lanosterol and FF-MAF either used two different methods to analyze them individually [2] or had extended retention time (>30min) with poor sensitivity (linear range from 67.5 to 5000 ng/mL) [3]. A rapid, sensitive, and highly selective liquid chromatography-tandem mass spectrometry method was developed and validated for simultaneous determination of lanosterol and FF-MAF in biofilm liquid. The analytes were separated on a pentafluorophenyl (PFP) column, and detected by tandem mass spectrometry with an APCI interface. The method renders a lower limit of quantification (LLOQ) of 1 ng/mL for both lanosterol and FF-MAF. The chromatographic run time was within 7.5 min. The standard calibration curves for both lanosterol and FF-MAF were linear over the ranges of 1.00–1000 ng/mL in biofilm liquid. The intra- and inter-run precisions, expressed as the relative standard deviation (RSD), were less than 7.7% and 7.8% (determined from QC samples for lanosterol and FF-MAF), and accuracy was within ± 3.9 and ± 2.7 % in terms of relative error, respectively. To our knowledge, no fully validated method for simultaneous detecting lanosterol and FF-MAF was reported before. The present method was already successfully validated and applied to evaluate CYP51 inhibition in early drug discovery. The developed method can support simultaneous quantification of lanosterol and FF-MAF in tissue or serum/plasma samples with minor changes, and the short run time makes it employable for high throughput analyses.

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P181 - METABOLITE PROFILING AND IDENTIFICATION OF GIPR PEPTIDE 1: A DUAL-[14C] LABELED LIPID-CONJUGATED PEPTIDE IN DOG PLASMA, URINE, AND FECES

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Background: GIPR Peptide 1 is a lipidated 31-amino acid peptide, which shows efficacy in alleviating nausea and vomiting by acting as an agonist of the glucose insulinotropic peptide receptor (GIPR). The compound shows potential to be developed as a preventative or treatment option for diabetes and obesity, as well as a medication to alleviate symptoms like vomiting and nausea that often accompany these illnesses.

Objective: The aim of this study was to elucidate the major elimination pathway and catabolite profiles of [14C] GIPR

Peptide 1 in plasma, urine, and feces following subcutaneous administration to male Beagle dogs at a dose of 18 mg/kg. Two [¹⁴C] radiolabels were positioned on a unnatural amino acid and on the lipid chain, respectively. Understanding the catabolism and elimination of GIPR Peptide 1 is pivotal for assessing its therapeutic potential and safety profile in the treatment of nausea and vomiting.

Methods: Plasma, urine, and fecal samples were collected post-administration and analyzed using liquid scintillation counting (LSC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). This comprehensive analysis aimed to quantify the recovery, identify, and understand the biotransformation pathways of GIPR Peptide 1 and its catabolites. Results: The study revealed significant elimination of GIPR Peptide 1 through urine (85%) and feces (6%) within 168 hours post-dose. GIPR Peptide 1 predominated in plasma, representing 96% of total drug-related materials, suggesting its high plasma stability. Urine and fecal analyses revealed no detectable parent drug, indicating substantial catabolism. Twelve catabolites were identified in urine while fecal analysis revealed four catabolites, with hydrolysis identified as the primary catabolism pathway.

Conclusion: Understanding the catabolic fate of lipidated peptides like GIPR Peptide 1 is essential for developing effective and safe treatments for nausea and vomiting. This study underscores the high plasma stability of GIPR Peptide 1. Biotransformation, primary through hydrolysis, was identified as the major elimination pathway in urine and feces. The study also offers valuable insights into the application of dual labeling for conducting ADME studies on peptide compounds.

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Conflict of Interest Disclaimer: All authors of this study are employees of Takeda Pharmaceuticals and may own stock or stock options in the company. This relationship could be perceived as a potential conflict of interest in the interpretation of the study's results and conclusions. The authors have strived to present all findings and analyses in an unbiased and rigorous manner, in accordance with the highest scientific standards.

P182 - A COMBINED QUANTITATIVE AND QUALITATIVE DMPK ASSAY USING POOLED SAMPLES FOR INCREASED THROUGHPUT IN MACROCYCLIC PEPTIDE DRUG DEVELOPMENT

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Macrocyclic peptides are rapidly emerging as novel drug modalities for challenging therapeutic targets, and to date several macrocyclic peptides have been approved as therapeutic agents to treat cancer, infection, and others. One of the main hurdles in developing successful peptide drugs is to enable their oral delivery. To that end, *in vitro* assays with simulated intestinal fluid have been used to assess drug stability in the gastrointestinal tract.

The bottleneck of these peptide stability assays is the LC-HRMS data acquisition time, which averages ~40 – 53 hours across all timepoints and replicates. Enabling accurate t_{1/2} determinations and metabolite identifications in a higher-throughput manner would accelerate the peptide optimization process. To increase throughput, pooling of up to ten peptides for each timepoint in one injection was investigated.

Although pooling increases the number of peptides analyzed per injection, it creates a complex mixture of varying concentrations of test peptides as well as a potentially substantial number of unknown metabolites generated from the matrix enzymes, necessitating instrumentation capable of sensitive and selective detection.

Peptides (10 µM) were incubated in 10 mg/mL simulated intestinal fluid and timepoints were quenched with acetonitrile:methanol:formic acid containing 1 µg/mL Daptomycin as ISTD. Plates were centrifuged at 4000 rpm before supernatant was transferred individually or pooled (n=4 or 10) for injection. Samples were separated on a 1 or 5 minute gradient using a Hypersil GOLD C18 column (2.1x50 mm, 1.9µm). Eluting compounds were detected with a Thermo Scientific Orbitrap Astral mass spectrometer using Orbitrap data for quantitation of the parent compound(s) and the Astral analyzer for MS2 spectral data acquisition in data-dependent fashion.

Using the Orbitrap Astral MS with its two complementary analyzers enabled to quantitate the macrocyclic peptide parent ions and their metabolites from high-resolution Orbitrap Full Scan data in parallel with fragmentation data acquisition of detected metabolites in the Astral analyzer. The latter enabled their structure elucidation and determination of transformation sites. Using a cycle time setting of 0.3 seconds, for each Orbitrap MS1 scan approximately 45 MS2 scan events could be acquired in the Astral analyzer using 10 ms maximum injection times.

Preliminary results demonstrate that the Orbitrap Astral MS was able to reasonably reproduce the stability t_{1/2} values of each cyclic peptide in the pooled approach when compared to LC-HRMS acquisition of individual incubations (one sample/well). This demonstrated the proof of concept that sample throughput can be increased without loss of data quality by pooling samples before data acquisition. Furthermore, parallelization of Orbitrap and Astral analyzers allowed acquisition of high-quality fragmentation spectra for metabolite identification without compromising scan speed or sensitivity of the MS1-based quantitation. This enabled the sequencing and identification of unknown cyclic peptide ring

opening metabolites using Molecular Discovery's MassMetaSite software.

In summary, the presented work will demonstrate the increased throughput of combined stability testing and metabolite identification using sample multiplexing to speed up the development of macrocyclic peptide drugs.

P183 - CHARACTERIZATION OF PYRAZINAMIDE METABOLISM *IN VITRO*: INSIGHTS INTO THE ROLE OF THE ALDEHYDE OXIDASE, AND XANTHINE OXIDASE

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Pyrazinamide (PZA) is an antimycobacterial agent employed as a first-line anti-tuberculosis (anti-TB) drug that exhibits unique sterilizing activity for both drug-sensitive and multidrug-resistant tuberculosis (MDR-TB) (1). Several aspects of the metabolism and pharmacokinetics of the PZA in humans remain unknown, even though it has been available for 70 years. Therefore, the aim of the study was to characterize the enzymes that are involved in the metabolism of PZA. Metabolite profiling and incubation of PZA with pooled Human liver cytosol (HLC) confirmed the formation of pyrazinoic acid (PA), 5-hydroxy-pyrazinoic acid (5-OH-PA), and 5-hydroxy-pyrazinamide (5-OH-PZA) using LC-MS/MS with the authentic standards. We found that PZA undergoes metabolism to 5-OH-PA through sequential metabolic steps involving the PA and 5-OH-PZA. Furthermore, we also identified the enzymes involved in the formation of the metabolites in humans using a combination of chemical inhibition and activity assays using recombinant enzymes. Amidase and Molybdoflavoenzymes such as xanthine oxidase (XO) have previously been implicated in the metabolism of this drug (2). The involvement of amidase, Aldehyde Oxidase (AO), and XO in the metabolism of PZA was established using specific inhibitors. Bis-p-nitrophenyl phosphate (BNPP), a selective amidase inhibitor, hindered the formation of the pyrazinamide metabolites. Moreover, Selective AO inhibitors such as raloxifene, chlorpromazine, estradiol, and amodiaquine, as well as selective XO inhibitors like allopurinol and oxypurinol, potentially inhibited the formation of the PZA metabolites in pooled HLC. These findings were further confirmed using human recombinant AO and XO. Kinetic analyses for PA and 5-OH-PZA from PZA in recombinant AO were confirmed using the Michaelis-Menten enzymatic model. The K_m and V_{max} values for PA and 5-OH-PZA were 10.57 μM and 136.5 nmol/min/mg protein, 13.90 μM , and 37.67 nmol/min/mg protein, respectively, resulting in a CL_{int} value of 12.91 and 2.71 ml/min/mg protein. Additionally, the formation of 5-OH-PA from 5-OH-PZA and PA in recombinant AO had an estimated CL_{int} of 12.70, and 3.83 ml/min/mg protein, respectively. In conclusion, both Amidase and AO participated in the metabolism of the PA and 5-OH-PA, whereas AO and XO were responsible for catalyzing PZA to 5-OH-PZA and PA to 5-OH-PA. Based on the findings of this study, we have evidence that three enzymes namely amidase, AO, and XO contribute to the PZA metabolism, among them amidase and AO are responsible for the formation of PA and 5-OH-PA, while only AO and XO were involved in the metabolism of 5-OH-PZA and 5-OH-PA in pooled HLC.

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P184 - BRIDGING TARGETED METABOLOMICS TO GLOBAL PROTEOMICS APPROACH TO CLARIFY RIBAVIRIN-INDUCED RESISTANCE IN BREAST CANCER MCF-7 CELLS

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INTRODUCTION: Ribavirin, a synthetic nucleoside extensively used for the treatment with hepatitis C infections, is already known to be effective in cancer therapy. However, it is reported that ribavirin treatment frequently induces resistance especially in breast cancer cells¹, which mechanism remains unclear. Phosphorylated ribavirin (Fig. 1), ribavirin-monophosphate (RMP) and -triphosphate (RTP), exert antitumor effects mediated by the inhibition against inosine monophosphate dehydrogenase (IMPDH) and eukaryotic translation initiation factor 4E (eIF4E). Therefore, integration of metabolomics and proteomics would be required for understanding detailed scheme of ribavirin-induced resistance in breast cancer. This study aimed to clarify the crucial factors associated with ribavirin-induced resistance focusing on the relationship between ribavirin-driven intracellular metabolic changes and comprehensive protein expression alteration. **METHODS:** MCF-7 (WT), a breast cancer cell line, was treated with 50 μM ribavirin, leading to establishment of Ribavirin-resistant MCF-7 (RES). Triple Quad LC-MS/MS system was used for analyzing ribavirin-associated metabolites, while SWATH (sequential window acquisition of all theoretical fragment ion spectra)-MS method was used for analyzing comprehensive protein expression levels, while **RESULTS/DISCUSSION:** RES cells were successfully established with 10-fold higher IC₅₀ value of ribavirin than that in WT. Metabolomics analysis revealed that the intracellular concentrations of RMP and RTP were significantly lower in RES than in WT under the treatment with 100

μM ribavirin. Additionally, the ratio of metabolites (xanthine monophosphate (XMP), guanine triphosphate etc.) derived from inosine monophosphate (IMP) to IMP were also high in RES. These findings were clearly supported by proteomics data such as downregulation of adenosine kinase (ADK), which converts ribavirin into its phosphate metabolites, and upregulation of IMPDH1/2, which converts IMP into XMP, in RES. Thus, phosphate metabolism of ribavirin and phosphorylated ribavirin-associated proteins would be closely related to ribavirin resistance. Considering the metabolomics data, to further identify the factors involved in ribavirin resistance, phosphorylated ribavirin-associated protein expression changes were highlighted. Using ADK inhibitor, the 107 proteins, which were downregulated in WT, while upregulated in RES were determined as phosphorylated ribavirin-associated protein. Pathway analysis revealed that the proteins annotated with Ribosome Biogenesis were greatly enriched in the protein group, indicating that these proteins would contribute to the development of phosphorylated ribavirin-driven resistance. CONCLUSION: The integrated metabolomics and proteomics analysis revealed that phosphorylated ribavirin-associated proteins would contribute to development of ribavirin resistance, and firstly proposed that Ribosome Biogenesis could be a possible target to overcome the resistance in breast cancer cells.

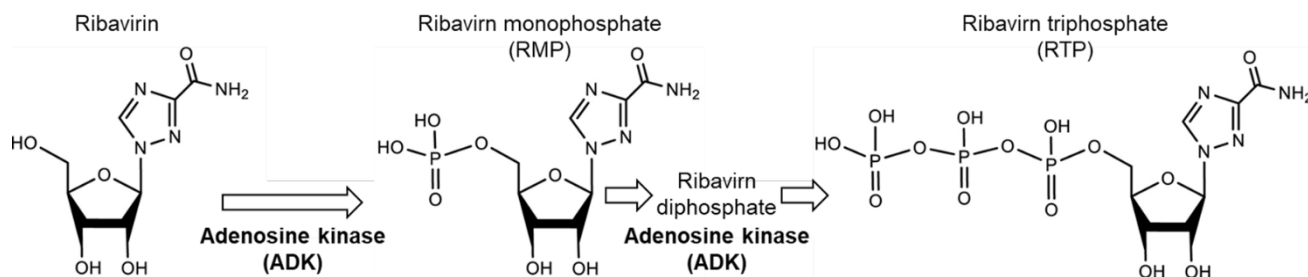


Fig. 1. Structures of ribavirin phosphate metabolites mediated by adenosine kinase activity

P185 - METABOLIC FLUX ANALYSIS UNVEILED TISSUE- AND SEX-SPECIFIC TRYPTOPHAN METABOLISM IN A GUT MICROBIOME DEPENDENT MANNER IN HUMANIZED PXR-TRANSGENIC MOUSE

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The host nuclear receptor pregnane X receptor (PXR) and the gut microbiome have each been shown to play major roles in xenobiotic biotransformation and metabolic diseases and are intertwined through microbial metabolite-mediated PXR activation. For instance, indole-3-propionic acid, a microbial metabolite of tryptophan, serves as a natural PXR ligand with anti-inflammatory and anti-obesity properties. In this study, we utilized ¹³C11-L-tryptophan isotope tracing-based metabolic flux analysis using humanized PXR transgenic (hPXR-TG) mice to explore the molecular basis underlying the regulation of tryptophan metabolism by gut microbiome, sex, and tissue specificity. We hypothesize that the gut microbiome regulates the incorporation of tryptophan-derived carbons into key metabolic pathways of the host in a tissue- and sex-specific manner. We orally gavaged ¹³C11-L-tryptophan (20 mg/kg) to 3-month-old adult conventional (CV) and germ free (GF) hPXR-TG mice (n=3-4 per group) and collected various tissues after 30 min. Liquid chromatography-mass spectrometry (LC-MS) was used to perform metabolic flux analysis in serum, liver, duodenum, jejunum, ileum, colon, and brain. Principal component analysis (PCA) revealed distinct gut microbiome-dependent variations in metabolic fluxes of tryptophan-derived ¹³C across all tissues and in both sexes. Colon exhibited the most significant regulation of gut microbiome-mediated metabolites derived from tryptophan, followed by the small intestine sections and liver, whereas the brain had minimal impact. Interestingly, GF male mice demonstrated elevated levels of indoxyl sulfate, a product of host sulfotransferases (Sults) from indole, most prominently in the liver and multiple intestinal segments. This aligns well with our previous findings that mRNA of gut microbiome diverted the incorporation of tryptophan-derived carbons into various amino acid metabolism pathways in the liver and intestinal sections, including metabolism of aspartate, tyrosine, glutathione, taurine, and hypotaurine, cysteine, glutamate, homocysteine, methionine, glycine, serine, valine, leucine, isoleucine, lysine, and histidine. Additionally, bile acid biosynthesis, and purine metabolism were impacted. In CV mice, PCA showed distinct sex-dependent variations in liver, duodenum, and colon. Most of the sex differences were observed within the metabolism pathways related to liver diseases and metabolic syndromes. In conclusion, our study elucidates the chemical foundation underlying the necessity of microbiome in tryptophan metabolism and its interplay with crucial intermediary metabolic pathways in a tissue- and sex-specific manner.

P186 - APPLICATION OF PROTEOMIC CORRELATION PROFILING TECHNIQUE BASED ON PROTEOMIC DATA TO IDENTIFY ENZYMES INVOLVED IN METABOLISM OF DRUGS OF CONCERN

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In drug discovery, identifying enzymes involved in the metabolism of drug candidate is crucial for predicting the degree of pharmacological effects and safety based on drug exposure. While various methods have identified enzymes responsible for drug metabolism, most of them target known drug metabolizing enzymes. Therefore, this study evaluated the usefulness of Proteomic Correlation Profiling (PCP) as a method to comprehensively search and identify unidentified enzymes involved in drug metabolism by assessing the correlation with enzyme activity using well-accepted probe substrates of human drug metabolizing enzymes 1). First, the metabolic enzyme activity were measured using a total of 18 substrates of cytochrome P450, uridine 5' diphospho-glucuronosyltransferase, hydrolase, and oxidoreductase in human liver cytosol and microsomes derived from 25 donors, and conducted proteomic analysis of these liver cytosols and microsomes. Then, proteins were classified considering their functions and performed correlation analysis between metabolic enzyme activities and protein expression levels of 294 oxidoreductase, 521 hydrolase and 506 transferase in microsome, and 260 oxidoreductase in cytosol. As a result, for 13 metabolic reactions, the correlation coefficients with the responsible enzymes were 0.7 or higher, and the correlation ranks were from 1st to 3rd. For the remaining 5 reactions with a correlation coefficient of less than 0.7, the ranks were from 2nd to 19th. Factors that lowered the correlation rank included small variations in the expression levels of the responsible enzymes among donors, accidental high correlations with other enzymes, the presence of inactive enzyme forms, or the influence of genetic polymorphisms. In conclusion, this method was useful for narrowing down candidates of unidentified enzymes involved in drug metabolism, and it was shown to enable timely and accurate identification. We introduce examples where we were able to identify the enzymes responsible for the metabolism of nintedanib and nabumetone with this methodology.

P187 - IN VITRO METABOLISM OF KOBUSIN IN HUMAN LIVER PREPARATIONS

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Kobusin, a tetrahydrofurofuranoid lignan with 1,3-benzodioxole group isolated from *Castilleja tenuiflora* and *Flos Magnoliae*, exhibits anti-inflammatory, antihypertension and cancer prevention activities. However, there was no report on the metabolism and pharmacokinetics of kobusin. This study was performed to identify *in vitro* metabolic pathways of kobusin in human hepatocytes and liver microsomes using liquid chromatography–high-resolution mass spectrometry. The hepatic extraction ratio of kobusin was 0.80, suggesting that it undergoes extensive degree of hepatic metabolism. Kobusin was metabolized to 4 phase 1 metabolites such as kobusin catechol (M1), O-desmethylnobusin (M2 and M3), and hydroxynobusin (M4) by human liver microsomal incubation of kobusin in the presence of nicotinamide adenine dinucleotide phosphate (NADPH), suggesting that cytochrome P450 (CYP) may be responsible for the formation of M1-M4 from kobusin. Hepatocyte incubation of kobusin produced 4 phase 1 metabolites (M1-M4) and 13 phase 2 metabolites including O-methyl-M1 (M5 and M6) via catechol O-methyltransferase (COMT), six glucuronides of M1, M2, M3, M5, and M6 (M7-M12), and 5 sulfates of M1, M2, M3, M5, and M6 (M13-M17). Two glutathione (GSH) conjugates of M1 were identified after incubation of kobusin with human liver microsomes in the presence of NADPH and GSH, but they were not detected in the hepatocytes. In conclusion, kobusin is extensively metabolized, producing 17 metabolites in human hepatocytes catalyzed by CYP, COMT, UDP-glucuronosyltransferase, and sulfotransferase. These results can help in clarifying the involvement of metabolizing enzymes in the pharmacokinetics and drug interactions of kobusin and in elucidating GSH conjugation associated with the reactive intermediate formed from M1 (kobusin catechol).

P188 - METABOLISM OF ZASTAPRAZAN IN HUMAN AND ANIMAL HEPATOCYTES

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Zastaprazan (JP-1366) is being developed as a novel potassium-competitive acid blocker and is undergoing clinical phase II studies. The purpose of the present study is to characterize the comparative metabolism of JP-1366 in human, dog, monkey, mouse, and rat hepatocytes by using liquid chromatography–high-resolution mass spectrometry and elucidate drug metabolizing enzymes responsible for JP-1366 metabolism. JP-1366 incubation with the hepatocytes from human, mouse, rat, dog, and monkey resulted in the formation of 18 phase I metabolites and 5 phase II metabolites [i.e., N-dearylation (M6), hydroxylation (M1, M19, M21), dihydroxylation (M7, M8, M14, M22), trihydroxylation (M13, M18), hydroxylation and reduction (M20), dihydroxylation and reduction (M9, M16), hydrolysis (M23), hydroxylation and glucuronidation (M11, M15), hydroxylation and sulfation (M17), dihydroxylation and sulfation (M10, M12), N-dearylation and hydroxylation (M3, M4), N-dearylation and dihydroxylation (M5), and N-dearylation and trihydroxylation (M2)].

CYP3A4 and CYP3A5 played major roles in the metabolism of JP-1366 to M1, M3, M4, M6, M8, M9, M13, M14, M16, M18, M19, M21, and M22 on the basis of the cytochrome P450 (CYP) screening test and immune-inhibition analysis with CYP antibodies. UDP-glucuronosyltransferase (UGT) 2B7 and UGT2B17 were responsible for the glucuronidation of M1 to M15.

P189 - UNVEILING THE MECHANISTIC INVOLVEMENT OF THE HUMAN MICROBIOME AND HUMAN DRUG-PROCESSING GENES IN MODULATING THE HEPATIC TRANSCRIPTOME IN MICE *IN VIVO*

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The gut microbiome acts as an important metabolic sensor that communicates with the liver to modulate xenobiotic biotransformation and nutrient homeostasis. The pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are xenobiotic-sensing nuclear receptors that also play important roles in regulating intermediary metabolism. The cytochrome P450 3A (CYP3A) enzymes are major Phase I enzymes involved in the metabolism of drugs, environmental toxicants, and various endogenous molecules. The goal of the present study was to determine how the gut microbiome and major drug-processing genes of human (h) origin modulate the hepatic transcriptome in mice *in vivo*. We utilized wild-type (WT) and hPXR-hCAR-CYP3A4/CYP3A7 humanized transgenic mice (hTg) to unveil the species-specific effect of the four drug processing genes on the hepatic transcriptome. In addition, we investigated three different microbiome communities, including the gut microbiomes from WT, hTg mice, and human donors. Bulk RNA sequencing was conducted on the livers of germ-free (GF, without microbiome) and conventional (CV, with microbiome) in 2-3 months old male and female hTg and WT mice (n=3-4 per group). Fecal microbiome of healthy male and female human donors (pooled from 4 donors per sex) was introduced to sex-matched hTg GF mice by fecal microbial transplant (FMT) with a one-month colonization period. Metagenomic shotgun sequencing (MGS) was performed using the large intestinal contents of CV and hTg mice. RT-qPCR confirmed the human-specific expression of the drug-processing genes in the hTg mice. The introduction of the four human drug-processing genes in male mice led to up-regulated pathways related to autophagy, oxidative stress, and pathogen response, while simultaneously decreasing pathways associated with ATP production. In female mice, this alteration also influenced cholesterol metabolism. FMT resulted in 61.6 – 75.9% match in the colonized gut microbes between the human donors and the mouse recipients. Compared to the mouse microbiome, we observed that species in the following genera were uniquely enriched by human stool FMT: Roseburia, Dorea, and Blautia in males and Parabacteroides, Akkermansia muciniphila, and Bacteroides in females. In males, the up-regulated hepatic pathways that were uniquely regulated by the human microbiome were involved retinol metabolism and lipid localization in males (no down-regulated pathways were observed). In females, the transplantation of human microbiome uniquely up-regulated genes involved in xenobiotic and lipid metabolism whereas the down-regulated genes were involved in immune response. Correlation analysis unveiled distinct human-derived microbes, including Dorea scindens, Blautia coecoides, and Roseburia intestinalis in males and Bacteroides uniformis, Bacteroides faecis, Parabacteroides distasonis, and Akkermansia muciniphila in females, that are candidate regulators of the hepatic metabolic pathways. In conclusion, using transcriptomic and metagenomic approaches, our results provide the first evidence of the how species differences in both the gut microbiome sources and the four drug-processing genes contribute to the regulation of major hepatic metabolic pathways involved in xenobiotic biotransformation and intermediary metabolism.

P190 - *IN VITRO* PLASMA OR WHOLE BLOOD STABILITY METHOD FOR ADCS

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Antibody-drug conjugates (ADCs) are typically designed to selectively target tumor cells to improve cell-killing efficiency and reduce toxicity in healthy tissues. For efficacy and safety concerns, they must be stable in circulation to carry the payload to the site of the targeted cell. Therefore, understanding the release of small molecule cytotoxic drugs from ADC in plasma or whole blood *in vitro* should help identify factors affecting *in vivo* payload release. In a previous study, lithium heparin was used as an anticoagulant rather than EDTA so as not to inhibit any enzymes in the biological matrix. Unfrozen plasma was also used in the ADCs study, but there was no experimental data to illustrate this choice. In our study, the effects of corresponding experimental conditions were explored.

In the study of *in vitro* stability, the factors that affect the release of payload from ADCs include 1) Anticoagulant (EDTA-K2 or heparin sodium), 2) Plasma status (fresh or frozen), 3) Sealed or breathable condition during incubation, 4) Plasma or whole blood, 5) Changes in enzyme activities after long incubation periods. Therefore, we carried out stability experiments comparing the payload concentrations for the five conditions using DS8201. Results showed that the plasma with heparin sodium as the anticoagulant was more suitable than EDTA-K2 anticoagulant plasma since EDTA-K2 may inhibit enzyme activity. There was no clear difference between fresh and frozen plasma and sealed and breathable conditions during incubation. The released concentration of Dxd in whole blood was higher than in plasma at the same timepoint, such as 24h. Propantheline, enalapril, bisacodyl, and procaine were selected as marker substrates of plasma hydrolases and incubated with fresh plasma or plasma previously incubated for 21 days to obtain the half-lives. The

changes of half-lives of these compounds were within 2-fold (between 1.01 and 1.48). The changes indicating that plasma enzyme activity is maintained after 21 days of incubations. The pH changes during incubation ranged from 7.4 to 8.1. Different pH values may lead to changed hydrolysis rates, but considering that the changes of half-lives for the 4 marker substrates were acceptable, we considered that the activity of their enzymes was not affected. Therefore, we propose to use the heparin sodium anticoagulant frozen plasma when assessing the release of payload for ADCs.

We also validated the method with brentuximab vedotin in plasma from rats, monkeys, or humans. The % theoretical release max of MMAE from monkey and human plasma was consistent with those reported in the literature. The much higher %theoretical release max of MMAE in in-house rat plasma study, when compared with that in literature, may be due to different plasma sources.

In conclusion, we evaluated the factors affecting the release of payload from ADCs in plasma stability experiments and determined the optimum incubation conditions to better evaluate *in vitro* ADC stability.

P191 - ANTISENSE OLIGONUCLEOTIDES' METABOLITE IDENTIFICATION USING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY—HIGH RESOLUTION MASS SPECTROMETRY: APPLICATION FOR NON-HUMAN PRIMATE STUDY SAMPLES OF TOFERSEN

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Metabolite identification (MetID) of synthetic oligonucleotides such as antisense oligonucleotides (ASO) provide insight into clearance mechanism. MetID data from nonclinical safety species samples vs. human plasma and urine samples may qualitatively show that overall human metabolites are exposed in animal safety testing. ASO MetID can be technically challenging: highly polar analytes with polyanionic backbone, broad charge state distribution and alkali metal cation adduct formation in mass spectrometry, and difficulty in sample extraction and preparation. An effective methodology for ASO MetID was established in the Biogen laboratories, consisting of: (1) sample preparation procedures developed at Ionis Pharmaceuticals, involving liquid-liquid extraction followed by ion-pairing reverse phase solid-phase extraction, (2) analysis of metabolite samples by hydrophilic interaction liquid chromatography (HILIC)—high resolution mass spectrometry and tandem mass spectrometry (HRMS and MS/MS), using an Orbitrap Fusion™ Lumos™ mass spectrometer, (3) automatic data processing to identify ASO metabolites, using Oligonucleotide Analysis of the software BioPharma Finder™ 4.0. Compared with ion-pairing reverse phase LC commonly used for ASO analysis, of which residual ion-pairing reagents in LC-MS such as tertiary amines would cause severe signal suppression in positive electrospray ionization, HILIC allows us to readily switch from ASO MetID to other applications without thoroughly cleaning the instrument. Furthermore, HILIC gives lower charge states than ion-pairing reverse phase LC. Examples to be presented in this poster include MetID in non-human primate (NHP) liver and kidney tissue samples collected one-week after the last of repeated intrathecal doses of tofersen (BIIB067). Tofersen is a 2nd generation, mixed backbone, 20-mer ASO. The ASO is a “gapmer” with five 2'-O-(2-methoxyethyl) (2'-MOE) nucleosides at the 5' and 3'-ends of the molecule, respectively, flanking a gap of ten 2'-deoxynucleosides. Metabolites in NHP tissue samples were unambiguously identified by deconvoluted mono-isotopic mass and LC-MS/MS CID & HCD spectra having sufficient fragment coverage. Semi-quantitative feature of the ASO MetID was assessed using several putative metabolite standards of tofersen. MS signal over-represents shorter mers, whereas UV signal over-represents longer mers. A total of 12 metabolites of tofersen, being truncated oligonucleotides presumably mediated by endonucleases and exonucleases, were identified in NHP tissue samples at greater than 1% but all lower than 10% of total drug-related LC-HRMS peak areas.

P192 - ELUCIDATION OF THE PROPERTIES OF FATTY ACID AMIDE HYDROLASE 2 AS A DRUG-METABOLIZING ENZYME

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Purpose: DS-8500a has been reported to undergo amide hydrolysis as one of its major metabolic pathways, and species differences in this hydrolysis have been observed [1,2]. It has also been suggested that fatty acid amide hydrolase (FAAH) 2 is the main enzyme responsible for this hydrolysis. However, there are no previous reports of FAAH2 functioning as a drug-metabolizing enzyme. Therefore, the main objective of this study was to investigate the potential of FAAH2 to hydrolyze novel synthetic compounds, using DS-8500a as a model compound. Methods: *in vitro* metabolic studies were conducted by incubating DS-8500a with FAAH1, FAAH2, carboxylesterase (CES) 1, and CES2 expression systems, as well as human liver microsomes. The hydrolysis metabolites of these samples were quantified using LC-MS/MS. Results and Discussion: The results of *in vitro* metabolism studies using FAAH and CES expression systems demonstrated that DS-8500a was predominantly hydrolyzed by FAAH2. It was also observed that this hydrolysis reaction mediated by FAAH2 was inhibited by URB597, a FAAH2 inhibitor. Furthermore, concentration-dependent formation of hydrolytic metabolite of DS-8500a was observed in human liver microsomes and various expression systems. In human liver microsomes, two Km values for a high-affinity site and a low-affinity site were obtained. The Km value of the high-

affinity site in human liver microsomes was found to be comparable to that obtained in the FAAH2 expression system. Additionally, the K_m value for the low-affinity site was close to those observed in the FAAH1 and CES1 expression systems. These findings suggest that the high- and low-affinity hydrolysis of DS-8500a in human liver microsomes corresponds to FAAH2, and FAAH1 and/or CES1, respectively. Conclusions: This study revealed that FAAH2 can function as a metabolic enzyme for DS-8500a, a xenobiotic compound. In future studies, we plan to further investigate species differences in the hydrolysis of DS-8500a using liver microsomes and examine whether similar hydrolysis occurs via FAAH2 in analogous compounds of DS-8500a.

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P193 - DETECTION OF HARD ELECTROPHILIC REACTIVE METABOLITES FORMED FROM ALICYCLIC AMINES USING GLUTATHIONE TRAPPING

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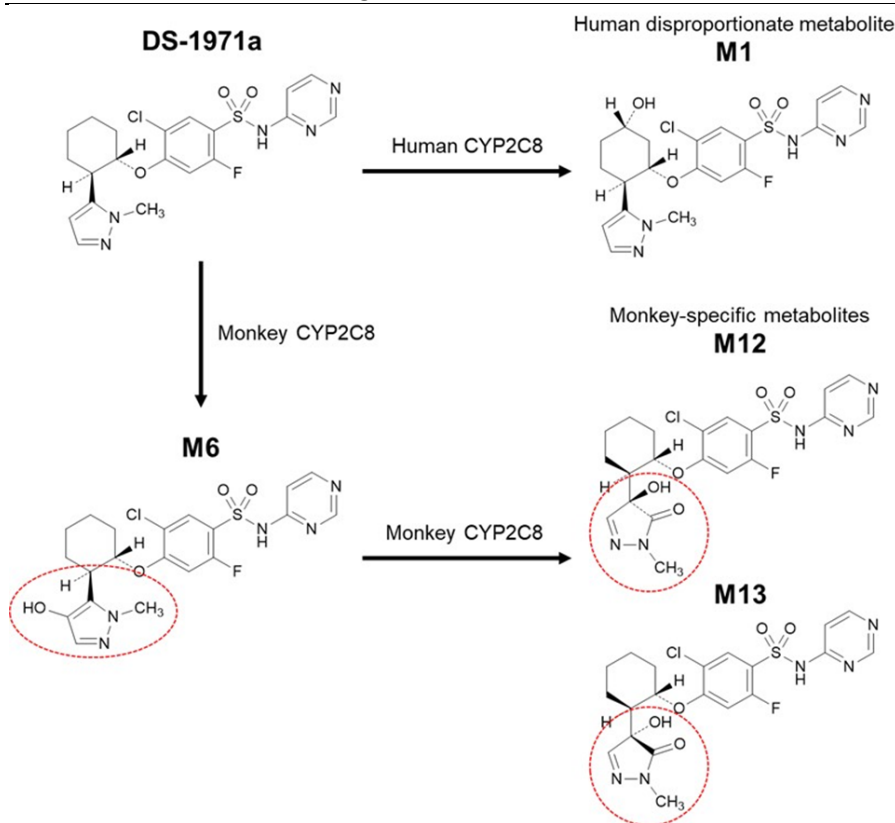
Alicyclic amines are ubiquitous compounds with manifold uses in medicinal chemistry. They can be metabolically activated to form reactive intermediates (e.g. iminium ion, aldehyde, epoxide, α,β -unsaturated carbonyl, iminolactam). Reduced glutathione (GSH) and its various derivatives have been often used to trap soft electrophilic intermediates (e.g. quinone, epoxide, α,β -unsaturated carbonyl). Across a large GSK GSH trapping dataset, some alicyclic amines were observed to form direct cysteine or cysteinylglycine conjugates, accounting for approximately 10% of the analyzed compounds with positive results. These results suggest that electrophilic intermediates were generated from the alicyclic amines (e.g. piperidine) and trapped as cysteine and/or cysteinylglycine conjugates. This is unusual since these conjugates are believed to be formed from hard electrophiles typically requiring hard nucleophile trapping agents (e.g. cyanide) and cannot be trapped by soft nucleophiles. Compound A and tofacitinib, both containing a piperidine group, were used as alicyclic amine examples to explore the bioactivation pathways generating hard electrophiles. High-resolution mass spectrometry was applied for metabolite identification and characterization. Not only were the direct cysteine and cysteinylglycine products observed, but also the aldehyde cysteine and cysteinylglycine conjugates were reported. These former products were proposed to be the result of reactions with hard electrophile iminium ion intermediates. The mechanisms to form the various cysteine and cysteinylglycine conjugates were investigated. This is the first report to show that glutathione can be applied to indicate hard electrophile formation from alicyclic amines.

P194 - MONKEY CYP2C8-MEDIATED FORMATION OF REARRANGED METABOLITES OF THE SELECTIVE Nav1.7 INHIBITOR DS-1971a

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DS-1971a is a potent and selective inhibitor of voltage-gated sodium channel Nav1.7. Our recent studies have shown that DS-1971a is a substrate of both aldehyde oxidase and cytochrome P450 (CYP), that there is a marked species difference in the metabolism of DS-1971a [1], and that a human disproportionate metabolite (M1) was generated by CYP2C8-mediated primary metabolism [2]. In addition, mass balance study of radiolabeled DS-1971a in humans and experimental animals (monkey, dog, rat, and mouse) suggested that monkey-specific metabolites (M12 and M13) were detected in plasma, urine, and feces [1, 2]. The aim of this study was to confirm the structures of M12 and M13 and characterize the CYP isoform responsible for the formation of M12 and M13. The structures of M12 and M13 were elucidated by X-ray analysis and NMR analysis of the metabolites to reveal that a rearrangement reaction occurs after di-oxidation of pyrazole ring. Incubation studies with monkey CYP-expressing Bactosomes indicated that M6 (a monoxide at the pyrazole ring) was generated by CYP2C8-mediated metabolism from DS-1971a, and M12 and M13 (dioxides at the pyrazole ring) were generated by CYP2C8-mediated metabolism from M6. These results suggested that the formation of rearranged metabolites M12 and M13 is predominantly catalyzed by CYP2C8 in monkey. Considering that M1 is also generated by CYP2C8-mediated metabolism in humans, species differences in CYP2C8-mediated metabolism may lead to the formation of a human disproportionate metabolite of DS-1971a.



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P195 - PHYSIOLOGICALLY BASED PHARMACOKINETIC ANALYSIS OF THE IMPACT OF CONTROLLED-RELEASE FORMULATIONS ON ORAL ABSORPTION OF CYP3A4 SUBSTRATES USING MODIFIED ILIAD MODEL

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Purpose: Controlled-release (CR) drug delivery systems have many advantages over immediate-release (IR) formulations from both clinical (e.g. safety and efficacy) and industrial (e.g. market expansion and product life-cycle management) perspectives. However, effective CR formulations are not necessarily easy to design and develop, because the controlled release of drugs from formulations may be affected by multiple factors, that is the physicochemical properties of the drug and the physiological states of the GI tract. Particularly, the expression and activity of metabolic enzymes and their regional differences may affect the nonlinear absorption and bioavailability of the substrate drugs in CR formulation. Indeed, the AUC of simvastatin, a substrate of CYP3A4, from a CR formulation was reportedly increased compared to that from an IR formulation¹), whereas the AUC of nifedipine was comparable between IR and CR formulations²). Therefore, the present study aims to quantitatively analyze and understand the effect of CR formulations on the oral absorption of CYP3A4 substrates using our ILIAD model with simvastatin and nifedipine as model substrates. Methods: Permeability and the intracellular unbound fraction of drugs were evaluated by the transport study with Caco-2 cells. CYP3A-catalyzed drug metabolism was measured using human intestinal microsomes. Rat *in vivo* studies were carried out to evaluate plasma concentration profiles and respective availability of drugs in the upper and middle small intestine, with/without 1-aminobenzotriazole (ABT), a non-specific CYP inhibitor. A PBPK analysis with a modified ILIAD model was performed using the Napp program. Results and Discussion: The modified ILIAD model predicted a significant increase in intestinal availability (F_g) of simvastatin and nifedipine in the CR formulation compared to the IR formulation. Because CYP3A4 is highly expressed in the upper part of the intestine, these results are likely due to decreased CYP3A4-mediated first-pass metabolism in the case of the CR formulation. Such a hypothesis is supported by the rat *in vivo* studies that AUC and C_{max} remarkably increased by administering simvastatin directly into the middle small intestine compared to the upper small intestine. This difference disappeared in the presence of ABT, implying the

higher expression and activity of the CYP3A in the upper small intestine. On the other hand, nifedipine in the CR formulation showed a decrease in fraction absorbed (Fa) along with an increase in F_g value, resulting in no change in AUC for the IR and CR formulations. These findings indicated that CR formulations may complicate drug absorption through changes in intestinal metabolism due to drug release. Conclusions: Our simulation approach revealed that the pharmacokinetic profiles of CYP3A substrates after oral administration are susceptible to CR formulation due to avoidance of intestinal first-pass metabolism. This also implied that CR formulations of CYP3A4 substrates can improve oral bioavailability.

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P196 - MECHANISM BY WHICH AADAC REGULATES HEPATIC LIPID LEVEL *IN VIVO*

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Non-alcoholic fatty liver disease (NAFLD), which is defined by the pathological accumulation of lipids in the liver without alcohol abuse, is the most common liver injury. Our group has characterized arylacetamide deacetylase (AADAC) as a drug-metabolizing enzyme catalyzing the hydrolysis of clinical drugs containing an acetyl moiety. Recently, it has been suggested that AADAC may be involved in lipid metabolism since overexpression of AADAC in culture cells results in decreased intracellular lipid accumulation. However, since AADAC prefers compounds containing an acetyl moiety, it is unlikely that AADAC hydrolyzes triglyceride (TG) and cholesterol esters that are esterified with long-chain fatty acids abundant in the body. This study aimed to clarify the mechanism by which AADAC regulates hepatic lipid level *in vivo*. Hepatic lipid level in Aadac knockout (KO) mice tended to be higher than that in wild-type (WT) mice (male, C57BL/6J, 8 weeks old). When a high-fat high-sucrose diet was fed for 16 weeks, hepatic TG and total cholesterol (TCHO) levels were more highly increased ($P = 0.16$ and 0.054 , respectively) in Aadac KO mice than in WT mice, whereas plasma TG and TCHO levels showed opposite trends ($P = 0.35$ and 0.044 , respectively). Hepatic mRNA levels of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Hmgcr) and squalene epoxidase (Sqle), which are cholesterol synthesis enzymes, were higher in Aadac KO mice than in WT mice. These mRNA levels are known to be positively correlated with hepatic iron level in mice, and our laboratory recently revealed that Aadac KO mice showed significantly higher hepatic ferrous iron level than WT mice due to lowering hepatic level of ceruloplasmin, which has a ferroxidase activity (oxidation of Fe²⁺ to Fe³⁺). Co-immunoprecipitation and proteomics revealed the interaction of AADAC with ceruloplasmin. Thus, it was suggested that the increased hepatic iron level by deletion of Aadac induces hepatic lipid accumulation. It has been reported that ceruloplasmin is degraded by endoplasmic reticulum (ER)-associated degradation, which is essential for clearing misfolded proteins from the ER to decrease ER stress. Considering the possibility that AADAC suppresses lipid accumulation by decreasing intracellular ferrous iron level via the protection of ceruloplasmin against degradation under ER stress, 1.0 mg/kg tunicamycin, an ER stress inducer was intraperitoneally administered to mice (female). As a result, the hepatic ferrous iron level was increased to a higher degree in Aadac KO mice than in WT mice by tunicamycin administration, with concomitant significantly higher hepatic TG and TCHO levels. Thus, we found that Aadac attenuates lipid accumulation via protecting ceruloplasmin against ER stress to prevent the increase in hepatic ferrous iron level. This study provides new insight into the mechanism by which AADAC regulates lipid metabolism via iron metabolism rather than directly hydrolyzing lipids.

P197 - IDENTIFICATION AND CHARACTERIZATION OF PHASE II METABOLITES OF RESOLVINS D1 and D5.

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Specialized pro-resolving mediators (SPMs) are oxidized lipid mediators derived from arachidonic acid, docosahexaenoic acid, or eicosapentaenoic acid primarily by the action of cyclooxygenase (COX) or lipoxygenases (LOX). This generates multiple classes of SPMs including lipoxins, resolvins, protectins, and maresins. SPMs can be produced by a wide variety of cell types including macrophages, neutrophils, and lymphocytes, and, as the name describes, SPMs function is to resolve inflammation. SPMs have been shown to reduce joint pain and stiffness from arthritis, reduce vessel inflammation in coronary heart disease, promote plaque stability, improve the metabolic parameters and complications of diabetes, and stimulate wound healing and tissue regeneration. Thus, SPMs and their biochemical precursors are now marketed and available for purchase as dietary supplements. Yet, despite these findings on their biological activities, the amount to which SPMs are formed *in vivo* is a question of intense debate. Direct measurement of SPMs in plasma and other biological samples revealed that concentrations are generally quite low. It is well understood that other oxylipins, including

prostaglandins and leukotrienes, exert their biological activity and are then rapidly inactivated via metabolic transformation. But, studies on the metabolism of SPMs are limited. SPMs are characterized by the presence of multiple hydroxy groups, which can be reduced by phase I metabolism. Cysteinyl-conjugated SPMs are also generated via mechanisms similar to the cysteinyl leukotrienes. Yet, despite evidence that SPMs can be transformed by both phase I and II metabolism, most studies that have sought to quantify endogenous SPM production have focused only on the measurement of unmetabolized SPMs. To that end, we have undertaken experiments to identify and characterize metabolites of the SPMs resolvin (Rv) D5 and RvD1 by human liver microsomes. Using targeted and untargeted metabolomic approaches, glucuronide and sulfate metabolites of resolvins have been identified by liquid chromatography-mass spectrometry. Further, the metabolism of these molecules by specific enzymes has been tested. We have also demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs), which are metabolized via glucuronidation, affect the formation of RvD metabolites. Collectively, our findings indicate that SPM metabolism is more complex than previously appreciated. We anticipate that the completion of this work will improve the understanding about SPM – and oxylipin– metabolism and outline a strategy to comprehensively evaluate the formation of these important signaling molecules *in vivo*.

P198 - METABOLIC STABILITY ASSESSMENT OF THE TAU PET TRACER [18F] GTP1

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[18F]GTP1 (1,1-dideutero-2-(1-(benzo[4,5]imidazo[1,2-a]pyrimidine -2-yl)piperidin-4-yl)-1-[18F]fluoroethane) is a tau specific PET tracer to monitor the accumulation of tau aggregates in the brain a hallmark of Alzheimer's disease. Cytochrome P450 mediated oxidative dehalogenation results in retention of 18F anion in bone tissue which complicates image analysis due to elevated background 18F levels. Deuterium substitution at the carbon bearing the 18F radionuclide was undertaken to mitigate enzymatic defluorination of the tracer. Incubations of the deuterated GTP1 with human liver microsomes and human hepatocytes followed by LC-MS/MS analyses were used to investigate the in-vitro metabolic profile. Eleven oxidative metabolites with higher polarity than the substrate were characterized. Only a small amount of a defluorinated carboxylic acid metabolite resulting from enzymatic oxidation at the adjacent carbon was noted. Overall, the results indicate that minimal 18F release from the deuterated tracer is observed in-vitro corroborating the PET scans. In addition, showing little 18F bone uptake. In addition, specific metabolism by CYP1A2 was also investigated because of the increased activity of CYP1A2 due to smoking and clinical concerns. GTP1 was incubated with recombinant CYP1A2 and also in HLM in the presence of CYP1A2 selective inhibitor α -naphthoflavone. The two most abundant oxidative metabolites were found to be non-CYP1A2 mediated as their formation was not affected by the addition of the CYP1A2 inhibitor nor were they formed in incubations with rCYP1A2. Two medium abundance metabolites were found to be produced by CYP1A2 as their abundance was suppressed by the presence of the CYP1A2 inhibitor. These results indicate that the effect of smoking on the use of the GTP1 tracer could be limited and not reduce its clinical effectiveness in smokers.

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P199 - PRE-SYSTEMIC METABOLISM OF EMIQ, A POTENTIAL THERAPEUTIC FOR MYOTONIC DYSTROPHY TYPE 1, IN HUMAN INTESTINAL S9

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Quercetin, a widely recognized supplement, has shown potential therapeutic benefits for various health conditions, including myotonic dystrophy type 1 (DM1), a genetic disorder marked by delayed muscle relaxation. Quercetin can selectively inhibit transcription of toxic CTG repeats in the DMPK gene, which is responsible for DM1 [1]. However, quercetin's low bioavailability limits its therapeutic efficacy. Enzymatically Modified Isoquercitrin (EMIQ), a glycosidic modification of quercetin with up to 10 glycosyl moieties, has been shown to improve quercetin's bioavailability and solubility. Despite its potential, there is a lack of concrete metabolic data available for EMIQ, which is crucial for establishing an oral dosing regimen for the treatment of DM1 in the future. EMIQ, isoquercitrin (a monoglucoside of quercetin), or quercetin (5 μ M) were incubated for 4 hours, with or without human intestinal S9 (HIS9) and/or uridine diphosphate glucuronic acid (UDPGA) as a cofactor for glucuronidation. A fit-for-purpose LC-MS method was developed for quantifying quercetin and isoquercitrin in biorelevant matrices. In the presence or absence of HIS9, the half-life of quercetin was approximately 5 hours. However, when both HIS9 and UDPGA were present, quercetin disappeared very rapidly, becoming undetectable after 60 minutes. When isoquercitrin was incubated with HIS9 in the absence of UDPGA, it rapidly decreased, while quercetin was steadily formed. In the presence of both HIS9 and UDPGA, isoquercitrin

disappearance was much more rapid, and quercetin was nearly undetectable at any time point. When EMIQ was incubated without HIS9 and UDPGA, isoquercitrin remained steadily at 1.5 μ M, and no quercetin was detected. However, when EMIQ was incubated with HIS9 in the absence of UDPGA, isoquercitrin reached a peak concentration of 5 μ M at 60 minutes, then decreased, while quercetin increased steadily at a rate of 430 nM/hr. In the presence of both HIS9 and UDPGA, EMIQ incubation resulted in isoquercitrin increasing to 3 μ M by 30 minutes, then decreasing, along with low and transient quercetin concentrations. These data show: 1) Rapid disappearance of quercetin, consistent with glucuronidation, as previously reported; 2) HIS9-mediated hydrolysis of isoquercitrin to form quercetin, followed by its disappearance in the presence of UDPGA due to glucuronidation; and 3) HIS9-mediated hydrolysis of EMIQ to reveal isoquercitrin and quercetin. This preliminary study provides comparable insights into the pre-systemic metabolism of EMIQ, isoquercitrin, and quercetin in the intestinal environment. Future research on the metabolic profile and enzyme kinetics of EMIQ can not only aid in the development of an effective therapeutic agent for DM1, but also contribute to the treatment of other conditions, given quercetin's wide range of potential applications.

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P200 - UNEXPECTED MAJOR CIRCULATING METABOLITE M219 DETECTED IN 14C-LABELED HUMAN ADME STUDY OF BRILAROXAZINE

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A notable advantage of utilizing radiolabeled drugs compared to their non-radiolabeled counterparts lies in their capability to generate quantitative data of all drug-related components without the necessity for metabolite standards. At Frontage Laboratories, we have established a systematic method development approach by continuous monitoring of radioactivity at different study stages, such as in-life mass balance, sample extraction, and column recovery. The systematic method will ensure the comprehensive detection of all drug-related components within complex matrices without missing any major metabolites. This advantageous methodology aligns with the FDA's recommendation for conducting human radiolabeled mass balance studies prior to phase III clinical trials. Human AME (hAME) studies can provide quantitative and comprehensive information on the absorption, distribution, metabolism, and excretion (ADME) of the drug in the humans, and give opportunity to identify any major circulating metabolites that may require further toxicity evaluation. This strategic approach was successfully employed in the development of brilaroxazine (RP5063) for the treatment of neuropsychiatric and inflammatory disorders by Reviva Pharmaceuticals. In healthy men following a single oral dose of [14C]brilaroxazine at 15 mg (~163 μ Ci), a total of 5 drug-related components were detected in plasma, and the most abundant metabolite (40.5% of total plasma radioactivity) was identified as M219 (N-[2,3-dichlorophenyl]glycine), generated through an unusual N-dealkylation and oxidation pathway. This metabolite had not been previously detected or reported in plasma of animals and humans during the preclinical or phase I clinical trial. Further investigations at Frontage, it is found that M219 was also the major circulating metabolite across multiple animal species, including rats, mice, and canines, constituting 16.2%, 53.5%, and 51.2% of total plasma radioactivity, respectively. One possible reason for missing the major metabolite in previous studies would be that the M219 metabolite was eluted after the parent compound under reverse-phase chromatographic conditions. This presentation will emphasize the importance of the systematic method for metabolite profiling and metabolite structure identification during drug development, and the high data quality of Frontage Laboratories as contract research organization.

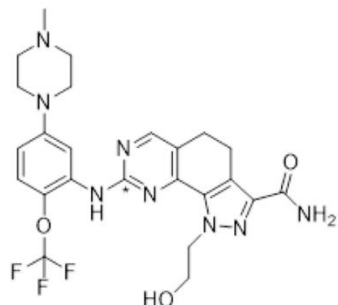
P201 - ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION STUDIES FOLLOWING A SINGLE ORAL DOSE ADMINISTRATION OF ONVANSERTIB TO RATS

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Background: Onvansertib, a small molecule PLK-1 inhibitor, is currently under development by Cardiff Oncology, Inc for the treatment of various cancers. The absorption, distribution, metabolism, and excretion of [14C]-Onvansertib was assessed following a single oral administration to male and female rats. Objectives: To determine the mass balance and the routes and extent of excretion of total radioactivity following a single oral administration of [14C]-Onvansertib to male and female Sprague Dawley rats. To determine the routes and extent of biliary excretion of total radioactivity following a single oral administration to male bile-duct cannulated Sprague-Dawley rats. To generate and quantify metabolite profiles

of radioactivity in plasma, urine, feces, and bile. To identify the major metabolites present in plasma, urine, feces, and bile. Methods: To enable drug related material to be readily traced and analyzed, [14C]-Onvansertib was used in this study. The radiochemical purity of the [14C]-Onvansertib was determined using radio-HPLC and was $\geq 95\%$ prior to use in the study. Male and female Sprague Dawley rats utilized for this study were in the weight range of 148 - 555 g and approximately 6 -12 weeks old at the time of dose administration. Each animal in the study received a single oral dose of 10 mg/kg [14C]-Onvansertib. Serial whole blood samples, urine, feces and bile were collected from each animal over a pre-defined time course post-administration. Structural identification analysis was conducted using tandem radio-HPLC and LC-MS/MS on the major metabolites identified in plasma, urine, feces, and bile. Results and Conclusions: Following a single oral dose of 10 mg/kg [14C]-Onvansertib to intact male and female rats, levels of total radioactivity in plasma were roughly two-fold greater in female rats vs. male rats and plasma levels in both sexes were below the limit of quantitation by 96 hours post-dose. Excretion was predominantly via the feces in both sexes, with mean recoveries of 96.94 and 94.11% of the administered radioactive dose observed for male and female rats, respectively. Urine accounted for a mean of 3.72 and 5.42% of the administered radioactive dose for male and female animals, respectively. The mean total recoveries of radioactivity were 101.81 and 101.20% in male and female animals, respectively. Following a single oral administration of [14C]-Onvansertib to male bile duct cannulated rats, excretion was predominantly via the bile and feces with a mean recovery of 49.17% and 36.19% of the administered radioactive dose respectively. Urine accounted for a mean of 5.93% of the administered radioactive dose. The mean total recovery of radioactivity was 92.98% in bile ducted cannulated male rats. The above data established that approximately 56% of radioactive material was absorbed following oral administration. The major metabolic pathways found in rats were N-oxidation, N-demethylation, aliphatic oxidation and alcohol oxidation of onvansertib. Structure of Onvansertib is uploaded.



* denotes position of radiolabel

P202 - VALIDATION OF LC-MS/MS METHOD FOR THE QUANTIFICATION OF DEHYDROEPIANDROSTERONE SULFATE (DHEAS) AND PHASE-2 METABOLITES OF SEVERAL HORMONES IN RAT'S PLASMA, AND BILE SAMPLES

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Phase II metabolites of androgenic and estrogenic sex hormones have been excreted through biliary excretion which is the part of enterohepatic recirculation (EHR). This process has been proposed as the homeostasis and recycling pathway of steroid hormones. Also, some of them such as dehydroepiandrosterone sulfate (DHEAS) have been used as a probe for drug-drug interaction (DDI) study. However, the quantification of these hormones in several biological samples in the recirculation process such as bile and other samples is challenging. In this work, we aimed to develop and validate a sensitive and robust liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for analyzing DHEAS and other phase II metabolites of hormones in bile and plasma samples.

A highly sensitive UHPLC-MS method was developed and validated according to the FDA guideline for quantifying DHEAS and other phase II metabolites of hormones in biological samples. Due to the lack of a steroid-free matrix, the charcoal-stripped bile and plasma samples were used as the matrix for the validation. An AcQuity UHPLC BEH C18 column (50 mm $\times$ 2.1 mm ID, 1.7 μ m) was used for UPLC under a gradient elution system composed of 0.2 mM ammonium fluoride in both methanol (solvent A) and water (solvent B) at 0.5 mL/min flow rate. Mass analysis was performed using an API 7500 Qtrap mass spectrometer and Multiple Reaction Monitoring (MRM) in negative mode with specific transitions. In the perfusion study, female rats were administered a cyanidin-3-glucoside through the portal vein infusion, 1 mM at 2 mL/hour flow rate, and bile and blood samples were collected at different time points.

The validated assay was successfully used for DHEAS and other phase II conjugates of steroid hormone concentration in rats for pharmacokinetics studies. The simple precipitation method exhibited good recovery in plasma and bile samples. Calibration curves exhibited linearity, with correlation coefficients exceeding 0.99. The limits of quantification (LLOQ) and lower limits of determination (LLOD) were in the nanomolar range. The intra- and inter-day precision and accuracy, represented by the relative standard deviation (%RSD) and relative error (%RE), were mostly within $\pm 15\%$.

In conclusion, the validated, sensitive, and simple LC-MS/MS method for DHEAS and other phase II conjugates of several sexual hormones here has further application for diverse sample matrices, enabling the measurement of these compounds in different biological specimens (including urine and fecal samples) in preclinical and clinical setting.

P203 - EXPLORING THE IMPACT OF PCB-18 (2,2',5-TRICHLOROBIPHENYL) EXPOSURE ON HEPATIC DRUG METABOLIZING ENZYMES AND XENOBIOTIC RESPONSES IN HUMANIZED MICE

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Polychlorinated Biphenyls (PCBs) are long recognized for their persistence in the environment and bioaccumulation potential and pose significant public health risks due to their widespread presence and enduring nature. PCBs are metabolized in the liver via oxidation and conjugation reactions and are known to activate distinct hepatic xenobiotic-sensing transcription factors. Among various PCB congeners, PCB-18 raises significant health concerns in neurotoxicity, immunotoxicity, and cancers. The goal of the present study was to determine how acute PCB-18 exposure modulates the hepatic transcriptome in a transgenic mouse model humanized with the major drug-processing genes, namely the xenobiotic-sensing nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR), as well as the phase-I drug-metabolizing enzymes cytochrome P450 (CYP) 3A4 and 3A7. We hypothesize that PCB-18 modifies important hepatic pathways involved in xenobiotic biotransformation of PCBs, which may modify the metabolism of important intermediary metabolites. Three-months-old adult humanized (h) PXR-hCAR-CYP3A4-CYP3A7 quadrupole transgenic mice were exposed orally to PCB-18 (6.0 mg/kg body weight) or vehicle (corn oil, 10 ml/kg) once daily for three consecutive days (n= 5 per exposure per sex), and livers were subjected to bulk RNA sequencing. In both sexes, PCB-18 differentially regulated pathways involved in P450-mediated xenobiotic metabolism, retinol metabolism, steroid hormone and fatty acid metabolism, protein digestion and absorption, bile secretion, arachidonic acid metabolism, and chemical carcinogenesis. In the livers of male mice, PCB-18 down-regulated the bile acid sensing nuclear receptor small heterodimer partner (SHP/Nr0b2) and the oxidative stress sensor Nrf2-target gene Nqo1 but up-regulated Cyp3a13. In livers of female mice, PCB-18 up-regulated Nqo1, the major bile acid uptake transporter sodium taurocholic co-transporting polypeptide (Ntcp/Slc10a1), as well as the bile acid/xenobiotic-uptake transporter organic anion transporting polypeptide (Oatp/Slco) 1a4 but decreased the rate-limiting bile acid efflux transporter bile salt export pump (Bsep/Abcb11). In this study, the roles of the two human transcription factors, pregnane X receptor (PXR) and constitutive androstane receptor (CAR), are central to understanding PCB-18's effects on the hepatic transcriptome, highlighting their pivotal function in mediating responses to environmental toxins. Specifically, these transcription factors are key to activating the liver's defense mechanisms against PCB-18 by modulating the expression of genes involved in xenobiotic metabolism, thereby influencing the liver's ability to process and detoxify PCBs. Therefore, the present study showed for the first time in this quadrupole humanized transgenic mouse model that PCB-18 oral exposure dysregulates the hepatic transcriptomic involved in xenobiotic biotransformation and chemical carcinogenesis, as well as modifies multiple intermediary metabolism pathways, especially bile acid transport.

P204 - HEPASH CELLS, A NOVEL PLATFORM TO ASSESS THE CLEARANCE OF LOW METABOLIC TURNOVER COMPOUNDS AND CYP INDUCTION

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Low clearance compounds, including Beyond Rule of 5 (bRo5) compounds, are very poorly metabolized in suspension metabolism assays that use cryopreserved primary human hepatocytes (PHH). Moreover, long-term culture metabolism studies, important for evaluating clearance, require complex culture systems and difficult-to-apply techniques. While PHH remain the gold standard for metabolism and induction assays, it is unfeasible to continuously use the same lot, there is variability in drug-metabolizing enzyme activity between lots, and the cost is high. Developed at the Central Institute for Experimental Medicine and Life Science, HepaSH cells, which are derived from TK-NOG mice transplanted with PHH, are a potential source of hepatocytes that may solve these problems. In this study, to evaluate the usefulness of HepaSH cells in drug discovery research, we investigated how long they could sustain CYP and Non-CYP activity, and whether they were responsive to typical CYP inducers. First, to obtain the basic drug metabolism profile for HepaSH cells, we conducted suspension metabolism assays using PHH from 3 donors and HepaSH cells from 5 donors. Using CYP (1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4) and non-CYP (UGT, AO, FMO, CES1) substrates, we calculated metabolite formation rates through time-course sampling. The drug metabolizing enzyme activity in HepaSH cells was comparable to or higher than that in PHH, and there was little difference in the metabolite formation rates between PHH and the HepaSH lots used in this assay. Metabolic enzyme activity across different experiments was consistent, and cell viability was high. The metabolite formation rates of PHH and HepaSH cells from the same donor showed a good correlation, indicating that the drug metabolism profile of HepaSH cells reflects that of the donor. Subsequently, we sought to verify their potential to induce CYPs (1A2, 2B6, 3A4) using an induction assay described in the ICH M12 Draft Guideline on Drug Interaction

Studies. The results confirmed the suitability of HepaSH cells for evaluating CYP induction. Finally, we conducted long-term culture metabolism assays in monolayer, sandwich, and spheroid cultures consisting solely of HepaSH cells to evaluate whether their CYP metabolizing capacity was sustained. HepaSH cells maintained major CYP activity for approximately one week across all culture methods tested. This outcome suggests that HepaSH cells are a viable hepatocyte source for assessing the metabolic clearance of low clearance compounds. HepaSH cells, in which drug-metabolizing enzyme activity is maintained for extended periods, offers a simple but versatile platform that can transition from short-term suspension metabolism assays to long-term culture metabolism assays, suggesting their potential for use in induction assays and clearance prediction. In the future, HepaSH cells are expected to be implemented in drug discovery research, serving as a new source of hepatocytes alongside PHH.

P205 - INFLUENCE OF LOCALLY BIOAVAILABLE HERBAL COMPONENTS ON THE REGULATION OF GUT HOMOEOSTATIS VIA EXPRESSION OF INTESTINAL DRUG METABOLIZING ENZYMES AND TRANSPORTERS IN IRINOTECAN-INDUCED INTESTINAL INJURY MODEL

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Drug induced intestinal toxicity is one of the common side effects of pharmacological treatments, chemotherapy being the most intensive. Apart from detrimental effects on defensive gut barrier, absorptive capacity and other important intestine functions, this toxicity can be dose-limiting and further compromising the treatment. Current standard care options are limited, non-specific and uncomfortable for the patients. In addition to that, so far, biomarkers are not found to be reliable, making it difficult to evaluate the level of toxicity and damage to gut. Whether acute or chronic, various molecular and cellular mechanisms are involved in response and possible resolving of the effects. The first response to drugs as xenobiotics is upregulation of metabolic and detoxifying enzymes, such as cytochrome P450, carboxylesterase and UDP-glucuronosyltransferase enzymes, and transporters involved in their transit through body. In this study, polyphenol-rich herbal formula ProwozTM was used to test the possibility of preventing irinotecan-induced intestinal damage. For that, 8-9 weeks old male (n=3) and female (n=3) F344 rats were administered saline (control group), Irinotecan (2x1150mg/m²) only, and Irinotecan (2x1150mg/m²) along with pretreatment (3 days prior to Irinotecan) and co-treatment (2 days with Irinotecan) of low dose (500) or high dose (1000 mg/kg) of ProwozTM or 1000 mg/kg of Chinese traditional medicine Xiao-Chai-Hu Tang (XCHT) as positive control. After Irinotecan dosing (day 3) rats were sacrificed and ileum tissue samples were tested for the expression of metabolizing and detoxifying enzymes (CYP2D1, CYP4B1, CYP3A9, CYP4F5, CYP51A1, CES2J, CES2E, CES2C, CES1C, CES1D, CES2H, UGT1A6, UGT1A2, UGT1A1, and UGT1A7) and transporters (ABCG3L1;ABCG3L3, ABCG2, ABCB11, ABCB1, and ABCB9), correlating them with biomarkers of gut homeostasis. Results show sex difference on treatment effect on expression of tested and transporters. It is hypothesized that protective effects of ProwozTM is achieved through induction of UGT1A1 and UGT1A2, CYP3A9 and CYP4F5 in both sexes, and in females additional upregulation of CYP2D1 and CYP4B1. However, ProwozTM treatment prevented downregulation of all CES1 in male rats, and all CES2 in female rats, keeping them at the homeostatic level. In addition, protective effects against irinotecan-induced gut toxicity could be due to upregulation of BCRP (ABCG2) and PGP (ABCB1) transporters, responsible for efflux of SN38, irinotecan active form, from intestine. In male rats it was caused by high dose, while in females by low dose of ProwozTM. Furthermore, significant correlations were found between MDA levels in feces and CYP2D1 (r=-0.4); between MDA levels in urine and CES1C (r=0.431), ABCG2 (r=-0.454) and ABCB9 (r=0.449); plasma MDA levels and ABCG3L1;ABCG3L3 (r=-0.3842); liver MDA levels and CES2E (r=0.414); ileum MDA levels and CES2E (r=0.478); cecum MDA levels and ABCB9 (p=-0.374). This study presents that ProwozTM, polyphenol herbal formula with locally bioavailable ingredients, is protecting intestine from irinotecan toxicity by supporting detoxification and maintenance of gut homeostasis.

P206 - CLARIFYING METABOLISM OF THE NATURAL PRODUCT DRUG TROXERUTIN *IN VITRO* AND *IN VIVO*

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Venoruton is used as an OTC drug (500 mg – 1500 mg/day) in Australia and Europe for treatment of edema and symptoms related to chronic venous insufficiency. It contains a standardized mixture of mono-, di-, tri-, and tetra-O-(β -hydroxyethyl)-rutosides (HR) in various isomeric forms. Troxerutin, the tri-hydroxyethyl derivative of rutin, also known as vitamin P4, is the major form accounting for > 45% of all ingredients. Rutosides can be deglycosylated by enzymes present in small intestine and colon to form less soluble aglycones and the oral bioavailability of HRs is low in preclinical species and human. Despite previous 14C studies of HRs in preclinical species and human, the mechanism of absorption, metabolism, and excretion and drivers of pharmacology remain unresolved and still a subject of debate. In the present study, stability of troxerutin and rutin in human feces was evaluated. Troxerutin, similar to rutin, can undergo rapid

hydrolysis to produce its corresponding aglycon. Subsequently, the stability and metabolism of troxerutin and its aglycon were studied in mouse, rat and human intestinal microsomes (IM) and liver microsomes (LM) in the presence of NADPH and UDPGA. In contrast to the high stability of troxerutin in IMs and LMs, troxerutin aglycone formed one predominant glucuronide in IMs and LMs within several minutes, which was almost the exclusive metabolite produced in HIM and HLM. The glucuronide was purified from the HLM incubation mixture for structural elucidation, where 2D NMR (COSY and HMBC) successfully determined the site of conjugation from the five possible hydroxyl groups present on troxerutin aglycone. Troxerutin was orally dosed at 5 mg/kg in mice and rats and had low oral bioavailability (< 1%) in both species. Systemic exposures of troxerutin aglycone and troxerutin aglycone glucuronide were also monitored. While troxerutin aglycone was not detected at the lower limit of quantification (0.1 ng/mL), troxerutin aglycone glucuronide had C_{max} concentrations comparable to troxerutin in mouse and rat plasma. Plasma T_{max} for troxerutin and troxerutin aglycone glucuronide were 0.5 h and 4 h, respectively. Based on the permeability, solubility, and results from the *in vitro* and *in vivo* studies, a mechanism of absorption, metabolism, and excretion for troxerutin is proposed.

P207 - PRODUCTION AND USE OF RECOMBINANT MIRNAS TO DISSECT THE INTRINSIC DIFFERENCES IN MIRNA BIOGENESIS AND REGULATION OF DRUG METABOLISM AND DISPOSITION

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Variations in the absorption, distribution, metabolism, and excretion (ADME) or pharmacokinetics of drugs may lead to therapeutic failure or adverse effects. Recent studies have demonstrated that epigenetic factors, such as noncoding RNAs, may affect ADME gene expression beyond transcriptional regulation. Specifically, microRNAs (miRNAs) are genome-derived small noncoding RNAs that regulate target gene expression through posttranscriptional gene regulation (PTGR) mechanisms. Some miRNAs have been identified to control PTGR of specific ADME genes, including those encoding drug-metabolizing enzymes (e.g., CYPs and UGTs), transporters (e.g., ABCs), and nuclear receptors (e.g., PXR). Notably, one miRNA might be processed to both 5p and 3p strands in cells, and one (e.g., hsa-miR-491-5p) is usually the predominant form. Therefore, this study is aimed at utilizing our novel RNA bioengineering technology to create and use model recombinant human miR-491-5p, -3p, and pre-miR-491 molecules to delineate the differences in miRNA biogenesis and function in the regulation of drug metabolism and disposition. To this end, we have successfully designed, cloned, expressed, and purified two sets (human tRNAGly and tRNA^{Leu} scaffolds) of recombinant pre-miR-491, miR-491-5p, and -3p molecules (BioRNAs). Our studies revealed that BioRNA/pre-miR-491 was mainly processed to the 5p strand in human HEK293 as well as Huh7, HepG2, and Hep3B cells, whereas BioRNA/miR-491-5p and 3p were selectively processed to 5p and 3p, respectively. Immunoblot and immunofluorescence confocal imaging studies demonstrated that miR-491-3p reduced the expression of ABCB1 transporter in HEK293 cells, while miR-491-5p suppressed the expression of BCL-XL. Future studies will critically define the impact of miR-491 on ABCB1-mediated drug transport and efficacy, as well as its regulation of UGT1A1 expression and consequent influence on drug metabolism. Our findings are expected to establish the intrinsic differences in miRNA biogenesis and role in ADME.

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P208 - A SIMPLE APPROACH FOR FACILE DETERMINATION OF METABOLIC SOFTSPOTS OF MACROCYCLIC PEPTIDES BASED ON METABOLITE GENEALOGY INFORMATION REVEALED IN THE METABOLITE PROFILES

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Macrocyclic peptides are emerging as a new class of therapeutically promising compounds that can modulate protein functions and disrupt protein-protein interactions. However, metabolic characterization of macrocyclic peptides has been challenging because of the complexity of the cyclic nature of the peptides. A derivatization approach has previously been reported facilitating the determination of softspots of the initial macrocycle ring-opened metabolites [1]. Here we report a differentiated label free approach that capitalizes on sequentially shorter metabolites after the initial macrocycle ring-opening to determine the metabolic softspots of macrocyclic peptides. These 'metabolite genealogies' are revealed in full scan LC/MS profiles after background subtraction to aid determining the initial sites of hydrolysis akin to ladder type sequencing in tandem mass spectra. Here the approach is demonstrated on model peptides after *in vitro* incubations with hydrolytic matrices.

In drug discovery, macrocyclic peptides are often subject to metabolic stability assays for SAR optimization. During the metabolic stability assays, aliquots of the incubation samples could be set aside for determining metabolic softspots of key compounds. The softspots are relayed to the chemistry team for structural modification of the chemotypes to increase metabolic stability. Typically, samples of early incubation timepoints are analyzed so that the obtained profiles would contain not only peaks of the parent drugs, but also their initial macrocycle ring-opening metabolites (i.e, parent plus 18 Da) and some of their further cleavage metabolites. A high-resolution LC/MS-based background subtraction technique

was used to maximize the purity of the obtained profiles [2]. With the parent drug sequences known, the genealogy information of the metabolites revealed in the profiles (i.e., the specific amino acid residues lost off the initial macrocycle ring-opening peaks to form the further cleaved peaks) points to the metabolic softspots of the initial macrocycle ring-opening sites. For example, the metabolite profile of a rat intestine luminal contents incubation of JB1 (H-Cys-Tyr-Ala-Ala-Pro-Leu-Lys-Pro-Ala-Lys-Ser-Cys-OH, cyclized through disulfide) yielded not only the JB1 peak and a JB1+18Da peak, but also peaks of the JB1+18Da further losing Ser, Lys, and Ser-Cys, respectively, pointing to the initial hydrolysis ring-opening at the Lys-Ser amide bond. MS/MS data of the JB1+18 Da ion was also acquired during the analysis, providing a secondary confirmation of the above proposed structure based on the b and y ions predicted from the proposed structure. This approach has been successfully applied to a complex bicyclic macrocycle program for facile determination of the metabolic softspots. This approach has led to a swift optimization of the SAR to generate metabolically stable compounds for acceptable pharmacokinetics which enabled proof-of-principle pharmacodynamic studies.

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P209 - COMPARATIVE METABOLITE PROFILING AND IDENTIFICATION OF GALNAC-CONJUGATED siRNA, siRNA01, IN PLASMA PREPARED WITH VARIOUS ANTICOAGULANTS, SERUM, AND *IN VIVO* PLASMA USING LC-UV-HRMS

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Following subcutaneous administration, GalNAc-conjugated siRNA enters the blood circulatory system, where it can be metabolized by nucleases. The metabolic stability of the siRNA in the blood before entering the liver is crucial for the effectiveness of siRNA-based drugs. Therefore, a reliable *in vitro* incubation system that can predict the *in vivo* metabolism of siRNA in blood is critical, especially for screening lead compounds with high metabolic stability. However, systematic research on this issue has not yet been reported in detail.

In this study, a comparative analysis of the metabolite profiles of siRNA01, a GalNAc-conjugated siRNA available on the market, was conducted in plasma prepared with various anticoagulants and in serum. A well-established LC-UV-HRMS method was used for the analysis. These results were then compared with *in vivo* plasma metabolite profiles. The main objective was to identify a suitable *in vitro* incubation system that could predict the metabolism of siRNA01 by nucleases in *in vivo* blood.

Metabolite profiles of siRNA01 in rat, monkey, and human plasma prepared with EDTA and sodium heparin anticoagulants, as well as in serum, revealed that the main metabolite of the AS chain in sodium heparin-anticoagulated plasma and serum is 3' n-1 across these three species, with a relative abundance of 4%-28%. A noteworthy result was that this major metabolite was not detected in EDTA-anticoagulated plasma of all three species. This may be due to the chelation of EDTA with divalent metal ions, such as Ca²⁺ and Mg²⁺, thereby inhibiting nuclease activity. No relevant metabolites of the SS chain were detected in these systems, indicating that the SS chain is more stable than the AS chain in plasma or serum.

In the rat plasma administered with siRNA01, the main metabolite of the AS chain was 3' n-1 (14.7%). In addition, a small quantity of the SS chain showing a loss of one GalNAc (3.36%) was detected. These results are similar to those of the *in vitro* nuclease metabolic profiles of rat sodium heparin-anticoagulated plasma and serum. This suggests that both sodium heparin-anticoagulated plasma and serum can be utilized for the metabolic study of siRNA01. They can predict nuclease metabolite profiles in *in vivo* blood, and help evaluate whether the related metabolites can be metabolized by the nucleases in the bloodstream.

Besides, the metabolite profiles of freshly prepared rat and monkey plasma anticoagulated with sodium heparin and serum were similar to those of their corresponding cryopreserved matrices, indicating commercially cryopreserved matrices are acceptable for siRNA01 metabolic studies, obviating the need for fresh preparation.

Similar comparative studies were also conducted on several other GalNAc-conjugated siRNAs. The results also suggested that *in vitro* metabolism in sodium heparin-anticoagulated plasma and in serum could effectively predict nuclease metabolites in *in vivo* blood (data not shown).

P210 - EXPLORING PROTEIN-OLIGONUCLEOTIDE CONJUGATE LYSOSOMAL CATABOLISM: A WORKFLOW UTILIZING LIQUID CHROMATOGRAPHY-HIGH RESOLUTION MASS SPECTROMETRY (LC-HRMS) AND ISOCRATIC LABELING TECHNIQUE

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Antibody-drug conjugates (ADCs) are generally composed of three major components: an antibody for the selective targeting of cell or tissue surface antigens, a cytotoxic drug payload, and a linker unit which connects these two moieties. In a novel approach, an antisense oligonucleotides (ASO) was substituted in place of traditional small molecules as the drug payload and conjugated to a large protein transport vehicle (TV), a novel platform technology that engineers blood brain barrier receptor binding into an Fc domain. This unique engineering was developed to overcome the rapid plasma clearance of an ASO while enhancing broader biodistribution including central nerve system (CNS) delivery via systemic delivery.

A critical component of ADC development is identification or verification of the active released entity upon cellular uptake and exposure to lysosomal enzymes. Our oligonucleotide transport vehicle (OTV) technology mimics this pathway, where the ASO must be liberated from the transport vehicle protein and traffic to the nuclear compartment to engage RNase H. Coupled LC/HRMS with commercial rat and human lysosomal preparations are used as an *in vitro* tool that allows exploration of the release characteristics of these new protein-oligonucleotide conjugates, and provides insights into potential metabolic or chemical liabilities of the ASOs. This study demonstrates comprehensive characterization and structure elucidation of transport vehicle protein and ASO catabolism. An isocratic labeling technique is employed to distinguish between endogenous and exogenous mechanisms, offering a general method for analyzing non-cleavable linked protein-oligonucleotide conjugates. Moreover, the potential for tailoring this method to specific cases is highlighted.

P211 - ASSESSING THE ADME PROPERTIES OF BELVARAFENIB (GDC-5573), A RAF INHIBITOR, IN MALE RATS

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Belvarafenib (GDC-5573) is in-licensed from Hanmi Pharmaceuticals, and it is a potent and selective Type II RAF dimer inhibitor, capable of inhibiting the kinase activity of wild type and mutant BRAF and RAF-1 (CRAF), thus blocking MAPK signaling downstream of activated RAS. In an effort to elucidate the ADME characteristics of belvarafenib, a study was carried out using Sprague Dawley male rats. This study involved the application of [¹⁴C]GDC-5573 mass balance (200 µCi/kg) to both intact and bile-cannulated (BDC) animals. These subjects were further divided into two groups receiving oral (PO, 10 mg/kg) and intravenous (IV, 5 mg/kg) administrations. Systematic collection and analysis of plasma, urine, feces, and bile samples from these animals were critical components of this study.

Following an oral dose in intact rats, belvarafenib was the most prominent drug derived material in circulation in 0-72 hours AUC pooled plasma, representing 89.5% of the total radioactivity. Additionally, M2 (oxidative metabolite (M1) plus glucuronidation) accounted for 9.75% of total radioactivity in the circulation. Fecal excretion was the major route of elimination and accounted for 88.5% of the administered dose and urine was a very minor route of elimination and counted for 1.23% of the administered dose. The two abundance components found in feces were M1 at 53.4% and belvarafenib at 32.8% of the administered dose. M2 and M10 (oxidation metabolite) were also detected at 1.12% and 0.673%, respectively. In BDC rats, radioactivity in bile accounted for 32.1% (PO), and 41.6% (IV) of the administered dose. Belvarafenib made up less than 1% of the administered, indicating extensive metabolism. In contrast, M2 was the major metabolite representing 22.9% following PO dosing and 28.0% following IV dosing.

In conclusion, belvarafenib was well absorbed in rats, and its primary elimination path was metabolism through oxidation to form M1, followed by glucuronidation to form M2. The processed drug was primarily excreted via bile in the form of M2, while M1 represented the majority of the compound detected in feces.

P212 - METABOLISM AND EXCRETION OF THE ADENOSINE A2A/A2B RECEPTOR INHIBITOR, [14C]-ETRUMADENANT, IN HUMANS

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Etrumadenant is an orally available, selective dual antagonist of both the adenosine 2a receptor (A2aR) and the adenosine 2b receptor (A2bR) and is in clinical development for the treatment of cancers. The metabolism and excretion of etrumadenant in humans were studied *in vivo* and *in vitro*. Following a single 150 mg oral dose of [¹⁴C]-etrumadenant to 8 healthy subjects, the mean cumulative recovery of the total radioactivity was 96.4% with 54.4% in urine and 41.0% in feces. More than 80% of the dose (ranging from 82.3 to 89.0%) was identified in excreta by liquid chromatography-high resolution mass spectrometry coupled with radiometric detection. [¹⁴C]-etrumadenant metabolite profiles were similar among the individuals. A direct O-glucuronide (M2) was the major metabolite in urine, accounting for 33.8% of the dose.

Intact etrumadenant was the most abundant component in feces (25.3% of the dose) but a minor one in urine (2.1% of the dose). The remaining etrumadenant-related molecules were mainly oxidative metabolites including N-dealkylated etrumadenant (M1), multiple mono-oxygenated products and secondary glucuronide metabolites. In plasma, the major metabolites were M1 (15% of total radioactivity) and M2 (43% of total radioactivity). *in vitro* reaction phenotyping revealed that the oxidative metabolism of etrumadenant was predominantly mediated by cytochrome P450 3A4, and formation of M2 was catalyzed by multiple UDP-glucuronyltransferase (UGT) isozymes including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT2B4, UGT2B7 and UGT2B15. The role of UGT1A1 in the clearance of etrumadenant was evaluated *in vitro* and *in vivo*. The M2 formation rates were similar in human liver microsomes from donors with different UGT1A1 genotypes. No correlation between UGT1A1 genotype and M2 urinary excretion was observed in the human mass balance study. The mean % of dose recovered as M2 in urine was 35.3%, 25.5%, and 44.2% in the normal, intermediate, and poor metabolizers, respectively. In summary, the current study indicates that etrumadenant is eliminated by multiple clearance pathways, therefore has a low potential for strong drug-drug interaction with an inhibitor of drug metabolizing enzymes. In addition, etrumadenant pharmacokinetics is not affected by UGT1A1 polymorphism.

P213 - COMPREHENSIVE METABOLITE PROFILING OF SH00302 IN HUMAN PLASMA, URINE AND FECES USING LC-HRMS AND AN ADVANCED DATA PROCESSING WORKFLOW: COMPARISON WITH RADIOCHROMATOGRAPHIC ANALYSIS

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Drug metabolite profiling that includes metabolite detection and structural characterization is an integrated part of drug metabolism and pharmacokinetics studies in the drug discovery and development process, which provides insights into its pharmacokinetics, pharmacodynamics, and toxicity. Currently, LC-HRMS is the single dominant analytical platform for drug metabolite profiling. In the analysis, accurate LC-MS and LC-MS/MS datasets of a test sample and a control sample are required. Then, commonly used extracted ion chromatography (EIC), mass defect filter (MDF), product ion filter (PIF), and/or neutral loss filter (NLF) are employed to process LC-HRMS datasets to find metabolite ions and their MS/MS spectra, which are utilized for metabolite structural elucidation. Data processed results from the control sample are often used to identify false positives from the data analysis of the test sample. The LC-HRMS data processing tools and workflows are included in all commercial drug metabolite identification software packages, such as Compound Discovery, MetabolitePilot, UNIFY, MassMata-Site, which are effective in rapid and sensitive profiling of *in vitro* metabolites. However, *in vivo* metabolite profiling using these software tools often lead to many false positives or miss uncommon or low abundance metabolites.

The primary objective of this study was to develop an advanced LC-HRMS data processing workflow (Xeno-Discovery) for comprehensive profiling of *in vivo* metabolites. Xeno-Discovery used a combination of an improved background subtraction algorithm (background subtraction filter, BSF) and MDF as the primary data processing tool, while EIC and PIF were employed as supplemental tools. To evaluate the performance of Xeno-Discovery, LC-HRMS datasets of human plasma, urine and feces samples from the human radiolabeled ADME study of SH00302 were processed using Xeno-Discovery and resultant metabolite profiling data were compared with those from radiochromatographic analysis. Results showed that unprocessed LC-MS profiles of human plasma, urine and feces did not display any metabolites of SH00302. Metabolite profiles generated by BSF-MDF were similar to radiochromatograms and had no significant false positives. Additionally, BSF-MDF processing detected a few more metabolites in urine and feces, which were not found by radiochromatographic analysis due to their low abundances. EIC analysis that searches for metabolites based on their predicted m/z values could generate some false positives hits, which was significantly reduced by using a combination of BSF and EIC. Furthermore, a combination of PIF and EIC provided additional capability for sensitively and selectively detecting certain types of metabolites that had predictable fragmentation pathways.

In summary, an advanced LC-HRMS data processing workflow, Xeno-Discovery, was developed for metabolite profiling of *in vivo* samples. The metabolite profiles of radiolabeled SH00302 in human plasma, urine and feces generated by Xeno-Discovery were consistent with the results from radiochromatographic analysis in terms of selectivity and sensitivity. This example demonstrated that BSF-MDF based Xeno-Discovery data processing workflow is a very powerful tool to support comprehensive profiling and structural characterization of *in vivo* metabolites by LC-HRMS.

P214 - SIMULTANEOUS DETERMINATION OF 7 BIO-TOXINS(PEPTIDE TYPE) IN FISHES BY UPLC-MS/MS

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Microcystins (MCs) and nodularin (NOD) are naturally occurring cyclic peptide-type biotoxins produced by cyanobacteria, mainly *Microcystis aeruginosa* and *Nodularia spumigena*. Due to their hepatotoxic effects, the accumulation of MCs and NOD in food has become a growing public health concern. The aim of this study is to establish a method for the occurrence of 6 types of MCs and NOD in fish using ultra-performance liquid chromatography tandem mass spectrometry

(UPLC-MS/MS). Internal standards (IS) were used to provide quantitative determination of the low levels of MCs and NOD in eel. We extracted 7 bio-toxins with 80% ethanol, eliminated lipids with hexane, and performed a clean-up stage using SPE cartridges. The validation procedure was conducted to determine recovery, linearity, selectivity, precision, limit of detection, and limit of quantification according to CODEX guidelines. To implement the method on different species of fish, yellow fish (*Pseudobagrus fulvidraco*), far eastern catfish (*Silurus asotus*), crucian carp (*Carassius auratus*), salmon (*Oncorhynchus keta*), and Mozambique tilapia (*Tilapia mossambica*) were validated and found to meet CODEX guidelines. Based on these results, the developed method can be applied for the detection and quantification of 6 MCs and NOD in freshwater, pelagic, and marine fish.

P215 - EFFECTS OF MIR-222-3P-MEDIATED DOWNREGULATION OF ARYLACETAMIDE DEACETYLASE ON DRUG HYDROLYSIS AND LIPID ACCUMULATION

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Arylacetamide deacetylase (AADAC) is involved in the hydrolysis of clinical drugs such as ketoconazole and rifampicin. Recently, it has been suggested that AADAC is also involved in lipid metabolism since overexpression of AADAC in culture cells suppresses the intracellular lipid level. In a panel of 23 human liver samples, no significant correlation was observed between AADAC mRNA (19.7-fold variation) and protein levels (137.6-fold variation), suggesting a significant contribution of post-transcriptional regulation to AADAC expression. This study aims to investigate whether AADAC is regulated by microRNA in the human liver and elucidate its biological significance. Computational prediction analysis raised two potential miR-222-3p recognition elements in the 3'-untranslated region (UTR) of AADAC. Luciferase assay revealed that one of the potential miR-222-3p recognition elements was functional in downregulating AADAC expression. In HEK293 cells transfected with an AADAC expression plasmid containing the full-length 3'-UTR, miR-222-3p overexpression decreased AADAC protein level and hydrolase activity for flutamide, a typical substrate of AADAC, whereas miR-222-3p inhibition increased them. Flutamide hydrolase activity in HEK293 cells transfected with an AADAC expression plasmid not containing 3'-UTR was not altered by miR-222-3p overexpression. Similar results as in HEK293 cells were obtained in human hepatoma-derived Huh-1 cells endogenously expressing AADAC and HepaSH cells that are hepatocytes from chimeric mice with humanized livers. In individual human liver samples, AADAC protein levels were inversely correlated with miR-222-3p levels. These results suggest that AADAC expression is downregulated by miR-222-3p in the human liver. The effect of the regulation of AADAC by miR-222-3p was evaluated by Oil red O staining. Overexpression of miR-222-3p resulted in increased lipid accumulation in Huh-1 cells, and that was reversed by AADAC overexpression. In contrast, miR-222-3p inhibition decreased lipid accumulation, and that was reversed by AADAC knockdown. In conclusion, we found that hepatic AADAC was downregulated by miR-222-3p, resulting in decreased drug hydrolysis and increased lipid accumulation. It has been reported that hepatic miR-222-3p levels in patients with nonalcoholic fatty liver disease including steatosis and nonalcoholic steatohepatitis are higher than in healthy subjects. Increased expression of miR-222-3p in the liver of these patients may cause lipid accumulation by suppressing AADAC expression.

P216 - IDENTIFICATION OF ENZYMES RESPONSIBLE FOR SULINDAC SULFIDE S-OXYGENATION

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Background: Sulindac sulfide, a pharmacologically active metabolite of sulindac, is a nonsteroidal anti-inflammatory drug. Flavin-containing monooxygenase 3 (FMO3), located in the membranes of the endoplasmic reticulum, catalyzes NADPH-dependent oxidation of sulindac sulfide. As an interesting characteristic of FMO, it has been reported that FMO activity is decreased by heating without NADPH [1]. To investigate the effect of this issue in S-oxygenation of sulindac sulfide, we examined the thermal stability of sulindac sulfide S-oxygenation activity by pre-heating without NADPH. However, sulindac sulfide S-oxygenation activity in human liver microsomes (HLM) were only decreased to approximately 60% by pre-heating at 37°C for 30 min without NADPH. This result suggested that enzymes other than FMO3 also S-oxygenate to sulindac sulfide. Thus, the purpose of this study was to clarify the enzymes responsible for the metabolism of sulindac sulfide. Methods: Sulindac sulfide S-oxygenation activities by HLM and recombinant human cytochrome P450s (CYPs) were measured by LC-MS/MS. Results: From the results of kinetic analysis in HLM, the Eadie-Hofstee plot for sulindac sulfide S-oxygenation activity was biphasic, indicating the involvement of multiple enzymes in this metabolism. The apparent Km values for high-affinity and low-affinity components were 6.42 ± 0.58 and 16.5 ± 1.4 μ M, respectively. To determine whether cytochrome P450s (CYPs) are involved in this metabolism, the activities by recombinant human CYPs and the inhibitory effects of specific inhibitor of CYP isoforms were evaluated. These studies suggested that sulindac sulfide is also metabolized by CYP1A2, 2C8, 2C9, and 2J2. To further analyze the contribution of each CYP isoform to sulindac sulfide S-oxygenation, the relative activity factor (RAF) method was employed. The contributions of CYP1A2, 2C8, 2C9, 2J2, and other enzymes, of which FMO3 might account for the most part, to the sulindac sulfide S-oxygenation

in HLM were calculated as 19%, 30%, 5%, 5%, and 41%, respectively. These results were consistent with our initial study, in which sulindac sulfide S-oxygenation activity was decreased by 40% by pre-heating without NADPH. Our investigation indicated that sulindac sulfide is mainly S-oxygenated by CYP2C8 and FMO3. Conclusion: In conclusion, this was the first study to clarify that sulindac sulfide is S-oxygenated by CYP2C8 in human liver microsomes. We provide useful information that sulindac sulfide is not a specific substrate for FMO3.

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P217 - LIVER HUMANIZED CHIMERIC PXB-MOUSE® AS A MODEL TO PREDICT HUMAN RENAL TOXICITY BY ALDEHYDE OXIDASE DEPENDENT METABOLISM OF SGX-523

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Aldehyde oxidase (AO) is a cytosolic molybdenum-containing hydroxylase that oxidizes a variety of azaheterocyclic-containing drug molecules and has gained the attention of drug discovery programs due to profound species differences in activity and challenges predicting clearance. In addition, metabolites generated by AO are poorly predicted from preclinical studies in rodents and canines, complicating safety assessment programs. SGX523, a selective drug candidate for MET receptor tyrosine kinase, was halted in the clinic due to acute renal failure. The observed clinical findings were unexpected due to SGX523 demonstrating an acceptable safety profile in preclinical toxicology studies in rat and dog. Metabolite profiling of plasma from human patients identified a key AO-mediated circulating metabolite (M11), which was not observed in rats and dogs. Retrospective toxicology studies in monkey (a species with high AO-activity) provided evidence of intratubular crystals of M11 and associated inflammatory changes in kidney tissue, consistent with crystallization of this poorly soluble metabolite in the kidney. As an alternative to utilizing non-human primates, the humanized PXB mouse® (Phoenix Bio) has been developed as a predictive model of human metabolism. In effort to assess the suitability of this humanized mouse model for recreating the renal toxicity observed by SGX523, PXB mice were exposed to SGX523 by single or repeat oral dose daily for four days up to 60 mg/kg, followed by analyses of blood chemistry, renal histopathology, and SGX523 and M11 exposure. In addition to measuring effects on serum creatinine and blood urea nitrogen (BUN) as markers of kidney injury, we expanded the biomarker panel to include urinary kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) as highly sensitive markers of tubular injury. Upon dosing PXB-mice with SGX-523, elevated levels of serum creatinine, BUN, urinary KIM-1 and NGAL were observed with increasing dose. This correlated with high M11 metabolite/SGX523 ratios in the urine and kidney tissue, in addition to higher circulating exposure of M11. Microscopically, obstructive/retrograde nephropathy was characterized by renal tubular dilatation in a linear distribution and multifocal birefringent amorphous crystals in tubules. This work showcases the utility of KIM-1 and NGAL for early risk assessment of kidney injury for drug candidates, in addition to the utility of a humanized liver mouse model to assess safety of specific human metabolites as an alternative to standard preclinical toxicology species, such as non-human primates.

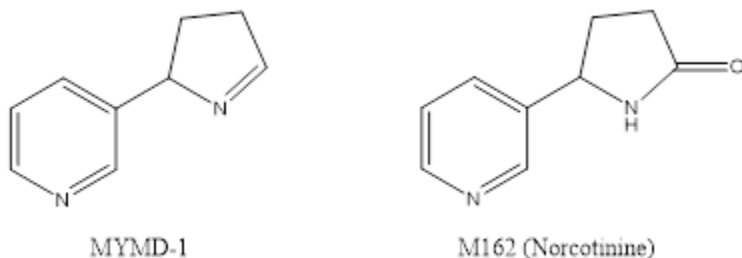
P218 - IDENTIFICATION OF THE MAJOR CIRCULATING METABOLITE OF MYMD-1 AND ELUCIDATION OF THE MECHANISM OF CLEARANCE OF MYMD-1 IN HUMANS. ROLE OF ALDEHYDE OXIDASE and CYP2A6

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MYMD-1 is an immunomodulator that is currently entering Phase II clinical development for the treatment of autoimmune diseases. In preclinical and clinical studies, MYMD-1 has been shown to inhibit the inflammatory cytokines TNF- α , IL-6 and IL-17A1,2. The objectives of the current study were to identify the human circulating metabolites in samples obtained following multiple doses of MYMD-1 and to determine the enzymes involved in their formation. Analysis of pooled plasma by LC/MS/MS indicated that MYMD-1 accounted for <0.1% of the drug related exposure and 95.5% of the exposure was represented by an oxygenated metabolite (M162). Subsequently, a radiolabeled disposition study was carried out in rats and M162 was found to be a major metabolite in plasma and excreta. Therefore, rats were dosed with MYMD-1 (400 mg/kg for 3 days) and M162 was isolated from urine, purified and analyzed by high resolution mass spectrometry (HRMS) and NMR spectroscopy. Based on HRMS and NMR data, and by comparison with a synthetic standard, M162 was unambiguously identified as the known compound norcotine. Following incubation of MYMD-1 (1 μ M) with a panel of human recombinant cytochrome P450 (rCYP) enzymes, MYMD-1 was metabolized by rCYP2A6 with an intrinsic clearance (CL_{int}) of 1.03 μ L/min/pmol. No turnover was observed with rCYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 or 3A5. MYMD-1 was metabolized in NADPH-fortified human liver microsomes (HLM) and human liver cytosol with CL_{int} of 36.1 and 91.2 μ L/min/mg, respectively. The depletion of MYMD-1 in cytosol was accompanied by robust formation of M162 and the formation of the metabolite was strongly inhibited by the aldehyde oxidase inhibitor menadione. No M162 was

formed by rCYP2A6. Three additional mono-oxygenated metabolites were identified at low levels in HLM, and their formation was shown to be catalyzed by CYP2A6. No meaningful turnover of M162 was observed in any of the rCYP incubations but a small amount of oxygenated M162 was formed by rCYP2A6. Studies with transporters indicated that MYMD-1 is not a substrate of the common uptake and efflux transporters, and there was minimal elimination of unchanged MYMD-1 in human urine. Thus, aldehyde oxidase is the main determinant of the clearance of MYMD-1 in humans, producing the major circulating and excretory metabolite M162, and CYP2A6 is predicted to play only a minor role in the disposition of MYMD-1.



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P219 - IMPACT OF G-QUADRUPLEX ON CARBOXYLESTERASE 2-DEPENDENT DRUG AND LIPID METABOLISM

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G-quadruplex (G4) is the higher-order structure found in the guanine-rich nucleic acid sequences. The G4 structure consists of two or more G-quartets formed by four guanines and is stabilized by a monovalent cation such as potassium ion. G4s have been reported to play critical roles in biological processes including gene transcription and RNA splicing by the regulation of the binding of transcriptional factors, the chromatin structure, and the modulation of splicing factor. We noticed that there are some potential sequences forming G4 structure in the upstream region and intron of carboxylesterase 2 (CES2) gene. In this study, we investigated whether G4 formation affects the CES2 expression and thereby regulates drug and lipid metabolism in the human liver. A qPCR stop assay, an experiment applying the principle that polymerase chain reaction is inhibited by the higher-order structure of the template DNA, to evaluate G4 formation *in vitro* showed that three guanine-rich sequences in the CES2 upstream region can form G4s. CES2 mRNA levels in human hepatocarcinoma-derived HepG2 and Huh-1 cells were significantly decreased by the treatment with pyridostatin (PDS), which is a G4 stabilizer. Consistently, CES2 protein level and procaine hydrolase activity in HepG2 cells were also decreased by PDS treatment. In addition, CES2 mRNA, protein levels, and procaine hydrolase activity in HepaSH cells, hepatocytes derived from human liver chimeric mice, were decreased by PDS treatment, suggesting that G4 formation would downregulate CES2 expression not only in hepatocarcinoma cells but also in normal hepatocytes. Oil red O staining in HepaSH cells treated with free fatty acids showed that lipid accumulation was increased by PDS treatment, suggesting that G4-mediated downregulation of CES2 caused the increased lipid accumulation. Collectively, we found that G4 formation in the 5'-flanking region of the CES2 gene downregulates the CES2 expression and decreases the hydrolase activity for drugs and lipid in hepatocytes. Thus, we found a CES2 regulatory mechanism that has been largely uncharacterized and even affects its biological function. G4s formed in the CES2 upstream region would affect the binding of transcriptional factors, inhibit the transcription, and result in decreased drug and lipid metabolism in the human liver.

P220 - PROTEIN ABUNDANCE OF HYDROLYTIC DRUG METABOLIZING ENZYMES IN DIFFERENT TISSUES AND ACROSS SPECIES

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Hydrolases (EC 3) constitute the largest and most diverse group of enzymes, encompassing over 200 members that catalyze the cleavage of various types of chemical bonds such as carbon-oxygen (C-O), carbon-nitrogen (C-N), and

phosphorus–nitrogen (P–N) bonds. Beyond their physiological significance, these enzymes also play a key role in drug metabolism, especially activating prodrugs. Differences in hydrolase abundance across tissues and species can influence extrapolation of drug metabolism from preclinical studies. In this study, we quantified a large number of hydrolases in S9 fractions isolated from different drug metabolizing organs (liver, intestine, kidney, and lung) in humans (pool of 4-50), Wistar Han rats (pool of 6 to 240), CD-1 mice (pool of 12), beagle dogs (n=3), and cynomolgus monkeys (n=4). Tissue S9 fractions were trypsin-digested and analyzed using high-resolution mass spectrometry-based total protein abundance (TPA) approach. In humans, out of 223 hydrolases, 147 were ubiquitously detected in all tissues, whereas 61 were present in 2-3 tissues, and 15 were tissue-specific. These hydrolases were categorized into esterases (n=48), peptidases (n=53), amidases (n=16), cathepsins (n=8), phosphatases (n=41), deacetylases (n=1), deaminase (n=6), glycosidases (n=8), proteases (n=4), sulfatases (n=5), and other hydrolases (n=33). Examples include carboxylesterases (CES1 and CES2), which exhibited high abundance in the human liver and intestine, respectively, while CES1 was also abundant in lungs and CES2 was also detected in the kidneys. In contrast, in monkeys, both CES1 and CES2 were predominantly detected in the liver, where CES1 abundance was approximately 3-fold lower than in humans. Interestingly, comparable abundances of CES5a and CES2 were seen in the liver and kidney in dogs, with no detectable expression in the intestine, while rodents displayed multiple CES isoforms across tissues. Paraoxonase 1 (PON1) was most abundant in cyno and mouse liver, followed by rat, human, and dog. PON2 on the other hand showed the highest abundance in dog kidney compared to the low levels in other tissues and species and no measurable levels were detected in cyno. However, PON3 showed comparable expressions in human, rat, and cyno liver. Epoxide hydrolase 1 (EPHX1) displayed high liver abundance compared to other tissues in humans and dogs, followed by rats, while mice predominantly expressed EPHX2 compared to EPHX1 in the liver, and in cyno only EPHX2 was detected. Arylacetamide deacetylase (AADAC) showed highest levels in cyno, followed by human and mouse, with lower expression in dog and rat. Cathepsin isoforms were ubiquitously expressed across tissues in all species, with the highest levels observed in human tissues. Dipeptidase 1 (DPEP1) exhibited the highest abundance in human kidneys, followed by dog, with cyno displaying the lowest levels. Intestinal alkaline phosphatase displayed the highest levels in rat intestine, approximately 9-fold higher than in human, followed by dog, mouse, and cyno. Our study presents the first comprehensive global quantitative analysis of inter-species and inter-tissue hydrolytic enzymes, crucial for translating preclinical data to humans and predicting species-specific drug metabolism of hydrolase substrates within tissues.

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P221 - NUCLEAR RECEPTOR PPAR γ IS A POTENT REGULATOR OF GPNMB GENE IN THE FATTY LIVER

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Purpose: The nuclear receptor peroxisome proliferator-activated receptor γ (PPAR γ), and glycoprotein nonmetastatic melanoma protein B (GPNMB) play key roles in hepatic lipid metabolism [1] and 2)]. This study was conducted to determine whether PPAR γ associates with the regulation of hepatic GPNMB expression in the fatty liver.

Methods: The expression of hepatokine genes in the fatty liver of type 2 diabetes model ob/ob mice was analyzed using the Gene Expression Omnibus (GEO) database. The expression of Gpnmb mRNA and GPNMB protein were analyzed by quantitative real-time PCR or western blotting. The reporter assay was performed in HEK293FT cells transfected with the luciferase reporter containing the PPAR γ -response elements (PPREs) of Gpnmb gene together with PPAR γ and RXR α -expression plasmids. The gel shift assay was performed using the 32P-labeled DNA oligonucleotide probes and *in vitro* translated PPAR γ and RXR α proteins.

Results and Discussion: Gpnmb was isolated as the hepatokine gene most highly expressed in fatty liver of ob/ob mice. The expression of Gpnmb mRNA were highly expressed in the liver of ob/ob mice and was induced by PPAR γ ligand treatment. The expression of Gpnmb mRNA or GPNMB protein in the liver of ob/ob-PPAR γ LKO mice (liver-specific PPAR γ knockout mice in the ob/ob background) was significantly lower than in the fatty liver of ob/ob-PPAR γ WT mice. We found three putative PPRE consensus sequences in the intron 1 of Gpnmb. The reporter assay indicated that PPRE-1, -2, and -3 are necessary for PPAR γ -dependent induction on Gpnmb transcriptional activity. Further, the gel shift assay indicated that PPAR γ /RXR α is capable of binding to each probe containing the PPRE-1, -2, or -3.

Conclusions: PPAR γ positively regulated Gpnmb expression through PPRE-1, -2, and -3 in the first intron of the Gpnmb. These results suggest that PPAR γ is a potent regulator of Gpnmb in the fatty liver.

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P222 - QUANTITATIVE ANALYSIS OF OLIGONUCLEOTIDE BY ION PAIRING REVERSED-PHASE LC-MS/MS

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Oligonucleotides (OGNs) have attracted massive attention as therapeutics due to their ability to regulate gene expression precisely and efficiently. OGN bioanalysis is critical to evaluate its efficacy and toxicity. Ion pairing reversed-phase liquid chromatography (IP-RPLC) and mass spectrometry-based assays are widely applied for the analysis of OGN for a couple of advantages: high specificity for intact OGNs/metabolites and wide dynamic range. Owing to their high polar and strong non-specific binding properties, the bioanalytical method development for OGNs can be complicated.

To achieve optimal results, quite a few influences need to be considered. For example, the selection of the ion pairing reagent and the organic modifier, the concentration of ion-pairing reagent/organic modifier and the pH value of mobile phases, the selection of column and temperature as well as the applied gradient. Due to multiple charge states of the OGN analyte and formation of cation adduct, it's challenging to separate and differentiate OGN analyte, metabolites, and potential interferences to achieve idea peak shape. In this study, an IP-RPLC-MS/MS method for the analysis of a 20mer OGN (FRO-024) was developed and qualified in rat brain tissue samples. Sample processing and LC-MS/MS parameters were optimized. Especially, peak tailing problem was addressed. N,N-Diisopropylethylamine (DIPEA) and 1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) were added in mobile phase A (1.0% HFIP, 0.1% DIPEA and 10µM EDTA in water) and mobile phase B (0.075% HFIP, 0.0375% DIPEA and 10µM EDTA) in water/acetonitrile (35/65). Column from Waters was used (ACQUITY Premier Oligonucleotide C18 Column, 130Å, 1.7 µm, 2.1 x 50 mm) and 65 °C was applied to column. The curve was linear within 1-2000 ng/mL and LLOQ of 1ng/mL was achieved. The qualified method was used to analyze rat brain samples.

P223 - EXPANDING PHARMACOGENOMICS PANEL ASSAY TO INCLUDE TRANSPORTER GENES AND MORE PHASE I METABOLISM VARIANTS

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Pharmacogenomics (PGx) is the study of how genes affect a person's response to drugs. Making use of PGx testing can be critical in determining the effective, safe dose of a given drug for an individual. Therefore, when developing drugs, it is important to test the effect of various pharmacogenes on drug disposition, response, and toxicity across a wide number of diverse people. In previous work we validated PGx testing for 69 different variants in genes such as CYP2D6, CYP2C9, and CYP2C19 but had not tested ABCB1 and SLCO1B1 transporters and important variants in the CYP3A4 and CYP3A5 genes. We hypothesized that using the Agena MassARRAY system we could validate robust, targeted, and cost-efficient PGx assays for testing in these other pharmacogenes. To this end, we validated a custom multiplexed assay testing for variants rs1128503, rs2032582, rs12721627, rs2242480, rs2740574, rs28371759, rs4986910, rs4986913, rs28365095, rs28371706, rs10264272, and rs2306283. We then used these validated assays to generate quality data from DNA samples procured from both whole blood and buccal swabs. Multiple single-nucleotide polymorphisms (SNPs) were simultaneously analyzed across multiple genes. The assays were based on deoxyribonucleic acid (DNA) polymerase chain reactions (PCR) that utilized oligonucleotide primers designed to target specific loci in the genome. The DNA was procured from whole blood or buccal swabs from human subjects, and isolated by a magnetic particle processing robot (KingFisher Flex with a 96-well tip comb) into a stable buffer medium. The molecular weights of single-base extension amplicons were determined via an Agena Bioscience MassARRAY Analyzer 4 MALDI-TOF with an automated 96 position chip prep module. The specific terminators added were determined by matching the molecular weights of the subject sample amplicons to those of known sequences from their respective loci. To validate these assays in buccal swabs we evaluated the range of sample dilution that still yielded better than a 90% call rate, allowing dilutions from 2 to 32-fold. Assay call rates of 100.0% overall demonstrated suitable extracted DNA and reaction chemistry (run criteria > 90%). Inter- and intra-run assay replicates agreed 98.1% of the time (run criteria > 95%). Positive control assays agreed 100.0% (criteria > 95%) with literature determinations from multiple references characterized by orthogonal techniques. Negative controls validation run data was 0.00% (run criteria < 10%). This study has demonstrated that this method is ideal for generating robust data and assessing pharmacogenomics in a targeted manner. These assays can be used in tandem with pharmacokinetic data to better understand the metabolism of drugs across a diverse landscape of genetics. Finally, while we validated twelve new assays using this approach, our study demonstrated the ability to customize multiplexed assays targeting whichever genes and variants may be of interest in a given study.

P224 - CHARACTERIZATION OF COMMON DPYD SINGLE NUCLEOTIDE VARIANTS ON 5-FLUOROURACIL CATABOLISM *IN VITRO*

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5-fluorouracil (5-FU) is a commonly prescribed antimetabolite chemotherapeutic used to treat various cancers. However, approximately 30% of treated cancer patients experience severe 5-FU toxicity. Dihydropyrimidine dehydrogenase (DPD, gene name DPYD) is the rate-limiting enzyme responsible for 5-FU catabolism to its inactive metabolite dihydrofluorouracil (DHFU). Single nucleotide variants (SNVs) in the DPYD gene that impair DPD activity reduce 5-FU clearance, leading to severe toxicities during 5-FU therapy. The Clinical Pharmacogenetics Implementations Consortium (CPIC) recommends DPYD genetic testing and 5-FU dose reductions for variants that have been linked to DPD deficiency and 5-FU toxicity, including c.1905+1G>A (rs3918290, DPYD*2A), c.2846A>T (rs67376798, p.D949V), c.1679T>G (rs55886062, DPYD*13, p.I560S), and c.1129-5923C>G (rs75017182, HapB3). Although DPYD genotype testing is highly informative when any of these 4 variants are identified, these variants account for only a minor proportion of observed toxicities, suggesting that current DPYD testing fails to capture the full extent of DPYD genetic variation responsible for 5-FU toxicity. The *in vitro* and *in vivo* relevance of a number of common (global minor allelic frequency >1%) DPYD SNVs on DPD activity and severe 5-FU toxicity remains conflicting. Thus, the objective of this study is to characterize the functional impact of several common DPYD SNVs on DPD-mediated 5-FU catabolism *in vitro*. Plasmids containing common DPYD SNVs c.85T>C (rs1801265, p.C29R; MAF = 21.9%), c.496A>G (rs2297595, p.M177V; MAF = 9.17%), c.1601G>A (rs1801158, p.S534N; MAF = 1.47%), and c.1896T>C (rs17376848, p.F632=; MAF = 4.17%) were generated. The DPYD variant plasmids as well as a wildtype DPYD (DPYD*1) plasmid were transfected into HEK293T cells. 48 hours post-transfection, cell lysates were incubated with 0.2µM or 20µM 5-FU for 30 minutes. DHFU formation was measured using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) and compared to DHFU formation in DPYD*1 plasmid transfected cells. Following incubation with 0.2µM 5-FU, DHFU formation was not significantly different between DPYD*1 transfected cells and cells transfected with the common DPYD SNVs c.85T>C (p > 0.9999), c.496A>G (p = 0.9981), c.1601G>A (p = 0.5319), and c.1896T>C (p = 0.8311). Similarly, no significant differences in DHFU formation were observed between DPYD*1 and the common SNVs after incubation with 20µM 5-FU (c.85T>C p > 0.9999; c.496A>G p > 0.9999; c.1601G>A p = 0.7325; c.1896T>C p = 0.9769). These findings suggest that the common DPYD SNVs c.85T>C, c.496A>G, c.1601G>A, and c.1896T>C do not significantly alter DPD-mediated 5-FU catabolism and likely are not major contributors to severe 5-FU toxicity in cancer patients. Accordingly, characterization of rare and ethnicity-specific DPYD variation may be a better approach to identifying genetic predictors of 5-FU toxicity.

P225 - THE *IN VIVO* PHARMACOKINETICS OF CROSS-LINKING ANTISENSE-MODIFIED NUCLEIC ACIDS RADIOLABELED BY FT-LABELLING METHOD

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Pharmacokinetic evaluations are crucially important in the development of nucleic acid drugs. In *in vivo* distribution studies, quantitative whole-body autoradiography (QWBA) is often used and it requires label nucleic acids with a radioisotope (RI). In this study, we introduced a new nucleic acid labeling technique, functionality-transfer (FT)-labeling, developed by RINAT Imaging. This technique achieves position-selective 3H-labeling of nucleic acids. Presently, 17 nucleic acid drugs are on the market, and most of them are antisense nucleic acids (ASOs). A variety of modified nucleic acids including methoxyethyl group (MOE) have been developed for ASO due to the problem of stability against nuclease *in vivo*. Moreover, Luxna Biotech has recently developed AmNA (amide-bridged nucleic acid) and scpBNA (spirocyclopropylene-bridged nucleic acid), which are bridged nucleic acids. These nucleic acids are not only nuclease resistant, but also have advantages in lowering hepatotoxicity and are expected to be applied to drug discovery research. However, the pharmacokinetic impacts of introducing these cross-linking modifiers into ASO are unknown. Therefore, we evaluated the *in vivo* pharmacokinetic impacts of introducing AmNA/scpBNA, using LX-A3945, an ASO introduced with AmNA/scpBNA, and LX-A8140, an ASO introduced with MOE, radio-labeled by the FT-labeling technique. LX-A3945 and LX-A8140 were radio-labeled by the FT-labeling technique and was administered subcutaneously to 8-week-old C57/BL6 mice. Then, QWBA was performed at 2, 24, 168, 336, and 672 h after administration to calculate the concentrations of radioactivity in tissues at each time point. Concurrently, the urine, feces, and expired air were sampled up to 672 h after administration to calculate the excretion of radioactivity. Moreover, for the urine, the production of 3H₂O was assessed. Furthermore, metabolite analysis was performed using an LC-RAD-MS/MS system. In QWBA, both ASOs distributed rapidly to the whole body with the liver showing the highest radioactivity concentration, followed by the renal cortex. There was a difference in the distribution of both ASOs in the tissues examined. The excretion study clarified that the main excretion route was via urine for both compounds, and the radioactivity was excreted by 71.5% for the LX-A3945 groups

and 77.9% for the LX-A8140 groups by 672 h after administration. The contribution ratio of the urine and feces were 7:3 for both compounds.

These results suggest that the *in vivo* pharmacokinetic impacts of both ASO introduced AmNA/scpBNA and MOE can be evaluated and FT-labeling technique used in this study will be useful for future nucleic acid drug discovery research.

P226 - DISCOVERY OF GENETIC BIOMARKERS ASSOCIATED WITH ANTITUBERCULOSIS DRUG-INDUCED LIVER INJURY IN THE KOREAN POPULATION: INSIGHTS FROM NUCLEAR AND MITOCHONDRIAL DNA VARIATIONS.

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Despite effective tuberculosis (TB) control programs in Korea, hepatotoxicity remains a major concern during TB treatment. The anti-TB drug-induced hepatotoxicity (ATT_DILI) results from a multifaceted interaction of genetic, environmental, and non-genetic factors. Our study aimed to identify genetic biomarkers for ATT_DILI among a Korean population, focusing on nuclear and mitochondrial DNA variations. A retrospective study was conducted on 58 ATT_DILI cases and 502 ATT_nonDILI controls, all recruited from the Center of Personalized Precision Medicine of Tuberculosis at Pharmacogenomics Research Center, Inje University. Peripheral blood samples were used for DNA extraction, followed by genotyping using the Infinium™ Global Diversity Array with Enhanced PGx-8 v1.0 microarray. We employed GenomeStudio v.2.0.5, PLINK v.2, and SNPtest v2.5.6 for quality control and genome-wide association analyses, adjusting for sex, age, and anti-TB drug use using multivariate logistic regression, with the Bonferroni correction applied for multiple comparisons. The significant SNPs were evaluated for their functional impacts, regulatory effects, and potential influence on gene expression through various platforms including SNPinfo, RegulomeDB, UCSC Genome Bioinformatics, and the GTEx Portal. All statistical analysis was performed using statistical packages in R v.4.3. Our investigation detected several mitochondrial DNA variants potentially linked to increased ATT_DILI risk, yet they did not retain statistical significance following multivariate adjustment. Analysis of 577,078 autosomal single nucleotide polymorphisms (SNPs) for their association with ATT_DILI identified variants within the mitochondrial fission factor (MFF) gene, especially rs6722598A>G, rs4420693C>T, and rs4442987C>T, which exhibited a significant association with reduced risk of ATT_DILI (Odds Ratio [OR]: 0.31; 95% Confidence Interval [CI]: 0.2 - 0.49; p = 1.63E-6). Functional analysis demonstrated the strong regulatory potential of these variants, particularly in liver tissue expression. Validation of previously reported SNPs with ATT_DILI revealed the protective effect of glutathione S-transferase Pi 1 (GSTP1) rs1695A>G (OR: 0.46; 95% CI: 0.24 - 0.88; p = 0.018) and the increased risk associated with solute carrier organic anion transporter family member 1B1 (SLCO1B1) rs4149056A>G (OR: 1.81; 95% CI: 1.12 - 2.93; p = 0.013) at normal significance. In conclusion, our study highlighted the significant association between MFF gene variants and lower risk of ATT_DILI development in Korean patients with tuberculosis. Further research is necessary to validate these findings.

P227 - ANALYTICAL VALIDATION OF A GENOTYPING TECHNIQUE FOR A SINGLE-NUCLEOTIDE POLYMORPHISM TO PREDICT OF THE ONSET SJS/TEN IN JAPANESE

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Stevens–Johnson syndrome and toxic epidermal necrolysis (SJS/TEN) represent rare but severe cutaneous adverse drug reactions. Specific human leukocyte antigen (HLA) types, such as HLA-B*58:01 and HLA-B*15:02 or HLA-A*31:01, have been associated with SJS/TEN onset related by allopurinol and carbamazepine, respectively. However, because HLA typing is time-consuming and expensive, a rapid and easy assay method has been expected in clinical situations. Previously, we reported that the single-nucleotide polymorphism (SNP) rs9263726 was in absolute linkage disequilibrium with HLA-B*58:01 in Japanese, offering a potential surrogate marker for this HLA type. In this study, we developed a novel genotyping method for this SNP utilizing the single-stranded tag hybridization chromatographic printed-array strip (STH-PAS) technique and performed an analytical validation. We recruited Japanese SJS/TEN patients from participating institutes of the Japan Severe Adverse Reactions research group and through a nationwide case-collecting network system. Genomic DNA was extracted from blood leukocytes, HLA-B type was determined and rs9263726 was genotyped using TaqMan SNP Genotyping Assay or STH-PAS. The analytical validation of STH-PAS was performed for several parameters referring to “Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of

Food, Feed, Cosmetics, and Veterinary Products” and “Accuracy Management Manual for PCR Tests for Novel Coronavirus Infection”. The reproducibility of the intra-laboratory repeatability was performed with three independent measurements. Accuracy was evaluated by the consistency of positive and negative controls and the genotypes of rs9263726 revealed by a TaqMan assay. A three-fold dilution series (0.04-90 ng) of a representative sample was made to determine a tentative detection limit, and then STH-PAS was performed using 0.37-30 ng of genomic DNAs as a template to obtain an accurate detection limit, and the lowest concentration at which 95% of the samples were detectable was set as the detection limit. The robustness of the method was evaluated with 10 ng of genomic DNA by adding minor modifications to the annealing temperature, master mix volume, and primer concentration. Furthermore, the effects of the types of thermal cyclers were determined. The results of genotyping rs9263726 in 28 allopurinol-related SJS/TEN patients using STH-PAS correlated well with those obtained using the TaqMan SNP Genotyping Assay for 15 HLA-B*58:01-positive and 13 HLA-B*58:01-negative patients (The concordance rate of rs9263726 as a surrogate marker for HLA-B*58:01 was 100%). Additionally, at least 1.11 ng of genomic DNA was sufficient to digitally detect positive signals on the strip. Robustness studies showed the importance of the annealing temperature (66°C) for consistent results. In conclusion, we have developed and analytically validated an STH-PAS method for identifying genetic variants associated with SJS/TEN using multiplex single-tube PCR and a membrane stick. The developed method may serve to avoid allopurinol-induced SJS/TEN at least in Japanese.

P228 - MECHANISMS UNDERLYING COGNITIVE ENHANCEMENT INDUCED BY ORAL INGESTION OF YEAST-DERIVED RNA USING COMPREHENSIVE TARGETED LIPIDOMICS

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Oral ingestion of nucleic acids exhibits various biological functions, but those in the brain are largely unknown. We have recently reported that the ingestion of DNA hydrolysate derived from salmon milt showed cognitive enhancement in healthy mice¹, while the effect of ingestion of RNA on cognitive function remains unclear. The purpose of the present study was to elucidate the effect of torula yeast-derived RNA (tyRNA), which contains ~70% of RNA consisting of 20-200 bases of oligoribonucleotides, on learning and memory, and its underlying mechanisms using comprehensive targeted lipidomics in healthy mice. First, seven-week-old ICR mice were fed a low-levels of nucleic acid diet (AIN-93M) or a diet that contained 2.5% tyRNA. Fourteen days later, the novel object recognition test (NORT) and the special recognition test (SRT) were performed to evaluate cognitive function, revealing that the tyRNA ingestion enhanced both object recognition and spatial memory. To understand the underlying mechanism for such cognitive enhancement effect of tyRNA, the biodistribution of orally administered tyRNA was then examined. Measurement of its putative nucleic acid-related metabolites by LC-MS/MS showed that brain concentrations of uridine, cytidine, deoxycytidine (dC), uridine monophosphate (UMP), and deoxycytidine monophosphate (dCMP) of tyRNA ingested group were significantly higher than those in the control group. In the plasma of tyRNA-administered mice, on the other hand, concentrations of uridine and cytidine were significantly higher than those of the vehicle group. Since certain nucleic acids-related metabolites including uridine and cytidine contribute to the synthesis of brain phosphatidylcholine (PC), which is a component of the neuronal membrane, via the Kennedy pathway³, and the synthesis of brain PC may promote neurite outgrowth⁴, we hypothesized that orally ingested tyRNA leads to an increase in certain phospholipids, thereby showing cognitive enhancement. Therefore, the comprehensive targeted lipidomics using LC-qTOFMS was performed in the brains of mice fed 2.5% tyRNA. PC18:0/18:1 level in the brain was significantly higher in tyRNA group compared to the control group, implying that ingestion of tyRNA may increase PC synthesis in the brain possibly via the Kennedy pathway. Then, the effect of RNA-related nucleotides on the expression of Synapsin 1 (Syn1), a presynaptic marker, was examined in primary cultured murine cortical neurons, showing that only uridine and cytidine significantly increased the expression of Syn 1. Thus, uridine and cytidine may promote synaptogenesis. Taken together, ingestion of tyRNA in mice may increase nucleic acids-related metabolites in the brain and improve cognitive function by the increase in PC in healthy mice.

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P229 - EVALUATING TRANSCARDIAL PERFUSION INTEGRITY IN BRAIN TISSUE: DEVELOPMENT OF A NOVEL HEMOGLOBIN QUANTIFICATION ASSAY

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In the realm of pharmacological research, accurately determining drug concentration within tissues is important. However, one significant obstacle to this accuracy is the potential overestimation of drug concentrations due to blood contamination within the tissue. To address this issue, our study focuses on the effectiveness of perfusion in removing blood contamination and the evaluation method of blood contamination in the brain. Previous methods have employed radioactive tracers, blood tracers, transferrin, and other biomarkers to assess the extent of blood contamination. However, selecting a specific biomarker already present in the tissue can introduce bias into the results. In light of this, hemoglobin was identified as an appropriate biomarker for assessing blood contamination in brain tissue, given its inherent presence and relevance. Our recent investigation has further explored the use of parallelism to indirectly assess the matrix effects of endogenous substances like hemoglobin in the brain. Obtaining a genuine blank matrix for such substances poses significant challenges, rendering traditional spike and recovery methods—commonly applied for exogenous substances—ineffective for evaluating matrix effects. To circumvent this issue, we employed a surrogate matrix, implementing additional dilution steps to ascertain the minimum required dilution (MRD). By leveraging parallelism to calculate MRD, our methodology aims to effectively evaluate the matrix effects of endogenous substances like hemoglobin. To prepare the brain samples for analysis, two distinct procedures were utilized for perfused and non-perfused brain samples. For perfused samples, mice were euthanized with Avertin (2,2,2 Tribromoethanol) and subsequently perfused transcardially with 1x PBS (Phosphate-buffered saline) at a flow rate of 3 mL/minute for 10 minutes. Non-perfused samples were obtained through cardiac puncture, with brains carefully collected using forceps. All brain samples were homogenized in a 1:10 ratio with 1x cell extraction buffer PTR. The homogenates underwent substantial dilutions of 810 and 65,610 times for perfused and non-perfused samples, respectively. The extent of blood contamination was quantified using an ELISA for mouse hemoglobin. This assay was pivotal in determining the influence of blood presence on the brain pharmacokinetic method. Our findings revealed a significant discrepancy in hemoglobin content between non-perfused and perfused brain tissues. Specifically, hemoglobin concentration in non-perfused brains averaged $509.40 \pm 35.16 \mu\text{g/g}$, whereas, in perfused brains, it markedly reduced to $8.88 \pm 3.03 \mu\text{g/g}$, indicating a residual hemoglobin content of merely 1.74% relative to non-perfused tissue. Through this study, we demonstrate the efficacy of perfusion in mitigating blood contamination in brain tissue samples. Moreover, our innovative approach to utilizing parallelism for matrix effect assessment of endogenous substances like hemoglobin offers a promising avenue for enhancing the accuracy of quantification of drugs in “true” tissue except blood.

P230 - CIRCULATING TUMOR CELLS KINETICS AFTER SHORT- AND LONG-TERM FOLFIRINOX AND GEM/NAB-PTX CHEMOTHERAPY IN XENOGRFT MOUSE MODELS OF HUMAN PANCREATIC CANCER

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To date, there has been no definite experimental evidence for the significance of circulating tumor cells (CTCs) as an indicator to estimate the chemotherapeutic effect in cancer patients. Mobilization of CTCs after various types of therapy, such as radiation therapy, has been reported, but systematic study of CTCs after chemotherapy remained quite limited. We previously reported a transient increase in CTC number 1 week after single-dose chemotherapy in human breast, lung, and gastric cancer xenograft mouse models [1, 2]. In this study, we sequentially examined CTC numbers after single-dose and repetitive-dose chemotherapy, including FOLFIRINOX (FFX) and Gemcitabine and nab-Paclitaxel (GnP) using two pancreatic cancer xenograft models. CTC was detected by the immunocytology-based microfluidic platform. We further examined the dynamic change in the histology of primary tumor tissues during chemotherapy. We confirmed a transient increase in CTCs 1–2 weeks after single-dose and repetitive-dose of FFX/GnP chemotherapy. Histological examination of the primary tumors revealed that the peak period of CTC at 1–2 weeks after chemotherapy corresponded to the maximal destructive phase consisting of cell cycle arrest, apoptosis of tumor cells, and blood vessel destruction without secondary reparative tissue reactions and regeneration of tumor cells. The early phase (1–2 weeks) after chemotherapy is a histologically unique time point of primary tumor tissue with the purest destructive phase without significant secondary histological reactions such as regeneration of tumor cells, repair of blood vessels, and formation of fibrous scar. These findings indicate that mobilization of CTCs early after chemotherapy is mediated by the shedding of degenerated tumor cells into the disrupted blood vessels driven by the pure destructive histological changes in primary tumor tissues. These results suggest that sequential monitoring of CTCs in terms of number and morphology (single or cluster) before and after chemotherapy using our immunocytology-based CTC detection platform could be a potentially powerful diagnostic tool for liquid biopsy to predict the response to anti-cancer therapy in various clinical settings.

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P231 - EFFECTS OF ANESTHESIA AND DIFFERENT LYMPHATIC FLUID COLLECTION METHODS ON THE LYMPHATIC TRANSPORT OF ORALLY ADMINISTERED DRUGS: A COMPARATIVE STUDY IN RATS

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The absorption of oral dosage forms in the body primarily involves intestinal and lymphatic absorption into the bloodstream. The investigation of drug absorption, distribution, and transportation through the lymphatic circulation gained significant attention in the mechanism study of bioavailability. This study aimed to evaluate the effects of anesthesia (with or without) and different lymphatic fluid collection methods on the lymphatic transport of orally administered drugs in rats. Vitamin D3 and halofantrine hydrochloride, highly lipophilic compounds, are significantly absorbed into the bloodstream through the lymphatic transport pathway. The lymphatic transport of Vitamin D3 was compared in mesenteric lymph duct cannulated rats under anesthesia and non-anesthetized rats. However, the anesthetized group exhibited approximately 30% reductions in lymph flow rate and lymph volumes compared to the non-anesthetized group. Therefore, selecting conscious rats, rather than commonly used anesthetized rats in most previous experiments, provides a more accurate reflection of drug lymphatic transport.

The study also examined the impact of two different lymph collection methods, specifically mesenteric lymph duct cannulation and thoracic duct cannulation, on the lymphatic transport of halofantrine hydrochloride following oral administration in conscious rats. The plasma AUC_{inf} of halofantrine hydrochloride in the mesenteric lymph duct cannulation group, thoracic duct cannulation group, and control group (non-surgical) were (7083 ± 1908) ng·h/mL, (11195 ± 5968) ng·h/mL, and (18815 ± 5249) ng·h/mL, respectively. This indicates that the recovery rates through mesenteric lymph duct cannulation and thoracic duct cannulation were (11 ± 3.42) % and (23 ± 4.76) % of the total recovery, respectively. Consequently, using different experimental models may result in slight differences in the pharmacokinetic evaluation of orally administered drugs through lymphatic transport in the body. Therefore, selecting an appropriate surgical model based on specific research objectives is necessary to accurately reflect the absorption of drugs through lymphatic transport in the body.

In addition to investigating the absorption kinetics of oral compounds through the intestine into the bloodstream, it is sometimes crucial to consider their lymphatic absorption pathways, particularly for compounds with high lipophilicity. Mechanistic studies on the lymphatic absorption, distribution, and transportation processes of oral drugs can provide new possibilities for optimizing the oral bioavailability of drugs.

P232 - THE IMPORTANCE FOR DETERMINING PLASMA/TISSUE CONCENTRATIONS AND HIGHLY SENSITIVE BIOANALYTICAL METHODS TO UNDERSTAND RELATIONSHIP BETWEEN EXPOSURE AND EFFICACY OF PHOSPHORODIAMIDATE MORPHOLINO OLIGONUCLEOTIDES

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Background: Up to date, four kinds of phosphorodiamidate morpholino oligomers (PMOs) such as viltolarsen have been approved for the treatment of Duchenne muscular dystrophy (DMD); however, it is unclear whether human efficacy can be estimated using plasma concentrations. In addition, the bioanalytical methods of PMOs using liquid chromatography with tandem mass spectrometry (LC-MS/MS) are not highly sensitive (lower limit of quantification [LLOQ]: 10 to 20 ng/mL in plasma according to clinical pharmacology and biopharmaceutics review of casimersen, golodirsen, and viltolarsen), and plasma concentration at later time points could not be determined because of their rapid decrease in plasma, which makes it difficult to obtain tissue (muscle) to plasma concentration ratio (Kp). The aim of this study was to develop highly sensitive bioanalytical methods and clarify the relationship between exposure and efficacy of PMOs. Methods: Firstly, we summarized the tissue distribution of ¹⁴C-viltolarsen in wild type mice, mdx model mice, and cynomolgus monkeys measured by Quantitative Whole-Body Autoradiography. We then developed highly sensitive bioanalytical methods of viltolarsen with LC-MS/MS, and plasma and tissue concentrations of viltolarsen in mdx model mice and cynomolgus monkeys were determined. Results: In the tissue distribution studies, all muscles in mdx model mice showed higher concentration of ¹⁴C-viltolarsen than those in the wild type mice and cynomolgus monkeys. Highly sensitive bioanalytical methods of viltolarsen with LC-MS/MS (LLOQ: 1 ng/mL in plasma) were successfully established by optimizing sample preparation step using protein precipitation and solid-phase extraction. By using these methods, the good correlation between the concentration of viltolarsen and the efficacy in skeletal muscles in cynomolgus monkeys was found. In

addition, the Kp values in mdx model mice could be determined at later time points. Conclusion: Tissue distributions of viltolarsen in mdx model mice, wild type mice and cynomolgus monkeys were clarified, and it was suggested that mdx model mice would be better to estimate the distribution of viltolarsen in DMD patients. By using highly sensitive bioanalytical method, the concentrations of viltolarsen in skeletal muscle were shown to correlate with skipping efficiency in cynomolgus monkeys. In addition, Kp value could be determined at later time points in mdx mice. These results suggested that pharmacokinetic (PK)/pharmacodynamics (PD) analysis can be conducted using human PK profile, and Kp values and skipping efficacy in mdx model mice, which is useful information for efficient and effective development of PMO including translation from nonclinical to clinical phase.

P233 - EFFECTS OF PHOSPHOLIPID TYPE AND PARTICLE SIZE ON LIPID NANOPARTICLE DISTRIBUTION *IN VIVO* AND IN PANCREATIC ISLETS

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Recently, lipid nanoparticles (LNPs) have been used as nanocarriers in a drug delivery system to deliver nucleic acid drugs. Their practical application is currently primarily limited to the liver and some specific organs. However, altering the type and composition ratio of phospholipids improves their distribution in organs other than the liver, namely, the spleen and lungs. This study aimed to elucidate the effects of LNP components and particle size on *in vivo* distribution through systemic circulation to the pancreatic islets, to achieve better targeting of islets, which is a fundamental therapeutic target for diabetes.

Fluorescence-labeled LNPs were prepared using three different phospholipids, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), at particle sizes of 30–160 nm (diameter) using the microfluidic device method. Baffled-structured iLNP devices with adjusted flow rate ratios and total flow rates were used. After intravenous administration the LNPs, the distribution of each LNP type to major organs including the pancreas and pancreatic islets was compared using ex vivo fluorescence imaging and observation of pancreatic tissue sections. DSPC-LNPs and DOPE-LNPs showed highest distribution in the spleen and liver, respectively. In contrast, DOPC-LNPs showed the highest distribution in the pancreas and the lowest distribution in the liver and spleen. Additionally, smaller particles showed better distribution throughout the pancreas. Furthermore, the most significant intrapancreatic LNP distribution in the islets was observed for DOPC-LNPs at 160 nm particle size. Furthermore, larger LNPs tended to distribute in the islets, whereas smaller LNPs tended to distribute in the exocrine glands. DOPC-LNPs were distributed to islets at all cholesterol concentrations with high distribution observed at > 40% cholesterol and > 3% PEG; the distribution was higher at 24 h than at 4 h.

In conclusion, we successfully elucidate the effect of LNP components and particle size on *in vivo* distribution, especially in the pancreas, after intravenous administration by performing fluorescence imaging and our original islet analysis technique. The results showed that selecting DOPC as the phospholipid enhanced the distribution of LNP to the islets and that this distribution was still observed 4–24 h after administration. Moreover, altering the particle size could be used as a means to alter the distribution of nanoparticles to the exocrine glands or endocrine tissues within the pancreas. In conclusion, the insights obtained from this study provide important information for the development of nanoparticles to address the unmet medical needs in pancreatic diseases.

P234 - PHARMACOKINETICS COMPARISON BETWEEN PRESSURIZED INTRAPERITONEAL AEROSOLIZED CHEMOTHERAPY (PIPAC) AND INTRAVENOUS INFUSION OF OXALIPLATIN IN PIG PLASMA AND TISSUES

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Although intravenous (IV) infusion is still the gold standard for treating peritoneal surface malignancies (PSMs), it is believed to have little effect because of the limited drug delivery to the peritoneal surface in spite of high systemic peak concentration. Therefore, pressurized intraperitoneal aerosol chemotherapy (PIPAC) is regarded as one of novel laparoscopic drug delivery systems that can induce regression of PSMs in rescue situations. In this study, our goal is to demonstrate that PIPAC delivers a higher concentration of the chemotherapeutic agent to the peritoneal surface while lowering the concentration at systemic circulation than an existing IV infusion by conducting pharmacokinetic studies using a pig model. Oxaliplatin was chosen as a chemotherapeutic agent because it is one of the three validated drugs administered in PIPAC, and oxaliplatin monotherapy is one of the regimens of intraperitoneal agents. The concentrations of oxaliplatin in plasma and five different tissues such as liver, kidney, spleen, pancreas, and peritoneum were quantified using LC-MS/MS method. Sample preparation was processed through a simple protein precipitation with acetonitrile containing carboplatin as an internal standard. As a result, the maximal concentration (C_{max}) of oxaliplatin in plasma after IV infusion for 5 min was 4030 ng/mL and the other points are detectable until 2 hours, whereas, the C_{max} of oxaliplatin

after PIPAC was 159 ng/mL and more than two points around the time for maximal concentration (Tmax) are under the lower limit of quantification (LLOQ). Additionally, the ratio of oxaliplatin concentration in tissue homogenates (liver, kidney, spleen, pancreas, and peritoneum) by IV infusion to PIPAC was 0.74, 0.76, 0.77, 0.76, and 0.46, respectively, indicating oxaliplatin penetrates into other tissues better when PIPAC is used, especially for the peritoneum. Finally, this study successfully applied to prove the effectiveness and safety of PIPAC surgery compared to IV infusion and can be used as a supplement for the development of further chemotherapeutic surgery.

P235 - CHARACTERIZATION OF AN 3D-PRINTED EXPANDABLE DRUG DELIVERY SYSTEM FOR TOFACITINIB IN BEAGLE DOGS

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Tofacitinib is a Janus kinase (JAK) inhibitor for the treatment of rheumatoid arthritis, which was developed as an oral dosage form. This study aimed to develop a 3D-printed expandable drug delivery system of tofacitinib and characterize the *in vivo* pharmacokinetics in beagle dogs. The expandable drug delivery system consisted of a 3D-printed capsular device designed to expand in the stomach and a sustained-release (SR) tablet of tofacitinib. Once in the stomach, the capsular device could be expanded and release the drug from the inner SR tablet within the stomach. After completion of drug release, the capsular device loses the expanding structure and is designed to move to the small intestine. Mixture design was applied to determine the gastric residence time of the expandable drug delivery system with the formulation compositions of the inner IR and SR tablets as control factors. While the capsular device was fabricated by fused deposition modeling (FDM) 3D printer, inner tablets were prepared by conventional wet granulation methods. The prepared expandable drug delivery system was orally administered to beagle dogs and the plasma concentrations of tofacitinib were determined using a validated LC-MS/MS. Following oral administration in beagle dogs, while the tofacitinib SR tablet showed the maximum plasma concentration (Cmax) after 2 h, the expandable system maintained stable plasma concentration up to 8 h. The area under the plasma concentration vs. time curve (AUC) was 752.8 ± 92.6 ng*h/mL for the SR tablet and 515.0 ± 130.3 ng*h/mL for the expandable system. The 3D-printed capsular device may provide a promising tool to develop novel controlled-release dosage forms with an optimized pharmacokinetics and therapeutic efficacy.

P236 - TRANSLATIONAL PHARMACOKINETIC-PHARMACODYNAMIC/TOXICODYNAMIC (PK-PD/TD) MODELING OF ANTITUMOR EFFECTS AND PERIPHERAL NEUROPATHY IN GEMCITABINE AND NAB-PACLITAXEL CHEMOTHERAPY

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Chemotherapy-induced peripheral neuropathy is often observed with gemcitabine and nab-paclitaxel (GnP) therapy, the standard first-line chemotherapy regimen for unresectable pancreatic cancer. The challenge to improve the prognosis and patient survival with GnP therapy is to develop evidence-based dosing strategies that can strike a delicate balance between therapeutic efficacy and toxicity management, and to continue treatment without interruption. The aim of this study is to develop pharmacokinetic (PK) and pharmacodynamic/toxicodynamic (PD/TD) model of antitumor effects and peripheral neuropathy in GnP therapy from rat data and to investigate whether pharmacometrics approach could translate animal data to clinical outcomes. A mouse xenograft model of human pancreatic tumor was prepared by SUIT-2 cell line in seven- to nine-week-old female athymic nude mice (KSN strain). The data of drug concentrations in the plasma and tumor, tumor volume, and paw withdrawal frequency in the von Frey test after GnP treatment to xenograft model mice were used for developing PK-PD/TD model. The Simeoni tumor growth inhibition model with tumor concentrations and empirical indirect response models were applied for the PD and TD models, respectively. The population PK-PD/TD model was sufficient to adequately predict individual tumor volume and paw withdrawal frequency. According to Wong et al. [1], the tumor growth inhibition value in mouse xenografts significantly correlated with the observed overall response rate, and a threshold %TGI of 60% at clinically relevant exposure was identified for clinical response. Then, employing the hybrid PK-PD/TD model based on the approach by Wong et al.[1], clinical outcomes were simulated by replacing rat PK parameters with reported population estimates of PK parameters in cancer patients. For the standard GnP regimen, the model predicted clinical overall response as 75.1% (95% CI: 72.6–78.1%), which was overestimated compared to that in a recent phase II study (42.1%) but lower than the observed disease control rate (96.5%) [2]. Model simulation indicated that reducing the GnP dose to less than 40% was ineffective; compared to a 60% weekly dose reduction, a change in dose schedule from every week for 3 weeks to every 2 weeks would be a plausible approach. In conclusion, the PK-PD/TD model-based translational approach may contribute to optimal dose determination to avoid severe peripheral neuropathy while maintaining antitumor effects during GnP chemotherapy.

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P237 - RADIATION THERAPY FOR BRAIN TUMORS: DOES IT INFLUENCE DRUG DELIVERY?

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Radiation therapy (RT) is an effective treatment for brain tumors [1] where it is difficult to remove all the infiltrative tumor cells through surgical resection. Given this limitation, RT was recognized as an adjuvant treatment for brain tumors. In recent decades, RT, in conjunction with the cytotoxic temozolomide, has been further refined; yet the combination only improves overall survival by a few months.[2] Intensive research is being conducted to improve the effectiveness of RT with concomitant drug therapy for brain tumors. It is critical to appreciate how RT may influence drug action through changes in drug delivery to the tumor. Therefore, the influence of RT on the blood-brain barrier (BBB), and how that may change drug delivery needs to be explored. Current opinions, even within the brain tumor community, hold that RT will lead to changes in the BBB that result in increased drug delivery in those areas that have been irradiated.[3] There is a distinct lack of data that support this opinion. The influences of radiation to the central nervous system (CNS) are mainly on brain vasculature (e.g., the BBB) and neuroglial cells. Since the BBB often hampers brain penetration of chemotherapeutics, the impact of radiation on the BBB becomes an important question for drug delivery to brain tumors. Studies have shown that radiation induces opening of tight junctions, increases proinflammatory mediators, and alters efflux transporters activities.[4,5] RT has been implicated with increased BBB permeability of radiolabels in both experimental animals and patients, with the results mainly quantified by imaging methods.[3,6,7] However, quantitative studies measuring drug distribution in irradiated brains have been lacking. We have investigated whether RT affects drug distribution across either the intact BBB or the more permeable blood-tumor barrier (BTB). Using two groups of compounds: 1) compounds that are limited by tight junctions, and, 2) compounds limited by efflux transport, we find that RT has no effect on the limitation of tight junctions on drug penetration into the normal brain. Also, we saw no effect of RT on drugs that are either liable to efflux transport or not. Then we investigated the impact of RT on the brain distribution of brigimadlin, a potent MDM2-p53 antagonist that is limited by BBB efflux, in both normal mouse brain and orthotopic tumor models. Consistently, we found no acute or delayed impact of RT on the distribution of this drug across either normal intact BBB or the more permeable BTB. In conclusion, our study demonstrated that RT does not have significant influence on drug distribution across the BBB or BTB in the mouse. Though previous reports showed that RT alters BBB physiology, we believe that these changes are insufficient to impact drug delivery to the brain. This study supports previous investigations, where the co-administration of chemotherapeutics with RT, with the intent of enhancing delivery, failed to improve patient survival,[8] and could provide insights into the development of combination radio-chemotherapy for brain tumors.

P238 - EVALUATION OF A CINNAMOMUM VERUM PRODUCT ON THE PHARMACOKINETICS OF THE CYP2A6 MODEL DRUGS NICOTINE AND LETROZOLE IN HEALTHY ADULTS

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Introduction: Cinnamon (Cinnamomum spp.) is used worldwide as a culinary spice and botanical dietary supplement. As a supplement, cinnamon is commonly used for multiple purported health benefits, including anti-inflammatory and blood glucose-lowering effects. The major constituents, cinnamaldehyde and 2-methoxycinnamaldehyde, were previously shown to be mechanism-based inactivators of cytochrome P450 (CYP) 2A6 through heme degradation and apoprotein modification. Using a mechanistic model, both constituents were predicted to increase the area under the plasma concentration vs. time curve (AUC) of the CYP2A6 substrates nicotine and anti-cancer drug letrozole by 4- to 5-fold.¹ The objective of the current study was to evaluate the effects of a well-characterized cinnamon (C. verum) product on the pharmacokinetics of nicotine and letrozole in healthy, non-smoking adults. Our hypothesis was that chronic exposure to C. verum will increase systemic exposure to nicotine and letrozole in humans. Methods: Healthy non-smoking adults (n=16) participated in this open-label crossover study. They were administered a single dose of nicotine gum (2 mg) or letrozole (2.5 mg tablet). Blood (5 mL) was collected from 0-24 (nicotine) or 0-240 (letrozole) hours to obtain baseline pharmacokinetics. After a washout period of at least 24 hours (nicotine) or 14 days (letrozole), participants self-administered the C. verum product (2 g thrice daily) at home for 5 consecutive days. On Day 6, they were administered

nicotine gum or letrozole with *C. verum*, and blood was collected from 0-24 (nicotine) or 0-240 (letrozole) hours. Plasma was analyzed for nicotine, cotinine (COT), and 3-hydroxycotinine (3HC) or letrozole using LC-MS/MS methods. The pharmacokinetics of each analyte were determined via non-compartmental analysis using Phoenix WinNonlin™. Results: The geometric mean plasma concentration vs. time profiles for nicotine and letrozole were nearly superimposable regardless of *C. verum* exposure (Figure 1). The geometric mean ratio (GMR) [90% confidence interval] for nicotine and letrozole AUC in the presence to absence of *C. verum* was 1.03 [0.96 – 1.12] and 1.11 [0.98 – 1.24], respectively. Geometric mean half-life for nicotine in the presence and absence of *C. verum* was 3.2 [3.0 – 3.5] and 3.7 [3.3 – 4.1] hours, respectively, and for letrozole was 43 [36 – 51] and 48 [35 – 65] hours, respectively. The GMR for the nicotine metabolic ratio (3HC/COT), a marker for CYP2A6-dependent metabolic activity, was 0.90 [0.75 – 1.09], indicating minimal CYP2A6 inhibition by *C. verum*. Conclusions: A typical dosage of a well-characterized *C. verum* product showed minimal inhibition of CYP2A6 activity in healthy adults. The general lack of effect of *C. verum* on the pharmacokinetics of nicotine and letrozole suggests that *C. verum* may be safe to consume with both drugs, as well as other CYP2A6 substrates. Further research is needed to assess the effects of *C. verum* on pharmacodynamic endpoints, including biomarkers of inflammation (e.g., C-reactive protein, erythrocyte sedimentation rate) and blood glucose (e.g., HbA1c).

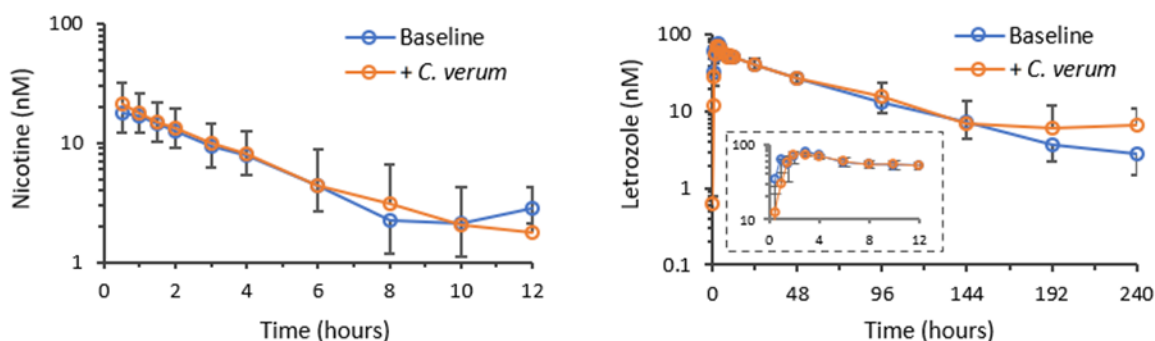


Figure 1. Geometric mean plasma concentration vs. time profile for nicotine (left panel; n = 16 participants) and letrozole (right panel; n = 11-14 participants) following administration of a single dose of nicotine gum (2 mg) or letrozole (2.5 mg tablet) alone (baseline; blue) and after chronic (orange) exposure to a well-characterized *C. verum* product (2 g thrice daily for 6 days). Error bars denote 90% confidence intervals. The inset in the right panel shows the 0-12 hour time period. The last two time points for both profiles represent < 6 participants.

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P239 - THE IMPACT OF GLUCOSE LEVELS ON EFFICACY OF THE FDA-APPROVED ANTISENSE OLIGONUCLEOTIDE DRUGS

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Antisense oligonucleotide (ASO) drugs are developed to selectively target messenger RNA (mRNA) of disease-causing genes via the mechanisms of ribonuclease H (RNase H) cleavage and steric hindrance, which ultimately inhibit or correct the translation of targeted proteins. Up to now, the US Food and Drug Administration (FDA) has approved 13 ASO drugs for the treatment of several rare and common diseases, and decades of ASO drugs are expected to be approved by the FDA in next 5-10 years. Nowadays, more patients are benefiting from ASO drugs. However, a significant proportion of these patients are undergoing one or multiple chronic disease conditions, such as diabetes, hypertension, and hyperlipidemia. Whether the chronic disease conditions alter therapeutic efficacy of the FDA-approved drugs and whether dose justifications are needed or not for those patients are unclear. Our study aims to determine the impact of glucose levels on efficacy of the FDA-approved drugs using mipomersen and inotersen as the model drugs, which are approved for the treatment of familial hypercholesterolemia and hereditary transthyretin (TTR) amyloidosis, respectively, by degrading apolipoprotein B (ApoB) and TTR mRNAs to reduce translation of the disease-related proteins. HepG2 cells were incubated with the culture media containing various glucose concentrations of 2.5 mM (low level), 5.5 mM (normal level), 10 mM (pre-diabetes level), or 25 mM (diabetes level) for 24 hours and the cells were further treated with a standard dose (100 nM) of mipomersen or inotersen for 48 hours. The silencing efficacy on ApoB and TTR at both mRNA

and protein levels decreased when the glucose concentration increased in a dose dependent manner. In the diabetic condition (25 mM glucose), nearly no silencing effects on targeted mRNA and protein production by the ASOs were observed, implying that diabetic patients may experience less benefit on the ASO drugs at the standard dose and may need to justify the dose for the desirable efficacy. In addition, an increase of metformin concentrations, a first-line antidiabetic drug, can significantly enhance the silencing efficacy of the ASO drugs. In order to further confirm the impact of glucose on efficacy of the ASO drugs, knocking down of glucose transporter (GLUT2, encoded by the SLC2A2 gene), the major liver transporter for glucose uptake, was performed prior to the treatment of the ASO drugs. The knockdown of GLUT2 resulted in a significant decrease in mRNA and protein levels of ApoB and TTR, which is similar to the treatment of ASOs with a low glucose level compared to a high glucose level. The data strongly suggest that glucose level has a direct impact on the therapeutic efficacy of the FDA-approved ASO drugs and antidiabetic drugs can help to improve the efficacy of the ASO drugs by controlling glucose levels. We are continuing to elucidate the underlying mechanism by which glucose levels influence the pharmacokinetic characteristics of FDA-approved ASO drugs with the ultimate aim to design safe and effective ASO drugs for patients suffering chronic diseases, particularly diabetes, in order to optimize treatment outcomes.

P240 - OPTIMIZATION OF ORAL DOSE OF 3-HYDROXYBUTYRATE BASED ON PHARMACOKINETIC MODELING FOR COGNITIVE FUNCTION AND MOOD

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The brain can use ketones, mainly 3-hydroxybutyrate (3-HB) as one of the energy sources, so supplying ketones is expected to maintain brain health. In this study, we aimed to evaluate the effects of 3-HB consumption on cognitive function and mood. A previous study has shown that 10 g of medium-chain triglycerides induces ketone metabolism in the brain, with the plasma C_{max} of 3-HB at 0.28 mM(1). Therefore, we hypothesized that, when it achieves 0.28 mM of C_{max}, 3-HB would improve cognitive function and mood. We first conducted a pharmacokinetic study with 10 healthy subjects aged 49.1±6.1 to measure the changes in serum level after intake of 2 g and 4 g of 3-HB. Based on the results, we then established a compartment model of oral 3-HB intake to estimate the minimum dose to achieve the 0.28 mM of 3-HB in serum. One-compartment model with saturable and non-saturable absorption processes sufficiently reflected the actual values of serum 3-HB concentration, and the minimum intake exceeding C_{max} of 0.28 mM was expected to be 3.5 g. We then performed a randomized, double-blind, placebo-controlled, crossover study to evaluate the acute effects of 3.5 g of 3-HB on cognitive function and mood in 40 healthy participants aged 52.1±6.4. A serial arithmetic test and mood assessment were performed before and after the 3-HB intake. The serial arithmetic test was used for a load to make the brain use energy as well as for an assessment of attentional function. Additionally, Cognitrax was performed afterward to assess various cognitive function domains. Regarding cognitive function, total responses and correct answers increased after the 3-HB intake in the serial arithmetic test, but no significant effects were observed in any of the cognitive domains measured by Cognitrax. As for effects on mood, 3-HB suppressed the increase in confusion-bewilderment, fatigue-inertia, total mood disturbance scores, and the decrease in vitality-activity score in the Profile of Mood States 2nd Edition (POMS2). Similarly, 3-HB suppressed fatigue in the visual analogue scale (VAS). In summary, we developed a compartment model of oral 3-HB intake and showed the efficacy of the minimum effective dose of 3-HB predicted by the model on attentional function assessed by the serial arithmetic test and various moods measured by POMS2 and VAS.

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P241 - A RETROSPECTIVE COMPARISON OF THE PK PROFILES OF LIPID 5, THE IONIZABLE LIPID IN LNP2, ACROSS RAT, NHP, AND HUMAN

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Lipid nanoparticles (LNP) have been recently used to encapsulate mRNAs for delivery to specific cell types and/or tissues. LNPs consist of an ionizable lipid, cholesterol, DSPC, and PEG. Lipid 5 is a proprietary ionizable amino lipid utilized in LNP2 formulations encapsulating mRNA for a range of mRNA therapeutics. The purpose of this study was to compare the pharmacokinetics of Lipid 5 in circulation across rat, cynomolgus monkey (NHP) and human. LNPs containing Lipid 5 (i.e., LNP2) were dosed as single or repeat intravenous bolus or infusions in rat, NHP, and human encapsulating a variety of mRNA payloads. A time course of plasma or serum samples were collected and analyzed for Lipid 5 using validated/qualified LC/MS/MS assays. Concentrations of Lipid 5 in systemic circulation were used to calculate pharmacokinetic parameters using Phoenix PK software using a non compartmental approach consistent with

the IV bolus or infusion route. When practical, the terminal elimination phase of each concentration versus time curve was identified using at least the final three observed concentration values. The slope of the terminal elimination phase was determined using log linear regression on the unweighted concentration data. Where available, a retrospective comparison of the PK profiles of Lipid 5 across rat, NHP, and human were conducted which included results from up to 6 different mRNA constructs/programs. As the LNP was delivered intravenously, for all species the time to the observed maximum concentration (T_{max}) was the first collection following injection. In rat and NHP, Lipid 5 was cleared quickly from the circulation with an effective or terminal half-life of roughly 1 or 4-6 hours, respectively. The exposure to Lipid 5 increased with increasing dose levels in roughly a dose proportional manner. No gender differences were observed and no accumulation was observed after 3 or 5 repeated weekly doses. Although very limited data from human clinical samples¹, Lipid 5 was also cleared quickly from the circulation with a terminal half-life of roughly 6-9 hours. Summaries regarding dose proportionality, gender difference and accumulation could not be determined from the available dataset. In conclusion, the biphasic pharmacokinetic profiles for Lipid 5 in rat, NHP, and human post intravenous injection suggest a very fast distribution phase followed by a slower terminal elimination phase. These preclinical and clinical data demonstrate consistency in the pharmacokinetic profiles of Lipid 5 in LNP2 formulations encapsulating mRNA which enable predictive PKPD modeling.

P242 - SURVEY ON PHARMACODYNAMIC MARKER USAGES IN BIOSIMILAR DEVELOPMENT AND EVALUATION

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A biosimilar is a biological product which is highly similar to an original (reference) biological product despite having minor differences without clinical meaning. Extensive comparisons in quality, non-clinical aspects and clinical pharmacokinetic characteristics between a biosimilar and the reference products are conducted, but further clinical efficacy (and safety) evaluation has been usually conducted for at least 1 indication toward the biosimilar approval in order to assess their comparability. Recently, several regulatory authorities including US FDA and UK MHRA publicized their considerations that clinical efficacy studies are not so sensitive to find potential differences between the biosimilar and the reference product and clinical pharmacokinetic (PK)/pharmacodynamic (PD) comparison is more suitable. However, there is no validated list of PD markers that could be used for each indication. We performed survey for used (potential) PD markers in the approval of both original and biosimilar drugs using publicized drug assessment reports from FDA, EMA and MHLW/PMDA and Common Technical Document Summaries. FDA stated potential evidence to address some characteristics (Relevance to the mechanism of action, time course and exposure response (dynamic range), sensitivity to differences between the proposed biosimilar product and the reference product, and analytical validity of the biomarker assay) for a PD marker to be used in the comparability studies (Strauss et al., Clin. Pharmacol. Ther., 113 (1) 55-61 (2023)), and we applied these criteria to evaluate candidate PD markers. Several biomarkers have already been used for PD evaluations of biosimilars: absolute counts of neutrophils and CD34+ cells (or area under the curve (AUC) values) for filgrastim, glucose infusion rate (AUC or maximum value) in the clamp study for insulin-related products, serum calcium concentrations, bone mineral density and bone metabolic biomarkers for teriparatide, AUC of erythrocytes and hemoglobin for erythropoietin, and serum globotriaosylceramide-3 concentration for agalsidase beta. We then explored and evaluated the candidate PD markers of biological products. Plasma concentrations of phenylalanine for pegvaliase, LDL-cholesterol and free PSCK9 for anti-PSCK9 antibody, and bone mineral density and serum C-terminal crosslinked telopeptides of type I collagen for anti-RANKL antibody are seemed to fulfill the 5 criteria. Thus, these biomarkers could be used for PD evaluations in novel biosimilar development in the future. We also listed to show potential PD marker candidates lacking necessary information for future evaluation. In addition, we will show a perspective on the pharmacokinetic evaluations for biosimilars.

P243 - EVALUATION OF RECEPTOR-MEDIATED UPTAKE AND TRANSCYTOSIS USING HUMAN IPS CELL-DERIVED BRAIN MICROVASCULAR ENDOTHELIAL CELLS

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Purposes: The blood-brain barrier (BBB) is a structure that tightly regulates the transport of molecules between the blood and brain parenchyma and, consequently, presents a challenging obstacle to the delivery of drugs targeting the central nervous system through the brain parenchyma. Therefore, *in vitro* BBB models with functions and drug permeabilities similar to those of the human BBB are crucial for evaluating the efficacy and safety of novel drugs under development. However, conventional BBB models have limitations, such as species differences and loss of BBB functionality. These problems are addressed by the development of human iPS cell-derived brain microvascular endothelial cells (iBMECs), which have robust tight junctions that function similarly to *x in vivo*. However, BBB functions in iBMECs have not been

fully evaluated, and there is a need to establish an antibody-based measurement system that can evaluate receptor-mediated transcytosis (RMT). Therefore, this study aimed to develop an antibody-based RMT evaluation system using capillary electrophoresis as the measurement method.

Methods: The expression of the transferrin, LRP1, LDL, and insulin receptors in iBMECs was confirmed by RT-qPCR and immunofluorescence staining. The uptake of the transferrin receptor was evaluated using pH-dependent fluorescent probe-conjugated transferrin and antibodies (MEM-189) and uptake of the LDL receptors was evaluated using Dil-labeled acetylated low-density lipoprotein (Dil-Ac-LDL). The amount of MEM-189 and control antibodies transported via transferrin receptors on iBMECs was measured by a Simple Western assay using capillary electrophoresis. RMT function was evaluated by calculating the coefficient of permeability.

Results and Discussion: We observed the expression of transferrin, LRP1, LDL, and insulin receptors by x. Moreover, we observed the uptake of transferrin, MEM-189, and Dil-Ac-LDL via their respective receptors. The permeability coefficient of MEM-189 was significantly higher than that of the control antibody, confirming that RMT in iBMECs was functional.

Conclusions: Various BBB receptors were expressed on iBMECs, which we successfully developed into a highly sensitive antibody-based system using electrophoresis to evaluate RMT.

P244 - CANCER TREATMENT METHODS IN S-1 PLUS OXALIPLATIN UTILIZING RENAL FUNCTION BIOMARKERS WITH CONCURRENT CHRONIC KIDNEY DISEASE IN RATS

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Cancer patients with chronic kidney disease (CKD) have elevated rates of both all-cause and cancer-specific mortality. It is important to investigate the influence of CKD on pharmacokinetics, as it could impact the efficacy of chemotherapy for cancer patients with CKD. S-1 combined with oxaliplatin (SOX) chemotherapy is widely used for gastric and colorectal cancer in Japan. Renal biomarkers are used to monitor CKD; however, it remains unclear which marker will be a predictive factor for optimal dosage. In this study, we examined the association between renal biomarkers and pharmacokinetics in SOX chemotherapy in CKD model rats. CKD model rats were prepared by oral administration of adenine (400 mg/kg) to male Wistar rats for 5 days. Blood and urine samples were collected over 30 days to measure renal biomarkers, including serum creatinine (sCr), plasma Cystatin C, NGAL, and L-FABP, and albumin levels in the urine. Creatinine clearance was calculated by sCr and urine creatinine. S-1 (2 mg/kg as tegafur, p.o.) and oxaliplatin (5 mg/kg, i.v.) were administered to control and CKD model rats. Blood and urine samples were collected for up to 48 hours after drug administration. Plasma concentrations of tegafur, gimeracil, and 5-fluorouracil (5-FU), intact L-OHP, and platinum (Pt) were determined by LC-MS/MS. Population pharmacokinetic model analysis of 5-FU and Pt was conducted to investigate the relationship between renal biomarkers and pharmacokinetic parameters using the Phoenix® NLME software version. 8.2. Plasma cystatin C levels in CKD model rats were elevated on Day 8 (1.5 ± 0.2 mg/L) and sustained until Day 29 (1.8 ± 0.2 mg/L) compared to control rats (0.4 ± 0.01 mg/L). Urine L-FABP levels transiently increased from Day 8 and reached peak levels on Day 29. The AUC_{0-48h} of 5-FU after S-1 administration was decreased dependently on CKD progression, whereas the values in CKD model rats were higher than in control rats. The AUC_{0-2h} of Pt was also increased in CKD model rats (control: 2.9 ± 0.7 , CKD day 8: 7.2 ± 1.4 , day 29: 17.7 ± 3.0 $\mu\text{g}\cdot\text{h/mL}$). The clearance of 5-FU and Pt was correlated with urine L-FABP levels and creatinine clearance in individual rats on Day 8 ($r = -0.83, 0.73$). These results suggest a correlation between urinary renal biomarkers and clearance of 5-FU after S-1 administration. In conclusion, urine L-FABP levels and creatinine clearance are associated with the pharmacokinetics of anticancer agents in SOX chemotherapy.

P245 - UNRAVELING THE IMPACT OF METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS ON THE THERAPEUTIC EFFICACY OF FDA-APPROVED SIRNA DRUGS

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The emergence of small interfering RNA (siRNA) drugs represents a paradigm shift in precision medicine, offering promising avenues for therapeutic intervention in a wide array of human diseases. Despite the approval of several siRNA drugs by the US Food and Drug Administration (FDA), our understanding of their therapeutic efficacy and safety under disease conditions, such as metabolic dysfunction-associated steatohepatitis (MASH), remains rudimentary. This study seeks to elucidate the intricate interplay between MASH pathology and the pharmacokinetics/pharmacodynamics of FDA-approved siRNA drugs, specifically those targeting systemic diseases of liver-origin. MASH, characterized by hepatic inflammation, fibrosis, and cellular stress, exerts profound effects on liver physiology, including alterations in the absorption, distribution, metabolism, and excretion (ADME) of small molecule drugs. Moreover, while the ADME of small molecule drugs in MASH conditions has been extensively studied, the impact of MASH on the ADME of siRNA drugs remains unexplored, representing a novel avenue for investigation. To address this critical knowledge gap, we established an *in vitro* MASH model using HepG2 and HepaRG cells subjected to fatty acid-induced steatosis, mimicking the disease microenvironment. After inducing steatosis with oleic acid and palmitic acid (2:1) at increasing concentrations of 250, 500,

750, and 1,000 μM , the MASH model cells were treated with 1 nM of patisiran or vutrisiran, which is approved by the FDA for the treatment of hereditary transthyretin (TTR) amyloidosis. Comprehensive assessments of therapeutic efficacy and cytotoxicity were conducted using MTT assays, TTR gene expression analysis via reverse transcription quantitative polymerase chain reaction, and quantification of target TTR protein levels using enzyme-linked immunosorbent assay. Furthermore, expressions of LPL, FABP4, ADIPOQ, PPAR γ , FASN, SCD1, PPAR α , IL-1 β and TNF- α genes were evaluated. Oil red O staining was used to stain oil droplets and intracellular triglyceride accumulation was later quantified using a spectrophotometer at absorbance of 490 nm. Our findings suggest a potential correlation between fatty acid accumulation in the MASH model and enhanced efficacy of patisiran, indicating a complex interplay between disease severity and siRNA drug response. We unraveled the mechanistic basis of MASH-induced alterations in siRNA drug pharmacokinetics and pharmacodynamics, with a particular focus on elucidating potential pathways linking fatty acid accumulation, subcellular trafficking dynamics, and therapeutic outcomes. By shedding light on these intricate interactions, this study aids in the development of tailored and efficacious siRNA-based therapies for individuals afflicted by liver diseases, ultimately improving patient outcomes and quality of life.

P246 - PROTECTIVE EFFECTS OF OMEPRAZOLE AGAINST OXALIPLATIN-INDUCED PERIPHERAL NEUROPATHY VIA OCTN TRANSPORTER-MEDIATED MECHANISMS IN A RODENT MODEL

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Oxaliplatin (L-OHP) induced peripheral neuropathy (OIPN) is a major clinical problem caused by sustained neuronal injury in the dorsal root ganglion (DRG) by platinum (Pt). Despite several treatment and prevention approaches for OIPN, there are still no effective coping strategy. Omeprazole (OPZ), a proton pump inhibitor drug, has been reported that it may suppress the onset of OIPN in clinical database analyze and expected to be a prophylactic drug for OIPN as drug repositioning. [1] However, the mechanism by which OPZ inhibits the development of OIPN remains unclear. We have previously reported the quantitative relationship between Pt concentration in DRG and the degree of OIPN, suggesting that prevention of Pt distribution to the DRG may be an efficacy approach to prevent OIPN. In present study, we examined the impact of OPZ on the distribution of Pt into DRG and ameliorative effect on OIPN from a pharmacokinetic perspective. Male Wistar rats were divided into four groups according to dose treatment and dosages: L-OHP monotherapy (5 mg/kg, i.v.), and OPZ combination groups (2, 10 or 20 mg/kg, p.o.). Rats were administrated with L-OHP once a week and OPZ five times a week for 3 weeks. Blood samples were collected for 2 hours after administration of L-OHP. DRG samples were isolated after blood sampling was completed. Plasma and DRG concentration of Pt were determined by LC-MS/MS. The expression levels of transporter, organic cation transporters (OCTs) and organic cation/carnitine transporters (OCTNs), which reported involved in Pt distribution into DRG were measured by real-time PCR. OIPN was evaluated by von Frey test from Day 0 to Day 18. In 2, 10 and 20 mg/kg of OPZ combination groups, each distribution volumes of Pt (1.05 ± 0.54 , 0.37 ± 0.05 , and 1.07 ± 0.57 L/kg, respectively) were significantly decreased compared to L-OHP monotherapy group (2.23 ± 0.83 L/kg). Pt concentration in DRG were significantly decreased to 14.9-28.7% compared to L-OHP monotherapy group on Days 7 and 14. The expression levels of OCTN1 on DRG in 20 mg/kg of OPZ combination group was significantly increased on Day 14 (1.85 ± 0.69 fold of control), whereas no differences were found in OCT expression levels. In von Frey test, the paw withdrawal thresholds in 10 and 20 mg/kg of OPZ combination groups (5.5 ± 3.3 g and 5.8 ± 1.2 g, respectively) were higher than that in L-OHP monotherapy group (2.0 ± 0.8 g) from Day 11 until end of the experiments. These results suggest that OPZ inhibits Pt influx to DRG through OCTN transporter, leading to suppress the onset of OIPN. In conclusion, OPZ would be a candidate drug to ameliorated OIPN in chemotherapy with L-OHP.

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P247 - TISSUE PHARMACOKINETIC-PHARMACODYNAMIC ANALYSIS OF PH-RESPONSIVE DOXORUBICIN-RELEASING MICELLES WITH ANTICANCER EFFECT

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Polymeric micelles are used as delivery carriers for anticancer drugs. It can incorporate unique chemical bonds and pilot molecules into the micelle's structural design. These contribute to tumor targeting and prevention of nonselective accumulation of the drug in other organs. These material designs have contributed significantly to the development of drug delivery systems. However, the relationship between material design effects and anticancer effects is rarely discussed objectively. We believe this is due to the lack of tissue pharmacokinetics (PK) evaluation of intracellular drugs and a tissue pharmacokinetic-pharmacodynamic (PK-PD) model that would allow discussion of the relationship. Therefore, this presentation aimed to develop a tissue PK-PD model to assess the impact of *in vivo* pH-responsive release and targetability of pilot molecules in folic acid (FA)-modified micelles on the pharmacological effects of pH-

responsive doxorubicin (DOX) release. To accomplish our objectives, we monitored the time profiles of intratumoral DOX concentrations using a microdialysis probe inserted into the tumors of Walker256 tumor-bearing rats, followed by compartmental analysis. Maximal DOX release from micelles occurred 350 min after the administration of pH-responsive DOX-releasing micelles. The time to peak drug concentration was shortened to 150 min owing to FA modification of the micelles. Additionally, FA modification resulted in a 27-fold increase in the tumor inflow rate constant. Subsequently, Walker256 tumor-bearing rats were treated with DOX, pH-responsive DOX-releasing micelles, and pH-responsive DOX-releasing FA-modified micelles to monitor the tumor growth–time profiles. We developed a PD model by introducing an intratumoral threshold concentration in the drug efficacy compartment and performed tissue PK-PD analysis of the tumor growth–time profiles. The intratumoral threshold concentration was 55–64 ng DOX/g tumor, indicating similar results among the three formulations. The time efficacy index, indicating the cell proliferation delay, exhibited delay depending on the number of drug delivery functions. The tissue PK model development using the microdialysis method revealed the pH-responsive DOX distribution profiles in the tumor and contributed to the intratumoral pharmacokinetic parameters estimation. The PD model with threshold concentrations contributed to the pharmacodynamic parameters estimation of the three formulations without any inconsistency in their action mechanism. We believe that our tissue PK-PD model can objectively assess the contributions of the material design effect such as pH-responsive release ability and pilot molecule targetability to pharmacological effects.

P248 - PHARMACOKINETIC AND PHARMACODYNAMIC INTERACTIONS BETWEEN ASTRAGALUS MEMBRANACEUS POLYSACCHARIDES AND METFORMIN IN DIABETIC MICE

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As one of the most used traditional Chinese medicinal herb for treatment of Type-II diabetes, Astragalus membranaceus is often used in combination with other first-line antidiabetic drugs in clinical practice [1]. However, the related pharmacokinetic and pharmacodynamic interactions have never been investigated. Previous studies [2-3] found that gut microbiota played an important role on the hyperglycemic effect of metformin (MET) and Astragalus membranaceus polysaccharides (AMP), which is the main constituent of the herb. Herein, we hypothesized there might be gut microbiota mediated pharmacokinetic and pharmacodynamic interactions between AMP and MET. The current study is designed aiming to evaluate interactions between AMP and MET in db/db mice from the pharmacokinetic and pharmacodynamic perspectives.

The db/db mice were orally treated with MET of high (250 mg/kg) and low (125 mg/kg) doses 2h after dosing AMP (600 mg/kg) once daily for consecutive 23 days. The pharmacokinetic interactions were evaluated by monitoring the steady state concentrations of MET on Day 23 via LC-MS/MS and the pharmacodynamic interactions were monitored by serial hypoglycemic tests including iTT (Day 22), ipGTT (Day 20), HOMA-IR index (Day 20) and GLP-1 measurement (Day 24). Furthermore, the underlying mechanisms were explored by monitoring tight junction protein expressions in small intestine, GPCR41/43 expressions in colon, gut microbiota composition and SCFA production in all treatment groups.

Significantly improved hypoglycemic effect (further decreased fasting blood glucose level (Figure 1A), ipGTT (Figure 1C), iTT (Figure 1D) and HOMA-IR index (Figure 1E)) and increased SCFA productions (Figure 1G) were only observed for the combination use of 600 mg/kg AMP and 125 mg/kg MET in comparison to that from single use of 125 mg/kg MET. In the meantime, the steady state concentrations of MET at either high (250 mg/kg) or low (125 mg/kg) dose were not affected by the co-administered AMP (Figure 1H). Further mechanistic studies found that the expressions of Claudin-1 in small intestine (Figure 2A) and GPCR41 and GPCR43 in colon (Figure 2B) were significantly increased after combination use of 600 mg/kg AMP and 125 mg/kg MET in comparison to that from single use of 125 mg/kg MET.

The current study for the first time demonstrated that the combined use of AMP (600 mg/kg) and MET at 125 mg/kg would lead to enhanced hypoglycemic effect without affecting the steady state concentrations of MET, which warranted further verification in clinical trial.

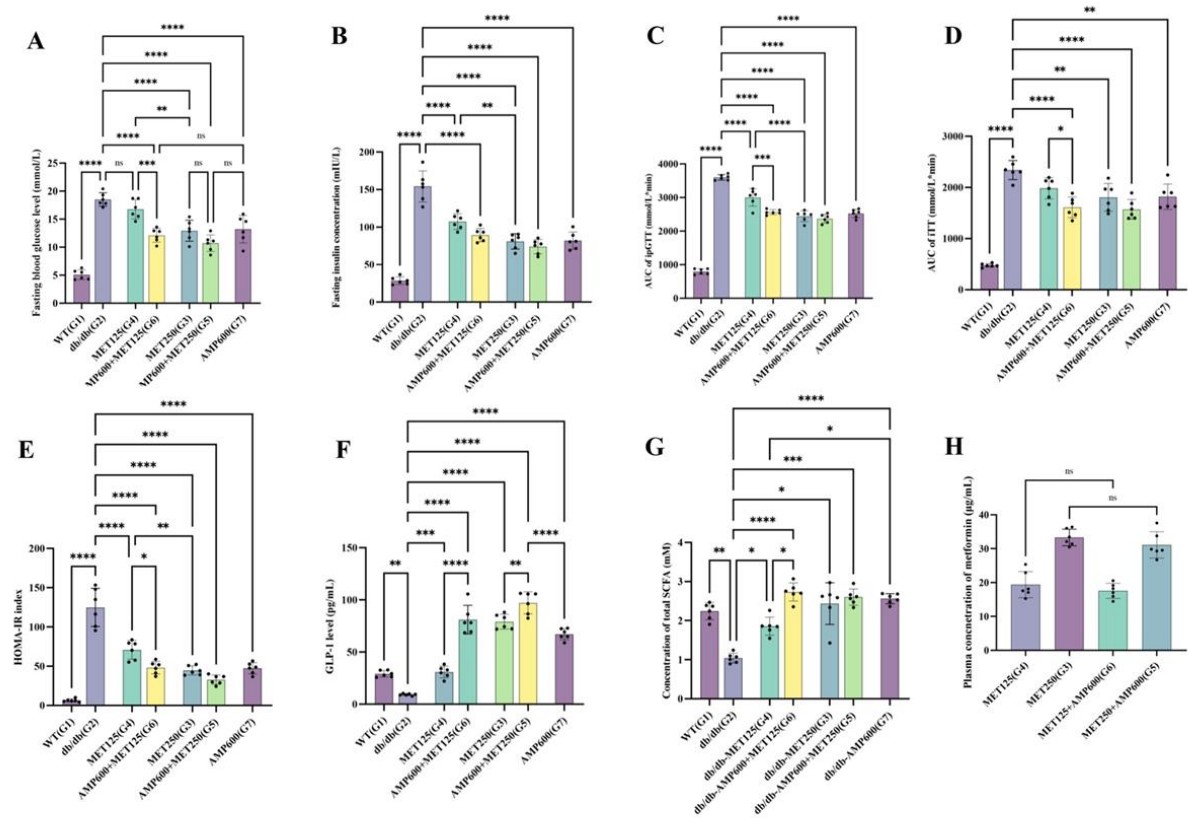


Figure 1 Comparison on hypoglycemic effect (A-F), SCFA production (G) and steady state concentration of MET (H) after single use of MET (125 and 250 mg/kg) and its combined use with AMP (600 mg/kg) in *db/db* mice

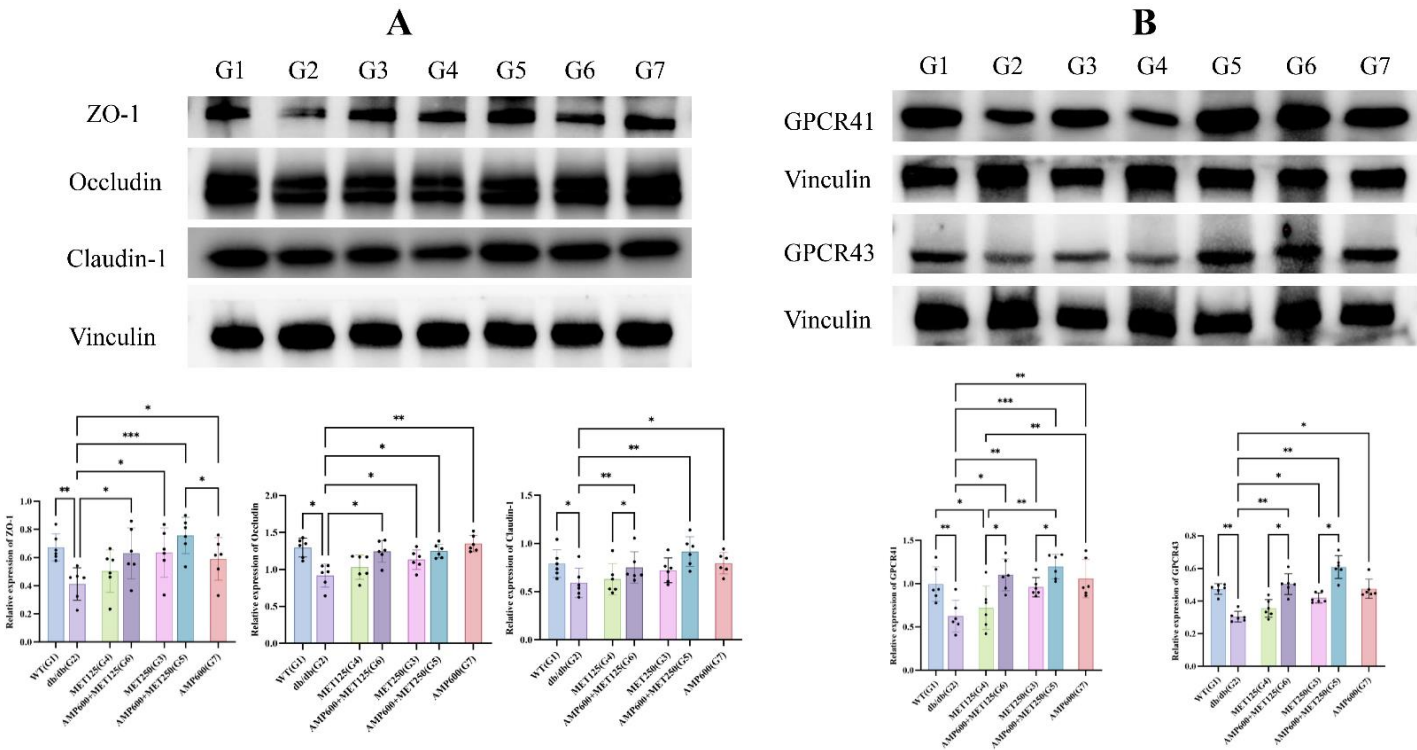


Figure.2 Comparison on tight junction proteins expression in small intestine (A) and GPCR41/43 expression in colon (B) between single use of MET (125 and 250 mg/kg) and its combined use with AMP (600 mg/kg) in *db/db* mice

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P249 - ANALYSIS OF INFLUX/EFFLUX KINETICS OF METFORMIN IN CACO-2 CELLS

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Metformin is one of the oral antidiabetic drugs classified as biguanides, which improves glucose metabolism by inhibiting hepatic gluconeogenesis based on the activation of AMP-activated protein kinases. In recent years, it has been reported that metformin has an enhancing effect on the glucose elimination into the intestinal lumen [1], suggesting that the intestine is an important target in the pharmacological efficacy of metformin. On one hand, Shirasaka et al. showed that the contribution of transporters such as organic cation transporter 3 (OCT3), thiamine transporter 2 (THTR2), and plasma membrane monoamine transporter (PMAT) is about 45% for the intestinal absorption of metformin [2]. Based on these reports, our group is trying to evaluate the correlation between the promoting effect of glucose elimination and intracellular concentration of metformin. Furthermore, we aim to develop a metformin formulation with a modified dosage form targeting the intestinal epithelia. In this presentation, we report the intracellular concentration of metformin and its influx/efflux clearance values. The cellular volume of the metformin-treated cells was measured by using antipyrine as a cell volume marker [3], and obtained cell volume was applied to the evaluation. In this study, 0.1 mM metformin and antipyrine were added to cultured Caco-2 cells, and the cells and media were recovered over time. An internal standard substance (acetaminophen) and methanol were added to samples to remove protein, and the supernatant was analyzed by LC-MS/MS. A mixture of 5 mM ammonium formate and methanol (8:2) was used as the mobile phase and a Shim-pack Arata C18 column was used as the column. Based on the intracellular and extracellular concentration of antipyrine, the intracellular volume of Caco-2 cells was estimated. In addition, kinetic parameters of metformin influx/efflux were calculated using the actual intracellular volume and intracellular concentrations of metformin. From the results of LC-MS/MS analysis, intracellular concentrations of metformin and antipyrine were clarified. The intracellular volume of Caco-2 cells was calculated to be 1.67 $\mu\text{L}/\text{mg}$ protein, and the influx/efflux clearances of metformin were 0.11 $\mu\text{L}/\text{min}/\text{mg}$ protein and 0.03 $\mu\text{L}/\text{min}/\text{mg}$ protein, respectively. The influx clearance of metformin was three times higher than that of efflux clearance, indicating the influx of metformin was dominant.

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P250 - DEVELOPING RENAL FUNCTION EVALUATION FOR PEDIATRIC DOSE SELECTION BASED ON PHARMACOMETRIC APPROACH WITH RENALLY ELIMINATED DRUGS

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Purpose: When administering renally eliminated drugs to pediatric patients including neonates, it is crucial to adjust the dosage considering the transitional phase of renal development [1]. Multiple equations have been evaluated for predicting renal function in children [2-4]. For drugs predominantly eliminated from the kidney, elimination from the circulatory system is expected to approximate renal clearance. In this study, aimed at contributing to the appropriate use of renally eliminated drugs in Japanese pediatric patients under 3 years of age, we constructed renal function estimation equations using the clearance of vancomycin and amikacin and investigated dosage adjustments considering renal function.

Methods: We targeted pediatric patients under 3 years old who received vancomycin or amikacin at the National Center for Child Health and Development from April 2014 to March 2020. Serum concentration, body weight, postnatal days, postmenstrual age (PMA), and serum creatinine (SCr) were extracted from electronic medical records to construct a Population Pharmacokinetic (PPK) model. The Schwartz formula for renal function estimation was introduced as a covariate in total clearance (CL), and the constant (k value) was estimated. Based on the finalized PPK model, dosing nomograms tailored to pediatric characteristics were created using Monte Carlo simulation. This study was conducted with the approval of the ethics committees of Musashino University (R4-5) and the National Center for Child Health and Development (2019-119).

Results and Discussion: Serum concentration data were obtained from 719 pediatrics (PMA: 24.2–195.6 weeks; body weight: 0.32–17.90 kg; SCr: 0.06–5.87 mg/dL) for vancomycin and 20 pediatrics (PMA: 37.0–195.0 weeks; body weight: 1.54–15.70 kg; SCr: 0.09–8.84 mg/dL) for amikacin, yielding 3648 and 66 data points, respectively. The pharmacokinetic model that best reflects the serum concentration profiles in pediatric patients is the two-compartment model. As covariates, body weight was incorporated for volume of distribution and body weight, PMA, and the Schwartz formula as renal function estimation for CL in the final PPK model. The estimated k value for vancomycin was 0.56, consistent with the k value of 0.55 reported for children aged 2–13 years [5]. Moreover, the proposed renal function estimation equation was also applicable to amikacin. These results suggest that utilizing body weight, PMA, and renal function may be valuable for predicting the clearance of renally excreted drugs in pediatric populations, including preterm and low-birth-weight Japanese children.

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P251 - ASSESSMENT OF MOXIFLOXACIN-INDUCED QTc PROLONGATION IN CANINE AND NONHUMAN PRIMATE USING A LINEAR MIXED EFFECT MODELING APPROACH

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Recent introduction of the International Council for Harmonisation (ICH) E14/S7B Questions and Answers (Q&A) has increased the opportunity to perform clinical QTc characterization during Phase 1 in place of a thorough QT (TQT) study and improve product QTc labeling in situations where a compound is negative in both the *in vitro* hERG and *in vivo* QTc assays. To take advantage of this new guidance, especially in the case of E14 Q&A 6.1, the *in vivo* QT assay must be powered to detect an effect similar to a human TQT study, and take interspecies QTc differences into account. Conduct of concentration-QTc (cQTc) modeling of large animal data can improve study power over that of a traditional by-time analysis. Moreover, cQTc analysis can be used to quantify the drug concentration required to induce a QTc effect at a target threshold (e.g. 10 msec) in a large animal species versus human to determine any species shift, as required by the new guidance.

In preparation for this guidance, we tested oral moxifloxacin in canine (10, 30, 90 mg/kg) and nonhuman primate (nhp; 25, 50, 100 mg/kg) orally using implanted telemetry in a Latin Square design (N=8) and derived QTc values. Plasma samples were collected in a separate phase in the same animals to characterize exposure. After verifying key assumptions which included no impact of the drug on heart rate (HR), independence of QTc from HR, no time delay between drug concentration and delta-QTc, and linearity of the cQTc relationship, linear mixed effect (LME) modeling was performed as per Garnett et al, 2018. Model coefficients and parameters were used to determine the linear relationship between QTc and Moxifloxacin concentration.

Moxifloxacin produced a 10 msec increase in QTc at 2208 (dog) and at 1983 (nhp) ng/ml of free concentration, which were 2.0- and 1.8-fold the reported human value of 1120 ng/mL of free concentration, respectively. Fisher's least significant difference (LSD) was calculated for total human moxifloxacin concentration ranging from 1866 ng/ml (from Q&A training material) to 3085 ng/ml (Florian et al, 2011). It was found to be 3-4 msec, in both species. These values are sufficient to support an integrative risk assessment under ICH E14/S7B Q&A.

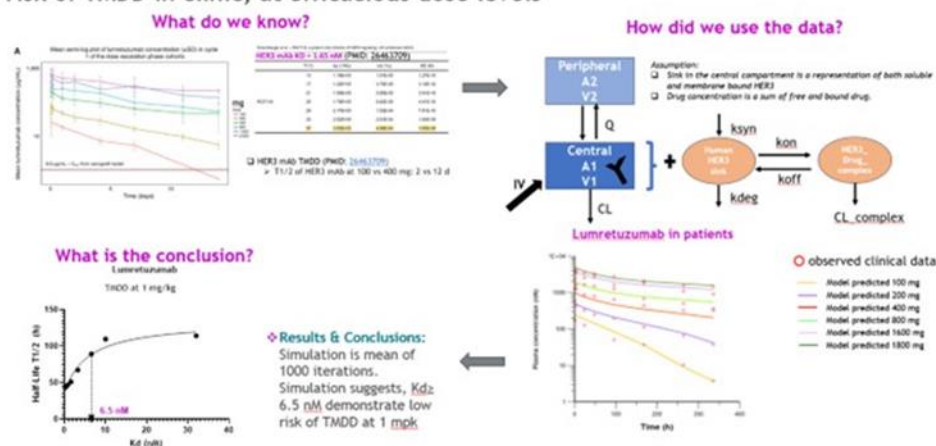
P252 - MODELING AND SIMULATION REVEALS THE IMPACT OF BINDING AFFINITY (Kd) ON TARGET-MEDIATED DISPOSITION: A CASE STUDY OF LUMRETUZUMAB

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The influence of binding affinity (Kd) on the target-mediated disposition of lumretuzumab, a monoclonal antibody against HER3, was studied using a semi-mechanistic model. A quantitative base model was built to describe the

pharmacokinetics of lumretuzumab with reference to data from a first-in-human study. Following intravenous administration of lumretuzumab in patients, the peak concentration (C_{max}) and area under the concentration-time curve (AUClast) increased in a greater-than-dose-proportional manner from 100 mg to 400 mg q2w, above which target-mediated disposition became saturated and linear increases were observed.¹ In the present reanalysis of this published data, a model-based calibration was performed using Phoenix NLME naïve pool (v.8.3.5), and simulations were run over a total of $n = 1000$ iterations. Plasma concentrations of lumretuzumab were captured using two compartments and linear elimination to explain the linear PK; the nonlinearity was accommodated by invoking a sink to represent the binding of the antibody to both membrane-bound and soluble HER3. The model accounted for binding parameters (k_{on} and k_{off}), which were measured by surface plasmon resonance, and *in vitro* HER3 protein turnover rate to explain the nonlinearity in exposure. Key assumptions of the model included the representation of both soluble and membrane-bound HER3 as a sink facilitating target-mediated disposition from the central compartment and the representation of drug concentration as a sum of free and bound antibody. The final model demonstrated acceptable precision, with thetas accompanied by coefficients of variation $\leq 40\%$. Additionally, visual predictive check (VPC) and residual plots of predicted plasma concentrations demonstrated the robustness of the model. Output from this simulation explains why a monoclonal antibody against HER3, which is characterized by $K_d \geq 6.5$ nM, would carry a low risk of target-mediated disposition at efficacious dose levels.

Modeling and simulation suggests $K_d > \text{single digit nM}$ for HER3 mAb reduces the risk of TMDD in clinic, at efficacious dose levels



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P253 - POPULATION PHARMACOKINETICS OF CEFTRIAXONE IN JAPANESE PATIENTS WITH HEMODIALYSIS.

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Background: Ceftriaxone is a third-generation cephalosporin antibiotic with a relatively long half-life of approximately 7 h. The pharmacokinetics of ceftriaxone is characterized by high protein binding with albumin and excretion via both bile and kidney. No dose adjustment is recommended even in patients with impaired renal function probably because biliary clearance can compensate for the decrease in renal clearance. However, there are concerns regarding significant changes in ceftriaxone exposure in hemodialysis patients who have extremely low levels of renal function and often suffer from hypoalbuminemia. Additionally, high ceftriaxone levels in the blood are believed to cause neurotoxicity. In the present study, we retrospectively investigated the unbound and total (unbound + bound) ceftriaxone concentrations in hemodialysis patients receiving an empiric regimen (e.g., 2g per 24h).

Methods: Hemodialysis patients with at least one set of measured unbound and total ceftriaxone concentration were enrolled. Unbound and total ceftriaxone concentrations were measured using stored residual plasma samples obtained at routine clinical practice, and patient demographics, including dosing and blood sampling times, were collected from electronic health records. The obtained unbound and total ceftriaxone concentrations were simultaneously fitted using a one-compartment model with saturable protein binding. Parameter estimation was conducted with a population pharmacokinetic approach based on a nonlinear mixed-effect model.

Results and Discussion: Unbound and total ceftriaxone concentrations were measured in 184 plasma samples collected from 58 hemodialysis patients with a median eGFR of 8 mL/min/1.73m² and a median albumin level of 2.8 mg/dL. The median unbound and total ceftriaxone concentrations were 27.5 and 143 mg/L, respectively. Out of a total of 184 observations, 48 total ceftriaxone concentrations in 30 patients exceeded the toxic level of 100 mg/L after 24 h post-dosing. The observed unbound fractions exhibited high variability (median 18%, ranging from 1.5 to 98.5%) and could not be fully explained by albumin levels, implying difficulties in estimating unbound concentrations from total concentrations in hemodialysis patients. The estimated population mean of unbound clearance and half-life in hemodialysis patients were 0.85 L/h and 19.5 h, respectively, suggesting no compensation from biliary clearance. This analysis suggested the need for ceftriaxone dose adjustment and monitoring unbound ceftriaxone concentration in hemodialysis patients.

P254 - IMPACTS OF ANTI-DRUG ANTIBODIES ON PHARMACOKINETIC AND PHARMACODYNAMIC ACTIONS OF CELL-PERMEABLE MIDDLE MOLECULE PEPTIDE DRUG

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The generation of anti-drug antibodies (ADAs) can be caused by the administration of biologic drugs, which may affect a drug's pharmacokinetic (PK) and pharmacodynamic (PD) properties and reduce its efficacy. This risk of immunogenicity is generally related to molecular size, where small molecule drugs are usually considered to be poorly immunogenic. Recently, efforts have been strengthened in the exploration of middle molecule drug candidates. Several peptide drugs with molecular weights of 1~2 kDa have also been reported with ADA issues, e.g., Etelcalcetide, Icatibant and Degarelix. However, these peptide drugs all target cell surface receptors; the case of cell-permeable middle molecules has not yet been adequately investigated. In the present study, we examined the impact of ADAs using a middle molecule compound named AP22151, an in-house cell-permeable 11-mer cyclic peptide inhibitor for RAS proteins. ADAs to AP22151 were prepared via immunization of rabbits by use of the KLH-conjugates of an AP22151 analogue, followed by equilibrium dialysis to estimate the binding affinity between AP22151 and the purified ADAs. A SimBiology PK model was then established to describe the plasma concentration time course of AP22151 in mice from pre-study data, and the estimated affinity between AP22151 and the ADAs was applied to the model. Thus, we were able to assess the ADA impacts on AP22151 at different ADA levels using the modeling approach. We predicted that higher ADA levels would more strongly increase the total plasma concentrations of AP22151 and extend its half-life, without apparently affecting the free drug concentrations. According to the free drug theory, it was assumed that the PD responses of AP22151 would remain unchanged despite the presence of ADAs. To verify the above predictions, we further performed *in vivo* studies using ICR mice. In the *in vivo* experiment, each mouse received an intravenous injection of AP22151 after a 24-hour continuous infusion of either ADA or PBS (control). Plasma samples were collected at a series of time points for the measurement of AP22151 and ADA concentrations. Additionally, the inorganic phosphorus (IP) levels in blood were examined as an indicator of PD response due to the inhibition of the RAS/RAF/MEK pathway. As a consequence, the *in vivo* PK/PD data were consistent with the predictions, which suggests the usefulness of model analysis and may provide a better insight into the future drug discovery for middle molecules.

P255 - RAPID DETERMINATION OF LIPOPHILICITY: EXPLORATION AND ESTABLISHMENT OF REVERSED-PHASE LIQUID CHROMATOGRAPHY (RPLC) METHODS

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The lipophilicity (Log P or Log D) of a compound plays a crucial role in drug development. It serves as a valuable parameter for evaluating various properties of compounds, including absorption, distribution, metabolism, and excretion-toxicity (ADMET). Understanding the lipophilic nature of a compound provides valuable insights into its pharmacokinetic behavior and aids in optimizing drug formulations for improved efficacy and safety.

There are several commonly used methods for detecting Log P values of compounds. The choice of method depends on factors such as the required accuracy of Log P values and the project stage (discovery, pre-clinical development, or clinical development). Among these methods, the shake-flask method, although low throughput, is widely considered the gold standard due to its precision. However, it is challenging to accurately determine the lipophilicity values of highly lipophilic compounds (Log P > 5) due to the extremely low solubility of compounds in the aqueous phase. To address this issue, the RPLC (reversed-phase liquid chromatography) method offers a wider measuring range. Moreover, this method is more efficient and user-friendly compared to the shake-flask method. With the increasing proportion of highly lipophilic compounds in drug discovery and development, the development of RPLC methods has gained significant interest among researchers.

In this study, we explored and established two RPLC methods that can efficiently and accurately detect the Log P values of compounds. The predictive ability of the method was investigated with linear correlation coefficients. Based on

considerations of cost, time efficiency, and predictive ability, it is believed that RPLC methods are promising choices for predicting the lipophilicity of tested compounds.

P256 - FIRST-IN-HUMAN DOSE ESTIMATION FOR A NEW COMPOSITION BEE VENOM, A CANDIDATE DRUG OF ALZHEIMER DISEASE, BASED ON PHARMACOKINETIC INTERSPECIES ALLOMETRIC SCALING FROM MICE, RATS, AND BEAGLE DOGS

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Bee venom is a traditional remedy employed for treating conditions related to the nervous system, musculoskeletal system, and autoimmune diseases. It consists of various peptides, including melittin (50–60%), bee venom phospholipase A2 (bvPLA2; 10–12%), apamin (1–3%), adolapin (0.1–0.8%), and mast cell degranulating peptide (1–3%), biologically active amines (e.g., histamine and epinephrine), and non-peptide components (e.g., minerals and amino acids). Among the various components contained in bee venom, from our previous research bvPLA2, may protect the brain from neurological disorders and have potential for use in the treatment of Alzheimer's disease. Although the dried crude honeybees usually consist only approximately 10–12% of bvPLA2, based on the proven efficacy of bee venom and bvPLA2 in Alzheimer's disease, new composition of bee venom (NCBV) was developed with a fortified content of bvPLA2 (up to 76.2%). It was lyophilized using an ultrafiltration manufacturing process by INISTst R&D center (Gyeonggi-do, Republic of Korea). These days, we are also planning a phase I clinical trial to administer NCBV subcutaneously in patients with Alzheimer's disease in Republic of Korea (INISTst R&D center). Quantitative estimations of first-in-human (FIH) doses are critical for phase I clinical trials in drug development. Thus, the objective of this study was to evaluate the interspecies scaling for NCBV to estimate the appropriate initial human dose in humans from animal pharmacokinetics data. Allometric equations have demonstrated their utility in extrapolating animal data to estimate pharmacokinetic parameters in humans. The allometric scaling of NCBV was developed using nonlinear mixed-effect modeling. The CL/F of bvPLA2 in humans was estimated, and a first-in-human dose proposed. The estimated drug clearance rate (CL/F) in the Brw method is about 1.52 L/h for a 70-kg human. To meet the area under the curve (AUC) required for effective Alzheimer's disease treatment, a dose of 0.3 mg of NCBV was finally proposed as the first dose for a 70-kg human. The first-in-human dose value, calculated based on the toxicological profile obtained from non-clinical studies using the No Observed Adverse Effect Level (NOAEL), ranged from 0.1 mg to 3 mg. Therefore, the first-in-human dose estimation value through allometric scaling is considered appropriate, as it aligns with the range obtained from the NOAEL-based approach.

P257 - PREDICTION OF THE *IN VIVO* PHARMACOKINETICS OF SUSTAINED-RELEASE INJECTIONS FOR SEMAGLUTIDE BY A NOVEL IVIVC MODEL COMBINED WITH DOE

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in vitro-in vivo correlation (IVIVC) is a predictive mathematical model that correlates the *in vitro* dissolution of a dosage form and the corresponding *in vivo* pharmacokinetics. This study aimed to develop an extended IVIVC model combined with the design of experiment (DoE) that allows the prediction of the pharmacokinetics of a dosage form from its formulation composition, and vice versa. Semaglutide, a potent glucagon-like peptide 1 (GLP-1) receptor agonist, was used as a model drug. The formulations of semaglutide sustained-release (SR) injections with different release rates were designed by applying the mixture design. The *in vitro* release profiles were determined using the test tube method. The maximum dissolution rates based on Michaelis-Menten kinetics (V_{max}) were obtained to describe the effects of the sustained-release injectable formulations. *in vivo* pharmacokinetics of three types of semaglutide SR injectable formulations (0.2 mg per injection) with different release rates were characterized in SD rats. Plasma concentrations of semaglutide were determined by a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. A population pharmacokinetic model was developed to estimate the *in vivo* dissolution profiles of semaglutide SR injections. By correlating the observed *in vitro* dissolution parameters and a pharmacokinetic model-estimated *in vivo* dissolution parameters, level A IVIVC was established for semaglutide SR injectable formulations. Finally, by connecting the DoE model and IVIVC model, the extended IVIVC model has been established. The prediction error (%) of the extended IVIVC model was 1.00% to 4.95% for C_{max} and 0.90% to 2.59% for AUC. The present model-based approach would provide a promising tool for more efficient and rational development of drug formulations to acquire an optimal pharmacokinetic profile.

P258 - PREDICTION OF HUMAN EFFECTIVE DOSE AND PHARMACOKINETICS-PHARMACODYNAMICS USING PRECLINICAL DATA**Sunyoung Lim**

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HM-A is an EZH1/2 inhibitor showing encouraging anti-tumor efficacy in the preclinical studies and was predicted using well-defined xenograft PK/PD model to support clinical effective dose prediction. The main objectives of this study were to 1) prediction of human PK by Wajima method using PK data from mice, rats and dogs 2) prediction of human effective dose and PK/PD from preclinical PK/PD model. For human PK prediction of HM-A, C_{ss}-MRT normalized profiles obtained from preclinical species were superimposed. Additionally, a PK model using saturable absorption and nonlinear two-compartment, and linked Simeoni (exponential to linear) with transit for efficacy model to describe the PK-PD relationship in xenograft mouse model. As a result of these predictions, PK/PD modeling was well described the relationship between PK and efficacy in the xenograft mouse model. Also, the optimal effective dose range for HM-A was predicted to dose from the PK/PD model. In conclusion, HM-A expected to have favorable pharmacokinetic properties for once daily dosing and sufficient to drive efficacy in efficacious clinical dose.

P259 - ESTABLISHMENT OF DOE-IVIVC MODEL FOR THE PHARMACOKINETIC PREDICTION OF RUXOLITINIB SUSTAINED-RELEASE DOSAGE FORMS**Soyoung Shin¹**, Tae Suk Lee², Keon Kim², Joonhee Kim², and Beom Soo Shin²¹Chung-Ang University, Seoul, South Korea, ²Sungkyunkwan University, Suwon, South Korea

This study aimed to demonstrate the utility of the *in vitro-in vivo* correlation (IVIVC) model combined with the design of experiment (DoE) to predict the *in vivo* pharmacokinetics of the sustained-release dosage form from its formulation composition. Ruxolitinib sustained-release formulations were designed by applying the DoE method and prepared by the wet granulation method. *in vitro* dissolution data were obtained using the paddle method. From *in vitro* dissolution profiles, the time of 80% dissolution (T_{80%}) of each formulation was calculated, and the maximum dissolution rate (V_{max}) was estimated by applying a modified Michaelis-Menten equation. DoE model was developed for both dissolution parameters, i.e., T_{80%} and V_{max}. Both models exhibited good predictability (R²_{adj} > 99.85%) and the average prediction error (%PE) was 4.81% for T_{80%} and 1.70% for V_{max}. To estimate *in vivo* dissolution of ruxolitinib SR formulations, *in vivo* pharmacokinetic profiles were obtained following oral administration of 3 different formulations to beagle dogs. The *in vivo* dissolution parameters were estimated by developing a population pharmacokinetic model. By correlating *in vitro* V_{max} and the corresponding *in vivo* V_{max}, a level A IVIVC was developed. Finally, the DoE-IVIVC model was developed by incorporating the mathematical equations of DoE and IVIVC models to predict *in vivo* pharmacokinetics from the formulation compositions. The average %PE was 7.90% and 9.43% for C_{max} and AUC, respectively. External validation resulted in %PE of 14.27% for C_{max} and 3.76% for AUC, demonstrating the acceptable predictability of the developed DoE-IVIVC model. The present DoE-IVIVC model could be used as a valuable tool for the development of SR formulations to achieve desired pharmacokinetic profiles.

P260 - PREDICTION OF INTESTINAL AVAILABILITY OF CYP3A AND UGT SUBSTRATE DRUGS FROM *IN VITRO* ASSAY WITH DIFFERENTIATED INTESTINAL CELLS DERIVED FROM CRYOPRESERVED HUMAN SMALL INTESTINAL MUCOSA (CHIM)**Chiaki Sugawara**, Keiichi Nakamura, Ryuto Tomacechi, and Kazuya Maeda

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In the small intestine, there are many examples in which various metabolic enzymes such as CYPs and UGTs suppress the intestinal absorption of substrate drugs. Caco-2 cells is now often used for the characterization of intestinal absorption of drugs in the process of drug development, but CYP3A expression in Caco-2 cells is reported to be almost negligible. Thus, more appropriate *in vitro* assay system has been desired to estimate intestinal availability (F_g value) of drugs. We have demonstrated that human crypt-derived differentiated intestinal epithelial cells originated from surgical specimens can predict F_g values of CYP3A substrate drugs¹). Recently, we have also prepared the differentiated intestinal cells derived from commercially-available cryopreserved human small intestinal mucosa (CHIM)²). Therefore, we investigated whether the CHIM-derived differentiated cells can accurately predict the F_g values of CYP3A and UGTs substrate drugs or not.

The metabolic activities of five CYP3A substrates and four UGT substrates with varied metabolic clearances were quantified with CHIM-derived cells. After 2-hour incubation, the amount of parent drugs and metabolites were measured with LC-MS/MS. Calculated *in vitro* intrinsic metabolic clearance was converted to *in vivo* intrinsic clearance by simple scale-up method. Then, F_g value was determined with Q_{gut} model³).

As a result, the rank order of human F_g values of CYP3A and UGT substrate drugs can be well reproduced by the estimated ones from *in vitro* metabolism assay and the absolute F_g values were well predicted within the range of about

2-fold difference. These results suggested that CHIM-derived human intestinal cells are useful for the accurate prediction of Fg values of CYP3A and UGT substrate drugs.

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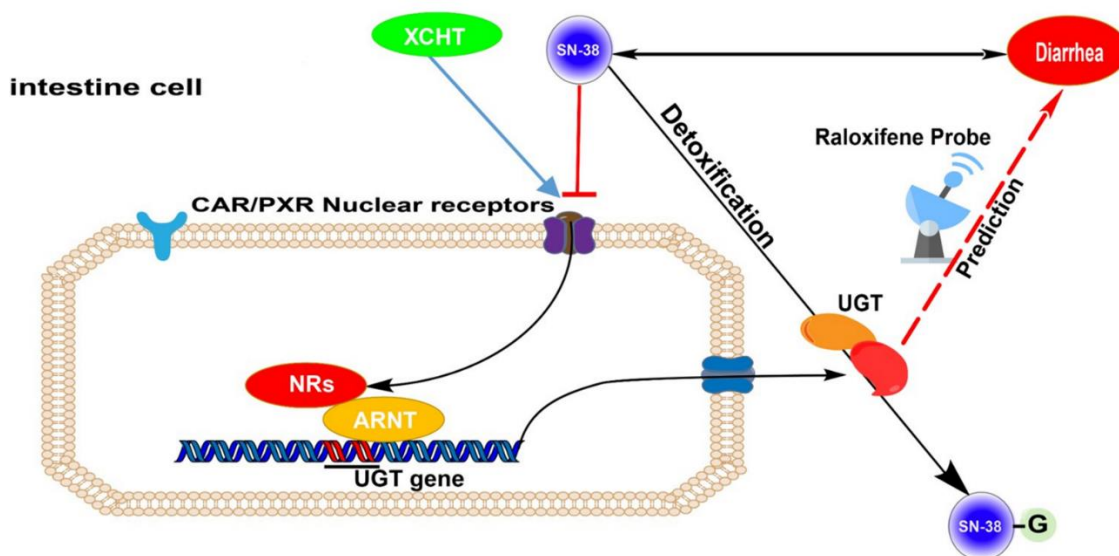
P261 - GUT UGT ACTIVITY LOSS PRECEDES THE DEVELOPMENT OF IRINOTECAN-INDUCED SEVERE DIARRHEA: MECHANISMS, BIOMAKER AND PREVENTION

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Irinotecan has been used widely for various cancer therapy, especially metastatic colorectal cancer. However, 15~40% of treated patients suffer from severe late diarrhea, which limits its application. The irinotecan induced severe diarrhea (IISD) have been attributed to local exposure of its active metabolite SN-38, and intestinal expression of the UGT proteins is critical toward the detoxification of SN-38. Time course study of IISD in mouse and rat after consecutive administration of irinotecan, we now report that the agonist activity of SN-38 at the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) could selectively impaired functions of Ugts in intestine, which accelerated the IISD with increased local accumulation of SN-38. Surprisingly, herbal formula Xiao-Chai-Hu-Tang (XCHT) and its bioactive components can stop the vicious circle, and improved impaired intestinal Ugts functional caused by SN38 through PXR/CAR-UGTs signaling. We also show that loss of UGT activities that precede IISD with human plasma Raloxifene-4'-Glucuronide levels can be used as biomarker to predict the development of IISD. Based on these data we suggest that XCHT and probe drug raloxifene could be used to protect against IISD and predict the incidence of the IISD, respectively.



P262 - PLASMA PROTEIN BINDING DETERMINATION CHALLENGES AND SOLUTIONS FOR VERY HIGHLY PROTEIN BOUND DRUGS: CASE STUDY OF CILOFEXOR AND ITS METABOLITES WITH CLINICAL APPLICATIONS

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Plasma protein binding (PPB) is used at various stages of drug development and may be relevant when interpreting drug exposure in populations with altered protein binding. Dose adjustment may be necessary to ensure proper drug exposure in special populations such as renal-impaired (RI) and hepatic-impaired (HI) patients. A pharmacokinetic (PK) study with PPB assessments can assist in the determination if dose adjustment is needed and therefore, accurate determination of PPB is important. Cilofexor (CILO) is a selective, oral, and nonsteroidal farnesoid X receptor agonist in clinical development for treatment of metabolic dysfunction-associated steatohepatitis (MASH). Preliminary PPB assessments have indicated that CILO, and its metabolites GS-716070 and GS-1056756, are very highly protein bound (>99.9%). The accurate determination of clinical PPB for such highly bound compounds has been very challenging. Good, reliable, and

accurate methods for determination of PPB were needed. As CILO and its active metabolite, GS-716070, were very highly protein bound, equilibrium was difficult to achieve using equilibrium dialysis (ED) methods and the low receiver side analyte concentration also hindered LC-MS/MS detection. Therefore, clinical plasma samples were diluted 1:10 with PBS buffer and were added to the donor side, while blank PBS was added to the receiver side. Samples were spiked with test article, and the LLOQ for the LC-MS/MS method was decreased from 1 ng/mL to 0.01 ng/mL for each analyte. Equilibrium was achieved in 7 hours. Undiluted fraction unbound (f_u) was calculated from Diluted f_u ,d. CILO's inactive metabolite, GS-1056756, was not only highly bound, but also very "sticky" and adhered to the dialysis membrane during ED; therefore an ultracentrifugation method was developed for its PPB determination. Equilibrium was established in 6 hours. Both methods were qualified before their clinical applications. The f_u of CILO and GS-716070 were reliably determined for mild, moderate, and severe HI cohorts using the ED method in the Phase I clinical CILO HI study. Data show different decreases in PPB compared with healthy matched controls (HMC) are associated with different HI grades. The f_u of GS-1056756 for the same impaired cohorts were also determined using the ultracentrifugation method. However, no clear differences were shown among the HI cohorts, or between the HI cohorts and the HMC. (Figure 1) In the RI study, f_u of CILO and GS-716070 were similar in the Severe RI group relative to HMC. (Table 1) For CILO and GS-716070, an ED PPB method was developed based on 1:10 diluted plasma; then undiluted f_u was calculated based on f_u ,d. Accurate PPBs were determined in the HI and RI study for CILO and GS-716070. For the highly protein bound and sticky (dialysis membrane adherent) metabolite, GS-1056756, an ultracentrifugation PPB determination method was developed and served the purpose for PPB comparison between HI and HMC cohorts. Details of the PPB methods are provided, and the data obtained provided important information for evolution of the drug exposure in the HI and RI populations and for dose selection decisions for future clinical trials.

P263 - EVALUATION OF *IN VITRO/IN SILICO* APPROACH FOR PREDICTING HUMAN ABSORPTION PARAMETERS AFTER SUBCUTANEOUS ADMINISTRATION OF ANTIBODIES AND FC-FUSION PROTEINS

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Background: Subcutaneous (SC) administration is commonly used as the administration route for monoclonal antibodies (mAbs) and Fc-fusion proteins due to its lower invasiveness and self-injection feasibility. Estimation of bioavailability (BA) and absorption rate constant (K_a) is crucial for predicting the pharmacokinetics (PK) after SC administration. However, translating these parameters from animals to humans remains challenging, and a reliable method for predicting human BA and K_a has not been established. Although a previous study reported validation of human BA prediction using an *in vitro* system called Subcutaneous Injection Site Simulator (Scissor) [1, 2], the number of tested mAbs has been limited and the complete usefulness for BA and K_a prediction have not been fully clarified. In this study, we aimed to assess the applicability of Scissor in predicting human BA and K_a of mAbs as well as Fc-fusion proteins, and to construct *in silico* models for predicting human BA and K_a using Scissor parameters. **Methods:** Scissor allowed us to investigate the diffusion kinetics in extracellular matrix mimic gels for commercial formulations of nine mAbs and three Fc-fusion proteins to provide Scissor parameters derived from diffusion data. Then, correlations between Scissor parameters and human BA and K_a were examined. Multiple regression analysis was also conducted using Scissor parameters and other PK parameters to construct *in silico* models for predicting human BA and K_a . **Results and Discussion:** The *in vitro* assay using Scissor failed to evaluate two Fc-fusion proteins probably due to adsorption to system devices caused by low formulation concentration. Therefore, correlation analysis was conducted for nine mAbs and one Fc-fusion protein that evaluated diffusion kinetics. The results showed no clear correlation between Scissor parameters and human BA. However, moderate correlation was observed between Scissor parameters and K_a , indicating that diffusion in the subcutaneous tissue may impact K_a but not directly affect BA. When constructing the human BA prediction model using multiple regression analysis, the predictive accuracy was low when only Scissor parameters were used as explanatory variables ($R^2=0.0358$). However, the addition of PK parameters (volume of distribution: V and elimination half-life: $t_{1/2}$) in humans significantly improved predictive performance ($R^2=0.852$). In the construction of the K_a prediction model, good predictive accuracy was observed even when only Scissor parameters were used as explanatory variables ($R^2=0.561$). **Conclusion:** Scissor has shown potential utility in predicting human K_a of mAbs and Fc-fusion proteins. It was challenging to predict human BA from Scissor, but predictive accuracy was improved by adding V and $t_{1/2}$. Further investigation is required to enhance reliability of predictive performance of human BA and K_a .

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P264 - ILLUSTRATING THE MECHANISTIC ABSORPTION AND DISPOSITION OF OATP1B AND BCRP PROBE SUBSTRATES AND THE TRANSPORTER-MEDIATED DRUG-DRUG INTERACTIONS USING PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODELING**Revathi Chapa¹**, Joyce Macwan¹, Jeremy Perrier², and Viera Lukacova¹*Simulations Plus, Lancaster, CA, United States, ²PhinC Development, Massy, France*

Statement of Hypothesis: Pravastatin and Rosuvastatin are among the most commonly used probe substrates in DDI risk assessment for new molecular entities (NMEs) that are potential OATP1B (pravastatin or rosuvastatin) or BCRP (rosuvastatin) transporter inhibitors. Their absorption and disposition in the body is governed by complex interplay of transporters and enzymes, making the quantification of the relative contribution of each of these pathways to the overall disposition very challenging. To delineate the role of their individual contributions, we developed a fully mechanistic PBPK model for each compound using a middle-out approach. The final PBPK models adequately reproduced the observed clinical data, and the contributions of individual transporters and enzymes were verified using DDI studies with perpetrators like rifampicin, gemfibrozil, itraconazole and fluconazole.

Methods: Mechanistic absorption and disposition model was developed for rosuvastatin by integrating 1) apical efflux by BCRP in the gut, liver, and kidney; 2) OATP1B1, OATP1B3, OATP2B1 and NTCP-mediated hepatic uptake; 3) OAT3-mediated active secretion in kidney; and 4) CYP2C9 mediated hydroxylation in liver and UGT1A1 mediated glucuronidation in gut and liver. Similarly, the model for pravastatin incorporated 1) apical efflux by MRP2 in the gut and liver; 2) OATP1B1 and OATP1B3-mediated hepatic uptake; 3) OAT3-mediated active secretion in kidney; and 4) CYP3A4 mediated hydroxylation in gut and liver. The models were implemented in GastroPlus® v.9.8.3 (Simulations Plus, USA) using the *in silico*, *in vitro*, and optimized parameter values. The models were developed using clinical data after intravenous and oral administration of each compound and were verified using clinical data across the dosing range. In addition, DDI studies with rifampicin, gemfibrozil, itraconazole, and fluconazole were used to verify the fraction transported by each transporter (ft), fraction of dose metabolized (fm) and fraction of dose excreted in urine (fe) as parent.

Results and Conclusions: The developed models adequately described the PK of pravastatin and rosuvastatin for all the doses. The simulated Cp-time profiles closely matched the observed mean profiles and the simulated C_{max} and AUC were within the bioequivalence (BE) limit of 0.8-1.25 for ~67% and ~71%, respectively, for 15 studies of rosuvastatin and ~68% and ~83%, respectively, for 16 studies of pravastatin. And the predictions were within 2-fold of the observed values for all of the rosuvastatin studies and ~95% of pravastatin studies. The predicted C_{max} and AUC DDI ratios for rosuvastatin and pravastatin with the inhibitors were within the limits of Guest criterion for 14 of the 15 simulated DDI studies. Overall, the PBPK models accurately predicted (i) the PK of pravastatin and rosuvastatin across the clinical dose range, (ii) the reported bioavailability and percent contributions of metabolism and excretion, and (iii) the impact of the clinical inhibitor's using the published *in vitro* inhibition constants. This work demonstrates the utility of PBPK approach to predict drug interactions involving complex interplay of multiple transporters in the absorption and disposition mechanisms and use of these victim drug/compound files for evaluating the inhibitory potential of investigational drugs that interact with transporters like OATP1Bs and BCRP and support regulatory decision making.

P265 - A FOUR-COMPARTMENT STOMACH REPRESENTATIVE OF THE FED GASTRIC pH PROFILE IN PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODELS CAN IMPROVE PREDICTIVE ACCURACY OF FOOD EFFECTS ON ORAL DRUG ABSORPTION**Lisa Cheng¹**, Po-Chang Chiang², and Harvey Wong¹*¹The University of British Columbia, Vancouver, Canada, ²Genentech Inc., South San Francisco, CA, United States*

Currently, physiologically-based pharmacokinetic (PBPK) models investigating food effects apply a static gastric pH 5 throughout the gastric emptying time, yet this approach inadequately reflects the dynamic changes in the fed stomach, potentially leading to inaccuracies in predicting the dissolution of ionizable compounds.¹ We hypothesize that the incorporation of a more physiologically representative fed gastric pH profile in PBPK models will improve food effect predictions. To address the absence of gastric re-acidification in the current approach, we modified the Advanced Compartmental Absorption and Transit (ACAT) model structure from a one-compartment to a four-compartment stomach while retaining the seven-compartment small intestine and one-compartment colon. The four-compartments correspond to gastric pHs 5, 3.5, 2, and 1.5, thereby mimicking gastric re-acidification after a meal. All physiological models were built using the Simulation, Analysis, and Modeling II software v2.3 (Charlottesville, USA). Posaconazole, a weakly basic antifungal agent exhibiting positive food effects, was selected as the model compound. Several pharmacokinetic parameters including the area under the plasma concentration-time curve (AUC), maximum plasma concentration (C_{max}), and time to reach C_{max} (T_{max}), were used for the evaluation of the models' performance. Clinical data from a 100 mg posaconazole dose showed a 2.85-fold increase in the fed to fasted AUC while the four-compartment stomach ACAT and static gastric pH 5 ACAT model predicted a 3.08- and 1.77-fold increase, respectively. The observed fed C_{max} was 189% greater than the fasted value which was comparable to a 157% increase predicted using the four-compartment stomach ACAT model. Conversely, the static pH 5 ACAT model predicted only a 6% increase in the C_{max} from fasted to fed state. At the 200 mg dose, the observed fed AUC and C_{max} were 3.91- and 3.88-fold greater than that of the

observed fasted exposure parameter, respectively. The four-compartment variable gastric pH model predicted a 4.43- and 4.13-fold increase, whereas the one-compartment static gastric pH model simulated a 2.59- and 1.68-fold increase in AUC and C_{max}, respectively. Lastly, at the 400 mg dose, observed AUC and C_{max} increased by 4.25- and 3.28-fold in the fed state. The four-compartment stomach ACAT model indicated a 6.76- and 5.94-fold increase in the predicted fed AUC and C_{max}, respectively, whereas the static gastric pH 5 strategy predicted a 3.96- and 2.59-fold increase. In all models, a delay in T_{max} was noted in the simulated fed profiles, which aligns with the observed fed data. These results suggest that the incorporation of gastric re-acidification could better predict the positive food effect trends of posaconazole when compared with the static gastric pH 5 approach. Overall, these findings demonstrate that a more physiologically representative fed gastric pH profile in mechanistic models could improve its ability to predict food effects on oral drug absorption.

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P266 - PHYSIOLOGICALLY BASED BIOPHARMACEUTICS MODELLING OF THE EFFECT OF A PERMEABILITY ENHANCER ON THE ABSORPTION OF A HIGHLY SOLUBLE AND POORLY PERMEABLE SMALL PEPTIDE IN HUMANS

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Compound A is a highly soluble, highly bound to proteins and poorly permeable small peptide (molecular weight 2955 g/mol). In preclinical species, distribution volume is low (cynomolgus monkey ~0.35 L, dog ~0.46 L). Transitioning to clinical settings where oral administration is preferred over parenteral routes, thus an enteric coated (EC) tablet was formulated for Compound A. Given its poor permeability, sodium salcaprozate (SNAC), a permeability enhancer, was incorporated into the formulation. SNAC is proposed to facilitate the opening of tight junctions within the small intestine, thereby promoting paracellular absorption [1]. It has been reported that *in vivo* dissolution of EC formulations might be delayed compared to their *in vitro* counterparts, which may influence exposure [2]. It was hypothesised that using Physiologically Based Biopharmaceutics Modelling (PBBM) focusing on the alteration of intestinal paracellular permeability induced by SNAC, could explain the absorption of Compound A. The aim of the study was to develop a PBBM model of Compound A based on permeability changes caused by SNAC; and by incorporating different dissolution delay times, to explain the variability of the SNAC effect leading to variability in Compound A exposure. The model was developed using PKSim/MoBi v10 [3]. EC tablets with 20 mg Compound A + 600 mg SNAC and 40 mg Compound A + 600 mg SNAC were administered as a single dose to healthy subjects (n= 6 and 9, respectively). A Weibull function was fitted to *in vitro* dissolution data of Compound A and used as dissolution input. It was assumed that both molecules dissolve simultaneously. The model was built initially in PKSim and then transferred to MoBi to setup the EC tablet and to define a linear relationship between SNAC concentration in the duodenum and the paracellular permeability of Compound A, using the subject with the highest exposure as a reference. The bi-exponential decline observed in human plasma concentrations was described using a 2-compartment model representing plasma and extracellular volume. Distribution and clearance parameters were fitted using human pharmacokinetics data. By incorporating different dissolution delay times dependent on exposure level, the model successfully explained individual plasma concentration profiles at both Compound A doses. Furthermore, the model was utilised to simulate a Compound A IV administration to estimate %F which was similar to that observed in preclinical species (~2%). This study introduces a method to simulate the activity of a permeability enhancer based on its intestinal concentration (as a function of dissolution) to explain the absorption and exposure of a poorly permeable small peptide in humans. The presented modelling approach can be used to predict the absorption and pharmacokinetics of poorly permeable compounds that depend on the activity of a permeability enhancer.

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P267 - EFFECT OF HEPATIC CYP3A4 ONTOGENY ON DRUG EXPOSURE IN CHILDREN VARIES DEPENDING ON THE ELIMINATION PATHWAYS AND ITS CONTRIBUTION TO TOTAL DRUG CLEARANCE**Chie Emoto** and Mikiko Nakamura*Chugai Pharmaceutical Co., Ltd., Chuo-ku, Tokyo, Japan*

CYP3A4 is involved in the metabolism of many drugs, and it has been reported that its expression in the liver increases with age (Emoto et al., PMID: 35953161). While there are two reports on hepatic CYP3A4 developmental profiles by Salem et al. (PMID: 24671884) and Upreti et al. (PMID: 26139104), no conclusion has been reached regarding the optimal profile. These two CYP3A4 expression profiles have been incorporated into Simcyp™ Pediatrics, where Profile 1 and Profile 2 are the Salem and Upreti models, respectively. According to Profile 1, the expression of CYP3A4, which was about 11% at birth compared to adults, reaches adult levels by approximately 2 years of age. Conversely, for Profile 2, it is about 15% at birth relative to adults, develops rapidly, surpassing adult levels until around 6 years of age before gradually declining to adult levels. Therefore, for CYP3A4 substrates, the hepatic CYP3A4 developmental profile could affect the prediction of exposure in children, especially for neonates and infants. However, the impact of the CYP3A4 developmental profile is expected to vary due to the contribution of hepatic CYP3A4 to total clearance, as well as age-dependent effects on the other elimination pathways. In this study, the impact of the CYP3A4 developmental profile on drug exposure in children was quantitatively evaluated through PBPK simulations using midazolam, a typical substrate of CYP3A4, and hypothetical compounds with modified elimination pathways such as biliary and renal excretions. Simcyp™ Pediatrics (version 22) was used as a simulation platform. For the tested substrates, hypothetical compounds based on midazolam were generated, with the fraction metabolized (fm) adjusted to 30% and 60% by reducing the V_{max} of P450-mediated oxidation and by enhancing the biliary and renal clearances. A fast ontogeny profile, one of the default settings in Simcyp, was applied to biliary clearance according to the report by Johnson et al. (PMID: 26862026). An age-dependent change in renal clearance was described by the BSA-based equation (PMID: 16928154). For midazolam, from birth to the age of 5 years, the predicted AUC using Profile 1 was more than 25% higher than that of Profile 2. This age range shortened as the contribution of biliary and urinary excretions in the total clearance increased. In the case of biliary excretion, the age range was shorter than for urinary excretion. This quantitatively demonstrates that the impact of CYP3A4 developmental profile on predicting pharmacokinetics in children varies according to the contribution of CYP3A4. Therefore, determining the elimination pathways of drug candidates and their contributions to total clearance is crucial before moving to PK prediction in children. This determination provides the age range for conducting sensitivity analyses, using the two developmental profiles of hepatic CYP3A4, in predicting pediatric exposure.

P268 - ROLE OF INTESTINAL TRANSPORTERS IN REGIONAL ABSORPTION KINETICS AND INTRICATE PHARMACOKINETIC PROFILES OF TALINOLOL**Kazuya Ishida**¹, Xiaomin Liang¹, Fulden Buyukozturk², Jia Hao¹, Christine Wan¹, and Yurong Lai¹¹Gilead Sciences, Inc, Foster City, CA, United States, ²MathWorks, Natick, MA, United States

Intestinal uptake and efflux transporters play a pivotal role in oral absorption dynamics to shape the plasma or blood concentration curves of drugs, influencing interindividual pharmacokinetic (PK) variability and drug-drug or drug-food interactions. Plasma concentration-time profiles of several drugs, such as talinolol, bedaquiline, irbesartan, and amisulpride, exhibit dual peaks, yet the mechanism underlying these phenomena remains elusive. While intestinal efflux transporters like P-glycoprotein (P-gp), a well-studied efflux transporter, are thought to cause these dual peaks PK phenomena, they are not the sole determinant. Here, we hypothesized that the interplay between intestinal transporters plays a role in contributing to these dual peaks, and selected talinolol as a model substrate to assess transporter contributions to the observed dual peaks in the plasma. Concentration- and pH-dependent transport of talinolol was evaluated using Caco-2 cells. A full physiologically based pharmacokinetic (PBPK) model with multi-layer gut wall within an advanced dissolution, absorption, and metabolism (M-ADAM) model was built in SimBiology® (MathWorks). Human talinolol PK data was also obtained from the literature. The global sensitivity analysis was performed to identify the transporter(s) that may significantly contribute to dual peaks of talinolol. The basolateral to apical permeability of talinolol in Caco-2 cells decreased as the extracellular pH at the donor side decreased. However, the apical to basolateral permeability did not change significantly, indicating that the transport of talinolol by P-gp is pH-dependent. Although incorporating regional P-gp activity difference slightly improved the plasma concentration profile of talinolol, it did not completely capture the dual peaks of the drug. Furthermore, the full PBPK model that incorporated regional expression of P-gp in the intestine cannot describe the dual peak PK of talinolol. The global sensitivity analysis revealed that the most important parameters for the talinolol plasma profile in the first 5 hours after drug administration are apical uptake, passive diffusion, and apical efflux. The findings in the present study suggest that regional differences in the extent of absorption in the intestine contribute to the complex absorption profile of talinolol. The interplay of uptake and efflux transporters in the enterocytes is one factor contributing to this absorption dynamics and attributed to the dual peaks in talinolol PK profiles.

P269 - DEVELOPMENT AND VALIDATION OF PBPK MODELS FOR PREDICTING HUMAN PHARMACOKINETICS OF ENAVOGLIFLOZIN**Hyeon-Cheol Jeong¹**, Min-Soo Kim², Yoon-Jee Chae², and Kyeong-Ryoon Lee¹¹Korea Research Institute of Bioscience and Biotechnology, Cheongju-si, Chungcheongbuk-do, South Korea, ²College of Pharmacy, Seoul National University, Seoul, South Korea

Enavogliflozin, a selective sodium-dependent glucose cotransporter 2 (SGLT2) inhibitor, has recently been approved in South Korea, and introduced a novel therapeutic option for diabetes management. This research aimed to establish and confirm physiologically based pharmacokinetic (PBPK) models for enavogliflozin and its significant metabolite, M1, facilitating the prediction of their behavior in the human body under varied physiological conditions. The study utilized a middle-out approach with *in vitro*, *in silico*, and clinical data to construct and validate PBPK models for enavogliflozin and M1 and mechanistic kidney model for enavogliflozin to account for its non-linear urinary excretion. Model parameters were derived from available clinical trial data, and predictions were validated against observed pharmacokinetic data from human studies. The PBPK models for enavogliflozin and M1 successfully predicted the concentration-time profiles in plasma and urine, demonstrating good agreement with observations. The key pharmacokinetic parameters, such as area under the curve (AUC) and maximum plasma concentration (C_{max}), fell within the acceptable two-fold error range when compared to observations, confirming the accuracy of developed model. The simulation results revealed an estimated oral bioavailability of enavogliflozin ranging from 78.9% to 79.0% across various dosing regimens, aligning with preclinical findings. Furthermore, the models estimated the metabolic ratio of M1, offering insights into enavogliflozin's metabolic pathways in humans. Constructed model was applied to predict pharmacokinetic profile in patients with hepatic impairment, utilizing changes in metabolic enzyme activities documented in literature for different Child-Pugh scores. Simulation results indicated significant variations in the AUC and C_{max} across varying degrees of liver function, suggesting altered pharmacokinetics in hepatically impaired patients compared to healthy individuals. The findings highlight the necessity for further investigation into dose adjustments for enavogliflozin in populations with moderate to severe hepatic impairment, emphasizing the utility of PBPK modeling in optimizing drug dosing across diverse patient populations. In conclusion, this study presents the first comprehensive PBPK models for enavogliflozin and its metabolite M1, providing a robust tool for predicting the pharmacokinetic behavior of these compounds in humans. The validated models underscore the potential of PBPK modeling in enhancing our understanding of pharmacokinetics/pharmacodynamics, supporting dose optimization, and facilitating the assessment of drug-drug interactions and population-specific pharmacokinetics with comorbidities. Furthermore, this work could be contributes to the informed clinical use of enavogliflozin in the treatment of diabetes, paving the way for more personalized and effective therapeutic strategies.

P270 - ENHANCING DRUG-DRUG INTERACTION PREDICTION BY INTEGRATING PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODEL WITH FRACTION METABOLIZED BY CYP3A4**Pin Jiang**

Medicilon, Shanghai, China

Abstract Background and Purpose: Enhancing the precision of drug-drug interaction (DDI) prediction is essential for mitigating potential drug interactions and enhancing drug safety and efficacy. This study aims to investigate the impact of *in vitro* and *in silico* approaches for calculating the fraction metabolized by CYP3A4 (fm) on DDI prediction accuracy and identified the most effective method for improving DDI prediction using physiologically based pharmacokinetic (PBPK) models.

Experimental Approach: Both *in vitro* and *in silico* methods were utilized to determine fm values for 33 approved drugs. These fm values were then integrated into PBPK models. Subsequently, the PBPK models were combined with a PBPK model of ketoconazole to predict potential DDIs. Finally, the accuracy of these predictions was assessed.

Key Results: The integration of *in vitro* fm had remarkable precision in predicting C_{max}R (C_{max} ratio) of 31 drugs and accurately predicting AUCR (AUC ratio) of 28 drugs out of 33 drugs, both within 2 times of the measured values.

However, using 100% fm and *in silico* fm resulted in lower prediction accuracy that was comparable to each other.

Conclusions and Implications: Our study highlights the importance of incorporating *in vitro* fm data into PBPK models to improve the accuracy of predicting DDIs. While *in silico* fm may have some potential, its influence on predictions appears to be limited. Additionally, our findings suggest that drugs with high C_{liver} levels (>15 L/h) and high fm (>75%) are particularly susceptible to the impact of CYP3A4 inhibitor ketoconazole.

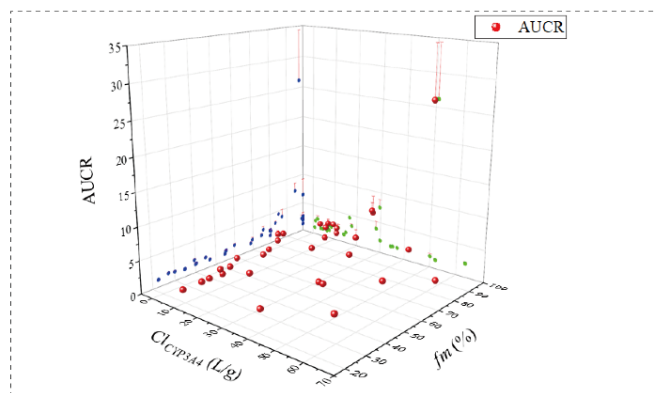


Figure 1. 3-D Scatter Plot of the Cl_{liv} , *in vitro* f_m , and the AUCR. Red balls in the 3-D scatter plot represent 33 compounds positioned according to their corresponding values of Cl_{liv} , *in vitro* f_m , and AUCR. Green points in the plot show the projection of the red balls onto the AUCR- Cl_{liv} plane, demonstrating the relationship between Cl_{liv} and AUCR, independent of f_m . Similarly, blue points in the plot indicate the projection of the red balls onto the AUCR- f_m plane, indicating the relationship between f_m and AUCR for each compound, independent of Cl_{liv} .

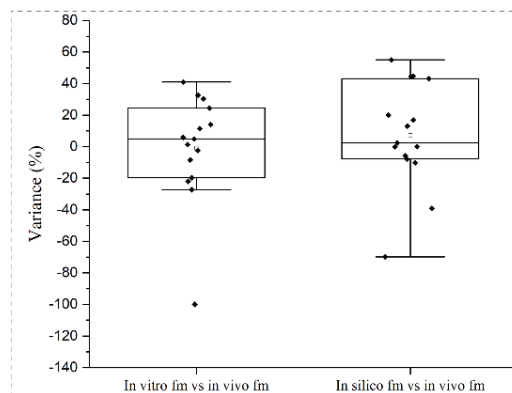


Figure 2. Comparison of *in vitro* and *in silico* predictions of f_m accuracy with *in vivo* values for compounds with a Cl_{liv} higher than 15 L/h. The boxplot on the left depicts the difference between f_m *in vitro* and f_m *in vivo*, while the boxplot on the right displays the difference between f_m *in silico* and f_m *in vivo*.

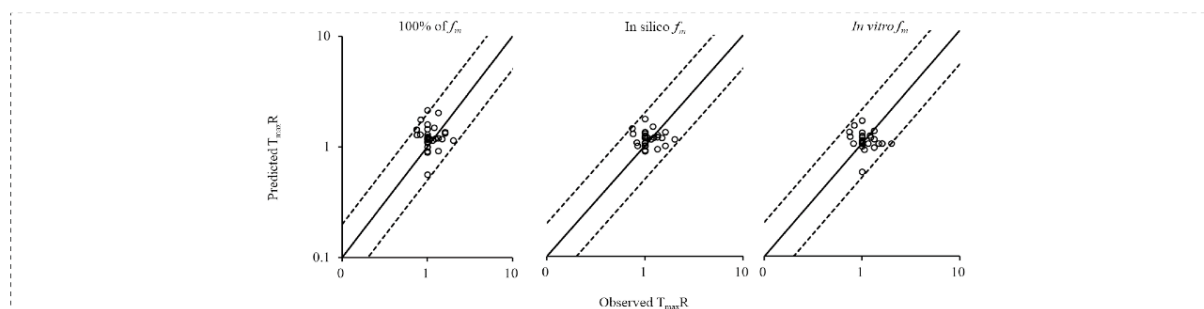


Figure 3. Comparison of $T_{max}R$ predictions made using three different f_m s with the measured $T_{max}R$ values. The left panel assumes a fixed f_m value of 100%, the middle panel uses an *in silico* f_m , and the right panel uses an *in vitro* f_m .

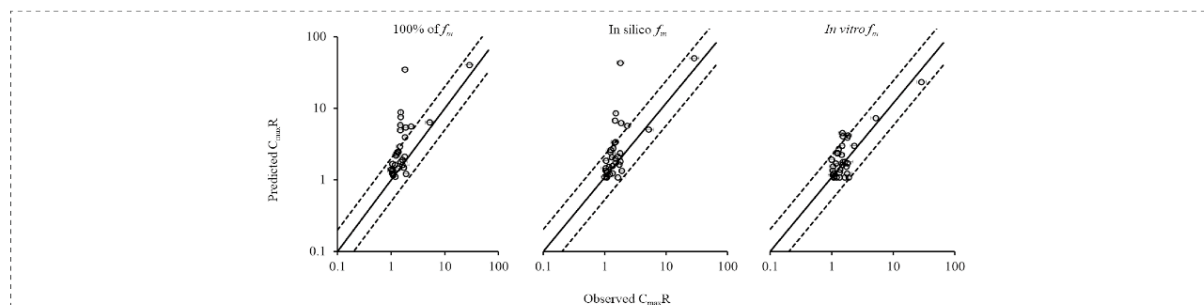


Figure 4. Comparison of $C_{max}R$ predictions made using three different f_m s with the measured $C_{max}R$ values. The left panel assumes a fixed f_m value of 100%, the middle panel uses an *in silico* f_m , and the right panel uses an *in vitro* f_m .

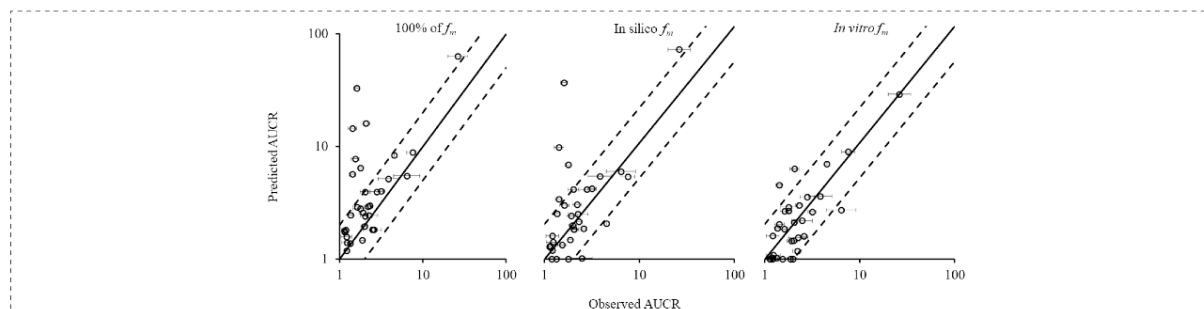


Figure 5. Comparison of AUCR predictions made using three different f_m s with the measured AUCR values. The left panel assumes a fixed f_m value of 100%, the middle panel uses an *in silico* f_m , and the right panel uses an *in vitro* f_m .

P271 - SYSTEMATIC EVALUATION OF UNDERLYING MODELS THAT IMPROVE PURELY IN-SILICO HIGH-THROUGHPUT MECHANISTIC PBPK PREDICTIONS ACROSS SPECIES**Jeremy Jones**¹, Rafal Bachorz², David Miller², Michael Lawless², Vladimir Chupakhin², and Robert Fraczekiewicz²¹*Simulations Plus, Murays Bay, New Zealand*, ²*Simulations Plus, Lancaster, CA, United States*

The ability to quickly and accurately predict key PK properties based solely on chemical structure can aid in several tasks, from triaging environmental chemicals that pose a risk to humans and other species to prioritizing HTS hits and especially drug design and optimization. The high-throughput Physiologically Based Pharmacokinetic (HTPK Simulation) module in ADMET Predictor® (AP) achieves this task by integrating ADMET property estimates to solve a deterministic system of differential equations with respect to human, rat or mouse physiology, based on a simplified version of the GastroPlus® Advanced Compartmental Absorption and Transit (ACAT) model. This ACAT model relies on several models for ADME property estimates and while the initial version of the HT-PK performed well, delivering very similar results to the full PBPK modeling predictions in GastroPlus1, a systematic investigation of the contribution of the underlying models to prediction accuracy has never been performed. We hypothesize that optimizing the combination of underlying ADMET property prediction models will lead to more accurate PK parameter predictions in humans, rats, and mice. We began by establishing a “ground truth” database of PK endpoints for benchmarking by carefully curating literature data. We focused primarily on Area Under the Curve (AUC), maximum plasma concentration (C_{max}), and oral availability (%F_b). These properties are dose dependent for some compounds, especially BCS Class II and IV chemicals. We used the HTPK module in AP version 11.0 to calculate the PK properties of compounds in the “ground truth” dataset. We included sub-analyses of non-pharmaceutical compounds as well. Observed versus predicted plots were created and RMSE and other statistical measures were tabulated. This process was systematically repeated during the optimization process of the HTPK module in AP version 11.5 with varying combinations of new and improved underlying ADME models to derive the most accurate predictions of PK properties. Our results demonstrate that a combination of new and improved ADME models, along with a new dosage form (IR solution) significantly improved AUC, C_{max}, %F_b and other PK predictions. This was true for pharmaceutical and non-pharmaceutical compounds, where our predictions were more accurate than other publicly available tools. In conclusion, the improvement of PK predictions was significant and led to the inclusion of new underlying models in the ADMET Predictor HTPK platform.

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P272 - ASSESSMENT OF CYP3A-MEDIATED DRUG INTERACTION VIA CYTOKINE (IL-6) ELEVATION FOR MOSUNETUZUMAB (LUNSUMIOTM) USING PHYSIOLOGICALLY-BASED PHARMACOKINETIC MODELING**Fang Ma**, Chi-Chung Li, Nick Jones, Chunzhe Li, and Yuan Chen*Genentech, South San Francisco, CA, United States*

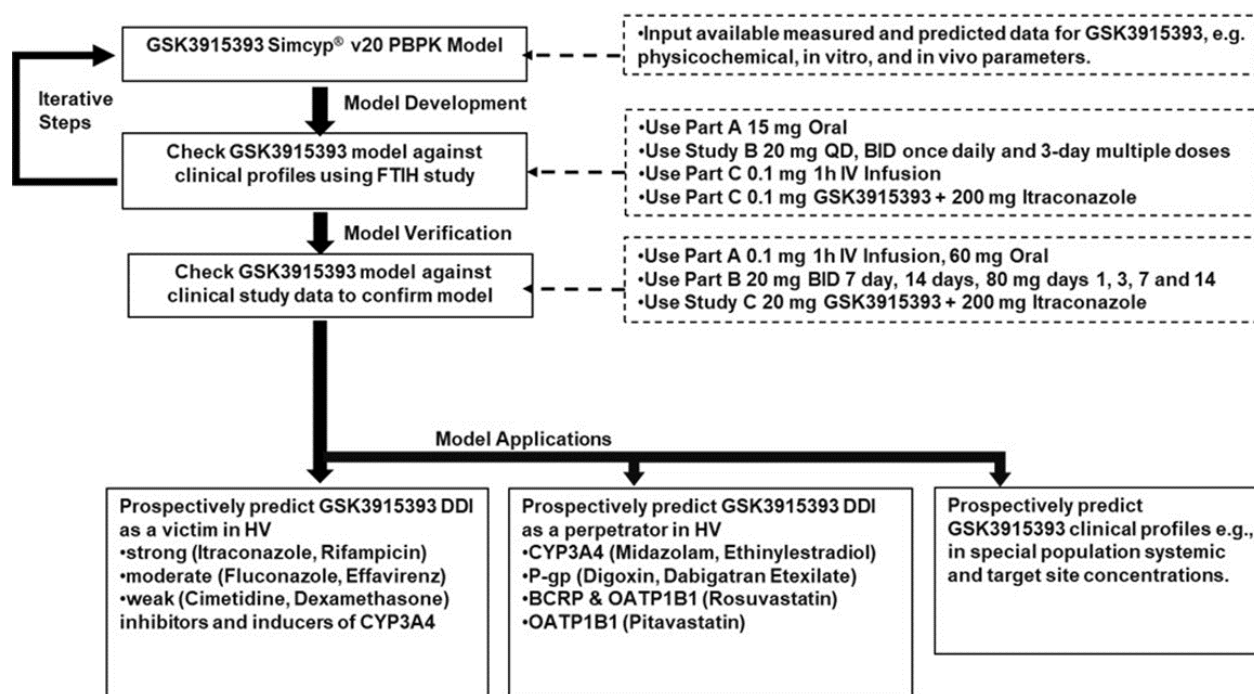
Mosunetuzumab ((LUNSUMIOTM) is a CD3/CD20 bispecific antibody. As an on-target effect, transient elevation of interleukin-6 (IL-6) occurs in early treatment cycles. A physiologically-based pharmacokinetic (PBPK) model was developed to assess potential drug interaction caused by IL-6 enzyme suppression on cytochrome P450 3A (CYP3A) during mosunetuzumab treatment. The model's performance in predicting IL-6 CYP3A suppression and subsequent drug-drug interactions (DDIs) was verified using existing clinical data of DDIs caused by chronic and transient IL-6 elevation. Sensitivity analyses were performed for a complete DDI risk assessment. The IL-6 concentration- and time-dependent CYP3A suppression during mosunetuzumab treatment was simulated using PBPK model with incorporation of *in vitro* IL-6 inhibition data. At clinically approved doses/regimens, the DDI at maximum CYP3A suppression was predicted to be a midazolam maximum drug concentration in plasma (C_{max}) and area under the plasma drug concentration-time curve (AUC) ratio of 1.17 and 1.37, respectively. At the 95th percentile of IL-6 concentration level or when gut CYP3A suppression was considered, the predicted DDI risk for mosunetuzumab remained low (<2-fold). The PBPK-based DDI predictions informed the mosunetuzumab product label to monitor, in early cycles, the concentrations and toxicities for sensitive CYP3A substrates with narrow therapeutic windows.

P273 - HIGH IMPACT APPLICATION OF A CONTINUOUS LEARN AND CONFIRM PBPK MODELING STRATEGY: EVALUATING THE DRUG-DRUG INTERACTION POTENTIAL FOR A TRANSGLUTAMINASE-2 INHIBITOR**Jennypher Mudunuru**¹, Farzaneh Salem², Debbie Thomson², Iain Uings², and Kunal Taskar²¹*GlaxoSmithKline, Collegeville, PA, United States*, ²*GlaxoSmithKline, Stevenage, United Kingdom*

GSK3915393 is an irreversible inhibitor of the enzyme transglutaminase 2 (TG2) and is predominately metabolized by CYP3A4. GSK3915393 is an inducer and inhibitor of CYP3A4, P-gp, BCRP and OATP1B1 *in vitro*. Weak induction of

CYP3A4 in human hepatocytes and metabolism-dependent inhibition of CYP3A4 in HLM was observed *in vitro*. Static equations per the latest regulatory guidance^{1,2} indicated that GSK3915393 may be an inducer and/or inhibitor of CYP3A4 and an inhibitor of P-gp, BCRP and OATP1B1 in the clinic. A Physiologically Based Pharmacokinetic Model (PBPK) was developed in Simcyp® v20 and healthy volunteer population using the available clinical data from the FTIH (First time in human) study to evaluate the DDIs (Drug-Drug interaction) potential for GSK3915393 as a substrate and perpetrator. The model described a mechanistic absorption (M-ADAM), systemic distribution (via full PBPK model) and a mechanistic clearance model (via enzyme kinetics). A fit-for-purpose perpetrator model was built for prospective DDI risk assessment. Plasma concentration-time profiles and PK parameters were predicted and compared against observed data. The validated model was then utilized for predicting DDI risk with a strong CYP3A4 inhibitor, itraconazole and compared against observed itraconazole DDI data. The predicted mean plasma concentration- time profiles and 5th and 95th percentiles captured the observed profile and variability across all dose levels and routes of administration. The predicted vs. observed AUC_{0-∞} and C_{max} were 188 vs 166 ng.h/mL and 96 vs 146 ng/mL, respectively. Co-administration of GSK3915393 with itraconazole resulted in 12.5 and 6.5-fold increases in geometric mean AUC and C_{max} in the clinical study. The simulated ratios were 12.7 and 5.5 for AUC and C_{max}, respectively. DDI simulations with weak (cimetidine) and moderate (fluconazole, erythromycin, and verapamil) index inhibitors resulted in 1.4 and 3.6, 4.4 and 4.4-fold increases in AUC of GSK3915393, respectively. A reduction in exposure of GSK3915393 was predicted with weak, moderate, and strong inducers dexamethasone, efavirenz and rifampicin (mean 0.61, 0.28 and 0.09-fold change in AUC respectively). The perpetrator DDI simulations were predicted over a dose range of 20 – 800 mg. GSK3915393 increased midazolam exposure and predicted AUC ratios of 1.37-4.17 respectively and ethinyl estradiol were predicted in the range of 1.08 to 1.26. GSK3915393 increased exposure of dabigatran etexilate, a sensitive P-gp substrate, with the AUC ratios ranging between 1.27 – 2.46. Simulations with OATP1B1 substrates predicted a <2-fold increase in the AUC of rosuvastatin, pravastatin and pitavastatin for the doses ranging up to 800 mg of GSK3915393, suggesting no potential DDI risk via this transporter inhibition pathway. A robust and verified GSK3915393 PBPK model for predicting the clinical exposure for single, multiple doses, food effect and DDIs was developed using the Simcyp® platform, which was then applied to prospectively predict both victim and perpetrator DDIs.

Figure 1: Workflow for GSK3915393 PBPK Model Development, Verification and Applications



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P274 - APPLICATION OF A PERMEABILITY-LIMITED MULTI-COMPARTMENT LIVER (PERMCL) MODEL TO PREDICT ZONAL HEPATOCELLULAR STATIN CONCENTRATIONS IN METABOLIC DYSFUNCTION-ASSOCIATED STEATOHEPATITIS (MASH)

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Statin therapy is frequently prescribed to treat hyperlipidemia¹, a common comorbidity in ~70% of patients with MASH. MASH-associated changes in hepatic drug metabolizing enzymes (DMEs) and transporters may alter the pharmacokinetics of pravastatin, rosuvastatin, and atorvastatin^{2,3}. However, current understanding of disease-mediated changes in the hepatocyte disposition of statins in patients with MASH is limited. This is an important knowledge gap because the statin target, 3-hydroxy-3-methylglutaryl coenzyme-A (HMG-CoA) reductase, is located within hepatocytes. This study used a physiologically based pharmacokinetic (PBPK)/PerMCL modeling framework that incorporated zonal transporter and DME data to predict the impact of MASH on the systemic and hepatocellular concentrations of pravastatin, rosuvastatin, and atorvastatin.

Membrane-localized zonal abundance (MZA)⁴ of hepatocyte sinusoidal uptake transporters that play a critical role in statin disposition (e.g., organic anion transporting polypeptide [OATP]1B1, OATP1B3) and unadjusted zonal abundance⁵ data for other transporters and DMEs were incorporated into the Simcyp™ Simulator's PerMCL model, encompassing six hepatic zones. Relevant hepatic transport and metabolism pathways were verified against published data for Healthy Volunteers and MASH virtual populations. OATP1B uptake pathways for statin PerMCL models were verified using clinical genotype and drug-drug interaction data, while atorvastatin CYP3A4 metabolism was verified using 2-hydroxy atorvastatin clinical data.

The verified PBPK/PerMCL models were used to simulate plasma and zonal hepatocellular concentration-time profiles for pravastatin, rosuvastatin, and atorvastatin in Healthy Volunteers and MASH for 100 virtual individuals aged 40-65 years. Simulations (Table 1) predicted >1.6-fold changes in maximal total plasma concentrations and area under the curve for pravastatin and rosuvastatin in MASH compared to Healthy Volunteers at steady state. For atorvastatin, changes in these parameters were predicted to be >3.2-fold. Furthermore, steady state maximal unbound hepatocellular statin concentrations increased up to 61% (range: 4-61%) in the periportal region (Zone 1) and decreased up to 51% (range: 6-51%) in the pericentral region (Zone 6) in MASH. These findings can be attributed to increased periportal and decreased pericentral OATP1B3MZA in MASH⁴ and suggest the potential for altered statin efficacy in this population.

This study provides novel insight into systemic and hepatic statin disposition in MASH. Future research will focus on translating these pharmacokinetic simulations into predictions of statin cholesterol-lowering efficacy through the application of a pharmacodynamic model.

Table 1. C_{max,ss} and AUC_{τ,ss} for pravastatin, rosuvastatin, and atorvastatin model simulations using Healthy Volunteers (HV) and MASH virtual populations in the Simcyp™ Simulator Version 23.

Virtual Population	Total Plasma				Hepatocellular Unbound Concentrations			
	C _{max,ss} (ng/mL)	AUC _{τ,ss} (ng*hr/mL)	C _{max,ss} Ratio ^a	AUC _{τ,ss} Ratio ^a	Zone 1 C _{max,ss} (ng/mL)	Zone 1 C _{max,ss} Ratio ^a	Zone 6 C _{max,ss} (ng/mL)	Zone 6 C _{max,ss} Ratio ^a
Pravastatin 40 mg PO QD, 14 days								
HV	52.3	169.7	-	-	401.6	-	368.6	-
MASH	85.5	282.4	1.63	1.66	543.3	1.35	347.6	0.94
Rosuvastatin 20 mg PO QD, 14 days								
HV	8.4	88.8	-	-	48.4	-	29.8	-
MASH	15.0	169.1	1.79	1.90	50.5	1.04	14.6	0.49
Atorvastatin 40 mg PO QD, 14 days								
HV	10.2	49.0	-	-	11.3	-	2.8	-
MASH	32.6	174.0	3.20	3.55	18.2	1.61	1.9	0.68

^aMASH/HV

τ = 312 to 336 hr

Pharmacokinetic parameters were obtained by simulating a fixed oral dose administered every 24 hours for 14 days in the fasted state.

Simulations were performed for 100 virtual individuals (age range of 40-65 years; female proportion of 0.5). The population geometric mean values are shown. PO, by mouth; QD, once daily.

Supported by 2020/2021 Certara Simcyp GPS; NIH T32GM086330.

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P275 - A NEW MODEL TO PREDICT DRUG ENTEROHEPATIC RECIRCULATION IN HEALTHY HUMANS WITH NALBUPHINE AS A MODEL DRUG

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Hypothesis: Nalbuphine (NAL) is a κ -agonist/ μ -antagonist opioid being developed as an oral extended formulation (ER) for the treatment of chronic cough conditions, including cough in idiopathic pulmonary fibrosis, refractory chronic cough as well as itch in prurigo nodularis¹. NAL is extensively glucuronidated and likely undergoes enterohepatic recirculation. We hypothesize that the enterohepatic recirculation (EHR) of NAL either as the parent or via glucuronidation will contribute significantly to its disposition. The purpose of this work is to develop pharmacokinetic models for NAL absorption and EHR.

Methods: Clinical PK datasets in healthy subjects from three trials that included IV, oral solution and ER tablets in fed and fasted state and two published trials^{2,3} were used to parameterize a novel partial differential equation (PDE) based model, termed 'PDE-EHR' model. Experimental inputs included *in vitro* dissolution and permeability data. The model incorporates a continuous intestinal absorption framework⁴, explicit liver and gall bladder compartments, and compartments for systemic drug disposition. The model was fully PDE-based, with well-stirred compartments achieved by rapid diffusion.

Results: The PDE-EHR model accurately reproduces NAL concentration-time profiles for all clinical datasets. NAL disposition simulations required inclusion of both parent and glucuronide recirculation. Inclusion of intestinal P-glycoprotein efflux in the simulations suggests that NAL is not expected to be a victim or perpetrator of P-glycoprotein mediated drug interactions.

Conclusions: The PDE-EHR model is a novel tool to predict EHR and food/formulation effects on drug PK. The results strongly suggest that even intravenous dosing studies be conducted in fasted subjects when EHR is suspected. The modeling effort is expected to aid in improved prediction of dosing regimens and drug disposition in patient populations.

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P276 - DEVELOPING A NOVEL RAT PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL TO IMPROVE THE PREDICTION OF CONCENTRATION-TIME PROFILES

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Hypothesis: We hypothesize that a permeability-limited PK model is necessary to evaluate underlying mechanisms of distribution kinetics of drugs. To this end, our lab has recently developed the human PermQ model¹. During the model development, it was found the concentrations of many drugs were greatly over-predicted at very early time points after IV bolus or infusion, particularly for hydrophobic bases with a high blood-to-plasma ratio. The addition of a shallow compartment improved the prediction of early timepoint concentrations. This needs further evaluation, and data collection in rats is facile. The purpose of the present study is to develop a rat PermQ model that can be used to evaluate mechanisms of distribution kinetics of hydrophobic bases.

Methods: This model is a permeability- and perfusion-limited PBPK model and was developed using the human PermQ framework briefly described as follows. Drug can reversibly distribute between capillaries and interstitial fluid by fenestra or discontinuities in capillaries, or by transcellular diffusion through endothelial cells. Passive membrane permeability and transporter mediated are considered. Drugs also can partition into intracellular phospholipids and neutral lipids except into aqueous volumes. A shallow distribution compartment was added particularly for basic drugs. For midazolam and glyburide, the fup and BP were collected in-house. C-t profiles were modeled for 7 drugs using the same experimental inputs for three different models¹⁻⁴: Rodgers and Rowland (RR), a perfusion-limited membrane-based model (Kp,mem), and rat PermQ. All models were built and evaluated using Mathematica version 13.1 (Wolfram Research Inc.).

Results: For metoprolol, the exposure overlap coefficients (EOC) values for RR, Kp,mem and rat PermQ are 0.833, 0.820 and 0.966, respectively. For atenolol, the EOC values for RR, Kp,mem and rat PermQ are 0.681, 0.675 and 0.868, respectively. For carvedilol, the EOC values for RR, Kp,mem and rat PermQ are 0.770, 0.779 and 0.947, respectively. For

digoxin, the EOC values for RR, Kp,mem and rat PermQ are 0.880, 0.826 and 0.848, respectively. For midazolam, the EOC values for RR, Kp,mem and rat PermQ are 0.469, 0.841 and 0.811, respectively. For atorvastatin, the EOC values for RR, Kp,mem and rat PermQ are 0.805, 0.890 and 0.877, respectively. For glyburide, the EOC values for RR, Kp,mem and rat PermQ are 0.439, 0.474 and 0.896, respectively.

Conclusion: Rat PermQ model improved the prediction of rat C-t profiles for basic drugs. Mechanisms of basic drug distribution will be evaluated next. Funding: Grants NIGMS 2R01GM104178 and 3R01GM114369.

P277 - DEVELOPMENT OF A PHYSIOLOGICALLY BASED PHARMACOKINETIC MODEL TO ASSESS POTENTIAL CAUSES FOR DIFFERENCES IN SIMVASTATIN PHARMACOKINETICS BETWEEN THAIS AND CAUCASIANS

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Ethnicity affects drug metabolism and disposition through genetic variation changes in drug-metabolizing enzyme and drug-transporter activity. Earlier studies suggested potential ethnic differences for substrates of CYP3A enzyme and OATP1B transporter between Caucasians and other populations, including Thais. In this study, we built a Thai healthy volunteer population physiologically based pharmacokinetic (PBPK) model based on the Chinese Healthy Volunteer default population model in Simcyp simulator version 20, but using over a thousand demographic and physiology dataset from the Thai Healthy Volunteer database and currently available Thai pharmacogenetic data from a public domain. The developed population model was successfully qualified with several CYP3A and OATP1B substrates using the midazolam, sildenafil, quetiapine, sirolimus, and atorvastatin default models in the Simcyp against pharmacokinetic studies in Thais. However, when using with a simvastatin drug model previously developed and qualified with several drug-drug interaction studies in Caucasian subjects, the Thai population model 3-5-fold underpredicted simvastatin acid but not simvastatin plasma concentrations in Thai healthy young and elderly subjects. Noteworthy that the plasma exposure ratio between simvastatin acid and simvastatin was found to be much higher in Thais than in Caucasians. Both simvastatin and simvastatin acid are substrates of CYP3A, but only simvastatin acid is a substrate of OATP1B. Ex vivo simvastatin plasma hydrolysis from Thai healthy volunteers showed increased esterase enzyme activity, when compared to that reported in Caucasians, but this alone could not explain the underprediction. However, the developed Thai population model with increasing relative abundance of the liver's esterase enzymes could adequately capture both simvastatin and simvastatin acid PK in the Thai subjects. These results suggested that the difference in the hepatic esterase enzyme activity and/or abundance between Thais and Caucasians could be a potential cause for the observed ethnic difference in the simvastatin acid PK. Further studies are warranted to confirm this possibility.

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P278 - FREEZ CONVERTED SUSPENSION-TYPE HUMAN PRIMARY HEPATOCYTE AS A NOVEL *IN VITRO* MODEL FOR PHARMACOKINETIC AND TOXICITY ASSAY AT AN AFFORDABLE PRICE

Raghda Shahin

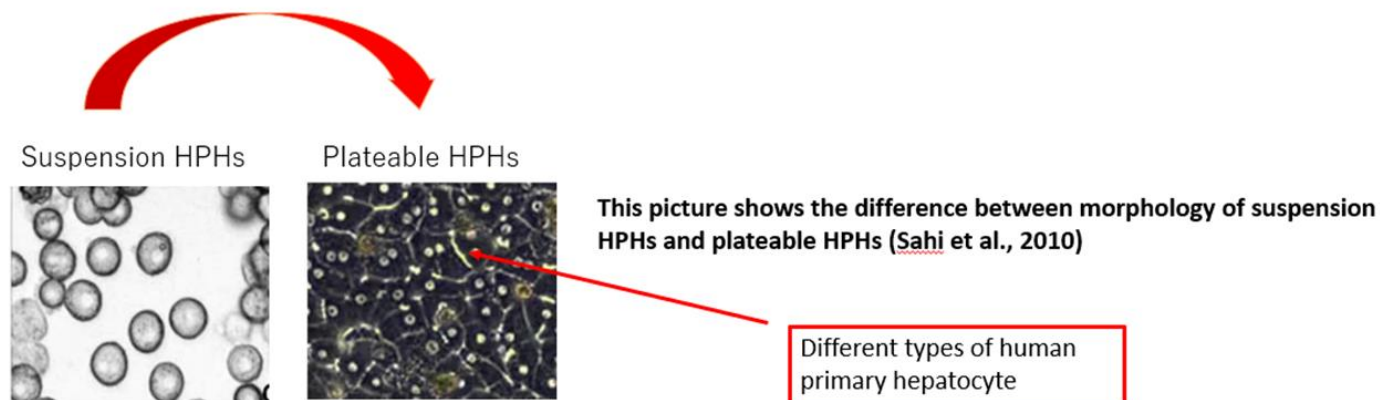
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The liver, one of the largest organs performs various crucial functions to maintain homeostasis of the human body. In addition, it has a crucial role in detoxifying xenobiotic compounds, such as medical drugs and chemicals. As a result, the liver's biotransformation is a significant consideration in developing medications. Despite considerable efforts to enhance the drug development process, the industry is still facing issues as the animal testing models that are commonly employed in pharmaceutical and industrial research to assess human toxicity cannot reliably predict drug safety in humans. Still, human primary hepatocytes (HPHs) are considered the gold standard for *in vitro* analysis of drug candidate metabolites and toxicity, as they recapitulate human liver characteristics *in vivo*. Unfortunately, the utilization of these HPHs is hindered by high costs and limited availability. In addition, although employing the identical isolation and cryopreservation technique for HPHs, the commercially available HPHs are divided into two types: plateable (pHPHs) and suspension (sHPHs) human primary hepatocytes which have different behaviors (1). For unknown reasons, the sHPHs are circular, cannot establish cell-matrix, cell-cell interactions, and render them unable to adhere to the plate surface and culture as monolayer. Consequently, they exhibit inefficient polarization and relocalization of efflux transporters and possess a limited usable time of only 2-4 hours. In contrast, pHPHs which are polygonal and considered the best *in vitro* hepatocyte models can generate a compact monolayer that lives for at least 4-6 days. We have been succeeded in inventing protocols that convert sHPHs to be similar to pHPHs that able to be plated as 2D and 3D. The objective of this study is to develop a protocol employing small molecules and cryopreserve cultured sHPHs enabling their use in induction and efflux transporter assays that are readily available for immediate use. Frozen cultured sHPHs were seeded in a

medium containing small molecules. gene expression and function were assessed. Frozen cultured sHPHs have been successfully cultured displaying similar morphology to pHPHs, including the formation of tight junctions, metabolizing enzymes such as CYPs, and efflux transporters were expressed. Furthermore, inducers led to the induction of CYP1A2, CYP2B6, and CYP3A4. This innovative study demonstrates the frozen functional cultured sHPHs, providing an economical new cell model alternative to expensive pHPHs for use in pharmacokinetics, and toxicity assay as a novel *in vitro* model.

This slide for explains our hypothesis in converting suspension human primary hepatocytes to plateable human primary hepatocyte

We made patent in Convert suspension human primary hepatocyte to palatable type human primary hepatocyte



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P279 - ESTABLISHING A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL TO SIMULTANEOUSLY PREDICT PHARMACOKINETIC VARIATIONS OF IRINOTECAN, SN-38, AND SN-38 GLUCURONIDE

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Irinotecan is widely used either alone or in combination to treat metastatic or advanced solid tumors, including colorectal and pancreatic cancers. Irinotecan is a prodrug and undergoes hydrolysis to form its active form, SN-38, catalyzed by human carboxylesterases (CES). The limited clinical use of irinotecan is due to the occurrence of neutropenia and diarrhea caused by SN-38, which is metabolized by UDP-glucuronosyltransferase (UGT) 1As. Thus, it is crucial to quantitatively predict the pharmacokinetic (PK) variation of SN-38 due to factors such as drug-drug interactions (DDIs) or genetic polymorphism of metabolizing enzymes to ensure its safe use. This work aimed to develop a full mechanistic physiologically based pharmacokinetic (PBPK) model simultaneously incorporating the processes of irinotecan hydrolysis, SN-38 metabolism, and the formation of SN-38 glucuronide, as well as their elimination in the urine and bile. Using the SimCYP simulator, the base model was constructed using available data from the literature, including enzyme and transporter kinetics recovered from recombinant systems. The base model incorporates the bioactivation of irinotecan to SN-38 by CES, the metabolism of SN-38 into SN-38 glucuronide by UGT1A1, the hepatic uptake by organic anion-transporting polypeptides (OATPs) 1B1 and 2B1 and the biliary excretion of SN-38 by P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP). The base model was further refined using clinical plasma concentration-time profiles and results from mass balance studies in the literature. We found that the base model initially overpredicted the clinical volume of distribution (V_{ss}) and underpredicted the clearance (CL) for both irinotecan and SN-38. By refining the partition coefficients and including an estimated maximum rate of metabolism (V_{max}) for CES-1 (irinotecan)/UGT1A1 (SN-38), the PBPK model was improved. This enhancement resulted in the successful capture of the PK profiles of all three components and enabled the accurate prediction of the plasma concentration-time curves (AUC) and the maximum plasma concentration (C_{max}) of all three components after intravenous infusion of a wide range of irinotecan doses. The C_{max} and AUC of all three compounds were within 2-fold in accordance with their clinical pharmacokinetics. Furthermore,

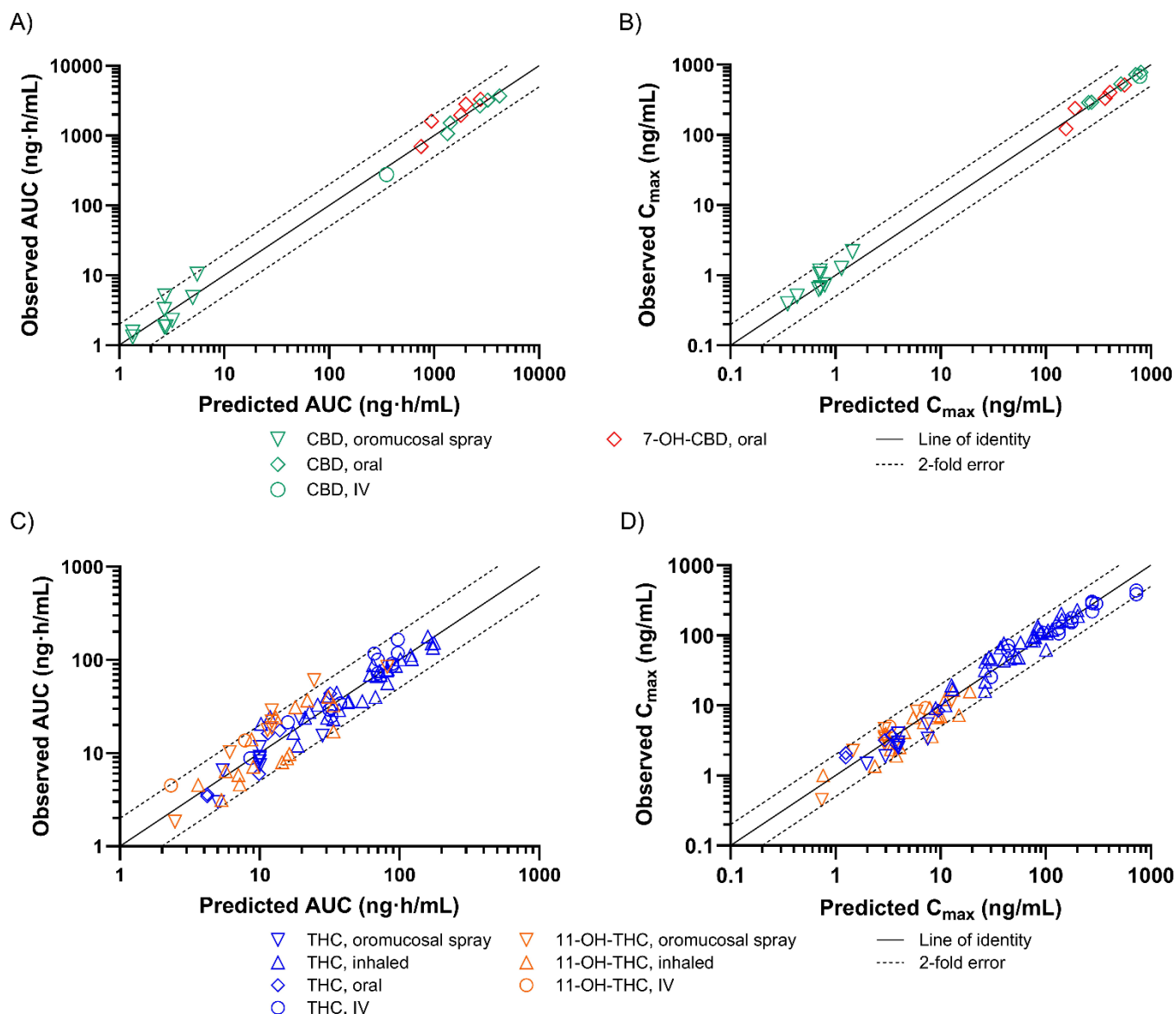
a simulation of SN-38 was performed on a virtual population with low (*28/*28) and reduced function (*1/*6, *1/*28) polymorphisms compared to the population with normal function (*1/*1). The simulation accurately reflected a decrease in CL and an increase in AUC that was observed in the clinic. In conclusion, a full mechanistic PBPK model was successfully developed for simultaneously simulating the PK variations of irinotecan, SN-38, and SN-38 glucuronide. This model can be utilized to quantitatively predict SN-38 exposure in various scenarios, such as the changes in dosage regimens, genetic polymorphism, and DDIs, for its safe use.

P280 - PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELING OF CANNABIDIOL (CBD), Δ-9-TETRAHYDROCANNABINOL (THC), AND THEIR ACTIVE METABOLITES IN HEALTHY ADULTS AFTER ADMINISTRATION OF CBD AND THC BY MULTIPLE ROUTES

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“Cannabis” represents several plants of the genus *Cannabis* (family Cannabaceae), particularly *C. sativa*, which contains >500 constituents, including >100 cannabinoids [1,2]. Cannabidiol (CBD) and Δ-9-tetrahydrocannabinol (THC) are the most extensively studied cannabinoids in cannabis [3]. CBD is eliminated mainly by cytochrome P450 (CYP) 2C19- and CYP3A-mediated metabolism to the primary and active metabolite, 7-OH-CBD [4]. THC is eliminated mainly by CYP2C9-mediated metabolism to the primary and active metabolite, 11-OH-THC, with a minor contribution by CYP3A [5]. CBD and THC have been reported to inhibit multiple CYPs *in vivo* and/or *in vitro* [6-8], raising concerns for potential cannabinoid-drug interactions. CBD and THC are available in many different formulations with multiple routes of administration. Collectively, a rigorous understanding of the pharmacokinetics of CBD, THC, and their active metabolites remains critical. The objective of this work was to develop and verify physiologically based pharmacokinetic (PBPK) models for CBD, THC, and their metabolites after various routes of administration of CBD or THC. Models for all of these cannabinoids were developed using a middle-out approach and the Simcyp™ PBPK Simulator (v22). Physicochemical and pharmacokinetic input parameters were either collected from the literature or optimized using Simcyp™. A step-wise workflow was applied. First, PBPK models were developed for intravenous (IV) CBD (1.6-20 mg) and THC (1.25-5 mg). Second, a model was developed for 11-OH-THC after IV THC administration but not for 7-OH-CBD due to lack of IV data. Third, models for the oral and oromucosal spray routes were developed for CBD (750-6000 mg and 5-20 mg, respectively) and for the oral, inhaled, and oromucosal spray routes for THC (4.25-45.7 mg, 1.6-86.0 mg, and 5.4-21.6 mg, respectively). Fourth, a model was developed for 7-OH-CBD after oral administration of CBD. Finally, the model for 11-OH-THC after IV THC administration was linked to the THC models for the inhaled and oromucosal spray routes. At each step, the models were optimized and verified using independent datasets obtained from published clinical studies involving healthy adults administered CBD or THC. PBPK model-predicted plasma AUC and C_{max} for CBD and THC at all doses and routes of administration were within 0.51- to 1.68-fold of the observed values (Figure 1). Model-predicted AUC and C_{max} for 7-OH-CBD and 11-OH-THC were within 0.58- to 1.26-fold (Figure 1A, 1B) and 0.40- to 2.28-fold (Figure 1C, 1D) of the observed values, respectively. In summary, PBPK models for CBD, THC, and corresponding active metabolites were developed and verified after multiple routes of administration of CBD or THC. These models can be used for other applications, including predicting changes in systemic exposure to CBD, THC, and active metabolites in combination with CYP inhibitors or inducers to assess potential safety and efficacy concerns.



P281 - EFFECT OF LIPOPROTEIN BINDING AND PLASMA DILUTION ON THE ACCURACY OF ULTRACENTRIFUGATION METHOD IN PLASMA PROTEIN BINDING

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Ultracentrifugation (UC) is the proposed methodology for studying the protein binding of challenging compounds, such as targeted protein degraders, covalent inhibitors, and those with severe nonspecific binding properties. However, UC is prone to lipoprotein contamination that can result in artificially elevated fraction unbound (f_u) values compared to other methods. A previous study reported that protein contamination does not give rise to a serious problem for practical estimation of PPB, based on the estimation by simulation that the extent of overestimation of f_u was 13% for a 99% binding compound[1]. However, our preliminary data showed that for certain compounds, the f_u values by UC are significantly higher than the other dialysis-based methods (eg. 24-hour equilibrium dialysis with diluted plasma). Furthermore, large variations were observed between neat and 10% plasma for certain compounds. Since the dilution method can provide reliable f_u estimations by equilibrium dialysis, this led to the question of whether plasma dilution can be applied to the UC method. In order to quantitatively estimate the effect of binding to low-density lipoproteins/very-low density lipoproteins (LDL/VLDL) as well as plasma dilution on the data accuracy of ultracentrifugation method, a set of 22 compounds covering a wide range of LogP (0.8-8.6) were selected with or without report to bind to LDL/VLDL, and the protein binding in human plasma was tested by the in-house UC protocol. Both neat human plasma and diluted plasma (10%, 5%, and 2%) were performed. The obtained f_u values (for diluted plasma, undiluted f_u were extrapolated) were

compared to literature values. Results showed that for the 4 highly lipophilic ($\text{LogP} \geq 5$) compounds that bind to LDL/VLDL, nearly all fu in neat plasma were at least 2.5-fold higher than in diluted plasma. Extremely lipophilic compounds such as amiodarone showed several hundred-fold higher fu values in UC compared to reported values from other methods. For the 4 highly lipophilic compounds with no lipoprotein binding evidence, similar trend in fu were observed that two (Itraconazole and Lapatinib) of the 4 compounds showed significantly higher fu in neat plasma. Data in diluted plasma were closer to reported values to different extents for all 8 highly lipophilic compounds. Fu of all the moderate lipophilic ($\text{LogP} < 5$) compounds with or without evidence of LDL/VLDL binding were within 3-fold of literature values in neat and 10% plasma except for cyclosporine A. Nine of the 14 compounds were within 2-fold of literature values in neat and 10% diluted plasma. The present results indicate that lipophilicity could play an important role in the data accuracy in UC. Fu could be over-estimated using neat plasma for highly lipophilic compounds. Appropriate plasma dilution fold should be selected according to compound's logP and estimated binding ratios when UC method should be used for protein binding during drug discovery. In the late stages, it is recommended that a different method should be used to verify the accuracy of fu values determined by UC.

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P282 - DOES THE FLUX COMPETITION METHOD ADD SIGNIFICANT VALUE IN SPECIES DIFFERENCE PROTEIN BINDING EVALUATION?

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The competition method is frequently evaluated in drug development to compare the fraction unbound (fu) differences between two species. In the experiment, plasma spiked with compound on both sides of the membrane are dialyzed for a long time to reach equilibrium (defined as the equilibrium competition method). Kalvass et al [1] reported the flux dialysis in 2018 in which the flux dialysis-based competition method (defined as flux competition) was compared with the equilibrium competition method. According to theoretical analysis, it was reported that the flux competition method showed the benefits of allowing for the calculation of actual fu values and not requiring equilibrium to be reached. The purpose of the present study was to evaluate whether the flux competition method has significant advantages over the equilibrium competition method in competition evaluation. Ten compounds with moderate or minimal species differences in fu, as well as large (>3-fold ratio) species differences, were selected for validation. In the equilibrium competition, human plasma and plasma of a second species spiked with compounds at both sides of the equilibrium dialysis device were tested at 2 μM in for multiple timepoints up to 48 hr or 96 hr. In the flux competition method, compounds spiked plasma at one side of the device was incubated for up to 120 hr, but experiments were carried out with spiking on different sides. Equilibrium dialysis of these compounds in the plasma of both species was performed to obtain control data. Results showed that for 6 of the 10 compounds, comparable fu ratios were obtained from both competition methods, with fu ratios within 2-fold of the mean. For 4 of the 10 compounds, inconsistent data were observed between the two flux competition experiments: compound spiked human plasma and animal plasma. Besides, 3 of the fu ratios spiked human plasma were significantly larger than spiked animal plasma, as well as the data from the equilibrium competition method. It can be found from the receiver/donor ratio-time curves (R-t curves) that for the 6 compounds exhibiting consistent data, all the curves reached a plateau within the incubation time. However, for pimozide, amiodarone, and nifedipine, the R-t curves did not reach a plateau within 120 hr of incubation. For cefpiramide, both curves reached a plateau, but the obtained fu ratios showed a 2.5-fold difference. After reviewing the two parameters Req and k obtained by nonlinear curve fitting, we found that most of the P values from statistical analysis were higher than 0.05, indicating that the fitted curves were not significant. We further calculated the Rslope values using linear regression from the early times points of the R-t curve, similar fu ratios were obtained. In conclusion, the present study indicated that the flux competition method does not necessarily add more value to competition PPB evaluation due to the much longer time required to reach equilibrium compared to the equilibrium competition method. Reaching equilibrium is required to obtain high-quality data for fu estimations on both sides.

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P283 - ENHANCED QUAN/QUAL APPROACH WITH SOFTWARE ASSISTED DATA ANALYSIS FOR TIER I SOFT SPOT IDENTIFICATION IN DRUG DISCOVERY

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Understanding compound metabolism in early drug discovery is crucial for medicinal chemists aiming to design molecules with improved safety and ADME properties. The metabolic stability assay in liver microsomes or hepatocytes is commonly employed as a tier I *in vitro* assay for optimizing hepatic clearance due to its high throughput and predictive capability. Analysis typically involves robust ultra high-performance liquid chromatographic separation and accurate-mass MS detection. Recent advancements in mass spectrometric technology have enabled sensitive, fast scanning, and data-dependent acquisition of high-resolution MS/MS, facilitating combined quantitative/qualitative (quan/qual) metabolic stability assays to simultaneously determine metabolism rates (quan) and metabolic sites (qual). Here, we present a quan/qual approach for tier I soft spot identification, supported by software-assisted structure elucidation and automatic report generation. This assay typically employs a substrate concentration of 1 μ M, with samples collected at multiple time points, followed by protein precipitation. The resulting injection samples undergo analysis using a generic LC gradient in less than 4 minutes, coupled with a high-resolution mass spectrometry instrument for both MS and MS2 acquisition. Intrinsic clearance is determined using Gubbs and internal data analysis tools, while metabolite identification is automated using Mass-MetaSite batch processor to generate report of soft spot identification and relative percentages based on MS response. These data have proven valuable across multiple projects, enabling earlier medicinal chemistry decisions and enhancing the efficiency and impact of the design-make-test cycle, thereby improving the translatability of early *in vitro* metabolism data. Additionally, limitations of this approach and strategies to overcome them will be also discussed.

P284 - ANALYSIS OF GOVERNMENT REGULATION EFFECTIVENESS BASED ON PHTHALATE RISK ASSESSMENT

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Phthalate is a substance that acts as a plasticizer that makes plastic flexible, and is widely used in food appliances, food containers, cosmetics, and household goods, and can affect the human body through various channels, confirmed human exposure through integrated risk assessment. Regulations began worldwide as it became known as an endocrine disruptor. In addition to limiting the use of phthalates, the Korean government not only regulates the use of phthalates, but also classifies and manages consumers such as food packaging materials, medical devices, sanitary products, cosmetics, and electronic products. In this study, a scenario-based exposure assessment and a bio-monitoring-based assessment using bio-monitoring data collected from 2015 to 2020 (the Ministry of Environment's National Environmental Health Survey (Korea KoNEHS) were conducted simultaneously. As a results, the total exposure of the seven phthalates was 0.005 to 1.145 μ g/kg b.w./day and adults have been found to be exposed to phthalates, which are mainly migrated from food containers to processed foods, and children (under the age of 7) have been found to be exposed through children's products such as toys and house dust. In order to confirm domestic and international biomonitoring according to released amount, the '15-'17 and '18-'20 period of the Ministry of Environment's National Environmental Health Survey(KoNEHS), US NHANES ('15-'18), Canada CHMS ('16-'19), and Germany GerES ('14-'17) was used. It was confirmed that the body concentration of phthalates (DEHP, DBP, BBP, and DINP) all decreased in the entire age of Korean, and the value of the group corresponding to the 95th also decreased. Compared with the bio-monitoring data of American, Canadian and German, DEHP was somewhat higher, and polymer phthalates such as DINP and DIDP were higher in the foreign population group. Different exposure pattern of each Phthalate depends on the banned substances and regulatory standards of each country.

P285 - REGULATION OF GLYCOLYSIS IN CD8+ T CELLS DETERMINES THE SUSCEPTIBILITY TO HLA-MEDIATED ABACAVIR-INDUCED SKIN TOXICITY

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Purpose: Idiosyncratic drug-induced autoimmune toxicity (IDT) associated with specific human leukocyte antigen (HLA) allotype does not occur in all subjects of a given HLA model population, suggesting the difficulties to predict the IDT risk without considering additional factors. We have generated a unique transgenic mouse line carrying HLA-B*57:01 (B*57:01-Tg) and successfully reproduced abacavir (ABC)-induced skin toxicity in B*57:01-Tg mice. On the other hand, we recently conducted serum metabolomics analysis and revealed that the glycolysis was the most significantly upregulated pathway in ABC-treated B*57:01-Tg mice. Here, we examined whether intracellular glycolysis level change in CD8+ T cells contributes to the susceptibility to ABC-induced skin toxicity in the B*57:01-Tg mouse model.

Results and Discussion: Our RNA-sequencing (RNA-seq) and proteomic data analysis using isolated CD8+ T cells

demonstrated that the expression levels of several glycolytic enzymes including hexokinase (HK) 2 were significantly upregulated in ABC-treated B*57:01-Tg mice, compared with that in the vehicle-treated group. In line with this observation, glycolytic rate in CD8⁺ T cells was also significantly increased by ABC treatment in B*57:01-Tg mice, resulting in increase of pyruvate production. Treatment of a HK inhibitor, 2-Deoxy-D-glucose, attenuated an increase of IFN- γ -secreting CD44^{high}CD62L^{low}CD8⁺ T cells (effector CD8⁺ T cells) in B*57:01-Tg mice, thereby abrogating plasma thymus and activation-regulated chemokine (TARC) level elevation and CD8⁺ T cell dermal infiltration. Under calorie restricted condition by diet change, ABC treatment also failed to activate CD8⁺ T cells and induce ABC-induced skin toxicity in B*57:01-Tg mice, where intracellular glycolysis rate and pyruvate production of CD8⁺ T cells were suppressed. These results suggested that glycolysis in CD8⁺ T cells might play a major role in the onset of ABC-induced IDT in B*57:01-Tg mice.

Conclusion: Individual differences in the energy metabolism status may affect the susceptibility to HLA-mediated IDT in humans.

P286 - OMICS DATA-BASED ANALYSIS OF MITOCHONDRIAL CHARACTERISTICS AND TOXICITY MECHANISMS

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Mitochondrial toxicity is a major mechanism underlying drug-induced liver injury (DILI). Mitochondrial permeability transition (mPT) is defined as an increase in inner membrane permeability, resulting in mitochondrial swelling and the leakage of various inflammatory molecules from mitochondria into the cytosol. Previously, using liver mitochondria isolated from db/db mouse model of non-alcoholic fatty liver disease (NAFLD), now renamed and classified as metabolic dysfunction-associated steatotic liver disease (MASLD), I demonstrated an increased susceptibility to mPT induced by troglitazone, a drug pulled from the market due to DILI. Furthermore, employing data-independent acquisition mass spectrometry, I showed alterations in the proteome of liver mitochondria derived from MASLD mice. This study aimed to gain further insights into toxic molecular mechanisms in MASLD mitochondria. First, I investigated whether the susceptibility of mPT changes in other hepatotoxic drugs. Next, alterations in mitochondrial characteristics and toxic mechanisms were explored through bioinformatics analysis of proteomic data from MASLD mitochondria. No enhanced susceptibility to mPT induced by diclofenac and ticlopidine, known to exhibit mitochondrial toxicity, or by pioglitazone, a drug with low DILI risk, was observed. An increase in the pathway of mitochondrial-derived reactive oxygen species generation, a known mPT inducer, along with various proteins regulating mPT pore opening, and upregulation of mitochondrial phospholipase PNPLA8/iPLA2 γ expression, reported as an off-target mechanism of troglitazone-mediated mitochondrial toxicity, were also identified. On the other hand, KEGG pathway analysis revealed an upregulation of cardiolipin biosynthesis, suggesting resistance to bile acids due to increased cardiolipin content. Subsequent analysis using deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA), both with mitochondrial toxicity, showed a decrease in mPT susceptibility in MASLD mitochondria, as predicted. mPT induction by both DCA and CDCA was minimal in the absence of Ca²⁺. The susceptibility to mPT induction by cholic acid, which is less hepatotoxic, did not show obvious changes in MASLD mitochondria. Finally, enrichment of gene ontology biological process terms like 'response to oxidative stress' and 'mitochondrial organization' was observed. In summary, alterations in the proteome of MASLD mitochondria cause differences in response to mitochondrial toxic compounds. Bioinformatics analysis using MASLD mitochondrial proteome data could be a valuable tool in predicting mechanisms of mitochondrial toxicity.

P287 - MATERNAL EXPOSURE TO 2,3,7,8-TETRACHLORODIBENZO-p-DIOXIN CAUSES EPIGENETIC EFFECTS: POSSIBLE MECHANISM BASED ON WHOLE-GENOME METHYLATION ANALYSIS

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Dioxins, particularly 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), are environmental pollutants known to cause developmental toxicity. Our preliminary studies suggested that TCDD reduces the content of α -lipoic acid and S-adenosylmethionine (SAM) in the male fetal hypothalamus, resulting in reduced adenosine triphosphate (ATP) levels. SAM is necessary for the biosynthesis of α -lipoic acid. Moreover, DNA methyltransferase 1 (DNMT1) expression was induced in the male fetal hypothalamus following exposure to maternal TCDD. We hypothesized that these effects can be ameliorated by maternal α -lipoic acid supplementation [1]. Therefore, our study aimed to examine the effect of maternal exposure to TCDD on the methylation levels and relevant transporters involved in transporting vitamin B12 and folic acid in the placenta. Female rats were exposed to 1 μ g/kg oral TCDD on gestational day (GD) 15. Fetal hypothalamus, liver, and serum were analyzed on GD 20 and α -lipoic acid was maternally supplemented on GD 15–20. Furthermore, male hypothalamic methylation levels were analyzed using whole-genome bisulfite sequencing (WGBS). The results showed that methylation levels of enzymes related to nicotinamide adenine dinucleotide (NAD) synthesis in the male hypothalamus were altered after maternal TCDD exposure. Moreover, reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that TCDD significantly reduced nicotinamide phosphoribosyl transferase (Nampt) and

nicotinamide mononucleotide adenyl transferase 3 (Nmnat3) mRNA levels. Following maternal α -lipoic acid supplementation, this suppression was significantly reversed. Furthermore, RT-PCR analysis showed that TCDD significantly reduced multidrug resistance-associated protein 1 (Mrp1) mRNA levels; this protein transports vitamin B12 into the placenta. Interestingly, maternal α -lipoic acid supplementation significantly increased Solute carrier family 46 member 1 (Slc46a1) mRNA levels; this protein is responsible for transporting folic acid. The present study showed that TCDD changes the pattern of whole-genome DNA methylation and these effects can be improved by the compensatory reaction of maternal α -lipoic acid supplementation. The observed disruption in NAD synthesis can be attributed to reduced ATP levels. Moreover, the α -lipoic acid concentration in the maternal serum did not change; however, the mRNA levels of the transporters for vitamin B12 and folic acid in the placenta significantly decreased or tended to be decreased, respectively. Therefore, the decreased transport of nutrients from the mothers to the fetuses may cause decreased α -lipoic acid and ATP levels in the fetal hypothalamus.

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P288 - PHYSICOCHEMICAL CHARACTERIZATION OF PARTICULATE MATTER FROM A COAL-FIRED POWER PLANT AND THE TOXICOLOGICAL PROFILE IN BALB/C MICE

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Coal-fired power plants are a major source of electricity throughout the world, but their use raises serious concerns regarding air pollution. We determined the physicochemical characteristics and toxicological profile of fly ash particulate matter (PM) obtained from the electrostatic precipitator of Mae Moh Coal-fired Power Plant (Lampang, Thailand) in mice. We determined the average PM size by using electron microscopy. We evaluated two major types of toxins in PM, heavy metals and polycyclic aromatic hydrocarbon, with inductively coupled plasma mass spectrometry and gas chromatography–mass spectrometry, respectively. We evaluated the inhalation toxicity of fly ash PM by subjecting mice to intratracheal instillation of 1–10 mg/kg/day for 7 consecutive days. After exposure, we performed histopathological examination and determined proteomic changes in the lungs. We compared toxicological effects of fly ash PM with a negative control (vehicle) and a positive control (standard PM 2.5, NIST, USA). The average size of the fly ash PM was $4.29 \pm 1.85 \mu\text{m}$ as determined by electron microscopy. The major heavy metal was iron ($101,067.31 \pm 8,246.12 \text{ mg/kg}$). Acenaphthene was the most abundant polycyclic aromatic hydrocarbon ($40.77 \pm 0.003 \text{ mg/kg}$). Fly ash PM and the standard PM significantly induced pulmonary inflammation in the lungs compared with the vehicle control. In addition, proteomics analysis of the lungs revealed three relevant inflammation-associated pathways: leukocyte transendothelial migration, phagosome, and neutrophil extracellular trap formation. This study is the first to report on the physicochemical characterization, histopathology, and proteomics-associated toxicological profiles of fly ash PM obtained from the largest coal-fired power plant in Thailand. The information obtained from this study should be useful for academic researchers, electricity enterprises, and policymakers to develop appropriate strategies to manage air pollution from coal-fired power plants throughout the world.

P289 - CHANGE IN TRANSPORT FUNCTION OF DRUGS INTO THE MULTICELLULAR INNER BLOOD-RETINAL BARRIER SPHEROIDS UNDER HYPOXIC CULTURE WITH ADVANCED GLYCATION END-PRODUCTS

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Pathophysiology of retinal disorder in diabetes are reported to involve disruption of the inner blood-retinal barrier (BRB). The inner BRB is formed by retinal capillary endothelial cells with being supported by retinal pericytes and Müller cells and regulates compound/drug transport between the circulating blood and retina by tight-junctions and membrane transporters (1). To elucidate the details of altering the tight-junctions and transporters in the inner BRB under diabetic conditions, such as physiological over-production of advanced glycation end-products (AGEs) and hypoxia, the *in vitro* tools are considered to be helpful. In this study, we evaluated function of compound/drug transport in the multicellular inner BRB spheroids, which was newly established previously (2), under diabetes-mimicked culture conditions. By co-incubation of bovine serum albumin (BSA) with D-(-)-ribose for a week, AGE-BSA was prepared (3). After 21-hour treatment with 200 $\mu\text{g/mL}$ AGE-BSA in the conventional culture, the inner BRB spheroids were additionally cultured for 3 hours under hypoxic

conditions in the presence of 200 µg/mL AGE-BSA. By this diabetic conditions, accumulation of FITC-dextran, a marker compound for paracellular transport³, into the spheroids was significantly increased to 1.5-fold compared to the regular culture conditions (control). In addition, mRNA expression of Zonula occludens-1, a tight junction-related molecule, in the spheroids cultured under the diabetic condition was significantly decreased by 25% compared with control, suggesting leakage of tight-junctions on the spheroids by this culture method. In the previous annual meeting of JSSX, we showed the functional attenuation of P-glycoprotein (P-gp) in the retinal capillaries prepared from streptozotocin-induced diabetic rats although expression of *mdr1a*, encoding P-gp, in the capillaries was significantly increased (#3P-165). Under the *in vitro* diabetic culture conditions, uptake of rhodamine 123, a fluorescent substrate of P-gp, by the inner BRB spheroids was significantly promoted compared to that under control conditions, indicating attenuation of P-gp function in the spheroids. Regarding *mdr1a* expression, mRNA level of *mdr1a* was significantly increased in the inner BRB spheroid cultured under the diabetic conditions. Since the expressional and functional profiles of P-gp in the inner BRB spheroids under diabetic conditions were similar to those in the retinal capillaries of streptozotocin-treated rats, it is implied that the culture conditions in this study help us to understand the regulatory mechanisms of transporters including P-gp at the inner BRB in *in vivo* streptozotocin-induced diabetes.

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P290 - DETERMINATION OF STEADY STATE BRAIN K_{p,uu} OF BIDAC(TM) DEGRADERS USING CHEMICAL KNOCK OUT ANIMAL MODELS.

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Targeted protein degradation [1] has emerged as a novel therapeutic modality with the potential to expand the range of druggable protein targets compared with conventional inhibitors including targets residing in the CNS [2]. Heterobifunctional or BiDAC degraders (typically MW 700-1000 Da) fall outside of generally accepted small molecule (MW~500 Da) chemical space. One challenge with such molecules is their permeability across biological membranes, compounded by efflux transporter effect at the gut and tissue level which specifically impacts oral bioavailability and exposure in the CNS compartment. Thus, an appropriate assessment of permeability with respect to oral and CNS exposure would aid in the design of protein degraders that achieve therapeutically relevant levels *in vivo*. While oral bioavailability data for BiDAC degraders is provided by early routine rodent PK studies, collecting *in vivo* blood brain barrier (BBB) permeability data needs special considerations. *in vivo* BBB permeability data can be obtained utilizing basic rodent *in vivo* models designed to measure brain-to-plasma ratio (K_{p,brain}), which reflects the extent of brain penetration approaching a steady state (SS) in the presence of efflux transporter at the BBB. An estimate of the extent of free drug concentration in brain (K_{p,uu,brain}) can then be performed by applying corrections for brain and plasma free fractions [3]. Such data alongside physicochemical property assessment of a BiDAC degrader can then be utilized to drive iterative SAR generation and optimization in the discovery stage. In advanced drug discovery stages, more sophisticated translational techniques predict K_{p,uu,brain} at SS. Such data can be incorporated in models to increase the accuracy of human pharmacokinetics and dose projections. These techniques include efflux transporter knock-out (KO) models including chemical KO in rat and mice [4,5]. The current work presents BiDAC degrader BBB permeability data from chemical KO experiments in rats and mice. In mouse, the chemical KO of efflux transporter at BBB was achieved by co-dosing, where elacridar, a pan efflux inhibitor, was administered by oral gavage. After 2h, Degradar-A, Degradar-B, loperamide (MDR1 substrate) and imatinib (BCRP substrate) were administered subcutaneously in a cassette dosing format. Mouse plasma and brain were collected at different time-points and concentrations of each analyte were determined by LC-MS/MS. For each condition, with and without inhibitor, the AUC (area under the concentration curve) was determined for plasma and brain. In rats, the chemical KO of efflux transporters at the BBB was achieved by a dual infusion approach [5]. In this approach, an IV (intravenous) infusion of elacridar was performed for 30min. This was immediately followed by a 4h infusion of Degradar-A, Degradar-B, loperamide and imatinib in a cassette dosing format. The plasma and brain samples were collected immediately at the end of infusion period. The plasma and brain concentrations of each analyte were determined by LC-MS/MS. The K_{p,uu,brain} at SS, indicated that the BiDAC degraders were brain permeable and demonstrated the extent of distributional impairment of these compounds in brain due to efflux transporters expressed at the BBB in rodent species.

P291 - ARE Δ^9 -TETRAHYDROCANNABINOL AND ITS MAJOR METABOLITES SUBSTRATES OR INHIBITORS OF THE HUMAN HEPATIC AND PLACENTAL SOLUTE-CARRIER TRANSPORTERS?**Xin Chen**¹, Zsuzsanna Gáborik², Qingcheng Mao¹, and Jashvant Unadkat¹¹University of Washington, Seattle, WA, United States, ²SOLVO Biotechnology, Budapest, Hungary

Δ^9 -Tetrahydrocannabinol (THC), the primary psychoactive component of cannabis, is used worldwide, including during pregnancy. Therefore, the potential for transporter- or metabolism-based drug interactions with THC needs to be considered. We have previously shown that THC is sequentially metabolized to its circulating metabolites 11-hydroxy-THC (11-OH-THC, psychoactive) and 11-nor- Δ^9 -carboxy-THC (THC-COOH, non-psychoactive), primarily by CYP2C9 and CYP3A4, respectively. We have also shown through animal, perfused human placenta as well as *in vivo* studies in pregnant women consuming cannabis that fetal exposure to THC is lower than the corresponding maternal exposure. Collectively, these data, as well as our *in vitro* studies with overexpressing cells, show that THC is effluxed by a placental transporter(s) other than P-glycoprotein (P-gp) or Breast Cancer Resistance protein (BCRP). The identity of this transporter as well as whether THC is transported by hepatic and placental Solute Carrier (SLC) transporters is unknown. Therefore, in this study, we investigated whether THC, 11-OH-THC and THC-COOH are substrates and/or inhibitors of hepatic or placental SLC transporters at their pharmacological-relevant concentrations. Using HEK293 cells overexpressing human OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OATP2B1 (SLCO2B1), OCT1 (SLC22A1), OCT3 (SLC22A3), OAT2 (SLC22A7), OAT4 (SLC22A11), or NTCP (SLC10A1), we evaluated the uptake of 5 μ M THC, 0.3 μ M 11-OH-THC, and 2.5 μ M THC-COOH in the absence or presence of a selective inhibitor of the respective transporter (for NTCP, in the absence of Na⁺), as well as their potential inhibitory effects on the cellular uptake of radio-labeled probe substrates of these transporters. Three to four independent experiments were conducted (each in triplicate). The uptake of cannabinoids was measured by LC-MS/MS and the uptake of the radio-labeled probe substrates was measured by liquid scintillation counting. Data (with and without the inhibitor or the cannabinoids) were compared using the paired t-test. OATPs or OATs did not transport any of the cannabinoids. In contrast, OCT1 transported THC and THC-COOH as evidenced by their significantly lower cellular uptake (49% and 62% of the control, respectively) in the presence of the OCT1 inhibitor, 100 μ M quinidine. THC-COOH uptake by OCT3 cells was modestly but significantly inhibited (17%) by the OCT3 inhibitor, 100 μ M corticosterone. Interestingly, in the absence of sodium, NTCP uptake of THC and 11-OH-THC was significantly stimulated (200% and 186% of the control, respectively). However, none of the cannabinoids, at their pharmacologically relevant concentrations, significantly inhibited the uptake of the prototypic substrates of the investigated transporters. Intriguingly, 11-OH-THC and THC-COOH significantly stimulated OATP2B1 uptake of 3H-estrone-3-sulfate (143% and 119% of the control, respectively). The placental transporter effluxing THC remains unknown and is not OCT3, OATP2B1 or OAT4, all located on the basal membrane of the placental syncytiotrophoblasts. These data contribute to our understanding of THC disposition and suggest potential drug-THC interactions with respect to OCT1. This work was supported by the National Institute on Drug Abuse [Grant P01DA032507]. We thank Gilead Sciences, Inc. (OAT1B1/1B3), Pfizer Inc. (OAT2) and SOLVO Biotechnology (remaining transporters) for generously sharing their cell lines.

P292 - EFFECT OF LIPOPOLYSACCHARIDE ON P-GLYCOPROTEIN AT THE INNER BLOOD-RETINAL BARRIER *IN VIVO***Kiyotaka Daikohara**, Shin-ichi Akanuma, Yuma Tega, and Ken-ichi Hosoya

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In diabetic patients, the serum concentration of lipopolysaccharide (LPS), an inflammatory agent, has been reported to be increased compared with that in non-diabetic patients [1]. Since it has been indicated that the elevation of systemic LPS triggers the onset of diabetic retinopathy (DR), which is a diabetic complication and a serious retinal disease [2], mechanisms of LPS-related DR pathogenesis should be understood. P-glycoprotein (P-gp, *mdr1a/1b*), an efflux transporter, has been reported to accept endogenous compounds which are involved in DR pathogenesis [3,4]. Through *ex vivo* analyses using freshly-prepared retinal capillaries, we demonstrated that LPS treatment reduces the P-gp-mediated efflux transport from the retina across the inner blood-retinal barrier (inner BRB) [5]. Thus, it is hypothesized that the retinal concentration of P-gp substrates related to DR onset is elevated because of the decrease in P-gp activity at the inner BRB by LPS, resulting in the pathogenesis of DR. To elucidate the *in vivo* importance of this attenuation of P-gp-mediated efflux transport at the inner BRB by LPS, activities of P-gp in the retinal capillaries of rats which received 5 mg/kg LPS intraperitoneally were examined. Luminal accumulation of NBD-CSA, a fluorescent P-gp substrate [6], in the retinal capillaries of LPS-treated rats was significantly reduced by 45% compared with that of saline-treated rats, suggesting that P-gp activity at the inner BRB in LPS-treated rats is attenuated. To test the effect of this attenuation on the retinal drug distribution from the circulating blood across the BRB, *in vivo* transport study using [3H]digoxin [7], a typical P-gp substrate, was performed. *in vivo* retinal uptake of [3H]digoxin in LPS-treated rats showed significant 1.31-fold increase compared to that in saline-treated rats. In addition, mRNA expression level of *mdr1a*, which encodes P-gp, was significantly decreased by 71% in the retinal capillaries of LPS-treated rats. Consequently, these results indicate that LPS causes the reduction of P-gp activity and expression at the inner BRB *in vivo*. It is expected that our findings lead to the understanding for mechanisms involved in LPS-induced DR pathogenesis.

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P293 - INTEGRATED TRANSPORTER ELUCIDATION CENTER: IDENTIFYING THE SLC AND ABC PROTEOME IN HUMAN PLACENTA

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Objectives: A key challenge confronting the transporter field is the lack of a complete proteomic profile of the SLC and ABC transporters enriched in the placenta. One goal of the Integrated Transporter Elucidation Center is to comprehensively define the SLC and ABC proteome in healthy and diseased human placentas using quantitative targeted proteomics (QTAP). In this study, we prioritized transport proteins of interest in human placental cell lines and healthy placental tissue and identified proteotypic peptides of these target proteins. **Methods:** mRNA data from placenta trophoblasts were used to guide the selection of SLC and ABC proteins for QTAP analysis using microLC-MS/MS. Transport proteins (n=22) with high mRNA expression (> 4 Fragments Per Kilobase of transcript per Million mapped reads [FPKM], placentome database) were selected if they were not already included in our QTAP panels. First, R code was designed to accelerate proteotypic analysis by identifying unique and missed cleavage-free peptides. Second, a placental tissue sample and three human placental cell lines (BeWo, JAR, and HTR-8/SVneo) were examined for the presence of these identified unique peptides using an M-Class Acquity LC coupled to a SCIEX Triple Quadrupole 7500. Human cells were seeded at 1x10⁶ and cultured for 48 hours. Membrane proteins were extracted from each sample by differential detergent fractionation(1), and extracts were analyzed by (label free) QTAP following trypsin digestion. Three MRMs per peptide were used for response assessment with up to two additional MRMs being used for confirmation. **Results:** R coding eliminated missed cleavages, peptides that were too hydrophilic or too hydrophobic (for chromatography purposes), and non-unique peptides. This approach reduced the large number of candidate peptides (>600) for the 22 selected proteins to ~220 peptides. As examples, OAT4 and OATP4A1 were detected in digested placenta membranes but not membranes from the three cell types. OAT4 (peptide: SVFTSTIVAK) combined peak areas for three MRMs (+2y8, +2y7, +2y6) in the placenta digest was 0.65x10⁶. Four MRMs for OAT4.GCFGISLTCLTIYK could also be detected. OATP4A1 (peptide: YEVELDAGVR) combined peak areas for three MRMs was 0.66x10⁶. Membrane marker Na⁺/K⁺ ATPase (peptide: VDNSSLTGESEPQTR) combined peak areas for two MRMs, which are consistently used in our laboratory as endogenous controls, were 1.44x10⁶ for the placenta digest, 3.64x10⁶ for the BeWo digest, 5.05x10⁶ for the JAR digest, and 1.81x10⁶ for the HTR-8/SVneo digest. In accordance with high mRNA expression, peptides were detected in placenta digest for other proteins including LAT4. **Conclusion:** These data support a novel approach to streamline the workflow for quantifying SLC and ABC transport proteins, and are useful in extrapolating *in vitro* mechanistic studies performed in placental cell lines to human pregnancies.

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P294 - IMPLICATION OF H⁺/PEPTIDE TRANSPORTERS (PEPTS) ON UPTAKE OF N-LACTOYLPHENYLALANINE (LAC-PHE) IN THE INTESTINAL AND RENAL EPITHELIAL CELLS

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[Objectives] Recently, it has been reported that N-lactoylphenylalanine (Lac-phe), a biomarker that suppresses feeding and obesity, is induced by exercise¹. Lac-Phe has been shown to be significantly induced in the blood by exercise, but its physiological role is unknown. Since Lac-Phe exhibits a pseudodipeptide structure, it is possible that a peptide transporter is involved in the pharmacokinetics of Lac-Phe. In this study, we investigated whether Lac-Phe is a substrate for PEPTs using the intestinal epithelial cell line Caco-2 cells expressing PEPT1, and the renal proximal epithelial cell line SKPT cells expressing PEPT2. Methods: Caco-2 cells and SKPT cells were grown in DMEM and DMEM/F12, respectively, containing 10% FBS, 1% nonessential amino acid and antibiotics. Lac-Phe was synthesized in house. Uptake of Lac-Phe was determined at 37°C as described previously². The amounts of Lac-Phe in the cells were determined by LC-MS/MS. Results and Discussion: First, we confirmed that PEPT1 and PEPT2 are functionally expressed in Caco-2 and SKPT cells, respectively, by using typical substrates cefadroxil and Glycylsarcosine (Gly-Sar). These compounds were pH-dependently taken up by Caco-2 and SKPT cells. The uptake of Lac-Phe was also pH-dependent, and Lac-Phe inhibited the uptake of Gly-Sar in its concentration manner in both cells. These results indicated that Lac-Phe might be a substrate for PEPTs. Very recently, orally administered metformin increases the blood levels of Lac-Phe in patients with type 2 diabetes³. Our preliminary experiment using Caco-2 cells cultured on Transwell revealed that metformin applied in the apical side increased the concentration of Lac-Phe in basal side. In this meeting, we are going to report on this result.

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P295 - MATE ARE BOUND TO A MULTIPROTEIN COMPLEX CONTAINING NHE3, AND IT'S CAUSING FUNCTIONAL INTERACTIONS

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Multidrug and toxin extrusion 1 (MATE1) is an H⁺-driven organic cation/H⁺ exchange transporter. It is expressed in the renal proximal tubular brush border membrane and is thought to be responsible for the final step of organic cationic drug excretion via counter transport with H⁺. Similarly, Na⁺/H⁺ exchanger 3 (NHE3) is expressed in the proximal tubular cells. It is possible that MATE1 and NHE3 are coexpressed in the proximal tubules of the kidney and form a membrane transporter complex with which they functionally interact; however, the details of this interaction have not been clarified. In this study, we examined whether MATE1 forms membrane transport complexes and functionally interacts with NHE3 in mouse kidney and MATE1/NHE3 co-expressing cells. Kidneys from 10-week-old male C57BL/6 mice were harvested and used for experiments. Plasmids containing either hNHE3 or hMATE1 were used to generate MATE1/NHE3 stable co-expressing cells. Immunohistological methods were used to examine the localization of MATE1 and NHE3 in mouse kidney and MATE1/NHE3 co-expressing cells. The membrane fractions of mouse kidney and MATE1/NHE3 co-expressing cells were extracted and subjected to western blotting and immunoprecipitation. The transport activity was measured under various conditions using MATE substrates. Western blotting confirmed MATE1 and NHE3 expression in the plasma membrane fractions of mouse kidney and MATE1/NHE3 coexpressing cells. Immunohistochemical staining confirmed the colocalization of MATE and NHE3 on the kidney brush border membrane. Furthermore, immunoprecipitation revealed a possible interaction between MATE1 and NHE3 in mouse kidney and MATE1/NHE3 co-expressing cells. Transport activity assays indicated that NHE3 activation may affect the transport activity of MATE1 in MATE1/NHE3 co-expressing cells. These results suggest that MATE1 and NHE3 form a complex and interact in mouse kidney tissues. In addition, MATE1 and NHE3 may functionally interact in MATE1/NHE3 co-expressing cells. Elucidation of the regulatory mechanism of membrane transporter complexes containing MATE1 is expected to have clinical applications, such as avoiding the risk of side effects of drugs that are substrates of MATE1 and predicting changes in the blood concentrations of target drugs when used in combination.

P296 - COPROPORPHYRIN I AS AN *IN VITRO* FLUORESCENT PROBE FOR MEASURING OATP1B1 ACTIVITY**Elizabeth Hayden** and Jason Sprowl*University at Buffalo, Buffalo, NY, United States*

The hepatic uptake transporter, organic anion transporting polypeptide 1B1 (OATP1B1), plays an integral role in mediating drug clearance. Recent investigations have highlighted the utility of coproporphyrin I (CPI) plasma and urine concentrations as a clinical biomarker of OATP1B1 activity, however use of CPI to measure OATP1B1 activity *in vitro* is limited. Considering its fluorescent properties [1], we hypothesized that measurement of CPI fluorescence can be used to measure OATP1B1 transport activity *in vitro* and identify inhibitors with potential to contribute to dangerous drug-drug interactions. To address this hypothesis, we measured cellular accumulation of CPI (0 – 25 μM) in HEK293 cells or OATP1B1 overexpressing HEK293 cells grown in 96-well plates via fluorescence with excitation and emission wavelengths of 390nm and 615nm, respectively. Fluorescence of CPI was measured in six samples per condition and normalized to protein concentration. Each experiment was replicated at least three times. We found that 6.22-fold increased CPI fluorescence was detected in OATP1B1 overexpressing HEK293 cells, compared to cells in the absence of the transporter ($p < 0.001$; determined using an unpaired student t-test) and that the reaction velocity (V_{max}) and substrate affinity (K_m) of CPI for OATP1B1 in this model was 1.962 μM and 0.0575 pmol/mg/min, respectively. To further explore the utility of this model in identifying OATP1B1 inhibitors, we assessed the potential of several tyrosine kinase inhibitors (TKIs) to inhibit transport activity by exposing cells to individual TKIs (10 μM) for 15-minutes, followed by co-incubation of the TKI with 10 μM CPI for 30 minutes and measuring fluorescence as described above. Data was analyzed using a One-way ANOVA and Tukey's post-test using Graphpad Prism. Data revealed that capmatinib, tepotinib, tucatinib, tivozanib, and nilotinib were potent OATP1B1 inhibitors ($p < 0.0001$) and the concentration of nilotinib required to inhibit maximum velocity by 50% is 0.9875 μM . Collectively, this work is the first to show that fluorescent measurement of coproporphyrin I is a sensitive and specific method to assess OATP1B1-mediated transport activity *in vitro*, and can be used to investigate potential candidates for drug-drug interactions. Additionally, this assay was able to validate nilotinib inhibitory properties that were previously reported [2].

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P297 - PHARMACOKINETIC INTERACTIONS OF NICLOSAMIDE IN RATS: INVOLVEMENT OF ORGANIC ANION TRANSPORTERS 1 AND 3 AND ORGANIC CATION TRANSPORTER 2**MinJu Kim**¹, Min-Ji Kang², Yoon-Jee Chae², and Kyeong-Ryoon Lee¹¹Korea Research Institute of Bioscience and Biotechnology, Cheongju-si, Chungcheongbuk-do, South Korea, ²Woosuk University, Wanju, Jeonbuk Stage, South Korea

Niclosamide, a well-established anthelmintic drug, is generally safe and well-tolerated in humans. Despite its conventional dosage resulting in low but detectable systemic levels, potential drug interactions with other concurrent medications should be considered. This study investigates the interaction between niclosamide and various drug transporters due to limited existing information. Niclosamide was found to inhibit the transport activities of OATP1B1, OATP1B3, OAT1, OAT3, and OCT2 *in vitro*, with strong inhibitory effects on OAT1, OAT3, and OCT2, evidenced by IC₅₀ values below 1 μM . In rats, the coadministration of 3 mg/kg of niclosamide led to increased systemic exposure to furosemide (an OAT1/3 substrate) and metformin (an OCT2 substrate), alongside a significant reduction in the renal clearance (CL_r) of these drugs. These findings indicate that niclosamide inhibits the renal transporters OAT1/3 and OCT2 both *in vitro* and *in vivo*, thereby increasing the systemic exposure to their substrates by significantly obstructing the urinary elimination pathway in rats. This study enhances the understanding of niclosamide's transporter-mediated drug interactions, promoting its effective and safe usage.

P298 - CARRIER-MEDIATED UPTAKE OF CAPRIC ACID IN HEPG2 CELLS**Takuma Kitamura**, Tomoya Yasujima, Takahiro Yamashiro, and Hiroaki Yuasa*Nagoya City University, Nagoya, Aichi, Japan*

Purpose: Orally ingested medium-chain fatty acids are known to be absorbed from the small intestine and, thereafter, taken up into hepatocytes in part before reaching the systemic circulation. Although the hepatic uptake process has been suggested to be facilitated by the involvement of carrier-mediated transport, the mechanism has little been clarified. Therefore, we examined the uptake of capric acid, one of medium-chain fatty acids, in the HepG2 cell, a human hepatocyte model, to help elucidate the carrier-mediated mechanism and explore its potential involvement in drug

disposition and also utilization for drug delivery.

Methods: The initial uptake of [^3H]capric acid was evaluated for 40 min at pH 7.4 and 37°C, unless otherwise indicated, in HepG2 cells.

Results and Discussion: The uptake of capric acid (3 nM) by HepG2 cells was highly dependent on temperature and Na⁺, and also saturable, suggesting the involvement of Na⁺-dependent carrier-mediated transport. Kinetic analysis indicated a maximum transport rate of 30.2 pmol/min/mg protein and a Michaelis constant of 8.86 μM for the carrier-mediated transport component, and a clearance of 0.73 $\mu\text{L}/\text{min}/\text{mg}$ protein for the accompanying, minor nonsaturable transport component. In experiments to examine the effect of saturated fatty acids with a range of carbon chain lengths of 2 to 20 carbon atoms at the concentration of 100 μM , capric acid uptake was inhibited by only those with 8 to 14 carbon atoms, suggesting that only those with the limited range of carbon chain lengths, which mostly belong to medium-chain fatty acids, may be specifically recognized by the carrier-mediated transport system. We also examined the effect of biotin, a substrate of sodium-dependent multivitamin transporter (SMVT/SLC5A6), which operates in a Na⁺-dependent manner for the monocarboxylic acid type of vitamins like biotin. However, capric acid uptake was not inhibited by biotin (1 mM), suggesting that SMVT is not responsible for the transport system. Thus, the present study indicated the involvement of a Na⁺-dependent carrier-mediated transport system in capric acid uptake in HepG2 cells. This transport system may represent the one for the hepatic uptake of medium-chain fatty acids, although the molecular mechanism remains to be clarified.

P299 - INVOLVEMENT OF CARRIER-MEDIATED TRANSPORT IN RIBAVIRIN TRANSPORT AT THE BLOOD-TESTIS BARRIER

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Ribavirin is used as an antiretroviral agent targeting the hepatitis C virus, and its male reproductive toxicity, including an alteration in the motility and the morphology of sperms, has been reported. This suggests the influx transport of ribavirin from the circulating blood to the seminiferous tubules of the testes, and it is assumed to be regulated by blood-testis barrier (BTB), where Sertoli cells act to be responsible. In the present study, the mechanism of ribavirin transport at the BTB was investigated by means of *in vivo* analysis in mice and *in vitro* analysis with a mouse-derived Sertoli cell line (TM4 cells). *in vivo* integration plot analysis in mice revealed that the net influx clearance of [^3H]ribavirin in the testis (31.6 $\mu\text{L}/(\text{min}\cdot\text{g testis})$), that was greater than that of [^{14}C]D-mannitol (8.79 $\mu\text{L}/(\text{min}\cdot\text{g testis})$) used as a non-permeable paracellular marker^{1,2}, suggesting the facilitative influx transport of ribavirin from the circulating blood to the testis. *in vitro* transport study with TM4 cells, the uptake of [^3H]ribavirin by TM4 cells showed a time-dependent increase at 37°C for at least 30 min. The uptake of ribavirin by TM4 cells was in a concentration-dependent manner, with a K_m , V_{max} , and K_d of 2.49 mM, 1.24 nmol/(min·mg protein) and 0.155 $\mu\text{L}/(\text{min}\cdot\text{mg protein})$, respectively, suggesting the carrier-mediated transport of ribavirin at the BTB. The uptake was significantly reduced in the presence of adenosine, inosine, thymidine, guanosine and adenine while it was not affected by uridine, cytidine, hypoxanthine, choline and MPP⁺. In addition, [^3H]ribavirin uptake by TM4 cells was significantly reduced in the presence of 10 μM dipyrindamole and 100 μM S-[(4-nitrophenyl)methyl]-6-thioinosine (NBMPR), while the uptake was not changed by 100 nM NBMPR, implying the involvement of equilibrative nucleoside transporter 2 (ENT2/SLC29A2) in the ribavirin transport at the BTB. The uptake of [^3H]ribavirin by mouse ENT2-expressing oocytes was in a time- and concentration-dependent manner with K_m and V_{max} values of 1.41 mM and 2.03 nmol/(h·oocyte), respectively, and was significantly reduced in the presence of 100 μM NBMPR, but not 100 nM NBMPR, supporting that ribavirin is recognized by mouse ENT2. Furthermore, TM4 cells transfected with mouse ENT2-specific siRNA revealed a significant reduction of [^3H]ribavirin uptake by 13%, and these results suggest the partial contribution of ENT2 to the influx transport of ribavirin across the BTB.

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P300 - CONSTRUCTION AND FUNCTIONAL OF HUMAN BBB MODEL DERIVED FROM MDR1 GENE-TRANSFECTED HUMAN iPS CELLS IN TWO- AND THREE-DIMENSIONAL CULTURE PLATE

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Background and Purpose: Establishment of an *in vitro* model of the human blood-brain barrier (BBB) would substantially benefit the development of central nervous system (CNS) therapeutics. Brain microvascular endothelial-like cells derived

from human iPS cells (hiPS-BMECs) could be used to establish *in vitro* human BBB models with high trans-epithelial/endothelial electrical resistance (TEER) values. However, the expression of multidrug resistance protein 1 (MDR1/P-gp/ABCB1) in hiPS-BMECs is insufficient [1], and we differentiated MDR1 gene-transfected human iPS cells into MDR1-expressing hiPS-BMECs (MDR1-BMECs). The present study was conducted to evaluate expression and function of MDR1 and other major transporters in MDR1-BMECs constructed in two- and three-dimensional (D) culture plates [2, 3].

Methods: The MDR1 gene was transfected into human iPS cells (IMR90-4), which were then differentiated into MDR1-BMECs [2]. Protein expression was analyzed using sequential window acquisition of all theoretical fragment ion spectra-mass spectrometry (SWATH-MS), and the transport functions in MDR1-BMECs were evaluated in transcellular transport analyses by 2D and 3D culture models constructed with Transwell® and the MIMETAS OrganoPlate® 3-lane plate [3], respectively.

Results and Discussion: SWATH-MS analysis revealed the markedly increased expression of MDR1 protein in MDR1-BMECs, and other transporters showed almost identical expression between MDR1-BMECs and mock cells, suggesting that MDR1 gene overexpression did not significantly affect the expression of other transporter proteins. In the 2D culture model, the basolateral-to-apical (B-to-A) transport of MDR1 substrates, such as [3H]vinblastine, [3H]digoxin, [3H]cyclosporin A, quinidine, loperamide, cetirizine, erlotinib, and verapamil, was higher than apical-to-basolateral (A-to-B) transport, and their efflux ratio (ER) values were in the range of 1.52–26.0. The efflux dominant transports were significantly attenuated by PSC-833, an MDR1 inhibitor, indicating MDR1-mediated efflux transport in MDR1-BMECs. In the 3D culture model, MDR1-BMECs were formed capillary structures, and the clear efflux-dominant transport of [3H]vinblastine, [3H]digoxin, [3H]cyclosporin A, and quinidine was observed, with respective ER values of 23.5, 10.1, 6.67, and 2.16. In addition, no significant change in the functions of other transporters, such as BCRP/ABCG2 and MCT1/SLC16A1, was observed in 2D or 3D culture models.

Conclusion: Thus, 2D- and 3D-cultured BBB models constructed with MDR1-BMECs could be useful experimental tools for developing novel CNS therapeutics.

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P301 - A SENSITIVE AND EFFICIENT LC-MS/MS METHOD FOR CREATININE-D5 ANALYSIS IN *IN VITRO* OCT2 INHIBITION ASSAY

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Purpose: *in vitro* transporter inhibition studies are designed to determine if the investigational drug is an inhibitor of a specific transporter. The Organic Cation Transporter 2 (OCT2), expressed predominantly in renal tubular epithelial cells, primarily transports hydrophilic, low molecular weight organic cations. Creatinine, a validated substrate of OCT2, serves as an *in vitro* indicator of OCT2 activity. However, as an endogenous substance with high polarity, creatinine poses challenges due to its difficult retention in reverse phase chromatography and high baseline in the matrix. To address these issues, hydrophilic interaction chromatography was employed to ensure good and robust retention, and creatinine-d5 was used as the substrate instead of unlabeled creatinine to avoid endogenous interference in the matrix. This approach not only facilitated efficient and rapid analysis but also achieved a lower limit of quantitation (LLOQ) of 0.02 nM, which is four times lower than those reported in the current literature. **Method:** The OCT2 transporter samples were extracted using a protein precipitation method with acetonitrile/methanol (95/5, v/v) containing an internal standard. The LC-MS/MS analysis was conducted using a SCIEX Triple Quad 6500 Plus, coupled with a Waters ACQUITY UPLC I-Class PLUS. The chromatographic separation was carried out on a Waters Atlantis Premier BEH Z-HILIC column (2.1 * 50 mm, 1.7µm). The mobile phases used were a mixture of 0.1% Formic Acid & 10mM NH4OAc in water and acetonitrile, respectively. The flow rate was set at 0.4 mL/min, and the total run time was 2.0 minutes. Quantitation was performed using the transition of m/z 119.1→49.1 for creatinine-d5. **[Results & Discussion]** The sensitivity and linearity of the method were evaluated. The results indicated that creatinine-d5 had good linearity within the concentration range of 0.02-20 nM (R² > 0.99). The accuracy and RSD of LLOQ (n = 6) were 92.6% and 8.4%, respectively. In terms of optimizing chromatographic conditions, 10 mM ammonium acetate was selected as an additive in the mobile phase, which resulted in an improved peak shape and signal-to-noise ratio. **Conclusions:** In this study, an efficient and sensitive LC-MS/MS method for creatinine-d5 was established. The lower limit of quantitation (LLOQ) is four times lower than that reported in

existing literature, and the analysis time is just 2 minutes. The utilization of creatinine-d5 avoids the impact of endogenous substances, thereby making the detection results more accurate. The establishment of this analytical method has successfully expedited the study of *in vitro* OCT2 inhibition.

P302 - REGULATION OF P-GLYCOPROTEIN EXPRESSION AND ROLE OF ERM PROTEINS IN LUNG CANCER CELL METASTASIS

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Purpose: Cancer invasion and metastasis are established in a two-step process: Epithelial-Mesenchymal Transformation (EMT) and Mesenchymal-Epithelial Transformation (MET). Cancer metastasis is associated with multidrug resistance (MDR). One of the causes of MDR is the increased expression of drug efflux transporters such as P-glycoprotein (P-gp). On the other hand, scaffold proteins (Ezrin, Radixin, and Moesin) are important to the expression of efflux transporters, and Ezrin (Ezr) and Moesin (Msn) are known to be scaffold proteins for P-gp in lung cancer. In this study, we aimed to elucidate whether the functions of P-gp and scaffold proteins are altered in EMT/MET-induced lung cancer cells and to clarify its mechanism. **Methods:** Lung cancer cell line HCC827 cells were treated with the transcriptional regulator Snail to induce EMT, and mRNA expression levels of scaffold proteins and P-gp were quantified by RT-PCR. In addition, protein levels were quantified by Western Blotting, P-gp activity was measured using Rho123, and cytotoxicity was examined using Paclitaxel. Furthermore, HCC827 cells were treated with dexamethasone (DEX) to induce MET. The mRNA expression levels of scaffold proteins and P-gp, and their protein expression levels on the membrane were quantified. The activity of P-gp was measured using Rho123, and cytotoxicity was examined using Paclitaxel. In addition, EMT and MET were continuously induced by DEX treatment after Snail treatment, and membrane expression and activity of P-gp were measured. **Results and Discussion:** The mRNA expression level of Msn was significantly increased when EMT was induced. The membrane protein expression level of P-gp was significantly increased, and its activity and cell viability were both increased. This suggests that the activation of P-gp in EMT-induced lung cancer cells may be regulated by Msn. On the other hand, the mRNA expression levels and membrane protein expression of Ezr and P-gp were significantly increased when MET was induced, and P-gp activity and cell viability were also increased. This suggests that membrane expression of P-gp in MET-induced lung cancer cells is regulated by Ezrin. The increase in membrane expression of P-gp was synergistic when EMT/MET was continuously induced. In the present study, we found increased P-gp expression and activity in both EMT and MET processes in cancer metastasis, suggesting that the mechanism differs at each step.

P303 - INVOLVEMENT OF EFFLUX TRANSPORTERS ABCG2 AND ABCB1 IN OVERALL DISPOSITION OF MULTIKINASE INHIBITOR PAZOPANIB

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Introduction: Pazopanib is an orally active second-generation tyrosine kinase inhibitor (TKI) approved for the treatment of advanced soft-tissue sarcoma and renal cell carcinoma. Pazopanib is a substrate of efflux transporters including breast cancer resistance protein (BCRP/ABCG2) and P-glycoprotein (P-gp/ABCB1), yet information on the involvement of these transporters in pazopanib disposition is limited. The purpose of the present study is to elucidate the involvement of Abcg2 in the disposition of pazopanib.

Methods: Pazopanib was orally/intravenously administered to wild-type, Abcg2^{-/-}, hepatic Abcg2 knock-down and hepatic ABCG2/Abcg2 knock-in mice. Such knock-down/knock-in was achieved using adeno-associated virus encoding short hairpin RNA (shRNA) and ABCG2/abcg2 genes in wild-type and Abcg2^{-/-} mice, respectively. Since mice exhibited extensive hepatic metabolism of this drug in contrast to humans showing minimal metabolism, a prior administration of cytochrome P450 inhibitor 1-aminobenzotriazole (ABT) was performed. Plasma and liver concentration of pazopanib was measured by LC-MS/MS.

Results and Discussion: Coadministration of elacridar, an inhibitor of both P-gp and BCRP increased plasma and liver concentration of pazopanib under the treatment of 1-aminobenzotriazole (ABT), suggesting potential involvement of these efflux transporters in pharmacokinetics of pazopanib. In addition, this result may also propose that P450 enzymes may be largely involved in overall disposition of pazopanib, and this may hinder observation of effect of these transporters inhibitors. In the absence of ABT, elacridar seemed to increase plasma concentration of pazopanib mostly at the absorption phase, suggesting the involvement of intestinal efflux transporters. After oral administration of pazopanib, Abcg2^{-/-} mice exhibited slightly higher but statistically insignificant plasma concentration of pazopanib in comparison to wild-type mice, while after the intravenous injection of pazopanib, the tested groups seemed to have the same profile. This may suggest the partial involvement of Abcg2 in the disposition of pazopanib. When P450 inhibitor ABT was pre-administered to avoid P450 metabolism in this case, plasma concentration of pazopanib in the elimination phase in

Abcg2^{-/-} mice was lower than wild-type mice, supporting that Abcg2 may be potentially involved in overall elimination of this drug. To confirm possible involvement of Abcg2 in elimination phase, plasma concentration profile was examined in knock-in of ABCG2 and ABCG2 with Q141K polymorphism in Abcg2^{-/-} mice and was slightly higher in ABCG2 with Q141K polymorphism knock-in mice at the elimination phase. On the other hand, biliary clearance of pazopanib in knock-in of Abcg2/ABCG2 in Abcg2^{-/-} mice was higher than that in Abcg2^{-/-} mice. Thus, Abcg2 may be partially involved in elimination of pazopanib although further studies are required for the demonstration.

Conclusion: Both Abcg2 and Abcb1 may contribute to the disposition of pazopanib, and this was suggested in rodents using cytochrome P450 inhibitor due to larger metabolic capacity compared to humans. Further analysis using more specific inhibitor and potential biomarkers of these efflux transporters is undergoing to clarify their role in pazopanib disposition.

P304 - ABCC4 GENETIC POLYMORPHISM MAY INDIRECTLY REGULATE DISPOSITION OF CAPECITABINE METABOLITE IN COLORECTAL CANCER PATIENTS

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Introduction: Oral anticancer drug capecitabine shows pharmacological effects after metabolizing to 5-FU via 5'-DFCR and 5'-DFUR. Carboxylesterase, cytidine deaminase (CDA), and thymidine phosphorylase (TYMP) sequentially activate this drug. In our prospective study, the AUC/dose of 5'-DFUR in colorectal cancer patients with ABCC4 gene polymorphism rs3742106 was significantly lower than that in the wild-type. The polymorphism rs3742106 is located in the 3'-UTR of ABCC4, and the T allele might lead to decreased gene expression of ABCC4. The purpose of the present study is to develop a PBPK model of capecitabine and metabolites in patients to clarify elements of the difference in their pharmacokinetics, and the evaluation of the possible effect of ABCC4 gene polymorphism rs3742106 on hepatic drug-metabolizing enzyme activities. Methods: The previously established PBPK model (Pharm Res. 18, 1190, 2001) was modified by the addition of a gastric compartment and GI transfer rate constant. Parameter optimizations by the nonlinear least square method and sensitivity analysis were performed using the Napp program. The mRNA level of drug-metabolizing enzymes and transporters in HepaRG cells was measured by qPCR after ABCC4 knockdown or exposure to cAMP analog 8-bromo-cAMP. Intracellular cAMP accumulation was measured by cAMP-Glo assay. Results and Discussion: The average plasma concentration time-profiles of capecitabine in all patients were successfully explained by the incorporation of the additional GI compartment, implying the slower absorption property of capecitabine tablets in the patients compared to the previous Phase I study (Pharm Res. 18, 1190, 2001). Fitting to plasma concentration time-profiles obtained in each ABCC4 rs3742106 genotype of the modified PBPK model yielded the higher intrinsic clearances for CDA and TYMP in G/T+T/T patients than those in G/G patients. The ABCC4 knockdown by siRNA and exposure to 8-Br-cAMP, an analog of endogenous MRP4 substrate cAMP led to increase in mRNA level of TYMP in HepaRG cells. Intracellular level of cAMP was also confirmed to be increased by the ABCC4 knockdown. Thus, functional decrease of ABCC4 may alter the metabolism of 5'-DFUR via accumulation of cAMP in hepatocytes, and this may at least partially explain the effect of ABCC4 gene polymorphism on AUC/Dose of 5'-DFUR. Also, the exposure to 8-Br-cAMP in HepaRG cells increased the metabolism of tolbutamide to 4-hydroxytolbutamide which is mainly governed by CYP2C9, but decreased the metabolism of phenacetin to acetaminophen which is mainly governed by CYP1A2, implying that indirect induction and/or downregulation by ABCC4 may also occur in cytochrome P450 enzymes. Conclusion: ABCC4 polymorphism may indirectly alter the pharmacokinetics of capecitabine metabolite by altering the expression of drug-metabolizing enzymes. Further analysis of such alteration on pharmacokinetics of other drugs may be needed to more deeply understand interindividual difference in drug disposition.

P305 - FUNCTIONAL CHARACTERIZATION OF ENT3/SLC29A3 AS A LYSOSOMAL URATE TRANSPORTER

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Gout is a disease caused by monosodium urate (MSU) crystals precipitated in joints. In the disease development, MSU crystals are phagocytosed by macrophages into lysosomes and induce lysosomal dysfunction that leads to inflammation. However, urate could be partially dissolved in lysosomes and cleared through the lysosomal membrane and then through the plasma membrane. Hence, we hypothesized that carrier-mediated mechanisms might be involved in the membrane transport processes to facilitate urate clearance and their functional levels, which could affect the lysosomal accumulation of MSU crystals, might be involved in interindividual differences in the development of gout. We focused in the present study on the lysosomal membrane transport process and examined the possibility that equilibrative nucleoside transporter 3 (ENT3/SLC29A3), which is highly expressed at the lysosomal membrane of macrophages and recognizes purine nucleosides and nucleobases as substrates, might be capable of transporting urate, which is structurally analogous to such ENT3 substrates. We prepared recombinant human ENT3 (ENT3-AA) by replacing the dileucine motif, which is responsible for lysosomal localization, with dialanine for redirection to the plasma membrane to enable functional analyses conveniently by cellular uptake assays. The uptake assays were performed in transiently ENT3-AA-transfected

HEK293 cells, using [^{14}C]urate (4 μM). The uptake of urate evaluated at pH 5.0, which was to mimic the acidic lysosomal environment, was greater in ENT3-AA expressing cells than in mock cells, indicating that ENT3-AA is capable of transporting urate. The specific uptake of urate by ENT3-AA was dependent on extracellular pH, being increased with a decrease in pH. These findings suggested that ENT3 can be involved in the efflux transport of urate from the acidic environment in the lysosome to the neutral environment in the cytosol. Kinetically, ENT3-AA-mediated urate uptake at pH 5.0 was saturable with a Michaelis constant of 1.15 mM, suggesting a characteristic of rather low-affinity transport. Separately, we found in THP-1 cells, which are monocytic cells and can be differentiated into macrophages, that the expression level of ENT3 mRNA was greater in differentiated cells than in undifferentiated ones, suggesting the potential role of ENT3 particularly in the differentiated, macrophage type of THP-1 cells. Taken all these results together, ENT3 could possibly operate as a lysosomal urate exporter and might play a role in urate clearance in macrophages, which might be related to the development of gout.

P306 - FUNCTIONAL CHARACTERISTICS OF A NEWLY IDENTIFIED VESICULAR MEMBRANE-LOCALIZED MONOAMINE TRANSPORTER FOR SEROTONIN TRANSPORT

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Purpose: A group of monoamines, such as dopamine, norepinephrine, and serotonin, play important roles as neurotransmitters. The disposition of such monoamines is known to be regulated by transporters at the plasma membrane and synaptic vesicular membrane of nerve cells and, hence, the functional levels of such transporters could affect neurotransmission. Therefore, those transporters have been of interest as therapeutic targets in the development of drugs to treat neuronal dysfunction and related diseases. While several monoamine transporters have so far been identified and exploited for therapeutic purposes, there still could be those that have not been identified yet. We here report on the functional characteristics of a newly identified one, which is localized to the vesicular membrane and tentatively named vesicular monoamine transporter 1 (NVMAT1), focusing on its serotonin transport function.

Methods: In the functional analysis, we used a recombinant NVMAT1 (NVMAT1-AA), in which the signal sequence for vesicular membrane localization (LL) was replaced with AA for plasma membrane localization. The cellular uptake of [^3H]serotonin (10 nM) was evaluated, unless otherwise indicated, for 2 min at pH 7.4 and 37°C, in HEK293 cells transiently expressing NVMAT1-AA. We also evaluated [^3H]serotonin uptake in HEK293 cells transiently transfected with serotonin transporter (SERT/SLC6A4), which operates for serotonin uptake at the plasma membrane, and vesicular monoamine transporter1 (VMAT1/SLC18A1), which operates for serotonin uptake intracellularly at the vesicular membrane, in combination with NVMAT1.

Results and Discussion: The uptake of serotonin was greater in cells expressing NVMAT1-AA than in mock cells, indicating that NVMAT1-AA can transport serotonin. The specific uptake of serotonin by NVMAT1-AA was reduced by the removal of Na^+ , but the reduction was only moderate and, hence, not indicative of the requirement of Na^+ as the driving ion. In addition, the specific uptake was not affected by the removal of Cl^- or dependent on pH. Therefore, it is likely that NVMAT1 operates in a facilitative manner. Kinetically, the specific uptake was saturable with the Michaelis constant of 10.6 mM, indicating a rather low affinity characteristic. In SERT-transfected HEK293 cells, in which serotonin uptake was elevated, compared with mock cells, the cellular accumulation of serotonin was increased by co-transfection of VMAT1, but this increase was counteracted by the additional co-transfection of NVMAT1. This result suggests that NVMAT1 operates for the efflux transport of serotonin at the vesicular membrane. Thus, the present study indicated that NVMAT1 transports serotonin in a facilitative manner with a rather low affinity and play a role in vesicular efflux.

P307 - FUNCTIONAL CHARACTERIZATION OF ATP13A3 AS A PLASMA MEMBRANE POLYAMINE TRANSPORTER

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Purpose: A group of ATP13A4 family transporters, ATP13A2, 3, and 4, have recently been reported to play a role as lysosomal polyamine transporters. 1), 2), 3) However, their functional characteristics have little been clarified yet. We here report our finding that ATP13A3 may be present at the plasma membrane, rather than the lysosomal membrane, and operate for cellular polyamine uptake. Clarifying polyamine uptake mechanisms involving such transporters should help explore their potential utilization for drug delivery and also address pathological issues related to polyamines, which are biologically active substances involved in various physiological processes. **Methods:** The initial uptake of [^3H]putrescine was evaluated for 5 min at pH 7.4 and 37°C, unless otherwise indicated, in COS-7 cells transiently expressing human ATP13A3. **Results and Discussion:** The uptake of putrescine, one of major polyamines, was greater in cells expressing ATP13A3 than in mock cells, indicating that ATP13A3 can transport putrescine. The specific uptake of putrescine by ATP13A3 was not affected by the removal of Na^+ or Cl^- , indicating that these ions are not involved in its mediated putrescine transport. However, it was dependent on pH, favoring near neutral pH conditions. This pH-dependent characteristic was in agreement with those reported for polyamine uptake systems. Kinetically, the specific uptake was

saturable with the Michaelis constant of 62.8 μ M, indicative of the carrier-mediated type of operation at the plasma membrane. These characteristics were similar to those of ATP13A4, which has also been suggested to play a role as a plasma membrane transporter, rather than lysosomal transporter, for polyamines in our recent study. 4) Conclusions: This study indicated the potential role of ATP13A3 as a plasma membrane transporter for cellular polyamine uptake. Its functional characteristics were similar to those of ATP13A4. Future studies should be warranted to clarify their respective roles. [Reference] 1) van Veen, Sarah et al. ATP13A2 deficiency disrupts lysosomal polyamine export. *Nature*, 2020; 578(7795), 419–424. 2) Hamouda, Norin Nabil et al. ATP13A3 is a major component of the enigmatic mammalian polyamine transport system. *The Journal of biological chemistry*, 2021; 296, 100182. 3) van Veen, Sarah et al. ATP13A4 Upregulation Drives the Elevated Polyamine Transport System in the Breast Cancer Cell Line MCF7. *Biomolecules*, 2023; 13(6), 918. 4) Morimoto, Haruka et al. Functional identification of ATP13A4 as a plasma membrane polyamine transporter: analysis of the spermidine transport function, 38th Annual Meeting of the Japanese Society for the Study of Xenobiotics, 2023

P308 - EFFECT OF THE HYPOXIA-INDUCIBLE FACTOR PROLYL HYDROXYLASE INHIBITOR ROXADUSTAT ON EXPRESSION OF BREAST CANCER RESISTANCE PROTEIN (BCRP/ABCG2) IN HUMAN KIDNEY EPITHELIAL CELL LINE HK-2

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Purpose: The member of the ATP-binding cassette transporter family breast cancer resistance protein (BCRP/ABCG2) is a drug transporter restricting absorption and enhancing excretion of various compounds. The cis-regulatory elements in the human BCRP/ABCG2 promoter include a hypoxia response element, the DNA binding site for hypoxia-inducible factor (HIF), major transcriptional regulator of cellular responses to hypoxia. We previously found that the hypoxia mimetic agent cobalt chloride enhanced the expression of HIF-1 α protein and BCRP/ABCG2 mRNA in human kidney epithelial cell line HK-2 [1]. In addition, we found that pioglitazone treatment induced the activation of HIF-1 and the increase in mRNA expression of BCRP/ABCG2 in HK-2 cells [2]. In this study, we examined the effect of roxadustat, a HIF prolyl hydroxylase inhibitor, on mRNA expression of BCRP/ABCG2 in HK-2 cells. **Methods:** HK-2 cells were cultured under an atmosphere of 95% air/5% CO₂ at 37°C. The cells were treated with roxadustat in the culture medium, and then total RNA was extracted from the treated cells and reverse-transcribed to cDNA. The relative mRNA expression levels were evaluated by real-time PCR analysis. **[Results]** The treatment with roxadustat at a concentration of 30 μ M for 3 hours markedly increased the mRNA expression of facilitative glucose transporter 1 (GLUT1) and vascular epithelial growth factor (VEGF), typical HIF target genes, in HK-2 cells. The observation indicated that roxadustat activated the HIF signaling pathway in HK-2 cells. On the other hand, the expression of BCRP/ABCG2 mRNA was not significantly modulated by the treatment with roxadustat, in contrast with the previous results that HIF-1 activation with cobalt chloride and pioglitazone significantly enhanced the expression of BCRP/ABCG2 mRNA in HK-2 cells. **Conclusions:** HIF activation by roxadustat might have distinct effects on mRNA expression from those by cobalt chloride and pioglitazone in HK-2 cells. **References:** [1] Nishihashi K, Kawashima K, Nomura T, Urakami-Takebayashi Y, Miyazaki M, Takano M, Nagai J. Cobalt chloride induces expression and function of breast cancer resistance protein (BCRP/ABCG2) in human renal proximal tubular epithelial cell line HK-2. *Biol Pharm Bull.* 40(1):82-87, 2017. [2] Urakami-Takebayashi Y, Kuroda Y, Murata T, Miyazaki M, Nagai J. Pioglitazone induces hypoxia-inducible factor 1 activation in human renal proximal tubular epithelial cell line HK-2. *Biochem Biophys Res Commun.* 503(3):1682-1688, 2018.

P309 - SEPARATE EVALUATION OF SYSTEMIC CIRCULATION TRANSITION AND BILIARY EXCRETION OF DRUG METABOLITES USING A NOVEL *IN VITRO* CULTURED HEPATOCYTE SYSTEM ICHEP

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Purpose: In drug development stages, the human safety of drug candidates cannot be sufficiently predicted using preclinical safety studies with experimental animals. One reason for this is production of disproportionate drug metabolites; the metabolites produced are specific to humans and higher levels of metabolites are found in humans than in experimental animals. In the guidance for Safety Testing of Drug Metabolites published by the U.S. Food and Drug Administration, the safety of such metabolites needs to be evaluated before large-scale clinical trials. To evaluate biliary excretion of drugs, *in vitro* evaluation using primary cultured human hepatocytes is investigated, but this methodology is cumbersome because drugs accumulate in the closed bile canaliculus lumen formed between hepatocytes, and their amounts cannot be directly measured. To overcome this concern, we have recently established a novel hepatocyte culture system with an induced open-form bile canaliculus (icHep), which enables a rapid evaluation of drug biliary excretion using conventional permeation assays¹. Here, we investigated the applicability of icHep as a system that enables separate evaluation of blood and biliary excretion of drugs. **Methods:** PXB-cells derived from liver-humanized mice

were cultured on a permeable support coated with claudin-1 proteins. The permeation assay was initiated by adding the buffer containing E217 β G or SN-38 to the cell culture insert. [Results] mRNA expression of drug transporters and metabolic enzymes including MRP2 and UGT1A1 are comparable to that in conventional sandwich cultured PXB-cells. Permeation assay in icHep system showed unidirectional transport of E217 β G and SN-38 (MRP2 substrates) from blood to bile side in time-dependent manner. Moreover, each transport was suppressed upon treatment with the MRP2 inhibitors. Furthermore, SN-38 glucuronide, which is mostly excreted into bile rather than blood in human *in vivo*, was distributed in the blood side (4.49%), bile side (49.4%), and hepatocytes (46.1%), respectively. Conclusions: icHep provides a simple and appropriate evaluation of the excretion process of drug metabolites into blood and bile using permeation assays. We are currently analyzing the excretion pathways of various drug metabolites using icHep.

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P310 - QUANTITATIVE ANALYSIS OF THE IMPACT OF C421A MUTATION ON BCRP ACTIVITY IN HUMAN ILEAL CRYPT-DERIVED DIFFERENTIATED CELLS

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Human intestinal crypt-derived cells can differentiate into epithelial cells that maintain the functions of transporters (TPs) and drug metabolic enzymes (DMEs). They are expected to serve as useful *in vitro* models for predicting the gastrointestinal absorption of drugs during drug development. Previously, we reported that Fg estimated from the apical-to-basal clearance of human jejunal crypt-derived differentiated cells showed a one-to-one correlation with that estimated from clinical data. In this study, we characterized human ileal crypt-derived differentiated cells to examine whether this experimental system can assess inter-individual variability in the activity of TPs and DMEs. As an example, we focused on the C421A mutation in BCRP, which has been reported to increase the AUC of sulfasalazine and rosuvastatin. We found human ileal spheroids carrying wild-type and C421A homozygous-mutated BCRP. These spheroids were cultured in L-WRN medium containing factors that are essential for the maintenance of stem cells. Spheroids were enzymatically dissociated, seeded on culture inserts or plates, and differentiated for four days in a medium without these factors. The transport of BCRP substrates (sulfasalazine, rosuvastatin, and dantrolene), P-gp probe (digoxin), ASBT probe (d5-TCA), CYP3A4/5 probe (midazolam), passive transport marker (antipyrine), and paracellular transport marker (lucifer yellow) was tested and analyzed by LC-MS/MS or fluorescence spectrophotometry. P-gp- and BCRP-mediated transport of digoxin and sulfasalazine was inhibited by inhibitors of P-gp and BCRP, and efflux ratios were reduced from approximately 15 and 10 to about 1, respectively. Moreover, in addition to the Na⁺-dependence in the uptake of d5-TCA, Papp in the apical-to-basal direction was reduced by up to approximately 25% by the ASBT inhibitor. In addition, Fg calculated from the apical-to-basal transport of midazolam and the amount of 1-OH' midazolam formed during the study was estimated to be 0.59-0.89. These results suggest that human ileal crypt-derived differentiated cells retain the gastrointestinal characteristics of the ileum. The Papp of BCRP substrates in the basal-to-apical direction across BCRPC421A-homozygous mutated cells was approximately 50-80% of that in wild-type cells. The activity of BCRP was calculated from the Efflux Ratio of each BCRP substrate, and the transport activity of sulfasalazine, rosuvastatin, and dantrolene in BCRPC421A-homozygous mutated cells were 88,19,45% of that in wild-type cells. These results suggest that this experimental system can functionally reflect the effects of genetic polymorphisms in BCRP. In conclusion, human intestinal crypt-derived differentiated cells are promising *in vitro* models for evaluating the impact of the C421A mutation of BCRP on intestinal absorption of BCRP substrates and the impact of mutations in other TPs and DMEs.

P311 - NON-TARGETED METABOLOMICS FOR THE IDENTIFICATION OF BIOMARKER CANDIDATES FOR BREAST CANCER RESISTANCE PROTEIN

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Naturally occurring endogenous and exogenous substrates can potentially serve as biomarkers for drug transporters. Such biomarkers could be used in transporter phenotyping and elucidating transporter-mediated drug-drug interactions. Our aim was to identify biomarker candidates for breast cancer resistance protein (BCRP, encoded by ABCG2) function using a hypothesis-free metabolomics approach. We analyzed fasting plasma samples from 356 unrelated healthy volunteers using non-targeted metabolite profiling by liquid chromatography high-resolution mass spectrometry. Based on the ABCG2 rs2231142 (c.421C>A, p.Gln141Lys) genotype, we stratified the volunteers to poor (n = 2), decreased (n = 57), and normal (n = 297) BCRP function groups. Linear regression analysis was employed to investigate associations of plasma metabolite features with BCRP function. Altogether 9,152 metabolite features were found. Preliminary results indicated that the peak areas of 19 metabolite features were significantly larger in individuals with poor or decreased BCRP function than in those with normal BCRP function after correction for multiple testing (P<10⁻⁵), suggesting that

these features represent BCRP biomarker candidates. These 19 molecular features were on average smaller than the other 9,133 molecular features, with a mean $\pm$ standard deviation molecular weight of 297.1 ± 158.0 g/mol compared to 488.5 ± 370.6 g/mol ($P = 0.024$). We are currently working to identify the biomarker candidates based on exact molecular weights, chromatography retention times, and mass fragmentation data. Taken together, these data indicate that several metabolite molecular features naturally present in human plasma associate with BCRP function. Further studies are required to identify the compounds and to investigate whether they are substrates of BCRP.

P312 - PLASMA CONCENTRATION OF LAT1 SUBSTRATE AMINO ACIDS AFFECTS THE SPINAL CORD TRANSFER AND THE ANALGESIC EFFECT OF PREGABALIN.

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Pregabalin is an anti-neuropathic pain drug that exerts its effect by inhibiting the voltage-dependent calcium channel in the spinal cord. We have suggested the involvement of L-type amino acid transporter (LAT) 1 in pregabalin uptake at the blood-brain barrier and blood-spinal cord barrier (BSCB) [1,2]. Since LAT1-mediated transport at the BSCB is saturated with plasma branched-chain amino acids (BCAA), endogenous substrates of LAT1, evaluating the influence of plasma BCAA concentrations on pregabalin transfer to the spinal cord is essential to comprehend the conditions under which pregabalin effectively exerts its action. This study aimed to examine the contribution of LAT1 to the permeation of pregabalin across the BSCB and its pharmacological effect in the spinal cord. The analgesic effect of pregabalin was evaluated by tail immersion test using an oxaliplatin-induced cold allodynia model of 7-week-old male SD rats. Plasma and spinal cord concentrations of pregabalin were quantified by LC-MS/MS. Intravenous administration of 6 mg/kg pregabalin (pregabalin alone group) significantly increased the AUC of tail withdrawal latency (expressed as a percent of maximal possible effect; %MPE) at 120 min after administration compared to the vehicle group, confirming the analgesic effect. On the other hand, intraperitoneal administration of BCAA 15 min before pregabalin administration (pregabalin+BCAA group) significantly decreased the AUC of %MPE by 39% compared to the pregabalin alone group, indicating the attenuation of the analgesic effect of pregabalin by BCAA administration. Although the plasma concentration profiles of pregabalin until 120 min after administration were identical between pregabalin alone and pregabalin+BCAA groups, the spinal cord concentrations of pregabalin were decreased to 75% as an AUC of 0 to 120 min by the pre-administration of BCAA. These results suggest that decreased LAT1 activity due to elevated plasma BCAA concentration affects the transfer of pregabalin to the spinal cord. In humans, residual LAT1 activity after oral administration of 5 g of BCAA or consumption of a high-protein diet was estimated to be decreased by 57% and 35%, respectively, according to the reported plasma amino acid concentrations [3,4]. Thus, fluctuations in plasma amino acid concentrations due to the medication and daily BCAA intake may affect the spinal cord transfer of pregabalin via LAT1.

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P313 - SCREENING OF AN FDA-APPROVED DRUG LIBRARY TO IDENTIFY ORGANIC ANION TRANSPORTER 4 INHIBITORS

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Purpose: Human organic anion transporter 4 (OAT4) is localized to the apical membrane of proximal tubules and has been shown to play a potentially important role in the renal excretion of anionic drugs. However, information on the role of OAT4 in pharmacokinetics is limited partly due to the lack of orthologous genes in rodent animal models. Identifying a potent inhibitor of OAT4 from approved drugs can help to define the role of OAT4 in pharmacokinetics. Therefore, the objective of this study was to screen the inhibitory effect of FDA-approved drugs on OAT4.

Method: This study evaluated the inhibitory effect of FDA-approved 2999 drugs on the uptake of 6-carboxyfluorescein (6-cf) in tetracycline-inducible OAT4 or OAT3-expressing HEK 293 cells through fluorescence measurement at λ_{ex} 495 nm/ λ_{em} 530 nm.

Results and Discussion: The uptake of 6-cf in OAT4 overexpressing cells was increased linearly up to 2 min and by decreasing extracellular pH. The K_m value for OAT4-mediated 6-cf uptake was determined to be 169 μM . Thus, the inhibitory effect of the drugs on OAT4-mediated uptake was assessed at an incubation time of 1 min, a 6-cf concentration of 20 μM , and an extracellular pH of 5.5, the condition under which approximately 10-fold higher uptake compared with mock cells was detected. Out of the 2999 drugs in the library, 261 and 7 drugs inhibited 6-cf uptake by OAT4 overexpressing cells by more than 30% at 20 μM or 20% at 10 μM , respectively. Out of the first 268 drugs screened, a total of 80 drugs were confirmed to be identified as OAT4 inhibitors since these drugs inhibit 6-cf uptake by OAT4

overexpressing cells by more than 50% at 20 μM or 30% at 10 μM . Many of the identified OAT4 inhibitors were anionic compounds with carboxy or sulfo groups. Some of the identified inhibitor drugs were reported as OAT4 substrates/inhibitors, including drugs targeting the renin-angiotensin-aldosterone system. The inhibitory effect of these 80 drugs on OAT3-mediated 6-cf uptake was also examined, and several drugs were found to potentially inhibit OAT4 more specifically than OAT3.

Conclusion: We found 80 OAT4 inhibitor candidate drugs through screening using an FDA-approved drug library. These drugs may be useful in evaluating the role of OAT4 in pharmacokinetics.

P314 - CHARACTERIZATION OF LONG-LASTING INHIBITION OF AMINO ACID TRANSPORTER LAT1 BY NANVURANLAT (JPH203) IN CHOLANGIOCELLULAR CARCINOMA

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Purpose: Nanvuranlat (JPH203) is being developed as a therapeutic agent for biliary tract cancer. JPH203 functions as an inhibitor of the L-type amino acid transporter 1 (LAT1/SLC7A5), which is highly and selectively expressed in tumors. The inhibitory effect of JPH203 on LAT1-mediated leucine uptake was reported to be maintained in colon adenocarcinoma HT-29 cells even after the washout, indicating potential long-lasting inhibition. However, both the mechanistic and quantitative characterization of its long-lasting inhibition have not yet been conducted, and its application to biliary tract cancer cells has not yet been fully elucidated. In the present study, we first attempted to demonstrate long-lasting inhibition of LAT1 by JPH203 in cholangiocellular carcinoma cells. We then hypothesized that the long-lasting inhibition of JPH203 is due to its cellular uptake to further demonstrate the potential application of this drug to biliary tract

cancer. Methods: Cholangiocellular carcinoma cell line HuCCT-1 cells were exposed to JPH203 at 37 °C for 2 hours. After three times washing with buffer, the cells were further incubated in JPH203-free buffer for an additional 4 hours. The cells were harvested using methanol, and the remaining amount of JPH203 within the cells was quantified by LC-MS/MS. After a similar JPH203 exposure and washout, the cells were incubated with sodium ion-free buffer containing [¹⁴C]leucine at 37 °C for 1 minute to assess the inhibition of the initial velocity of the uptake by JPH203. The radioactivity of [¹⁴C]leucine within the cells was determined by a liquid scintillation counter after solubilizing the cells with 0.2 M NaOH. Results and Discussion: The presence of 10 and 100 μM JPH203 reduced [¹⁴C]leucine uptake in HuCCT-1 cells to 34.6% and 12.3% of that in vehicle-treated cells, respectively, indicating the inhibitory effect on LAT1 in cholangiocellular carcinoma cells. Notably, a decrease in [¹⁴C]leucine uptake in HuCCT-1 cells was also observed even when JPH203 was washed out after the exposure to JPH203, and [¹⁴C]leucine uptake was reduced to 73.2% and 59.2% of vehicle-treated cells in the presence of 10 and 100 μM JPH203, respectively. These findings suggest the long-lasting inhibitory effect of JPH203 on LAT1 in cholangiocellular carcinoma cells. Further, the measurement of the remaining amount of JPH203 within the cells revealed the negative correlation between the cellular uptake amount of JPH203 and reduced [¹⁴C]leucine uptake in the cells. This implies that after being taken up by the cells, JPH203 may remain within the cells, thereby providing long-lasting inhibition of LAT1.

P315 - PROTEIN-MEDIATED UPTAKE EFFECT AND ITS IMPLICATIONS FOR OATP1B1/1B3-MEDIATED DRUG-DRUG INTERACTIONS INVESTIGATIONS

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Protein-mediated uptake effect (PMUE) has been hypothesized to fill the gap in *in vitro* to *in vivo* extrapolations (IVIVE) of transporter-mediated drug disposition. To date, several studies investigating this phenomenon have yielded inconsistencies, with some concluding that plasma protein-mediated uptake may be an artifact resulting from non-specific binding or residual-drug protein complex phenomena while others concluded its clinical significance. In this study, we explored the effect of plasma protein on organic anion transporting polypeptide (OATP)-1B1 and 1B3 mediated victim DDI investigation for three ECCS 1B/3B drugs (atorvastatin, pitavastatin and repaglinide). First, we confirmed uptake using primary human hepatocytes (PHH) and then estimated transport kinetics using HEK293 cells transfected with either transporter in the absence and presence of 4% w/v bovine serum albumin (BSA). IC₅₀ values for cyclosporine on these OATP substrate drugs +/- BSA were then determined. To evaluate the IVIVE, DDI predictive power, R values were estimated following the FDA and ICH M12 guidelines. Overall, the fold uptake over time in the presence of 4% BSA was found to be significantly lower (>10 fold) for all substrates tested than the absence of BSA in both primary human hepatocytes and transfected cells. In the presence of 4% plasma protein, steady-state uptake in PHH was 15, 20, and 52 fold lower for atorvastatin, pitavastatin, and repaglinide, respectively, compared to the absence of plasma protein. The uptake kinetics data shows a 6- to 10-fold reduced K_m for pitavastatin and repaglinide, whereas no significant changes were observed for atorvastatin in the presence of 4% BSA compared to the absence of BSA. Furthermore, in the presence of 4% BSA, unbound IC₅₀ values of cyclosporine were increased up to 12-fold for atorvastatin, pitavastatin, and repaglinide, respectively. Moreover, the predicted DDI (OATP1B R-value) of cyclosporine for atorvastatin, pitavastatin and repaglinide were found to be closer to observed clinical DDIs when using the unbound IC₅₀ estimated from the

experiments conducted in the presence of 4% BSA than its absence. Together, these findings suggest the importance of evaluating different experimental conditions for *in vitro* DDI studies independent from different chemical platforms (i.e., ECCS).

P316 - EXAMINING THE EXPRESSION AND ACTIVITY RELATIONSHIP OF HUMAN HEPATIC TRANSPORTERS USING TARGETED PROTEOMICS

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Purpose. Proteomics-based transporter abundance data is widely used in the prediction of transporter-mediated hepatic clearance of drugs in humans using the relative expression factor (REF) approach. However, reproducibility of transporter abundance data, which affects the predicted hepatic clearance, remains challenging due to differences in reported surrogate peptides, sample preparation techniques, and mass spectrometry instrumentation. For instance, OATP1B1 and OATP1B3 abundance were highly variable (>80-fold) in the same hepatic tissue samples analyzed across six laboratories.¹ To improve the accuracy of the proteomic assays and the prediction of transporter-mediated clearance, the goal of this work was to validate proteomic methods (e.g., surrogate peptide selection) by examining the expression-activity relationship of several hepatic transporter substrates using a REF-based approach. **Methods.** Suspended, pooled, mixed gender human hepatocytes (lots UEC and CFM) were used to measure transporter-mediated uptake clearance (CL_{uptake}) of substrates in the presence and absence of a cocktail of inhibitors, which is known to completely inhibit major hepatic uptake transporters. Similarly, cell lines expressing the transporter of interest (e.g., OATP1B1, OATP1B3) were used to measure transporter-specific uptake clearance. Membrane enrichment and digestion workflows were optimized to measure transporter abundance in hepatocytes and cell lines using targeted proteomics, which was used to calculate REF values (abundance in human hepatocytes/abundance in cell line). The selection of surrogate peptides for specific transporters, e.g., OATP1B3, was investigated based on REF and functional activity data. **Results.** Functional studies revealed the observed OATP1B3-specific CL_{uptake} of cholecystokinin octapeptide (CCK8) in HEK293-OATP1B3 cells and human hepatocytes (lots UEC and CFM) as 68.0, 16.2, and 17.8 $\mu\text{L}/106 \text{ cells}/\text{min}$, respectively. Additionally, OATP1B3 abundance in HEK293-OATP1B3 cells and human hepatocytes (lots UEC and CFM) was measured as 0.85, 0.19, and 0.16 pmol/106 cells, respectively, using surrogate peptide NVTGFFQSLK, and as 6.56, 0.88, and 0.81 pmol/106 cells, respectively, using IYNSVFFGR. The REF-based predicted CL_{uptake} of CCK8 in human hepatocytes (lots UEC and CFM) were 74-96% of the observed CL_{uptake} using NVTGFFQSLK and 47-56% of observed CL_{uptake} using IYNSVFFGR. Similar analyses were conducted for OATP1B1 and other hepatic transporters to establish expression-activity relationship. **Conclusions.** Contrary to the common practice of reporting transporter abundance from the peptide with higher values (IYNSVFFGR), these results suggest that the peptide with lower OATP1B3 abundance (NVTGFFQSLK) provided more accurate REF-based prediction of CCK8 CL_{uptake} in human hepatocytes. Further examination into the expression-activity relationship and the peptide selection process may be warranted to decrease variability and improve accuracy of transporter abundance measured by proteomics.

P317 - TO BE THERE OR NOT TO BE THERE: DOES PRESENCE OF HUMAN OATP2B1 IMPACT CYP3A EXPRESSION?

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The cytochrome P450 enzyme CYP3A4 and its rat orthologue rCYP3A1 are well known for their inducibility upon exposure to a variety of exogenous molecules including the glucocorticoid dexamethasone. So far, little is known about endogenous molecules acting as their transcription-modulation factors. Interestingly, when assessing the *in vivo* relevance of the membranous organic anion transporting polypeptide 2B1 (OATP2B1, gene name SLCO2B1) in Slco2b1^{-/-} knock-out and SLCO2B1^{+/+} knock-in rats, we found increased hepatic rCYP3A1 expression in the humanized animals. Furthermore, a previous study showed species specificity of OATP2B1 in substrate recognition especially for endogenous sulfated steroids. Taken together, we assume an OATP2B1-mediated cellular uptake of an endogenous molecule which modulates rCYP3A1 expression. It is the aim of this study to further investigate this assumption using rat H4IIE as well as human HepaRG cells in hepatic cell models. At first we validated our previous observation on rCYP3A1 in the liver of Slco2b1^{-/-} and SLCO2B1^{+/+} rats by Western blot analysis. The same method was applied to confirm successful introduction of the human OATP2B1 into H4IIE cells using the transfection reagent jetPRIME®. To test whether the transcription modulating factor originates from serum we used culture medium either supplemented with FCS or with charcoal stripped FCS. Notably, independent of the treatment, rCYP3A1 protein expression was higher in OATP2B1-expressing cells compared to H4IIE control cells. Whether this also applies to the human CYP3A4 was tested in human hepatic HepaRG cells. Given the endogenous presence of OATP2B1, expression of the transporter was reduced by transient transfection of small interfering RNA. Quantitative real-time PCR revealed a 7 times lower mRNA expression of OATP2B1 while no influence on CYP3A4 was observed, independently if cells were cultured in FCS-containing medium for 24 or 72 hours post silencing. However, CYP3A4 mRNA expression was increased in both control and OATP2B1

silenced cells, cultured for 72 hours in medium containing charcoal stripped FCS. Importantly, increased CYP3A4 expression was also observed in cells which were not exposed to silencing RNA and cultured in charcoal stripped FCS-containing medium. In summary, our results suggest that presence of OATP2B1 influences rat CYP3A1 expression independently of FCS ingredients, however, expression of human CYP3A4 seems to be highly sensitive towards changes in FCS composition. Additional studies are needed to further investigate these findings.

P318 - FUNCTIONAL CHARACTERISTICS OF A NEWLY IDENTIFIED PLASMA MEMBRANE URATE TRANSPORTER

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Purpose: Carrier-mediated membrane transport mechanisms have been suggested to be involved in the disposition of urate in various organs, including the liver, kidney, and small intestine as the major ones. Clarifying the mechanisms with identifying transporters responsible for them should be of help in devising strategies to control the level of urate in plasma, of which the elevation could lead to the development of various diseases, such as gout. Identification of such transporters has been indeed in progress, facilitated in part by recent development of genome wide association studies. However, there still could be urate transporters that have not been identified yet. As a search attempt for such yet-to-be identified urate transporters, we examined several transporter-like proteins with unknown function and transporters for their urate transport activity. We here report on the functional characteristics of the one that was newly identified in the attempt and tentatively named NURAT (novel urate transporter).

Methods: The initial uptake of [¹⁴C]urate was evaluated for 3 min at pH 5.0 and 37°C, unless otherwise indicated, in HEK293 cells transiently expressing human NURAT.

Results and Discussion: The uptake of urate was greater in cells expressing NURAT than in mock cells, indicating that NURAT can transport urate. The specific uptake of urate by NURAT was not affected by the removal of Na⁺ or Cl⁻, whereas it was dependent on pH, favoring lower and acidic pH conditions. Based on this, a possibility is that urate may be transported in its unionized form, of which the fraction increases with a decrease in pH, by a facilitative mechanism. Kinetically, the specific uptake was saturable with a K_m of 6.06 mM, indicating a rather low affinity. According to the Human Protein Atlas database, NURAT is expressed ubiquitously in various organs. Although its localization within those organs is yet to be identified, NURAT may be able to operate for the cellular urate uptake from or efflux to plasma, in which the concentration of urate (about 240 ~ 350 μM) is much lower than the K_m, or other body fluid efficiently without showing saturation. Finally, the specific uptake was found to be inhibited by benzbromarone and several anionic compounds, indicating that these compounds are recognized by NURAT.

Conclusions: Findings in this study suggest that NURAT could play a role in urate uptake or efflux as a facilitative transporter at the plasma membranes, although future studies should be warranted to determine its expression profile and clarify its physiological role.

P319 - TRANSPORT CHARACTERISTICS OF EXTRACELLULAR VESICLES DERIVED FROM HUMAN TROPHOBLAST STEM CELLS IN HUMAN BLOOD-BRAIN BARRIER ENDOTHELIAL CELLS

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The delivery of central nervous system-acting macromolecular drugs such as nucleic acids and antibodies to the brain is restricted due to the presence of the blood-brain barrier (BBB). On the other hand, the BBB possesses various carrier proteins including transporters and receptors, which make it possible to assist the brain delivery of these macromolecular drugs across the BBB. The extracellular vesicles have gained attention as the carriers of the nucleic acids such as microRNAs and messenger RNAs and proteins for the targeted delivery to specific sites via carriers-mediated transport systems. Especially, the stem cells-derived extracellular vesicles are reported to exhibit the unique characteristics of the transport to the targeted cells and the pharmacological effects such as multilineage differentiation capacity and anti-inflammatory properties. In the present study, we focused on the extracellular vesicles derived from human trophoblast stem cells. The purpose of this study was to investigate the transport characteristics of human trophoblast stem cells-derived extracellular vesicles at the human BBB. The extracellular vesicles were collected from culture medium of human trophoblast stem cells by ultracentrifugation. The particle size distribution was analyzed using a Zetasizer Nano. The uptake of extracellular vesicles was evaluated using human brain microvessel endothelial cells (hCMEC/D3 cells), which are the model cells of human BBB. The particle size distribution of the extracellular vesicles derived from trophoblast stem cells was consistent with that of the exosomes. As hCMEC/D3 cells were incubated with the PKH67-labeled extracellular vesicles for 6 hours at 37 °C, the fluorescence signals derived from the extracellular vesicles were detected in the cytoplasm. Uptake of the PKH67-labeled extracellular vesicles was significantly reduced at 4°C compared to that at 37 °C. The extracellular vesicles uptake was also inhibited in the presence of excess unlabeled extracellular vesicles. The

neutralizing antibody against an adhesion molecule significantly decreased the extracellular vesicles uptake by hCMEC/D3 cells. These results suggest that the BBB has an active transport system for the extracellular vesicles derived from trophoblast stem cells and the adhesion molecule would be involved in the internalization of trophoblast stem cells-derived extracellular vesicles by human microvessel endothelial cells. The human trophoblast stem cells-derived extracellular vesicles would have the potential as a drug carrier which can be crossing the BBB.

P320 - ANALYSIS OF DRUG-ENDOGENOUS SUBSTRATE INTERACTIONS USING *IN VITRO* AND *IN VIVO* METHODS

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Purpose: Every year, more than 700,000 people worldwide die by suicide, making it an important issue in public health. Natural causes of suicide have been suggested to include the presence of genetic polymorphisms of P-glycoprotein (P-gp) and a significant increase in the brain concentration of its biological substrate, testosterone (TES). We hypothesized that drug-endogenous substrate interaction (DEI) at the blood-brain barrier (BBB) mediated by P-gp may be behind this increase in TES concentration in the brain. Therefore, we analyzed DEI using *in vitro* and *in vivo* methods with the aim of constructing "molecular preventive suicidology" as a new natural science approach to suicide prevention. **Methods:** Using P-gp overexpressing LLC-GA5-COL150 cells, we identified drugs that inhibit TES efflux from the cells. In addition, transport studies were performed using a rat BBB kit (Pharmacocell) to estimate drugs that interact with TES. The selected test drugs were administered intraperitoneally to rats, and whole brain and plasma samples were collected after the final administration to calculate TES concentrations and brain/plasma drug concentration ratios (Kp values). **Results and Discussion:** In the uptake study using LLC-GA5-COL150 cells, a significant increase in intracellular accumulation of TES was observed with several drug combinations. Furthermore, when the transport activity of TES in rat BBB kits was confirmed using these drugs, a significant decrease in transport activity was observed for flunitrazepam, oseltamivir, and cyclosporine. The *in vivo* interaction of these three drugs with TES in the rat BBB showed a significant increase in the Kp value of TES under the combined use of these drugs. This study suggests that the drugs and the endogenous substrate TES may cause P-gp-mediated interactions in the BBB *in vitro* and *in vivo*. The clinical side effects of these drugs were thought to involve DEI, at least in part, in the development of abnormal behavior and depression, which are consistent with the involvement of TES. The results of this study are expected to provide an opportunity for personalized medicine based on a new natural science approach for suicide prevention.

P321 - INVESTIGATION OF CREATINE PHARMACOKINETICS AFTER CHRONIC ORAL ADMINISTRATION IN CREATINE TRANSPORTER-DEFICIENT MICE

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Purpose: The creatine transporter (CRT, SLC6A8) is a membrane protein that transports creatine. It is expressed in the small intestine, brain, and kidneys. CRT-deficiency syndrome is characterized by reduced brain creatinine concentrations. Repeated oral creatine administration enhances the muscles and treats cerebral creatine deficiency syndromes caused by deficits in creatine synthesis enzyme genes. To date, oral creatine administration has not shown therapeutic efficacy in CRT-deficiency syndrome, and the influence of oral creatine administration on creatine pharmacokinetics remains unclear. Thus, our aim was to evaluate changes in creatine concentration in the plasma, brain, and urine of CRT knockout (Slc6a8-/-, CRT-KO) mice after 30 d of chronic oral creatine administration.

Methods: CRT-KO and wild-type (WT) mice were orally administered drinking water containing 8.0 mg/mL creatine for 30 d (n = 5-8). Control mice received water without creatine (n = 6/group). Creatine concentrations were measured by liquid chromatography-tandem mass spectrometry.

Results and Discussion: In the control group, the plasma creatine concentration in CRT-KO mice was 55% lower than that in WT mice. After 30 d of creatine administration, plasma creatine was increased by 9.1- and 2.3-fold in CRT-KO WT mice, respectively, compared to the concentrations in the control group. Plasma creatine concentrations in CRT-KO mice treated with creatine were significantly higher than those in control WT mice, suggesting that creatine is absorbed from the intestine, even in CRT-KO mice. In the control group, cerebral creatine concentrations were significantly lower by 83% in CRT-KO mice than in WT mice, suggesting that CRT is involved in supplying creatine to the brain. Despite the increase in plasma creatine concentrations following administration, cerebral creatine concentrations were not significantly different

between the creatine administration and control groups in KO or WT mice. Therefore, in WT mice, the creatine supplied to the brain by CRT reaches saturation with normal plasma creatine concentrations. In control group urine, the creatine concentration in CRT-KO mice was 7.9-fold greater than that in WT mice. Creatine administration increased urinary creatine concentrations by 15- and 51-fold in CRT-KO and WT mice, respectively, suggesting that CRT participates in creatine reabsorption by the kidneys.

Conclusion: CRT is involved in supplying creatine to the brain and creatine reabsorption by the kidneys. A mechanism other than CRT mediates intestinal creatine absorption in CRT deficiency; however, increasing blood creatine concentrations does not recover brain creatine concentrations. This study demonstrates that oral creatine administration alone is insufficient to remedy cerebral creatine deficiency. Thus, developing alternative therapeutic approaches for creatine transporter deficiency is necessary.

P322 - TAURINE TRANSPORT MECHANISM IN AN *IN VITRO* HUMAN BLOOD-BRAIN BARRIER MODEL CELL LINE

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Taurine is one of the most abundant amino acids in the body, and plays an important role in cellular osmoregulation, neurotransmission, and neuroprotection in the brain. For sufficient supply to the brain, taurine must cross the blood-brain barrier (BBB). To clarify the transport mechanisms for taurine at the BBB in humans, we analyzed characteristics of taurine transport using an *in vitro* human BBB model cell line (hCMEC/D3 cells). [³H]Taurine uptake by hCMEC/D3 cells increased in a time-, extracellular Na⁺ and Cl⁻-dependent manner. In addition, the uptake was inhibited by co-exposure of substrates for taurine transporter (TauT) and γ-aminobutyric acid (GABA) transporter (GAT). Moreover, the uptake was saturable with a Michaelis constant (Km) of 94 μM, which is similar to Km values of human TauT-mediated taurine transport (7-18 μM¹, 2). On the other hand, the half maximal inhibitor concentration (IC₅₀) value of GABA was 324 μM, which is close to Km values of human TauT-mediated GABA transport (564-982 μM³, 4). These results suggest that TauT is a major contributor to taurine uptake by hCMEC/D3 cells. After being taken up into the cells, taurine must undergo efflux from the cells in order to be distributed in the brain. To identify the transporter mediating the taurine efflux transport from the cells, we focused on monocarboxylate transporter (MCT) 7 since MCT7 has recently been reported as a facilitative taurine transporter⁵. In efflux transport analyses, taurine efflux from hCMEC/D3 cells increased for at least 60 min, and the treatment of siRNA targeting MCT7 significantly reduced MCT7 mRNA levels and the [³H]taurine efflux from the cells by 93% and 12%, respectively. These data suggest that MCT7, at least partly, contributes to the taurine efflux from hCMEC/D3 cells. Taken together, the present study suggests that TauT and MCT7 are involved in taurine supply at the human BBB. Our findings are expected to enhance our understanding of maintaining homeostasis of taurine levels in the human brain.

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P323 - CHARACTERIZATION OF AGE-DEPENDENT ABUNDANCE OF DRUG TRANSPORTER PROTEINS AND CONJUGATING ENZYMES IN HUMAN HEPATOCYTES: IMPLICATION FOR PEDIATRIC DRUG DOSING

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Approximately 50% of the drugs administered to children lack age-appropriate dosing guidelines and are used "off-label" in clinic. Age-dependent differences in drug elimination processes pose a serious challenge in predicting pediatric pharmacokinetics and dosage. Legislation like the Best Pharmaceuticals for Children Act (BPCA) and the Pediatric Research Equity Act (PREA) has spurred global pediatric clinical trials. In the absence of clinical data, the FDA recommends using physiologically-based pharmacokinetic (PBPK) modeling as a potential alternative or a complementary tool for predicting drug pharmacokinetics in children. These models utilize physiological differences in pediatric populations, including age-dependent differences in the abundance of drug-metabolizing enzymes and transporters

(DMET), to mechanistically extrapolate adult pharmacokinetic data to children. In the past decade, abundances of hepatic DMET proteins have been quantified in various subcellular fractions isolated from frozen tissues¹⁻³. These data are prone to high technical variability due to manual tissue processing and fraction enrichment. Therefore, we quantified hepatic drug transporters and conjugating enzymes in 50 pediatric and 8 adult human hepatocytes using global quantitative proteomics to investigate the association of age and sex with the abundance of these proteins. Out of the 34 studied proteins, OAT7, P-gp, UGT2B17, and SULT1E1 exhibited the highest variability among uptake transporters, efflux transporters, UGTs, and SULTs with a %CV of 95, 80, 280, and 214%, respectively. Proteins were divided into three groups (Class 1-3) based on their age-dependent abundance patterns. Class 1 proteins (total, 5) showed the highest abundance after birth and their levels decreased with age. Class 1 included MRP6, OAT2, OAT7, SULT1E1, and UGT2B17 proteins. MRP6, OAT7, and SULT1E1 were highest in <1 year old samples and reached minimal levels in >1 year age samples. Class 2 included proteins (total, 5) that did not change with age (MRP2, OATP1B1, OATP1B3, OATP2B1, and PAPST1); whereas class 3 proteins (total, 24) were expressed at low levels at birth and increased to reach adult levels. For example, the abundance of P-gp, MRP3, and UGT1A6 was negligible in <1 year age samples and increased significantly after 1 year of age. Analysis of the association of the protein abundance with sex yielded four proteins, OAT2, UGT1A3, UGT2B7, and SULT1B1, which had significant differences between males and females ($p < 0.05$). The current study strives to improve the knowledge base of the ontogeny of hepatic DMET proteins using cryopreserved human hepatocytes. The generated data can be incorporated in PBPK models to improve the prediction of age-dependent drug pharmacokinetics and dose, leading to safer drug pharmacology in children.

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P324 - IDENTIFICATION AND VALIDATION OF SELECTIVE ENDOGENOUS SUBSTRATES OF ORGANIC ANION TRANSPORTER 1 (OAT1) VERSUS OAT3 USING METABOLOMICS AND IN-VITRO MECHANISTIC STUDIES

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Endogenous substrates of drug transporters can serve as potential phenotypic biomarkers to predict transporter-mediated drug-drug interactions (DDIs) and interindividual variability in transport function.¹ Renal organic anion transporter 1 (OAT1) and OAT3 are expressed on the basolateral membrane of kidney proximal tubule cells and facilitate the active secretion of several drugs and endogenous substrates. Glycochenodeoxycholate-3-sulfate, 6 β -hydroxycortisol, and pyridoxic acid are more selective substrates for OAT3, whereas taurine is considered more selective for OAT1. However, these biomarkers are confounded by the contribution of other drug-metabolizing enzymes and transporter (DMET) proteins, high interindividual variability, and/or show non-significant changes in plasma concentrations upon OAT1/OAT3 inhibition.² The goal of the present study was to identify and validate selective clinical biomarkers of OAT1 and OAT3 using a state-of-the-art metabolomics-based DMET biomarker discovery approach. For biomarker identification, human plasma (pooled samples collected at 1, 1.5, and 2 h) and urine (0-4 h) samples from a clinical pharmacokinetic DDI study ($n=16$ healthy adults) involving oral furosemide (5 mg, OAT1/3 substrate) and probenecid (1000 mg, OAT1/3 inhibitor), were analyzed by LC-HRMS, followed by data analysis using XCMS Online software (xcmsonline.scripps.edu). Untargeted metabolomics analysis yielded >10,000 features (m/z values) that were either increased or decreased in pooled plasma and urine samples in the presence of probenecid. Features that were significantly increased in the pooled plasma (1.45- to 10-fold) and exhibited either a decrease or no change in urine samples, along with a renal clearance ratio of < 0.8, were identified as putative OAT1/OAT3 biomarkers. The putative OAT1/OAT3 biomarkers (m/z range: 130-467) were predicted to be fatty acids, glucuronide conjugates, peptides, steroids, and tryptophan metabolites. The selectivity of these potential clinical biomarkers was validated using mechanistic in-vitro assays, wherein the uptake of endogenous substrates measured in the processed human urine was compared between transporter over-expressing cells and corresponding mock cells. The putative biomarkers were screened as substrates for OAT1, OAT2, OAT3, organic anion transporting polypeptide 1 (OATP1B1), and OATP1B3. Of the 63 putative OAT1/OAT3 biomarkers identified in the clinical samples, 4 compounds (m/z values: 130, 159, 328, and 337) were confirmed as selective OAT1 substrates. Similarly, 12 features (m/z range: 184-467) were confirmed as selective OAT3 substrates. The intraindividual and interindividual variability of validated biomarkers was assessed by analyzing the full pharmacokinetic profiles (0-24 h) for these biomarkers in 4 participants. The structures of these validated biomarkers will be elucidated using an MS/MS fragmentation approach and synthetic standards, followed by a detailed pharmacokinetic analysis (0-24 h) in 16 participants. These biomarkers may have utility for determining the selectivity of new chemical entities towards inhibiting OAT1 versus OAT3 in the clinic as well as their interindividual variability. The integrated metabolomics and in-vitro

approaches used in this study can be extended to determine selective biomarkers of other drug transporters (such as OATP1B1 versus OATP1B3) and drug-metabolizing enzymes.

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P325 - FUNCTIONAL IDENTIFICATION OF A LYSOSOMAL TRANSPORTER SLC46A3 AS A NOVEL GLYCOSIDE TRANSPORTER

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Flavonoid is a kind of polyphenolic compound having a C6-C3-C6 backbone structure. They exhibit various pharmacological effects including anti-inflammatory, antioxidant, anti-cancer, and anti-obesity properties, making them promising drug candidates. Generally, most flavonoids exist in plants as sugar-conjugated forms, flavonoid glycosides. Due to their physicochemical properties, flavonoid glycosides typically have low membrane permeability, which limits their pharmacological effects on intracellular targets. Therefore, it is believed that the molecular entity of pharmacological effects of flavonoids on intracellular targets is almost based on aglycones, and the effects of flavonoid glycosides might be underestimated. To appropriately evaluate the intracellular pharmacological effects of flavonoid glycosides, a methodology to deliver flavonoid glycosides into the cytosol is required; however, such methodology has not been established because a transporter for flavonoid glycosides in mammals has not been identified. Preliminary examinations showed that flavonoid aglycones markedly inhibited the uptake of 5-carboxyfluorescein, a fluorescent probe, by SLC46A3. Therefore, in this study, we investigated whether SLC46A3 transports flavonoid aglycones and their glycosides. The uptake of flavonoid aglycones (hesperetin, naringenin, and quercetin) and their glycosides (hesperidin, naringin, and rutin) was measured at pH 5.0 in MDCKII cells expressing SLC46A3 dC (MDCKII/SLC46A3 dC), which is mutated for the altered localization at the plasma membrane from lysosomes by lacking a tyrosine motif in the C-terminus region of SLC46A3. The uptake of hesperetin, naringenin, and quercetin were comparable between MDCKII/SLC46A3 dC cells and mock cells, suggesting that these flavonoid aglycones were not recognized as substrates, albeit having potent inhibitory effects on SLC46A3. Surprisingly, the uptake of hesperidin, naringin, and rutin were significantly increased in MDCKII/SLC46A3 dC cells in a time-dependent manner, while the uptake in mock cells remained minimal throughout experimental period. The uptake of these glycosides mediated by SLC46A3 dC was pH-sensitive and significantly decreased at 4°C compared to 37°C. These results suggest that flavonoid glycosides tested in this study were transported into the cells in a proton-dependent manner. In conclusion, our results indicated that a lysosomal transporter SLC46A3 is a novel flavonoid glycosides transporter. SLC46A3 could be useful as a tool for delivering flavonoid glycosides into the cytosol to appropriately estimate their intracellular pharmacological effects.

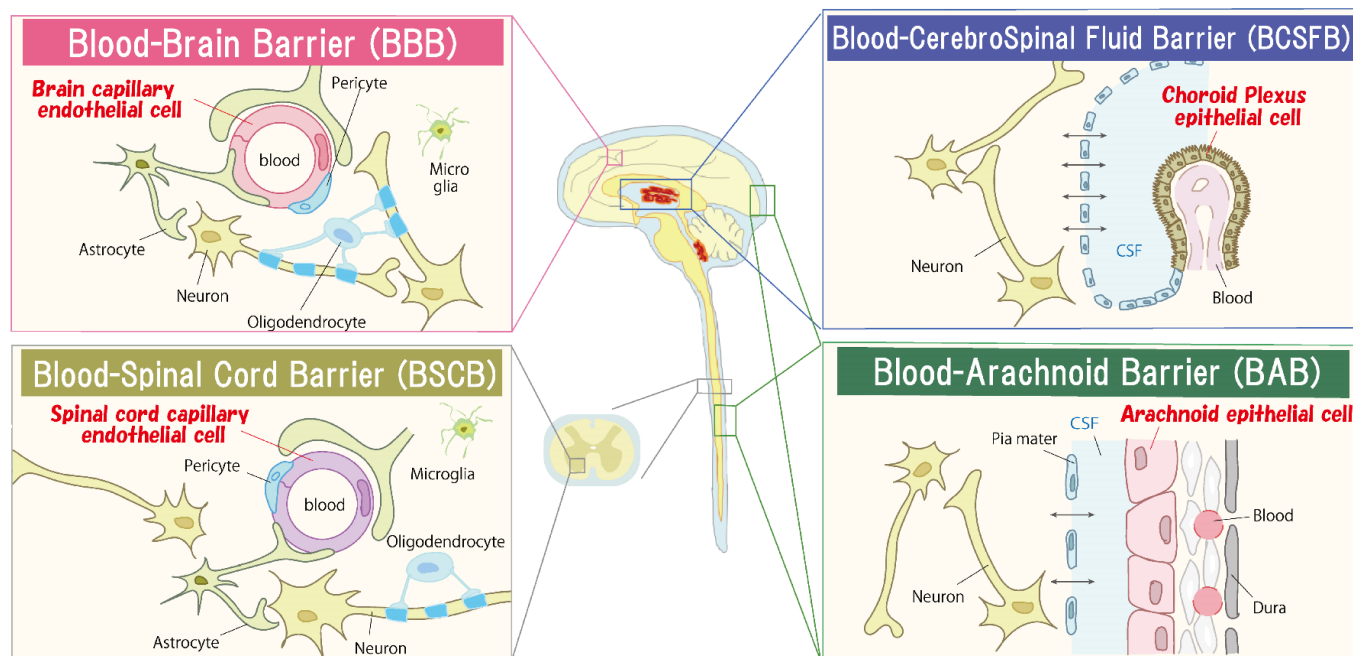
P326 - NEXT GENERATION QUANTITATIVE PROTEOMICS-BASED CENTRAL NERVOUS SYSTEM BARRIER STUDIES

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In 2011, using the quantitative Targeted Absolute Proteomics (qTAP) method, we reported that, unlike rodents, the protein expression levels of known organic anionic and organic cationic drug transporters are under limits of quantification in the human blood-brain barrier (BBB). It raised the question how we can understand the pharmacokinetics of anionic and cationic drugs in the human brain. Using the qTAP method, we also reported that, among the known receptors, the protein expression level of transferrin receptor was the greatest. It also raised the question "Is not really there a more rapid pathway for the delivery of macromolecular drugs into the brain than transferrin receptor?". To answer these questions, we have developed a new technique to comprehensively reveal the absolute expression levels of proteins (quantitative Global Absolute Proteomics, qGAP method), have comprehensively searched for transporter and receptor candidates expressed in the human BBB, and comprehensively determined their absolute protein expression levels. For the most highly expressed transporter candidate molecule, we generated the gene knockout cell line (hCMEC/D3 cells) and mouse, and revealed for the first time that it is a novel drug transporter in the BBB. Furthermore, for the first time, the transport mechanisms of anionic and cationic drugs, which could not be explained by the BBB transporters, were found to abundantly exist in the blood-arachnoid barrier (BAB). As for the receptors, we identified the receptors whose protein expression levels are about 10-fold higher than that of the transferrin receptor, and is going to demonstrate its usefulness

for drug delivery to the brain. In my presentation, I would like to introduce the new CNS barrier studies opened by comprehensive absolute quantification.



P327 - MONOCARBOXYLATE TRANSPORTERS 8 AND 10: SIBLINGS WITH DIFFERENT PERSONALITIES

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Monocarboxylate transporters 8 (MCT8) and 10 (MCT10) are members of the SLC16A family (SLC16A2 and SLC16A10, respectively) with high amino acid identity. To compare properties of these two transporters, we used OPTI-EXPRESSION™ technology to develop assays for MCT8 and MCT10 in MDCK-II cells. An advantage of this assay system is that it allows investigation of transport into polarized MDCK-II cells from either the apical side or the basolateral side, as well as transcellular transport through the cell monolayer, providing flexibility in assay design. MDCK-II cells transfected with MCT8 showed uptake of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) across both the apical and basolateral membrane, with a preference for the basolateral membrane. The K_m values were determined to be 24 μM for T3 and 28 μM for T4. MCT8 also enhanced the transcellular transport of T3 and T4 through the cell monolayers by more than 10-fold. MCT8 appears specifically transport thyroid hormones as no transport of amino acids was observed. In contrast, MCT10 did show affinity towards amino acid substrates such as tyrosine, phenylalanine, and levodopa. However, rather than increased uptake, 4-fold lower accumulation was seen in MDCK-II cells transfected with MCT10 than in matched control cells, suggesting that MCT10 functions as an efflux transporter rather than an uptake transporter towards these amino acids. Efflux activity was confirmed by examining the basolateral to apical (B->A) transcellular permeability, where MCT10 increased the transport through the cell monolayer approximately 5-fold for these amino acids. MCT10 also enhances the transcellular transport of T3, with 20-fold higher apical to basolateral (A->B) than in the matched control cells. In addition, MCT10 can function as an uptake transporter towards T3, increasing accumulation of T3 by 4-fold over that seen in control cells, with a K_m value of 30 μM . No indications of sodium-dependent transport or pH-dependent transport were observed for either MCT8 or MCT10, and addition of accessory proteins like CD147 or PDZK1 that are important for other members of the SLC16A family also had no effect on MCT8 or MCT10 activity. MCT8 and MCT10 are similar in that both can transport thyroid hormones in either an uptake or efflux mode, and both are expressed on both apical and basolateral membranes, although MCT8 appears to have a preference for T4 while MCT10 has a preference for T3. In contrast, MCT8 cannot transport amino acids, while MCT10 functions as an efflux transporter for several aromatic amino acids. Thus, while these two closely related transporters share many properties, they also demonstrate key differences.

P328 - INVESTIGATING THE ROLE AND FUNCTION OF OATP DRUG TRANSPORTERS IN RELATION TO TAMOXIFEN THERAPY

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Breast cancer is one of the most frequently diagnosed cancers worldwide. Patients with estrogen receptor positive (ER+) breast cancer are commonly prescribed tamoxifen, an antiestrogen therapy. Therapeutic outcomes have mainly focused on characterizing the extent of tamoxifen metabolism into its principal active metabolite endoxifen. However, up to 40% of patients will develop *de novo* or acquired tamoxifen resistance during therapy. Furthermore, therapeutic failure in patients who attain normal endoxifen plasma levels suggests that there are contributing factors independent of tamoxifen metabolism. Based on previous studies, we propose that altered expression of drug uptake transporters may impact estrogen as well as tamoxifen and its metabolite disposition and thereby modulate the extent of ER activation in target breast cancer tissues. It has been previously shown that certain organic anion transporting polypeptides (OATPs; OATP1A2 and OATP2B1) have altered expression in malignant breast cancer tissue compared to healthy breast tissue. Therefore, the objective of our study is to characterize the impact of tamoxifen and endoxifen on OATP-mediated estrogen transport. Cellular uptake assays were conducted by transiently transfecting HEK293T cells with vector-only control (pcDNA), OATP1A2, or OATP2B1 plasmids. To determine if endoxifen and tamoxifen inhibit transport of estrone 3-sulfate (E1S), cells were treated with 1µg/mL of E1S alone and in combination with 1µM endoxifen, tamoxifen, or rifampicin (OATP inhibitor) in a 10-minute co-incubation. Relative intracellular E1S concentration was measured using ultra-high performance liquid-chromatography tandem mass-spectrometry (UHPLC-MS/MS). HEK293T cells transfected with OATP1A2 and OATP2B1 showed a 224.6% ($p=0.0015$) and 521.7% ($p=0.0001$) increase in E1S uptake compared to control, respectively. As expected, rifampicin treatment markedly inhibited E1S transport by 52.6% ($p=0.02$) and 73.2% ($p=0.0001$) in both OATP1A2 and OATP2B1, respectively. Endoxifen treatment also significantly inhibited E1S transport in OATP1A2 and OATP2B1 transfected cells by 44.0% ($p=0.0395$) and 31.2% ($p=0.0017$), respectively compared to OATP transfection alone. Interestingly, tamoxifen treatment significantly decreased E1S transport in OATP2B1 ($p=0.0001$), but not in OATP1A2 ($p>0.05$) transfected cells. Our study demonstrates that tamoxifen and endoxifen can directly modulate OATP-mediated transport of E1S. Accordingly, altered OATP transporter activity could play a significant role in modulating ER activation and possibly tamoxifen therapeutic response. Studying the functional implications of drug transporters in breast cancer could provide important insights to their clinical relevance.

P329 - NIEMANN-PICK C1-LIKE 1 REGULATES *IN VIVO* BEHAVIORS OF SPHINGOLIPIDS

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Background and Aim: Niemann-Pick C1-Like 1 (NPC1L1) is a transmembrane protein highly expressed in the intestine, particularly in the luminal membrane of intestinal epithelial cells. NPC1L1 functions as a cholesterol importer to mediate intestinal cholesterol absorption and is reported to be a target molecule of ezetimibe, a cholesterol absorption inhibitor used clinically for dyslipidemia. Our previous studies demonstrated that NPC1L1 is involved in intestinal absorption of fat-soluble vitamins, such as vitamin E and vitamin K1, in addition to cholesterol (1, 2). Given the large difference in the chemical structures of these vitamins and cholesterol, NPC1L1 may have relatively broad substrate specificity and regulate the intestinal absorption of various dietary lipids. The aim of this study was to find new NPC1L1 substrates and reveal additional physiological roles of NPC1L1.

Methods and Results: Based on our previous observations that very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) increase after high-fat diet (HFD) feeding in wild-type (WT) mice but not in *Npc1l1* knockout (KO) mice, we hypothesized that NPC1L1-related molecules are in VLDL/LDL. Comprehensive analyses of NPC1L1-dependent VLDL/LDL components revealed that NPC1L1-dependent increases in sphingosine-1-phosphate (S1P), a bioactive lipid, and apolipoprotein M, an S1P binding protein, in VLDL/LDL from mice fed an HFD. Because S1P is a metabolite of sphingomyelin (SM) and SM is abundant in HFD, we hypothesized that NPC1L1 is involved in the intestinal absorption of SM. *in vitro* SM uptake studies using NPC1L1-overexpressing cells and *in vivo* SM absorption studies with WT and *Npc1l1* KO mice demonstrated that, consistent with our hypothesis, SM is a substrate of NPC1L1 and intestinal absorption of SM is controlled by the NPC1L1-mediated pathway.

Conclusion and Discussion: Our results suggest that the cholesterol importer NPC1L1 is involved in the intestinal absorption of SM and regulates VLDL/LDL-S1P levels. These findings would contribute to a better understanding of molecular mechanisms of intestinal absorption of sphingolipids and provide novel insights into sphingolipids and S1P homeostasis.

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P330 - ANIMAL SPECIES DIFFERENCES IN THE AMILORIDE TRANSPORT FUNCTION OF SLC19A3

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SLC19A3, known as thiamine transporter 2 (THTR2), has been of increasing interest for its multispecific transport function for several basic compounds, including pyridoxine as a newly identified substrate, in addition to thiamine as the originally identified one (1). This transporter is also notably unique in its animal species differences in the transport function. As revealed in our recent study, the rat and mouse orthologs of SLC19A3 can transport thiamine but not pyridoxine, whereas human SLC19A3 can transport both of them (2). It was subsequently attributed to the presence of seven human SLC19A3-specific amino acid residues that are involved in pyridoxine transport but not in thiamine transport (3). To further clarify the multispecific and animal species-dependent transport function of SLC19A3, we examined in the present study its functional characteristics for the transport of amiloride, a newly found fluorescent substrate, focusing on the aspect of species differences. We first conducted a series of experiments using MDCKII cells stably expressing human SLC19A3, evaluating the initial uptake of amiloride (10 μ M) for 10 min at 37°C by fluorometric detection. SLC19A3 was found to transport amiloride in a pH-dependent manner, favoring neutral to basic conditions over acidic conditions. This characteristic was similar to that of thiamine transport, different from pyridoxine transport, which is more efficient under acidic conditions (1). Kinetically, when evaluated at pH 7.4, the K_m of SLC19A3-mediated transport of amiloride was 63.1 μ M, being higher than those of thiamine and pyridoxine (2.36 μ M and 18.5 μ M, respectively) and, hence, indicating a low affinity characteristic. In the subsequent assessment of species differences in the amiloride transport function using transiently transfected HEK293 cells, the rat and mouse orthologs of SLC19A3 were found to lack the function, whereas human SLC19A3 was confirmed to have that. This species-dependent characteristic was similar to that in the pyridoxine transport function, suggesting a possibility that amiloride and pyridoxine may share the substrate recognition site that includes the human SLC19A3-specific amino acid residues determined to be involved in pyridoxine transport. It should be noted, however, that amiloride transport was found to be similar to thiamine transport but not to pyridoxine transport in pH sensitivity and, hence, there may be another set of amino acid residues that determine the differences in pH sensitivity. Caution may be needed when selecting model animals in studies related to SLC19A3-mediated transport, although more detailed studies should be needed to fully clarify the unique characteristics of SLC19A3.

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P331 - XENOBIOTIC TRANSPORTER SLC22A4 EXPRESSED IN MICROGLIA AND NEURON AS A POTENTIAL TARGET FOR NEUROGENESIS IN ALZHEIMER'S DISEASE MODEL MICE

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Introduction: Microglia are resident immune cells in the brain that have attracted attention as a target for Alzheimer's disease (AD) treatment because they may play an important role in the progression/suppression of AD pathology by phagocytosis of amyloid- β (A β) and altering inflammatory/anti-inflammatory polarity. Xenobiotic transporter SLC22A4/OCTN1 is functionally expressed in microglia, as well as neural stem cells and neurons in the brain, and accepts a food-derived amino acid ergothioneine (ERGO) as a substrate. ERGO is well absorbed and distributed to the brain parenchyma by OCTN1 despite its hydrophilicity and low membrane permeability, enhancing cognitive function at least partially by promoting neurogenesis/neuronal maturation. Thus, this transporter/substrate combination may be a promising target to develop therapeutic agents for the treatment of neurodegenerative diseases. This study aimed to clarify the uptake and biological effects of ERGO via OCTN1 in the microglia of AD model AppNL-G-F mice.

Methods: Functional expression of OCTN1 in primary cultured microglia (PMG) derived from neonatal mice was evaluated by [³H]ERGO transport study. To evaluate the effect of ERGO on microglia under the pathological condition of AD, PMG was isolated from adult AppNL-G-F mice. To examine the effect of ERGO on cognitive deficiency in AppNL-G-F mice, ERGO or vehicle was orally administered once a week to 5-week-old to 7-month-old AppNL-G-F mice.

Results and Discussion: The uptake of [³H]ERGO into PMG increased in a time-dependent manner and decreased in the presence of unlabeled ERGO. PMG isolated from AppNL-G-F mice treated with ERGO exhibited significantly higher gene expression of Trem2 which is known to promote phagocytosis. Correspondingly, the phagocytic index assessed as microglial bead-phagocytic activity tended to be increased in the ERGO-treated group. Thus, ERGO is taken up by PMG

and may promote phagocytosis. Oral administration of ERGO in AppNL-G-F mice prevented cognitive decline assessed in the spatial recognition test. Immunohistochemical analysis in the hippocampus showed that the area of doublecortin-positive cells, a marker of newborn neurons, was significantly higher in ERGO-treated mice than in control mice, suggesting the promotion of neurogenesis. Thioflavin staining showed a significant reduction of A β plaques in the cerebral cortex after ERGO treatment. This may be consistent with the immunofluorescent images showing that the ratio of anti-inflammatory (Cd206) to inflammatory (Cd86) markers in microglia was increased in the ERGO-treated group.

Conclusion: The SLC22A4/ERGO axis may be a promising target for another type of therapeutic activity, possibly by promoting neurogenesis and anti-inflammatory microglial polarization.

P332 - FUNCTIONAL IDENTIFICATION OF DISRUPTED IN RENAL CARCINOMA 2 (DIRC2/SLC49A4) AS A LYSOSOMAL EXPORTER FOR CATIONIC AMPHIPHILIC DRUGS

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Our recent study has revealed that SLC49A4, known as disrupted in renal carcinoma 2, is an H⁺-coupled lysosomal exporter for pyridoxine (vitamin B6), a cationic compound.¹⁾ We examined in the present study the possibility that this transporter might also transport cationic amphiphilic drugs (CADs) that are known to undergo lysosomal trapping, using pyrilamine, an H₁-antagonist, as a model CAD. The COS-7 cell line was used as a model cell system for transient introduction of human SLC49A4 and a recombinant SLC49A4 protein (SLC49A4-AA), in which the N-terminal dileucine motif involved in lysosomal localization was removed by replacing with dialanine for redirected localization to the plasma membrane. Functional analysis was carried out by the evaluation of the SLC49A4-AA-mediated cellular uptake of [3H]pyrilamine (10 nM) for 10 sec in the initial phase in transiently transfected COS-7 cells. We also examined the effect of the modulation of SLC49A4 expression on the accumulation of [3H]pyrilamine (10 nM) in the intracellular compartments after 30 min of incubation in the presence or absence of digitonin (50 μ M) for plasma membrane permeabilization. Functional analysis using the SLC49A4-AA mutant, which operates for cellular uptake at the plasma membrane in transiently transfected COS-7 cells, demonstrated its H⁺-coupled operation for pyrilamine transport, which was saturable with a Michaelis constant of 132 μ M at pH 5.5. In addition, many CADs that may potentially undergo lysosomal trapping, which include imipramine, propranolol, verapamil, and some others, were found to inhibit SLC49A4-AA-mediated pyrilamine transport, suggesting their affinity for SLC49A4. The CADs that inhibited SLC49A4-AA-mediated pyrilamine transport could also be SLC49A4 substrate candidates. Further, the introduction of SLC49A4 into COS-7 cells induced a significant decrease in the accumulation of pyrilamine in the intracellular compartments in the cells treated with digitonin for permeabilization of plasma membranes, suggesting its operation for lysosomal pyrilamine export. These results suggest the involvement SLC49A4 in the lysosomal trapping of pyrilamine as a lysosomal exporter. The functional level of SLC49A4 may have an impact on the disposition and therapeutic effect of lysosomotropic CADs. It should be noted, however, that the conclusions are based on findings in model cell systems expressing SLC49A4-AA, and we cannot exclude the possibility that such recombinant SLC49A4 proteins might be functionally modulated. The function of FLAG-SLC49A4 may also be affected by the digitonin treatment to permeabilize plasma membranes for the assessment of its operation at the lysosomal membrane. Therefore, it is necessary to examine the function of unmodulated SLC49A4 under physiologically relevant conditions in the future.

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P333 - DRUG-DRUG INTERACTION OF PACLITAXEL AND TAS-119, A NOVEL AURORA A KINASE INHIBITOR, MEDIATED BY ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B1

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TAS-119 is a novel, selective, and orally active inhibitor of Aurora A, a key regulator in mitotic cell division. Aurora A is associated with spindle assembly checkpoint and regulation of the transition from G2 to M phase, and its overexpression associated with poor disease progression and survival in various cancers. Furthermore elevated levels of Aurora A are reported to override the mitotic spindle checkpoint activated by treatment with chemotherapeutic agents like taxanes, and are linked to taxane resistance. To overcome this taxane resistance, coadministration of TAS-119 with paclitaxel has been investigated in clinical study. On the other hand, Mori et al¹⁾ reported that paclitaxel could inhibit organic anion transporter polypeptide (OATP) transporters and cause drug-drug interaction at the clinical dosage. In this study we investigated the substrate specificities of OATP transporters for TAS-119 and evaluated the OATP transporter-mediated drug-drug interaction between paclitaxel and TAS-119. And we also evaluated the effect of gene polymorphisms in SLC01B1 (the gene coding for OATP1B1) on the pharmacokinetics of TAS-119. The time-dependent uptake of TAS-119 into OATP1B1- and OATP1B3-expressing HEK293 cells was observed. Concentration-dependent uptake of TAS-119 into OATP1B1-expressing HEK293 cells was saturated with the K_m value of 0.60 μ M. And the OATP1B1 mediated uptake of TAS-119

was inhibited by paclitaxel, but that of paclitaxel was not inhibited by TAS-119. The clinical study suggested that the concomitant dosing of TAS-119 and paclitaxel caused the increase of exposure of TAS-119, but the mean values of any pharmacokinetic parameters of paclitaxel were comparable among the same dose groups of paclitaxel. On the other hand, in this clinical study, no obvious differences on oral clearance of TAS-119 among SLCO1B1 genetic variants, which were detected by restriction fragment length polymorphism analysis and DNA sequencing, were observed. These results suggested that TAS-119 was a substrate for OATP1B1 and 1B3 transporter. In clinical study, the exposure of TAS-119 was increased by coadministration with paclitaxel due to the inhibition of OATP1B1-mediated transport of TAS-119 by paclitaxel, but no impact of TAS-119 on pharmacokinetics of paclitaxel was found. And no obvious effect of SLCO1B1 variants, which were detected in this study, on the exposure of TAS-119 was observed.

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P334 - *IN VITRO* ASSESSMENT OF THE INHIBITION OF HUMAN ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B1/1B3 BY HIGHLY PLASMA PROTEIN BOUND COMPOUNDS IN THE PRESENCE OF PHYSIOLOGICAL CONCENTRATION OF ALBUMIN

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The objective of the study was to establish *in vitro* assay for organic anion transporting polypeptide (OATP1B1/OATP1B3) transporters which enable the assessment of transporter inhibition by highly plasma protein bound compounds that is relevant to the *in vivo* situation. One approach to predict *in vivo* drug-drug interaction is the use of 4% human serum albumin (4% HSA: approximating human plasma albumin concentration)^{1,2}. The use of Human Embryonic Kidney (HEK) 293 cells overexpressing OATP1B1/OATP1B3 in protein-free Hank's balanced salt solution buffer (HBSS) versus HBSS with 4% HSA was explored for the inhibition of OATP1B1/1B3 by rifampin with the following OATP substrates: estradiol 17- β -glucuronide (E17G) for OATP1B1 and pravastatin for OATP1B3. In protein-free HBSS buffer, rifampin (100 μ M) decreased OATP1B1-mediated uptake of E17G (2 μ M) by 99.2%. In contrast, the expected inhibition of OATP1B1-mediated E17G uptake by rifampin was not observed in the presence of 4% HSA. To improve inhibition of OATP1B1-mediated E17G uptake by rifampin in the presence of 4% HSA, E17G was equilibrated with 4% HSA at 37°C shaking water bath for 5 hours prior to adding to OATP1B1 incubations. In protein-free HBSS buffer, rifampin (100 μ M) decreased OATP1B1-mediated uptake of E17G (2 μ M) by 94.8% and OATP1B1-mediated uptake of equilibrated E17G (5 μ M plus 4% HSA) by 68.3%. Positive control rifampin behaved as expected with and without 4% HSA under new experimental conditions. OATP1B3 substrate was examined under similar experimental conditions: rifampin (100 μ M) decreased OATP1B3-mediated uptake of pravastatin (10 μ M) by 90% in the presence or absence of 4% HSA. A key question is whether our findings apply to other highly plasma protein bound compounds. Suramin (a drug used in the treatment of African trypanosomiasis and river blindness) showed concentration-dependent inhibition of OATP1B1 activity (substrate E17G, 5 μ M) with an IC₅₀ value of 0.80 μ M in the absence of 4% HSA, while no inhibition was observed in the presence of 4% HSA. Results from Liraglutide (a glucagon-like peptide-1 agonist (GLP-1A) and other compounds will be shared in the meeting. In summary, a new assay has been established to predict potential *in vivo* inhibition of OATP1B1/B3 by highly plasma protein bound compounds using specific substrates (E17G, OATP1B1) and (pravastatin, OATP1B3) in the absence and presence of 4% HSA with positive control inhibitor rifampin.

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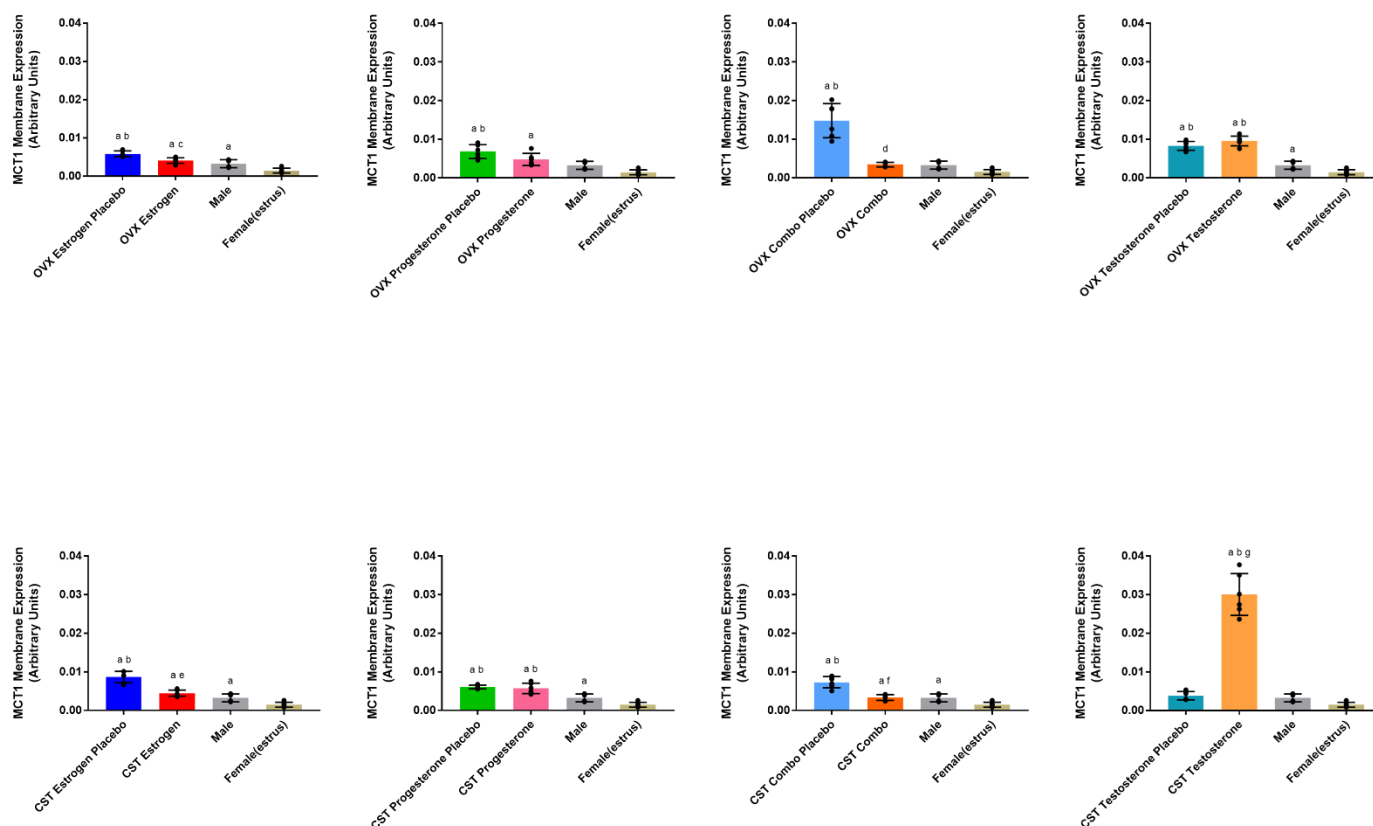
P335 - REGULATION OF RAT HEPATIC MONOCARBOXYLATE TRANSPORTER EXPRESSION BY SEX AND CROSS-SEX HORMONE TREATMENT

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Proton-dependent monocarboxylate transporters (MCTs) are involved in monocarboxylate uptake and efflux in a range of biologically important tissues and barriers, including the liver, kidney, and blood-brain barrier (BBB) (1). MCTs are involved in the transport of L-lactate and monocarboxylate drugs, including gamma-hydroxybutyrate, valproic acid, gabapentin, enacarbil, non-steroidal anti-inflammatories, and β -lactam antibiotics (1). Prior studies with intact females, males and ovariectomized females have elucidated the hormonal regulation of hepatic monocarboxylate transporter

expression, prompting investigation into the regulatory role of individual hormones (3). The hepatic expression of MCTs in response to exogenous male and female sex hormones among cis- and trans-gender populations remains poorly understood. In this study, we aim to address this knowledge gap by investigating the effect of exogenous sex and cross-sex hormones on hepatic MCT1, MCT4, and CD147 membrane-bound protein expression. Ovariectomy and castration surgeries were conducted on prepubertal Sprague Dawley rats, followed by administration of estrogen, progesterone, testosterone, or a corresponding placebo for 21 days prior to liver collection. The quantitative measurement of membrane-protein levels was conducted using Western blot. Quantitative analysis revealed estrogen alone or combination with progesterone reduced membrane MCT1 expression, while testosterone administration increased MCT1 membrane-protein expression. This aligns with the significantly lower MCT1 membrane protein expression observed in intact females when compared with intact males. The membrane expression of MCT4 exhibited a significant increase in intact females compared to intact males, whereas significance was not observed between the hormone-treated and placebo-treated groups, suggesting a lack of association with sex hormone regulation. No significance was observed between the hormone-treated and placebo-treated groups for hepatic CD147 protein expression. In summary, hepatic MCT1 expression is influenced by both sex and cross-sex hormone treatment, with regulation contingent upon exposure to sex hormones. In contrast, hepatic MCT4 regulation appears to be solely dependent on biological sex and is not affected by sex hormone treatment. These findings will support a comprehensive understanding of sex hormone-dependent regulated hepatic drug disposition and elimination of MCT substrates across various populations.



P336 - INVESTIGATION IN THE PREDICTIVE ACCURACY OF BRAIN KPUU ACROSS SPECIES USING *IN VITRO* AND *IN VIVO* METHODS

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Achieving adequate penetration across the blood-brain barrier (BBB) is paramount for the efficacy of central nervous system (CNS)-targeted therapeutics. Efflux transporters, notably P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), are critical determinants in modulating drug bioavailability within the brain. The parameter K_{puu}, the ratio of unbound drug concentrations in brain parenchyma to plasma at steady state, serves as a key indicator for assessing drug permeability and facilitating the optimization of CNS-active compounds. Enhancement of K_{puu} predictability, leveraging *in vitro* assays and efflux transporter proteomics at the BBB, has shown promise in refining CNS-targeted therapeutics. Our study aims to investigate the predictive accuracy of K_{puu} across species, utilizing efflux ratio (ER)-

based assessments and inter-species scaling, for the robust identification of BBB-permeable drugs. To this end, we evaluated the K_{puu} predictability for a cohort of 66 compounds, encompassing literature reported K_{puu} values in humans, non-human primates (NHP), rats, and mice. Each drug's apparent permeability (P_{app}) was measured at 1 μ M using NIH MDCK cells over-expressing human MDR1 in the presence of 5 μ M elacridar. ER values were determined at 1 μ M in NIH and in-house MDCK cells over-expressing human MDR1 and BCRP, respectively. In addition, K_{puu} values for each species were predicted based on either *in vitro* ER values corrected by relative expression factor or the measured K_{puu} value in a different species. For weak or non-P-gp substrates (ER in NIH cell line <10), P-gp ER-based K_{puu} prediction are within 3-fold of observed K_{puu} for highly permeable compounds (P_{app}>20) in human (81%), NHP (88%), rats (78%) and mice (73%). For compounds with P_{app}<5, P-gp ER-based method over-estimates K_{puu}. For good P-gp substrates (ER in NIH cell line >10), P-gp ER-based K_{puu} prediction are within 3-fold of observed K_{puu} in human (68%), NHP (60%), rats (58%) and mice (71%). Interestingly, rat observed K_{puu}-based prediction works well for human (75% and 88%), NHP (100% and 65%), and mouse (90% and 74%) for weak or non-P-gp substrates and good P-gp substrates, respectively. In addition, for compounds that are substrates for both P-gp and BCRP, incorporating BCRP ER in ER-based K_{puu} prediction does not improve categorization of compounds in brain penetration. In conclusion, for highly permeable compounds, ER determined at 1 μ M in NIH cell line is a useful *in vitro* model for K_{puu} brain prediction for rodents, NHP and human. For poorly permeable compounds, the *in vitro* model is at risk of over-predicting brain K_{puu}. The inter-species scaling method exhibits comparable prediction accuracy to the *in vitro* ER-based method generally, but demonstrates higher prediction accuracy for compounds with K_{puu} lower than 0.1. This study provides a strategic framework for the discovery of small molecule therapeutics with enhanced brain accessibility.

P337 - THE IMPACT OF UPTAKE AND EFFLUX TRANSPORTERS ON THE DISPOSITION AND CHOLESTEROL LOWERING EFFECT OF EZETIMIBE

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Purpose: Ezetimibe is a cholesterol-lowering agent by inhibiting cholesterol absorption in the GI tract. The drug-drug interaction between ezetimibe with other drugs (e.g., rifampicin) has been sporadically reported previously. The purpose of this study is to determine the impact of uptake (i.e., OATPs) and efflux (i.e., Pgp, MRP2) transporters on the disposition and cholesterol-lowering effect of ezetimibe.

Method: The Caco-2 cell culture model was used with a specific chemical inhibitor (rifampicin for OATPs, verapamil for Pgp, and MK-571 for MRP2) to determine the metabolism, uptake, and efflux of ezetimibe. An *in vivo* model using rats with high cholesterol diet was used to determine the impact of these transporters on the plasma cholesterol levels. A UPLC method was used in the Caco-2 study to determine ezetimibe's disposition and an LC-MS method was used to quantify the ezetimibe and its glucuronide in the plasma. A colorimetric kit was used to measure the plasma cholesterol level.

Results: The results showed that ezetimibe-glucuronide is a good substrate of MPR2 and OATP1B1 in the Caco-2 cell culture model, where the efflux and uptake ratio increased dramatically in the presence of specific inhibitors. When ezetimibe (1.0 mg/kg) was co-administered with OATP1B1 inhibitor rifampicin (10, and 50 mg/kg), the total exposure of ezetimibe (parent + glucuronide) in the plasma increased significantly, while the plasma cholesterol level increased 17% and 25%, respectively. Similarly, when the MRP2 inhibitor probenecid (10 and 50 mg/kg) was co-administered with ezetimibe (1.0 mg/kg), the plasma exposure of the total ezetimibe was increased but plasma cholesterol level also increased by 15%, and 20%, respectively.

Conclusion: These findings showed that ezetimibe plasma exposure is affected by OATP and MRP2 transporters. More importantly, the results showed that the cholesterol-lowering effect (i.e., plasma cholesterol exposure) is inversely related to the plasma exposure of the total ezetimibe.

P338 - ALLEVIATING MYCOPHENOLIC ACID-INDUCED TOXICITY IN THE GI TRACT USING WOGONIN AND CHRYSIN

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Purpose: Gastrointestinal side effect is a serious concern of mycophenolic acid (MPA), an active metabolite of the prodrug mycophenolic mofetil (MMF) that is used as an immunosuppressive agent in clinical settings. This study is to develop safe and effective approaches to prevent/treat Mycophenolic acid-induced diarrhea (MID) in patients with organ transplantation. **Method:** MID model was established in rats by given seven consecutive doses of 70 mg/kg/day MMF from day 1 to day 7, pretreated with wogonin or chrysin at 100 mg/kg from day -3 to day 7. Bodyweight, stool consistency were monitored daily, conduct the PK study on day7, and collect the tissues to measure the tissue distribution of MAP and its metabolites. Histology study of intestinal to evaluate the intestinal damage caused by MMF, plasma and tissue expression of TNF- α was measured as markers of MID-induced inflammation using ELISA Kit. Conduct the vitro experiments using the Caco-2 cell model. **Results:** The body weights and the fecal condition results showed that wogonin significantly

protected body weight loss and reduced diarrhea score. Chrysin's efficacy is mild. wogonin and chrysin didn't alter the exposure of MPA and MPAG in the plasma. However, wogonin significantly reduce the exposure of MPA in the ileum. wogonin and chrysin were mainly distributed in the ileum and the colon, suggesting that these two compounds are locally bioavailable. Inflammatory cytokine TNF- α was regulated by wogonin, suggesting that wogonin protect the tissue via anti-inflammatory pathway. Histology study show that in the treatment group, despite a remarkable thinning of the intestinal walls, epithelial morphologic of the ileum and colon did not display significant alternations. In the vitro study, wogonin and chrysin could induce UGT activity in the Caco-2 cell model. conclusion: MID model in rat was established successfully, and Wogonin could alleviate diarrhea induced by MPA without affecting the plasma exposure of MPA, probably due to the reduced MPA exposure in the ileum.

P339 - DETERMINATION OF DRUG-TO-ANTIBODY RATIO OF T-DXD IN PLSAMA SAMPLE BY LBA-LC-MS

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The *in vivo* drug-to-antibody ratio (DAR) value and drug load distribution on an ADC were very crucial to the drug efficacy and safety. The circulation stability of ADC had two important part, the first one was the free payload release rate, which was achieved based one the release mechanism result of linker-payload; and the other one is DAR charge. During linker payload screening stage, the payload release rate showed the extent of linker cleavage in plasma, and the change of DAR value given more information of deconjugation. The commonly used detection methods for DAR values were UV/Vis spectroscopy[2], hydrophobic interaction chromatography (HIC)[3], RP-HPLC[4], et al. Most of the methods were only suitable for experiments with simple sample components such as solution. There were many challenges in the analysis of DAR values by LC-MS in biological samples. A highly specific and sensitive LBA-LC-MS method was successfully developed for the detection DAR value of Trastuzumab deruxtecan (T-DXD) in biological samples. The T-DXD was enriched by immuno-capture by biotinylated antigen from human plasma and by anti-human IgG Ab from other nonclinical species plasma. After elute the ADC from the immune-capture beads, 20 mM TECP was add to the elution; a Thermo QE Plus was used to detect the intensity of LC and HC. The intensity of the top three glycosylation HCs were summarized. The average DAR was calculated based on the summarized intensity. The results showed that the DAR values of T-DXD in mouse stability sample after Day 0, 3 and 7 day incubation were 7.9, 6.8 and 3.6, respectively. The method had been used to determine both *in vitro* and *in vivo* ADC plasma stability. The workflow of the method also can be used for both conventional conjugated ADC and site-specific conjugated ADC, which could help researchers understand stability of ADC much better.

P340 - LC-HRMS BASED KINETIC CHARACTERIZATION PLATFORM FOR IRREVERSIBLE COVALENT INHIBITOR SCREENING

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Covalent inhibitors are recognized as an important component in drug discovery and therapeutics. The irreversibility of covalent conjugation makes MS a suitable tool for covalent screening characterization. MS allows label-free, direct detection of the native protein and the covalent adduct in the sample, providing relative quantification of target engagement. Several studies using MS to covalent screening by intact protein MS or peptide mapping have developed by ICE Company researchers; such as covalent binding rate, binding site mapping and measurement for Kinact/Ki value. Using KRASG12C inhibitors AMG510 as a positive control, the platform was demonstrated to be effective and accuracy for fast binding compounds. In this study, the final protein concentration was 1.5 mM and the concentrations of AMG510 were from 0.49 to 4 mM, and the total incubation time was 100 seconds(s). A HR-MS thermos QE Plus coupled a C4 column was used as to measure the intensity of the covalent protein.

In this case study, the high binding rate was found after incubation for 100s; and binding site mapping were investigated and the sequence coverage was larger than 75%. Kinact/Ki values was fitted using more than six concentrations using five timepoints within 100s. This method was used for several compounds screening and Kinact/Ki measurement.

P341 - DEVELOPMENT OF ORGANOID-DERIVED ENTEROCYTES FROM HUMAN IPS CELLS OR BIOPSIED TISSUE FOR PHARMACEUTICAL RESEARCH

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Purpose: Intestinal tissues derived from rodents and Caco-2 cells are widely used for *in vitro* pharmacokinetic studies. However, both entail problems such as species differences from humans and low expression levels of specific drug

metabolizing enzymes and transporters, respectively. Monolayers prepared from human intestinal organoid derived from human iPS cell-derived enterocyte-like cells (ELCs) or human intestinal tissue have attracted attention as a novel *in vitro* pharmacokinetic assessment system because they have gene expressions and functions similar to those of living organisms. In this study, we evaluated the functions of the monolayers prepared from ELCs or human intestinal tissue. Methods: Using our laboratory protocol, we generated ELCs and established organoids from them. Human intestinal organoids were also established from biopsied tissue or primary cryopreserved human enterocytes. Monolayers were produced from these organoids. We evaluated the usefulness of the monolayers by comparing their functions with those of the original ELCs, the organoids, or primary cryopreserved human enterocytes. Results and Discussion: Intestinal organoids were successfully established from ELCs, biopsied tissue or primary cryopreserved human enterocytes. The organoids could be cultured for more than 1 year with passages (53 passages), and they can form a tight monolayer in 3 days. Gene expressions of many drug metabolizing enzymes and transporters were enhanced in the monolayers compared to organoids. Their levels were similar to those of human adult intestine, and the trend did not change over 53 passages (1 year of culture). The CYP3A4 activity level was comparable or higher than that in primary cryopreserved human enterocytes. Furthermore, the monolayers had the efflux activity of P-gp and BCRP. Conclusions: We have successfully developed highly functional monolayers prepared from ELCs or intestinal tissue-derived organoids that can be applied to drug discovery research. Both had similar gene expression levels and activities of most drug metabolizing enzymes and transporters, similar to primary cryopreserved human enterocytes or human adult intestines.

P342 - ANALYTICAL METHODS FOR PK ANALYSIS OF ANTIBODY DRUG CONJUGATES

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Antibody-drug conjugates (ADCs) leverage the high specificity of monoclonal antibodies (mAbs) to precisely deliver potent payloads to target tissues, while minimizing adverse effects. ADCs combine a mAb with high specificity to the target and a biologically active payload (often a small molecule compound) with a stable linker. The PK properties of the ADC derive from both the antibody and the linker chemistry. A thorough PK evaluation of ADCs can include analysis of various forms of the ADC; e.g. total mAb, total ADC, the drug-to-antibody ratio (DAR), and concentrations of released payload and total conjugated drug (Zhu et al, 2020). The purpose of the work was to develop robust analytical methods for ADC PK determination utilizing Adcetris (Brentuximab vedotin) as a model ADC.

Mice were administered Adcetris (10 mg/kg i.v.), and terminal blood samples were collected into K3-EDTA tubes (n=3) over 21 days at 8 different time points. Different analytical methods were developed for the sample analysis of each form of the model ADC. Human IgG ELISA was used for analyzing the total antibody concentrations in plasma. LC/HR-MS/MS was applied for the identification of the released payload, which was quantified using LC/MS/MS. To determine the DAR profile as a function of time, the ADC was purified, using immunocapturing with biotinylated anti-human IgG antibody and streptavidin coated paramagnetic beads, followed by TCEP reduction and eventually analyzing the ADC heavy and light chains by LC/HR-MS.

Total mAb plasma level was 897nM one hour after administration, decreasing to 475nM at 24h and to 175nM over 21d. The initial DAR of 3.53 decreased to 1.10 at 7d and further to 0.735 at 21d. Monomethyl Auristatin E (MMAE) was identified as the released payload with a mean concentration of 7.1nM at 1 hour and 0.07nM at 21d. Total ADC and conjugated drug concentrations were calculated from the total mAb and DAR data.

Integrating LC/HR-MS, LC/MS/MS, and ELISA methodologies, in conjunction with tailored sample preparation and antibody reduction strategies, were shown to be useful in the PK assessment of ADCs with payload conjugation via native interchain cysteines. This approach allows quantification of total mAb, released payload, and the drug-to-antibody ratio (DAR) profile across the PK time course, thereby facilitating a comprehensive evaluation of ADC dynamics.

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