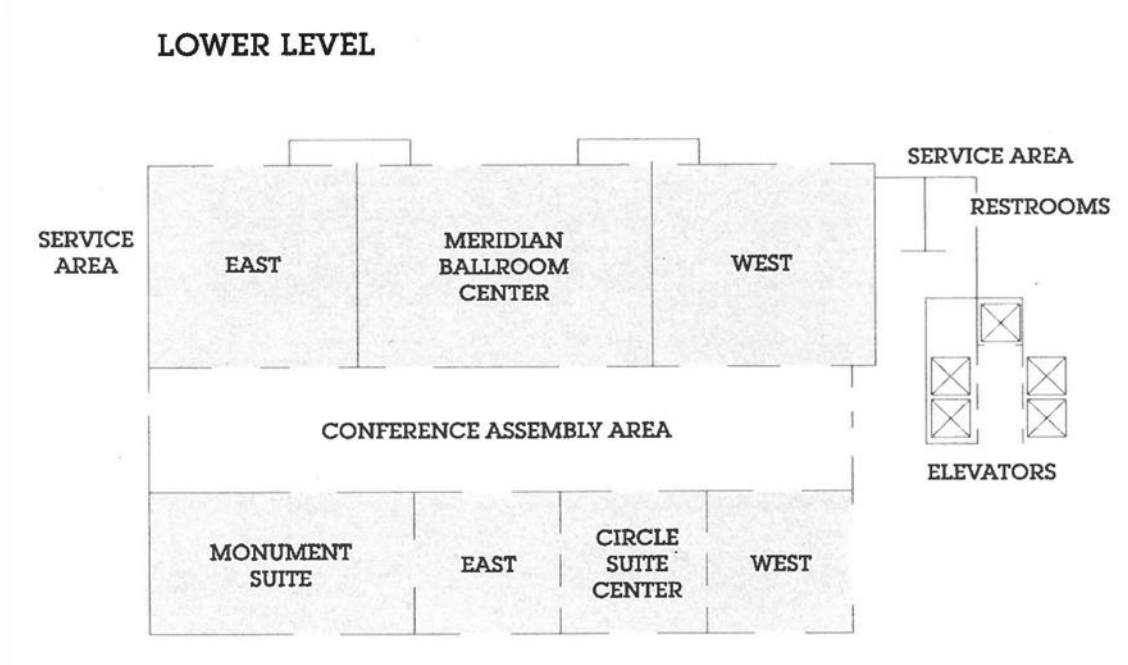
Great Lakes
Drug Metabolism & Disposition Group

13th Annual Meeting of the Great Lakes Drug Metabolism & Disposition Group

May 10-11, 2018

Sheraton Indianapolis City Centre Hotel
Indianapolis, Indiana

Hotel Map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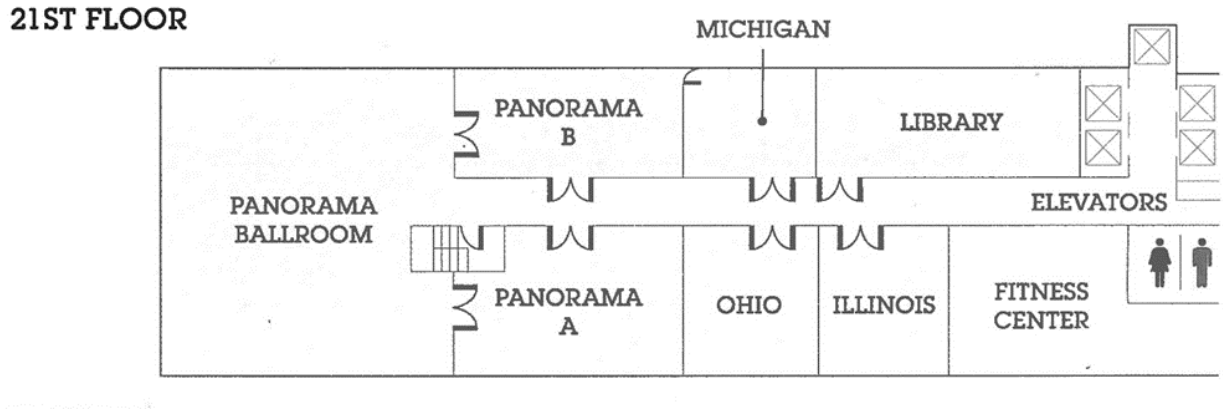
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Schedule

Thursday, May 10, 2018

10:00 AM	**Registration** (Lower Foyer) **Poster Setup, & Sponsor Booth Setup** (Circle Suites and Lower Assembly Area)
11:00	**Welcome** (Meridian Ballroom), Stephen Hall, Ph.D., Senior Research Fellow, Eli Lilly & Company
11:10	*Moderator: Rich Voorman, Ph.D. RMLV Partners* **Applications of Translational Science in Drug Development and Regulatory Agencies – Challenges and Opportunities**, Larry Lesko, Ph.D., Professor of Pharmaceutics, College of Pharmacy, University of Florida, Orlando, FL
11:50	**The Impact of Cellular Binding Proteins on Metabolic Enzyme Activity**, Nina Issoherranen, Ph.D., Professor and Vice-Chair of Pharmaceutics, University of Washington, Seattle, WA
12:30 PM	**Lunch** (Panorama Ballroom, 21st Floor) **Students/Postdocs Networking Lunch with Industry Scientists** (Panorama A/B) **Poster Viewing and Sponsor Booths** (Circle Suites and Lower Assembly Area)
2:00	*Moderator: Laura Furge, Ph.D., Professor of Chemistry, Kalamazoo College* **Protein Structure Based Approaches for Reducing Potential P450 Drug-Drug Interactions**, Eric Johnson, Ph.D., Professor of Molecular Medicine, Scripps Research Institute, La Jolla, CA
2:40	**Allosteric Mechanism of Drug-Drug Interactions Mediated by Human Cytochrome P450 CYP3A4**, Ilia Denisov, Ph.D., Senior Research Scientist, Department of Biochemistry. University of Illinois, Urbana, IL
3:20	**Break (**Lower Assembly Area)
3:40	*Moderator: J. Matt Hutzler, Ph.D., Director of ADME Services, Q2 Solutions* **Projection of Plasma Metabolite-to-Parent Drug Ratio in Human**, Scott Obach, Ph.D., Senior Research Fellow, Pharmacokinetics, Dynamics, & Drug Metabolism Department, Pfizer Inc., Groton, CT
4:20	**Drug Discovery Challenges for Developing a Treatment for Glioblastoma**, Laurent Salphati, Pharm.D., Ph.D. Principal Scientist, Drug Metabolism and Pharmacokinetics, Genentech, South San Francisco, CA
5:00	**Poster Session (Author Available), Sponsor Booths, & Mixer** (Circle Suites and Lower Assembly Area)
7:00	**Dinner and Special Presentation** (Panorama Ballroom, 21st Floor) *Introduction: Laura Furge, Ph.D., Professor of Chemistry, Kalamazoo College* **My Life as a NIH/NGIMS Program Director - Introducing Alan Alda, Discussing Stephen Colbert with Francis Collins, Meeting the Kennedy Clan and Trying to Tell NIH Why Drug Metabolism Enzymes, Drug Transporters, and Pharmacokinetics are Really Important Research Topics**, Richard Okita, Ph.D, Program Director, Division of Pharmacology, Physiology, and Biological Chemistry, NIGMS, Bethesda, MD

Schedule

Friday, May 11, 2018

7:30 AM	**GLDMDG Business Meeting** (TBD) **Continental Breakfast** (Lower Foyer) **Poster Viewing & Visit Sponsor Booths** (Circle Suites and Lower Assembly Area)
9:00	*Moderator: Hyun-Young Jeong, Ph.D., Professor, Department of Pharmacy Practice, University of Illinois at Chicago* **Solute Carriers as Modulators of Chemotherapy Toxicity**, Alex Sparreboom, Ph.D., Professor of Pharmaceutics, The Ohio State University, Columbus, OH
09:40	**The Dose Makes the Drug: Strategies to Assess and Improve the Probability of Success for Small Molecule and Antibody Drug Candidates**, Cory Kalvass, Ph.D., Senior Principal Research Scientist, AbbVie, Chicago, IL
10:20	**Break** (Lower Assembly Area)
10:40	*Moderator: Kate Hillgren, Ph.D., Senior Research Advisor, Eli Lilly and Company* **Non-CYP Metabolism: Lost in Translation, Michael Mohutsky**, Ph.D., Principal Research Scientist, Eli Lilly and Company, Indianapolis, IN
11:20	**Differential Microbiota in the Gut Modulates Susceptibility to Acetaminophen-Induced Hepatotoxicity in C57BL /6 Mice**, Sungjoon Cho, Graduate Student, University of Illinois at Chicago
11:35	**Physiologically-Based Pharmacokinetic Model Incorporating Extrahepatic Glucuronidation Predicts Integrase Inhibitor Pharmacokinetics**, Stephanie Liu, Pharm.D., Clinical Pharmacology Fellow, Indiana University School of Medicine
11:50	**Closing Remarks**, Yoichi Osawa, Ph.D., Professor, Department of Pharmacology, University of Michigan
12:15	**Eli Lilly Tour for Students and Postdocs**, Tour Coordinator: Maria Posada, Ph.D., Senior Research Scientist, Eli Lilly and Company

Posters

1. Alternative Splicing Contributes to Carboxylesterase1 Variability

Xinwen Wang, Jian Shi, Hao-Jie Zhu

Department of Clinical Pharmacy, University of Michigan, Ann Arbor, MI 48109

Carboxylesterase1 (CES1) is a primary human hepatic hydrolase involved in the hydrolytic biotransformation of numerous widely used clinical medications. Considerable interindividual variability in the responses to its substrate drugs has been extensively reported, which is essentially attributed to its genetic polymorphisms. CES1 consists of four mRNA and protein isoforms produced by alternative splicing (AS), which might have distinct contribution to the overall CES1 function. However, the activity and expression of each CES1 isoform and the overall impact of the AS on total CES1 function in human liver remain unclear. In this current study, we examined the expressions and functions of four CES1 isoforms formed by AS using transfected cells and individual human livers. The CES1 isoforms 3 and 4 showed significantly impaired activity on hydrolyzing enalapril and clopidogrel while having no significant effect on CES1 mRNA or protein expressions compared to the isoforms 1 and 2. In individual human liver samples, the correlation between the CES1 activities with the normal function isoform group 1-2 protein expressions was stronger than the impaired function isoform group 3-4, indicating the CES1 isoform group 1-2 contributes more to the total CES1 activity than the isoform group 3-4. In conclusion, the study results suggest that the different CES1 isoform compositions may affect the CES1 activity and consequently lead to the varied responses to the medications mediated by CES1. Further study is warranted to examine the impact of diseases on the CES1 AS and its consequent effect on the metabolism of CES1 substrate medications in patients.

2. Commensal gut bacteria metabolize tacrolimus

Yukuang Guo, Camila Crnkovic, Jimmy Orjala, John Lee, Hyunyoung Jeong, Hyunwoo Lee.

University of Illinois at Chicago

Tacrolimus is a commonly prescribed immunosuppressive drug used after solid organ transplantation. Tacrolimus is a narrow therapeutic index drug, but maintaining blood drug concentrations within the therapeutic range has been difficult due to large inter- and intra-individual variability in tacrolimus disposition. The objective of this study was to examine the role of gut microbiota in tacrolimus disposition. To explore the possibility of gut bacteria directly metabolizing tacrolimus, tacrolimus was incubated with C57BL/6J mouse cecum content or human stool samples anaerobically for 24 hours, and the mixture was analyzed by HPLC-UV. The results showed that tacrolimus amount decreased by up to 80% upon incubation, and this was accompanied by appearance of two new peaks on the chromatogram. Such results were not observed when tacrolimus was incubated with boiled cecum content or hepatic microsomes. Based on a recent report showing a positive correlation between fecal abundance of *Faecalibacterium prausnitzii* (*F. prausnitzii*) and tacrolimus oral dose needed to maintain therapeutic concentration in kidney transplantation patients, the ability of *F. prausnitzii* to metabolize tacrolimus was also tested. Incubation of tacrolimus with *F. prausnitzii* led to production of the same metabolites. Results from mass spectrometer analysis of the metabolites suggest that the metabolites are likely reduction products of tacrolimus. Together, these results indicate that gut bacteria, specifically *F. prausnitzii,* can metabolize tacrolimus. The extent of its contribution to overall tacrolimus disposition remains to be defined.

3. Differential microbiota in the gut modulates susceptibility to acetaminophen-induced hepatotoxicity in C57BL/6 mice.

Sungjoon Cho[1], Kyoung-Jae Won[2], Ashutosh Tripathi[1], Vanessa Leone[3], Nathaniel Hubert[3], Eugene B. Chang[3], Hyunwoo Lee[1], Hyunyoung Jeong[1,2]

[1]Department of Biopharmaceutical Sciences, [2]Department of Pharmacy Practice, College of Pharmacy, University of Illinois at Chicago, [3]Department of Medicine, University of Chicago, Chicago, IL, USA

Drug-induced liver injury (DILI) is the leading cause of acute liver failure in the US. Risk factors for developing DILI remain unclear, and thus identification of the individuals highly susceptible to DILI has been difficult. The objective of this study is to investigate the role of gut microbiota in modulating susceptibility to DILI using an acetaminophen (APAP) as a model drug.

C57BL/6 mice from two different vendors [Jackson (JAX) and Taconic (TAC)] were cohoused with mice from the same or the other vendor for 4 weeks. After overnight fasting, mice were dosed with APAP (300 mg/kg, i.p) and sacrificed 24 h after dosing. Hepatotoxicity was determined by measuring serum levels of alanine aminotransferase (ALT). TAC mice showed 4.7-fold higher ALT level compared to JAX mice, and this was abrogated upon cohousing. To further verify the role of differential gut microbiota in APAP hepatotoxicity, cecum materials from JAX or TAC mice were inoculated to C57BL/6 germ-free (GF) mice and APAP toxicity were measured after 4 weeks. Mice given TAC cecum exhibited 2.8-fold higher ALT level than mice that received JAX cecum, recapitulating the toxicity difference in conventional JAX and TAC mice. To identify the underlying mechanisms, a time-course experiment was performed where JAX and TAC mice were sacrificed at 0, 0.5, 2, 6, 12 or 24 h after APAP dosing. TAC mouse liver exhibited faster and prolonged presence of APAP-protein adducts. The extent of glutathione depletion after APAP dosing was greater in TAC mouse liver while the expression of CYP2E1, the major enzyme mediating APAP bioactivation was similar between JAX and TAC mice. Taken together, these results suggest that differential gut microbiota modulates susceptibility to APAP-induced hepatotoxicity potentially by altering APAP-protein adduct formation.

4. SAR study of flavonoids/isoflavonoids on estrogen receptor α and β selectivity: prenylation enhances flavonoid estrogenicity but decreases isoflavonoid activity.

Mbachu, O., Howell, C., Hajirahimkhan, A., Simmler, C., Dong, H., Chen, S. N., Pauli, G. F., Dietz, B. M., Bolton, J. L.*

UIC/NIH Center for Botanical Dietary Supplements Research, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago, 833 South Wood Street, Chicago, Illinois 60612-7231, USA

Use of botanical dietary supplements have increasingly become a preferred option in women's health because of perceived efficacy and safety. Some of the compounds in these botanicals are estrogenic *flavonoids* and *isoflavonoids* that share a common structural backbone. Studies show that estrogen receptor b (ERβ) ligands generally provide better safety and cytoprotective properties than estrogen receptor a (ERα) ligands. A structure-activity relationship (SAR) on estrogenic potencies of the following *flavonoids* and *isoflavonoids* found in dietary supplements was conducted to determine which molecular structures favor ERβ potency and selectivity: *8-prenylnaringenin (hops)*, *8-prenylapigenin (licorice), apigenin (ubiquitous), naringenin (ubiquitous), icaritin (horny goat weed), desmethylicaritin (horny goat weed)*; isoflavonoids: *genistein (soy), dihydrogenistein (soy; via gut microbiota), 8-prenylgenistein (licorice, lupin bean)*. Alkaline phosphatase assay was conducted using endometrial carcinoma cell lines (ERα+, Ishikawa) as *in vitro* biological endpoints to determine ERα potency. ERE-luciferase assays were used to quantify ERβ potency using breast carcinoma cells (ERβ+ transfected, MDA-MB-231:β41). Evaluations for these flavonoids showed A-ring *prenylation* at *C8* position and C-ring *saturation* at *C2-C3* position resulted in significant ERα potency, while A-ring *prenylation* at *C8* position and *unsaturation* at *C2-C3* position provided significant ERβ potency and selectivity. Methylation of 4' hydroxyl group at the B-ring significantly *reduced* overall estrogenic potency. In contrast, *C8-prenylation* and C-ring *unsaturation* at *C2-C3* on *iso*flavonoids significantly *decreased* overall estrogenic potency. These results indicate that *C8-prenylation*, *C2-C3 unsaturation* (resulting in a more planar molecule), and *4' hydroxylation* in these flavonoids are important for robust ERβ potency and selectivity, and can potentially provide favorable biological outcomes *in vivo.*
Supported by NIH P50AT000155 and T32AT007533.

5. Enhancing the Degradation of Misfolded Nitric Oxide Synthase P450 by Activating Heat Shock Protein 70

Amanda Davis[1], Haoming Zhang[1], Miranda Lau[1], Sumita Chakraborty[1], Yoshihiro Morishima[1], Andrew Lieberman[2], & Yoichi Osawa[1]

Departments of Pharmacology[1] and Pathology[2] University of Michigan, Ann Arbor, MI

The heat shock protein 90 and 70 (Hsp70/90) chaperone system provides an essential protein quality control mechanism for the cell. Heme containing enzymes, such as cytochrome P450s and neuronal nitric oxide synthase (nNOS), are susceptible to damage by xenobiotics and inactivation by specific environmental toxins or drugs leading to the selective degradation of these proteins. In the course of studies on the inactivation of nNOS, we discovered that covalent alteration of the heme binding cleft leads to misfolding of the cleft which is selectively recognized by Hsp70. Hsp70 recruits c-terminus of Hsp70 interacting protein (CHIP) leading to ubiquitination and proteasomal degradation of nNOS. In an analogous manner the Hsp90/70 chaperone system also regulates other client proteins that are prone to misfolding and aggregation, such as the polyglutamine-androgen receptor (polyQ-AR) a genetic mutant whose misfolding and aggregation leads to muscular- and neuro-degeneration in Kennedy's disease. We have demonstrated that genetic and pharmacological activation of Hsp70 increases the ubiquitination and degradation of misfolded nNOS and polyQ-AR in cells and alleviates neurotoxicity in a *Drosophila* model of Kennedy's disease. To advance the development of Hsp70 activation as a therapeutic strategy for the treatment of neurodegenerative diseases, we have established a workflow to identify novel Hsp70 modulators that enhance degradation of misfolded Hsp90/70 client proteins, utilizing nNOS as a model. We have developed a thermal shift high-throughput screen to identify novel compounds that bind and thermostabilize Hsp70. As a secondary assay we have developed an ELISA to measure the *in vitro* ubiquitination of nNOS and identify compounds that increase Hsp70- and CHIP-dependent ubiquitination. Preliminary results suggest that this method can be utilized to identify compounds that thermostabilize Hsp70, increase nNOS ubiquitination *in vitro*, and promote degradation of nNOS in HEK293 cells. This workflow has the potential to advance the development of therapeutics for the treatment of Kennedy's disease and other neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's disease which are caused by misfolding and aggregation of the Hsp90/70 client proteins tau, huntingtin, and α-synuclein.

This work was supported, in whole or in part, by National Institutes of Health Grant R01-GMO77430 and R21-NS101030, the University of Michigan Medical School's Protein Folding Diseases Initiative, and University of Michigan's Center for the Discovery of New Medicines. AD is an awardee of a PhRMA Foundation Pre-Doctoral Fellowship in Pharmacology/Toxicology and a trainee of the University of Michigan, Pharmacological Sciences Training Program T32-GM007767.

6. Use of Peptides to Disrupt NO Synthase Recognition by the Hsp90/Hsp70 Chaperone Machinery

Dana Felker[1], Miranda Lau[2], Yoshihiro Morishima[2], Amanda Davis[2], Haoming Zhang[2], Rudy J. Richardson[1], Yoichi Osawa[2]

[1]Department of Environmental Health Sciences, The University of Michigan School of Public Health, Ann Arbor, Michigan 48109-0632
[2]Department of Pharmacology, The University of Michigan Medical School, Ann Arbor, Michigan 48109-0632

The Hsp90/Hsp70 chaperones are upregulated during stress and regulate the quality control of proteins during toxicant injury. Although it is clear that chaperones specifically recognize damaged proteins in a sea of proteins in their native conformations, it remains unknown exactly how this occurs. We utilized neuronal NO synthase (nNOS) as a model Hsp90/Hsp70 chaperone client to study this process. Specifically, we used synthetic peptides (8-12 mer) that correspond to important domains on nNOS to block recognition by chaperones. Such an approach successfully identified a domain recognized by chaperones on the epidermal growth factor receptor that is exposed only on the monomeric protein. We chose sites near the heme domain that are thought to become exposed upon damage and subsequent monomerization of nNOS. To assess the extent to which peptides interfered with chaperone action, we utilized a convenient assay that measures the chaperone-mediated insertion of heme into the heme-deficient monomeric form of the nNOS. Thus, by simple measurement of nNOS activity, we could readily assess chaperone recognition of nNOS. We tested 20 peptides and found that targeting three regions within the nNOS dimerization interface (the BH_4 cofactor binding site, the Zn^{2+} binding loop, and the active site cleft opening) interfered with chaperone action on nNOS. As a control, peptides with sequences derived from regions outside the dimerization interface did not affect the heme insertion. We are currently testing the peptides to see if they directly block association of Hsp90 and Hsp70 to nNOS. Identifying the site of binding will greatly aid in understanding how chaperones recognize damaged nNOS and may provide a useful method to pharmacologically control levels of nNOS.

This work was supported by NIEHS training grant T32-ES007062, NIH grants GM077430 and GM007767, NINDS grant NS055746, and the University of Michigan Medical School's Protein Folding Disease Initiative.

7. Oxidative Inhibition of Cytochromes P450 Through Heme-Thiolate Sulfenylation

Matthew E. Albertolle, Thanh Thanh Phan, Ambra Pozzi, F. Peter Guengerich.

Vanderbilt University

The lumen of the endoplasmic reticulum (ER) provides an oxidizing environment to aid in the formation of disulfide bonds, which is tightly regulated by both antioxidant proteins and small molecules. On the cytoplasmic side of the ER, cytochrome P450 (P450) proteins have been identified as a superfamily of enzymes that are important in the formation of endogenous chemicals as well as in the detoxication of xenobiotics. Proteomic analyses of murine kidney and liver microsomes led to the finding that a number of other drug metabolizing enzymes located in the ER are also redoxregulated in this manner. We expanded our analysis of sulfenylated enzymes to human liver and kidney microsomes. Evaluation of the sulfenylation, catalytic activity, and spectral properties of P450s 1A2, 2C8, 2D6, and 3A4 led to the identification of two classes of redox sensitivity in P450 enzymes: heme thiolate-sensitive and thiol-insensitive. These findings provide evidence for a mammalian P450 regulatory mechanism, which may also be relevant to other drug-metabolizing enzymes. Testing this oxidative sensitivity in mouse and human primary isolated hepatocytes will be performed to identify relevance in vivo. This oxidative mechanism may have relevance to recent reports involving disease-drug interactions.

8. Rolapitant is not an inactivator of CYP2D6 in vitro, but in silico rolapitant is sequestered in the enzyme by a flexible helix F'

Sabrina Leddy, Sarah M. Glass, Michael Orwin, and Laura Lowe Furge.

Kalamazoo College

Rolapitant is an NK1 receptor antagonist used for chemotherapy-induced nausea and vomiting. In clinical studies, rolapitant was shown to inhibit the activity of CYP2D6 for at least a week after one dose. In the present study, rolapitant's interaction with CYP2D6 was investigated *in silico* and *in vitro*. Rolapitant does not appear to be a mechanism-based inactivator or a tight-binding inhibitor of CYP2D6. Instead, rolapitant behaved as a mixed inhibitor in assays, with a K_i of 34 μM or 20 μM with dextromethorphan or bufuralol as the reporter substrate, respectively. While this may account for a fraction of the inhibition, these values are below clinical relevance and therefore could not cause the observed long-term inhibition *in vivo*. During MD simulations, rolapitant was able to move into a position away from the heme iron and into a groove above helix I where it formed pi-pi stacking, but was unable to interact with the heme. Furthermore, during 1 μsec simulations, the rare escape of rolapitant from the enzyme required movement of helix F'. Finally, free energy landscapes for the MD simulations indicated that there are multiple low energy binding conformations for rolapitant in the active site area. Overall, rolapitant's *in vivo* inhibitory potency may be due to non-productive binding modes in CYP2D6, sequestering in the enzyme, and to the drug's long mean elimination half-life of seven days.

9. Interactions of Cytochrome P450 3A4 with Azole Inhibitors

Malika P. Godamudunage[1], Anne M. Grech[1], and Emily E. Scott[1,2]

Departments of Medicinal Chemistry[1], Pharmacology[2], and Biophysics[2], University of Michigan, Ann Arbor, MI, 48109

Cytochrome P450 3A4 (CYP3A4) is the major enzyme responsible for adult drug metabolism. Drug oxidation by CYP3A4 is inhibited by azole compounds, potentially leading to adverse drug/drug interactions, but structural information regarding human CYP3A4/azole interactions is limited to the antifungal ketoconazole. Herein CYP3A4/azole drug interactions are interrogated using both spectral binding assays and X-ray crystallography.

While titration of CYP3A4 with ketoconazole results in type II spectral binding shifts typical for heme coordination and the data is readily fit to a single-site, hyperbolic binding curve, the previously available structure of this complex reveals two ketoconazole molecules in the CYP3A4 active site. One molecule coordinates the heme iron via its nitrogen heterocycle, consistent with the observed spectral shifts, while the second is more distant from the heme and therefore may not be detectable in spectral binding titrations. In contrast, titrations of CYP3A4 with fluconazole and letrozole provide spectroscopic evidence for two distinct, concentration-dependent binding modes. While the absorbance shifts at high concentration were consistent with heme coordination, the question is raised whether the low concentration spectral shifts might derive from detection of another ligand molecule or whether one ligand molecule might reorient in the CYP3A4 active site. X-ray structures of CYP3A4 obtained under saturating concentrations showed only one copy of each of these ligands, coordinating the heme iron. The absence of evidence for a second copy of either ligand may support ligand reorientation. Finally, although titration of CYP3A4 with fosfluconazole showed very little spectral change, the X-ray structure indicates ligand does bind in the CYP3A4 active site. Overall, this data suggests that CYP3A4 interactions are better elucidated by parallel use of multiple techniques and demonstrates a diversity of CYP3A4/azole ligand interactions.

10. A Novel QconCAT-Based Proteomics Method for Determining Allele-Specific Protein Expression (ASPE): a New Approach to Identify Cis-regulatory Genetic Variants

Jian Shi [1] , Xinwen Wang [1] , Huaijun Zhu [1, 2], Hui Jiang [3] , Danxin Wang [4] , Hao-Jie Zhu [1]

[1] Department of Clinical Pharmacy, University of Michigan, Ann Arbor, MI 48109 [2] Department of Pharmacy, Drum Tower Hospital Affiliated to Medical School of Nanjing University, Nanjing, Jiangsu, China [3] Department of Biostatistics, University of Michigan, Ann Arbor, MI 48109 [4] Center for Pharmacogenomics, Ohio State University, Columbus, OH 43210

Cis-acting variants regulate gene expression in an allele-specific manner, and measuring allele-specific expression (ASE) is thus a powerful approach for identifying cis-regulatory elements. Most ASE studies are conducted at the mRNA level; however, the correlation between mRNA and protein expressions is very poor for many genes, and genetic variants affecting expression at the post-transcriptional level cannot be determined by measuring mRNA ASE. In the present study, we developed a novel targeted proteomics approach for quantification of allele-specific protein expression (ASPE) based on scheduled high resolution multiple reaction monitoring (sMRM-HR) with a heavy stable isotope-labeled quantitative concatamer (QconCAT) internal protein standard. This strategy was applied to the determination of the ASPE of UGT2B15 in human livers using the common UGT2B15 nonsynonymous variant rs1902023 (i.e. Y85D) as the marker to differentiate expressions from the two alleles. The QconCAT standard contains both the wild type tryptic peptide and the Y85D mutant peptide at a ratio of 1:1 to ensure accurate measurement of the ASPE of UGT2B15. The results from 18 UGT2B15 Y85D heterozygotes revealed that the ratios between wild type Y allele and mutant D allele varied from 0.60 to 1.46, indicating the presence of cis-regulatory variants. In addition, we observed no significant correlations between the ASPE and mRNA ASE of UGT2B15, suggesting the involvement of different cis-acting elements in regulating the transcription and translation processes of the gene. This novel ASPE approach has the potential to be widely utilized for the identification of cis-genetic variants capable of regulating gene expression at the protein level.

11. Physiologically-Based Pharmacokinetic Model Incorporating Extrahepatic Glucuronidation Predicts Integrase Inhibitor Pharmacokinetics

Stephanie Liu, Jessica Bo Li Lu, Zeruesenay Desta, Brandon T. Gufford. Indiana University School of Medicine.

Background: Integrase inhibitors now dominate HIV treatment regimens due to clinical efficacy, excellent safety, and limited drug interactions. Dolutegravir metabolism is dependent upon hepatic glucuronidation (major, UGT1A1) and oxidation (minor, CYP3A4). However, the contribution of extrahepatic glucuronidation to dolutegravir PK has not been quantitatively evaluated.

Methods: Dolutegravir was incubated with human liver, intestinal, and kidney microsomes and appropriate co-factors (2 mM UDPGA, 5 mM, $MgCl_2$, 50 µg alamethicin/mg protein) to recover enzyme kinetic parameters (see table). Stepwise PBPK model development using SimCYP® first incorporated hepatic metabolism only (CYP3A4 and UGT), followed by inclusion of intestinal and then renal glucuronidation parameters.

Results: PBPK models underpredicted observed dolutegravir apparent oral clearance by 3.5-fold, 1.5-fold, or 15% by incorporating hepatic only, hepatic and intestinal, or hepatic, intestinal, and renal glucuronidation, respectively. The model incorporating glucuronidation in all three tissues predicted observed dolutegravir C_{max} and AUC_{0-24} within 6% and 29%, respectively.

Conclusion: A mechanistic PBPK model incorporating extrahepatic glucuronidation can accurately predict dolutegravir kinetics using *in vitro* data alone.

Enzyme Source	**K_m**	**V_{max}**	**Cl_{int}**
	µM	pmol/min/mg protein	µl/min per mg protein
HLMs	140 ± 9.08	601 ± 12.9	4.3
HIMs	260 ± 18.9	1,170 ± 33.4	4.5
HKMs	161 ± 26.6	291 ± 16.2	1.8

Values represent the parameter estimate S.E. by fitting the simple Michaelis-Menten equation ($v=V_{max} *[S]/K_m+[S]$) to metabolite formation velocity using Phoenix WinNonlin (version 7.0). Cl_{int} calculated as the ratio of V_{max} to K_m.

12. Time Series Transcriptome and Flow Cytometric Analysis of Cultured Human Cell Lines: Implications for Genomic Integrity in Response to Proteasome Inhibition

Thomas De Luca, Jared Thompson, David Hendrickson, Joseph Pomerening. Indiana University School of Medicine.

The eukaryotic somatic cell cycle is divided into four phases: mitosis (M), first gap (G1), DNA synthesis (S), and second gap (G2). Phase transitions are characterized by irreversible and switch-like signaling dynamics primarily governed by the post-translational modification and rapid destruction of periodically expressed gene products. Exit from mitosis is controlled by the anaphase-promoting complex (APC), which ubiquitylates and targets M and S phase regulators for degradation by the proteasome. The APC cofactors Cdc20 and Cdh1 govern the cell's transition through mitosis, and Cdh1 remains active throughout the subsequent G1 phase to suppress continued translation of mitotic cyclins. We previously observed that ablation of the APC cofactor Cdh1 results in both premature and prolonged DNA replications, suggesting that it more generally participates in the precise dynamics of genome replication by the dividing cell. To further characterize this, we exposed three human cell lines to the proteasome inhibitor MG132 and interrogated the G1 protein levels of several mitotic regulators using flow cytometry. Using varying concentrations of MG132 in combination with transcriptional (actinomycin D) and translational (cycloheximide) inhibitors, we demonstrated that translation of these proteins occurs due to remnant transcript carried over from mitosis. While high concentrations of MG132 prevent exit from mitosis, suboptimal concentrations permit synchronized cells to exit mitosis where they aberrantly accumulate mitotic regulators and proceed into another round of DNA replication. To identify genes that may be aberrantly expressed in G1 by compromised proteasome activity, we devised a flow cytometric method to specifically sort G1 from mitotic cells and obtain sufficient high-quality RNA for next-generation sequencing without pre-amplification. We identified cell cycle-regulated genes and established their kinetic profiles by hierarchical clustering using triplicate data from six-hour time series of synchronized human cells (HeLa, RPE-1, U2OS). We then used a group-based prediction system algorithm (GPS-ARM 1.0) to identify Cdh1 recognition motifs in the human UniProt library and crossreferenced this list with published proteomic databases to identify genes in our dataset that encode known and putative targets of APC-mediated proteolysis. Functional annotation revealed significantly enriched pathways related to mitotic progression, DNA repair, and DNA synthesis.

13. Improving selectivity of abiraterone analogs for steroidogenic cytochrome P450 17A1 over cytochrome P450 21A2

Rahul Yadav[†], Caleb D. Vogt[‡], Charlie Fehl[‖], Jeff Aubé[‡], and Emily E. Scott[†,§]

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Human cytochrome P450 17A1 (CYP17A1) operates at an intersection between the production of mineralocorticoids, glucocorticoids, and sex steroids. It catalyzes two essential types of reactions: hydroxylation of pregnenolone and progesterone at C17, and C□C bond cleavage of 17α-hydroxypregnenolone (17,20-lyase reaction). The hydroxylated products of the first reaction serve as precursors for glucocorticoid production, while C-C bond cleavage yield androgens or sex steroids. Androgen deprivation therapy is clinically effective for the treatment of androgen-responsive prostate cancer. CYP17A1 has been successfully targeted by inhibitor abiraterone. Although clinically efficacious against prostate cancer, abiraterone also causes hypertension, hypokalemia, and edema, likely partly due to off-target inhibition of a second steroidogenic enzyme, cytochrome P450 21A2 (CYP21A2), which hydroxylates progesterone and 17-hydroxyprogesterone at C21. Therefore, reduction in off-target abiraterone inhibition of CYP21A2 without compromising CYP17A1 inhibition is desirable.

A series of abiraterone analogs were designed and evaluated for improved selectivity for CYP17A1 over CYP21A2 based on available structural information for CYP17A1/CYP21A2 in complex with ligands. Differences in the orientation of ligands and spatial organization of the active sites CYP17A1 vs. CYP21A2 suggested that substituents on the B-ring of the steroid core might favorably interact with polar residues in CYP17A1 and sterically clash with CYP21A2. Determining IC_{50} values for such analogs demonstrated that the best analogs in this series significantly increased selectivity by decreasing CYP21A2 inhibition up to 84-fold compared with 6.5-fold inhibition for abiraterone. Crystal structures of some of these analogs verify the intended new contacts with CYP17A1 residues, which conserve the tight binding properties of abiraterone. However, docking of these analogs into CYP21A2 identified several steric clashes that are likely the origin of decreased binding and inhibition of this enzyme. Moreover, most selective analog show higher IC50 than CYP17A1 (IC50 > 640 nM and 8.4 µM for CYP3A4 and CYP2D6 inhibition, respectively). Overall, reducing off-target inhibition of CYP21A2 is expected to reduce the undesirable clinical side effects currently observed for abiraterone in prostate cancer treatment by reducing disruption of glucocorticoid and mineralocorticoid biosynthesis.

14. Implementing Data Mining and Machine Learning Techniques to Drug Development in Schizophrenia

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Keywords: PANSS, Neuropsychiatric

BACKGROUND: Schizophrenia is a chronic debilitating mental illness that commonly begins in late adolescence. We applied machine learning algorithms to the clinical data to develop a classification system to identify disease factors that contribute to patient's symptom complex and what subset of patients is more likely to improve with medical therapy utilizing the ACLAIMS (A Comparison of Long-Acting Injectable Medications for Schizophrenia) and CATIE (Clinical Antipsychotic Trials of Intervention Effectiveness) studies.

METHODS: Using WEKA, we developed a disease severity classifier for PANSS symptoms using selected patient attributes. The study was conducted using a retrospective data mining approach with existing data from published studies collected by NIMH's Database. The study population included 2076 patient data records & 11 antipsychotic agents in injectable or oral dosage forms. Machine learning was conducted in two phases, a training (ACLAMIS) set followed by a validation (CATIE) phase using unique datasets.

RESULTS: 70% of the instances were correctly classified by the algorithm. The baseline cognitive symptoms attribute contributed the most information gain with 0.278bits. Patients with higher baseline cognitive symptoms benefited the most from the treatment. Patient age was also significantly associated with the patient's outcome with an information gain of 0.109bits. Patients > 40years are less likely to benefit from the treatment compared to their younger counterparts. The subjects receiving their first antipsychotic prescription in their 20s showed maximum improvement.

CONCLUSIONS: Data mining and machine learning algorithms are promising tools that facilitate relationships between diseases and patient factors that can be used to predict patient outcome.

15. atRA induces cholesterol accumulation through CYP7A1 repression in the liver

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All-trans retinoic acid (atRA) is one of the most frequently used retinoids to treat certain cancers or dermatological diseases. Common side effects of atRA include hypercholesterolemia, leading to discontinuation of atRA uses. Previous studies have shown that atRA downregulates CYP7A1 (the rate-liming enzyme converting cholesterol to a bile acid intermediate), potentially responsible for hypercholesterolemia. However, the detailed molecular mechanisms in atRA-induced hypercholesterolemia is unclear. atRA increased cholesterol levels in a dose dependent manner in HepaRG cells, and this was accompanied by dramatic decrease in CYP7A1 mRNA and protein levels. CYP7A1 overexpression by lentiviral system reversed atRA-induced cholesterol accumulation in HepaRG cells, suggesting that the cholesterol accumulation is mediated by CYP7A1 repression. CYP7A1 promoter reporter assays in HepG2 cells revealed that the HNF4α binding site in the promoter (-149/-118) is essential for atRA-mediated CYP7A1 repression. HNF4α depletion by siRNA abrogated CYP7A1 repression by atRA in HepaRG cells. Western blot analysis revealed that atRA activates MAPKs signaling pathways including JNK, ERK, and p38 MAPKs in HepaRG cells. Pharmacological inhibition of JNK and ERK pathways but not p38 pathway by using a specific inhibitor (i.e., SP600125, PD98059, and SB203580 for JNK, ERK, and p38, respectively) attenuated atRA-mediated CYP7A1 repression and cholesterol accumulation. Overexpression of AP-1 (c-Jun/c-Fos), a downstream target of JNK and ERK, repressed CYP7A1 expression in HepG2 cells. In DNA pull-down assay, AP-1 exhibited sequence-specific binding to the HNF4α binding site in CYP7A1 promoter following atRA treatment in HepaRG cells. Chromatin immunoprecipitation assay also showed that AP-1 binding to the CYP7A1 promoter (-181/-34) was increased following atRA treatment, whereas HNF4α binding was decreased. Collectively, results from this study indicate that atRA-activated JNK and ERK pathways and downstream target AP-1 represses HNF4α transactivation of CYP7A1 promoter. These results suggest an important role of CYP7A1 in atRA-induced cholesterol accumulation and provide a basis to establish future strategy in cholesterol disorder by atRA.

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16. Steroidogenic Cytochrome P450 11B Binding of Redox Partner Adrenodoxin Promotes Substrate Binding to the P450

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The human mitochondrial cytochrome P450 (P450) enzymes CYP11B1 (11b-hydroxylase) and CYP11B2 (aldosterone synthase) are responsible for the final steps of cortisol and aldosterone biosynthesis in the adrenal gland. Both are drug targets for Cushing's syndrome, characterized by cortisol and aldosterone overproduction. While the majority of existing studies focus on CYP11B interactions with their natural substrates and potential inhibitors, the regulatory impact of their redox partner protein adrenodoxin (Adx) on steroidogenesis is not well understood. The current study uses recombinant, purified human CYP11B1, CYP11B2, Adx, and adrenodoxin reductase (AdR) in functional assays. Initial spectral substrate binding assays examined the effect of Adx on CYP11B substrate binding. In presence of Adx, the affinity of the physiological substrate 11- deoxycorticol to CYP11B1 doubled, while the affinity for the substrate 11-deoxycorticosterone (DOC) to CYP11B2 increased 7-fold. Thus, Adx allosterically promotes substrate interaction with the P450 active site. Since Adx had more prominent effects on CYP11B2 substrate binding but the interaction between these proteins is of transient nature, several Adx-CYP11B2 fusion proteins were designed, expressed, and purified to permit more detailed study of their interactions. These fusion proteins exhibit substrate affinity for DOC similar to that observed for a 1:1 mixture of CYP11B2 and Adx. Furthermore, turnover of DOC depends on whether free Adx is provided. In reactions with the fusion protein and AdR catalysis is impeded, which would be consistent with the fused Adx domain not dissociating from the P450 to receive electrons from AdR. However, the addition of high concentrations of free Adx does support DOC turnover, suggesting that the system can be catalytically active if the fused Adx is outcompeted by free Adx. Thus, the system is catalytically active under the right conditions but promotes more stable interaction between Adx and CYP11B2. Therefore, these fusion proteinsshould facilitate to future studies defining the AdxCYP11B interaction in more detail, thus providing valuable information about the regulation of human gluco- and mineralocorticoid biosynthesis.

17. Revisiting CYP2C8, CYP2C9 and CYP2C19 induction testing in vitro: Demonstration of substantial increases in assay dynamic range with ≥ 4 day treatment periods

Jun Sun, Paul R. Lesniak, Kelly E. Desino, David M. Stresser

AbbVie, Inc.

Regulatory agencies (e.g. FDA, EMA) recommend evaluation of CYP1A2, 2B6 and 3A4 induction in vitro. These enzymes are sensitive markers of compound interaction with PXR/CAR (CYP3A4, CYP2B6) and AhR (CYP1A2). FDA guidance goes on to state that the sponsor should evaluate CYP2C8, 2C9 and 2C19 if a compound exhibits CYP3A4 induction in vitro or in vivo, since these enzymes share a regulatory mechanism similar to CYP3A41,2. However, the dynamic range for CYP2C enzyme induction in plated human hepatocytes assay has long been regarded as inadequate, necessitating evaluation of CYP2C enzyme induction in vivo. In the current investigation, we describe significant increases in CYP2C induction assay dynamic ranges when treatment periods were extended beyond the typical 2-3 day period. The rifampin treatment period of 4 or 6 days increased the assay window for all three inducible CYP2C enzyme mRNA and activity to ≥ 6-fold, a value suggested as a minimum to ascertain that a < 2-fold induction response from a test compound would be considered as a negative finding. These data suggest that treating hepatocytes for ≥ 2-fold longer than the historical convention may offer the capability of robust evaluation of CYP2C enzyme induction in vitro. The primary mechanism of increased fold induction appears to be declining target gene in vehicle controls (a process occurring with most CYPs by day 2), while test compound treatment increased and/or stabilized target gene levels.

18. Accelerating Discovery: Implementation of a human hepatocyte CYP3A4 mRNA induction screen using 24 hour exposure times

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The purpose of this study was to evaluate and implement a higher throughput CYP3A4 mRNA induction screen. Streamlining efforts focused on reducing total treatment time, but other time-saving activities were also incorporated including using 1-step RT-PCR. Cryopreserved human hepatocytes from 2 donors (VJX and ACB) were plated in a 96-well sandwich culture format and treated with 10 µM test compound for either 24 hours or 48 hours (2 X 24h). The test set consisted of model and in-house compounds from diverse chemistries within AbbVie. Total RNA from hepatocytes was extracted followed by one-step or two-step rtPCR in 96 or 384-well format, respectively. The magnitude of the response was evaluated as the fold induction of the test compound over vehicle control (0.1% DMSO). Rifampin (10 µM) was selected as the positive control (PC), omeprazole (50 µM) as a moderate-inducing control (MP), and probenecid (10 µM) as a negative control (NC) for this study. The % of PC for the test compound was calculated as: % of PC = (fold of test compound-1)/(fold of positive control -1) * 100. For the purpose of this analysis, < 20% PC was considered a "non-inducer" and ≥ 20% PC was deemed as a "likely inducer". One-step rtPCR data showed high correlation with the two-step rtPCR. Donors ACB and VJX, exhibited 95% and 85% similar classification as inducers or non-inducers, respectively at 24h compared to 48h. Overall, there was an 84% concordance observed between donor VJX and ACB. The 24 hour CYP3A4 mRNA induction assay provided a robust dynamic range to screen for CYP3A4 mRNA induction potential while demonstrating good reproducibility between different human hepatocyte donors. Importantly, project teams experiencing induction concerns receive data on a weekly basis thereby accelerating decision making. By implementing this new induction screen method we have increased our assay capacity from 1288 compounds to 2576 annually. In addition, the new 24h method has resulted in a 50% reduction in cycle time, annual consumable savings of $79,000, and a labor reduction of 207 hours.

19. In Vitro Assessment of Transporter Interactions of PI3K/mTOR Inhibitor, LY3023414, for Potential for Clinical Implications

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Purpose: The class I phosphatidylinositol-3-kinase-protein kinase B-mammalian target of rapamycin (P13K/mTOR) pathway regulates the cell cycle and is altered in cancer cell growth and survival. LY3023414 is an orally available, potent, small molecule dual kinase inhibitor of PI3K/mTOR, and being investigated as a potential treatment for patients with non-small cell lung cancer or prostate cancer in clinical trials. LY3023414 is primarily cleared by liver. Therefore, substrate potential of LY3023414 for hepatic solute carrier (SLC) transporters, OATP1B1, OATP1B3, and OCT1 was investigated. Additionally, the ability of LY3023414 to inhibit a set of clinically relevant hepatic and renal SLC transporters was assessed using a panel of in vitro cell lines.

Methods: Stably transfected human embryonic kidney (HEK) cells expressing human Organic Cation Transporter 1 (OCT1), OCT2, Organic Anion Transporter 1 (OAT1), OAT3, Multidrug and Toxin Extrusion protein 1 (MATE1), MATE2-K, and virally transduced HEK cell lines overexpressing human Organic Anion Transporting Polypeptide 1B1 (vOATP1B1), and vOATP1B3 were used to determine if LY3023414 is a substrate and/or inhibitor of these transporters. MATE1 and MATE2-K cells were purchased from Corning. Additionally, selected inhibitors of OCT1-mediated LY3023414 uptake were compared with inhibition of prototypical OCT1 substrates, metformin and sumatriptan, to evaluate 1) inhibition potency of these inhibitors, and 2) if inhibitions are substrate-dependent.

Results/Conclusion: Clinically relevant LY3023414 inhibitions were observed for efflux transporters, MATE1 and MATE2K, hepatic uptake transporters, OATP1B1, OATP1B3, and OCT1, and renal uptake transporters, OAT3 and OCT2. No clinically relevant inhibition was observed with renal uptake transporter OAT1. LY3023414 is a substrate of hepatic uptake transporter, OCT1, but not a substrate of hepatic uptake transporters, OATP1B1 and OATP1B3. Several clinically relevant OCT1 inhibitors were tested to investigate the inhibition potential of OCT1-mediated uptake of LY3023414 and varying degrees of inhibition were observed. Ondansetron and Palonosetron had different OCT1 IC_{50} values when using LY3023414 as a substrate compared to metformin or sumatriptan. Conversely, verapamil showed similar OCT1 IC_{50} values when metformin, sumatriptan, and LY3023414 were used as substrates. Substrate specificity should be considered when evaluating interaction potential as some inhibitor/substrate combinations appear to show specificity in the OCT1 transporter.

20. Mechanism-Based Pharmacokinetic-Pharmacodynamic (PK/PD) Model Describing Striatal and Cortical Dopamine Response in Rats Following a Single Dose by Several Routes of Dextroamphetamine.

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Purpose

Amphetamine is a commonly prescribed drug to manage attention-deficit hyperactivity disorder (ADHD). The drug and its derivatives also have high abuse liability. Though its exact mechanism of action is not known, we do know that it increases dopamine concentrations by inhibiting reuptake of dopamine through the dopamine transporter (DAT) and by stimulating dopamine outflow through this transporter. Neuropsychological and imaging studies indicate that the striatum is implicated in the reward and reinforcing effects of amphetamine, while the PFC is associated with symptoms of inattention in ADHD patients. The objective of this study was to define PK/PD models in the rat that relate d-amphetamine exposure in plasma and brain to its effects on dopamine in these two brain regions following single doses by IV, IP and SC routes of d-amphetamine over a range of dose strengths.

Methods

Concentrations of d-amphetamine in plasma, brain extracellular fluid (by microdialysis) and cerebrospinal fluid were determined following a 0.1 mg/kg dose. Extracellular dopamine levels normalized to pre-dose levels were also measured in PFC and striatum using quantitative microdialysis following this dose. To support PK/PD model development, a range of doses of d-amphetamine (0, 0.5, 2.5 and 5.0 mg/kg) were obtained from mean published data using Engauge digitizer. Pharmacokinetic analyses of d-amphetamine concentrations in the various matrices, and pharmacodynamic analyses of dopamine response were conducted using nonlinear mixed-effects modeling with first-order conditional estimation. A moderator function was incorporated in the model to account for development of tolerance via negative feedback, thought to be mediated by presynaptic dopamine D2 receptors. The primary pharmacokinetic parameters were fixed to serve as a driving function for the pharmacodynamic model relating d-amphetamine exposure to dopamine response. Different model structures were evaluated based on reduction in the objective function, precision in parameter estimates, and examination of goodness-of-fit plots.

Results

An indirect response PK/PD model was built to capture the delay in dopamine response in striatum and PFC relative to d-amphetamine exposure in brain extracellular fluid. The model considered both inhibition of loss of dopamine via d-amphetamine competitive inhibition of DAT, and stimulation of dopamine release by reverse transport through DAT. Under non-stimulated conditions, dopamine reuptake was faster in the striatum than the PFC; this regional difference is consistent with known higher transporter density in striatum versus PFC. The model also predicted a greater d-amphetamine stimulation in the striatum than the PFC. This larger stimulation is consistent with the known stronger dopamine response elicited by d-amphetamine in the striatum versus the PFC. In both brain regions, including an inhibition of dopamine reuptake by DAT did not improve model performance, thus, d-amphetamine`s ability to stimulate reverse transport of dopamine was the model-derived mechanism by which the drug increased synaptic dopamine. In both brain regions, incorporation of a moderator function improved model performance, consistent with development of acute tolerance to the d-amphetamine challenge.

Conclusion

A PK/PD model that relates d-amphetamine exposure to dopamine response in PFC and striatum was developed in rats. Model differences in the two brain regions are consistent with known differential effects of d-amphetamine`s ability to increase dopamine in these two regions. This model may be employed to further our understanding of the mechanism of amphetamine in abuse and ADHD.

21. Metabolite Profile Comparison using a HμREL Co-culture Hepatocyte Model vs. a Conventional Hepatocyte Suspension for Low Turnover Drugs

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Q^2 Solutions

Intro: In vitro incubations are commonly used in drug discovery in order to provide an early prediction of in vivo human metabolism. Conventional systems employ hepatocyte platforms which are typically viable out to only 4 hours. For low turnover compounds, this may be insufficient to provide an adequate assessment of human metabolism. For this study, four commercially available drugs, meloxicam, timolol, linezolid, and XK-469 were investigated using a conventional hepatocyte suspension as well as the HμREL co-culture model. These compounds have been reported to exhibit low metabolic turnover using conventional systems. The primary goal of this study was to determine whether the HμREL model could produce metabolites consistent with that observed for in vivo human studies.

Methods: The conventional in vitro model employed for this work was a pooled hepatocyte suspension (0.5×10^6 hepatocyte/mL), with a 4 hour incubation time. The hepatocyte co-culture 24-well plates were generated at HμREL using a plateable pool of cryopreserved hepatocytes. Test compounds (10 μM) were incubated for 4 hr, 24 hr, 3 days, and 7 days in a 37°C incubator with moderate shaking. Samples were analyzed using a Thermofisher LTQ Orbitrap coupled to a Shimadzu Nexera HPLC. Metabolite identification was conducted using accurate mass MS and MS/MS data, acquired via data-independent acquisition (DIA). The MS data was searched for predicted biotransformations, and the MS/MS data for any metabolite-related fragment ions. Next, targeted MS/MS data was utilized to assign metabolite structures.

Preliminary Data: Human In vitro incubations for meloxicam, timolol, linezolid, and XK-469 were first conducted using hepatocytes in suspension. For all of these drugs, minimal to no metabolic turnover was observed, in agreement with literature findings, which characterize these compounds as low turnover. Incubations employing the HμREL co-culture model greatly enhanced overall levels of metabolic turnover for all four compounds with time-dependent generation of metabolites consistent with that reported in human in vivo studies. With regard to meloxicam, the primary metabolic pathway (oxidation of the methyl substituent of the methylthiazole moiety) was reported to represent nearly 70% of the dose in human subjects. Both the hydroxymethyl and subsequent carboxylic acid (major metabolite) of meloxicam were readily generated using the HμREL hepatocyte co-culture model. In the case of XK-469, the reported primary metabolic pathway involves aldehyde oxidase mediated mono-oxidation of the quinoxaline ring moiety, and accounts for 54% of the total urinary excretion in human. While the major mono-oxidative metabolite was not produced using conventional hepatocyte suspensions, the HμREL model produced the metabolite starting at ~24 hours, and by day 7 was abundant at over 50% of parent. In addition, low-level taurine and glycine conjugates were identified. With timolol, in vivo human data reveals that 22% of the dose is transformed via oxidative opening of the morpholine ring moiety, but was not observed with conventional hepatocyte suspensions. Again, the HμREL model generated strong turnover with major morpholine ring-opening metabolites being detected. Finally, while little metabolic turnover was observed for linezolid in suspensions, incubation with the HμREL model again generated substantially greater levels of metabolism. For each of the four drugs examined, continuous metabolic turnover was observed out to 7 days of incubation in the HμREL model, covering a range of metabolic mechanisms, including cytochrome P450, aldehyde oxidase, and numerous phase II pathways.

Novel Aspect: Through the use of the HμREL hepatocyte co-culture model, key human metabolites were identified for numerous low turnover drug molecules.

22. The full-length Cytochrome P450 Enzyme CYP102A1 Dimerizes at Its Reductase Domains and Has Flexible Heme Domains for Efficient Catalysis*

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The cytochrome P450 enzyme CYP102A1 from *Bacillus megaterium* is a highly efficient hydroxylase of fatty acids, and there is a significant interest in using CYP102A1 for biotechnological applications. Here we used size-exclusion chromatography-multiangle light scattering (SEC-MALS) analysis and negative-stain EM to investigate the molecular architecture of CYP102A1. The SEC-MALS analysis yielded a homogeneous peak with an average molecular weight of 235 ± 5 kDa, consistent with homodimeric CYP102A1. The negative-stain EM of dimeric CYP102A1 revealed four distinct lobes, representing the two heme and two reductase domains. Two of the lobes were in close contact, whereas the other two were often observed apart and at the ends of a U-shaped configuration. The overall dimension of the dimer was ~130 Å. To determine the identity of the lobes, we FLAG-tagged the N or C terminus of CYP102A1 to visualize additional densities in EM and found that anti-FLAG Fab could bind only the N-tagged P450. Single-particle analysis of this anti-Flag Fab:CYP102A1 complex revealed additional density in the N-terminally tagged heme domains, indicating that the heme domains appear flexible while the reductase domains remain tightly associated. Effects of truncation on CYP102A1 dimerization, identification of cross-linked sites by peptide mapping, and molecular modeling results all were consistent with the dimerization of the reductase domain. We conclude that functional CYP102A1 is a compact globular protein dimerized at its reductase domains, with its heme domains exhibiting multiple conformations that likely contribute to the highly efficient catalysis of CYP102A1.

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23. A rapid cocktail LC-MS/MS Method to assess inhibitor Selectivity for CYP phenotyping in human liver microsomes and hepatocytes

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Phenotyping to estimate the fractional contribution of cytochrome P450 enzymes to the metabolism of drug molecules ($f_{m,\ CYP}$) is a common practice in drug discovery and development. These experiments are performed to identify metabolic pathways and assess the risk of victim drug-drug interactions (DDIs) for new drug candidates. A rapid and robust discovery CYP phenotyping method where the selectivity and effectiveness of each chemical CYP inhibitor could be monitored in parallel (i.e. cocktail method) for early victim DDI risk assessment with minimal sample analysis would be of value to discovery project teams. In an attempt to develop a streamlined phenotyping method for control substrates and inhibitors, experiments were conducted to determine the formation rate of selective metabolites from a cocktail of seven CYP probe substrates in both pooled HLM and cryopreserved human hepatocytes. Utilizing a Tecan automation platform, the CYP probe cocktail mixture (tacrine, bupropion, amodiaquine, diclofenac, *S*-mephenytoin, dextromethorphan, and midazolam) was co-incubated ($\leq K_m$) in the presence of either no inhibitor, or CYP-specific chemical inhibitors at reported selective concentrations. Aliquots were collected at six time points and quenched with organic solvent containing internal standard. The incubated samples from these experiments were analyzed using a cocktail analytical method (LC-MS/MS on a Sciex API4000, 1.2 minutes per injection, ESI+) to enable rapid assessment of the effectiveness and selectivity ($f_{m,\ CYP}$) of each inhibitor.

Estimated $f_{m,\ CYP}$ in Human Liver Microsomes								
Probe Metabolite	Inhibitor (concentration)	1A2	2B6	2C8	2C9	2C19	2D6	3A
1'-Hydroxytacrine	a-NF (1 µM)	**0.93**	0.042	0.055	0.20	0.036	0.00	0.15
Hydroxybupropion	PPP (30 µM)	0.10	**0.70**	0.050	0.30	0.00	0.00	0.20
Desethylamodiaquine	Montelukast (3 µM)	0.17	0.025	**0.85**	0.18	0.071	0.00	0.12
4'-Hydroxydiclofenac	Sulfaphenazole (10 µM)	0.15	0.16	0.19	**0.97**	0.081	0.00	0.18
4'-Hydroxymephenytoin	N3BP (1 µM)	0.00	0.00	0.00	0.33	**0.83**	0.00	0.00
Dextrorphan	Quinidine (2 µM)	0.054	0.24	0.14	0.19	0.027	**0.89**	0.00
1`-Hydroxymidazolam	Ketoconazole (1 µM)	0.00	0.00	0.00	0.094	0.00	0.00	**0.90**
Estimated $f_{m,\ CYP}$ in Human Cryopreserved Hepatocytes								
Probe Metabolite	Inhibitor	1A2	2B6	2C8	2C9	2C19	2D6	3A
1'-Hydroxytacrine	a-NF (1 µM)	**0.97**	0.13	0.00	0.00	0.00	0.00	0.00
Hydroxybupropion	PPP (30 µM)	0.00	**0.93**	0.00	0.00	0.00	0.00	0.17
Desethylamodiaquine	Montelukast (3 µM)	0.00	0.00	**0.87**	0.00	0.00	0.00	0.31
4'-Hydroxydiclofenac	Sulfaphenazole (10 µM)	0.00	0.060	0.00	**0.75**	0.00	0.00	0.00
4'-Hydroxymephenytoin	N3BP (1 µM)	0.00	0.00	0.00	0.00	**0.80**	0.00	0.00
Dextrorphan	Quinidine (2 µM)	0.00	0.21	0.00	0.00	0.00	**0.86**	0.00
1`-Hydroxymidazolam	Ketoconazole (1 µM)	0.00	0.29	0.024	0.00	0.00	0.00	**0.93**

$f_{m,\ CYP}$ = (Formation Rate$_{no\ inhibitor}$ – Formation Rate$_{inhibitor}$) / Formation Rate$_{no\ inhibitor}$

Based on estimated $f_{m,\ CYP}$ values in HLMs and hepatocytes, the probe substrates and chemical inhibitor conditions selected for this experimental design demonstrate selectivity towards the target CYP enzyme. Development of a robust cocktail incubation and analytical method to simultaneously analyze all seven probe substrate metabolites results in a 7-fold decrease in analysis time per study, thus enabling rapid verification of the effectiveness and selectivity of each inhibitor while estimating $f_{m,\ CYP}$ for new drug candidates.

24. Effect of Delayed Gastric Emptying on the Pharmacokinetics of Warfarin

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Objectives: Glucagon-like peptide-1 agonists (GLP-1) are used in the treatment of diabetes and have been shown to slow gastric emptying in both healthy and diabetic subjects. Thus, GLP-1 agonists may affect the exposure of concomitantly administered oral medications. The objective of this work was to evaluate the effect of delayed gastric emptying time on the pharmacokinetics of warfarin.

Methods: The effect of dulaglutide, a GLP-1 agonist, on gastric emptying time was established based on the results of a scintigraphy study, acetaminophen absorption test, and subsequent PopPK modeling. A physiologically-based pharmacokinetic (PBPK) model for s-warfarin, coupled to a mechanistic absorption model, was implemented in Simcyp V16 using physicochemical and in vitro biological data and was used to predict the effect of delayed gastric emptying on warfarin pharmacokinetics. The PBPK model was verified using observed clinical data of warfarin alone and in combination with dulaglutide.

Results: The PBPK model reproduced the maximum concentration in plasma (Cmax) and area under the concentration time curve ($AUC_{0\text{-}inf}$) of s-warfarin with predicted values within 1.1-fold of the observed values. The model also predicted the observed time at maximum concentration (Tmax) within 2-fold of the observed value. Moreover, the s-warfarin PBPK model reproduced the effect of the gastric emptying delay following administration of 1.5 mg dulaglutide, with a predicted Cmax ratio of 0.9, an AUC ratio of 1.0, and Tmax difference of 2 hours. All predicted values were within 1.1 fold of the observed values. Furthermore, a sensitivity analysis showed that varying gastric emptying time up to 5 hours did not have a clinically meaningful effect on the AUC and Cmax of s-warfarin, but increased Tmax.

Conclusions: The delay in gastric emptying with the administration of 1.5 mg dulaglutide did not affect the AUC or Cmax of s-warfarin, but increased Tmax. The PBPK model reproduced the effect of dulaglutide on the pharmacokinetics of s-warfarin.

25. Mechanism of FASN regulation of NF-kB/p65 expression in drug resistance of breast cancers

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Fatty acid synthase (FASN), the sole cytosolic enzyme responsible for de novo palmitate synthesis, is critical for cancer cell survival and is a marker of poor prognosis. FASN overexpression has been shown to confer resistance to both drug and radiation resistance by inhibiting drug-induced ceramide production and by increasing DNA repair activities. Recently, it was found that FASN up-regulation of non-homologous end joining (NHEJ) repair of double strand DNA breaks was due to FASN up-regulation of Poly (ADP-ribose) polymerase 1 (PARP1) by potentially inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB/p65). However, how FASN regulates NF-kB/p65 remains unknown. In this study, we tested the hypothesis that, palmitate, the end product of FASN catalysis, plays an important role in regulating p65 expression and activity through decreasing its protein stability. Using paired isogenic breast cancer cell lines with FASN overexpression and knockdown, we found that FASN expression is negatively correlated with p65 protein level as well as NF-kB activity, whereas FASN expression has no effect on p65 at the transcriptional level. Similarly, we found that FASN pharmacological inhibition with two small molecule inhibitors resulted in a concentration-dependent increase in p65 protein level but did not affect p65 at the transcript level. Further, we found that exogenous addition of palmitate in cell culture resulted in a reduction p65 protein level, indicating a role for palmitate downstream of FASN in this process. Finally, inhibition of protein synthesis in conjunction with FASN inhibition resulted in increased p65 protein level to FASN inhibition alone, indicating a role for FASN in decreasing p65 protein stability. We are currently working to further characterize the role of FASN and, more specifically, palmitate in affecting p65 protein stability in the drug resistance pathway.

26. Exploring Structural Conformations of Human P450 1A1 with Diverse Ligands

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The human P450 enzyme 1A1 (CYP1A1) is an extrahepatic P450 often expressed in tumor tissues where it may be clinically significant in procarcinogens and toxin bioactivation. Prototypical CYP1A1 ligands are planar and polycyclic aromatic compounds, such as the procarcinogen benzo[a]pyrene found in environmental combustion and tobacco smoke. The single crystal structure of CYP1A1 is with the polycyclic, planar molecule alpha-napthoflavone (αNF) and reveals a narrow active site that is consistent with planar CYP1A1 ligands. However, a number of known CYP1A1 substrates and inhibitors do not adhere to these typical ligand features, including promising pro-drugs in cancer therapy based on a duocarmycin scaffold and clinically used tyrosine kinase inhibitors. Such CYP1A1 ligands could not be accommodated in the active site of the CYP1A1/αNF structure. This study aims to determine how CYP1A1 binds other CYP1A1 substrates and inhibitors using co-crystallization.

Potential ligands for co-crystallization trials with CYP1A1 were selected based on structural diversity, focusing on those that are not compatible with the 1A1/αNF crystal structure active site dimensions. These ligands were ranked based on their ability to inhibit CYP1A1 metabolism of a pro-luciferin substrate. Anticancer tyrosine kinase inhibitors (erlotinib, imatinib, and ponatinib) displayed nanomolar to low micromolar IC50 values. Antifungal azoles, which are generally potent P450 inhibitors, resulted in a range of inhibitory responses: sub-nanomolar potency for compact clotrimazole, miconazole, and tioconazole; nanomolar for larger ketoconazole and posaconazole; and micromolar for fluconazole and letrozole. Anticancer pro-duocarmycin analogs and the benzothiazole GW 610 inhibited CYP1A1 in the sub-micromolar to nanomolar range. The natural furanocoumarin and antimutagen bergamottin demonstrated a submicromolar IC50. Several of the potent inhibitors were cocrystallized and X-ray structures determined, including erlotinib, bergamottin, and a duocarmycin analog. These structures identify CYP1A1 active site features that are both conserved and modified across ligands, thereby providing a more robust framework for prediction of CYP1A1 activity with diverse compounds.

27. Mammospheres as Models for Predicting P450 1A1/1B1 Metabolism

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Since the Women's Health Initiative reported that hormone replacement therapy directly correlated with increased risk of breast cancer and heart disease, many American women have turned to botanical supplements to seek relief from menopausal symptoms. Little is known about how these extracts modulate the chemical carcinogenic effects of estrogens. In the genotoxic pathway, P450 1B1 performs 4-hydroxylation of estrone/estradiol whereas P450 1A1 catalyzes detoxification of estrogen through 2-hydroxylation. These pathways are classically regulated by the aryl hydrocarbon receptor (AhR), and estrogen receptor alpha (ERα) regulates P450 1A1 epigenetically. Botanical supplements can affect both ERα and AhR, causing differential effects within the same supplement. The ethoxyresorufin-O-dealklase (EROD) assay measures activity of P450 1 family of enzymes. Unfortunately, in 2D MCF-7 cells, EROD signal was low and the AhR-mediated effect could not be separated from the ERα-mediated effect. 3D-Mammospheres are considered to be better models of humans than 2D monolayers. qPCR showed increased CYP1A1, but not CYP1B1 expression in 3D models, and with the P450 1B1 selective inhibitor, 2,3',4,5'-tetramethoxystilbene (TMS), ERα-mediated effects can be separated from those mediated by AhR. These results indicate that MCF-7 mammospheres, not monolayers, can be utilized to screen for modulation of estrogen chemical carcinogenesis and should be investigated in other assays as a way to achieve *in vitro* results more similar to humans. *Supported by NIH Grant* P50AT000155.

28. OCTN1-MEDIATED TRANSPORT OF CYTARABINE IN ACUTE MYELOID LEUKEMIA

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BACKGROUND:
Cytarabine is used in the treatment of acute myeloid leukemia (AML) and requires functional transporters to enter cells. Using heterologous expression models, we recently reported that cytarabine is a substrate of OCTN1 (Drenberg *et al*, Cancer Res 2017). Here, we tested the hypothesis that OCTN1 facilitates the accumulation of cytarabine in AML cells, a prerequisite to anti-leukemic activity.

METHODS:
Uptake and cytotoxicity studies were performed in 10 AML cell lines and primary diagnostic AML blasts isolated from patients in the presence and absence of the transporter inhibitor NBMPR or agents co-administered clinically with cytarabine. Pharmacokinetic studies were performed in wild-type and OCTN1-deficient mice.

RESULTS:
Cytarabine (1 µM; 15 min) uptake in AML cell lines varied 6-fold (17-101 pmol/mg) and was highly sensitive to inhibition by NBMPR. Uptake values in blasts were in the same range as those observed in cell lines, with higher uptake and increased sensitivity observed in samples with higher OCTN1 protein levels. Among other AML-directed drugs evaluated, we found that the anthracyclines daunorubicin and idarubicin inhibited cytarabine uptake in a concentration-dependent manner ($P<0.05$). In contrast, exposure to the hypomethylating agent 5-azacytidine increased uptake, consistent with the notion that basal epigenetic regulation of OCTN1 was inversely related to cytarabine uptake. Plasma levels of cytarabine were unchanged in OCTN1-deficient mice, suggesting that variability in host OCTN1 function does not substantially impact disposition properties.

CONCLUSIONS:
These results identified OCTN1 as a contributor to the cellular uptake and efficacy of cytarabine, with broad implications for the future design of combination chemotherapy regimens.

29. ABCG2 expression contributes to CC-115 resistance, a dual mTOR/DNA-PK inhibitor

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Introduction: A major obstacle in cancer treatment is intrinsic or acquired resistance. ABC (ATP-binding cassette) transporters such as, ABCB1, ABCC1, and ABCG2 have been shown to contribute to multidrug resistance (MDR) and are predictors of clinical outcomes. CC-115 is a dual mTOR/DNA-PK inhibitor, which is currently in clinical trials for treating cancers including prostate, ewing's osteosarcoma, chronic lymphocytic leukemia, squamous cell carcinoma of head and neck, and glioblastoma. Here we evaluate the mechanisms of CC-115 resistance by investigating the possible role of ABCG2 in transporting CC-115.

Methods: Western blots, methylene blue and MTT assays, and FACS were used to determine ABCG2 expression, cell survival/viability with and without inhibitors, and accumulation of CC-115, respectively.

Results: The drug-resistant MCF7/AdVp3000 (M3K) cells have been shown to have a significant increase in ABCG2 as compared to parental MCF7 cells. M3K cells had a 50-fold increase in IC50 to CC-115 compared to MCF7. Use of ABCG2 inhibitors FTC and C8 decreased the IC50 of CC-115 in M3K cells by 10-fold. Accumulation studies using FACS showed that M3K cells had significantly lower accumulation of CC-115 as compared to MCF7 and addition of FTC and C8 significantly enhanced accumulation of CC-115 to the same extent as seen in MCF7 cells. Next, HEK293/ABCG2 stable clone with overexpression of ectopic ABCG2 and its control HEK293/vec cells were used to determine ABCG2's direct impact on CC-115 resistance. HEK293/ABCG2 cells had a 10-fold increase in resistance to CC-115 with an IC50 of 2.05 µM as compared to 0.23 µM in HEK293/vec cells. Addition of FTC and C8 decreased the IC50 by 10-fold in HEK293/ABCG2 cells. Accumulation of CC-115 in HEK293/ABCG2 cells was also significantly lower than that in HEK293/vec cells and addition of FTC and C8 significantly increased its accumulation only in HEK293/ABCG2 cells.

Discussion: The results from this study suggest that ABCG2 contributes to and possibly is responsible for CC-115 resistance and that inhibiting ABCG2 could lead to reversal of CC-115 resistance, increased response rate, highlighting the importance of personalized therapies with a potential to increase patient survival.

30. Comprehensive assessment of cytochromes P450 and transporter genetics with paclitaxel time above threshold

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Background: Efficacy and toxicity of paclitaxel treatment have been associated with the amount of time a patient's paclitaxel concentration remains above 0.05 μM ($T_{c>0.05}$). Our aim was to confirm that patients with low-activity CYP2C8 genotype have longer $T_{c>0.05}$ and to discover additional clinical and genetic predictors of paclitaxel $T_{c>0.05}$ using a comprehensive genotyping approach of functionally consequential SNPs in paclitaxel-relevant genes.

Methods: Sixty female patients with breast cancer scheduled to receive 12 weekly doses of 80 mg/m^2 paclitaxel infused over 1-hour were enrolled on a prospective observational clinical trial (NCT02338115). Plasma samples were collected 16-26 hours after infusion. Paclitaxel concentrations were measured via LC-MS and entered into MyCare™ Dose Exposure Calculator to estimate $T_{c>0.05}$. Germline DNA was genotyped using the iPLEX® ADME PGx Pro Panel. Six genes relevant to paclitaxel metabolism or transport: *CYP2C8*, *CYP3A4*, *ABCB1*, *ABCC2*, *ABCG2*, *SLCO1B1*, and *SLCO1B3* were chosen for analysis. For the primary analysis, *CYP2C8*, poor (PM) and intermediate (IM) metabolizer phenotypes were compared with normal metabolizers (NM) via linear regression. All other genes were tested similarly, followed by attempted inclusion of clinical variables: age, race, body surface area, and liver function tests in multivariable models.

Results: *CYP2C8* PM/IM status was associated with shorter $T_{c>0.05}$ (β= -11.31%, 95% CI: -21.15% - -0.24%, P=0.047). *SLCO1B1* PM/IM status was associated with longer $T_{c>0.05}$ (β = 17.35%, 95% CI: 4.33% - 32%, P=0.013). None of the other candidate genes or clinical variables were associated with $T_{c>0.05}$.

Conclusion: Contrary to what was expected, low-activity *CYP2C8* metabolizer phenotype was associated with shorter $T_{c>0.05}$. *CYP2C8* and *SLCO1B1* genetic polymorphisms could account for some of the inter-individual variability in paclitaxel exposure.

31. CYP2D6 Allelic Variants *1, *34, *17-2, *17-3, *53 and a Thr309Ala Mutant Display Altered Kinetics and Susceptibility to Inactivation

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CYP2D6 with over 100 allelic variants metabolizes ~20% of all pharmaceuticals and exhibits a wide-range of drug responses among individuals including cases of adverse drug events. Four CYP2D6 allelic variants - three with a series of mutations distal to the active site (*34, *17-2, *17-3) and one ultra-metabolizer with mutations near the active site (*53), along with reference *1 and an active site mutant of *1 (Thr309Ala) were expressed, purified, and studied for interactions with the typical substrates dextromethorphan and bufuralol and with the inactivator SCH 66712. Decreased activity was observed for *34, *17-2, and *17-3 while the increased activity of *53 confirmed it as an ultra-metabolizer. The Thr309Ala mutant displayed similar kinetic values as *1. The inactivation of 2D6 variants by a known-mechanism based inactivator, SCH 66712, was investigated in a time- and concentration-dependent manner for *1, *53, and T309A. Though *1 was inactivated by SCH 66712, *53 and Thr309Ala were less susceptible to inactivation. These findings support the designation of Thr309Ala as the nucleophilic target for inactivation of CYP2D6 by SCH 66712. Overall, these results suggest that kinetic and metabolic analysis of individual CYP2D6 variants is required to understand their possible contributions to variable drug response and the complexity of personalized medicine.

32. NADPH Coupling and Metabolite Distribution are Affected by Amino Acid Changes in CYP2D6 Allelic Variants *1, *34, *53, *17-2, *17-3, and in a Thr309Ala Mutant

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Human CYP2D6 is responsible for about ~15% of drug metabolism and has the most polymorphisms among P450s. CYP2D6 polymorphisms result in varying metabolic activity as well as product distribution. One possible explanation for varying activity is that certain allelic variants have reduced ability to couple NADPH consumption with product formation. The purpose of this study was two-fold: to determine the product distribution of several CYP2D6 variants with regard to metabolism of three substrates, dextromethorphan, bufuralol, and SCH 66712; and, to determine the percent coupling of NADPH consumption of each of the variants during metabolism. The CYP2D6 variants *1, *34, *53, *17-2, *17-3, and a Thr309Ala mutant were used in the present studies. Analysis of metabolite profiles revealed notable novel products formed from dextromethorphan metabolism. The oxygen surrogate CuOOH did not support the same distribution of products as NADPH. Variants *34, *17-2, and *17-3 displayed reduced enzyme activity and NADPH coupling while producing the same metabolites as *1 suggesting a possible role for Arg296 in NADPH coupling. Though a higher activity variant, *53 displayed similar NADPH coupling to *1 suggesting that increased activity is not due to increased NADPH coupling. Conversely, the Thr309Ala mutant showed similar activity as *1 but with greatly reduced NADPH coupling. Also, the reduced coupling of the Thr309Ala mutant may lead to an increased formation of H_2O_2 - an inactivator of CYP2D6, and support a role for Thr309 in oxygen activation in the mechanism of CYP2D6.

33. Mechanism-Based Pharmacokinetic/Pharmacodynamic (PK/PD) Model Describing Striatal and Cortical Dopamine Response in Non-Human Primates Following a Single Intravenous Dose of Dextroamphetamine

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Purpose

Amphetamine is a commonly prescribed drug to manage attention-deficit hyperactivity disorder (ADHD), but with attested abuse potential. Though its exact mechanism of action remains unclear, it is thought to act by elevating extracellular dopamine in the striatum and prefrontal cortex (PFC) by inhibiting reuptake of dopamine by the dopamine transporter (DAT) and by promoting dopamine outflow through this transporter. Neuropsychological and imaging studies indicate that the striatum is implicated in the reward and reinforcing effects of amphetamine, while PFC is associated with symptoms of inattention in ADHD patients. The objective of this study was to define PK/PD models that describe striatal and PFC dopamine responses in non-human primates (NHPs) following a single intravenous (I.V) dose of d-amphetamine.

Methods

Extracellular dopamine levels normalized to baseline (pre-dose) levels were measured in caudate (striatum) and PFC using quantitative microdialysis following administration of 0.1 mg/kg I.V bolus dose of d-amphetamine in male cynomolgus monkeys. To support PK/PD model development, a range of I.V bolus doses of d-amphetamine (0.3 ,0.5, 1 mg/kg), in male rhesus macaques were obtained from mean published data using Engauge digitizer. Dopamine and d-amphetamine concentrations were determined by HPLC MS/MS using appropriate internal standards. Pharmacokinetic analyses of d-amphetamine concentrations in various matrices and pharmacodynamic analyses of dopamine response in striatal and cortical extracellular fluid were conducted. A nonlinear mixed-effects (NLME) modeling approach with first-order conditional estimation in Phoenix NLME (Pharsight Corporation) was used to estimate population parameters and their between-occasion variability. A moderator function was incorporated in the model to account for development of tolerance via negative feedback, mediated by presynaptic dopamine D2 autoreceptors. The primary pharmacokinetic parameters were fixed to serve as a driving function for the pharmacodynamic model relating d-amphetamine exposure to dopamine response. Both the pharmacokinetic and pharmacodynamic parameters were estimated in the final PK/PD model. Different model structures were evaluated based on reduction in the objective function, precision in parameter estimates, and examination of goodness-of-fit plots.

Results

An indirect response PK/PD model was built to capture the delay in dopamine response in striatum and PFC relative to d-amphetamine exposure in brain extracellular fluid of NHPs. The model considered both inhibition of loss of dopamine via d-amphetamine competitive inhibition of DAT, and stimulation of dopamine release by reverse transport through DAT. Under non-stimulated conditions, dopamine reuptake was faster in the striatum than PFC; this regional difference is consistent with known higher transporter density in the striatum versus PFC. In the presence of d-amphetamine, dopamine was increased to a greater extent in the striatum versus the PFC at all doses. The model also predicted a stronger d-amphetamine stimulation in the striatum than the PFC. This larger stimulation is consistent with the known stronger dopamine response elicited by d-amphetamine in the striatum versus PFC. In both brain regions, inhibition of dopamine reuptake by d-amphetamine was a small percentage of d-amphetamine`s ability to stimulate reverse transport of dopamine. Thus, d-amphetamine`s effects on dopamine appeared to be primarily mediated through stimulation of reverse transport. In both brain regions, a clockwise hysteresis was observed at all doses, suggesting development of acute tolerance to the d-amphetamine challenge. This observation justified the incorporation of a moderator function in the model.

Conclusion

A PK/PD model that relates d-amphetamine exposure to dopamine response in striatum and PFC was developed in NHPs. Model differences in the two brain regions are consistent with known differential effects of d-amphetamine`s ability to increase dopamine in these two regions. This model may be employed to further our understanding of the mechanism of amphetamine in abuse and ADHD, and extend this understanding to humans.

34. Pharmacokinetics, Mass Balance and Metabolism of a PPARδ agonist, MBX-8025

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MBX-8025 is a selective PPARδ receptor agonist that is in development for the treatment of primary biliary cholangitis (PBC; primary biliary cirrhosis) leading to cholestasis. Previous studies have indicated that in intact rats, a major route of clearance of MBX-8025 is through the bile. Therefore, it is important to understand the adsorption, distribution, metabolism and elimination (ADME) properties of MBX-8025 in a model in which elimination through the bile is not available. In this study, radioactivity was measured in plasma and excreta following the oral administration of [14C]-MBX-8025 to bile duct ligated (BDL) rats and sham-operated controls. In addition, the metabolite profiles were determined by LC radiochromatography and the mass spectral characteristics of radioactive components were determined.

After an oral dose of [14C]MBX-8025, radioactivity appeared in plasma of BDL rats at higher levels compared to controls (2.4x at 2 hr, 7.7x at 8 hr) and primarily in the form of MPI-23 (O-dealkyl MBX-8025, CymaBay M2), unchanged MBX-8025 and MPI-13 (glucuronide conjugate of MPI-23, CymaBay M12). In the control sham operated rats, the radioactive dose was primarily eliminated via the feces as MBX-8025 and 10 metabolites. In BDL rats, in contrast, the radioactive dose was eliminated primarily in the urine as 12 metabolites with trace amounts of unchanged MBX-8025. In summary, blocking the clearance mechanism of drug resulted a shift from excretion from the feces to the urine. Although the relative amounts of some metabolites changed with BDL, the qualitative drug metabolite profile was unchanged.

35. Evaluation of the *In Vitro* Metabolism Capability of MetMax™ Rat Hepatocytes for Chemicals (Butoxyethyl Benzoate and Tricyclazole)

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The liver is the most important site of chemical/pharmaceutical metabolism in the body. The majority of chemicals or pharmaceuticals are metabolized by hepatic enzymes such as those from the cytochrome P450 superfamily (CYPs). Hepatocytes contain a full complement of enzymes and cofactors within the intact cell. Therefore, hepatocytes are one of the best *in vitro* sources for determining metabolites of a compound of interest. Traditionally, hepatocytes are provided in a cryopreserved form in which specialized handling procedures (i.e. cell plating) and equipment (i.e. CO_2 incubator) are needed to prepare for an experiment. These additional requirements increase the length of time and resources needed for a metabolism study and thus increase overall study cost. Recently, microsome-like hepatocytes (MetMax™) were developed by *In Vitro ADMET Laboratories, Inc.* (IVAL). These unique hepatocytes are reported to have similar, if not greater, metabolic enzyme activity than fresh or traditional cryopreserved hepatocytes and they are regularly used in metabolism studies for pharmaceuticals. However, whether this type of hepatocyte (MetMax™) can be applied to metabolism studies of industrial chemicals has yet to be explored. In this study, two representative chemicals (butoxyethyl benzoate and tricyclazole) were selected and incubated with both MetMax™ rat liver hepatocytes and rat liver microsomes under physiological conditions (pH 7.4 and 37°C). The final metabolite profiles were compared with their corresponding *in vivo* rat urinary metabolite profiles. Overall, rat MetMax™ metabolite profiles of both test chemicals were much closer to their corresponding rat urinary metabolite profiles than metabolite profiles generated with rat liver microsomes. These results suggest that rat MetMax™ hepatocytes may be more relevant than traditional rat hepatic microsomes for *in vitro* metabolism applications with chemicals and are promising as a potential alternative for expensive *in vivo* rat metabolism studies.

36. Characterization of tienilic acid as an inhibitor of CYP2C9 in human hepatocyte clearance assays: Filling the gap left by the pan-CYP inhibitor 1-aminobenzotriazole

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A drug eliminated by a single pathway is at higher risk of becoming a “victim drug” of a drug-drug interaction (DDI) event. When a co-administered drug blocks (inhibits) this elimination pathway, the victim drug may accumulate causing toxicity. By contrast, drugs eliminated by multiple pathways are less susceptible to such interactions.

Cytochrome P450 enzymes (CYPs) are responsible for clearance of approximately 75% of marketed, small molecule drugs. An HT pooled human hepatocyte clearance assay in use at AbbVie includes azamulin and 1-aminobenzotriazole (1-ABT) arms to assess CYP3A4 and total CYP contribution to hepatic clearance, respectively. Understanding total CYP (and by inference, nonCYP) and CYP3A4 contribution to clearance provide early assessment of victim DDI risk. However, 1-ABT is only a partial inactivator of CYP2C9, which may lead to overestimation of nonCYP involvement in clearance of CYP2C9 substrates. We investigated inhibition activity of tienilic acid, a CYP2C9 specific, mechanism based inhibitor, on phase I (CYPs) and phase II (UGTs) metabolism to obtain optimal concentration to couple with 1-ABT for overall $f_{m,cyp}$ determination in HT-chemical inhibition assay. Using both model and in-house compounds with known $f_{m,CYP2C9}$ from HT-phenotyping assay, the HT-chemical inhibition assay correlated well when tienilic acid (3 µM) was added to augment pan-CYP inhibition by 1-aminobenzotriazole in human hepatocytes. By supplementation of 1mM 1-ABT with 3 µM tienilic acid in human hepatocyte clearance assays, total hepatic cytochrome P450 clearance and nonCYP hepatic metabolism clearance could be better assessed and allows project team for improved DDI assessment at drug discovery early stage.

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37. Targeting Survivin to Overcome Docetaxel Resistance in Prostate Cancer

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Background: Despite therapeutic advancements, castration-resistant prostate cancer (CRPC) remains the second most common cause of cancer-related mortality in men. Docetaxel is the first cytotoxic agent to show modest improvements in overall survival rate in patients with CRPC. Unfortunately, over half of these patients do not respond to treatment and ultimately all develop resistance. The mechanism mediating docetaxel resistance remains unknown. However, survivin, an inhibitor of apoptosis (IAP) family member, and known mediator of chemo-resistance has been previously associated with docetaxel resistance, as inhibition of survivin expression sensitized prostate cancer cells to docetaxel in vitro, and a small molecule inhibitor targeting survivin expression led to a significant regression in prostate cancer xenograft tumors when given in combination with docetaxel. However, how survivin may mediate docetaxel resistance in prostate cancer remains unknown.

Methods and Results: In this study, we tested the hypothesis that overexpression of nuclear survivin contributes to docetaxel resistance in prostate cancer cells. First, utilizing western blot to assess protein level and methylene blue assays to determine ability to proliferate under cytotoxic conditions, we found that survivin expression and docetaxel IC50 correlates strongly in five prostate cancer cell lines. Furthermore, using paired parental drug sensitive and stepwise selected docetaxel resistant cell lines, we determined that resistant cells overexpress survivin as compared to parental cells, and overexpression of survivin increases resistance to docetaxel. Stable overexpression of survivin increases prostate cancer cells resistance to docetaxel while transient siRNA knockdown of survivin decreases resistance to docetaxel. A small molecule inhibitor of survivin dimerization reduces survivin protein level and inhibits prostate cancer cell growth in vitro.

Future Directions: Our studies suggest that survivin is likely implicated in CRPC and treatment with a direct survivin inhibitor may sensitize resistant cells to docetaxel. We are in the process of determining how this IAP may mechanistically mediate docetaxel resistance.

38. Human Cytochrome P450 17A1 and Peroxoanion Mediated Carbon-Carbon Bond Scission

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Human cytochrome P450 17A1 is a key enzyme involved in steroid hormone biosynthesis that catalyzes the 17-alpha hydroxylation of pregneolone and progesterone as well as a subsequent 17,20 carbon-carbon scission of these hydroxylated products that is the first committed step of androgen formation. While the first reaction is expected to proceed through a straightforward Cpd I mechanism, recent evidence has emerged suggesting involvement of a nucleophilic peroxoanion in this enzyme's carbon-carbon bond scission chemistry. A distinguishing feature between Cpd I and peroxoanion mediated chemistry is involvement of at least two protons in formation of Cpd I, which permits observation of kinetic solvent isotope effects to differentiate between the two possible operating mechanisms. Here we report observation of a normal isotope effect in P450 17A1's hydroxylase activity, but an unusual inverse isotope effect in its cleavage of the 17,20 carbon-carbon bond. These results show that while a Cpd I intermediate operates in the first reaction, a peroxoanion is responsible for the carbon-carbon lyase chemistry. These initial findings led us to conduct a series of resonance Raman experiments in which the peroxo- states were characterized following radiolytic cryoreduction. In these studies, when hydroxypregnenolone or hydroxyprogesterone were substrates we identified a new species with Raman spectra indicating an iron ligated hemiketal intermediate that is on the pathway for product formation.

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39. Interaction of CYP450-FMN domain Complex in Lipid Bilayer

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Structural interactions that enable electron transfer to cytochrome-P450 (CYP450) from its redox partner CYP-reductase (CPR) is a vital prerequisite for its catalytic mechanism. Here, we report the first structural model for the membrane-bound functional complex to reveal interactions between the full-length CYP450 and a FMN binding domain (minimal domain of CPR, FBD). Our results suggest anchorage of the proteins in a lipid bilayer to be a minimal requirement for CYP450 catalytic function. Akin to cytochrome-b_5 (cyt-b_5), Arg125 on the C-helix of CYP450s is found to be important for effective electron transfer, thus supporting the competitive behavior of redox partners for CYP450s. We report a general and holistic approach to study protein-protein interactions combining the use of nanodiscs with NMR and SAXS. Linking structural details to the mechanism will help unravel the xenobiotic metabolism of diverse microsomal CYPs in their native environment and facilitate the design of new drug entities

40. CYP2D6 Allelic Variants *17-2 & *17-3 Metabolize the Same Substrates as *1 but are Less Susceptible to Inactivation by SCH 66712

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The recreational use of high doses of dextromethorphan in American teens as a means of “getting high” is a public health concern. Dextromethorphan is metabolized almost exclusively by CYP2D6, a drug metabolizing CYP with more than 100 allelic variants. Studies show that there is a ~10% higher incident of dextromethorphan abuse among African Americans and more frequent critical clinical events. Individuals of African descent frequently posses one of the reduced activity alleles of CYP2D6 -*17-2 or *17-3, that compromise their ability to metabolize dextromethorphan and come down from their high. The goal of this project was to understand how the *17-2 and *17-3 variants interact with the prototypical substrates dextromethorphan and bufuralol and the mechanism-based inactivator, SCH 66712, and to compare *17-2 with *17-3. Spectral binding titrations with dextromethorphan and *1, reference CYP2D6, yielded a Ks of 80 ± 21μM. The Ks with *17-2 was similar at 120 ± 87 μM. Metabolism of test substrates by *17-2 and *17-3 resulted in the same products as *1. In inactivation assays, the allelic variants *17-2 and *17-3 exhibited less inactivation than *1 and required a higher concentration of SCH 66712 than *1 for the enzymes to be inactivated. Finally, our findings suggest that the Arg295Cys change present in both *17-2 and *17-3 decreases activity and that the Thr107Ile change leads to decreased enzyme stability. No differences were observed for the Ser486Thr change that is present only in *17-3.

41. Impact of growth on the metabolizing enzymes in liver, lung and kidney of beef cattle

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Beef cattle is an important livestock species that we are targeting in both growth performance and disease treatment. The cattle grows almost linearly from the new born to its slaughter age (~18 months). The animals experience significant physiological and biochemical changes, including metabolizing enzymes in many organs during the growth. In this work, impact of age on the major metabolizing enzymes, cytochrome P450 (CYP450) in liver, lung and kidney of beef cattle has been evaluated. Liver, lung and kidney were collected and processed to microsomes from the ages of 4-days, 3-, 7-, 10- and 14-months (n=3/age group) of Augus beef cattle. The microsomal CYP450 contents or activity from those organs were assessed. CYP450 contents in cattle livers were much higher than those in its kidneys and lungs. Between the bovine kidney and lung, the unit microsomal CYP450 1A activity was higher in the lung although microsomal proteins were 5 times more in the bovine kidney. Microsomal CYP450 content or activity was low in the new born calf and increased with age in the cattle liver, lung and kidney. This content or activity reached a constant level in bovine liver, lung and kidney at about 7-months old of cattle. Because of the percent ratios of liver, lung and kidney to body weights changed with age in cattle, the metabolizing capacity of each organ contributing to the whole body of the animals changed with age. The cattle had the maximum metabolizing capability in all three organs at the age of around 7 to 10 months. These findings will be helpful for in vivo study design and data evaluation in bovine drug discovery and development.

42. Preparation of 4-Methylumbelliferyl Glucuronide Using UGT-Express™ with In Situ Generated UDP-Glucuronic Acid

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The cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT) families of enzymes play critical roles in the metabolism of drugs and other xenobiotics. Evaluation of the roles of CYPs and/or UGTs in the metabolism of drug candidates presently involves complex microsomal enzyme preparations or membrane fragments containing the specific recombinant isoform — all of which are labile and require addition of expensive cofactors. We have previously reported the development of an enzymatic system – CypExpress™ – which express high levels of a specific human CYP, P450 oxidoreductase and an NADPH regenerating system, with greater catalytic stability for the preparation of drug metabolites. Here, we report the development of an analogous system — UGTExpress™ — in which a specific UGT isoform is combined with recombinant UDP-glucose dehydrogenase to provide the cost-effective production of glucuronide metabolites. Given the cost and instability of UDP-glucuronic acid in solution, the capability of generating this co-substrate in situ is especially appealing. Using 4- Methylumbelliferone with added UDP-glucuronic acid as a model system, RPHPLC was used to confirm the production of the 4-methylumbelliferyl glucuronide at levels of 0.5 mM over a 24-hour period. Combining the UDP-glucose dehydrogenase with the UGTExpress™ produced lower concentrations of the glucuronide, and is being optimized. We will be evaluating a multi-expression system containing the CYP3A4, oxidoreductase, UGT2B7 and UDP-glucose dehydrogenase in one platform, allowing for the sequential oxidation and glucuronidation of a substrate in one pot. *Supported by NIGMS grant R43GM122129.*

43. Antibody Quantification Using Immunoaffinity Purification in High-Throughput Bioanalysis

Drew Sparks, Kirk Knotts

Q2 Solutions

Monoclonal antibody (mAB) quantification in animal models by LC-MS/MS typically use long digestion times (≥18 hours) and chromatographic cycle times ranging from 5 minutes to 20 minutes. We present here a ‘bottom-up’ method adapted to high-throughput quantification of mABs spiked into monkey serum using a Sciex 4000 triple quadrupole instrument and Shimadzu UPLC pumps. Peptides were generated by digesting a generic human antibody with trypsin (rapid digestion kit) after an immunocapture clean-up step employing streptavidin magnetic beads. The quantification range was from 0.1 ug/ml to 100 ug/ml. The timeframe from initial sample handling to sample analysis was reduced to under six hours. Chromatographic cycle time was reduced to 2 minutes per sample. Accuracy values were less than 15% and all precision values were less than 20%.

44. Effect of the Plasticizer DEHP in Blood Collection Bags on Human Plasma Fraction Unbound Determination for Alpha-1-Acid Glycoprotein (AAG) Binding Drugs

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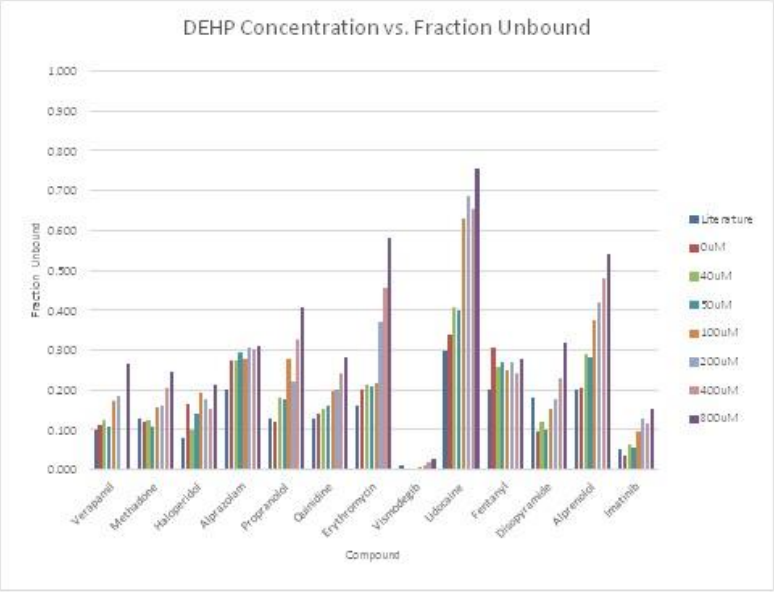
[1] Q2 Solutions, [2] H3 Biomedicine

Purpose
To investigate the cause of overestimation of human plasma fraction unbound for alpha-1-acid glycoprotein (AAG) binding drugs and to discover a practical solution suitable for high-throughput screening. Methods
Methods
Drugs with varying degrees of AAG binding (verapamil, methadone, haloperidol, alprazolam, propranolol, quinidine, erythromycin, vismodegib, lidocaine, fentanyl, disopyramide, alprenolol, imatinib) were evaluated using 96-well equilibrium dialysis (HTDialysis) protein binding methodology. Human plasma was obtained through 1) storage in Terumo blood bags until time of request, 2) collection in Terumo blood bags and immediately shipped, and 3) collection in heparinized vacutainer tubes. Fraction unbound (fu) values in plasma from each collection method were calculated following 4.5 hour incubations with the HT Dialysis blocks shaking at 167 rpm and 37°C. Relative recovery was determined by comparison of the donor and receiver concentrations to calculated concentrations of the T0 samples. Results obtained were compared to literature values. In addition, extractions of diethylhexyl phthalate (DEHP), a reported plasticizer in blood bags, were performed (3:1:1 ACN: 1N NaOH: Human Plasma) from each plasma collection sample, as well as DEHP-free plasma placed into Terumo bags over 7 days. Extractants were diluted with ACN containing internal standard (labetalol). Standard curves for DEHP and test compounds were generated, and analysis was conducted using LC/MS/MS on either an ABSciex 4000 or 5000 triple quadrupole mass spectrometer. Lastly, equilibrium dialysis experiments with varying concentrations (0-800 µM) of DEHP spiked into DEHP-free plasma were performed.
Results
Published literature fu values were compared to experimental fu values calculated from different collection methods for human plasma. Blood stored in Terumo bags for up to 28 days yielded DEHP concentrations between 300 µM and 1000 µM, which correlated with higher fu values for AAG binding drugs. Meanwhile, blood collected in Terumo bags and immediately transferred to tubes yielded DEHP concentrations of 1 µM to 10 µM and blood collected directly in vacutainer tubes yielded low DEHP concentrations of 0.1 µM to 2 µM, resulting in fu values more consistent with literature. DEHP spiked at varying concentrations into DEHP-free plasma yielded fu values increasing between 2- to 5-fold for all drugs tested. In addition, DEHP was discovered to leach from the Terumo bag over time, with increases in DEHP concentration from 33 to 300 µM over a 7 day period.
Conclusion
We were able to demonstrate the effect of DEHP leaching from commercially available blood collection bags on in vitro fu values, leading to drastic overestimation of fu by 2- to 5-fold for AAG binding drugs. Blood immediately transferred from collection bags to minimize the DEHP concentration yielded binding data that are nearer to accepted literature values. Ideally, a DEHP-free bag should be utilized to improve the accuracy of fu determinations for efficacious dose and/or safety margin predictions for drugs that bind to AAG with high affinity. Since AAG levels are known to increase in most disease states, obtaining accurate fu values may be criticalfor PBPK modeling.



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