2023
Great Lakes Drug Metabolism & Disposition Group Meeting

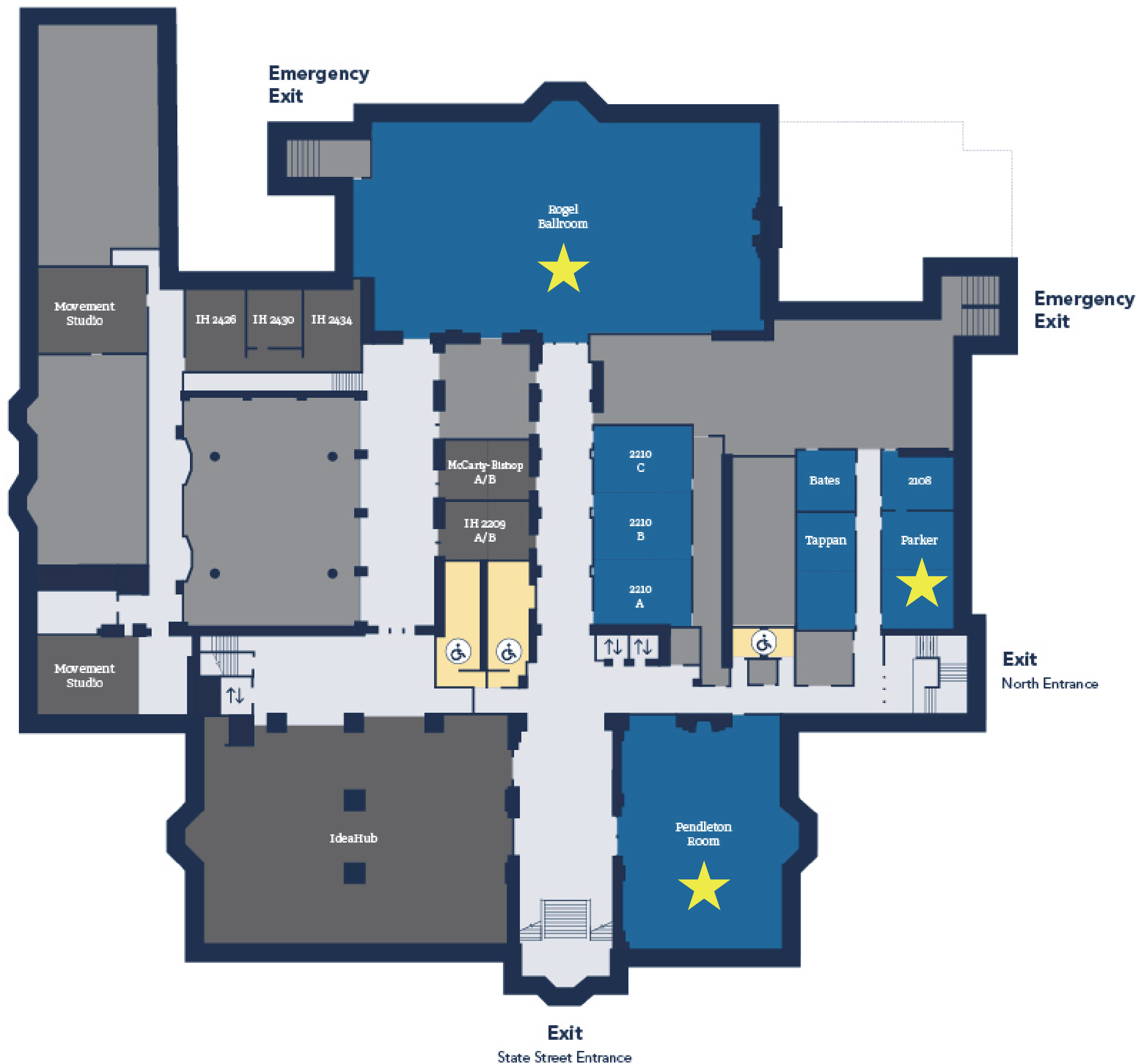
Ann Arbor sunset 2018 (JuwanGOAT/Wikimedia, Creative Commons)

Michigan Union, University of Michigan
Ann Arbor, Michigan

May 4 - 5, 2023

Maps

Michigan Union – Second Floor



Parker Room: GLDMDG business meeting

Pendelton Room: Oral presentations

Rogel Ballroom: Posters, vendor exhibits, and meals

Michigan Union – First Floor

Kuenzel Room: Thursday student/scientist networking lunch

Public Parking and Hotels

1. **Maynard Structure:** 324 Maynard Street (0.3 miles from the Michigan Union)
2. **Liberty Square Structure:** 510 E. Washington Street (0.4 miles from the Michigan Union)
3. **Liberty Lane Parking Garage:** 319 S. Fifth Avenue (0.4 miles from the Michigan Union)
4. **Fourth and William Structure:** 115 E. William Street (0.5 miles from the Michigan Union)
5. **Graduate Ann Arbor:** 615 E Huron Street
6. **Residence Inn by Marriott Ann Arbor Downtown:** 120 W Huron Street

Thursday, May 4, 2023

10:00 AM	**Check in, Coffee, Poster Setup, and Sponsor Booth Setup** (Rogel Ballroom)
11:00	**Welcome** (Pendelton Room), Yoichi Osawa, Ph.D., Dept. of Pharmacology, University of Michigan, Ann Arbor, MI.
11:10	*Moderator: Michael Mohutsky, Ph.D., Senior Director, Eli Lilly, Indianapolis, IN.* **Investigations of P450 MBI: The Value of Understanding Mechanisms Beyond K_{inact}.**, Larry Wienkers, Ph.D., Wienkers Consulting, LLC, Seattle, WA.
11:50	**Extracellular Vesicle (EV)-Based Liquid Biopsy in DMPK: Where Are We Now and Where Are We Going?**, David Rodrigues, Ph.D., Senior Scientific Director, Head of Drug Transporter & ADME Biomarker Sciences, PDM Medicine Design, Pfizer, Groton, CT.
12:30 PM	**Lunch, View Posters, and Visit Sponsor Booths** (Rogel Ballroom) **Students and Postdocs Networking with Industry Scientists** (Kuenzel Room)
2:00	*Moderator: Anthony Lee, Ph.D., Senior Director, Quantitative Pharmacology and Disposition, Seagen, Seattle, WA.* **The Cancer Target CD73 Regulates Hepatocyte Function and is Necessary for Long-term Liver Homeostasis.**, Natasha Snider, Ph.D., Associate Professor, Dept. of Cell Biology and Physiology, School of Medicine, University of North Carolina, Chapel Hill, NC.
2:40	**DMET Applications of a Kidney Microphysiological System.**, Edward Kelly, Ph.D., Associate Professor, Dept. of Pharmaceutics, School of Pharmacy, University of Washington, Seattle, WA.
3:20	**Break** (Rogel Ballroom)
3:40	*Moderator: Rich Voorman, Ph.D., RMLV Partners* **Empagliflozin: Identification of ADME-related Differences in Human vs. Toxicology Species.**, Mitchell Taub, Ph.D., Director of Non-Clinical DMPK, Boehringer Ingelheim Pharmaceuticals, CT.
4:20	**ADME Tactics in the Discovery & Development of the Orally Active SARS-COV-2 Inhibitor Nirmatrelvir.**, Amit Kalgutkar, Ph.D., Senior Research Fellow, DMPK Lead, Rare Disease and Antivirals Therapeutics, Pfizer, Cambridge, MA.
5:00	**Poster Session (Author Available), Sponsor Booths, and Mixer** (Rogel Ballroom) Authors at odd numbered posters (5:00-6:00 PM). Authors at even numbered posters (6:00-7:00 PM).
7:30	**Dinner** (Rogel Ballroom)

Friday, May 5, 2023

7:30 AM	**GLDMDG Business Meeting** (Parker Room) **Continental Breakfast, View Posters, and Visit Sponsor Booths** (Rogel Ballroom)
9:00	*Moderator: Gary Jenkins, Ph.D. Senior Director, AbbVie Inc, Grayslake, IL.* **A League of Their Own: Translational ADME for PROTACs.**, Marjoleen Nijsen, Ph.D., Vice President, DMPK, AbbVie, Chicago, IL.
9:40	**Understanding the Disconnect of In Vitro CYP3A4 Time Dependent Inhibition.**, Luc Rougee, Ph.D., Senior Advisor, Drug Disposition, Eli Lilly, Indianapolis, IN.
10:20	**Break** (Rogel Ballroom)
10:40	*Moderator: Young Jeong, Pharm.D., Ph.D., Professor, Dept. of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University, West Lafayette, IN.* **Multi-omic Discovery Used Drug Perturbation in African American Hepatocytes.**, Minoli Perera, Pharm.D., Ph.D., Associate Professor, Dept. of Pharmacology, Northwestern University, Chicago, IL.
11:20	**Multi-omic Analysis of Drug Metabolism in African Americans by Leveraging Primary Human Hepatocytes to Capture Interindividual Variability.**, Carolina Lee Clark, M.S., Northwestern University, Chicago, IL. *Student Abstract Award Winner.*
11:35	**What's all the HUpLA? Proof of Concept for a Novel Assay for Obtaining Uptake, Efflux, and Intrinsic Metabolic Clearance Based on the Extended Clearance Concept.**, Julia A. Schulz Pauly, Ph.D., Quantitative, Translational & ADME Sciences, AbbVie Chicago, IL. *Postdoc Abstract Award Winner.*
11:50	**Closing Remarks,** Young Jeong, Pharm.D., Ph.D., Professor, Dept. of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University, West Lafayette, IN.

1. Redox Partner Adrenodoxin Allosterically Alters the Function of the Human Steroidogenic Cytochrome P450 11B Enzymes

CARA L. LOOMIS (1), Sang-Choul Im (2), Michelle Redhair (3), Emily E. Scott (1,4,5)
Departments of Biological Chemistry (1), Medicinal Chemistry (4), and Pharmacology (5), University of Michigan, Ann Arbor, MI 48104, USA; Division of Metabolism, Endocrinology, & Diabetes, Department of Internal Medicine, University of Michigan, Ann Arbor, MI (2); Veterans Affairs Medical Center, Ann Arbor, MI. (2) Department of Medicinal Chemistry, University of Washington, Seattle, WA. (3)

Cytochrome P450 (CYP) enzymes are a class of heme monooxygenases with diverse functions, including steroid hormone synthesis. The human CYP11B enzymes play key roles in steroidogenesis by producing cortisol (CYP11B1) or aldosterone (CYP11B2) and are drug targets for Cushing's disease and primary aldosteronism, respectively. Development of selective drugs for either disease state has been hindered by the 93% sequence identity between the two enzymes. Further structural and functional characterization of these enzymes is necessary to identify differences between the two enzymes that can be exploited to improve drug selectivity. Both enzymes require reduction during two transient interactions with the iron-sulfur protein adrenodoxin per catalytic cycle. Previously, equilibrium and stopped flow studies determined that adrenodoxin binding on the P450 surface allosterically modulates ligand binding in the CYP11B1 and CYP11B2 active sites ~18 Å away (1,2). Adrenodoxin increases both substrate affinity and saturation by increasing the *on* rate and decreasing the *off* rate. Recently an orthogonal stopped-flow experiment cross-validated adrenodoxin suppression of substrate release.

The current focus is to determine the mechanism by which adrenodoxin causes these effects for both CYP11B1 where substrate binding is monophasic and CYP11B2 where substrate binding is biphasic. Thus, kinetic modeling experiments in MATLAB are being used to fit the data to specific mechanisms. These models range from adrenodoxin affecting conformational selection or induced fit, as well as more complex models considering ligand depletion, substrate reorientation, or a combination of conformational selection and induced fit. Fitting the initial data available suggests that the data likely follows a conformational selection model, but further work was needed to solidify this finding. The data fitting process suggests optimal stopped-flow experimental conditions that can be used to better resolve model ambiguity. Thus cyclical integration of experimental and computational approaches are being used to elucidate differences in adrenodoxin effects on the substrate binding mechanism between CYP11B1 and CYP11B2. Understanding how these enzymes work and the differences between them have the potential to drive selective drug design strategies.

1. S. Brixius-Anderko, E.E. Scott, Structural and functional insights into aldosterone synthase interaction with its redox partner protein adrenodoxin, J. Biol. Chem. (2021).
2. CL Loomis, S. Brixius-Anderko , and EE Scott . Redox partner adrenodoxin alters cytochrome P450 11B1 ligand binding and inhibition, JIB. (2022).

Funding: NIH R01 GM135346 and F31 HD111338

2. Modification of the Prostate Cancer Drug Abiraterone to Improve Selectivity for CYP17A1 Over CYP21A2

SARAH BURRIS-HIDAY[1], Dr. Caleb Vogt[6], Anna Welton-Arndt[7,9], Dr. Jeffrey Aube[7,8,9], Dr. Emily Scott[1,2,3,4,5]

From the Departments of Medicinal Chemistry[1], and Biological Chemistry[2], and Pharmacology[3] and the Programs in Chemical Biology[4] and Biophysics[5], University of Michigan, Ann Arbor, Michigan, USA. The Medicinal Chemistry Section of the National Institute of Health, National Institute of Drug Abuse Intramural Research Program, Baltimore, Maryland, USA[6]. The Department of Chemistry[7] and the Center for Integrative Chemical Biology and Drug Discovery Profession[8], UNC Eshelman School of Pharmacy[9] at the University of North Carolina at Chapel Hill, Chapel Hill North Carolina, USA

According to the Centers for Disease Control and Prevention, prostate cancer is the second most common cancer among men in the United States. In 2011 the Food and Drug Administration approved the first-in-class prostate cancer prodrug abiraterone acetate (Zytiga®). The active form of abiraterone effectively inhibits cytochrome P450 17A1 (CYP17A1), a key enzyme in androgen production. However, abiraterone also suppresses glucocorticoid and mineralocorticoid production, partially through inhibition of cytochrome P450 21A2 (CYP21A2). This latter action contributes to side effects including hypertension, hypokalemia, and peripheral edema. It has since been shown that the abiraterone metabolite 3-oxo-Δ^4-abiraterone not only inhibits CYP17A1 effectively, but can also beneficially inhibit other targets in the androgen pathway[1].

Compounds based on this 3-oxo-Δ^4-abiraterone metabolite were designed to determine if modifications of its pyridine ring could contribute to inhibition selectivity for CYP17A1 over CYP21A2. Both CYP17A1 and CYP21A2 natively metabolize the substrate progesterone, into either 17-hydroxyprogesterone or 21-hydroxyprogesterone respectively. These reactions were used to quantitatively evaluate the ability of increasing concentrations of 3-oxo-Δ^4-abiraterone analogs to inhibit catalysis. Progesterone substrate, the respective products, and internal standard were separated by HPLC followed by detection using either UV-vis absorbance (CYP17A1) or mass spectrometry (CYP21A2) to determine IC_{50} values. As expected, removal of the nitrogen from the pyridine ring substantially disrupts inhibition of both enzymes, but is more critical for CYP17A1, supporting the importance of the coordinate covalent bond between the compound nitrogen and the P450 heme iron in driving affinity. CYP17A1 and CYP21A2 are sensitive to both the position and composition of substitutions to the pyridine ring. Specifically, introduction of a methyl group at position 5 results in the desired selectivity for CYP17A1 over CYP21A2, whereas a methoxy group at the same position is tolerated by both enzymes but does not result in substantial selectivity. In contrast, introduction of the same methyl group at position 4 results in more selective inhibition of CYP21A2. An inhibitor with selectivity for CYP17A1 over CYP21A2 should reduce detrimental side effects, resulting in improved prostate cancer patient outcomes.

1. Li, Z. et al. *Nature* **2016,** *533* (7604), 547.
2. McKay, R.R et al. Cancer. *Clin. Cancer Res.* **2017**, *23* (4), 935.

Funding from NIH R37 GM076343

3. Discovery and Development of Small Molecule Sarcoplasmic Reticulum Ca^{2+}-ATPase (SERCA) Activators for the Treatment of Diastolic Dysfunction

ADAM ARD[1], Sherrice Zhang[1], Xinmin Gan[1], Carlos Cruz Cortes[2], Guadalupe Guerrero-Serna[2], Martin Clasby[1], Michel Espinoza-Fonseca[2]
[1]Department of Medicinal Chemistry, University of Michigan, Ann Arbor, MI. [2]Department of Internal Medicine, University of Michigan, Ann Arbor, MI.

Heart failure (HF) is a major cause of morbidity and mortality in the United States, accounting for 1 in 9 deaths and costing over $32 billion per year. Despite advancements in the clinical management of HF, the overall 5-year mortality rate has remained at over 50% for the past 10 years. This is, in part, due to current HF therapies being focused on addressing peripheral symptoms (e.g., blood pressure) instead of targeting the molecular defects within the cardiomyocyte directly. The Sarcoplasmic Reticulum (SR) Ca^{2+}-ATPase (SERCA) enzyme is a transmembrane protein responsible for pumping two Ca^{2+} ions from the cytosol into the SR lumen to relax muscle cells (diastole), which is regulated endogenously by phospholamban (PLN). A key abnormality in HF involves insufficient SERCA expression and impaired PLN phosphorylation, synergistically leading to SERCA inactivation, and thus, decreased Ca^{2+} transport in the cardiomyocyte resulting in diastolic dysfunction. It has been shown that reactivation of Ca^{2+} transport resulted in improved cardiac function in HF models, validating SERCA activation as a therapeutic approach for HF. Preliminary studies conducted by the **Espinoza-Fonseca** and **Herron** groups at the University of Michigan led to the discovery and validation of **HF600**, a potent small molecule SERCA activator that stimulates intracellular Ca^{2+} transport to reverse calcium mishandling in diseased human cardiomyocytes (EC_{50}= 2 mM), while also protecting the system against arrhythmia, and no apparent long-term cardiotoxicity. Through data-driven, computer-aided, fragment-based drug design, we are currently performing several hit-to-lead optimization structure-activity relationship campaigns based on **HF600** to develop diverse classes of safe, effective, pharmacologically viable small molecule SERCA activators directed at cardiomyocytes to restore diastolic function in the failing heart. Our goal is to develop several small molecule, orally bioavailable HF therapeutic candidates for administration in both an acute hospital intervention and a chronically reduced cardiac function setting.

4. Meta-Iodobenzylguanidine as an Exogenous Biomarker of Cardiac OCT3 Function

MIKE BOECKMAN[1], Zahra Talebi[1], Yan Jin[1], Erin K. Hertlein,[2] Sharyn D. Baker[1], Daniel Addison[3] and Alex Sparreboom[1]

[1]Division of Pharmaceutics and Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH, USA; [2]College of Medicine, University of Cincinnati, Cincinnati, OH, USA; [3]Division of Cardiovascular Medicine, Department of Medicine, The Ohio State University, Columbus, OH, USA.

Background: Doxorubicin (DOX) is an anthracycline used in the treatment of various malignancies, including breast cancer and certain leukemias, but its use is limited by a debilitating cardiotoxicity. We previously reported that the uptake of DOX into cardiomyocytes is mediated by the organic cation transporter OCT3, and that inhibition of this mechanism ameliorates cardiotoxicity without affecting antitumor activity. Since OCT3 inhibition is not associated with altered plasma levels of DOX, alternative cardiac biomarkers of OCT3 are needed to optimize dosing strategies of OCT3 inhibitors used with DOX. We hypothesize that meta-iodobenzylguanidine (mIBG), an analog of norepinephrine used to image neuroendocrine tumors, can serve this purpose as it a known substrate of OCT3 that accumulates in cardiac tissue.

Methods: Pharmacokinetic studies were performed in wild-type mice and age- and sex-matched mice with a genetic deficiency of OCT3 or the related transporters OCT1 and OCT2 (OCT1/2), MATE1, or OCT1/2 and MATE1 (OCT1/2/MATE1). Non-radiolabeled mIBG was injected i.v. as a bolus dose at 15 mg/kg. Plasma and heart samples were collected at various time points (up to 4 hours), and pharmacokinetic parameters were calculated using Phoenix WinNonlin. In separate experiments, mice received 0.5 mCi of iodine-123 labeled mIBG for single-photon emission computerized tomography (SPECT-CT) scanning. Images from the axial, coronal, and sagittal perspectives were gathered 30-60 min after injection, and signals were quantified in cardiac tissue.

Results: While the observed AUC of mIBG in plasma was similar between wild-type mice and transporter-deficient animals, the levels of mIBG in hearts of OCT3-deficient mice were statistically significantly reduced compared to wild-type mice with mean heart-to-plasma ratios of 3.5 ± 0.598 and 38 ± 8.05, respectively $P<0.001$). Levels of mIBG in the hearts of mice deficient in OCT1, OCT2, and/or MATE1 were either unchanged or slightly increased compared to results obtained in wild-type mice. In the SPECT-CT images, we verified that radiolabeled mIBG accumulates in cardiac tissue. The signal was quantified and normalized to dose and decay, and ranged from $6.19\text{-}19.3 \times 10^5$ Bq/mL. The observed reading at 30 min, corresponding with the peak cardiac levels observed for unlabeled mIBG as measured by LC-MS/MS, was robust and easy to visualize, and will serve as an optimized condition to perform future validation studies aimed at guiding the use of OCT3 inhibitors as an adjunct to DOX therapy.

Discussion: These findings confirm that radiolabeled mIBG can be utilized in conjunction with SPECT-CT scans as a non-invasive cardiac biomarker of OCT3 function. We are currently performing a library screen to identify novel small-molecule inhibitors of OCT3 that can be tested for OCT3-modulatory properties in vivo using a newly developed transgenic mouse model with cardiomyocyte-specific expression of human OCT3. It is expected that the proposed strategy can ultimately be translated to patients with cancer requiring treatment with DOX-based regimens to ameliorate cardiotoxicity.

5. High-Resolution LC-MS/MS Workflow for Quantification of Disulfide-Bridged Cyclic Peptides in Rat Plasma

RANDY ARNOLD, Eshani Nandita, Zoe Zhang, Lei Xiong, Elliott Jones
SCIEX

Cyclic peptides are polypeptides in the configuration of a ring formed by chemically stable bonds, such as disulfide bonds between 2 cysteine residues.

Cyclic peptides have been identified as critical therapeutic candidates given their structural stability and conformational rigidity.

With emerging interest in the advancement of cyclic peptide therapeutics, there is an equivalent drive toward developing highly robust and sensitive quantitative methods.

However, current bioanalytical methods for the quantification of cyclic peptides in biological matrices still present challenges.

For LC-MS-based methods, high baseline interference and resistance to CID, given the complex tertiary structure of cyclic peptides, impact overall sensitivity.

This study evaluated the quantitative performance of cyclic peptides on the ZenoTOF 7600 system. The Zeno trap enabled MS, CID and EAD modes were evaluated for quantification. The Zeno MS approach enabled low-level quantification of cyclic peptides compared to Zeno CID and Zeno EAD. In addition, the Zeno MS workflow facilitated an overall reduction in method development time with minimal ion path tuning, offering a simple quantitative workflow for cyclic peptides.

6. Implementation of High-Throughput SFC-MS/MS Assay for Determination of Experimental Polar Surface Area (EPSA)

DREW ZIELONKA, Jason Hulen, Stella Doktor, Edward Price, Nicole Richwine, Patrick Pazerunas, Justin Sheldon, Skyler Tiarks, Yueting Wang, David Stresser, Qin Ji, Estelle Maes
AbbVie

Topological polar surface area (TPSA) in drug compounds correlates to passive membrane permeability. However, this correlation for non-classical drug candidates such as beyond-rule-of-5 (bRo5) compounds is less predictive. A key feature of certain large and lipophilic bRo5 compounds that unexpectedly exhibit permeability is their ability to change shape – exhibiting chameleon-like behavior and flexing into structures that internalize polar atoms via intramolecular hydrogen bonding. By doing so, they effectively hide surface polarity that would otherwise render compounds impermeable in lipid bilayers. Experimental polar surface area (EPSA) accounts for intramolecular hydrogen bonding, providing further insight to the permeability profiles of bRo5 analytes such as degradomers. To meet the demand for a better permeability prediction for bRo5 compounds, we implemented a high-throughput assay to measure EPSA using SFC-MS/MS. Original methods utilized SFC-UV, a 16-minute LC method, and measured discrete analytes; whereas the high-throughput (HT-EPSA) method uses SFC-MS/MS, a 6-minute gradient, and cassette analysis of compounds (n=6). The HT-EPSA assay obtains EPSA values for 576 compounds in less than 11 hours.

HT-EPSA allows us to quickly screen compounds earlier and prioritize compounds before more intensive, costly, and lower-throughput assays. EPSA values correlate with Caco-2 apparent permeability for a structurally diverse set of compounds. The HT-EPSA assay delivers a key measurement for candidate selection in early drug discovery, offering a low-cost, high-throughput assessment of permeability, particularly for projects in the bRo5 space.

7. Development of a High Throughput Screen for Cytochrome P450-Ligand Binding Assays

ELYSE FRYDENDALL[1] and Emily E. Scott[1,2]
Department of Pharmacology[1] and Departments of Medicinal Chemistry[2], Biological Chemistry[2] and the Biophysics[2] and Chemical Biology[2] Programs, University of Michigan, Ann Arbor

Human cytochrome P450 enzymes are membrane-embedded monooxygenases responsible for a variety of functions including xenobiotic metabolism, steroidogenesis, fatty acid metabolism, and vitamin metabolism. Thus many P450 enzymes are either drug targets or drug metabolizing enzymes. To effectively design drugs targeting a P450 and/or avoiding metabolism by other P450 enzymes, it is important to understand interactions between the structural features of an individual P450 active site and small molecule physicochemical features. One method to investigate P450 structure-activity relationships is to profile the binding of individual compounds. Spectral binding assays can be used to identify binders vs. non-binders and determine dissociation constants. Historically, such ligand binding assays are performed using a spectrophotometer to track changes in P450 spectral characteristics with increasing concentrations of ligand. This low throughput experiment is effective and informative but requires significant hands-on time. A high throughput version of this binding assay has now been developed which increases the number of compounds that can be examined by 15-fold in an equivalent timeframe and with significantly less manual effort. This binding assay was validated for use in a 384 well plate for type I shifts normally observed for substrates and type II shifts often observed for heme-coordinating inhibitors. As a test, an initial library of about 90 primarily azole compounds was created and tested for the two drug-metabolizing P450 enzymes, CYP1A1 and CYP2D6. Each interaction was probed by collecting absolute spectra over an 11-point binding curve. As expected, many of these azoles bound both P450 enzymes. Of the compounds tested, 94% caused a measurable spectral shift with CYP1A1 and 70% with CYP2D6. Current analysis is yielding the range of dissociation constants observed this screen. Selected examples will be presented. The expectation is that this high throughput screen will be useful to rapidly identify new P450 ligands for other P450 enzymes that are less well studied by using larger and more diverse libraries. The resulting information should be useful in the development of pharmacophores, expansion of known ligand profiles for P450 deorphanization, and screening of potential drugs either to establish P450s as drug targets or avoid P450 metabolism.

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8. A High-throughput Acyl Glucuronide Stability Assay Based On 18O-Labeling

JOSH YU, Hoa Le, Jennifer Tang, Qin Yue, Jingyu Zhang, Bernard Murray, Xingrong Liu, Bill Smith, and Raju Subramanian

Drug Metabolism, Gilead Sciences, Foster City, CA, USA

Introduction

1-β-Acyl glucuronides are common metabolites of carboxylic acid containing compounds and are suspected to be involved in drug toxicity due to their potential liability arising from glucuronic acid moiety – also referred to as acyl migration. The current conventional assay for assessing acyl glucuronide liability measures migration half-lives in potassium phosphate buffer (KPB) and requires baseline chromatographic separation of the unmigrated 1-β-acyl glucuronide from the migrated isomers. This entails laborious LC method development which needs to be optimized for individual acyl glucuronide. Herein, we report an improved acyl glucuronide stability assay based on ^{18}O-labeling compatible with high-throughput bioanalytical LCMS workflow.

Methods

Acyl glucuronide reference standards (1 µM final concentration) or *in situ* generated acyl glucuronide (final concentration varied) from human liver microsome were incubated in 100 mM $H_2{}^{18}O$-KPB at 37 ºC for 36 hours. Sample at 0, 1, 2, 4, 8, 12, 18, 24, and 36-hour time points was analyzed by a UHPLC-HRMS system. The areas under the curve (AUCs) of unlabeled, singly ^{18}O-labeled, and doubly ^{18}O-labeled species (if any) of the same test article were summed to obtain the AUC_{total}. The percent of each individual species was calculated as the corresponding AUC divided by AUC_{total}. The change of unlabeled species over time during migration incubation was used as the indicator of migration potential.

Results

A total of 19 acyl glucuronides of known drugs, with acyl group migration half-life from 0.3 hour (unstable) to 79.0 hours (stable), were examined using the ^{18}O-labeling method. The unlabeled acyl glucuronides decreased over time during the 36-hour incubation in $H_2{}^{18}O$-KPB at 37 ºC, giving rise to the ^{18}O-labeled species. The rate of ^{18}O-labeling corelated well with the rate of acyl migration - ^{18}O was incorporated faster with glucuronides that were more prone to migration. For acyl glucuronides with reported migration half-lives greater than 3.6 hours, more than 30% remained unlabeled at 24-hour incubation; on the other hand, no more than 20% unlabeled remained for glucuronides with shorter half-lives. Therefore, the fraction unlabeled at 24 hours can be used to assess migration potential of acyl glucuronides. Similar results were obtained using acyl glucuronides generated *in situ* from human liver microsome, eliminating the need of using reference standards.

To further investigate the labeling mechanism, an O-glucuronide was studied, and no labeling was observed. suggesting that a free anomeric hydroxyl group (via the hemiacetal formation) is required for the incorporation of ^{18}O to the glucuronic acid moiety. Notably, no over-labeling was observed for all 19 acyl glucuronides studied, indicating that oxygen exchange did not occur at glucuronyl carboxylic acid or other functional groups present in the aglycone, including sulfonamide, amide, conjugated ketone, demonstrating the specificity and robustness of this ^{18}O-labeling method. Furthermore, 4-O-methylglucuronic acid was used as a model compound to examine the oxygen exchange kinetics. The disappearance of unlabeled 4-O-methylglucuronic acid followed first-order kinetics with a half-life of 8.1 hours, indicating that oxygen exchange is the rate limiting step for acyl glucuronides with short half-lives.

Novel Aspect

No known reports on the use of ^{18}O-labeling for glucuronide stability assessment – a simplified workflow compared to the conventional method.

9. Accelerating Drug Discovery Through An Automated, Multi-Parametric Optimization Approach: Spotlighting A high-throughput, fully automated Compound Screening Station

PATRICK PAZERUNAS, Sarah Andrews, Jason Hulen, Diana Mendoza, Nicole Richwine, Justin Sheldon, Skyler Tiarks, Drew Zielonka, Carlo Rosales, Estelle Maes, Kelly Desino, David Stresser, and Stella Doktor
AbbVie

Rapid delivery of high-quality Absorption- Distribution- Metabolism- Excretion (ADME) data is a key element of AbbVie's Drug Discovery effort. This is achieved by efficient compound delivery logistics coupled with automated workstations. Early identification of compounds with promising pharmacokinetic and physiochemical properties fuel our multi-parameter optimization model, which entails generation of ADME and potency data concurrently. Within the Quantitative Translational ADME Sciences department (formerly DMPK-BA), our mode of operation has evolved from an all-in-one automation platform established a decade ago to one supplemented with modular platforms and semi-automation. This has enabled more flexibility in assay timing while serving to accommodate overflow, simultaneous operation and overcoming downtime in other systems. This poster describes the evolution of this capability.

Affiliations: All authors are employees of AbbVie and may own AbbVie stock. The design, study conduct, and financial support for this research were provided by AbbVie. AbbVie participated in the interpretation of data, review, and approval of the publication.

10. Harmonization and Cross-site Benchmarking for Biologics LCMS-based Bioanalysis

JUNLI MA[1], Shuai Niu[1], Phil Krueger[1], Yuting Wang[1], Yihan Li[1], Vikram Shenoy[1], Khader Awwad[2], Ramona Rodila[3], David Rizzo[3], Hetal Sarvaiya[1]
[1]DMPK-ADME-BATS; [2]DMPK-LU; [3]Regulated Bioanalysis, DMPK-BA, AbbVie Inc.

As a global organization, we have multiple bioanalytical groups across the globe committed to supporting Biologics LCMS-based bioanalysis ranging from early development to late-phase GLP and clinical studies. Therefore, it is imperative to have a truly harmonized workflow that summarizes the consensus best practices, as well as data consistency with benchmarking that enables a seamless global operation model with high data quality and productivity that meets targeted turnaround times. The goal of our work described in this poster is to gain operational excellence across global site-based framework for biologics bioanalysis by harmonizing workflows and benchmarking studies.

Towards this we showcase the following in this poster-

- Global Biologics Bioanalytical (BA) teams established harmonized workflows and consensus on best practices across sites.
- Cross-site benchmarking study was conducted with ADC dosed residual cyno tox study samples that yielded highly comparable BA data.
- ADC/TAb ratio can be used as a quick checkpoint to help interpret in vivo linker-payload stability.

11. Data Processing Tools Used for High Throughput ADME Assays

SKYLER TIARKS, Patricia Stuart, Patrick Pazerunas, Justin Sheldon, Nicole Richwine, Jason Hulen, Drew Zielonka, Sarah Andrews, Diana Mendoza, Carlo Rosales, Estelle Maes, Stella Doktor, David Stresser, and Gary Jenkins
AbbVie Inc.

Critical project decisions often rely on fast, reliable and easy access to data. The use of electronic laboratory notebooks (ELNs) and Laboratory Information Management Systems (LIMS) for accurate recording of assays is a fast and effective way to provide easy access to data and has gained popularity in recent years. Here we describe AbbVie's data processing workflow using IDBS's E-Workbook (BioELN) and Dotmatics to analyze, record and "publish" data to a company-wide database, where it can be accessed by project representatives. An array of 5 high throughput early screening ADME assays include stability in human and mouse liver microsomes (HLM, MsLM), microsomal binding with human liver microsomes (FuMic), eLogD, and PAMPA. All assays are run weekly on up to 768 compounds. Using the BioELN and Dotmatics software, the data is analyzed and made available within one day after compounds are received and processed in the lab. Additionally, the BioELN templates are "global", allowing for the same templates to be utilized for similar assays at multiple sites. Using predetermined acceptance criteria and filters (in BioELN and Dotmatics respectively) for each template, only the compounds with results that do not meet the criteria need to be reviewed, which are highlighted automatically in ELN by the software and filtered in Dotmatics by the user for easy identification. This process is internally referred to "review by exception," which standardizes and streamlines the review process across similar assays. This makes data analysis faster and more accurate between users.

12. AURA: A Data Visualization Tool for Early-Stage Assessment of Endpoints in Drug Discovery

EDWARD PRICE, Virginia Saulnier, Cory Kalvass, Stella Doktor, Manuel Weinheimer
Research and Development, AbbVie Inc., 1 North Waukegan Road, North Chicago, Illinois 60064, United States

Biopharmaceutical companies produce substantial amounts of data on a regular basis. These data include collections of in silico and in vitro endpoints that cover the span of physicochemical properties, machine learning, high-throughput screening, and lower-throughput in vitro assays. As this data is generated, they are often placed in internal databases with scientists from various teams performing independent analysis with diversified conclusions and recommendations. As additional models are built, more assays are developed, physicochemical properties deviate, the need for easier data access, transparency, and utility assessment becomes more apparent across project teams. This becomes critical for teams to pivot and select compounds judiciously for further development along the pipeline. This work focuses on the development of AURA (Accuracy, Utility, and Rank-order Assessment), a simple and streamlined Spotfire® data visualization tool that allows end users to study the utility and predictability of any endpoint that potentially affects absorption (fafg) across the entire physicochemical space. We show the use of AURA across all company-wide projects for 35 endpoints relevant to absorption. AURA provides access, transparency, and accelerates the understanding of endpoint utilities within the permeability and absorption drug pipeline. This allows end-users to make well-informed data-driven decisions for their chemical space.

13. A Streamlined Confidence Assessment of In Silico ADME Models at AbbVie

STELLA DOKTOR, Majdi Hassan, Xiaofeng Li, Mamata Parab, Cory Kalvass, Abhishek Pandey, David Stresser and Edward Price
AbbVie

High volume, routine in vitro ADME assays provide excellent data sets on which to apply machine learning. We have continued to update our in silico ADME models based on in-house data that can be used by chemists pre-synthetically to guide compound design, thereby increasing probability of success. For in silico models to be effective they must be built on high quality and well-curated data sets. Furthermore, multiple predicted endpoints can be used in combination to enable multi-parametric optimization. The robustness of the models is statistically assessed both globally (all compounds) and at the project level by endpoint on an internal Spotfire® dashboard to help teams decide whether to use predicted values and assess the robustness of the model for specific projects. AbbVie has 19 statistically validated models including In vitro clearance in multiple species for microsomes and hepatocytes; efflux ratio in a stably transfected cell line and Caco-2; free fraction in brain, plasma and microsomes; In vivo clearance in rat; and volume of distribution in rodent. The use of these tools saves valuable time and directs synthesis of only the most promising compounds, thereby reducing the need for wet work and In vivo testing.

14. Atypical Compound Space Requires Atypical Solutions

CARLO I. ROSALES, Kenneth Ruterbories, Jason Hulen, Estelle Maes
AbbVie.

Rapid production and screening of novel compounds is vital to any drug discovery campaign. Much effort has gone towards developing high throughput chemical and biochemical assays, but obstacles continue to arise during the LC-MS/MS analysis of DMPK samples. Maintaining efficiency in any high-throughput workflow requires the use of robust, standardized methods. And due to the ever-evolving nature of drug discovery, these methods should be highly adaptable, allowing modifications to be made without the need for developing new methods from scratch. Our ADME and PK workflow contains three primary LC-MS/MS methods: APCI with ion pairing, ESI with acidic mobile phase, and ESI with alkaline mobile phase. A typical issue in LC-MS/MS *in vivo* sample analysis is ionization enhancement or suppression due to co-eluting components from the biological matrix and/or dose formulation. These matrix effects create uncertainties that may affect data quality and accuracy and stress our workflow by increasing analysis time and decreasing efficiency. Systematically choosing which compounds are best analyzed with which method dramatically increases both quality and throughput. Moreover, the increasing diversity and complexity of chemical spaces typically found in drug pipelines have caused additional difficulties in our standardized approach.

New compound spaces require changes to established paradigms. Here we describe a series of nitrogen-containing compounds for which matrix effects have been a persistent issue on our alkaline ESI method. APCI is known to be less susceptible to matrix effects, making it a good solution for typical instances of matrix effects. That said, some new chemical spaces are not suitable for APCI. Sensitivity for these compounds on our APCI method was consistently poor and, upon further investigation, was found to be a direct result of fragmentation during the ionization process. Due to the diversity of compounds we routinely screen, finding a solution that was flexible and did not require custom LC-MS/MS parameters was critical to maintaining our high-throughput workflow. Removing a transfer step after our sample clean up procedure and injecting straight from the protein precipitation crash plate mitigated ESI Internal Standard (IS) variability without the need to deviate from our standardized ESI method. The information gleaned from this case study has proven useful both for solving IS variability in various projects and for general method prediction. Subtle modifications to our workflow, guided by experimental observation, has allowed our group to keep pace with constantly evolving projects, while maintaining the heavy workload associated with high-throughput analyses.

15. Cathepsin A and High Cell Permeability Determine the Efficiency of Intracellular Bioactivation of Remdesivir in Lung Cells

JIAPENG LI, Shuxin Sun, Yueting Liu, Weiwen Wang, Hao-Jie Zhu*
Department of Clinical Pharmacy, College of Pharmacy, University of Michigan

Remdesivir (RDV) is a phosphoramidate prodrug (ProTide) of nucleoside analog GS-441524 (Nuc). RDV and Nuc exert their antiviral activity via their intracellularly generated active metabolite, triphosphate nucleoside (RDV-TP). Carboxylesterase 1 (CES1) and cathepsin A (CatA) are the major enzymes for activating RDV. However, it is still inconclusive to what extent the prodrug form RDV can improve the intracellular bioactivation of Nuc in human lung cells and the associated mechanisms.

In this study, we evaluated the bioactivation profiles of RDV and Nuc in various human lung cell models on a molar equivalent dose basis. The cell models were those commonly used in SARS-CoV-2 studies, including the human lung carcinoma cell lines (A549, A549-ACE2-TMPRSS2, Calu-3), normal bronchial epithelial cells (BEAS-2B), human induced pluripotent stem cell-derived alveolar epithelial type II-like cells (iPSC-AT2), and the African green monkey kidney epithelial cells (Vero E6). After incubation with RDV or Nuc for 6, 12, and 24 hours, the intracellular concentrations of the RDV, Nuc, and their metabolites were determined by an LC-MS/MS assay. The expression profiles of activating enzymes and related drug transporters were retrieved from public proteomics databases and previously published reports. The contribution of CatA and CES1 to RDV activation in BEAS-2B cells was further evaluated using CES1 and CatA inhibitors.

Compared with Nuc, RDV showed significantly higher intracellular concentrations of RDV-TP in A549, A549-ACE2-TMPRSS2, BEAS-2B, and iPSC-AT2 cells with 11- to 75-fold larger AUC_{0-24h} of RDVTP. Vero E6 cells generated the lowest levels of RDV-TP among the cell lines tested, likely due to the lack of CES1 and the high P-gp expression in Vero E6 cells. Like the alveolar epithelial cell type II, the major SARS-CoV-2 infection target *in vivo*, the human lung cell lines exhibit a very low expression of CES1 but a considerable expression of CatA. Moreover, our CES1 and CatA inhibition study confirmed the predominant role of CatA in activating RDV in BEAS-2B cells. Interestingly, for RDV, the AUC_{0-24h} of RDV-TP was highly associated with the total drug entry, indicating that cell permeability is another important factor affecting the efficiency of intracellular bioactivation of RDV.

In conclusion, RDV is more efficient than Nuc in intracellular activation in various human lung cell models, mainly due to its high susceptivity to CatA and great cell membrane permeability.

16. Species-Dependent Alpha 1-Acid Glycoprotein Binding and the Impact on Plasma Protein Binding of PF-07321332 (Nirmatrelvir), an Orally Bioavailable SARS-CoV-2 3CL Protease Inhibitor

SIENNAH R. GREENFIELD[1], Heather Eng[2], Qingyi Yang[1], Alyssa L. Dantonio[2], R. Scott Obach[2], Li Di[2], Amit S. Kalgutkar[1]

Medicine Design, Pfizer Worldwide Research, Development & Medical, [1]Cambridge, MA and [2]Groton, CT

The SARS-CoV-2 3C-like protease inhibitor PF-07321332 (nirmatrelvir), in combination with ritonavir (Paxlovid™), was granted emergency use authorization in December of 2021 by the U.S. Food and Drug Administration for the treatment of mild-moderate COVID-19 in adults and children who are at high risk for progression to severe COVID-19. Preclinical studies revealed considerable species differences in plasma protein binding. The unbound fraction of nirmatrelvir in plasma, albumin, and alpha 1-acid glycoprotein (the two main plasma binding proteins) from animals and human is reported herein. Nirmatrelvir demonstrated concentration-independent (0.3-10 μM) plasma protein binding in rat, monkey, and human. In contrast, nirmatrelvir was highly bound at low drug concentrations and moderately bound at high drug concentrations in rabbit and dog plasma. The species differences were predominantly driven by binding to dog and rabbit alpha 1-acid glycoprotein, and to a degree, dog albumin.

17. Cell Type-Dependent Activation of Nucleotide Prodrugs Across a Selection of Cell Lines and the Implications in Antiviral Studies

YUETING LIU, Shuxin Sun, Jiapeng Li, Weiwen Wang, Hao-Jie Zhu*
Department of Clinical Pharmacy, University of Michigan College of Pharmacy, 428 Church Street, Ann Arbor, Michigan 48109, United States

The prodrug design is a powerful tool to improve cell permeability and enhance the intracellular activation of nucleoside/nucleotide antiviral analogs. Previous in vitro studies showed that the activation of nucleoside/nucleotide prodrugs varied in different cell lines. In the present study, we investigated the activation profiles of two antiviral prodrugs tenofovir alafenamide (TAF) and sofosbuvir (SBV) in five cell lines commonly used in antiviral research, namely Vero E6, Huh-7, Calu-3, A549, and Caco-2. We found that TAF and SBV were activated in a cell-dependent manner, with Vero E6 being the least efficient and Huh-7 being the most efficient cell line for activating the prodrugs. We also demonstrated that TAF was activated at a significantly higher rate than SBV. We further analyzed the protein expressions of the activating enzymes carboxylesterase 1, cathepsin A, histidine triad nucleotide-binding protein 1, and the relevant drug transporters P-glycoprotein, organic anion-transporting polypeptides 1B1 and 1B3 in the cell lines using the proteomics data extracted from the literature and proteome database. The results revealed significant differences in the expression patterns of the enzymes and transporters among the cell lines, which might partially contribute to the observed cell-dependent activation of TAF and SBV. These findings highlight the variability of the abundance of activating enzymes and transporters between cell lines and emphasize the importance of selecting appropriate cell lines for assessing the antiviral efficacy of nucleoside/nucleotide prodrugs.

18. Utilizing Mass Spectrometry-Based Proteomics to Characterize Antiretroviral Drug Metabolism and Transport Potential in Blood Brain Barrier-Resident Cells

HANNAH N. WILKINS[1], Namandjé N. Bumpus[1], Ben C. Orsburn[1], Dionna W. Williams[2]
[1] The Johns Hopkins University School of Medicine, Pharmacology and Molecular Sciences, Baltimore, MD [2] The Johns Hopkins University School of Medicine, Molecular and Comparative Pathobiology, Baltimore, MD

A major obstacle to a cure for human immunodeficiency virus (HIV) is the viral reservoir that persists in the brain despite antiretroviral therapy (ART) administration. Neurocognitive deficits associated with HIV infection demonstrates the need to eliminate viral reservoirs in the brain. Challenges in ART penetrability of the brain that lead to HIV viral reservoir persistence arise from the protective capabilities of the blood brain barrier (BBB). It is well understood that firstline ART drugs, including the integrase inhibitor, dolutegravir, and reverse transcriptase inhibitors, tenofovir and emtricitabine, are substrates of ATP-binding cassette and equilibrative transporter families at the BBB and are modified by major drug metabolizing enzymes, including phase I, phase II, and nucleoside kinase enzymes. Transporter proteins and metabolizing enzymes at the BBB may instrumentally influence ART availability in the brain, yet their protein abundance is largely uncharacterized in the cells that comprise the BBB, specifically brain endothelial cells, pericytes, and astrocytes. To investigate the proteomic landscape of ARTrelevant metabolizing enzymes and transporters at the BBB, both global and targeted proteomics by mass spectrometry were performed on human monocultures of primary hepatocytes and BBB-resident cells, including brain endothelial cells, pericytes, and astrocytes. Mass spectrometry-based proteomics identified differential abundance of efflux transporters for tenofovir and emtricitabine and an influx transporter for dolutegravir with pericytes and brain endothelial cells having the highest overall transporter abundance. Heterogenous abundance of tenofovir and emtricitabine metabolizing enzymes was also observed with highest overall abundance occurring in astrocytes and pericytes. Novel UDP-glucuronosyltransferase (UGT) enzyme abundance was also identified in pericytes. Our findings indicate that differences in protein abundance of ART-relevant metabolizing enzymes and transporters in BBB-resident cells may reveal cell-dependent heterogeneous ART metabolizing or transporting capabilities. We propose that differential ART availability in the brain based on BBB-resident cell composition may serve as a foundation for considerations in ART dosing and delivery to the brain in the treatment of HIV.

19. Improvements in Quantitative Tissue Mimetic Models for Mass Spectrometry Imaging

ANDREW BOWMAN, Junhai Yang, David Wagner
AbbVie, Inc.

Quantitation for targets of interest is one of the most important issues within drug metabolism. While the technology and standards are generally well-accepted for liquid-chromatography-based methods, those for imaging-based modalities are less defined. Several types of tissue mimetic models have been published, but one of the confounding factors for these models has been the increased difficulty in sectioning caused by destroying cellular structure in the homogenization process. Further, the tissue homogenate has a different freezing profile than the surrounding gelatin, which causes it to pull free from the gelatin block that supports it. In this work we offer an improvement to the tissue mimetic model by the introduction of small amounts of gelatin to the homogenized tissue. This increases section-by-section reproducibility, while not impacting the mass spectral response of spiked targets of interest. Additionally, the more uniform tissue sections improve the limit of detection and limit of quantitation by decreasing variability in the homogenate created by fracturing in cryosectioning.

20. Drug Pharmacokinetics and Toxicity Study by Mass Spectrometry Imaging

JUNHAI YANG, Andrew Bowman, Wayne Buck, David Wagner
AbbVie Inc.

Introduction:

Spatial interrogation of tissue provides information how drug compounds interact with different cells or functional area of an organ and can reveal toxic information and related properties of drug candidates, assisting the drug screening and development. ABT-515 was an HCV protease inhibitor whose development was stopped due to biliary toxicity. It is being used here as a tool compound to compare mechanisms of biliary toxicity with distinct mechanism of action but similar physicochemical properties to select compounds that experience biliary toxicity.

Method and material:

Male Sprague-Dawley Rats were co-dosed with ABT-515 and ritonavir at 100 mg/kg and 15 mg/kg, respectively for 5 days. The livers were harvested at 6, 24, 48, 72 and 96 hours after last dose. Livers were sectioned at 10 µm thickness and coated with 2,5-dihydroxyacetophenone. MALDI Mass spectrometry imaging was applied on the coated liver sections at 10 µm spatial resolution on Bruker timsTOF Flex at negative ion mode. MS ion images were processed by Bruker SCiLs Lab and the corresponding H&E optical images of the same sections were obtained after the removal of matrix followed by H&E staining.

Preliminary results:

Neutrophil infiltration around bile ducts was observed after 5 days of dosing, Mass spectrometry imaging did not reveal retention of the compound in the bile ducts relative to the liver. Furthermore, Mass spectrometry imaging shown some concentration of the compound in the bile ducts, but the clearance from the bile ducts is as good as the clearance from the surrounding hepatocytes based on the time course study.

21. Quantitative Image-Based Drug Screening of Neurofilament Accumulation in Giant Axonal Neuropathy iPSC-MNs

MARYAM FARIDOUNNIA[1], Rachel Battaglia[2], Emma Bouck[3], David Zhu[4], Ariana Gonzalez[5], Adriana Beltran[5], Diane Armao[6], and Natasha Snider[1]*
[1]Department of Cell Biology and Physiology, [3]Department of Pathology and Laboratory Medicine, [5]Department of Pharmacology, UNC Human Pluripotent Stem Cell Core, and [6]Department of Radiology, UNC School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599; [2]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston; [4]Department of Biochemistry, Duke University School of Medicine
**Corresponding author*

Giant Axonal Neuropathy (GAN) is a fatal pediatric neurodegenerative disease caused by loss-of-function mutations in *KLHL16*, which encodes the ubiquitin ligase adaptor gigaxonin. Gigaxonin promotes the degradation of intermediate filament (IF) proteins, which accumulate in the cells of GAN patients. Localized build-up of neurofilament IFs within “giant” axon swellings render peripheral nerves especially susceptible to *KLHL16* mutations. The objectives of this study were to design a quantitative image-based drug screening assay and identify small molecules that can reduce the accumulation of neurofilaments in GAN. Induced pluripotent stem cells (iPSCs) with different *KLHL16* mutations were generated by reprogramming skin fibroblasts from seven GAN patients. Corresponding isogenic controls of two mutant lines (Y89S and G332R) were derived via CRISPR/Cas9 gene editing. The iPSCs were differentiated to neural progenitor cells (NPCs) and motor neurons (MNs) using previously established protocols. Spontaneous axonal neurofilament aggregates larger than 1.5µm in diameter formed in the mutant, but not the isogenic iPSC-MNs axons ($p<0.05$), validating the *in vitro* GAN iPSC-MN system. Mutant iPSC-MNs were subsequently used to conduct an image-based screen of ~1600 FDA-approved small molecule compounds. The cells were treated with each compound at 10µM for 72 hours, fixed, stained with pan-neurofilament antibodies, and imaged at 20X magnification. Drug effects were binned based on neurofilament organization into three categories: filaments, aggregates, fillaments+aggregates. For wells with filaments>aggregates, multiple areas were imaged, where the diameter of aggregates was determined using the Imaris spot function. To normalize for area occupied by axons, the raw images were subjected to segmentation to separate axon and aggregate area, and the area was measured using CellProfiler (Global thresholding with minimum cross-entropy method). The top 12 hits represented drugs that disrupted neurofilament aggregates and normalized neuronal morphology. Estradiol valerate and tazarotene, which act on estrogen and retinoic acid (RA) signaling pathways, respectively, exhibited the strongest effects at reducing aggregates and normalizing filaments by more than 2-fold ($p<0.01$) and were validated in further studies. The pharmacological effects of the RA receptor agonist tazarotene were further supported by mechanistic evidence revealing that GAN iPSC-NPCs and -MNs had a 6-fold upregulation of CRABP1, which buffers the receptor-binding ability of RA in cells. Taken together, our findings establish a clinically relevant *in vitro* drug screening assay and identify several candidate drugs that reduce neurofilament accumulation and aggregation in GAN patient iPSC-MNs.

22. *Fusobacterium nucleatum* Metabolites Promote Colorectal Cancer Progression through Activation of Aryl Hydrocarbon Receptor

KYOUNG-JAE WON[1], Pei-Ru Jin[1,#], Lydia J. Davis[2], Iain A. Murray[3], Gary H. Perdew[3], Jimmy Orjala[2], Hyunwoo Lee[1,4,*] and Hyunyoung Jeong[1,*]
[1]Department of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University. [2]Department of Biopharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago. [3]Center for Molecular Toxicology and Carcinogenesis, Department of Veterinary and Biomedical Sciences, The Pennsylvania State University. [4]Department of Comparative Pathobiology, College of Veterinary Medicine, Purdue University

Gut microbiota has emerged as a key contributor to host health and disease. However, the biological activities of gut microbial metabolites and how the host responds to these metabolites remain largely unknown. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcriptional factor binding diverse chemicals, and its dysregulation is associated with cancer proliferation and metastasis. We hypothesized that AhR could sense previously unknown gut bacterial metabolites and tested culture extracts of 10 gut bacterial species representing six major phyla in the gut for AhR activation using a promoter reporter assay in HepG2 cells. *Fusobacterium nucleatum* (*Fn*) exhibited the strongest activation, and from its culture extracts, We identified three compounds X, Y and Z as AhR agonists through activity-guided fractionation and structure elucidation. Cell-based ligand competition assay revealed that these compounds directly bind to AhR, with compound X being the most potent AhR activator. Given that *Fn* is enriched in colorectal cancer (CRC) tissues, we investigated whether *Fn* promotes CRC progression through activating AhR. *Fn* promotes the proliferation, invasion, and migration of human CRC cells (LIM1215, HCT-8, and H508) in vitro. An *Fn* deletion mutant which was unable to produce compound X, Y, and Z failed to enhance the invasion and migration of the CRC cells. Compound X increased the CRC proliferation in vitro, which was abrogated upon AhR inhibition (by siRNA-mediated knockdown or a chemical inhibitor). Altogether, we have identified novel AhR-activating *Fn* metabolites that promote CRC progression. These results lay the foundation for a better understanding of the role of *Fn* in CRC carcinogenesis, progression, and metastasis.

23. Gut Bacteria-Produced Metabolite 3,4-Hydroxy-Phenylpropionic Acid Alleviates Acetaminophen-Induced Liver Injury by Decreasing JNK Activation

XIAOTONG YANG, Yeonju Ko, Hyunwoo Lee, Hyunyoung Jeong
Department of Biopharmaceutical Sciences (X.Y.), College of Pharmacy; University of Illinois at Chicago, IL, USA. College of Pharmacy Institute of Pharmaceutical Science and Technology (Y.K.), Hanyang University, Ansan, Republic of Korea. Department of Comparative Pathobiology (H.L.), College of Veterinary Medicine; Department of Industrial and Physical Pharmacy (X.Y., Y.K., H.J.), College of Pharmacy, Purdue University, IN, USA

Acetaminophen (APAP) overdose can cause fulminant liver injury. The liver injury is caused by Hepatic Cytochrome P450-mediated APAP metabolism leads to the generation of a highly-reactive metabolite N-acetyl-p-benzoquinone imide (NAPQI), which initiates a series of events and eventually hepatocyte necrotic death. Our previous unbiased metabolomics analysis identified a gut microbiota-associated metabolite 3,4-hydroxy-phenylpropionic acid (HPPA) whose abundances differ between two groups of mice with altered susceptibility to APAP hepatotoxicity. This study aims to examine the effects of HPPA on APAP-induced liver injury and explore the underlying mechanisms. Supplementing antibiotics in drinking water completely depleted HPPA in mouse serum, indicating the host exposure of HPPA is determined by gut bacteria**.** Intraperitoneal administration of HPPA (50 mg/kg) two hours before APAP (300 mg/kg, i.p.) alleviated APAP-induced liver injury in mice. HPPA did not alter the steps of APAP metabolism that contribute to NAPQI amount, which include the hepatic glucuronidation and sulfation, the protein expression of CYP2E1 and CYP1A2, as well as hepatic glutathione levels. These results indicate minimal effects of HPPA on the early events of APAP hepatotoxicity (i.e., NAPQI formation and detoxification). Excessive NAPQI initiates oxidative stress and activation of c-Jun N-terminal (JNK) kinase that plays important role in APAP hepatotoxicity. HPPA decreased the APAP-induced JNK activation in mouse liver, which rendered mice more resistant to APAP-induced liver injury. In summary, this study revealed a gut bacterial metabolite HPPA has protective effect against APAP hepatotoxicity by reducing JNK activation, which expanded the knowledge of the gut microbiota and host interaction in the context of drug-induced liver injury.

24. Multi-omic Analysis of Drug Metabolism in African Americans by Leveraging Primary Human Hepatocytes to Capture Interindividual Variability

CAROLINA CLARK, Tanima De, Guang Yang, Cristina Alarcon, Juan Avitia, Minoli Perera
Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago IL

Background: Variation within drug metabolizing enzymes (DMEs), their regulatory regions, or regulatory proteins can contribute to interindividual variability of drug response and adverse drug reactions (ADRs). Genome-wide association studies (GWAS) have identified genetic variants associated with drug responses by linking individual phenotypic differences (i.e., ADRs) to corresponding genomic data. GWAS results alone are difficult to interpret as over 90% of results fall in non-coding regions. Integrating other –omic layers (i.e., transcriptomics) has proven beneficial in deciphering variant regulatory functions and prioritizing GWAS hits, but such studies continue to underrepresent minority populations. In doing so, prediction of drug response in minority populations, such as African Americans (AAs), using genetic variants discovered in other populations has been poor. This study aims to comprehensively survey the genetic, transcriptomic, and DNA methylation landscapes in AA-derived hepatocytes, for association to variation in drug metabolism using a novel and clinically relevant phenotype.

Method: Hepatocytes were extracted from 75 AA cadaveric livers and subjected to a panel of validated probe substrates for DMEs (Midazolam, Bupropion, Mephenytoin, Diclofenac, Phenacetin, Acetaminophen) to measure metabolite formation rate (MFR). Hepatocytes underwent genome-wide genotyping, mRNA sequencing, and DNA methylation profiling. MFR was collected via LC-MS/MS and linked to the donor's genetic, transcriptomic, and epigenetic data. A GWAS was conducted for the MFR of each probe substrate with donor age, sex, and the first two genomic principal components as covariates.

Results: Wide variability in all MFRs were seen between individuals, reflecting the known variation in AA drug metabolism. Loci reaching suggested significance level were identified, some with known links to DME and DME regulation including *CYP2C* (Mephenytoin, $p=4.1 \times 10^{-7}$) and *CYP4X1* (Bupropion, $p=7.5 \times 10^{-6}$), and *NCOR2* (Diclofenac, $p=5.3 \times 10^{-6}$). Other results may provide novel links to DMEs, such as the genome-wide association at the *ANGPT2* locus (Bupropion, $p=1.8 \times 10^{-8}$). Transcriptomic, epigenetic and fine-mapping analyses are ongoing.

Conclusion: This first of a kind study of MFR has found novel and established links to DME and liver function. CYP2C enzymes have known roles in the metabolism of probe substrates, and members within the CYP4 family have been implicated in xenobiotic metabolism. NCOR2 is a co-repressor of known CYP-transcription factors. ANGPT2 is a growth factor involved in inflammatory pathways, which have known roles in CYP regulation, but ANGPT2 itself has not been previously associated with CYP regulation. These results provide needed African-ancestry pharmacogenomic data that will be critical for individualizing drug dosing and drug-type selection in AAs and in understanding the regulation of these important enzymes.

25. Complementary Targeted Multi-Omics Workflows

Wendi Hale, Karen E. Yannell, Daniel Cuthbertson, Cathyrin Simmermaker, Genevieve Van de Bittner, Mark Sartain and JOHN SAUSEN
Agilent Technologies, Inc., Santa Clara, CA, USA

Metabolomics, lipidomics, and proteomics are all informative and provide biological insights to drug discovery and life science research. Specifically, this data is needed to understand therapeutic mode of action, identify any off-target effects, and for correlating efficacy and toxicity in cohort subgroups (precision medicine). This poster will cover sample prep and LC/TQ tools that provide reproducible and robust measurements for your studies- discussion of both validated quantitative analysis of a few targets or profiling hundreds or more biomarkers in a discovery study. Advantages of using an LC/TQ with large MRM databases for discovery or quantitation are additional sensitivity, precision in the measurement, linearity, and ease of data analysis. With a TQ everything is pre-annotated in the MRM database for easy data analysis and pathway interpretation. Discussed here are also details of three developed Omic workflows to reduce the method development for your lab. These workflows include automation geared to plasma and cell analysis. We can also extend the targeted work into most tissue types and can customize the targeted panels. These targeted workflows are complimentary to the untargeted HRAM workflows typically in use. This targeted approach enables the scientist to see lower detection limits and tighter precision, finding biomarkers we could otherwise miss with just HRAM workflows.

26. Optimizing a Microcavity Plate-Based Human Hepatocyte Spheroid Model for In Vitro Clearance Studies

David A. Kukla, Lance Heinle, Yueting Wang, and DAVID M. STRESSER
Quantitative & Translational ADME Sciences, AbbVie Inc, North Chicago, Illinois, United States

Background: Evaluation of in vitro intrinsic clearance (CL_{int}) is routinely conducted in drug discovery. Certain compounds exhibit very low CL_{int} (e.g., < 15% loss over 4-hour incubation, 1E6 hepatocyte/mL) necessitating alternative approaches. Self-assembled aggregates of hepatocytes (spheroids) exhibit suitable morphology, viability and drug metabolizing enzyme activity for weeks *in vitro*. Extended incubation times in this model could permit metabolic loss sufficient to obtain reliable measurements. To overcome limited number of cells (~1.5-2k/well) and lower turnover with single spheroids, multiple spheroids in a single well would be desirable. Elplasia® microcavity plates (Corning) enable culturing ~80 spheroids in a single well on a 96-well plate. **Objective:** With microcavity plates, we systematically evaluated seeding strategies, spheroidal size, and culturing techniques to assess suitability for clearance prediction. The effect of co-culturing hepatocytes with nonparenchymal cells was also evaluated. **Methods:** Spheroids were fabricated with initial seeding density of hepatocytes (~16-160k) per well within 96-well microcavity plates. As comparisons, single spheroids were fabricated by seeding 1.5k hepatocytes/well in 96-well ULA plates and monolayers were formed by seeding 50k hepatocytes/well in collagen coated 96-well plates. Albumin/urea production and cytochrome P450/FMO activities were assessed regularly with probe substrates. Finally, clearance was assessed with a diverse set of compounds. **Results:** Spheroids formed within ~3-5 days. Generally, decreasing hepatocytes per spheroid, promoted albumin/urea production and improved P450 activities on a per cell basis. Unlike monolayers that declined in viability and hepatic functions within days, conventional and microcavity spheroids displayed prolonged viability for at least 13 and 21 days, respectively. However, microcavity spheroids functionally outperformed single spheroids with ~2-fold higher albumin production, 1.3-1.6-fold higher urea production, and elevated cytochrome 450 (i.e., ~3-4-fold higher 3A4 activity) on a per cell basis after 1-2 weeks. We speculate that the higher performance with microcavity spheroids is due to the smaller spheroid diameter compared to conventional spheroids, which may improve nutrient exchange and reduce the likelihood of necrotic core formation. As anticipated, elevating the spheroid density per well in the microcavity model resulted in greater substrate depletion for low turnover compounds, enabling the measurement of intrinsic clearance. This optimized model is expected to be suitable not only for intrinsic clearance measurements but also applications requiring long term incubations (toxicity, metabolite ID, induction, etc).

Disclosures:
All authors are employees of AbbVie and may own AbbVie stock. AbbVie sponsored and funded the study; contributed to the design; participated in the collection, analysis, and interpretation of data, and in writing, reviewing, and approval of the final publication.

27. Beyond Intrinsic Toxicity: Defining Metabolite-Induced Hepatotoxic Cell Communications in Human Liver Organoids

SOPHIA MEYER[1], Charles Zhang [1], Jonathan Sexton[1,2]
[1]Department of Medicinal Chemistry, University of Michigan; [2]Department of Internal Medicine, University of Michigan

Drug-induced liver injury (DILI) is a rare hepatic disease and yet the leading cause of acute liver failure in the United States. Intrinsic DILI, caused by the drug itself, is often the consequence of hepatocyte toxicity from a metabolite rather than the parent drug itself. Preclinical models of hepatotoxicity are often unable to predict DILI from metabolites due to non-existent or declining metabolic activity of in vitro systems. Here, we employ a multicellular human liver organoid (HLO) microfluidic chip platform as our DILI model with a majority cell population of metabolically active hepatocytes as evidenced by cytochrome P450 expression and parent compound turnover. We hypothesize that our multicellular model demonstrates identifiable cell communications that are unique to each drug. Through single-cell RNA sequencing, we identified discrete transcriptomic profiles of hepatic stellate cells which account for ~20% of the HLO population. Activated, quiescent, and recovering fibrotic phenotypes of hepatic stellate cells were identified at distinct ratios between acetaminophen and fialuridine drug treatments and with corresponding markers of inflammation, motility, and metabolic processes. Further work is being done to quantify correlating markers of cell signaling at the protein-level and characterize cell phenotypes through morphological profiling.

28. Large Volume Injections: Using a Trap-And-Elute Chromatography System to Improve Sensitivity for Metabolite Identification from ADC and Low Dosage Samples

DANIEL LADROR, John Savaryn, Elyse Freiberger, David Wagner
AbbVie

Characterization of drug metabolites in vivo routinely involves organic liquid extraction of biologic fluids and tissue homogenate, drying the extract under nitrogen, resuspending the sample in a small volume, and injecting the sample onto an LC column for HRMS analysis. However, biological samples have a limit to the extent that they can be concentrated, as attempting to resuspend extract from a large volume of plasma in a small volume of solvent results in a viscous sample that is incompatible with injection on an LC column. As a result, the amount of material that can be loaded on column is limited by the autosampler loop volume. This restriction can cause sensitivity issues for identifying drug metabolites at low abundance, especially for antibody-drug conjugate (ADC) samples where the released free drug is a small fraction of the total drug load. Even when large volumes of in vivo samples are available for testing, only a limited amount of material can feasibly be processed for LCMS analysis.
One potential solution is to increase the injection volume using a trap-and-elute chromatography system. By employing a trap column and two additional LC pumps, multiple 100 µL injections can be sequentially loaded onto the trap column, after which the accumulated material is eluted onto an analytical column for LCMS analysis. Here, we demonstrate the utility of C8, C12, and C18 trap columns for analysis of low concentrations of analytical standards and common ADC payloads in large volumes of plasma. By injecting up to 800 µL of sample extract, we can enable characterization of metabolites that would be too low in abundance to be characterized in conventional injection volumes.

29. Assessing the Utility of AcquireX Workflows for Metabolite Profiling

ESSENCE B. UNDERWOOD, Elyse C. Freiberger, David S. Wagner
AbbVie

When profiling drug metabolites in a biological matrix using traditional DDA LC-MS methods, background signal from endogenous material can complicate detection and fragmentation of low-level metabolites. Common methods used to mitigate this interference include sample preparation techniques, optimizing mass spectrometric conditions, and use of internal standards. Recently, more software-based techniques have become available to optimize analysis *in silico*. One type of acquisition software from ThermoFisher Scientific, AcquireX Intelligent Data Acquisition, has the capability to perform dynamic exclusion automated workflows which use a matrix sample to remove peaks that are unrelated to the compound of interest. Here we provide an analysis of the dynamic exclusion workflow to increase the probability of selecting peaks for fragmentation that are drug-related over matrix-related peaks related to endogenous compounds. To test the utility of the method, we used commercial compounds of known metabolism and spiked the metabolites generated from *in vitro* systems into extracted plasma to mimic the matrix environment for circulating metabolites. Samples were run with AcquireX dynamic exclusion workflow and compared to the same samples run with traditional workflows to determine whether AcquireX improved success rate of selecting drug-related peaks for MS/MS fragmentation and whether this method is more efficient than the conventional process with a manual background exclusion.

30. Meeting the Challenges of in Vivo Studies and Timed Data Collection

Andrew Lemon, Ted Hawkins
Instem, 161 Washington Street, 8 Tower Bridge, Conshohocken, PA 19428, United States

Working in vivariums to accurately dose and sample animals at the correct timepoints can be a difficult challenge at the best of times. contract research laboratories have the added pressure of commercial deadlines and high activity. The pressure to outperform competitors and maintain quality and accuracy leads many organizations to look up digitization of these laboratories. We present an example of leveraging new technologies developed as a part of the internet of things (IOT) with scientific knowledge and experience to address these challenges head on. Design and schedule activities across multiple studies, different vivariums, and workstations. Digitally capture animal weights, syringe weights and record study days hands free. Automatically record data and pass samples to bioanalysis for concentration determination by mass spectrometry. This results in faster study execution, maintaining accuracy and quality.

31. Identification of Pazopanib as a Mediator in Drug-Induced Liver Injury (DILI) Via Competitive Counterflow

THOMAS DRABISON, Mike Boeckman, Eric Eisenmann, Sharyn Baker, Shuiying Hu, Alex Sparreboom, and Zahra Talebi

Division of Pharmaceutics and Pharmacology and Division of Outcomes and Translational Sciences, College of Pharmacy, The Ohio State University, Columbus, OH, USA

BACKGROUND

Tyrosine Kinase Inhibitors (TKIs), commonly used as a chemotherapeutic, inhibit the function of protein tyrosine kinases (PTKs) by binding and reducing their downstream signaling cascade. These TKIs are extensively metabolized in the liver, with CYP3A4 being the major contributor to enzyme-mediated degradation; however, the mechanism for hepatic uptake is still unclear. This may be, in part, by experimental challenges in traditional, direct uptake assays brought upon by a high level of non-specific, extracellular membrane binding of TKIs. By circumventing this issue, we have systemically addressed this utilizing a competitive counterflow assay, assessing the ability of TKIs to be transported by OATP1B1, a major hepatic-uptake transporter. This screen provides justification to explore these hits *in vivo* – specifically, we have identified pazopanib as a key mediator in drug-induced liver injury (DILI), based on the thesis that hepatic uptake is the initiating event which results in this injury, verified here in this *in vitro* assay. We have further explored this to identify OATP1B-mediated transport as a key contributor to this side-effect *in vivo*.

METHODS

The competitive counterflow method was optimized and validated using OATP1B1 overexpressing HEK cells, with estradiol-β-glucuronide as a prototypical substrate. Following development of a functional CCF, we screened the current FDA approved TKIs through OATP1B1 to determine which molecules are substrates for this transporter. *In vivo* studies were conducted with pazopanib to investigate its role in hepatotoxicity by monitoring liver-specific biomarkers, AST and ALT, in wild-type and OATP deficient mouse models.

RESULTS

Pazopanib showed up as a hit compound in our TKI screen, implicating it as a substrate for OATP1B1. This provided justification for further *in vivo* studies to determine its role in the development of hepatotoxicity, mediated by transport via OATP1B1. We have shown a significant increase in both ALT and AST in wild-type mice treated with pazopanib, both markers of DILI. OATP deficient mice are protected from this liver damage, implicating OATP1B1 as a key mediator of Pazopanib-induced liver injury.

CONCLUSIONS

We have successfully validated and optimized a competitive counterflow method for detection of substrates in OATP1B1 overexpressing HEK cells. In doing so, we have also identified OATP1B1 as a key moderator in pazopanib-induced liver injury, done so by facilitating its transport into hepatocytes. We have shown that protection to DILI can be achieved in mice deficient in the OATP1B1 proteins.

KEYWORDS

OATP1B1, Pazopanib, Hepatotoxicity

32. Role of Organic Anion Transporting Polypeptide 1B (OATP1B) In Hepatic Uptake and Adverse Effects of Aromatase Inhibitors (AIs)

HANIEH TAHERI [a, c]; Yang Li [a, c]; Yan Jin [a]; Alice A. Gibson [a]; Maryam B. Lustberg [b]; Alex Sparreboom [a]; and Shuiying Hu [a.c]

[a] Division of Pharmaceutics and Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH; [b] Yale Comprehensive Cancer Center, Yale School of Medicine, New Haven, CT; [c] Division of Outcomes and Translational Sciences, The Ohio State University, Columbus, OH.

Background

AIs (Anastrozole, Letrozole, Exemestane) are widely used in post-menopausal women with breast cancer. Despite their specificity, significant toxicities including arthralgia affect quality of life and limit effectiveness. The mechanism(s) by which AIs are taken up into liver and thereby regulate plasma levels, tissue distribution, as well as toxicity remains unknown. In this study, we explored the hypothesis that OATP1B-type transporter activity affects the pharmacokinetics of AIs and their dose-limiting side effect arthralgia.

Methods

In vitro uptake studies were performed in cells expressing human OATP1B1/1B3 or mouse OATP1B2. Biomarkers identified from untargeted metabolomics analysis in samples from wild-type and OATP1B2(-/-) mice were measured in baseline plasma samples from 20 patients treated with AIs with or without arthralgia symptoms. Pharmacokinetic studies were performed in wild-type, OATP1B2(-/-), and humanized transgenic mice receiving AIs (1-20 mg/kg; p.o.).

Results

Hepatic levels of several endogenous OATP1B2 substrates, including chenodeoxycholate-24-glucuronide (CDCA-24G), were reduced in OATP1B2(-/-) mice. Levels of CDCA-24G were also significantly associated ($P<0.01$) with higher risk of arthralgia in patients. The interaction of the AIs letrozole and exemestane with OATPs was verified in our *in vitro* and *in vivo* models.

Conclusions

In this study, we demonstrated that OATP1B activity as reflected by endogenous biomarkers is directly related to plasma levels of AIs and treatment-related arthralgia. Our currently ongoing studies are focused on the influence of OATP1B inhibitors on the plasma and liver levels of AIs, as well as the influence of OATP1B-deficiency on AIs-associated arthralgia using imaging, mechanical, and behavioral tests.

Keywords

OATP1B, Endogenous biomarkers, AIs, Pharmacokinetics, arthralgia

33. What's all the HUpLA? Proof of Concept for a Novel Assay for Obtaining Uptake, Efflux, and Intrinsic Metabolic Clearance Based on the Extended Clearance Concept.

JULIA A. SCHULZ, Mei Feng, Elizabeth Sande, Yue-Ting Wang, David M. Stresser, J. Cory Kalvass
Quantitative, Translational & ADME Sciences (QTAS), AbbVie Inc., North Chicago, Illinois, United States

Hepatic clearance (CL_H) prediction is a critical step in estimating first in human dosing. However, CL_H underpredictions are common especially for low-turnover compounds. Root causes for CL_H underpredictions attributable to properties (poor solubility/permeability, non-specific binding, low-turnover) that pose challenges for traditional *in vitro* ADME assays, resulting in non-valid data and inferior *in-vitro*-to-*in-vivo* correlations. Other processes, including organ and cellular distribution, can also play significant roles in CL_H. Recent advances in understanding the interplay of metabolism and drug transport for clearance processes have aided in the development of the Extended Clearance Model (ECM). Per the ECM, unbound intrinsic hepatic clearance ($CL_{int,u}$) depends on metabolic clearance (CL_{met}) and distribution processes (passive diffusion (CL_{pd}), active uptake (CL_{uptake}) and efflux (CL_{efflux})).

Our objective was to develop *in vitro* methods to measure CL_{met} and distribution parameters within a single assay. To this end, we designed a novel two-step assay enabling measurement of multiple kinetic parameters from a single experiment in plated human primary hepatocytes without and without transporter and CYP inhibitors – the Hepatocyte Uptake and Loss Assay (HUpLA).

Hepatocytes are incubated with drug-containing media to assess CL_{uptake} and CL_{pd}. Once the system reaches steady-state, drug-containing media is rapidly replaced with drug-free media. CL_{efflux} is determined by measuring appearance of drug in the media. CL_{met} is measured as parent loss from the incubation. Analytical sensitivity is substantially increased for low-turnover compounds, since drug loss is measured as loss from the system only after preloading hepatocytes and replacing drug-containing media with drug-free media.

We evaluated 9 drugs with known CL_H with different enzyme and transporter affinities. CL_H was predicted from the HUpLA results and compared to the observed *in vivo* CL_H. 89% of drugs were predicted within 2-fold of the observed CL_H. Indomethacin, a low CL_H compound, was the only outlier in the dataset (>3-fold).

HUpLA enables the measurement of enzymatic and transport processes concurrently within the same system, alleviating the need for intersystem scaling. The use of primary human hepatocytes enables physiologically relevant exploration of transporter-enzyme interplay. Most importantly, HUpLA shows promise as a highly sensitive measure for low-turnover compounds. Further evaluation of low-clearance compounds is needed to demonstrate the robustness of the method.

Disclosure: All authors are (or were) employees of AbbVie and may own AbbVie stock. AbbVie contributed to the design; participated in the collection, analysis, and interpretation of data, and in writing, reviewing, and approval of the final abstract, which contains no proprietary AbbVie data. (Mei Feng is a former AbbVie employee and has no conflict of interest.)

34. Exploring Small Molecules Absorption in Polydimethylsiloxane (PDMS)-based Microphysiological Systems

AGNES BADU-MENSAH, Trent Abraham, Scott Tallman, Kate Newell, Michael Mohutsky
Eli Lilly and Company

Human-based microphysiological systems (MPS), also known as organ-on-a-chip systems have been demonstrated to closely recapitulate *in vivo* microenvironment of human tissues and translate to human conditions. Thus, the human-based intestine, liver, kidney MPS models have been proposed as platforms for drug discovery-related drug disposition studies, where relevant models are currently lacking. Many of MPS models are developed in housings fabricated from polydimethylsiloxane (PDMS), a soft polymer known to absorb many small molecules to varying extents. This poses profound experimental challenges, especially in studies where decreased compound exposure may result in erroneous outcomes. This may confound downstream interpretation of endpoints such as receptor binding, enzyme inhibition/activation etc.

In this study, we explored the feasibility of adopting PDMS-based organ-on-chip in drug disposition studies by investigating the extent of PDMS absorption of small molecule compounds routinely used in *in vitro* metabolic, transporter, and drug interaction experiments. Of the 26 control compounds studied, eight had less than 50% recovery including CYP3A substrate, midazolam, and pan transporter inhibitor, cyclosporine. Eleven compounds were found to have recovery of 80% or more, while six (e.g., verapamil a P-gp inhibitor and CYP3A substrate, along with the CYP3A inhibitor, itraconazole) were observed to have greater than 30% PDMS absorption. Interestingly, 4 compounds demonstrated absorption both to reservoir plasticware and the PDMS, resulting in less than 40% of compound recovered in the chip outflow.

The findings highlight the need for careful consideration in compound selection and experimental design for ADME-related studies in human MPS models. Moreover, physicochemical property comparison of these ADME assay control compounds revealed trends associated with the LogP, polar surface area and hydrogen-bond donor values. Futures studies will explore incorporating these parameters into in silico models that may aid in predicting the absorption behavior of other compounds in PDMS-housed models.

35. Estimation of Fraction Metabolized by Cytochrome P450 Enzymes of Low Turnover Compound Diazepam Using HUREL® Human Pool™ Liver Hepatocyte Model

VINCENT C. PETERKIN, Yue-Ting Wang, and David M. Stresser
Quantitative, Translational & ADME Sciences, AbbVie Inc., North Chicago, Illinois, United States

During drug discovery and development, it is often desirable to advance compounds that are slowly metabolized, but this can present challenges in determining other properties downstream. For example, predicting clearance, understanding fraction metabolized (fm) by enzymes of interest and assessing victim drug-drug interaction (DDI) risk prior are all hampered by analytical challenges of not observing adequate metabolic depletion in conventional test systems. This inability to quantify DDI risk elevates uncertainty and ultimately may decrease probability of successful outcomes. Hepatocyte clearance assays typically provide reliable intrinsic clearance (CL_{int}) for values ≥ 3.0-3.5 mL/min/kg. However, to effectively phenotype a clearance pathway, CL_{int} should be several fold (~ 10X) higher to create a suitable clearance activity window to inhibit by CYP selective inhibitors[1]. Model systems for low clearance compounds such as hepatocyte suspension relay[2] and long term co-cultured hepatocyte[3,4] have been available for several years, but only recently have these systems been evaluated for suitability as reaction phenotyping models[5]. The great benefit of these systems is that some can measure intrinsic clearance up to 10 times lower than the standard approaches[4]. One system that has been tested for understanding low clearance but still in early stages for assessing fm in hepatocyte is the HμREL® Human Pool™ liver hepatocyte model[6,7]. The aim of our study was to perform a reaction phenotyping study in this long-term culture system for a known low clearance compound (diazepam) that cannot be assessed using the conventional hepatocyte suspension assay using ritonavir and fluoxetine to determine fmCYP3A4 and fmCYP2C19, respectively. In addition, we tested seven CYP marker compounds, to ensure a proper functioning system. We were able to predict clearance within 3-fold of what is observed in vivo for diazepam but more importantly we observed fmCYP2C19 contribution of 0.49. This is similar to the estimated in vivo fmCYP2C19 of 0.42-0.84. With additional adjustments in concentration and choice of inhibitor, we aim to evaluate fraction metabolized for other well-characterized compounds and ultimately to use this system for calculating fmCYP in low clearance compounds.

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36. Computational Design of Highly Stereoselective CYP102A1 Variants with UniDesign for Hydroxylation of Omeprazole

YUDONG SUN, Xiaoqiang Huang, Yoichi Osawa, Eugene Chen, Haoming Zhang
Department of Pharmacology and Department of Internal Medicine, The University of Michigan Medical School, Ann Arbor, MI 48109

CYP102A1 is a prototypic biocatalyst that has great potential in chemical synthesis, drug discovery, and biotechnology. CYP102A1 variants engineered by directed evolution and/or rational design are capable of catalyzing oxidation of a wide range of organic compounds. However, it is difficult to foresee the outcome of engineering CYP102A1 for a compound of interest. Here we apply a computer-aided approach to design CYP102A1 variants for regio- and stereo-selective hydroxylation of omeprazole using UniDesign Software. UniDesign comprises resource-friendly components of a rotamer library, efficient energy function and fast optimization algorithm to survey the vast space of CYP102A1 sequence that affords selective oxidation of omeprazole. Three CYP102A1 variants were identified and characterized including A82F/F87V/L188Q/L75I (UD1), A82F/F87V/L188Q/A264G (UD2), and A82F/F87V/L188Q/A328V (UD3). All three variants exhibit high NADPH oxidation rates of 660-1306 min^{-1} and hydroxylate omeprazole to produce 5-hydroxyomeprazole (5OHOMP) with UD2 being most active at 70.6±2.0 min^{-1}. When OMP enantiomers were used as substrates, they show high stereo-selectivity for R-enantiomer to produce R-5OHOMP with a turnover rate of 55±4.7, 84±9.5 and 79±11 min^{-1} whereas the corresponding rates for S-enantiomer are 2.1±0.19, 10±0.45 and 14±1.0 min^{-1} respectively. The corresponding ee values are 92, 87 and 70% respectively. These results suggest the critical roles of L75I, A264G and A328V in steering OMP in the optimal orientation for stereo-selective oxidation and demonstrate the utility of UniDesign for engineering CYP102A1 to produce drug metabolites of interest. The results are discussed in the context of protein structures.

37. Predicting Clinical Effects of CYP3A4 Perpetrators on Pirtobrutinib Using Physiologically-Based Pharmacokinetic Modelling

DAN-DAN TIAN, Stephen D. Hall, Kathleen M. Hillgren, Sonya C. Chapman, Maria M. Posada
Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN, 46285

BACKGROUND

Pirtobrutinib, a highly selective and non-covalent (reversible) Bruton tyrosine kinase (BTK) inhibitor, is metabolized via CYP3A4 and UGTs. In vitro, pirtobrutinib has high passive permeability, is a reversible and time-dependent inhibitor as well as an inducer of CYP3A4. Clinically, pirtobrutinib AUC was increased by 49% with itraconazole (capsule) and decreased by 71% with multiple dose rifampin; intravenous and oral midazolam AUC were increased by 12% and 70%, respectively, with pirtobrutinib.

METHODS

A pirtobrutinib PBPK model was developed and verified using physiochemical properties, in vitro biological data, and clinical data. The literature and Simcyp verified PBPK models of strong and moderate CYP3A4 inhibitors [itraconazole (solution), ritonavir, ketoconazole, clarithromycin, fluconazole, diltiazem, and verapamil], moderate and weak CYP3A4 inducers (bosentan and modafinil), were used for predicting CYP3A4-mediated effects on pirtobrutinib pharmacokinetics in healthy volunteers.

RESULTS

The PBPK model reproduced observed pirtobrutinib AUC and C_{max} after single and multiple doses of pirtobrutinib alone, with predicted/observed ratios of 1.06-1.38 and 0.77-1.07, respectively. The model also captured effects of itraconazole (capsule) and rifampin, with predicted AUC and C_{max} ratios within 0.91- to 0.96-, and 1.05- to 1.08-fold, respectively, of observed. Intravenous and oral midazolam AUC ratios in the presence of pirtobrutinib were predicted within 1.02- to 1.16-fold of observed. The model predicted pirtobrutinib AUC ratio of 1.33-1.73 with strong and moderate CYP3A4 inhibitors, 0.72-0.84 with moderate and weak CYP3A4 inducers.

CONCLUSION

The pirtobrutinib PBPK model was verified and can be used for predicting interactions between pirtobrutinib and CYP3A4 perpetrators.

38. Evaluating the Active Hepatic Scalar as a Surrogate for Transporter Kinetics to Predict Influence of Transporter/Enzyme Interplay in PBPK Model: Case Example – Atorvastatin and Pitavastatin

ALIX F LEBLANC, Md Masud Parvez, Julius O Enoru, Daniel AJ Bow and Ryota Kikuchi
QTAS, AbbVie Inc., USA.

It has been recognized that human clearance (hCL) is often underpredicted from *in vitro* assays, even for small molecules mainly cleared by metabolism regardless of species and test systems (hepatocytes or liver microsomes). A potential cause of the underprediction may be a difficulty to capture the transporter-enzyme interplay.

In a PBPK model development, kinetics data for the main enzymes and transporters involved in the elimination are key components required for an accurate clearance prediction. However, unlike enzyme kinetics, it is often a challenge and/or labor intensive to generate quantitative kinetics data for transporters. In order to still be able to account for the potential impact of hepatic transporters on elimination in the absence of kinetics data, "Active Hepatic Scalar" (AHS) is available as an option in the elimination part of the compound file in Simcyp (since ver. 18), where it is defined as a "multiplier on any scaled hepatic clearance to assess the net effect of uptake or efflux". AHS is factored into the well-stirred liver model where it multiplies to the whole organ intrinsic clearance.

In the present study, a simple method to obtain AHS is proposed using time course uptake in human hepatocytes. The performance of AHS was evaluated against clinical data in existing PBPK models by disabling the permeability limited liver model. The method was first developed with atorvastatin, then tested with pitavastatin PBPK model, both of which are hepatic transporter substrates.

The AHS of atorvastatin was 2.1, which improved the prediction comparing to clinical data. However, an additional scaling factor (SF) of 3, multiplying the AHS, was required in order to completely explain the clinical data. The AHS of pitavastatin was 4.0. By applying the additional SF (3) determined with atorvastatin, the predicted Cmax and AUC of pitavastatin were within 3-fold with clinical data. Addition of 4% BSA in human hepatocytes uptake study could not account for the additional SF since the AHS was lower than the value in its absence (2.5 vs 4.0). In conclusion, the simple method proposed in the current study to determine AHS may improve the prediction of compound elimination when hepatic transporters are involved. Further evaluations with additional PBPK models are warranted to optimize the use of AHS.

39. Incorporating Mechanistic Absorption into a Physiologically Based Pharmacokinetic Model of Caffeine

JESSICA REHMEL and Maria M Posada
Eli Lilly and Company

A physiologically based pharmacokinetic (PBPK) model of caffeine was modified in Simcyp® (version 20) to apply the advanced dissolution absorption and metabolism mode to absorption of caffeine administered orally as a solution. The original caffeine model used a first order absorption (non-mechanistic) approach. The model updates include incorporating an in vitro in-house measurement of permeability, elimination of the upper limit on permeability, and a change in the method of predicting volume of distribution at steady state to be consistent with the value observed following intravenous caffeine dosing.

The updated caffeine model was verified by comparing simulated concentration-time profiles and pharmacokinetic parameters with observations following intravenous dosing, oral dosing, and in drug-drug interactions with caffeine as the victim drug.

Following verification, multiple scenarios were explored, including prediction of 1) various assumptions regarding non-study caffeine consumption and 2) delayed gastric emptying of up to 3 times baseline gastric mean residence time. The caffeine model that incorporates a mechanistic absorption approach will enable investigation of scenarios where drugs affect GI transit time and could affect the rate or extent of caffeine absorption and help distinguish changes in absorption from changes in metabolism.

40. Reconstitution of Human Cytochrome P450 17A1 and Steroid 5α-Reductases in Phospholipid Liposomes, Amphipol and Nanodiscs

NEIKELYN BURGOS-TIRADO[1,2], SANG-CHOUL IM[1,2,3], Juan Valentín-Goyco[1,2], Hwei-Ming Peng[1], and Richard J. Auchus[1,2,3]

[1]Division of Metabolism, Endocrinology, & Diabetes, Department of Internal Medicine, and [2]Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109, [3]Veterans Affairs Medical Center, Ann Arbor, MI 48105, United States

Membrane-bound proteins account for over 30% of human proteins, including all of the 57 human cytochromes P450. These proteins are difficult to express in bacteria and to purify in their active forms, as detergents are often needed for isolation from the membrane and other proteins. In parallel, activity reconstitution requires the presence of phospholipid, which necessitates the incorporation of the purified membrane-bound P450s into lipid bilayers and/or detergent removal. The activity and catalytic properties of the P450s, however, can vary substantially depending on the specific phospholipids used and type of bilayer, including liposomes, bicelles, and nanodiscs. Furthermore, various lipid encasement forms can be more suitable for specific experiments, such as ligand binding titrations, addition of redox partners for activity determination, and biophysical studies. Finally, amphipathic polymers have been used to surround membrane-bound proteins and remove them from membranes without detergent or phospholipid. This study compares liposomes, nanodiscs, and amphipol for activity reconstitution of purified human P450 17A1 and steroid 5α-reductase type 2, which are transmembrane and integral membrane proteins, respectively.

For P450 17A1, the best activity is reconstituted with P450-oxidoreductase in liposomes; however, active enzyme is also obtained with nanodiscs and amphipol. For steroid 5α-reductase type 2, we have reconstituted activity with liposomes and nanodiscs but not amphipol. The advantages and disadvantages of these model membrane systems can be leveraged to optimize further experiments with these enzymes.

41. The Structural and Functional Characterization of Human Cytochrome P450 7B1 Missense Mutations that Cause Spastic Paraplegia Type 5

ALEXI CHABEZ[1] and Dr. Emily Scott[1,2,3,4,5]

From the Departments of Biological Chemistry[1], and Medicinal Chemistry[2], Pharmacology[3] and the Programs in Chemical Biology[4] and Biophysics[5], University of Michigan, Ann Arbor, Michigan, USA

Human cytochrome P450 enzymes are heme-containing monooxygenases that are membrane-embedded primarily in the endoplasmic reticulum or mitochondria. Their main function is to conduct the biotransformation of both endogenous and exogenous compounds, such as steroids and xenobiotics, respectively. Cytochrome P450 7B1 (CYP7B1) – a steroidogenic P450 enzyme – is commonly expressed in liver and brain and has a functional role in the 7α-hydroxylation of 25-hydroxycholesterol and 27-hydroxycholesterol to ultimately generate bile acid. Biallelic, missense mutations in *CYP7B1* can result in the loss of CYP7B1 enzymatic function, causing the accumulation of 25-hydroxycholesterol and 27-hydroxycholesterol in plasma, and 27-hydroxycholesterol accumulation in cerebrospinal fluid. Such accumulation results in a neurological disorder called spastic paraplegia type 5 (SPG5). SPG5 patients suffer from severe progressive spasticity and weakness of the lower limbs due to the degeneration of their lower motor neurons. Current efforts are directed towards biochemically characterizing five missense mutations commonly known to cause SPG5 to determine their individual effects on the CYP7B1 enzyme.

A synthetic, codon-optimized construct was generated to recombinantly express human CYP7B1 with truncation of the N-terminal helix and addition of a C-terminal His-tag, and the wild type enzyme successfully purified as a control enzyme. Site-directed mutagenesis was used to generate five common mutations correlated with SPG5: H285L, T297A, R417C, R417H, and R486C. Initial analysis to date suggests a variety of impacts from protein folding/stability to substrate binding. The R486C mutant in the loop between the β4 strands on the C-terminal end has significantly lower yield than the wild type enzyme and appears to have significant loss of heme during purification, suggesting that the folding and/or heme incorporation may be detrimental. However, the small amount of purified protein obtained has spectral characteristics very similar to the wild type enzyme and does indicate a Type I spectral shift upon binding with 27-hydroxycholesterol. In contrast, the T297A mutation in the I helix appears to have both yield and spectral characteristics similar to wild type, but ligand binding assays indicate a lower binding affinity for 27-hydroxycholesterol, suggesting that the mutation interferes with CYP7B1 substrate interaction. Further biochemical characterization of the effects of SPG5 mutations is currently underway. Collectively, we expect the information gathered from these five mutations will begin to facilitate understanding of the molecular basis for spastic paraplegia type 5.

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42. Pharmacological Modulation of Hsp70 to Selectively Remove Misfolded Neuronal Nitric Oxide Synthase

ANTHONY M. GARCIA[1], Emily Xu[2], Miranda Lau[1], Andrew Alt[3], Andrew Lieberman[4], and Yoichi Osawa[1]
[1]Department of Pharmacology, [2]Department of Chemistry, [3]Center for Chemical Genomics, [4]Department of Pathology, University of Michigan, Ann Arbor

The accumulation of misfolded proteins can result in various diseases, including neurodegenerative disorders. Hsp90/Hsp70 chaperone system regulates protein quality and thus represents a promising target for the development of treatments for these disorders. Over 20 inhibitors of Hsp90 have been developed but all clinical trials to date have failed, in large part, due to toxicity issues. In that Hsp90 inhibition targets client proteins in near native states, we hypothesize that targeting Hsp70 may be more selective for misfolded proteins. Studies with purified chaperones indicate that Hsp70 modulators target the misfolded nNOS. In the current study, we have developed a cellular model stably expressing native nNOS or C331A mutant of nNOS, which represents a slightly misfolded but functionally active nNOS, to better test this hypothesis. We used some known Hsp70 modulators, such as YM-1, as well as a novel agent discovered through screening efforts at the CCG. We showed that these Hsp70 modulators caused a time- and dose- dependent decrease in C331A mutant of nNOS while the native nNOS was not affected under the same conditions. Interestingly, the Hsp90 inhibitor, radicicol, caused a dose-dependent decrease in both wild type and C331A nNOS. These results suggest that Hsp70 modulation selectively removes the misfolded client protein leaving the natively folded client proteins untouched. Future studies will focus on developing and characterizing other Hsp70 modulators to identify small molecules that may better enhance the ubiquitination and degradation of misfolded nNOS

43. Deciphering the Role of Fatty Acid Metabolizing CYP4F11 in Lung Cancer and Its Potential as Drug Target

HUITING JIA, Sutapa Ray, Bjoern Brixius, Simone Brixius-Anderko
Department of Pharmaceutical Sciences, Center for Pharmacogenetics, University of Pittsburgh School of Pharmacy, Pittsburgh, USA

Lung cancer is the leading cause of cancer deaths worldwide with tobacco smoke as a major cause. The lipid mediator 20-hydroxyeicosatetraenoic acid (20-HETE) is known to regulate the blood pressure and promotes angiogenesis in healthy individuals. 20-HETE is generated by members of the cytochrome P450 family 4A/F. Intriguingly, inhibiting 20-HETE-producing CYP4 enzymes reduces the lung cancer tumor growth in xenograft mouse models. We conducted a bioinformatic analysis and found that the isoform CYP4F11 is significantly overexpressed in patients with lung squamous carcinoma. However, the exact role of CYP4F11 in lung cancer and its potential as drug target has not been established yet.

We hypothesize that CYP4F11-mediated 20-HETE production promotes lung cancer cell proliferation and migration. We first conduct cell studies to examine the role of CYP4F11 in lung cancer. We performed a transient knockdown of CYP4F11 in lung cancer cell lines to assess the impact of CYP4F11 on proliferation and migration. We found that the proliferation of lung cancer cells was significantly decreased after knocking down CYP4F11, indicating a pivotal role of CYP4F11 in lung cancer. The addition of exogenous 20-HETE to CYP4F11 knockdown cells could rescue proliferation, suggesting 20-HETE production impacts cancer cell proliferation. We then conducted transwell-migration assays and observed that a CYP4F11 knockdown attenuates the migration of lung cancer cells.

Second, we perform a preliminary screening for compounds inhibiting CYP4F11 to provide valuable information for lung cancer drug design. With recombinant human CYP4F11, we first conduct spectroscopic ligand binding assays to determine compounds with a high affinity to CYP4F11. Subsequently, the half-maximal inhibitory efficiency (IC_{50}) of selected compounds will be determined. Lead compounds will then be cross evaluated in lung cancer cell lines to test their impact on proliferation. We successfully evaluated the CYP4A/F inhibitor HET0016 which inhibits 20-HETE production. HET0016 shows high affinity to recombinant CYP4F11 and significantly attenuates lung cancer cell proliferation in a dose dependent manner. Additional compounds such as azoles and fatty acid amides, are currently evaluated.

We aim to further establish the role of CYP4F11 in lung cancer and the underlying mechanism. Furthermore, we will investigate to promote the exploitation of CYP4F11 as therapeutic target for a transformative lung cancer treatment option.

44. Increased Renal Elimination of Endogenous and Synthetic Pyrimidine Nucleosides in Concentrative Nucleoside Transporter 1 Deficient Mice

Avinash K. Persaud[1], Matthew C. Bernier[,2], Michael A. Massey[1,3], Shipra Agrawal[4], TEJINDER KAUR[1], Debasis Nayak[1], Zhiliang Xie[1], Brenna Weadick[1], Ruchika Raj[1], Kasey Hill[5], Nicole Abbott[5], ARNAV JOSHI[1], Nadeen Anabtawi[1], Claire Bryant[6], Arpad Somogyi[2], Zobeida Cruz-Monserrate[7], Foued Amari[8], Vincenzo Coppola[8,9], Alex Sparreboom[1], Sharyn D. Baker[1], Jashvant D. Unadkat[10], Mitch A. Phelps[1,5], and Rajgopal Govindarajan[1,10*]

[1]Division of Pharmaceutics & Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH 43210, USA. [2]Campus Chemical Instrument Center Mass Spectrometry and Proteomics Facility, The Ohio State University, Columbus, OH 43210, USA. [3]The Center for Life Sciences Education, College of Arts and Sciences, The Ohio State University, Columbus, OH 43210, USA. [4]Division of Nephrology & Hypertension, Renaissance School of Medicine, Stony Brook University, Stony Brook, NY 11794, USA. [5]Pharmacoanalytic Shared Resource (PhASR), The Ohio State University, Columbus, OH 43205, USA. [6]Center for Clinical & Translational Research, Nationwide Children's Hospital, Columbus, OH 43210, USA. [7]Division of Gastroenterology, Hepatology, and Nutrition, College of Medicine, The Ohio State University, Columbus, OH 43210, USA. [8]Genetically Engineered Mouse Modeling Core, Ohio State University Comprehensive Cancer Center, The Ohio State University, OH 43210, USA. [9]Department of Cancer Biology and Genetics, College of Medicine, The Ohio State University, Columbus, OH 43210, USA. [10]Department of Pharmaceutics, College of Pharmacy, University of Washington, Seattle, WA 98195, USA. [10]Translational Therapeutics, Ohio State University Comprehensive Cancer Center, Ohio State University, Columbus, OH 43210, USA

*Corresponding Author:
Rajgopal Govindarajan, Ph.D.
496 West 12th Avenue
Columbus, OH 43210-1291 Email: govindarajan.21@osu.edu; Phone: 614-247-8266

Concentrative nucleoside transporters (CNTs) are active nucleoside influx systems, but their in vivo roles are poorly defined. By generating and characterizing global CNT1 knockout (KO) mice, we identified a role of CNT1 in the renal reabsorption of pyrimidine nucleosides. CRISPR/Cas9 deletion of CNT1 in mice increased the urinary excretion of endogenous pyrimidine nucleosides with putative compensatory alterations in purine nucleoside metabolism but without impairment of fertility or survival. In addition, CNT1 KO mice exhibited high urinary excretion of the intravenously administered nucleoside analog drug gemcitabine (dFdC), which resulted in decreased systemic drug exposure. However, the increased urinary clearance of dFdC rendered this chemotherapeutic drug less effective in controlling tumor burden and preventing mortality in CNT1 KO mice orthotopically implanted with syngeneic Kras/p53-mutated mouse pancreatic ductal adenocarcinoma cells. Interestingly, increasing the dFdC dose to attain an area under the concentration-time curve level equivalent to that achieved by wild-type (WT) mice rescued antitumor efficacy and survivability in CNT1 KO mice. These findings provide new insights into how CNT1 regulates reabsorption of endogenous and synthetic nucleosides in murine kidneys and suggest that the functional status of CNTs may account for the optimal action of pyrimidine nucleoside analog therapeutics in humans. In addition, our studies propose that CNT1 KO mice are an excellent model to further study the role of CNT1 in nucleoside drug-drug interactions, CNT1 genetic polymorphisms, and *SLC28A1*-mutated human inborn errors of metabolism.

45. Exploring the Influence of Cholesterol on the Catalytic Activity of Human Aromatase

NIRUPAMA SUMANGALA[1,2], Sang-Choul Im[1,3,4], and Richard J. Auchus[1,2,3,4]
[1]Division of Metabolism, Endocrinology, & Diabetes, Department of Internal Medicine, and [2]Program in Biophysics, University of Michigan, [3]Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109, [4]Veterans Affairs Medical Center. Ann Arbor, MI 48105, United States.

Aromatase inhibitors are effective therapeutic agents for breast cancer, as the enzyme human aromatase (P450 19A1) is the only enzyme that converts androgens to estrogens. Abnormalities in P450 19A1 expression or activity have also been associated with various disorders, such as ovarian cancer, endometriosis, and infertility. P450 19A1 is widely expressed in different tissues, including the ovary, placenta, liver, adrenal gland, fat tissue, and brain, each of which has a unique lipid membrane composition and cholesterol content. We investigated the influence of cholesterol as a lipid on the catalytic activity of P450 19A1 in nanodiscs. Our findings demonstrate that cholesterol-containing nanodiscs exhibited similar rates and affinity of androstenedione binding to phospholipid-only nanodiscs. However, the rate of the first electron transfer from P450-oxidoreductase (POR) to P450 19A1 was three-fold faster in cholesterol-containing nanodiscs. The estrone formation rates were not significantly different between the two conditions. Furthermore, we performed computational docking of cholesterol to the P450 19A1-POR complex to understand the possible impact of cholesterol binding. Our results indicate that cholesterol may influence some aspects of the POR interaction with P450 19A1, thus representing an additional regulatory mechanism in this catalytic system.

46. Steroidal Isonitriles are Potent and Selective Ligands of Cytochrome P450 Enzymes

ALAINA M. RICHARD[1], Nathan R. Wong[2], KURT L. HARRIS[3], Reethy Sundar[2], Thomas C. Pochapsky[2,4], Emily E. Scott[1,3,5]

Program in Chemical Biology[1], Departments of Medicinal Chemistry[3], Pharmacology, Biological Chemistry, and Biophysics[5] at the University of Michigan; Departments of Biochemistry[2] and Chemistry[4], Brandeis University

The prostate cancer drug abiraterone (Zytiga) inhibits androgen synthesis by the human steroidogenic P450 enzyme CYP17A1. This drug is an effective CYP17A1 inhibitor because it consists of a substrate-like steroidal core with a nitrogen heterocycle appended at the site of metabolism. Appropriately positioned nitrogen heterocycles, such as the pyridine of abiraterone or imidazole, coordinate the heme iron to prevent oxygen binding and subsequent catalysis. Here we present a novel steroid-derived CYP17A1 inhibitor with an isonitrile moiety as the hemecoordinating group (3β-formyl-(R)-(20-isonitrilo)-pregnane[1]). This new ligand causes spectral changes consistent with its isonitrile carbon coordinating the heme iron of CYP17A1, and does so with a very high (<pM) affinity similar to abiraterone. A crystallographic structure of the complex confirms this type of interaction, with the isonitrile group forming a coordinate covalent bond and rising ~90° above the plane of the heme. Comparative spectral studies with other steroidisonitrile analogs demonstrated that changing the location of the isonitrile on the steroid resulted in negligible binding, likely because the appropriate positioning of the isonitrile above the heme iron was impeded by sterics. A potential advantage of isonitrile compounds is that while imidazole- and pyridine-based inhibitors only coordinate the ferric (Fe3+) heme iron, isonitriles can interact with the enzyme in both the ferric and ferrous oxidation states. Spectral studies furthermore demonstrated that the CYP17A1 heme-isonitrile coordination remained stable after heme reduction (to Fe2+), with limited reoxidation occurring slowly even under aerobic conditions. This same 3β-formyl-(R)-(20-isonitrilo)-pregnane compound demonstrates a reduced, but still relatively high affinity for promiscuous drug metabolizing enzymes CYP3A4 and CYP2D6 (k_d ~300 nM and 250 nM, respectively), while the isonitrile-CYP3A4 complex appears to reoxidize much more rapidly than the isonitrile-CYP17A1 complex. Studies comparing steroidal isonitrile inhibition of CYP17A1 and CYP3A4 are ongoing. Taken together, however, these results suggest that the isonitrile group provides an alternative moiety for the design of P450 ligands, with unique redox properties and tunable selectivity.

This work is funded by NIH grant R01 GM130997.

1) US patent application 63/237,421 filed 08/26/2021, Selective isonitrile inhibitors of cytochrome P450 subtypes, T.C. Pochapsky, N.R. Wong and R. Sundar.

47. CYP3A Deficiency Does Not Influence Vincristine Disposition and Neurotoxicity

YUE XU[1,5], Yang Li[1,5], Grace Taylor[1,5], Yasuhiro Kazuki[2], Kaoru Kobayashi[3], Ken-Ichi Fujita[4], Alice A. Gibson[1], Yan Jin[1], Alex Sparreboom[1], and Shuiying Hu[1,5]

[1]Division of Pharmaceutics and Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH, USA; [2]Division of Genome and Cellular Functions, School of Life Science, Faculty of Medicine, Tottori University, Tottori, Japan; [3]Department of Biopharmaceutics, Meiji Pharmaceutical University, Tokyo, Japan; [4]Division of Cancer Genome and Pharmacotherapy, Department of Clinical Pharmacy, Showa University School of Pharmacy, Tokyo, Japan; [5]Division of Outcomes and Translational Sciences, College of Pharmacy & Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA.

BACKGROUND

The dose-limiting side effect of many common anti-cancer drugs, including vincristine, is peripheral neuropathy. The incidence of vincristine-induced peripheral neuropathy (VIPN) is lower in patients who functionally express CYP3A5, a polymorphic liver enzyme that can metabolize vincristine *in vitro*, and VIPN is increased in patients concurrently treated with azole antifungals such as ketoconazole. The assumed mechanism for these interactions is through modulation of CYP3A-mediated vincristine metabolism, leading to decreased vincristine clearance and increased side effects. In this study, we tested these hypotheses in genetically-engineered mouse models with a deficiency of the entire murine *Cyp3a* locus and humanized transgenic mice with hepatic expression of human CYP3A5 variants.

MATERIALS & METHODS

Pharmacokinetic studies of vincristine (1 mg/kg, i.p.) given with or without ketoconazole (50 mg/kg) were conducted in wild-type mice, Cyp3a-deficient mice lacking all 8 murine CYP3A genes [CYP3A(-/-)], or humanized transgenic mice carrying the functional CYP3A5*1 variant or the non-functional cryptic splice variant CYP3A5*3. VIPN was evaluated by mechanical allodynia assessment (Von Frey Hair's test) before and after vincristine treatment. Vincristine concentrations in plasma samples were determined by LC-MS/MS.

RESULTS

Compared to wild-type mice, the systemic exposure to vincristine was increased by ~20% in CYP3A(-/-) mice, suggesting that the clearance of vincristine is largely independent of hepatic CYP3A function. Since CYP2C isoforms are up-regulated in the liver of CYP3A (-/-) mice and potentially provide a compensatory mechanism of elimination for some xenobiotics, the murine pharmacokinetics of vincristine were evaluated in the presence of the dual CYP2C/CYP3A inhibitor, ketoconazole. This experiment demonstrated lack of a profound ketoconazole-mediated change in the handling of vincristine in CYP3A(-/-) mice, suggesting that CYP2C does not serve as an escape mechanism for vincristine in these animals. In line with these observations, we found that CYP3A-deficiency did not influence the severity and time course of VIPN, and that exposure to vincristine was not substantially altered in humanized CYP3A5*3 mice (1.21-fold change) or humanized CYP3A5*1 mice (1.37-fold change) compared to CYP3A(-/-) mice.

CONCLUSIONS

Our study suggests that the contribution of CYP3A5-mediated metabolism to vincristine elimination and the associated drug-drug interaction potential are much smaller than held previously, and that plasma levels of vincristine are unlikely to be strong predictors of VIPN. These findings raise concerns about the causality of previously reported relationships between variant CYP3A5 genotypes or concomitant azole use with the incidence of VIPN and their dependence on altered drug disposition profiles.

48. Erythrocyte Targeted IdeS Selectively Cleaves RBC-Bound Antibodies and Protects Against IgG Mediated Hemolysis

BOYA ZHANG[1,2], Sachith Vidanapathirana[2], Michaela Dodd[2], Laura Cooling[3], and Colin F. Greineder[1,2]
Department of [1]Pharmacology, [2]Emergency Medicine, and [3]Pathology, University of Michigan, Ann Arbor, MI

Background: The destruction of erythrocytes by cell-bound IgGs occurs in Warm Autoimmune Hemolytic Anemia and limited therapies exist for arresting hemolysis once cells are decorated with auto- or alloantibodies. IdeS is an S. pyogenes enzyme that cleaves IgGs at a specific site in the hinge region and offers some protection against IgG-mediated disease, albeit without any selectivity for pathogenic antibodies. We created RBC-targeted IdeS fusion proteins (anti-RBC-Fab-IdeS) with superior pharmacokinetics, selectivity towards RBC-bound IgGs, and greater potency in a murine model of IgG-mediated hemolysis.

Methods: Murine anti-RBC-Fab-IdeS fusion were expressed, purified, and tested for affinity to RBC membranes and IdeS enzymatic activity. Agglutination assays were used to compare the ability of anti-RBC-Fab-IdeS vs. untargeted IdeS to cleave multiple RBC-bound IgGs. The selectivity of Fab-IdeS was measured by comparing the cleavage of RBC-bound vs. soluble IgG. Pharmacokinetics were evaluated using radiotracing. *In vivo* efficacy was tested in a murine passive immunization model, induced by an injection of 2mg/kg anti-mouse RBC IgG.

Results: Anti-RBC-Fab-IdeS demonstrated high affinity to RBC membranes (K_d = 24.3 ± 3.6nM) and prevented agglutination of murine RBC by multiple IgGs. Anti-RBC-Fab-IdeS was selective for RBC-bound IgG, cleaving 92.2% of RBC-bound IgG vs. 23.1% of soluble IgG after 2 hours at 20nM. Fab-IdeS showed improved pharmacokinetics, compared to untargeted IdeS. Finally, anti-RBC-Fab-IdeS offered more potent protection than untargeted IdeS in the passive immunization model, based on 48hr hemoglobin, and hematocrit.

Conclusion: Anti-RBC-Fab-IdeS showed improved pharmacokinetics, enhanced selectivity for RBC-bound IgG, and increased potency in a murine passive immunization model.

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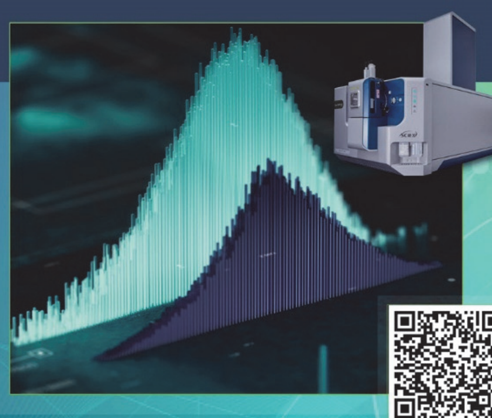

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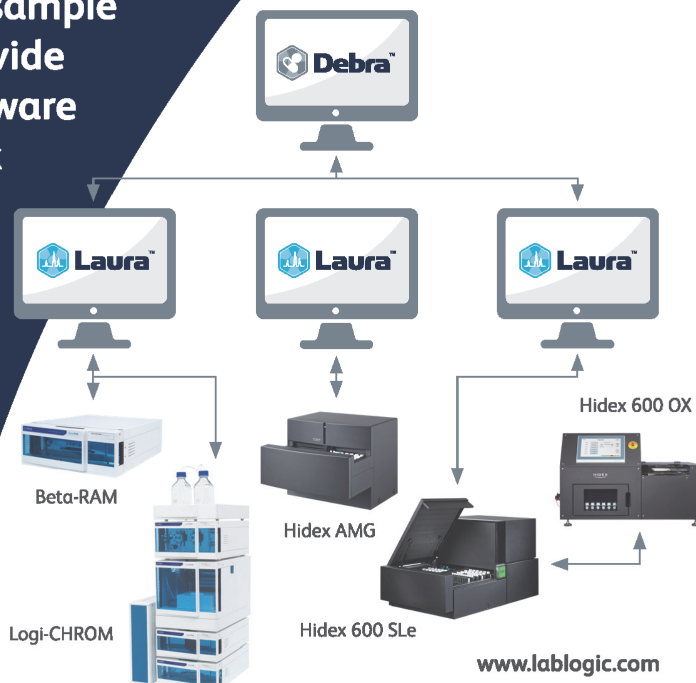

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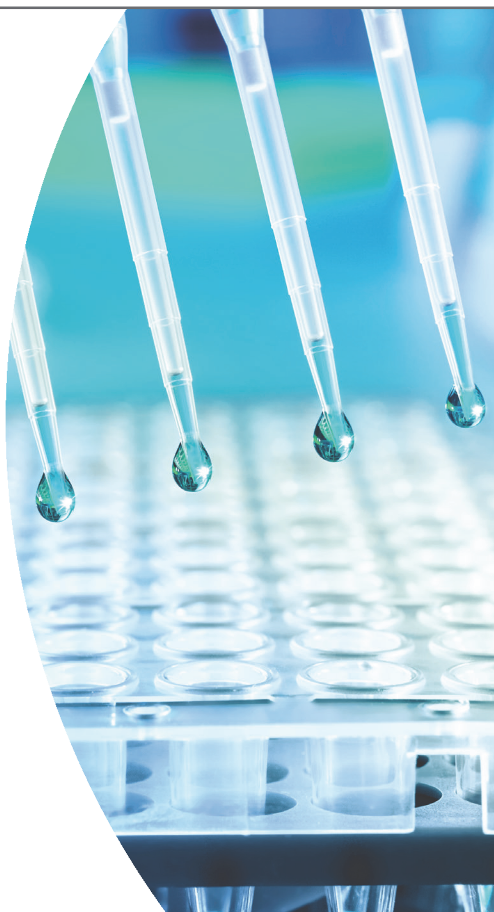

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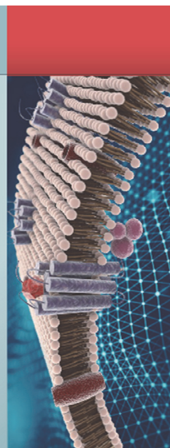

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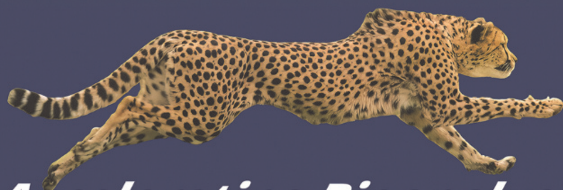

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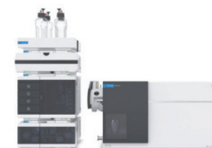

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