Great Lakes
Drug Metabolism Discussion Group

6th Annual Meeting of the Great Lakes Drug Metabolism Discussion Group

Monona Terrace Convention Center
Madison, Wisconsin

May 10-11, 2011

Schedule

Tuesday, May 10, 2011

10:00 AM **Registration, Continental Breakfast, Poster Setup, and Sponsor Booth Setup.**

11:00 AM **Welcome.**

11:10 AM **Investigation of the Role of Reactive Metabolites in the Mechanism of Idiosyncratic Drug Reactions.**, Jack Uetrecht, M.D., Ph.D., University of Toronto, Toronto, Ontario.

11:50 AM **Drugs Dancing Together: The Origins of "Cooperativity" in Drug-Drug Interactions.**, Stephen Sligar, Ph.D., University of Illinois, Urbana, Illinois

12:30 PM **Lunch, Poster Viewing, and Sponsor Booths.**

2:00 PM **Translating Genotype to Phenotype: Genetic Variability of DMEs.**, Andrea Gaedigk, Ph.D., Children's Mercy Hospital, Kansas City, Missouri.

2:40 PM **Pharmacogenetics in Anti-Cancer Drug Development.**, Jill M. Kolesar, Pharm.D., BCPS, FCCP, University of Wisconsin, Madison, Wisconsin.

3:20 PM **Break.**

3:40 PM **Regulation of Drug Metabolism in Infectious Diseases. Are Cytokines the Whole Story?**, Edward T. Morgan, Ph.D., Emory University, Atlanta, Georgia

4:20 PM **Ontogeny of Human Hepatic Drug Metabolizing Enzymes.,** Ron Hines, Ph.D., Medical College of Wisconsin, Milwaukee, Wisconsin and Children's Hospital and Health Systems, Milwaukee, Wisconsin.

5:00 PM **Poster Viewing, Sponsor Booths, and Mixer.**

7:00 PM **Dinner.**

Schedule

Wednesday, May 11, 2011

07:30 AM **Business Meeting.**

08:00 AM **Continental Breakfast and Poster Viewing.**

09:00 AM **New Models in Drug Development: Human Induced Pluripotent Stem (iPS) Cells and their Derivatives.**, Vanessa Ott, Ph.D. Cellular Dynamics International, Madison, Wisconsin.

09:35 AM **Impact of Protein Binding on Cell Penetration and PK/PD of Small Molecules.**, J. Cory Kalvass, Abbott Laboratories, Abbott Park, Illinois.

10:10 AM **Break.**

10:30 AM **Optimizing ADME Properties of Molecules in Early Drug Discovery.**, Jeffrey W. Cramer, Lilly Research Laboratories, Indianapolis, Indiana.

11:05 AM **Cytochrome P450 2E1 Competes with Cytochrome P450 2B4 for Limited NADPH-Cytochrome P450 Reductase.**, Cesar Kenaan, University of Michigan, Ann Arbor, Michigan.

11:30 AM **Individual Variability in the Detoxification of Carcinogenic Arylhydroxylamines in Human Breast.**, James Sacco, University of Wisconsin, Madison, Wisconsin.

11:55 AM **Closing Remarks.**

Posters

1. **Aspirin or Vitamin D Does Not Prevent Colon Tumors in Rodent Models of Colon Cancer.**

Amy A. Irving†, James Amos-Landgraf , Dawn Albrecht, Lori Plum, Kathleen Krentz, Linda Clipson, Norman Drinkwater, Richard Halberg, Hector DeLuca, and William Dove†.

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Colon cancer risk varies greatly around the world, indicating an environmental component to its pathogenesis. Epidemiological studies suggest that vitamin D and aspirin are associated with a reduced risk of colon cancer. Individuals with lower sunlight exposure, resulting in reduced 25(OH)D serum levels, have increased rates of colon cancer. Some studies have shown that individuals who take aspirin regularly have a reduced risk of developing colon cancer. However, short-term studies in humans show conflicting efficacy of these compounds. We have used two genetic models of familial colon cancer, the *Apc*$^{Min/+}$ mouse and the *Apc*$^{Pirc/+}$ rat, to investigate the effect on colonic tumors of aspirin and 25-hydroxyvitamin D_3 [25(OH)D_3]. Rats and mice were randomized to one of three test diets: aspirin, 25(OH)D_3 or the combination. Some of the mice and rats were also treated with dextran sodium sulfate, a colon-specific inflammatory agent, to test these agents on tumors with two potentially different etiologies. Longitudinal endoscopic monitoring allowed us to test these compounds both in preventing newly arising colonic tumors and against established tumors. Terminal intestinal tumor counts, as well as serum salicylate, calcium and 25(OH)D_3 measurements, were obtained at study termination. Despite significant increases of the relevant serum levels, aspirin and 25(OH)D_3, singly or in combination, failed to reduce tumor numbers or the number of growing colonic tumors in the Min mouse or the Pirc rat. Notably, a high daily dose of 25(OH)D_3 resulted in a statistically significant increase in the multiplicity of colonic tumors in the Pirc rat. This is a timely contribution to a current public health issue, as stated recently by the Institute of Medicine, “Serum [25(OH)D_3] above 75 nmol/L are not consistently associated with increased benefit… [with] reason for concern at serum 25(OH)D_3 levels above 125 nmol/L.” Future studies are needed to determine the range over which vitamin D may be detrimental and to explore mechanisms that may account for resistance to these compounds.

2. **Quantitative Model Based Approach for In-vitro Assay Selection.**

Lisa Zhang, Anjaneya Chimalakonda, Christine Huang, and Punit Marathe.

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Purpose: Application of quantitative model-based approaches could maximize drug discovery and development efficiency. These concepts are routinely implemented in early and late phase drug development, but not so routinely in early drug discovery. In this study, we report the application of quantitative model-based approaches for *in-vitro* assay selection in an early discovery program.

Methods: Three program compounds were run in two *in-vitro* assay systems (Assay 1 and 2). Pharmacokinetic-pharmacodynamic (PK-PD) studies were conducted in rats wherein samples were collected following oral dosing of these compounds. For each compound, 3-4 dose levels (between 0.3 - 100 mg/kg) were included. Plasma samples were analyzed for compound concentrations as well as biomarker response. The pharmacokinetic data were fitted to a compartment model. An inhibitory sigmoid Emax model was used to relate the plasma concentration to biomarker response and to determine the *in-vivo* EC50. The *in-vitro* assay determined- and *in-vivo* model estimated- EC50 values were compared.

Results: The two *in-vitro* assays provided different potency measurements for these compounds both in terms of rank ordering and absolute EC50 values (Table below). This presented a challenge in terms of using these assays for compound selection and triage. An inhibitory sigmoid Emax model was able to adequately describe the *in-vivo* exposure-response relationship for all three compounds. As shown in the table below, the *in-vivo* model determined EC50's correlated well with the EC50 determined using Assay 2. Therefore, Assay 2 was determined to be a better surrogate of *in-vivo* potency of this class of compounds and was subsequently selected as a screening assay.

Conclusion: This study demonstrates the application of quantitative model-based approaches to aid in *in-vitro* assay selection in an early discovery program.

Compound	Assay 1 EC50)M	Assay 2 EC50)M	*In-vivo* Model-determined EC50)M
1	0.019	0.86	0.87
2	0.011	3.0	1.7
3	0.0037	6.8	18

3. **Detection of CYP2D6, SULT1A1 and UGT2B17 Copy Number Variation (CNV) Using Multiplex PCR A.**

Andrea Gaedigk, Greyson P Twist, and J Steven Leeder.

Children's Mercy Hospital and Clinics, Section of Developmental Pharmacology and Experimental Therapeutics, Kansas City, MO 64108.

4. **Individual Variability in the Detoxification of Carcinogenic Arylhydroxylamines in Human Breast.**

Keelia Rhoads, James C. Sacco, Nicholas Drescher, Amos Wong, andLauren A. Trepanier

University of Wisconsin, Madison, WI 53706.

Cytochrome b_5 (b5) and NADH cytochrome b_5 reductase (b5R) detoxify reactive hydroxylamine metabolites of known arylamine and heterocyclic amine mammary carcinogens. The aim of this study was to determine whether hydroxylamine reduction for the prototypic arylamine, 4- aminobiphenyl, (4-ABP-NHOH) was present in human breast, and to determine whether variability in activity was associated with single nucleotide polymorphisms (SNPs) in the coding, promoter, and 3'UTR regions of the genes encoding b5 (*CYB5A*) and b5R (*CYB5R3*). 4-ABP-hydroxylamine (4-ABP-NHOH) reduction was readily detected in pooled human breast microsomes, with a K_m (280 μM) similar to that found with recombinant b5 and b5R, and a V_{max} of 1.12 ± 0.19 nmol/min/mg protein 4-ABP-NHOH reduction varied 75-fold across 70 individual breast samples, and correlated significantly with both b5 (80-fold variability) and b5R (14-fold) immunoreactive protein. In addition, wide variability in b5 protein expression was significantly associated with variability in *CYB5A* transcript levels, with a trend toward the same association between b5R and *CYB5R3*. Although a sample with a novel cSNP in *CYB5A*, His22Arg, was found with low reduction and b5 expression, no other SNPs in either gene were associated with outlier activity or protein expression. We conclude that b5 and b5R catalyze the reduction of 4-ABP-NHOH in breast tissue, with very low activity, protein, and mRNA expression in some samples, which cannot be attributed to promoter, coding, or 3' UTR SNPs. Current research is focusing on the influence of epigenetic changes on the transcriptional regulation of *CYB5A* and *CYB5R3*.

5. **Computational Site of Metabolism Prediction in CYP2C9.**

Matthew L. Danielson and Markus A. Lill.

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Cytochrome P450 enzymes are responsible for metabolizing many endogenous and xenobiotic molecules encountered by the human body. It has been estimated that 75% of all drugs are metabolized by cytochrome P450 enzymes. Thus, predicting a compound's potential sites of metabolism (SOM) is highly advantageous early in the drug development process. We have combined molecular dynamics, AutoDock Vina docking, the neighboring atom type (NAT) reactivity model, and a solvent-accessible surface-area term to form a reactivity-accessibility model capable of predicting SOM for cytochrome P450 2C9 substrates. To investigate the importance of protein flexibility during the ligand binding process, the results of SOM prediction using a static protein structure for docking were compared to SOM prediction using multiple protein structures in ensemble docking. The results reported here indicate that ensemble docking increases the number of ligands that can be docked in a bioactive conformation but only leads to a slight improvement in predicting an experimentally known SOM in the top-1 position for a ligand library of 75 CYP2C9 substrates. Using ensemble docking, the reactivity-accessibility model accurately predicts SOM in the top-1 ranked position for 49% of the ligand library and considering the top-3 predicted sites increases the prediction success rate to approximately 70% of the ligand library. Further classifying the substrate library according to Km values leads to an improvement in SOM prediction for substrates with low Km values. While the current predictive power of the reactivity-accessibility model still leaves significant room for improvement, the results illustrate the usefulness of this method to identify key protein-ligand interactions and guide structural modifications of the ligand to increase its metabolic stability.

6. **Simultaneously LC-MS-MS Analytical Method Development and Metabolism Study of Established Anti-Tuberculosis Compounds.**

Yang Song[a,b], Kuanwei Peng[a], Nan Zhang[a], Richard van Breemen[b], Scott G. Franzblau [a*].

a. Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St., Chicago, IL 60612; b. Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 S. Wood St., Chicago, IL 60612.

Introduction

Anti-tuberculosis drugs are typically used in combinations in order to preclude the emergence of drug resistant mutants. However, there are yet no analytical methods available to simultaneously detect the established drugs within a single one run for clinical plasma samples. Meanwhile, there is also a lack of retrospective studies of metabolic stability of those established anti-tuberculosis drugs, especially the grandfathered drugs. Herein, a LC-MS/MS based method was established to simultaneously quantify fifteen anti-tuberculosis drugs (moxifloxacin, ethambutol, isoniazid, levofloxacin, pyrazinamide, linezolid, LL3858, PA-824, rifampicin, ethionamide, thioridazine, TMC 207, clarithromycin, clofazimine, OPC 67683) in human plasma. Metabolic stability of those drugs in human and mouse microsomes are then evaluated by the developed LC-MS/MS method.

Methods

Standard solutions: The final concentrations of the plasma standards are 50, 20,10, 5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002, 0.001 μg/mL for moxifloxacin, ethambutol, isoniazid, levofloxacin, pyrazinamide, linezolid, LL3858, PA-824, rifampicin; 5, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002, 0.001, 0.0005, 0.0002, 0.0001 μg/mL for ethionamide, thioridazine, TMC 207, clarithromycin, clofazimine, OPC 67683.

Sample preparation procedures : 400 μL of acetonitrile containing 200 ng/mL of internal standards were added to a 100μl aliquot of plasma. The sample mixture was vortexed for 1 min and put in -20 °C for 30minutes before being centrifuged at 10,000 g for 30 min to remove the protein precipitate.

LC-MS/MS : Applied Biosystems 4000 Q TRAP LC/MS/MS

Preliminary data

1) The lower limit of quantitation (LLOQ) of each compound in human plasma: Moxifloxacin: 0.05μg/mL, Ethambutol: 0.01 μg/mL, Isoniazid: 0.05 μg/mL, Levofloxacin: 0.02 μg/mL, Pyrazinamide: 0.5 μg/mL, Linezolid: 0.05 μg/mL, LL3858: 0.05 μg/mL, PA-824: 0.05 μg/mL, rifampicin: 0.02 μg/mL, Ethionamide: 0.02 μg/mL, Thioridazine: 0.001 μg/mL, TMC 207: 0.2 μg/mL, Clarithromycin: 0.005 μg/mL, Clofazimine: 0.001 μg/mL, OPC 67683: 0.01 μg/mL.

2) The half-lives of each compound in human microsomes: Moxifloxacin: >300min, Ethambutol:>300min, Isoniazid:262min, Levofloxacin:>300miin, Pyrazinamide: >300min, Linezolid: 50min, LL3858: 40min, PA-824: 67min, rifampicin: 62min, Ethionamide: 31min, Thioridazine: 35min, TMC 207: 193min, Clarithromycin: 89min, Clofazimine: >300min, OPC 67683: 92min.

3) The half-lives of each compound in human microsomes: Moxifloxacin: 287min, Ethambutol: 280min, Isoniazid: 59min, Levofloxacin:>300min, Pyrazinamide: 221min, Linezolid: 172min, LL3858: 36min, PA-824: 79min, rifampicin: 171min, Ethionamide: 6min, Thioridazine: 18min, TMC 207: 63min, Clarithromycin: 125min, Clofazimine: 281min, OPC 67683: 103min.

Novel Aspect
For the first time, a simultaneous quantitation method to analysis fifteen established anti-tuberculosis drugs in clinical plasma samples was established.

7. **Characterization of DNA Glycocalyx Interactions.**

Michael J. Palte and Ronald T. Raines.

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Understanding the interactions between nucleic acids and the glycocalyx is crucial for the development of efficient siRNA and gene-delivery systems. Herein, we report on the utilization of fluorescently labeled, lipid–oligonucleotide conjugates to characterize the interactions between DNA and the cellular glycocalyx. To our knowledge, this study is the first to analyze directly the interaction of DNA with the glycocalyx. We find that the main anionic components of the glycocalyx, sialic acid and glycosaminoglycans, hinder the association of oligonucleotides with cells. Moreover, the amount of negative charge associated with an oligonucleotide correlates directly with a reduced affinity for the cell surface, and that negative charge can be overcome by installing cationic lipids in the glycocalyx. These data support the hypothesis that cells evolved an anionic glycocalyx as a barrier to nucleic acids, thereby enhancing genomic stability. As *Caenohabditis elegans* lacks the enzymes for sialic acid biosynthesis and is readily amenable to RNAi-mediated knockdown, we propose that inhibiting the biosynthesis of sialic acid could facilitate the entry of siRNA into mammalian cells.

8. **Cytochrome P450 2E1 Competes with Cytochrome P450 2B4 for Limited NADPH-Cytochrome P450 Reductase.**

Cesar Kenaan, Erin V. Shea, Haoming Zhang, Matthew Pratt-Hyatt, and Paul F. Hollenberg.

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For efficient catalysis to occur, cytochromes P450 (CYPs or P450s) require an interaction with their physiological redox partner, cytochrome P450 reductase (CPR). However, CPR is in limited supply as it exists in a 1:10 to 1:25 ratio to P450 *in vivo*. Furthermore, studies in both microsomal and reconstituted systems have shown that the presence of one P450 isoform can significantly influence the catalytic activity of another isoform. In this study, we assessed whether CYP2E1 could influence CYP2B4's catalytic properties under steady-state turnover conditions. The results show that CYP2E1 can inhibit the CYP2B4-mediated *N*-demethylation of benzphetamine (BNZ) with a K_i of 0.4 μM. However, CYP2B4 was incapable of inhibiting CYP2E1-mediated p-nitrophenol hydroxylation. When these inhibition studies were performed with an artificial electron donor, tert-butyl hydroperoxide, CYP2B4 was resistant to inhibition by CYP2E1. Determining the apparent K_M of CYP2B4 for CPR in the presence of increasing concentrations of CYP2E1 revealed the dual competitive and non-competitive nature of CYP2E1 inhibition since at ratios of 3:1 (CYP2E1:CYP2B4), CYP2E1 increased CYP2B4's K_M by 13-fold but had no effect on k_{cat}. Yet at higher ratios of CYP2E1:CYP2B4 (6:1), K_M decreased to similar levels that were observed with CYP2B4 in the absence of CYP2E1; however, k_{cat} also decreased by 10-fold. Additionally, CYP2E1 increased the K_M of CYP2B4 for BNZ by 8-fold, although the K_M was recovered when saturating concentrations of CPR were used. When CYP2B4 and CYP2E1 were incubated together in the presence of a chemical cross-linker (bis(sulfosuccinimidyl)suberate, BS^3), no CYP2E1-CYP2B4 cross-links were observed indicating that CYP2B4 and CYP2E1 do not interact with each other and suggesting that CYP2E1's inhibitory properties are not likely due to direct P450-P450 interaction. Further studies are under way to investigate the effect of CYP2E1 on the rate and extent of CYP2B4 reduction by CPR under pre-steady state conditions. (Supported in part by NIH grant CA16954).

9. **From Tier 1 and Beyond: Establishing a System to Identify Mass Spectrometry Parameters for Every Drug Candidate for Use Globally at Abbott.**

Lance Heinle, Mira Hinman, and Gary Jenkins.

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At every step in the drug discovery process drug candidates are put through a series of experiments to determine various properties critical to an effective and safe marketable drug including: potency, metabolism, elimination pathways, toxicity, etc…. These data are subsequently used to promote candidates with acceptable properties to the next stage in the discovery funnel and to help influence the next round of synthesis. In many of these experiments, the candidate is detected via Multiple Reaction Monitoring (MRM) - Mass Spectrometry (MS). In order to employ MRM-MS; the mass spec parameters for the candidate must first be optimized in order to obtain conditions that are unique to each candidate. With a number of different groups performing these experiments at different times, and at various locations, the optimization process is repeated anywhere from three to ten times during the drug candidates' life-time. Since the majority of these groups are utilize the same type of Mass Spectrometer, efforts are duplicated across groups obtaining the same parameters. An initiative to eliminate this replication of effort was started by a group of Scientists within GPRD. Our goal was to establish a '1-time' optimization process that could be shared across groups and divisions. This poster outlines those steps including: when/how to carry-out the optimization, determining acceptable optimization criteria, creating/accessing a Global database, and establishing a network of users.

10. **Time-Dependent Inactivation of Neuronal NO-Synthase, a P450-Like hemeprotein, by Cobalt Protoporphyrin IX.**

Sharraya Aschemeyer, Kelly M. Clapp, Miranda Lau, Hwei-Ming Peng, Yoshihiro Morishima, and Yoichi Osawa. Department of Pharmacology,

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Administration of cobalt protoporphryin IX (CoPPIX) to animals causes a loss of liver micrsomal heme and cytochrome P450 content, which is believed to be due to the induction of heme oxygenase. Interestingly, a single intracerebroventricular administration of CoPPIX at 1/100th the dose needed for effects on liver P450, leads to large reduction in body weight for up to 300 days, which is related to the loss of neuronal NO-synthase (nNOS) activity in the hypothalamus. The exact mechanism for the loss of nNOS activity is unknown. We have discovered that CoPPIX causes the time- and concentration- dependent loss of nNOS in in vitro studies with the purified enzyme. Cofactors, such as tetrahydrobiopterin or NADPH, are not required for the inactivation and substrates do not protect from inactivation. Analysis of the reaction mixtures by reverse phase HPLC under acidic conditions indicates that there is a loss of nNOS heme that corresponds to the loss of nNOS activity. Furthermore, a new peak on the HPLC chromatograph at 420 nm is formed in reaction mixtures of CoPPIX-inactivated nNOS. Although this new peak is spectrally highly similar to CoPPIX, the peak does not elute at the retention time of free CoPPIX but co-elutes with the apoprotein of nNOS. This peak may represent CoPPIX that is tightly associated with the nNOS protein. Studies with the use of HEK293 cells stably expressing nNOS confirm that CoPPIX causes the inactivation of nNOS and the formation of a protein adduct in nNOS expressing cells but not in the non-transfected cells. These studies indicate that CoPPIX can directly cause the decrease in heme and inhibit nNOS by mechanisms independent of heme oxygenase induction. Further studies with other NO-synthases and P450 enzymes are currently in progress to further define this direct mechanism of inactivation of P450 enzymes. Supported in part by NIH grants GM077430, DA022354, and F022271-05.

11. **Shifting Catalysis of Yersinia Protein Tyrosine Phosphatase.**

Shan Ke, Hua Deng and Robert Callender.

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Residue Q357 in the YopH WPD loop was reported to stabilize the thiol phosphate intermediate through the intermolecular hydrogen bonding during the YopH catalysis. The mutation at Q357 residue was performed according to the hydrophobicity of the amino acids. The Michaelis parameters of YopH wild type and its three mutants at Q357 residue were evaluated using pNPP as the substrate. The results demonstrate that YopH Q357F has a kcat value of 1800 per second, the most efficient PTPase characterized to date. The dynamic T-jump demonstrates that the chemical reaction is the decisive step and the WDP loop motion rate decides the YopH enzymatic activity.

12. ***In Vitro* Studies with Human Renal Transporters Predict No DDI Potential of Veliparib.**

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Poly (ADP-ribose) polymerases (PARP) are key enzymes involved in the repair of DNA single-strand break through base excision repair pathway. Veliparib is a novel PARP inhibitor currently under development for the treatment of tumors combined with DNA-damaging chemotherapeutics. It is largely eliminated as parent drug in the urine (70% of the dose) and renal unbound clearance exceeds glomerular filtration rate, suggesting the involvement of transporters in renal elimination. Organic Anion Transporter (OAT) 1, OAT3, and Organic Cation Transporter (OCT) 2 are the three transporters of known clinical relevance in kidney. Veliparib inhibited the uptake of reference substrates by OAT1, OAT3, and OCT2 with IC50 values of 2473 μM, >1000 μM, and 1286 μM, respectively. The clinical unbound plasma concentration of Veliparib is significantly lower than the IC50s determined for OAT1, OAT3, or OCT2, suggesting that in vivo drug-drug interaction on renal transporters is unlikely. Veliparib was found to be a substrate of OCT2, i.e. the uptake of Veliparib by OCT2-expressing cell line was significantly higher than that by mock cell line and inhibited by OCT2 inhibitor quinidine. These data suggest that OCT2 is at least partly involved in the renal elimination of Veliparib in human.

13. **Identification of the Modified Residue in CYP3A4 by a Bergamottin Reactive Intermediate.**

Hsia-lien Lin, Cesar Kenaan, Haoming Zhang, and Paul F. Hollenberg.

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Previous studies have demonstrated that bergamottin (BG), a component of grapefruit juice, is a mechanism-based inactivator of CYP3A4 and contributes, in part, to the grapefruit juice-drug interaction. Although the covalent binding of [^{14}C]BG to CYP3A4 apoprotein was evidenced by SDS-polyacrylamide gel electrophoresis, the reactive intermediate species of BG, which contribute to the mechanism of inactivation have not been reported. In the BG-inactivated CYP3A4, a GSH conjugate with MH^+ at *m/z* 696 was detected by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS analysis of BG-inactivated 3A4 revealed two deconvoluted spectra with the control and the modified apo-3A4 exhibiting a mass of ~56268 Da and ~56656 Da, respectively. The mass increase of the adduct in the modified apo-3A4 and the GSH conjugate with MH^+ at *m/z* 696 is 388 Da, equivalent to the mass of 6',7'-dihydroxybergamottin (DHBG) plus one oxygen atom. To identify the adducted residue, BG-inactivated 3A4 was digested with trypsin and the digest was then analyzed by LC-MS/MS. A mass shift of 388 Da was used for the SEQUEST database search, which revealed a mass increase of 388 Da for the peptide sequence ^{272}LQLMIDSQNSK^{282} of 3A4 and that the adducted residue is Gln273. Mutagenesis studies showed that Gln273 mutant was almost resistant to inactivation by BG and did not generate two major metabolites of BG metabolism as compared to 3A4 wild type. In conclusion, we have identified that the reactive intermediate, oxygenated DHBG, covalently binds to Gln273 and may contribute to the mechanism-based inactivation of CYP3A4 by BG.

14. **Metabolism and Pharmacokinetics of Anti-Tuberculosis Active Coumarin-3-Carboxamides with Nonselective CYP450 Inhibitor, 1-Aminobenzotriazole.**

Kuan-wei Peng, Yang Song, Nan Zhang, Rajan Giri, Zhiqiang Wang, Valentina Petukhova, Larry L. Klein, Scott G. Franzblau

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Drug-resistant tuberculosis (TB) poses an increasing challenge for TB control. Treatment of multidrug-resistant TB (MDR-TB) is difficult and takes at least 18-24 months with significant drug toxicity and less favorable outcomes. New drugs that are active against MDR-TB are urgently needed. A series of compounds containing a coumarin-3-carboxamide core structure were synthesized and analyzed for anti-TB activity. ITR685 (6-chloro-N-(2,5-dimethoxy-phenyl)-2-oxo-2H-chromene-3-carboxamide), a lead compound, from this series, was highly active in vitro (MIC:0.26uM), but low metabolic stability precluded the possibility of observing in vivo efficacy. The major metabolites were found to be hydroxylated on the 2,5-dimethoxybenzene ring. The non-specific CYP450 inhibitor 1-aminobenzotriazole (ABT) inactivates CYP450 enzymes by covalent modification of the heme prosthetic group and is orally available. The half-life of ITR685 in liver microsomes was increased 2-fold by ABT. In mice, the $AUC_{0\text{-}24h}$ for ITR685 (200 mg/kg) in mouse plasma and lung were increased 14-fold and 86-fold, respectively, after 2h prior treatment with a single oral dose of ABT at 100 mg/kg. ABT was therefore used to improve in vivo exposure of ITR685.

15. **Pharmacokinetics, Tissue Distribution, Excretion Balance, and Metabolite Profiling following a Single Intravenous or Oral Dose of [14C]-Forodesine in Rats.**

Dragomir Draganov[1], Daniel Sved[1], Leigh Harman[1], and Barry S. Levine[3].

[1]WIL Research Laboratories, LLC, [2]BioCryst Pharmaceuticals, Inc., [3]Levine & Associates, LLC.

Forodesine (BCX-1777) is a rationally designed, potent transition-state analog inhibitor of purine nucleoside phosphorylase (PNP) currently being evaluated for hematologic malignances. This GLP study determined the pharmacokinetics, tissue distribution, mass balance, and metabolite profile of [14C]-forodesine after a single intravenous (IV, 10 mg/kg, 200 µCi/kg) or oral (100 mg/kg, 200 µCi/kg) dose in male (M), non-pregnant female (F) and pregnant female (PF; dosed on gestation day 6) Sprague Dawley rats. Following IV dosing, pharmacokinetic parameters for forodesine equivalents were comparable among individual animals, between the genders, and between F and PF rats. Terminal T1/2 was 19, 16.2, and 13.3 h for M, F, and PF, respectively. The apparent Vd (>7 L/kg) indicated extensive tissue binding (PNP inhibitors bind tightly to PNP in numerous tissues including RBCs). Tissue distribution of forodesine equivalents was evaluated by quantitative whole body autoradiography (QWBA). The concentration of forodesine equivalents in most tissues was highest at 2 hrs post-dosing and then gradually decreased through 24 or 72 h. Following IV dosing, ≈90% of the dose was accounted for in urine and cage rinse; total recovery in excreta was >93%. At 72 h following IV dosing, ≈2% of the dose was recovered in the carcass. [14C]-forodesine gavage administration resulted in oral bioavailability of ≈5% for M, F, and PF rats. Tmax was 2 hrs post-dosing. Following oral dosing, ≈90% of the dose was accounted for in feces; total recovery in excreta was >94%. At 72 hrs following oral dosing, <1% of the dose was recovered in the carcass. Radio-HPLC and LC-MS/MS analysis of plasma pools following IV or oral administration detected only parent drug indicating that forodesine was not metabolized under the conditions of the study. In summary, forodesine was slowly eliminated from plasma into urine (IV) or feces (PO), and was not metabolized prior to excretion.

16. **Mechanism Based Inactivation of CYP2B6 by Selegiline.**

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Selegiline, the R-enantiomer of deprenyl, is used in the treatment of Parkinson's disease. The effect of selegiline on the enzymatic activity of bacterially expressed human cytochrome P450 2B6 in a reconstituted system has been investigated. Bupropion, a widely used antidepressant and a smoking cessation agent, is metabolized mainly by CYP2B6. Bupropion is metabolized to its primary metabolite, hydroxybupropion, by CYP2B6 and metabolism of this substrate in presence and absence of selegiline was examined. The K_m value of CYP2B6 in the presence of selegiline (12 μM) was approximately 88 μM whereas the K_m in the absence of selegiline was approximately 15 μM. The 7-ethoxy-4-(trifluoromethyl)coumarin *O*-deethylation activity of CYP2B6 was inactivated by selegiline in a mechanism-based manner. The inactivation was time-, concentration-, and NADPH-dependent. The inactivation was characterized by a K_I of 0.14 μM, a K_{inact} of 0.022 min^{-1} and a $t_{1/2}$ of 31.5 min. The reduced carbon-monoxide difference spectrum revealed 35% loss in the P450 spectrum in the inactivated sample that had lost about 70% activity. However, no loss in heme was observed when determined by HPLC. Elucidation of the molecular structure of the reactive metabolite was determined using glutathione (GSH) to trap the reactive intermediate that was generated when selegiline was incubated with CYP2B6. This led to the isolation of a GSH-selegiline conjugate with a *m/z* 528 that could be explained by the initial hydroxylation of the selegiline on the ring structure followed by the addition of glutathione at the propargyl moiety of the compound via the ketene intermediate. A proteomic approach was used to further characterize the binding of the reactive metabolite of selegiline to CYP2B6. The inactivated samples were digested with trypsin and analyzed by LC/MS and Sequest. The analysis revealed that the peptide DVFTVHLGPR was the tryptic peptide modified by the reactive metabolite formed by the metabolism of selegiline. The site of adduction was postulated to be aspartic acid-64. (Supported by grant CA16954 from the NIH).

17. **Ultra High-Throughput Microsomal Stability Assay with 7.5 Second Analysis Time per Sample.**

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To increase the probability of success of new chemical entities entering our pipeline a multi-parametric approach is taken in which chemistry iterations are driven by both potency and ADME properties. Historically in the industry, potency assays are positioned very early in the screening cascade, and ADME assays tend to be positioned later due to their higher cost and lower throughput. With the use of 384-well format coupled with cassette analysis and ultra fast LC/MS/MS, we have validated a microsomal stability assay that affords us 7.5 second analysis time per sample. This increased throughput allows us to assess the microsomal stability of all newly synthesized compounds with cycle times aligned with initial potency determinations, and enables a multi-parametric optimization approach.

A Tecan Freedom Evo liquid handling platform is used to perform the assay and an offset pipetting technology allows pooling of six compounds from each time point for cassette analysis by LC/MS/MS. The use of this unique offset pipetting feature has provided a hands-off pooling technique that is completely automated, fast and reliable. LC/MS/MS analysis is performed using a Shimadzu's NEXERA high pressure LC system with an AB Sciex 5500, and Discovery Quant software. Validation of the Tecan 384 head robot has shown robust pipetting accuracy with small volumes, clean sample transfers, and precise manipulation for all steps of the assay.

18. **Molecular Mechanisms Regulating Drug Metabolizing Enzyme Ontogeny.**

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The study objective was to evaluate changes in histone marks and the associated changes in chromatin structure as a mechanism controlling drug metabolizing enzyme ontogeny. The relative enrichment of histone marks was determined using chromatin immunoprecipitation with pooled chromatin isolated from 1st trimester primary human fetal or adult hepatocytes with quantitation by qPCR. CYP3A4 expression increases at least 20-fold between the third trimester and the adult (mean ± SD = 4.5± 2.1 versus 89.6 ± 64.0 pmol/mg microsomal protein, respectively). Evaluating the *CYP3A4* CLEM4 domain located from position -11,420 to -10,883 and containing 3 USF1, 2 HNF4α, 1 HNF1α, and 1 AP1 elements revealed a 6- to 14-foldenrichment of the H3K27me3 histone mark in fetal versus adult hepatocytes. In contrast, a 1.5- to 3-fold enrichment of theH3K4me1 histone mark was observed in adult versus fetal hepatocytes. A 100-fold difference in USF1 enrichment was observed in adult versus fetal hepatocytes. Similar results were observed for the presence of these same histone marks and the binding of C/EBPβ in the regulatory domain located between *CYP3A4* position -5900 and -5700. Given the association ofH3K27me3 and H3K4me1 with condensed and open chromatin, respectively, these data are consistent with changes in chromatin structure having a dominant role in regulating *CYP3A4* ontogeny. Supported in part by PHS grant GM081344.

19. **Mechanism-Based Inactivation of Cytochrome P450 2B6 by Methadone.**

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Methadone is a μ-opioid receptor agonist widely used in the treatment of narcotic addiction and in chronic pain conditions. Methadone is metabolized predominantly in the liver by cytochrome P450s (CYPS) to its pharmacologically inactive primary metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine. Initial in vitro data suggested that CYP3A4 is the major isoform responsible for the in vivo clearance of methadone in humans. However, recent clinical data have identified that CYP2B6 is actually the major isoform responsible for in vivo methadone metabolism and clearance.

In this study, methadone was analyzed for its ability to act as a mechanism-based inactivator of CYP2B6. Methadone inactivates CYP2B6 in a time, concentration and NADPH dependent manner with a K_I = 13.0 μM and k_{cat} = 0.01 min^{-1}. The activity loss of 2B6, in the presence of methadone and NADPH occurred with a concurrent loss of the reduced CO spectrum of the P450. Moreover, a good correlation between the loss of 2B6 activity and the loss of CO-binding was observed. HPLC analysis of P450-associated heme demonstrated that approximately 80% loss of native heme is accompanied by a comparable inactivation of 2B6. A labeled protein or stable heme adducts were not observed by LC/MS/MS analysis. These results suggest that covalent modification or destruction of heme is the major mechanism leading to the inactivation of 2B6 by methadone.

(Supported in part by the grant CA 16954 from the NCI)

20. **Cytochrome P450 2J2 Metabolizes the N-Acyl-Ethanolamine, Anandamide.**

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Cytochrome P450 2J2 (CYP2J2) is a heme containing monooxygenase that is highly expressed in hematologic malignant diseases and several human tumor tissues and cell lines. CYP2J2 metabolizes arachidonic acid (AA) to four epoxyeicosatrienoic acids (EETs) which promote tumor cell growth by increasing cell proliferation and inhibiting apoptosis. Studies show that the concentration of anandamide (AEA), an N-acyl-ethanolamine related to AA, can be increased, decreased, or unchanged in cancer cells. Conflicting reports exist regarding AEA's ability to increase or inhibit cancer cell proliferation. Moreover, AEA has been shown to induce apoptosis in certain cancer cells and it is involved in the metastatic processes of migration, invasion, and angiogenesis. In addition, anandamide is metabolized by several P450s, including CYP3A4, CYP2D6, and CYP2B6. As a result, it was hypothesized that CYP2J2 might play an important role in the metabolism of AEA in tumors. This study was carried out to determine if CYP2J2 could metabolize AEA. Using an LC/MS, initial studies demonstrate that expressed human CYP2J2 metabolizes AEA to form the 20-hydroxyeicosatetraenoic acid ethanolamide (HETE-EA) and several epoxygenated products, including 14,15-, 11,12-, and 8,9-epoxyeicosatrienoic acid ethanolamides (EET-EAs), in the reconstituted system. Preliminary kinetic studies suggest that the KM values for these products range from 14-103 μM and the kcat ranges from 1-5 pmol/min/pmol of P450. Further studies are needed to determine the effects of anandamide and its metabolites on the proliferation and apoptosis of cancer cells.

21. **Ribonucleotide-Prodrugs for Timed-Release Pharmacokinetics and Improved Water Solubility.**

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Many drug candidates demonstrate therapeutic potential in vitro but fail in vivo due to poor pharmacokinetics. To rescue these drug candidates, prodrug technology has been developed to increase favorable ADME characteristics of the drug candidate, often by attachment of a pro-moiety. Possible improvements to a drug candidate include timed-release of the drug to sustain therapeutic concentrations as well as improving its water solubility (or in some cases, lipophilicity). We chose to investigate ribonucleotides as a pro-moiety to endow these characteristics onto a model drug, hydroxytamoxifen. Ribonucleotide-prodrugs may offer many advantages to alternative pro-moieties. First, improvements to nucleotide chemistry over the last half century have made the synthesis of these prodrugs readily accessible on a laboratory to industrial scale. The catalytically efficient human pancreatic ribonuclease is known to circulate in plasma at approximately 0.4 mg/L, providing a ready mechanism for activation of the prodrug. Further, by choosing the appropriate ribonucleotide, or by appending multiple sequential ribonucleotides from a drug, the rate of activation of the prodrug could be tailored to meet the specific needs of the drug. Finally, the hydrophilic nature of the ribonucleotide (or multiple ribonucleotides), including the ionizable phosphoryl group(s), should increase the water solubility of the prodrug. Here, we report on an efficient route for the synthesis of uridine-hydroxytamoxifen, and on the kinetics of prodrug activation at the approximate physiological concentration of human pancreatic ribonuclease. These results demonstrate proof-of-principle for the exploitation of plasma ribonucleases as useful prodrug activators.

22. **P-Glycoprotein-Mediated Interaction of Lenalidomide and Temsirolimus in a Phase 1 Study in Patients with Multiple Myeloma.**

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Division of Hematology, Departments of Internal Medicine and Center for Biostatistics, College of Medicine, the Divisions of Pharmaceutics and Medicinal Chemistry, College of Pharmacy, the Comprehensive Cancer Center, Arthur G. James Cancer Hospital & Richard J. Solove Research Institute, The Ohio State University, Columbus, OH and Division of Hematology, School of Medicine, Indiana University.

Combined lenalidomide (an Immunomodulatory Drug, IMiD) and temsirolimus (mTOR inhibitor) was synergistic for cytotoxicity in multiple myeloma (MM) cell models, providing a rationale for clinical evaluation of this combination in multiple myeloma (MM). The current study aimed to investigate the pharmacokinetics of lenalidomide and temsirolimus in a phase I clinical trial for treatment of patients with relapsed MM. Patients were treated with oral lenalidomide (15-25 mg) days 1-21 of a 28 day cycle and weekly x 4 intravenous temsirolimus (15-20 mg). Pharmacokinetic parameters for both agents were calculated with non-compartmental and compartmental methods, and the potential role of P-glycoprotein (P-gp, ABCB1) in drug-drug interactions was evaluated using in vitro assays. An apparent clinical interaction was observed between lenalidomide and temsirolimus as demonstrated by statistically significant changes in systemic exposure and clearance for constant doses of one drug with increased doses of the other. In vitro studies indicated a higher basolateral-to-apical (BL-to-AP) flux compared to AP-to-BL flux for lenalidomide in MDCKII monolayers, suggesting an apically directed active transport of lenalidomide. In addition, HL-60 cells demonstrated an approximately 2-fold higher intracellular accumulation of lenalidomide than HL-60/VCR cells overexpressing P-gp. Co-incubation with temsirolimus, a known P-gp substrate and inhibitor, resulted in increased lenalidomide accumulation in HL-60/VCR cells. Further, siRNA knock-down of ABCB1 in HL-60/VCR cells led to more than a two-fold increase in lenalidomide uptake. These results suggest that lenalidomide is a P-gp substrate, contributing to its observed clinical pharmacokinetic interaction with temsirolimus. Clinical characterization of potential interactions of lenalidomide with other P-gp substrate co-medications will be critical for the continued optimal development of lenalidomide as an anti-tumor and immunomodulatory agent.

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