The Role of Drug Metabolism in Immune-Mediated Drug Toxicity:

Molecular, Clinical and Mechanistic Aspects

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Poster Abstracts

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P1. RAPID AND AUTOMATED METABOLITE STRUCTURE ELUCIDATION USING UNIFI FROM HRMS QTOF DATA

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Waters

High resolution mass spectrometry (HRMS) is an essential tool for metabolite elucidation of xenobiotics. The demand for greater and more routine use of HRMS in routine and high throughput assays is also increasing due to the rich information that can be provided and better quality decisions that can be made in discovery with a more complete picture of metabolism. HRMS workflows are often limited by the vast amounts of information these techniques produce. Combining HRMS with rapid data acquisition puts an increasing demand for rapid data elucidation and confirmation of potential metabolites formed. Traditional data elucidation taking hours to days for each compound cannot keep up with the speed of HRMS data generation and requires innovation in software to keep pace. The latest QTof instrument offers greater than 32,500 resolution, mass accuracy better than 1 ppm RMS, and UPLC compatible fast scan rates. HRMS, coupled with the high resolution chromatography power of UPLC, makes rapid data acquisition possible under generic conditions. MS^E spectra for 100 compounds can be obtained in an overnight run, a speed comparable to high throughput microsomal incubation where 96 compounds are typically grouped in one run. In this presentation, we wish to demonstrate the newly developed Unifi software for automated metabolite structural identification of HRMS QTof data. By providing structural information of parent (precursor) compound, Unifi analyzes the MS and MS^E fragment of potential metabolite and compares those with precursor MS and MS^E, a process which replicates manual elucidation. Each precursor or fragment structure is labeled in corresponding observed m/z in MS or MS^E spectrum. A structure heat map is displayed where the most likely site for biotransformation is highlighted. The analysis is automatically performed upon completion of a run. In the case of an overnight completed analysis, the elucidated structure is available for analyst inspection the next morning. In this way, metabolite structural confirmation and report generation can be completed within minutes, enable rapid turnaround for project scientists. This presentation will demonstrate the use of Unifi in metabolite identification of literature compounds that are known to undergo phase I and/or phase II biotransformation, including the following compounds: carbamazepine, diclofenac, glyburide, omeprazole, nefazodone, and many others. These compounds were incubated using human liver microsomes in the absence or presence of GSH for trapping of potential reactive metabolites. After protein precipitation and centrifugation, the supernatant were analyzed using Waters Xevo G2-S QTof and Acquity I-Class UPLC. The column used was an Acquity BEH C18 1.7 um, 2.1 x 100 mm. After data acquisition, metabolites are identified via the automated process as discussed above. Results show that Unifi was able to identify major metabolites of each compound as reported in the literature, including both phase I and phase II metabolism with diverse biotransformations.

P2. LIABILITY OF N-METHYLENE IMINIUM ION FORMATION FROM ALICYCLIC SECONDARY AMINES, A REACTIVE METABOLITE INTERMEDIATE OR A METABONATE?

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Drugs that are metabolized to generate electrophilic reactive intermediates which can form covalent adducts with macromolecules carry an increased risk of ADRs or idiosyncratic toxicity and drug-drug interactions caused by mechanism-based inactivation of P450. Glutathione (GSH), potassium cyanide (KCN), methoxyamine and semicarbazide have become widely used as trapping agents to evaluate the metabolic activation potential of drug molecules. In the liver microsome cyanide trapping assays piperazine containing compounds were found to form significant N-methylated piperazine cyanide (CN) adducts. Two pathways for the N-methyl piperazine CN-adduct formation were proposed. (1) The α-carbon in the N-methyl piperazine is oxidized to form a reactive iminium ion which can react with cyanide ion. (2) N-dealkylation occurs followed by condensation with formaldehyde and dehydration to produce N-methylene piperazine iminium ion which then reacts with cyanide ion to form the N-methyl CN-adduct. The CN-adduct from the second pathway was believed an artifact or metabonate. In this study, several approaches were applied to investigate the mechanisms involved in the formation of 4'-N-methylated piperazine CN-adducts. The first approach was the use of 4'-N-[13C]methyl piperazine to determine the extent of pathway 2 involvement. The second approach was to determine the relationship between 4'-N-dealkylation and 4'-N-methylated piperazine CN-adduct formation. In the third experiment, different [13C]-labeled one carbon donors such as methanol, formaldehyde or potassium carbonate were evaluated to determine the carbon source for the 4'-N-methylated piperazine CN-adduct formation. In the first study following microsomal incubations in the presence of cyanide ions, a significant percentage of 4'-N-[13C]methyl group in the CN-adduct was replaced by the unlabeled natural methyl group suggesting that the second pathway was predominant. The second experiment showed that in 4'-N-alkyl piperazine, the level of 4'-N-methyl piperazine CN-adduct formation was limited by the extent of prior 4'-N-dealkylation. The third experiment

demonstrated that formaldehyde, methanol or carbonate can be the one-carbon source for the formation of piperazine N-methylene iminium ion. When 4'-NH-piperaziens were incubated with KCN and [13C]-labeled formaldehyde, 4'-N-[13C]methylated piperazine CN-adduct was formed without NADPH or liver microsome suggesting a direct Mannich reaction is involved. However, when [13C]-labeled methanol or potassium carbonate was used as the one-carbon donor, 4'-N-[13C] methylated piperazine CN-adduct was detected only with the presence of liver microsome and NADPH, suggesting the requirement for P450. Since formaldehyde, methanol and carbonate are form in vivo, significant human exposure is expected. These naturally abundant one-carbon sources could potentially fuel the bioactivation of secondary amines to form N-methylene iminium ions in vivo. The resulting reactive N-methylene iminium ions could potentially bind to macromolecules which is a concern because of potential liability for mechanism based inactivation and/or idiosyncratic toxicity. Therefore, pathway 2 is relevant to in vivo bioactivation and warrant further investigations on its association to adverse drug reactions.

P3. THYMIC STROMAL LYMPHOPOEITIN AND INTERLEUKIN-4 MEDIATE HALOTHANE-INDUCED LIVER INJURY IN MICE BY INDUCING HEPATIC EOSINOPHILIA

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Liver eosinophilia has been associated with incidences of drug-induced liver injury (DILI) for more than 50 years, though its role in the etiology of this disease has remained unclear. We recently reported for the first time a pathogenic role of eosinophils in DILI using a murine model of halothane-induced liver injury (HILI). When female Balb/cJ mice were administered halothane, eosinophils were detected in the liver within 12 hours and increased thereafter proportionally to liver damage. Eosinophil associated chemokines, eotaxins, and the activator/growth factor interleukin-5 (IL-5) increased in response to halothane-treatment. Immunohistochemical staining for major basic protein (MBP), a cytotoxic eosinophil granule protein, revealed that eosinophils accumulated exclusively around areas of hepatocellular necrosis and appeared to show signs of degranulation as MBP staining was more diffuse than in the livers from vehicle controls. The severity of HILI was decreased significantly when the study was repeated in wild-type mice partially depleted of eosinophils and in the eosinophil lineage-ablated ΔdblGata-/- mice. Conversely, animals with selective depletion of neutrophils, which have been previously reported to play a pathogenic role in this model, failed to reduce the extent of HILI when levels of eosinophils remained unchanged. These findings indicate that eosinophils, not neutrophils, have a pathologic role in HILI in mice. The epithelial derived cytokine, thymic stromal lymphopoetin (TSLP), and its corresponding receptor TSLPR play an important role in priming a CD4+ T Cell and NKT cell inflammatory diseases in the lung, skin, and intestine that involve the interactions of IL-4, IL-5, and eosinophils. We now have found a similar role for TSLP in mediating hepatic inflammatory diseases. TSLP mRNA was constituently expressed by murine hepatocytes and levels increased during HILI. Moreover, the severity of HILI was reduced in both TSLPR-/- and IL-4-/- mice and was accompanied by decreases in serum levels of IL-5 and eotaxins and hepatic eosinophilia. In addition, we found that treatment of primary cultured murine hepatocytes with IL-4 caused gene induction and secretion of eotaxins, supporting a role of IL-4 on hepatic eosinophil infiltration. In conclusion, our findings demonstrate a pathogenic role of eosinophils in HILI that appears to be mediated by TSLP and IL-4 and suggest that aberrant levels of TSLP, IL-4 and/or other eosinophil-associated proteins may serve as risk factors for DILI. This is the first report of TSLP in vivo function in liver inflammation. Based on these findings, we propose that TSLP functions as a key signaling molecule from hepatocytes, akin to a danger signal, that initiates eosinophil infiltration and liver injury. The initiating event is the formation of drug-protein adducts and subsequent injury/stress that leads to secretion of TSLP from hepatocytes. This cytokine in turn activates hepatic CD4+ T Cells and NKT cells to secrete IL-4 and IL-5, which promote hepatic eosinophilia by inducing eotaxin secretion from hepatocytes and activating/maturing eosinophils in the bone marrow to express the eotaxin receptor, respectively. In the liver, eosinophils degranulate, releasing cytotoxic proteins including MBP or secrete pro-inflammatory cytokines, such as interferon-γ, leading to necrosis/apoptosis of hepatocytes.

P4. MECHANISMS -- BIOACTIVATION

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Rationale: Nevirapine (NVP) treatment is associated with significant idiosyncratic immune-mediated skin rash and hepatotoxicity in humans. NVP causes a very similar rash in female Brown Norway rats, and we had previously shown that 12-hydroxylation of NVP is required to induce the rash. In this study, we further examined the metabolism and covalent binding of NVP in the rat model and in human skin. Early immune activation through the NLRP3 inflammasome, and the role of IL-1β

and IL-18 in the skin in response to covalent adducts, was also examined. Methods: An anti-NVP antibody was produced and used in immunoblotting studies to detect covalent binding of NVP metabolites to skin and liver proteins from rodents and humans. Various sulfation inhibitors were administered to rats in order to prevent covalent binding and then the effects on the rash were determined. Cutaneous IL-1β and IL-18 levels in the skin were examined by ELISA. Anakinra, an IL-1 receptor antagonist, was given to rats to determine its involvement in the skin rash. Human keratinocytes or human dermal fibroblasts were incubated with NVP, 12-OH-NVP, or 12-OH-NVP sulfate and the production of IL-1β or IL-18 was measured. The NLRP3 activation inhibitors glyburide and z-VAD-FMK were used in keratinocyte cell cultures and the effects on IL-1β and IL-18 were determined. Results: Covalent binding was observed in the epidermis of NVP or 12-OH-NVP-treated rats. Major modified bands appeared between 40K-60K. Topical administration of 1-phenyl-1-hexanol (a sulfotransferase inhibitor) prevented rash and covalent binding where applied, and also prevented covalent binding of 12-OH to cytosolic skin fractions in vitro. IL-1β levels were significantly up-regulated in skin fractions of rats with a rash as well as in whole skin isolates. Dermal IL-1β spiked on day 1 indicating a potential early role for IL-1β. Anakinra treatment markedly decreased the skin rash in rats and also decreased cutaneous IL-1β levels. Glyburide prevented the increase in IL-1β produced from human keratinocytes and the prevention was maximal with 12-OH-NVP sulfate. Conclusions: In contrast to covalent binding in the liver, which involves direct oxidation to a quinone methide, the reactive metabolite that covalently binds in the skin is a benzylic sulfate. The sulfate responsible for the rash is formed in the epidermis. NLRP3 and IL-1β appear to play a role in early immune activation leading to NVP-induced skin rash. Analysis of IL-1β production by dermal fibroblasts or IL-1β and IL-18 production by z-VAD-FMK treated keratinocytes is currently being performed and will be presented. Further studies are examining the role of NLRP3 and IL-1β and IL-18 in the liver of NVP-treated C57 mice, which develop hepatic injury, but do not sulfate NVP and do not develop skin rash. NLRP3 is expressed in stellate cells and Kupffer cells and may play a role in initiating liver damage. Funding: Canadian Institutes of Health Research.

P5. COMPUTATIONAL EXPLORATION OF THE ROLE OF A PROTOTYPICAL DAMAGE-ASSOCIATED MOLECULAR PATTERN (DAMP) MOLECULE IN ACETAMINOPHEN HEPATOTOXICITY

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Drug-induced liver injury (DILI) is a major source of acute liver failure and is one of the leading causes of drug development failures. As such, there remains an unmet need for earlier identification and mitigation of DILI risk. The DILIsym™ model is a mechanistic representation of DILI in preclinical species and humans designed to address this need. The first generation model focused on hepatocyte drug interactions, with limited representation of inflammation. To facilitate the quantitative investigation of innate immune responses in DILI, we have expanded the representation to include liver macrophages, liver sinusoidal endothelial cells, and various mediators. The model represents the current understanding of mechanistic links between hepatocyte death, immune cell activation, mediator production, and mediator effects on hepatocyte death and regeneration. Selection of model parameters was informed by the literature. Mediator profiles and local accumulation of macrophages in the liver were aligned with reported data from acetaminophen (APAP) hepatotoxicity. HMGB1 is a classic alarmin or damage-associated molecular pattern (DAMP) molecule that can induce immune cell activation and is represented in the model. HMGB1 can be released from dying cells, including acetaminophen-exposed hepatocytes 1,2 and can also be produced by activated immune cells 3. Several reports demonstrate that in mice, neutralization of HMGB1 reduces APAP-mediated ALT elevation 4–6. In the model, HMGB1 neutralization reduces APAP-mediated ALT elevation (0.7x of max ALT) in the baseline simulated mouse. The simulated improvement fell within the range reported in the experimental literature (0.3-0.9x APAP ALT). The model parameters were varied to create earlier or higher HMGB1 profiles. An earlier HMGB1 profile did not improve the effect of HMGB1 neutralization on APAP hepatotoxicity. In contrast, HMGB1 neutralization was more effective in alleviating APAP hepatotoxicity when HMGB1 levels were higher, largely due to an increase in HMGB1-mediated immune activation. Lastly, model parameters were varied such that HMGB1-mediate immune cell activation led to a greater proportion of immune (e.g., TNF-α) mediated hepatocyte death relative to reactive-metabolite mediated hepatocyte death. It was initially surprising to observe that while the degree of liver injury was similar, this change led to slower progressing liver injury. Closer examination revealed that less reactive-metabolite mediated cell death reduced the level of DAMP release, which slowed immune cell activation and the subsequent immune-mediated injury. HMGB1 neutralization was most effective in alleviating APAP hepatotoxicity in this scenario (0.4x of max ALT). This research reports on how different HMGB1 profiles are predicted to translate to HMGB1 neutralization response and illustrates the application of the DILIsym™ model to test hypotheses regarding the role of the innate immune response in DILI.

P6. A MICROPATTERNED CULTURE WITH PRIMARY HEPATOCYTES AND KUPFFER MACROPHAGES FOR STUDYING INFLAMMATION-DRUG INTERACTIONS

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The appearance or relief of inflammation through drug therapy could differentially affect levels of enzymes involved in metabolism of co-administered drugs with potential pharmacological and toxicological consequences. An in vitro model that mimics liver inflammation may provide better predictive data in preclinical testing. We have developed a micropatterned co-culture of primary hepatocytes and embryonic fibroblasts (HepatoPac™) that retains high levels of phenotypic functions such as drug metabolism enzymes for 4 weeks in vitro. Here, we supplement the HepatoPac platform with primary Kupffer macrophages in order to mimic one component of inflammation. Species-matched Kupffer cells were added to human and rat HepatoPac at multiple ratios (to mimic both the normal and inflamed state of the liver) after stabilization to generate a tri-culture with primary hepatocytes and embryonic fibroblasts (HepatoPac- Kupffer cell co-culture). Recent evidence suggests that interaction between inflammatory stress and certain drugs may precipitate toxic responses. Here, we assess whether stimulation of HepatoPac- Kupffer cell co-cultures with LPS sensitizes the cultures to trovafloxacin (TVX) toxicity. Rat or human HepatoPac- Kupffer cell co-cultures were treated with increasing concentrations of TVX (+/- LPS) and assessed for changes in hepatic ATP content. TVX caused a concentration-dependent toxicity in the HepatoPac-Kupffer cell co-cultures which was potentiated by addition of 50ng/mL LPS to the cultures (TC50= 87.29 vs 27.77 Cmax for the rat platform and 68.24 vs 30.26 Cmax for the human platform). This effect was not observed with the non-toxic analog, levofloxacin. Treatment with pentoxifylline (an inhibitor of TNFα transcription) significantly decreased TVX/LPS- induced rat HepatoPac toxicity suggesting a synergistic effect between TNFα and trovafloxacin (TC50= 19.73 vs. 76.36 Cmax). In conclusion, rat or human HepatoPac-Kupffer cell co-cultures may be used to predict drug induced liver injury mediated by inflammatory stress.

P7. CRYOPRESERVED HUMAN PRIMARY HEPATOCYTES FOR ASSESSMENT OF ACUTE AND REPEAT DRUG-INDUCED-LIVER-INJURY (DILI). EFFECT OF 3D FORMAT AND INFLAMMATION SIGNALS

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Cryopreserved primary human hepatocytes (human CryoHeps) were cultured as 2D-cultures overlaid with Matrigel (2Dsw) or as spheroids (MT) and treated with various test drugs for 1 to 7 days. Acute cytotoxicity profiles were established in both culture formats and repeat-toxicity was assessed at the highest non-cytotoxic concentrations Immunofluorescence staining. Cytotoxicity profiles of paracetamol, chlorpromazine, troglitazone and trovofloxacine in the absence of NPC and/or LPS were equivalent in human fresh and CryoHeps, in 2D and Spheroid MT cultures. APAP cytotoxicity profile was equivalent in the presence or absence of inflammatory signals, whereas trovafloxacine cytotoxicity was increased in the presence of LPS or in NPC-hepatocyte co-cultures; in the latter case no synergic effect was observed in hepatocyte-NPC co-cultures + LPS. We demonstrate that human plateable CryoHeps represent a valuable tool for mechanistic evaluation of DILI in the presence (co-culture with NPCs and/or addition of LPS) or absence of inflammatory signals. The spheroid system allows further downscaling of cultures thus reducing the number of human CryoHeps to be used.

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This work was funded by the EU-EFPIA Innovative Medicine Initiative (IMI) project #115336-2 “Mechanism-Based Integrated Systems for the Prediction of Drug-Induced Liver Injury MIP-DILI”

P8. HEPATOCYTE-BASED IN VITRO MODEL TO IDENTIFY DRUG CANDIDATES CAUSING CHOLESTASIS

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Human and rat SCH were used to establish and validate the model. A mixture of the most relevant bile acids present in human plasma was composed and SCH were exposed to different concentrations of this mixture. Hepatocytes were first incubated with test compounds for two hours to provide the test compound with the opportunity to interfere with transporters and enzymes involved in BA disposition. Subsequently, the test compound and the concentrated BA mixture were co-incubated for 22 hours. The reduction reduction in urea formation by the hepatocytes (when additionally challenged with BA mixture as opposed to the test compound alone) was assessed as a quantitative measure of cytotoxicity exerted. The cholestatic potential of the compounds was expressed by calculating drug induced cholestasis index (DICI) values. Compounds with clinical reports of cholestasis such as cyclosporine A, troglitazone, chlorpromazine, bosentan, ticlopidine were found to be cholestatic in this in vitro assay. For these compounds, DICI values < 0.8 were obtained in multiple batches of hepatocytes and at incubation concentrations that only marginally exceeded therapeutic plasma concentrations (safety margin < 30). Moreover, the model detected cholestatic compounds such as chlorpromazine, troglitazone, ticlopidine, that require metabolism to exert their cholestatic effect. On the other hand, compounds such as diclofenac, valproic acid, and amiodarone, which cause hepatotoxicity by mechanisms other than altering BA disposition, were not cholestatic in our assay (DICI > 0.8 at all concentrations tested). The in vitro model proposed here, relying on a relatively simple toxicity assessment in SCH, appears to be a promising in vitro tool for early identification of drug candidates with clinical cholestasis risk.

P9. HUMAN PRECISION-CUT LIVER SLICES AS AN EX VIVO MODEL TO STUDY IDIOSYNCRATIC DRUG-INDUCED LIVER INJURY

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Idiosyncratic drug-induced liver injury (IDILI) is a major problem during drug development and has caused drug withdrawal and black-box warnings. Due to the low concordance of the hepatotoxicity of drugs in animals and humans, robust screening methods using human tissue are needed to predict and to develop biomarkers for IDILI in human . According to the inflammatory stress hypothesis, the effects of inflammation interact with the effects of a drug or its reactive metabolite(s) precipitating the toxic reactions to the liver. As a follow–up of our recently published mouse precision-cut liver slices (PCLS) model1, an ex vivo model involving human precision-cut liver slices (hPCLS), co-incubated for 24h with IDILI-related drugs (clozapine, ketoconazole, diclofenac, troglitazone, carbamazepine) and lipopolysaccharide (LPS), was developed to study IDILI mechanisms related to inflammatory stress in humans and to detect potential biomarkers. As comparator non-IDILI-related drugs voriconazole and olanzapine were included. LPS induced the production of inflammatory and anti-inflammatory cytokines, whereas none of the drugs caused a cytokine release. LPS exacerbated the toxicity of ketoconazole and clozapine but not of their non-IDILI-related comparators, voriconazole and olanzapine. However, the IDILI-related drugs diclofenac, carbamazepine and troglitazone did not show synergistic toxicity with LPS after 24h incubation. Coincubation of ketoconazole and clozapine with LPS decreased the glutathione levels in hPCLS, which did not occur with the other drugs. All drugs affected LPS-induced cytokine release, but interestingly, only ketoconazole and clozapine increased the LPS-induced TNF release. Decreased glutathione- and cysteine-conjugates of clozapine were detected in IDILI-responding livers following cotreatment with LPS. In conclusion, we identified ketoconazole and clozapine as drugs that exhibited synergistic toxicity with LPS, while glutathione and TNF were found to be potential biomarkers for IDILI-inducing drugs mediated by inflammatory stress. hPCLS appear suitable to further unravel the mechanisms of inflammatory stress-associated IDILI.

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P10. EXPOSURE-RESPONSE ANALYSIS OF THE IN VITRO LIGAND BINDING ENHANCEMENT TO HLA-DRB1*0701 BY LAPATINIB

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Introduction: Lapatinib-induced liver injury has been reported to be associated with HLA-DRB1*0701.1) In this study, we evaluated the effects of lapatinib on the enhancement of the binding of ligand peptides with HLA-DRB1*0701 in vitro. Also, to compare the in vitro effective concentrations to the exposure of lapatinib to liver in clinical settings, we simulated PK profiles of lapatinib in humans by a Simcyp population based simulator. Methods: The HLA binding study was performed at EpiVax,

Inc. (Providence, RI, USA) and the data was analyzed by the Emax model. Also, concentration-time profiles of lapatinib in humans were simulated by a Simcyp population based simulator (ver.12, Simcyp). Results & Discussion: Lapatinib enhanced the binding of the ligand peptide to HLA-DRB1*0701 with the EC50 value of 8.8 μM. The simulated maximum liver concentrations of lapatinib in humans exceeded the EC50 value at therapeutic doses. These results indicate that lapatinib could enhance the ligand binding to HLA in patients with HLA-DRB1*0701. Conclusion: These findings suggest that lapatinib may function as a ligand binding enhancer to HLA-DRB1*0701 in humans and lapatinib-induced liver injury might be triggered by this mechanism.

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P11. INDUCTION OF CYP3A4 BY DEXAMETHASONE ENHANCES THE CYTOTOXICITY OF LAPATINIB IN HEPARG CELLS

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Lapatinib is an orally active dual tyrosine kinase inhibitor currently used in the treatment of locally advanced or metastatic HER2-positive breast cancer. The lapatinib label contains a black-box warning for severe, potentially life-threatening hepatotoxicity. Lapatinib-induced liver injury is idiosyncratic in nature with at least a partial association with HLA-II alleles;1 however, the mechanism(s) of this toxicity remain unclear. Lapatinib is extensively metabolized by cytochrome P450 (CYP) 3A4/5 to yield an O-debenzylated, phenolic metabolite, which can undergo further oxidization to an electrophilic quinone imine intermediate.2 Quinone imines can form covalent adducts with cellular proteins, potentially triggering an immune response and subsequent toxicities. A recent retrospective clinical study reported that concomitant use of lapatinib with the CYP3A4 inducer dexamethasone significantly increased the risk of lapatinib-induced hepatotoxicity in metastatic breast cancer patients.3 Based on these findings, we hypothesized that induction of CYP3A4 by dexamethasone enhances the metabolic activation of lapatinib to form the reactive, potentially toxic quinone imine. To test this hypothesis, we examined the effect of dexamethasone on the cytotoxicity and metabolism of lapatinib in the HepaRG human liver cell line. HepaRG cells were pre-treated with dexamethasone (100 mM) for 72 h, followed by incubation with lapatinib (100 mM) for 24 h. Cell viability was monitored using WST-1 assays, and drug metabolites were quantified by LC/MS/MS analysis utilizing MRM. Induction of CYP3A4 by dexamethasone resulted in approximately a 50% decrease in cell viability, compared to cells treated with lapatinib alone ($n = 6$, $p < 0.01$). Similarly, pre-treatment of cells with dexamethasone increased the formation of the debenzylated metabolite of lapatinib by >2-fold, compared to treatment with lapatinib alone ($n = 6$, $p < 0.0001$). Co-incubation with the CYP3A4 inhibitor ketoconazole partially rescued the viability of cells treated with dexamethasone plus lapatinib and reduced debenzylated metabolite formation. A direct comparison of the cytotoxicity of debenzylated lapatinib vs. lapatinib was also made. Results from this analysis indicated that debenzylated lapatinib (LD50 = 38.5 ± 1.1 mM) was significantly ($n = 6$, $p < 0.0001$) more cytotoxic to HepaRG cells than lapatinib itself (LD50 = 84.9 ± 1.1 mM). Further, glutathione (GSH) adducts of the putative quinone imine were readily apparent in cells treated with debenzylated lapatinib. The levels of GSH adducts increased approximately 5-fold in HepaRG cells treated with dexamethasone plus debenzylated lapatinib, compared to cells treated with debenzylated lapatinib alone. This effect was attenuated by co-incubation with ketoconazole. Collectively, these data suggest that CYP3A4 induction potentiates the hepatotoxicity of lapatinib due to increased reactive metabolite formation. This study provides new mechanistic insights into the clinically important drug-drug interaction between lapatinib and dexamethasone. Moreover, these findings support the contention that CYP3A4-mediated bioactivation plays a pivotal role in lapatinib-induced hepatotoxicity.

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P12. PARTICIPATION OF CYP2E1 AND NADPH OXIDASE IN ISONIAZID-INDUCED NEUROTOXICITY IN GLUTATHIONE DEPLETION CEREBELLAR GRANULE NEURONS

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Xenobiotic metabolism within the brain constitutes a field of recent intensive research. Although low concentrations of these compounds could reach brain tissue, in situ xenobiotic metabolism could produce reactive, toxic metabolites that cause irreversible neuronal damage. Isoniazid is one of the anti-tuberculosis drugs that have been widely prescribed. It is relatively harmless but some patients under treatment with high doses of isoniazid develop peripheral neuropathy and cerebellar ataxia. It is proposed that oxidants could participate in isoniazid-induced cytotoxicity. In the presence of buthionine sulfoximine, an agent that reduces glutathione levels, isoniazid treatment of cerebellar granule cultured neurons resulted in reactive oxygen species production and cell death from 12 hours to 24 hours. DAS, a CYP2E1 inhibitor, rests the reactive oxygen species production and cell death by isoniazid and BSO treatment at 12 hours but not at 24 hours. Nicotinamide adenine dinucleotide oxidase (NOX) inhibitors and antioxidants prevent the ROS and cell death generated after 24 hours of isoniazid and BSO treatments. These results suggest that CYP2E1 is a potential promoter of neuronal oxidative damage and probably can work together with other enzymes such as NADPH oxidase during xenobiotic-induced cell death.

P13. THIOLACTONE SULFOXIDES AS NEW REACTIVE METABOLITES ACTING AS BIS-ELECTROPHILES: IMPLICATION IN CLOPIDOGREL AND PRASUGREL BIOACTIVATION

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The anti-thrombotics of the thienopyridine series, clopidogrel and prasugrel, are prodrugs that must metabolized in two steps to become pharmacologically active. The first step is the formation of a thiolactone metabolite. The second step is a cytochrome P450 (P450)-dependent oxidation of this thiolactone resulting in the formation of a sulfenic acid that is eventually reduced into the corresponding active thiol. It has been postulated that the sulfenic acid metabolite resulted from a nucleophilic attack of water on a highly reactive thiolactone sulfoxide derived from P450-dependent oxidation of the thiolactone primary metabolite. Here we show that it was possible to trap these thiolactone sulfoxides by a series of N-, S- or C-nucleophiles such as amines, thiols or cyclopentane-1,3-dione (CPDH), an equivalent of dimedone which is used as a sulfenic acid trapping agent. HPLC-MS studies showed that various bis-adducts having incorporated two nucleophile molecules were formed in these reactions. One of them that resulted from the oxidation of 2-oxo-prasugrel by human liver microsomes in the presence of ethanolamine and CPDH was isolated and completely characterized by 1H and 13C NMR spectroscopy in addition to MS and MS2 spectrometry. All metabolites derived from an attack of H2O or an amine at the CO carbon of the intermediate thiolactone sulfoxide existed as a mixture of two diastereomers having a cis configuration of the double bond, whereas those deriving from an attack of thiols appeared as a mixture of four diastereomers with a cis or trans configuration of the double bond. The results described in this article showed that thiolactone sulfoxides are formed as reactive metabolites during metabolism of clopidogrel and prasugrel and are able to react as bis-electrophiles with a variety of nucleophiles. The possible implications of these reactive metabolites in the pharmacological and/or secondary toxic effects of these drugs remain to be studied.

P14. DETERMINING METABOLISM DIFFERENCES IN PRECLINICAL SPECIES AND HUMAN IN VITRO AND IN VIVO FOR GSK2485852, A NS5B HCV INHIBITOR

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Differences among preclinical species and human in metabolite identification studies are always a concern in drug development. GSK2485852 was under investigation as a site IV inhibitor of the viral polymerase NS5B for treatment of Hepatitis C (HCV) infections. In vitro MetID studies of GSK2485852 identified three main metabolites, N-debenzyl, deborolation to phenol, and glucuronide of the deborolated product. The most abundant metabolite in rat hepatocytes was the deborolated glucuronide, while in dog, monkey and human hepatocytes the N-debenzyl product was the most abundant product. Human hepatocytes produced more N-debenzyl metabolite than the other species. In vitro CYP3A4 experiments suggested that CYP3A4 was the major isozyme responsible for the formation of N-debenzyl product. This was confirmed by the absence or large decrease in production of N-debenzyl formation in the presence of ritonavir (RTV), a potent 3A4

inhibitor. In vivo pharmacokinetics were measured in rats (GSK2485852 administered 1 mg/kg intravenously and 5 mg/kg orally) and dogs (GSK2485852 administered 5 and 25 mg/kg orally) with and without the addition of RTV (5 mg/kg rats and 10 mg/kg dogs). The RTV boost in rats and dogs showed an increase in GSK2485852 AUC of 30% in rats and 3 to 4 fold in dogs. These results provided an in vivo/in vitro correlation with respect to CYP3A4 involvement in N-debenzyl formation. Human studies in HCV infected patients indicated an over-predicted abundance of the N-debenzyl product where the AUC of N-debenzyl metabolite was approximately 50% higher than those of the parent, GSK2485852, and exceeded preclinical cover from toxicology studies. Secondly, a by-product of the N-debenzylation could be a benzaldehyde which is a potential reactive intermediate. Development of GSK2485852 was halted due to toxicity findings in repeat dose studies and poor human PK.

P15. WHICH TECHNIQUE FINDS MORE REACTIVE METABOLITES: ON-THE-FLY MASS DEFECT FILTERING, NEUTRAL LOSS OF 129 SCREENING, OR ISOTOPIC LABELING?

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Reactive metabolites have been shown to induce immune response in sensitive individuals1. Thus, screening for reactive metabolite has taken on a new importance. The most common screen for reactive metabolites uses glutathione (GSH) as a trapping agent. With newer accurate mass instrumentation becoming more commonplace, several options for finding GSH adducts have become available. The objective of this poster is to show which of the various available methods finds GSH adducts most reliably. We have incubated several drugs known to form high and low levels of GSH adducts at several concentrations and screened for them using three information-dependant analysis schemes. The first scheme uses mass defect to trigger MS/MS for confirmation that the presumed GSH adduct is indeed a GSH adduct. Most information-dependant analyses are based solely on intensity. This technique allows low-level metabolites to move to the top of the list for generating MS/MS based on their relationship to the parent drug as signaled by their mass defect. The second technique uses the signature Neutral Losses of 129 and/or 273 from the GSH adduct. However, this technique is not limited in how many neutral losses can be monitored; therefore, compound-specific neutral losses can also be used. These first two techniques are more suitable for screening or use in the drug discovery stage as they do not require any labeled compound. They also can find non-GSH metabolites. A third technique uses incubation with a mix of labeled and unlabeled GSH and triggering on the defined mass difference between the labeled and unlabeled adducts. A limitation of this technique is that it will only generate confirmatory MS/MS on GSH adducts (ignoring even the parent drug). The results section will summarize which techniques find the most metabolites and discuss which methods are most useful at various stages of the drug development process.

Reference

1. Naisbitt, et al., Curr. Opin. Allergy Clin. Immunol. 2001 1, 317-25.

P16. CAN WE BENCHMARK IN VITRO BODY BURDEN OF THE COVALENT DRUGS AS A TOOL TO ASSESS TOXICITY CAUSED BY NON-SPECIFIC COVALENT BINDING OF COVALENT DRUGS?

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An advantage of covalent inhibitors is that they can irreversibly block the activity of biological targets leading to a prolonged pharmacodynamic effect if the target resynthesis rate is slow. Although covalent inhibitors can show excellent efficacy compared to their reversible counterparts, there has been some reluctance in the pharmaceutical industry to use covalent modalities as clinical agents. The reason for this reluctance is largely due to potential safety concerns that could be caused by non-specific covalent binding across the proteome. Covalent drugs contain at least one reactive electrophilic moiety. The electrophilic moiety can react with biological nucleophiles such as -SH, -OH or –NH2 groups present in protein/DNA thereby causing toxicity either by immune responses or by loss of biological activity of the proteins/DNA that irreversibly bind with electrophile. However there are a number of covalent drugs used in clinical practice that do not appear to be associated with high frequencies of toxicity. While some of these are used in cancer, an indication wherein the benefit can far outweigh the risk of toxicity, others such as aspirin and omeprazole are used in non-life-threatening indications. We hypothesized that there may be a threshold limit for non-specific covalent binding, above which a covalent binding drug may be more likely to cause toxicity. Estimation of in vivo covalent binding burden from in vitro data has previously been used as an approach to distinguish those agents more likely to cause toxicity (e.g. hepatotoxicity) via metabolic activation to reactive metabolites. We have extended this approach to drugs containing an electrophilic moiety to determine the in vitro non-specific covalent binding burden for covalent drugs using a small library of clinically used covalent drugs selected from different indications and spanning different electrophilic structures. In vitro covalent binding burden was determined by incubating radio-labeled drugs

with pooled human hepatocytes. It was found that in vitro daily body burden of some covalent drugs, such as aspirin, is as high as 14 mg/day. The daily body burden of covalent drugs will be presented and the implication of covalent binding on toxicity will be discussed.

P17. EXAMINING THE MITOCHONDRIAL TOXICITY PROFILES OF VIRAL REVERSE TRANSCRIPTASE INHIBITORS VIA A MULTIPLEX, BRANCHED DNA PLATFORM

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The development of therapeutics that inhibit viral reverse transcriptases through nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs/NtRTIs) can be hampered by adverse events that are due to mitochondrial toxicity. Strategies for de-risking mitochondrial toxicity include examining potential changes in the transport of nutrients into the mitochondria, mitochondrial biogenesis/protein translation, metabolism, genomic DNA, and specific enzymatic activities in the electron transport chain. While these assays answer important functional questions, they may not necessarily detect subtle changes in mitochondrial DNA transcription and replication, which can lead to the downstream functional changes, leading to adverse events. Additionally and due to the fact that mitochondrial genes have differential localization in the mitochondrial genome, there may be differences in the relative expression of mitochondrial proteins, indicating that surveying one or two proteins from the mitochondrial compartment together with the nuclear compartment may not be sensitive to detect the breadth of initial expression changes. We hypothesize that compounds designed to inhibit viral reverse transcriptases may have varying binding affinities for human mitochondrial proteins, leading to differential mitochondrial gene transcriptional regulation. To address this hypothesis, we have developed a multiplex, branched DNA (bDNA) assay to ascertain transcriptional regulation in the mitochondria, as well as in the nucleus. This assay has the advantage of detecting eight transcripts in a single lysate, making it superior to standard real-time quantitative PCR methods. This is due to the fact that this assay amplifies the signal—and not the transcript—of an hybridized, gene-specific probe. The assay has been developed to examine the expression of nuclear-encoded: COX8A (Cytochrome c oxidase subunit VIIIA (ubiquitous)) UCP1 (Uncoupling protein 1 (mitochondrial, proton carrier), and ISCU (Iron-sulfur cluster scaffold homolog); it detects mitochondrial-encoded: MTDN6 (NADH dehydrogenase subunit 6—part of Complex I), MTCO1 (Cytochrome oxidase C), MTRNR1(12S rRNA), MTDN1 (NADH dehydrogenase subunit 1—part of Complex I), and CYTB(Cytochrome B). In the assay, HepG2 cells were treated daily with a selected group of NRTIs, including 2',3'-dideoxyinosine (ddI), 2',3'-dideoxy-3'-thiacytidine (3TC), and 3'-azido-3'-deoxythymidine (AZT) over the course of ten days. At the end of the ten days, initial cytotoxicity screening revealed anticipated trends whereby ddI >> 3TC = AZT. Additionally, in the case of ddI, bDNA data revealed a reduction in all of the mitochondrial-encoded genes, which was detected at an IC_{50} that was much lower than the IC_{50} cytotoxicity value. Depending on the anticipated exposures and peak concentration (C_{max}) for therapeutics, this assay may be applied to support candidate selection in pharmaceutical programs that are interested in pursuing the inhibition of reverse transcriptases, as well as other mechanisms of action, which may be related to mitochondrial DNA replication, repair, and transcription.

P18. SCANNING THE SERINE HYDROLASE PROTEOME FOR OFF-TARGET ACTIVITY OF PENICILLIN BINDING PROTEIN INHIBITORS (PBPIS) AND B-LACTAMASE INHIBITORS (BLIS)

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Most PBPIs and BLIs (both marketed and in development) utilize chemical warheads to form covalent bonds with active-site serine residues. Off-target activities of these inhibitors likely include mammalian serine hydrolases, which also utilize a serine in enzyme catalysis. Assessing selectivity against these enzymes is challenging due to their number (>200) and structural diversity: the traditional one-target-one-assay approach is not economically viable. Activity-Based Protein Profiling (ABPP) is being used as an alternative approach. ABPP utilizes an enzyme family-specific and active site-directed fluorescent probe to detect catalytically active serine hydrolases and their inhibition by test compounds. Multiple serine hydrolases can be detected in complex biological samples using SDS-PAGE and in-gel fluorescence. Inhibitor selectivity against purified enzymes was similar using ABPP and biochemical enzyme assays. ABPP data using rat tissue extracts (brain, heart, liver, lung, kidney, testes) revealed 5 proteins that are off-targets for novel PBPIs and BLIs. To help gauge safety risks associated with inhibiting these enzymes, these proteins were isolated and their identity determined. ABPP studies can be performed ex vivo to determine whether off-target binding (seen with tissue extracts) occurs in vivo and thereby help de-risk off-target activity of BLIs and PBPIs in early drug discovery.

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