# 2024
# Great Lakes Drug Metabolism & Disposition Group Meeting

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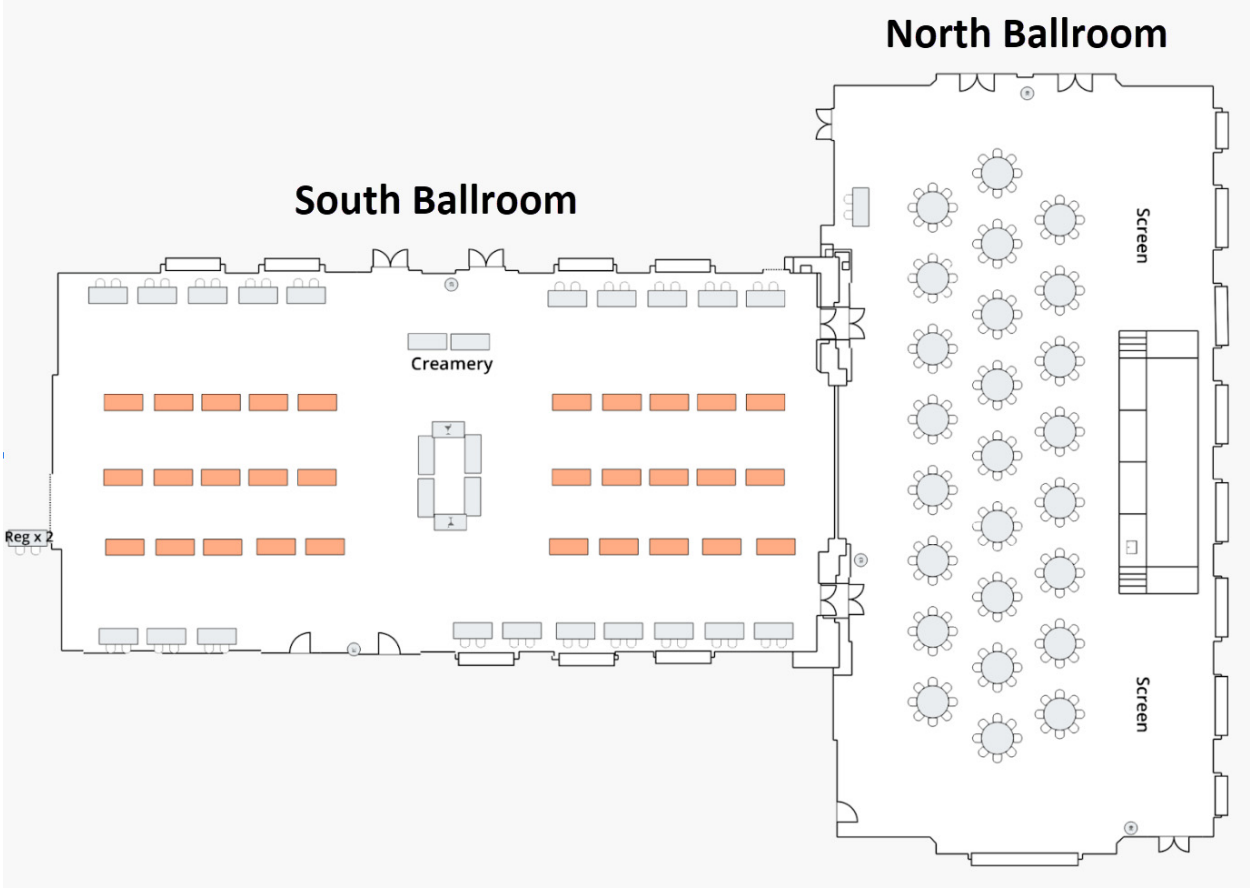
## Purdue Memorial Union, Purdue University
## West Lafayette, Indiana

## May 2 - 3, 2024

# Maps

## Purdue Memorial Union




**South Ballroom:** Posters, sponsor booths, Friday breakfast, breaks (coffee/tea)

**North Ballroom:** Oral presentations, Thursday lunch and networking lunch, Thursday dinner

**Room 263AB:** GLDMDG business meeting

## Thursday, May 2, 2024

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|---|---|
| 10:00 AM | **Check in, Coffee, Poster Setup, and Sponsor Booth Setup** (South Ballroom) |
| 11:00 | **Welcome** (North Ballroom), Young Jeong, Pharm.D., Ph.D., Professor, Dept. of Industrial and Physical Pharmacy, College of Pharmacy, Purdue University, West Lafayette, IN |
| 11:10 | *Moderator: Stephen Sligar, Ph.D., Professor, Depts. of Biochemistry and Chemistry, University of Illinois Urbana-Champaign, Urbana-Champaign, IL*<br>**Review of Structural Approaches to Drug Metabolism Studies**, Eric Johnson, Ph.D., Professor Emeritus, Dept. of Molecular Medicine, Scripps Research, La Jolla, CA |
| 11:50 | **Biochemical and Biophysical Studies of Cytochrome P450s and Transporters**, William Atkins, Ph.D., Professor and Chair, Dept. of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle, WA |
| 12:30 PM | **Lunch** (North Ballroom)<br>**View Posters and Visit Sponsor Booths** (South Ballroom)<br>**Students and Postdocs Networking with Industry Scientists** (North Ballroom) |
| 2:00 | *Moderator: Leslie Dickmann, Ph.D., M.P.H., Program Director, Faculty Associate, Division of Pharmacy Professional Development, School of Pharmacy, University of Wisconsin, Madison WI*<br>**Interindividual Variability in Tyrosine Kinase Inhibitor Metabolism in Ethnically Diverse Populations**, Klarissa Jackson, Ph.D., Assistant Professor, Division of Pharmacotherapy & Experimental Therapeutics, Eshelman School of Pharmacy, University of North Carolina, Chapel Hill, NC |
| 2:40 | **Drug Transporters in Chemotherapy-Induced Neuropathy**, Shuiying Hu, Ph.D., Assistant Professor, Division of Outcomes & Translational Sciences, College of Pharmacy, The Ohio State University, Columbus, OH |
| 3:20 | **Break** (South Ballroom) |
| 3:40 | *Moderator: Gary Jenkins, Ph.D. Senior Director, AbbVie Inc, Grayslake, IL*<br>**DESI-MS Application in Drug Discovery and Development of an Automated High-Throughput Drug Discovery Mass Spectrometry Platform**, Graham Cooks, Ph.D., Henry B. Hass Distinguished Professor, Dept. of Chemistry, Purdue University, West Lafayette, IN |
| 4:20 | **Proteomics-Based Research in Non-P450 Enzymes and Transporters**, Bhagwat Prasad, Ph.D., Associate Professor, Dept. of Pharmaceutical Sciences, Washington State University, Spokane, WA |
| 5:00 | **Poster Session (Author Available), Sponsor Booths, and Mixer** (South Ballroom)<br>Authors at odd numbered posters (5:00-6:00 PM). Authors at even numbered posters (6:00-7:00 PM). |
| 7:30 | **Dinner** (North Ballroom) |

# Agenda

## Friday, May 3, 2024

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|---|---|
| 7:30 AM | **GLDMDG Business Meeting** (Room 263AB)<br>**Continental Breakfast, View Posters, and Visit Sponsor Booths** (South Ballroom) |
| 8:00 | **Vendor Presentations** (North Ballroom)<br>**Improving Throughput of Bioanalytical Research through Advancements in QQQ Technology, and Expansion of Multi-omic Panels for Targeted Analysis of Biomarkers**, John Sausen, B.S., M.B.A., Director of Strategic Initiatives - Mass Spectrometry, Agilent Technologies<br>**Cyclic Ion Mobility - High Resolution Mass Spectrometry for the Rapid Profiling of Drug Metabolites in Biofluids,** Iggy Kass, Ph.D., HRMS Business Development Executive, Waters Corporation<br>**Plateable Hepatocytes in Non-Small Molecule Drug Development**, Christopher Bohl, Ph.D., Senior Manager Technical Support, BioIVT |
| 9:00 | *Moderator: Michael Mohutsky, Ph.D., Senior Director, Eli Lilly and Company, Indianapolis, IN*<br>**ADME of RNA-Based Therapy**, Julie Lade, Ph.D., Scientific Associate Director, Pharmacokinetics & Drug Metabolism, Amgen. |
| 9:40 | **Leveraging In Silico ADME Modeling in Drug Discovery – A Learning Journey**, Stella Doktor, M.S., Principal Research Scientist, AbbVie, North Chicago, IL. |
| 10:20 | **Break** (South Ballroom) |
| 10:40 | *Moderator: Shuiying Hu, Ph.D., Associate Professor, Division of Outcomes & Translational Sciences, College of Pharmacy, The Ohio State University, Columbus, OH*<br>**ADME Bioanalytical Strategies and Workflows to Quantify and Evaluate Oligonucleotide Therapeutics**, Ryan Hill, B.S., Principal Research Scientist, ADME Drug Disposition, Eli Lilly and Company, Indianapolis, IN |
| 11:20 | **The Blood Brain Barrier Demonstrates Capability to Influence Heterogenous Tenofovir and Emtricitabine Metabolism and Transport in the Brain**, Hannah Wilkins, B.S., The Johns Hopkins University School of Medicine, Baltimore, MD, *Student Abstract Award Winner.* |
| 11:35 | **A Physiologically Relevant Intestinal Model for Mechanistic Assessment of Oral Bioavailability**, Agnes Badu-Mensah, Ph.D., Eli Lilly and Company, Indianapolis, IN, *Postdoc Abstract Award Winner.* |
| 11:50 | **Closing Remarks**, Shuiying Hu, Ph.D., Associate Professor, Division of Outcomes & Translational Sciences, College of Pharmacy, The Ohio State University, Columbus, OH |

**1. The First in-human Study to Evaluate the Antiplatelet Properties of DT-678 in Acute Coronary Syndrome Patients and Healthy Volunteers**

HAOMING ZHANG[1], Y. Eugene Chen[2], Caixia Yang[3], Yanjun Gong[4], Jianping Li[4]

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**Background and Purpose**
DT-678 is a novel antiplatelet prodrug capable of releasing the antiplatelet active metabolite (AM) of clopidogrel upon exposure to glutathione. In this work we aimed to investigate factors responsible for clopidogrel high on-treatment platelet reactivity (HTPR) in acute coronary syndrome (ACS) patients and to evaluate the capacity of DT-678 to overcome the HTPR.

**Experimental Approach**
A total of 300 consecutive ACS patients naive to P2Y12 inhibitors were recruited and genotyped for CYP2C19 alleles. Blood samples were drawn before and after administration of 600 mg clopidogrel. Platelet reactivity index (PRI) and plasma AM concentrations were determined and grouped according to their CYP2C19 genotypes. DT-678 was applied ex vivo to whole blood samples to examine its inhibitory effects. To further examine the antiplatelet effectiveness of DT-678 in vivo, twenty healthy human subjects were recruited in a Phase I clinical trial, and each received a single dose of 3 mg DT-678 or 75 mg clopidogrel. The pharmacokinetics and pharmacodynamics in different CYP2C19 genotype groups were compared.

**Key Results**
Statistical analyses revealed that CYP2C19 genotype, body mass index, hyperuricemia, and baseline PRI were significantly associated with higher risk of clopidogrel HTPR in ACS patients. Addition of DT-678 ex vivo decreased baseline PRI regardless of CYP2C19 genotypes, overcoming clopidogrel HTPR. This observation was further confirmed in healthy volunteers receiving 3 mg of DT-678.

**Conclusion and Implications**
These results suggest that DT-678 effectively overcomes clopidogrel HTPR resulting from genetic and/or clinical factors in Chinese ACS patients, demonstrating its potential to improve antiplatelet therapy.

*This work was in part supported by a grant from the University of Michigan-Peking University Joint Institute for Translational and Clinical Research (UM/PUSC Award).

**2. Strategic Fluorination Improves the Metabolic Stability and Physicochemical Properties of Dextromethorphan (DXM) and Leu-Enkephalin**

TREVOR TROMBLEY, Jacob Sorrentino, Brett. Ambler, Krishna K. Sharma, and Ryan A. Altman

*Department of Chemistry, Purdue University, West Lafayette, IN*
*Terray Therapeutics, Los Angeles, CA Merck, West Point, PA*
*Department of Chemistry, Iowa State University, IA*
*Borch Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN*

The incorporation of fluorine and fluorinated substructures into a druglike molecule can profoundly influence the compound's pharmacology, distribution, metabolism, and pharmacokinetic profile (DMPK). This strategy has been leveraged in the lead-optimization of numerous drug candidates across a variety of therapeutic areas, particularly in the context of central nervous system (CNS) therapeutics. Our group identified Leu-enkephalin (δ-opioid agonist) and dextromethorphan (σ1 agonist, DXM) as prototypical examples of central nervous system (CNS) agents whose DMPK profiles could be improved via strategic fluorination. Specifically, Leu-enkephalin shows poor CNS permeability and possesses a Tyr amide bond that is rapidly cleaved by aminopeptidase N, leading to insufficient CNS concentrations in vivo. Meanwhile, the aryl methyl ether of DXM is rapidly dealkylated by the 2D6 isoform of cytochrome P450, generating the neuroactive metabolite DXO, which has considerable off-target activity at the N-methyl-D-Aspartate (NMDA) receptor and produces dissociative hallucinations in vivo. Our group employed synthetic fluorination strategies to deliver fluorinated analogues of DXM and Leu-enkephalin that showed increased metabolic stability and CNS exposure, while maintaining similar on-target activity. Of note, fluoroalkyl analogues of DXM ablated undesired off-target activity at NMDA.

**3. Investigation of YTE Time Extension Mutations on Monoclonal Antibody PK in Rats**

RENJIE ZHAN[1], Luc Rougee[1], Alex Miller[2], Jeffrey Boyles[2], Jason Robarge[1], Ryan Hansen[1]

*[1]Global PK/PD & Pharmacometrics, Quantitative Clinical Pharmacology and Discovery PK/PD, Eli Lilly and Company, Indianapolis IN*
*[2] Biotechnology Discovery Research, Eli Lilly and Company, Indianapolis IN*

Prolongation of monoclonal antibody (mAb) therapeutic exposure continues to be of interest to the pharmaceutical industry. One strategy to accomplish this consists of a triple mutation [Met(M)252Tyr(Y)/Ser(S)254Thr(T)/Thr(T)256Glu(E)] on the crystallizable region (Fc) of mAbs, also known as the YTE mutation. The underlying mechanism for the prolonged half-life with the YTE mutation involves altered pH-dependent binding affinity of the mAb to the neonatal Fc receptor (FcRn), which is a key player in the recycling mechanism for endogenous or exogeneous immunoglobulin G (IgG) molecules in monocytes and endothelia cells.

For wild type mAbs (non-Fc engineered), cynomolgus monkeys are the most widely utilized model for studying and projecting human pharmacokinetics (PK) for mAbs. Recently, rodent models (including rats and humanized FcRn mouse strains) have also been explored as alternative models for evaluating PK properties in discovery and for projecting human PK. However, the utility of pre-clinical species (rat or monkey) to study the impact of YTE mutations on mAb PK remains unknown, and no established approaches for translation of PK from preclinical species to humans for this time extension strategy exist.

The current study aimed to understand the impact of introducing YTE mutations on the PK of mAb in rats with a series of paired parental and YTE-mutated mAbs. PK of these paired mAbs was determined following IV administration to Sprague Dawley rats through a compartmental Pop-PK approach using Nonlinear Mixed-Effects Modeling (NONMEM software program, version 7.5.0).

It was observed that the increase in volume of distribution (Vd) and clearance (CL) did not result in any significant improvement in half-life of YTE-mutated mAbs compared to wild type mAbs. Therefore, our knowledge based on currently generated PK data suggested that rats are not an optimal species for evaluating the half-life extension benefit introduced by YTE mutations.

**4. The Blood Brain Barrier Demonstrates Capability to Influence Heterogenous Tenofovir and Emtricitabine Metabolism and Transport in the Brain**

HANNAH N. WILKINS[1], Ben C. Orsburn[1], Dionna W. Williams[2]

*[1]Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD*
*[2]Department of Pharmacology and Chemical Biology, The Emory University School of Medicine, Atlanta, GA*

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

**5. Exploration of Key Variables for Brain Uptake of Antibody Therapeutics**

DAVID BUSSING and LUC ROUGEE

*Eli Lilly and Company*

A physiologically-based pharmacokinetic (PBPK) model of receptor mediated transcytosis (RMT) of monoclonal antibodies (mAbs) from the plasma to the brain has been previously published for mAbs targeting the anti-transferrin receptor (TfR)[1]. The PBPK model incorporates current knowledge of brain anatomy and physiology with key mechanisms for brain uptake of mAbs, including RMT across the blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB), paracellular diffusion across said barriers, and neonatal fragment crystallizable (FcRn)-mediated recycling. The model provides a theoretical framework to explore the molecular properties of mAbs targeting brain receptors for the

purposes of uptake via RMT including affinity and valency. Furthermore, hypothetical properties of a receptor mediating the transcytosis are also investigated, including expression level, transcytosis rate, and turnover rate. The presented modelling exercise explores the effects of modulating the above mAb and receptor properties within the model with the intention of determining their effect on brain uptake using the current model structure.

1. Chang, HY., Wu, S., Chowdhury, E.A. et al. Towards a translational physiologically-based pharmacokinetic (PBPK) model for receptor-mediated transcytosis of anti-transferrin receptor monoclonal antibodies in the central nervous system. J Pharmacokinet Pharmacodyn 49, 337–362 (2022).

**6. Characterization and Potential Applications of Novel in vitro Triculture Blood-Brain Barrier and Neurovascular Unit Models for Antiviral Screening**

Z. MONICA XU,[1] Charlie Zhang,[1] James Sluka,[2] and Gregory T. Knipp[1]

*[1] Purdue University Department of Industrial and Molecular Pharmaceutics, West Lafayette, IN*
*[2] Indiana University, Biocomplexity Institute, Bloomington, IN*

The ongoing challenge of treating neurological complications arising from viral infections, such as those caused by SARS-CoV-2, necessitates innovative approaches to model the human neurovascular unit (NVU). We introduce a novel *in vitro* triculture model comprising astrocytes, pericytes, and brain microvessel endothelial cells cultured with neurons on a Transwell® filter to mimic the blood-brain barrier (BBB) and NVU. This NVU-enhanced direct contact triculture model is designed to explore the differential permeability of antiviral drugs and the expression of tight junction proteins and efflux transporters, which are crucial for maintaining BBB integrity. Our findings indicate differences in permeability and protein expression between our triculture model and NVU-enhanced models, suggesting that the NVU-enhanced triculture model can closely simulate *in vivo* conditions. The model provides a promising platform for high-throughput antiviral screening, elucidating the mechanisms of viral neuropathology, and facilitating the development of targeted antiviral therapies.

**7. Impact of Heterotropic Allosteric Modulation of Progesterone on Time Dependent Inhibition of CYP3A4 by Macrolide Molecules**

POOJA HEGDE[1]; Kaitlin Shin[1]; Trent L. Abraham[1]; Alec Bell[1]; Stephen D. Hall[1]; Luc R.A. Rougée[1]

[1]*Eli Lilly and Company, Indianapolis, IN, USA*

Common practice in drug discovery is to use in vitro determined values of inhibition parameters for a new chemical entity to predict the potential of a drug-drug interaction (DDI) with other clinical drugs, especially CYP3A4 substrates. While predictions of reversible inhibition have been successful, predictions of CYP3A4 time-dependent inhibition (TDI) consistently overpredicted or miss-predict (i.e. false positives) the interaction that is observed in vivo. While efforts have been undertaken to attempt to improve predictions by altering the concentration of inhibitor used, there remains a disconnect between in vitro to in vivo. Recent findings demonstrated that that the presence of the allosteric modulator progesterone (PGS) in the assay could alter the in vitro kinetics of CYP3A4 TDI with inhibitors whose mechanism is with the heme, such as metabolic-intermediate complex (MIC) forming inhibitors.

In the current work we evaluated, in human liver microsomes, the impact of the presence of 100 µM PGS on the TDI of molecules in the class of macrolides typically associated with MIC formation. Presence of PGS resulted in varied responses across the inhibitors tested. The TDI signal was eliminated for five inhibitors, and unaltered in the case of one, fidaxomicin. The remaining molecules erythromycin, clarithromycin, and troleandomycin, were observed to have a decrease in both potency and maximum inactivation rate ranging from 1.7-fold to 6.7-fold. These changes in TDI kinetics led to a >90% decrease in inactivation efficiency. In order to determine in vitro conditions that could produce in vivo inhibition, varied concentrations of PGS were incubated with clarithromycin and erythromycin. Resulting in vitro TDI kinetics were incorporated into dynamic physiologically based pharmacokinetic (PBPK) models to predict clinically observed interactions. The results suggested that a concentration of ~45 µM PGS would result in TDI kinetic values that could reproduce in vivo observations and could potentially improve predictions for CYP3A4 TDI.

**8. Solvent Isotope Effects as a Probe of Cytochrome P450 Mechanism**

G. DENISOV[1], Y. V. Grinkova[1], Stephen G. Sligar[1,2,3]

*Departments of [1]Biochemistry, [2]Chemistry and [3]Center for Biophysics and Quantitative Biology, University of Illinois at Urbana-Champaign, Urbana, IL, USA*

The catalytic cycle of the cytochromes P450 requires two electrons from a protein redox partner and two protons from water to generate the main catalytic intermediate, a ferryl-oxo complex with π-cation on the heme porphyrin ring, termed Compound 1. The protonation steps are at least partially rate-limiting, therefore the steady-state rates of P450 catalysis are usually slower in deuterated solvent ($D_2O$) by a factor of 1.5 - 3. However, in several P450 systems a pronounced inverse kinetic solvent isotope effect (KSIE ~ 0.4 – 0.8) is observed, where the reaction is faster in $D_2O$. Using numerical modeling of the P450 steady-state kinetics we demonstrate that a significant inverse KSIE cannot be obtained for a pure Compound 1 driven catalytic cycle of P450. An alternative protonation-independent catalytic intermediate needs to be introduced.

The most extensively documented inverse KSIE in the human steroid biosynthetic P450 CYP17A1 and several mutants confirmed the involvement of a heme peroxo anion intermediate, which has been trapped and characterized by resonance Raman and UV-VIS spectroscopy. The hydroxylation is catalyzed by Compound 1, while C-C lyase reaction mainly proceeds *via* unprotonated ferric-peroxoanion intermediate. Comparison of proton inventory measurements for the wild-type CYP17A1 and E305G mutant for hydroxylation and lyase reaction also validate a different reaction mechanism and predominant product formation via peroxo-ferric intermediate for the C-C bond scission.

*Supported by a MIRA grant from NIGMS R35 GM118145 (S.G.S).*

**9. Development of a Novel Fluorescence-Based Approach for Solute Carrier-Focused CRISPR/Cas9 Knockout Screening in Breast Cancer Cells**

YUE XU[1,4], Digamber Rane[2], Dario Palmieri[3], Mahesh Nepal[1,4], Alex Sparreboom[1], Blake R. Peterson[2] and Shuiying Hu[1,4]

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*[2]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, The Ohio State University, Columbus, OH*
*[3]Department of Cancer Biology and Genetics, College of Medicine, The Ohio State University, Columbus, OH*
*[4]Division of Pharmaceutics and Pharmacology, College of Pharmacy, The Ohio State University, Columbus, OH*

**BACKGROUND**: Paclitaxel is among the most widely used anticancer drugs and is known to cause a dose limiting peripheral neurotoxicity via a solute carrier (SLC) transporter, OATP1B2, in the dorsal root ganglion. However, the roles of SLCs in paclitaxel uptake by breast cancer cells remain largely unknown. The traditional endpoint of CRISPR/Cas9 library screening evaluated by cytotoxicity may not directly reveal the process of transmembrane influx. The main purpose of this study is to develop an alternative fluorescence-based approach for SLC-focused screening and identify solute carrier(s) responsible for paclitaxel uptake in cancer cells.

**METHODS**: Uptake studies of a novel fluorescent probe 6FC-Taxol (0.1 μM), a derivative of paclitaxel linked to a drug-like fluorophore related to Pacific Blue, were optimized with unlabeled paclitaxel by confocal microscopy and flow cytometry in various breast cancer cell lines. Cell lines with the greatest decrease in fluorescence were selected for cellular competition binding and cytotoxicity MTT assay before optimized further for CRISPR/Cas9 library edition and high-throughput cell sorting.

**RESULTS**: Confocal microscopy assay revealed MDA-MB-231 and MDA-MB-468 cells with greatest reduction of fluorescence intensity of 6FC-Taxol upon co-incubation with unlabeled paclitaxel. 6FC-Taxol showed similar cytotoxicity ($IC_{50}$=77-171 nM) compared to paclitaxel towards these cell lines. Cellular competition binding assays and uptake assays demonstrated comparable inhibitory potency between 6FC-Taxol and paclitaxel with similar lower nanomolar $IC_{50}$. Finally, conditions for cell sorting using 6FC-Taxol were optimized to obtain maximal separation of fluorescent cell clusters which can be used to distinguish uptake of 6FC-Taxol with and without inhibitors in both cell lines, with over a 4-fold difference in intracellular 6FC-Taxol fluorescence.

**CONCLUSION**: The novel fluorescent probe 6FC-Taxol was validated as a sensitive probe for CRISPR/Cas9 knockout screening in breast cancer cell lines MDA-MB-231 and MDA-MB-468 with optimal inhibition profiles. Collectively, we developed a novel fluorescence-based approach with the potential to fill a knowledge gap identifying uptake transporter for the important anti-cancer drug paclitaxel in cancer cells.

Keywords: fluorescent molecular probes, SLC transporters, CRISPR/Cas9

**10. Rapid LC-MS/MS Analysis of Extracellular Matrix Protein Crosslinking in Fibrotic Disease**

ELIZABETH J. SANDE[1], Sarah M. Lloyd[2], Kenneth Ruterbories, Yue-Ting Wang[1], Stephen O'Brien[2], Tracy Carr[2], Meghan Clements[2], Lisa Hazelwood[2], Yu Tian[1], Yupeng He[2], and Qin C. Ji[1]

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*[2]Discovery Immunology, Pharmacology & Pathology, Fibrosis, AbbVie Inc., 1 N Waukegan Rd., North Chicago, IL 60064*

Fibrotic medical conditions have been a focal area of many pharmaceutical efforts due to their debilitating and chronic progression. Idiopathic pulmonary fibrosis (IPF) begins with inflammation or injury in the lungs, then progresses to scarred lung tissue from an increase in extracellular matrix (ECM) which impairs lung function. IPF has very limited treatment options, thus novel therapies are needed for this high mortality disease. Changes to the lung ECM proteins may reveal the keys to unlocking these therapies. However, existing bioanalytical methodologies are often labor intensive and time consuming, incapable of returning results of discovery treatments quickly. Addressing this, we established a rapid liquid chromatography mass spectrometry (LC-MS/MS) assay to evaluate ECM crosslinking products including lysinenorleucine (LNL), hydroxylysylnoreleucine (HLNL), dihydroxylysylnoreleucine (DHLNL), pyridinoline (Pyr), deoxypyridinoline (DPyr), desmosine (Des) and isodesmosine (IsoDes). The development effort was focused on improving the efficiency of both sample preparation and LC-MS/MS analysis. We reduced the initial processing steps as well as removed the typical solid phase extraction (SPE) step which lessened the amount of hands-on sample preparation time. LC-MS/MS conditions were optimized to allow simultaneous analysis of seven crosslinks within two minutes to support high throughput laboratory practices. Using this methodology, we successfully assessed collagen crosslinking in human IPF lung tissue compared to healthy lung. We showed an increase in immature collagen crosslinks, especially DHLNL, in IPF tissue which correlates to published findings. In conclusion, a highly efficient LC-MS/MS assay was developed and its value in studying ECM protein crosslinking in a discovery setting was demonstrated. Using this approach to monitor crosslinks is the first step in establishing new therapeutic target identification and could eventually be applied to a range of fibrotic diseases.

**11. A High-Throughput 2D-UPLC-MS/MS System for the Analysis of Endogenous Molecules in Biological Matrices**

FENG JIN, Kenneth Ruterbories, Qin C. Ji

*AbbVie Pharmaceuticals*

Two-dimensional liquid chromatographic separation interfaced with tandem mass spectrometric detection (2D-LCMSMS) has emerged as a significant assay platform for simplifying sample preparation and enhancing the LCMS assay sensitivity. Various configurations including immunocapture cartridges, reverse phase-based stational cartridges and columns with different mobile phases for the first-dimensional separation, along with reverse phase second-dimensional analytical column separation, have been reported in literature. While some systems are designed for proteomic research with nano/micro flow rate [1], others are tailored for bioanalysis applications using regular LC flowrates.

In this study, we present the development of a high throughput, high-sensitivity 2D-LCMSMS platform in our lab for the bioanalysis of endogenous molecules. Our system employs reverse phase LC with a basic mobile phase for the first-dimensional separation, followed by UPLC with an acidic mobile phase for the second-dimensional separation. To demonstrate the performance, myostatin was selected as model target, as it is an important therapeutic target for muscle degeneration-related diseases. The results reveal a significant enhancement in the signal-to-noise ratio of analyte detection, ranging from a 3-fold to an impressive 26-fold improvement compared to one-dimensional separation approaches.

In addition, our assay platform achieves a lower limit of quantification (LLOQ) of 0.5 ng/mL. which is 5 times improvement over the most sensitive 2D-LCMS assay reported in the literature [2]. With its high throughput and exceptional sensitivity, this platform is being utilized in our laboratory to develop LCMSMS assays for endogenous molecule bioanalysis, supporting various drug development programs.

Ref:
1. He, J.; Meng, L.; Ruppel, J.;Yang, J.; Kaur, S.; Xu, K.; Anal. Chem. 2020, 92, 9412-9420.
2. Zhang, G.; Chen, S.; Song, K.; Pan, P.; Qiu, Y.; Amaravadi, L.;Wu, J. Bioanalysis 2019, 11, 957−970.

**12. Developing a High-Throughput SFC-MS/MS Method to Determine EPSA and Predict Human Permeability**

YUE-TING WANG, Mei Feng[1], Edward Price, Jason Hulen, Stella Doktor, David M. Stresser, Estelle M. Maes, Qin C. Ji, and Gary J. Jenkins
These authors contributed equally.

*Quantitative, Translational, ADME Sciences (QTAS), AbbVie Inc., 1 N. Waukegan Rd., North Chicago, IL 60064*
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Permeability is an important parameter affecting the absorption of orally administered drugs. Evaluating compound permeability using *in vitro* approaches is an essential step in early discovery and a common practice within the pharmaceutical industry. The high demand of the drug discovery pipeline, coupled with growing interest in exploring non-traditional chemical spaces (such as compounds that violate Lipinski's Rule of 5 or Ro5), makes the efficient and reliable evaluation of permeability a challenge. Herein we present a high-throughput supercritical fluid chromatography tandem mass spectrometry (SFC-MS/MS)-based methodology to measure the experimental polar surface area, which we term HT-EPSA, as a surrogate for assessing compound permeability. Compared to earlier methods, HT-EPSA features optimized SFC conditions, enhanced method sensitivity, selectivity, and efficiency, with enhanced quality control measures. Upon the integration into an existing high-throughput workflow, the HT-EPSA assay enabled a 6-fold increase in compound screening capacity, a 16-fold decrease in data acquisition time per compound, over 400-fold decrease in compound consumption, and a wider range of EPSA values to determine. Additional to assay development and validation, the predictability of EPSA to Caco-2 cell and human permeability was also evaluated, followed by the establishment of translational strategies attempting to bridge EPSA and clinically relevant permeability. The results demonstrate that the HT-EPSA method is highly beneficial for guiding early-stage compound rank-ordering and facilitating quick decision-making. EPSA is a valuable descriptor for predicting *in vitro* or human intestinal permeability, particularly for compounds that fall beyond the Ro5, such as Proteolysis Targeting Chimeras (PROTACs).

**13. High-Throughput Label-Free Opioid Receptor Binding Assays using Automated Desorption Electrospray Ionization Mass Spectrometry (DESI-MS)**

YUNFEI (VERONICA) FENG, Nicolás M. Morato, Kai-Hung Huang, and R. Graham Cooks

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The identification of novel small-molecule candidates with high analgesic properties but reduced side effects is of high importance in the context of the current opioid epidemic. To facilitate the discovery of such new non-addictive analgesic candidates, and overcome the multiple drawbacks associated with current radioligand binding assays (e.g. cost, safety concerns, versatility), we focused on the development of a label-free approach for probing the relative binding affinity of small molecules towards opioid receptors. In these assays, leucine enkephalin (LeuEnk, utilized as a competitive ligand), test compounds, and commercial membrane preparations of opioid receptors are incubated together (1 h, 37 °C) in Tris buffer pH 7.4. After incubation, DADLE (a methylated analog of LeuEnk used as an internal standard for quantification) is added, and the mixture is rapidly filtered. The amount of free LeuEnk is finally quantified by high-throughput (<1 second per sample) automated desorption electrospray ionization mass spectrometry (DESI-MS) using minimal sample volumes (50 nL). This parameter is used as an indicator of the binding strength of the test compounds. Here we demonstrate a competitive ligand binding assay towards μ and δ opioid receptors developed using an automated high-throughput platform based on DESI-MS. We first optimized the amount of LeuEnk and receptors for each assay such that the effects of the test compounds can be maximized: without test compounds, little free LeuEnk is present. Then, we successfully adapted the filtering process with a liquid handling robot, which significantly increased experimental efficiency. Several dose-response relationships for multiple known opioids (e.g. naloxone, naltrindole, PZM-21) demonstrate the agreement between our novel label-free methodology and traditional approaches based on scintillation counting. Among all compounds that we have tested, naltrindole has the strongest binding affinity towards δ opioid receptors, while naloxone is the strongest μ opioid receptor binder. This DESI-MS based method has also shown good reproducibility with relative standard deviations lower than 10%. It is then utilized to test the binding affinity of several late-stage-functionalized opioids.

**14. Real-Time Library Search on Orbitrap IQ-X Tribrid MS enhances metabolite profiling**

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**Purpose:** To demonstrate a use case of the "Real-Time Library Search" intelligent data acquisition strategy on the Thermo Scientific™ Orbitrap IQ-X™ Tribrid™ Mass Spectrometer for enhanced small molecule structure identification.

**Methods:** Model compounds were incubated in human hepatocytes and metabolites identified using an Orbitrap IQ-X Tribrid mass spectrometer and the Met-IQ workflow via the new "Real-Time Library Search" (RTLS) filter.

**Results:** The Met-IQ workflow enhanced $MS^2$ sampling while simultaneously retaining generation of $MS^3$ scans for ions of interest to assist in the structural characterization of metabolites.

**15. Next Generation Mass Spectrometry Fragmentation Modes to Answer Key Questions in Drug Metabolism and Disposition**

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DMPK groups typically perform in vitro and in vivo biotransformation studies which often generate mixtures containing conjugated metabolites. Definitive structure identification and quantitation of these phase-II metabolite species are required for regulatory filings, which necessitates high-quality analytical separation and fragmentation. Currently, CID and HCD are the most often used fragmentation modes in mass spectrometry, and these techniques often fail to determine the site of conjugation based on fragmentation. High energy and nonergodic techniques such as UVPD, ETD, ECD, and EAD have become increasingly popular in proteomics workflows that afford labile PTM site localization.

Alternative fragmentation strategies have not yet been applied widely to small molecules and may provide additional details for accurate and reliable metID. In this work, we compare HCD, UVPD, and EAD fragmentation modes for a series of common phase-II metabolite standards (i.e., O-glucuronidation, acyl-glucuronidation, N-oxidation, and sulfation). This will help determine which modes provide richer, diagnostic MS/MS spectra for improved conjugate site localization. Data presented here are collected on multiple MS platforms (Thermo Orbitrap Exploris 480 using HCD, Thermo Orbitrap Fusion Lumos using UVPD, and Sciex 7600 Zeno ToF using EAD) and processed using vendor and custom homebuilt software. The systematic evaluation of multiple techniques will lead to an analytical roadmap to guide future studies and workflows in the discovery and development metID space.

Abbreviations list
CID – collision-induced dissociation
DMPK – drug metabolism and pharmacokinetics
EAD – electron activated dissociation
ECD – electron capture dissociation
ETD – electron transfer dissociation
HCD – higher-energy collisional dissociation
metID – metabolite identification
MS – mass spectrometry
PTM – post-translational modification
ToF – time of flight
UVPD – ultraviolet photodissociation

**16. Orbitrap Exploris 240 Mass Spectrometer with Novel Data Acquisition Features Ensures Confident Metabolite Identification and Structure Elucidation**

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High resolution mass spectrometry (HRMS) is the gold standard for metabolite identification. A modified Orbitrap MS with ultra-high resolution and automated data acquisition features further improves the data quality by triggering MS/MS of low-abundant metabolites masked by matrix, facilitating overall confident metabolite identification and structural elucidation. In addition, rapid positive/negative polarity switching, and automatic background ion subtraction feature ensure data quality and enhance usability.

The modified quadrupole-Orbitrap mass spectrometer with automatic background subtraction through inclusion/exclusion list generation and MS/MS acquisition provided a wealth of information for metabolite identification of model compounds. The ultra-high resolution ensured high mass accuracy and isotopic fidelity, which provide confident elemental composition determination. This elemental composition determination, combined with the HR HCD MS/MS fragmentation, enabled confident metabolite ID and structural elucidation. Using the background exclusion feature in data acquisition, the high resolution data captured and revealed the high fidelity chlorine and sulfur isotopic patterns of Ticlopidine and Timolol metabolites, and these metabolites were readily identified using the chlorine and sulfur isotopic patterns as filters in Compound Discoverer.

**17. Re-imagining Drug Discovery using Automated Ambient Mass Spectrometry**

NICOLÁS M. MORATO, Veronica Feng, Kai-Hung Huang, Beinan Yang, Andrew D. Mesecar, and R. Graham Cooks

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The early drug discovery workflow relies heavily on high-throughput experimentation, both in terms of organic synthesis as well as analysis of complex biosamples. The identification of new biological targets through large-scale biospecimen studies, the generation of large sets of drug candidates and their rapid bioactivity screening, as well as the in vitro and cell-based confirmation of hits followed by lead optimization, all rely on high-throughput strategies which are typically spread out across diverse technologies in specialized facilities. The efficiency of this workflow could benefit from the consolidation of these activities in a single closed-loop platform. Mass spectrometry (MS) is an attractive technique to achieve such consolidation due to the inherent speed of mass analysis, however this advantage is rarely fully utilized due to the widespread use of sample purification approaches (e.g. chromatography) prior to MS.

Here we describe an automated system that achieves the consolidation of the early drug discovery pipeline by leveraging the advantages of desorption electrospray ionization (DESI), an ambient ionization technique that allows for the rapid and direct analysis of complex samples, both in qualitative and quantitative manner, without any need for workup. This system results from the combination of custom and commercial software, robotics, and analytical instrumentation, and its capable of achieving throughputs better than 1 Hz using high-density arrays (up to 6,144 samples per array) and 50-nL samples (<5 ng analyte). More significantly, inherent reaction acceleration in microdroplets allows reaction time to be reduced from minutes or seconds to just milliseconds.

The workflow of this platform involves automated sample preparation or manipulation using a fluid handling workstation, generation of microarrays on PTFE-coated slides using a pin-tool, automated transfer and analysis of spotted slides using high-throughput DESI-MS, and real-time processing of the spectral data. This methodology has been extensively demonstrated for the screening of organic reactions for identification of optimal synthesis conditions and the selective late-stage functionalization of complex molecules, as well as label-free quantitative biological assays using purified targets (e.g. enzymes, receptors), cell cultures, microorganisms, or tissue biopsies, all with no sample cleanup. Examples of all these capabilities will be provided and framed within the overall context of drug discovery, where the untargeted analysis of biospecimens using DESI-MS led to the identification of the sulfotransferase 2B1b as a potential enzymatic target for cancer treatment, and the same ambient MS technology was utilized for the kinetic characterization of this enzyme, a large-scale inhibitor screening campaign and the subsequent cell-based hit confirmation, as well as synthetic strategies towards lead optimization.

This presentation will showcase both the original iteration of this automated platform, developed throughout the DARPA Make It program and currently available as a facility service through the Bindley Bioscience Center at Purdue University, as well as a new-generation system built within the ASPIRE initiative of the US National Center for Advancing Translational Sciences.

**18. Application of Drug-Induced Growth Rate Inhibition and Intracellular Drug Exposures for Comprehensive Evaluation of Cellular Drug Sensitivity**

Dolonchampa Maji, MALERIE WOLKE, Shamim Khaja, John P. Savaryn, John C. Kalvass, Gary J. Jenkins, Malerie Wolke

*AbbVie*

*In vitro* cellular assays are indispensable tools for preclinical understanding of therapeutic candidates. Herein, we have outlined methods for robust determination of cellular sensitives by adapting drug-induced growth-rate inhibition analysis combined with intracellular drug exposure measurement. Using three auristatins as tool molecules, we demonstrate wide-spanned cellular response in sensitive versus resistant cancer cells, as well as in a toxicity-relevant cell type. Cellular response analysis generates metrics describing efficacious extracellular concentrations of drug as well as the phenotype of response – cytotoxic versus cytostatic. Cell associated drug measurements bridge the gap between extracellular drug concentrations and exposure at intracellular site required for a desired pharmacodynamic response. Such methods can complement rational drug design by providing thorough understanding of drug mechanism of action, guide mechanistic selection of target indication and inform exposure-response analysis at various stages of drug discovery.

**19. High-Throughput In Vitro Stability Assessment of Biologics**

ROY HENG, Jason Hulen, Yihan Li, Nicole Richwine, Malerie Wolke, Vikram Shenoy, Stella Doktor, Estelle M. Maes, Hetal Sarvaiya, Gary J. Jenkins

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*Acknowledgements: AbbVie QTAS Preanalytics and in vitro BA teams*

Antibody-based therapeutics have revolutionized the field of drug development by achieving therapeutic responses that were previously unattainable with small molecule drugs. However, these therapeutics are susceptible to various modifications and degradation pathways during circulation, which may impact their stability and efficacy. Moreover, the increasing complexity of protein therapeutics requires additional effort to understand and assess their stability liabilities during all stages of drug development. Therefore, understanding the stability of these molecules in vitro is valuable, not only to accurately predict in vivo behavior, but also to proactively identify potential liabilities that will assist discovery teams in generating successful candidates.

To generate candidate protein therapeutics with ideal physiological and functional properties, the QTAS Biologics Bioanalytical team is committed to provide a high-quality screening workflow to enable project teams to scrutinize potential liabilities arising from incubation in biological matrices. Herein, we present a suite of various in vitro characterization assays, for identification of liabilities such as aggregation, cleavage, and other modifications. This screening of biologics and drug conjugates workflow, guides project teams to select the best candidates within a short timeline.

1) High-throughput serum aggregation assay provides an early and efficient assessment of aggregation and stability.

2) High-resolution Mass Spectrometry (MS) in vitro serum stability (IVSS) assay offers in-depth characterization of intactness as well as post-translational modification liability.

3) Payload release assay for Antibody Drug Conjugates (ADC) in serum and lysosomes assesses the stability of ADCs.

**20. Development of a High Throughput Screen for Cytochrome P450 Ligand Binding Assays**

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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 most P450 enzymes are either drug metabolizing enzymes or drug targets. Characterizing interactions between a P450 active site and the physiochemical features of a small molecule is key to understanding enzymatic functionality and effectively designing drugs for either class of P450 enzyme. The most common method for characterizing small molecule binding is by quantifying absorbance changes that commonly occur when substrates bind the P450 active site and cause spin stage changes in the heme iron. Traditional ligand titrations monitored by a spectrophotometer require significant manual time and increasing solvent concentrations. This assay has therefore been adapted for semi-automated high throughput screening, which increases the number of compounds examined 50-fold with the improvement of being able to keep solvent concentrations constant, while keeping total required protein equal. This 384-well assay was validated for both type I and II shifts typically observed for substrates and heme-coordinating inhibitors, respectively. A library of ~100 azoles was assembled and screened with three human drug- and sterol-metabolizing P450 enzymes: CYP2A6, CYP2D6, and CYP8B1. Absolute spectra were collected across an 11-point titration for each compound. An R script corrects spectra for baseline absorbance and generates difference spectra by subtracting ligand-free P450 absorbance from each absolute spectrum, illustrating the change in absorbance due to ligand binding. As azoles frequently cause spectral shifts with P450 enzymes, it is not surprising that 42% to 57% bound to the different P450 enzymes. Absorbance differences plotted against ligand concentration yield dissociation constants (Kd). Kd values are used to generate pharmacophores for each P450 active site, for comparison to known structures for these three enzymes in the Protein Data Bank. The high throughput screen is thus useful for efficiently identifying and ranking ligands for P450 enzymes, facilitating generation of pharmacophores, identifying ligand profiles for P450 deorphanization, and screening potential drugs either to establish P450s as drug targets or to avoid P450 metabolism.

Supported by NIH R37 GM076343 and T32 GM007767

**21. Identification of OATP1B1 Substrates by Competitive Counterflow Screen**

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Tyrosine kinase inhibitors (TKIs) belong to a drug class of small molecules that inhibit the function of key protein kinases involved in the pathogenesis of a broad range of human diseases. Although the primary elimination pathway for most TKIs involves hepatic CYP3A metabolism, the mechanism by which these agents are taken up into hepatocytes remains unclear. Furthermore, the presence of high levels of extracellular binding creates experimental challenges in identifying a transporter-mediated process. Here, we optimized and validated a competitive counterflow (CCF) approach to examine TKIs as substrates of the hepatic uptake transporter OATP1B1. The CCF method was based on the stimulated efflux of radiolabeled estradiol-17β-glucuronide after steady state in HEK293 cells engineered to overexpress OATP1B1. A panel of 63 approved TKIs was examined for transport by OATP1B1, in which pazopanib was identified as a representative hit for further verification studies. The transport of pazopanib by OATP1B1 was demonstrated by decreased kinase activity of vascular endothelial growth factor receptor 2 (VEGFR2), its target protein kinase, in OATP1B1 overexpressing cells but not cells lacking OATP1B1. Similarly, the activity of VEGFR2 in hepatocytes isolated from wild-type mice harboring functional OATP1B1 was also decreased compared to mice with OATP1A/1B-deficiency. Furthermore, the molecular docking and interaction of pazopanib with OATP1B1 exhibited a binding pattern similar to estrone-3-sulfate, a well-established OATP1B1 substrate. Taken together, these findings further support the notion that OATP1B1 mediates the transport of pazopanib. The clinical utility of pazopanib is limited by severe and potentially fatal hepatotoxicity. Thus, we further hypothesized that this toxicity phenotype is dependent on OATP1B1. In a series of i*n vivo* approaches, higher liver accumulation of pazopanib that is accompanied by elevated liver transaminases (ALT and AST) was observed in wild-type mice compared to OATP1A/1B-deficient mice. Moreover, these findings occurred without pharmacokinetic differences between the two genotypes. Taken together, our study provides utility of using the CCF method for direct comparison of substrate affinity for OATP1B1 with a large set of agents in the class of TKIs.

**KEYWORDS**:
OATP1B1, Competitive Counterflow, Tyrosine Kinase Inhibitors

**22. A Universal Method to Screen Nanobodies from a Yeast-Display Platform for Cytochrome P450 Enzymes**

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Nanobodies (Nbs) are important tools for structure-function studies of proteins and have potential therapeutic applications owing to its specificity, stability, and small size. The conventional method of acquiring antigen-specific Nb through immunization in camelids is expensive and time consuming. In this study, we developed an approach to select Nbs for P450 enzymes from a highly diverse yeast-display library (McMahon et al, Nat Struct Mol Biol, 2018, 25:289-296). To avoid costly primary and secondary antibodies for Nb selection, we utilized biotin-streptavidin interactions instead. We biotinylated the antigen and conjugated the streptavidin with fluorescent probe molecules. We tested our method on three P450 enzymes including CYP102A1, CYP2B4, and neuronal nitric oxide synthase (nNOS). The Nb-biotinylated antigen binders were selectively enriched using magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS). Following two rounds of MACS, the population of positive binders was enriched by >5-fold compared to the naïve library. Subsequent FACS selection with a gating of 0.2% identified 215, 634, and 270 binders for CYP2B4, CYP102A1, and nNOS, respectively. Further selection based on EC50 determined at various antigen concentrations yielded 120 and 80 top binders for CYP102A1 and nNOS respectively. To characterize the functionality of Nbs, the top 30 binders of CYP102A1 were sequenced, resulting in 26 unique clones. Eight of them with low EC50 values were selected for expression and characterization. They were found to inhibit CYP102A1 activities with IC50 values ranging from 0.2 μM to 2.4 μM. These results demonstrate the utility of our approach which may be applicable to many protein targets for rapid selection of specific nanobodies.

**23. Gut Bacterial Metabolite Phenylpropionic Acid Alleviates Acetaminophen-induced Hepatotoxicity by Inhibiting Cytochrome P450 2E1 Expression**

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Acetaminophen (APAP)-induced hepatotoxicity has been a major cause of acute liver failure worldwide and is caused by APAP bioactivation into a toxic metabolite by hepatic drug-metabolizing enzymes. The gut microbiota has emerged as a modulator of the hepatic expression of drug-metabolizing enzymes and may contribute to inter-individual variability in APAP-induced hepatotoxicity. Our group previously reported that a gut bacterial metabolite phenylpropionic acid (PPA) alleviates APAP-induced hepatotoxicity in mice. This was accompanied by decreased hepatic protein (but not mRNA) levels of cytochrome P450 2E1 (CYP2E1), the major enzyme responsible for APAP bioactivation. To elucidate the mechanisms of PPA action, PPA effects on APAP toxicity and CYP2E1 expression were investigated in primary mouse hepatocyte culture and AML-12 mouse hepatoma cells. PPA, at physiological concentrations, decreased APAP-induced cytotoxicity (determined by the lactic dehydrogenase, LDH, release) in both primary mouse hepatocytes and AML-12 cells. Furthermore, we observed a decreased CYP2E1 protein expression in the PPA-treated hepatocytes, suggesting that the previous in vivo findings in PPA-supplemented mice are attributable to the direct effects of PPA on CYP2E1 expression. Unlike in mice, however, PPA treatment decreased the mRNA levels of CYP2E1 in primary mouse hepatocytes. Blocking protein synthesis through co-treatment with cycloheximide minimally impacted the decrease in CYP2E1 protein levels induced by PPA, supporting the minimal effects of PPA on the degradation rate of CYP2E1 protein. These results suggest the role of PPA in the transcriptional regulation of CYP2E1 in primary mouse hepatocytes. Elucidation of detailed molecular mechanisms underlying PPA action in primary mouse hepatocytes and AML-12 cells is underway.

**24. Gut Microbial Metabolites Upregulate Hepatic CYP3A4 Expression via Activation of Pregnane X Receptor**

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

**25. Pharmacokinetic Model for the Indirect Metabolism of Two Drugs by Gut Bacteria**

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The bioavailability of many important oral drugs, such as tacrolimus and sulfasalazine, shows clinical variability, leading to the conundrum: Why is it difficult to predict the kinetics of these approved drugs? Variations in bioavailability lead to toxicity or low clinical efficacy. Alterations in drug transporters and metabolic enzymes influence drug bioavailability. We provide evidence that another factor, indirect metabolism by gut microbes, can also influence important transporters and enzymes that can affect the bioavailability of oral drugs. We model the pharmacokinetics of two oral drugs, tacrolimus and sulfasalazine, under varying host colonic expressions induced by bacterial species: E. coli Nissle 1917 and Bifidobacterium adolescentis. We provide insight into how sensitive the pharmacokinetics of tacrolimus and sulfasalazine are to the bacterial influence on intestinal protein expression of drug transporters and cytochrome p450 enzymes. Bacteria-mediated changes decrease the peak blood concentration of tacrolimus compared to those in healthy renal transplant patients. Moreover, the bacteria lead to a two-fold increase in the peak plasma concentration of sulfasalazine in patients compared to that in healthy subjects. Our results suggest that the pharmacokinetic variability can be explained by accounting for the bacteria-induced changes to the host colonic transporters and enzymes. The results could lead to better PK models and address variability in bioavailability.

Keywords: Pharmacokinetics, Gut Bacteria, Indirect Metabolism, Tacrolimus, Sulfasalazine, Microfluidics

**26. Glutathione S-Transferase Kinetics and Inhibition in Cat and Dog Blood**

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Glutathione S-transferases (GSTs) eliminate reactive xenobiotics by conjugation with glutathione (GSH). GST substrates include chemotherapeutic agents and environmental chemicals. The importance of GST activity in blood (eGST) in drug metabolism, as well as its potential as a biomarker, has not been extensively studied in these animals. This study compared eGST activity and kinetics in cat and dog blood, further investigated this reaction using chemical GST inhibitors.

Venous blood was collected from 40 cats and 69 dogs. eGST activity and kinetics using chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates were calculated. The inhibitors used were ethacrynic acid (EA), auranofin, quercetin (QUE), atrazine, Cibacron Blue (CIB), triethyltin bromide (TEB), triphenyltin chloride (TPC), hematin (HEM), bromosulfophthalein (BSP) and N-ethylmaleimide. The strength of the inhibition was quantified by the IC50, and its mechanism was determined versus CDNB and GSH.

The eGST activity and apparent Vmax at 1.0 mM CDNB and 1.0 mM GSH was 6-12 times higher in cats than dogs. For CDNB, eGST activity in cats fitted the Michaelis-Menten model better than in dogs although in dogs this model was still a better fit than the allosteric sigmoidal model. Cat eGST had a higher affinity for CDNB, while dog eGST had the higher affinity for GSH. A wide variation in KM CDNB was seen in dogs. Inhibition was observed in the nanomolar range for CIB in dogs. For both species, the most potent inhibitors (IC50 < 5µM) were EA, QUE, CIB and HEM, while the weakest inhibitors (IC50 > 25µM) were BSP and TEB. In cats, non-competitive inhibition of EA and QUE versus CDNB was observed as opposed to a competitive mechanism in dogs.

The higher Vmax in cats may be due to increased expression of GSTP in erythrocytes. The dissimilarities in the [CDNB] versus activity relationship, together with differences in the KM values and in their distribution between cats and dogs indicate different GST isoforms in the blood of these species. While lower sensitivity to TPC, CIB, BSP and HEM indicates that the major isoform in cat blood may be GSTP, in dog blood, the inhibition profile (higher sensitivity to CIB, TPC and HEM; low sensitivity to BSP) may indicate a homo-or heterodimeric mix of GSTs (e.g. GSTP1, GSTP2, GSTA).

In conclusion, differences exist between the magnitude and kinetics of eGST activity in cats and dogs, which may be ascribed to variations in the expression and specificity of GST isoforms in their blood.

**27. Encequidar is a Gut-Restricted P-gp Specific inhibitor that Boosts Oral Bioavailability and Inhibits Intestinal Secretion in Rats, Dogs and Monkeys**

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*Gilead Sciences*

P-glycoprotein (P-gp, MDR1) mediated efflux of compounds can reduce oral bioavailability and/or increase biliary and intestinal clearance. Quantifying the role of P-gp-mediated efflux is helpful in drug design and predicting human pharmacokinetics (PK) parameters and exposure. Encequidar (ENC) is a recently identified potent and selective P-gp inhibitor (J. Med. Chem. 2021, 64, 3677−3693). *In vitro*, ENC inhibited P-gp-mediated digoxin efflux with an $IC_{50}$ of 5.8 µM but did not inhibit breast cancer resistance protein (BCRP)-mediated prazosin efflux up to highest tested concentration of 100 µM. ENC is also a poorly orally bioavailable gastro-intestinal (GI) tract restricted P-gp inhibitor. Thus, orally administered ENC can only inhibit P-gP present in the enterocytes. ENC was explored as an in vivo GI restricted P-gp inhibitor in Sprague-Dawley rats, beagle dogs, and cynomolgus monkeys to determine the extent of P-gp mediated efflux in limiting oral bioavailability or for clearance mediated by intestinal secretion.

The extent of P-gP efflux in limiting oral bioavailability was assessed with digoxin and paclitaxel in rats, paclitaxel in dogs and talinolol in cynomolgus monkeys (Table 1). In all cases, 5 mg/kg of oral encequidar mesylate was orally administered at 5 mg/kg 30 min prior to the oral administration of the substrate. In rats, ENC-mediated P-gp inhibition increased the $C_{max}$ and $AUC_{infinity}$ by 10-15 and 20-25-fold, respectively, for digoxin and paclitaxel compared to controls (Figures 1 and 2). In dogs, ENC-mediated P-gp inhibition increased paclitaxel $C_{max}$ and $AUC_{infinity}$ by 4-5-fold (Figure 3). In monkeys ENC-mediated P-gp inhibition increased talinolol $C_{max}$ and $AUC_{infinity}$ by 10 and 4.5-fold, respectively. Thus, ENC is a useful tool in determining the extent of PgP-mediated efflux in limiting oral bioavailability (Table 2).

The extent of P-gP efflux in intestinal secretion and its contribution to systemic clearance was assessed with paclitaxel in rats. ENC was orally dosed at 5 mg/kg 30 min prior to 1 mg/kg intravenous (IV) infusion administration of paclitaxel. Paclitaxel plasma PK and its excretion in feces were quantified. In feces, 20% of the administered dose was excreted as intact paclitaxel in absence of ENC and no paclitaxel was detected in the presence of ENC. The plasma clearance values of paclitaxel were reduced by 18% consistent with the change in fecal amount of paclitaxel. This suggests that intestinal secretion contributes 18-20% to systemic clearance of paclitaxel in rats, and intestinal secretion is completely inhibited in the presence of ENC.

In summary, ENC is a gut restricted P-gP specific inhibitor that can be formulated as a precipitation resistant solution. Co-administration of encequidar reveals the loss in oral bioavailability due to PgP-mediated efflux. In addition, the gut-restricted nature of encequidar reveals information on the extent of intestinal secretion towards total systemic clearance.

## 28. Physiologically-Based Pharmacokinetic Modeling for the Prediction of BCRP-Mediated Drug Interactions Using R Statistical Software

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The Breast Cancer Resistant Protein (BCRP) is expressed widely throughout the body, including in the intestine and liver, and impacts the absorption and disposition of many drugs which are BCRP substrates. Thus, coadministration of a BCRP inhibitor and BCRP substrate can lead to clinical drug-drug interactions (DDIs). Rosuvastatin is a widely prescribed statin and is often used as a clinical index substrate to assess BCRP inhibition as BCRP limits its intestinal absorption and mediates its biliary efflux. Pirtobrutinib is a BCRP inhibitor in the clinic and was shown to increase the AUC of orally administered rosuvastatin by 2.18-fold. Physiologically-based pharmacokinetic models (PBPK) can be leveraged to predict such BCRP-mediated DDIs using in vitro data.

To this end, a PBPK modeling platform was developed using the mrgsolve package in R statistical software. Rosuvastatin PBPK input parameters were obtained from literature, and PK predictions were compared with observed data. Population variability in rosuvastatin pharmacokinetics was modeled by introducing variability on transporter expression, renal clearance, and body weight. Furthermore, the model was used to predict the impact of single dose pirtobrutinib on rosuvastatin pharmacokinetics. The measured in vitro BCRP Ki of 1.3 µM was incorporated into a pirtobrutinib PBPK model, and the predicted extent of interaction was compared with observed clinical data.

As summarized in Table 1 below, the rosuvastatin model showed excellent concordance with observed data and the interaction between pirtobrutinib and rosuvastatin was well captured. Additionally, the model closely captured observed rosuvastatin plasma-concentration time profiles following a 20 mg oral dose and predicts extensive enterohepatic recirculation as the apparent fraction absorbed (0.48) was over 50% greater than the fraction absorbed (0.30).

A fully customizable modeling platform combining mechanistic absorption, disposition, and population variability was developed using the mrgsolve package in R statistical software. The model reproduced the PK of rosuvastatin and its DDI with pirtobrutinib. This flexible PBPK platform can be utilized for modeling in drug discovery and development.

| | Percent of rosuvastatin dose recovered in urine | Percent of rosuvastatin dose recovered in feces | Rosuvastatin oral bioavailability | Rosuvastatin AUC ratio following oral dosing +/- pirtobrutinib |
|---|---|---|---|---|
| **Predicted** | 31 | 68 | 0.24 | 2.29 |
| **Observed** | 28 | 72 | 0.20 | 2.18 |
| **Ratio (Pred/Obs)** | 1.11 | 0.94 | 1.20 | 1.05 |

Table 1: Summary of rosuvastatin model performance

## 29. A Chemical Biology Strategy for Predicting OATP1B-Type Transporter-Mediated Drug-Drug Interaction (DDI) Liabilities

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**INTRODUCTION:** Organic anion transporting polypeptides 1B1 and 1B3 (OATP1B) are pivotal hepatic drug transporters. Inhibiting OATP1B can disrupt drug clearance, elevate plasma concentrations, and induce therapy-related side effects. While endogenous OATP1B substrates are well-explored, most prescription drugs inhibitory potential remains elusive. We employed high throughput screening (HTS) of FDA-approved drugs to evaluate their OATP1B inhibitory effects and validate their potential for DDIs in translationally relevant rodent models.

**METHODS:** High Throughput Screening (HTS) of the Prestwick library of 1520 drugs was conducted using OATP1B3-overexpressing HEK293 cells. The assay system was validated with positive and negative controls employing PB-Gly-Taxol as a fluorescent substrate of OATP1B. Chemicals inhibiting OATP1B activity >50% were selected and further characterized on 96- and 12-well plate formats using the fluorescent substrate 8-fluorescein-cAMP (8-FCA) and the radiolabeled substrate estrone-17b-glucuronide (EbG). The potency of inhibition, uptake kinetics, and substrate transport properties were determined using concentration-dependent, Dixon plot, and competitive counter flow (CCF) analysis. The hit compound rifapentine was evaluated in mice by measuring an OATP1B-associated biomarker. DDI potential of rifapentine was tested with OATP1B substrate, pravastatin. Pharmacokinetic profiling was evaluated with LC-MS/MS-based methods and calculated with Phoenix WinNonlin software.

**RESULTS:** Forty-one drugs were initially identified from the screen and further narrowed to 10 drugs based on their physicochemical and toxicological properties by literature survey. The inhibition potency of these 10 drugs was assessed in OATP1B1, 1B2, and 1B3-overexpressing cells using 8-FCA as a substrate. Four drugs, including rifapentine, pyrvinium pamoate, dihydroergotamine, and dipyridamole with the greatest inhibition potency were further confirmed with the secondary substrate EbG. Rifapentine was identified as the most potent inhibitor among the four with IC50 <1 µM. Dixon plot and CCF analysis confirmed pyrvinium pamoate as a competitive inhibitor and substrate of OATP1Bs and the other three drugs were categorized as non-competitive inhibitors. In vivo studies showed that rifapentine (20 mg/kg, oral) significantly increased the exposure of the OATP1B biomarker CDCA-24G in mice plasma, with a >2-fold increase in AUC compared to vehicle-treated mice. Further DDI studies with pravastatin caused a >2-fold AUC increase in WT mice, whereas no significant changes were observed in OATP1A/1B KO mice.

**CONCLUSIONS:** This study systematically evaluated FDA-approved drugs for their potential to inhibit OATP1B-mediated transport. Rifapentine was identified as a novel potent inhibitor both in vitro and in vivo, highlighting its clinical relevance. This research bridges a knowledge gap regarding OATP1B-related DDIs and offers insights into potential strategies for minimizing harmful interactions in patient treatments.

KEYWORDS: OATP1B, drug transport, drug-drug interactions, rifapentine, pharmacokinetics

**30. Evaluation of Commercial Software for Prediction of Metabolic Structures and Site-of-Metabolism: In Silico Predictions Versus In Vivo Data**

DANIEL LADROR, Elyse Freiberger, Rich Burton, David Wagner

*AbbVie*

The investigation of drug metabolism is essential for understanding the behavior of a drug *in vivo*. Biotransformation can significantly reduce a drug's pharmacokinetic half-life and affect the therapeutic activity. Furthermore, metabolites can be transformed into moieties which add unexpected activity or toxicity for ongoing drug projects. As a result, it is critical to understand metabolism (typically by employing *in vitro* and *in vivo* metabolism assays) and identify metabolic properties as early as possible in a drug's development cycle. *In silico* tools are an intriguing option for supporting this workflow, as they can be used to predict the metabolism of drug structures prior to committing resources for synthesis and testing, potentially providing the same information without the use of animals.

Here, we present our evaluation of three commercial software platforms for metabolism prediction: MetaSite (Molecular Discovery), StarDrop (Optibrium), and Meteor Nexus (Lhasa). We specified two goals for this assessment: to understand whether *in silico* tools could predict unexpected, problematic metabolites prior to *in vivo* studies, and whether *in silico* tools could assist in the design of new drug structures with improved metabolic stability. We compared the *in silico* predictions with reported *in vivo* human data for 8 different compounds with published ADME experiments that include quantitative radiolabeled metabolism data.

In order to assess the utility of a platform's *in silico* prediction of metabolic structures, both the accuracy and precision were measured. The accuracy was defined as the number of experimental metabolites successfully predicted by the software divided by the total number of experimental metabolites observed. The precision, defined as the total number of experimental metabolites observed divided by the total # of metabolites predicted by the software, was a measurement of how judicious a software's predictions were. Our evaluation found that StarDrop was slightly superior to MetaSite and Meteor Nexus in accuracy but at the cost of lower precision. This is in part due to StarDrop's recent added phase II metabolism support and ability to run 2 generations of metabolism, which may give them significant improvement over previous experiments. All three software platforms were insufficiently powerful to inform ongoing drug development projects.

Site-of-metabolism is a more permissive assessment of the software's capabilities; a successful prediction does not require an accurate metabolite structure, just an accurate assignment of metabolic hotspots irrespective of biotransformation pathway. This can provide useful information for medicinal chemists who are looking to modify a drug's structure to improve its metabolic stability. Our evaluation found that this approach was viable for all three software platforms, offering potential for integration into ongoing drug development workflows.

**31. Predicting Human Intestinal Metabolism and its Significance to Oral Drug Absorption at Early Stage in Drug Discovery**

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Accurate predictions of human bioavailability (*F*) at early stage in drug discovery is essential for the development of orally administered drugs. *F* can be mechanistically defined as the product of *fa* (fraction absorbed through intestinal epithelia), *Fg* (fraction bypassing intestinal first-pass metabolism) and *Fh* (fraction bypassing hepatic first-pass metabolism). In a previous study, a good correlation was established between rat and human *fa*. Recently however, it has been recognized that there is often a species difference observed from in vivo *fa*Fg*, which can be ascribed to a species difference in the intestinal metabolism. The aim of the present study is to understand the role of intestinal metabolism and its impact on oral absorption by comparing various prediction methods for *Fg* in rats and humans. Here, we propose a novel linear regression model to predict *Fg* using in vitro liver microsomal clearance. This new method predicted human *Fg* within 3-fold of observed values for 93% of compounds (n=30), in contrast to the conventional $Q_{gut}$ model, which achieved only 70% with notable outliers. By applying the same method to predict rat Fg and deconvoluting fa from in vivo *fa*Fg* values obtained from oral PK studies, an improvement was observed in the in vitro to in vivo correlation (IVIVC) of Caco-2 permeability to rat *fa*. Collectively, our research provides a deeper understanding of the intricate relationship between permeability and metabolic stability in drug absorption, offering a valuable tool and insights for early stage prediction of human oral absorption including the translation of in vitro permeability to *fa* and potential explanation of species differences.

**32. A Physiologically Relevant Intestinal Model for Mechanistic Assessment of Oral Bioavailability**

AGNES BADU-MENSAH, Scott Tallman, Christopher Moreland, Geri Sawada, Robert Van Horn, Michael Mohutsky

*Eli Lilly and Company*

In advancing promising oral drug candidates, early delineation of the contribution of intestinal drug metabolizing enzyme (DME) and efflux transporter to oral absorption is crucial. *In vitro* models that can support the mechanistic assessment of intestinal DME and efflux transporter interplay effect on permeability would be crucial to earlier prediction oral bioavailability and possible drug-drug interactions (DDIs) in the drug discovery.

In practice, intestinal fraction metabolized (Fg) is extrapolated from in vitro studies with liver microsomes, while fraction absorbed (Fa) is estimated from in vitro solubility and permeability measures from Caco-2 and MDCK-MDR1 monolayer cultures in transwell inserts. However, none of these models recapitulate *in vivo* intestinal cytoarchitecture, DME and transporter expression,

The aim of this study was to develop a physiologically relevant intestinal model, suitable for determining the effect of intestinal efflux transporter and DME interplay on Fg and Fa for oral drug bioavailability prediction.

Intestinal models were developed by incorporating biopsy-derived duodenal organoid fragments and intestinal microvascular endothelial cells into the two channel microphysiological system (MPS) from Emulate Inc. The human duodenal MPS model was equipped with unidirectional flow and intermittent vacuum suction to mimic *in vivo* tissue perfusion and peristaltic contraction. Co-cultures were monitored and confirmed for appropriate intestinal cell differentiation, maturation, and polarity via immunocytochemical staining and confocal microscopy. Membrane formation and integrity were monitored with paracellular probe substrate markers lucifer yellow and Dextran.

DME and efflux transporter activities were evaluated using P-glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4) using probe substrates, inhibitors, and inducers. Concomitant dosing of P-gp probe substrate and inhibitor resulted in an increase in the apparent permeability of the luminal to basolateral movement of probe substrates. Additionally, metabolite formation was observed with or without CYP induction, thus confirming the metabolic activity in duodenal MPS systems.

This study demonstrates the expression of functional DME (CYP3A4) and efflux transporter (P-gp) in this duodenal MPS model, making it a suitable platform for the assessment of Fa and Fg in bioavailability estimation.

**33. Benchmarking drug metabolism gene expression signatures from human primary jejunum-derived cells against Caco2 to establish new preclinical models of intestinal DMPK.**

Jarrett Bliton[1], Bailey Zwarcyz[1], Bryan McQueen[1], Benjamin Lai[2], Eugene Chen[2]

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Oral administration of therapeutics requires transport across the single-cell thick epithelium of the small intestine to enter systemic circulation. In the small intestine, the jejunum is responsible for the majority of intestinal absorption and expresses drug transporters as well as phase I and II metabolism enzymes. Cancer-derived or immortalized cell lines are often used as a model of human small intestinal physiology to predict oral bioavailability and to establish safety and efficacy in humans. However, these models differ from healthy human physiology in many respects. Here we present gene expression data comparing a primary human jejunum-derived model and Caco2 cells with respect to drug metabolism and transport in the gut. The RepliGut® Planar-Jejunum Model was used as a source of primary stem cell-derived jejunum cells. Primary jejunum cultures were sampled at distinct time points during growth and maturation phases to generate RNASeq gene expression for comparison to publicly available Caco2 gene expression datasets. Following differential gene expression analysis, 4036 genes were significantly enriched in RepliGut® Planar-Jejunum cultures over Caco2, including genes that encode for major drug transport and metabolism proteins such as CYP3A4, CES2, P-gp, PEPT1, BCRP, and many others. Furthermore, primary jejunal epithelial cells were enriched for many gene sets associated with physiologically-relevant processes such as mucus production, nutrient absorption, and cell-cell junction assembly. 4189 genes were found to be upregulated in Caco2 cells with many enriched genes associated with processes for DNA replication, DNA repair, mitosis, and RNA processing. These data demonstrate the ability of the RepliGut® Planar to accurately represent the metabolic characteristics of the human intestine and highlight the impact of using primary intestinal cells in preclinical development of orally administered medications.

**34. Optimization of Bottom-Up PBPK Model Development Using Retrospective Analysis**

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Oral human fraction absorbed is often underpredicted in Simcyp, leading to underprediction of plasma concentrations and overprediction of efficacious dose to achieve a given target exposure. In addition, bottom-up prediction of human elimination profiles based on preclinical in vitro and in vivo data does not always match the observed clinical data. To interrogate possible causes of these disconnects and optimize bottom-up physiologically based pharmacokinetic (PBPK) model development, we evaluated the impact of three system parameters on PBPK model performances: GI physiology, P-gp Relative Expression Factor (REF), and recombinant CYP enzyme (rCYP) Intersystem Extrapolation Factor (ISEF).

Bottom-up PBPK models of AbbVie clinical assets (ABBV-X1-X8), with available first-in-human pharmacokinetic data, were built in Simcyp (V21) using preclinical data (*in vitro/vivo*) with the ADAM MechPeff model. Simulations were run matching the clinical trial design and the predicted exposures were compared to the observed values by calculating the AUC and $C_{max}$ ratio (observed/predicted). The impact of Original and New GI physiology, P-gp REF values (MDCK-MDR1 1.5 vs. 0.5) and rCYP ISEF values (BD Supersome Default vs. Adjusted (matching rCYP to hepatocyte $CL_{int,u}$)) on the AUC and $C_{max}$ prediction performance were tested independently and in combination.

New GI physiology, decreased P-gp REF (0.5), and default rCYP ISEF improved the AUC and $C_{max}$ predictions after oral administration in general but to varying degrees between compounds. For example, the predicted absorption of ABBV-X1 significantly increased when using New GI physiology, decreasing $C_{max}$ ratio from >10 to <3, while $C_{max}$ of ABBV-X2 was predicted within 2-fold regardless of the GI physiology parameters. Overall, the application of New GI physiology resulted in a considerable improvement in the prediction of oral absorption for most compounds compared to the Original GI physiology (76% vs. 43% within 3-fold). Decreasing P-gp REF yielded additional improvement in the predictions of $C_{max}$ for P-gp substrates (62% vs. 8% within 3-fold). The observed plasma profiles and AUC are better predicted using the default rCYP ISEF values instead of individually adjusted values (52% vs. 33% within 3-fold). The combination of optimized parameters (GI physiology, P-gp REF and rCYP ISEF) predicted the plasma exposures within 3-fold for 86% of the tested simulations while the original parameters only predicted 33% within 3-fold.

**35. Development And Validation of a Nifedipine Controlled Release Physiologically Based Pharmacokinetic Model in Pregnancy**

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Nifedipine is a first-line agent for the treatment of hypertension in pregnancy. However, there is little data to inform the dosing of controlled release (CR) nifedipine in pregnancy. We developed a physiologically based pharmacokinetic (PBPK) model to predict steady-state plasma concentration of nifedipine CR in pregnancy to compare the pharmacokinetics of 30mg CR twice daily (BID) and 60mg CR once daily (QD).

We constructed a PBPK model for the CR nifedipine using Simcyp V23. The default Sim-Nifedipine compound file was modified to employ the ADAM model drug dissolution and absorption and a full PBPK model for distribution. Model validation was performed by comparing simulated data with clinical studies in healthy volunteers, considering factors such as ethnicity, gender distribution, age, and dose regimen. Following successful validation in healthy populations, we utilized the model to simulate steady-state plasma concentrations in pregnant patients.

Predicted pharmacokinetic parameters for nifedipine CR were consistent with published data from clinical studies in healthy volunteers. The ratios of predicted versus area under the curve (AUC), maximum plasma concentration (Cmax), and time to reach maximum concentration (Tmax) of nifedipine, were from 0.96-1.56 of the observed values. Cmax, AUC, and concentration at steady-state of 30mg BID and 60mg QD CR nifedipine pregnant populations was ~50% lower than in nonpregnant, which is consistent with a 2-fold increase in CYP3A4 activity during pregnancy. Steady-state plasma concentrations in both dosing regimens are similar i.e., 13 ng/ml. However, the 60 mg QD peak-to-trough ratio is substantially larger.

This model holds promise for enhancing our understanding of nifedipine CR pharmacokinetics in pregnancy, ultimately contributing to more informed and effective management of hypertensive disorders during pregnancy. It is unclear whether the pronounced peak-tot-rough ratio with 60 mg QD will result in a subtherapeutic effect. Data from clinical studies is needed to verify the pregnancy predictions.

Key words. Nifedipine, Pregnancy PBPK model

**36. Reducing the Risk of Drug-Induced QT Interval Prolongation: Population Pharmacokinetic/ Pharmacodynamic Modeling**

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**Background:** Torsades de pointes (TdP) is a life-threatening polymorphic ventricular tachycardia associated with QT interval prolongation, which can be inherited or acquired (induced by over 150 commonly used drugs). Preclinical and clinical evidence indicates that progesterone has protective effects against drug-induced QT interval prolongation. The objective of this study is to assess the effect of oral progesterone on ibutilide-induced QT interval lengthening and identify sources of variability using population Pharmacokinetic/ Pharmacodynamic (PK/PD) modeling.

**Methods:** Data from three clinical trials with similar study designs in premenopausal women during menses phase (NCT01929083), premenopausal women during ovulation phase (NCT03834883) and postmenopausal women (NCT04675788), were pooled together for the analysis. These were randomized, double-blind, placebo-controlled, two-way crossover trials. Subjects were randomized to receive progesterone 400 mg or matching placebo daily for 7 days. On the 8th day of each phase, the QT-lengthening drug ibutilide (0.003 mg/kg) was administered via a 10-minute infusion. Time-matched serum ibutilide concentrations and heart-rate corrected QT intervals (QTc) were obtained serially at 13-14 time points over 8-12 hours. A population PK model of ibutilide and a PK/PD model of QTc interval were developed sequentially using NONMEM 7.5. Age, weight, race were tested as covariates on PK parameters. The PD model included three components, namely pre-ibutilide baseline QTc, circadian rhythm, and drug effect. The treatment group was tested as a categorical covariate on baseline QTc and ibutilide drug effect parameters.

**Results:** Preliminary data from 15 premenopausal women during menses, 11 premenopausal women during ovulation and 7 postmenopausal women were included in current analysis. Ibutilide PK was best described by a three-compartment model with first-order elimination from the central compartment. None of the covariates were selected for the PK model. The ibutilide effect on QTc intervals was best characterized by an Emax model with effect compartment. Treatment group was tested as an additive categorical covariate on baseline, Emax and EC50. Adding on baseline led to the best model fit. Progesterone group had 3.99 ms shorter pre-ibutilide baseline QTc than the placebo group.

**Conclusion:** Our model adequately characterized ibutilide PK and QTc interval change in premenopausal and postmenopausal women. The shorter pre-ibutilide QTc for the progesterone group indicated that progesterone showed protective effect against ibutilide-induced QTc interval lengthening. Enrollment is still ongoing, and sample size will be increased to improve model robustness and allow analysis of other potential covariates on PD parameters.

**37. Changes in QTc Interval across Different Trimesters of Pregnancy and Preeclampsia: A Systematic Review and Meta-analysis**

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**Background and Objectives**

- Pregnancy induces adaptations in the cardio-autonomic nervous system including increased heart rate, cardiac hypertrophy, and escalated sympathetic activity.[1]
- Preeclampsia causes additional cardiac stress due to increased cardiac workload and electrolyte imbalances that may affect cardiac repolarization.[2]
- The extent of the heart rate-corrected QT (QTc) interval changes, an important biomarker for drug safety, throughout pregnancy remains unclear.

This study aims to quantify the changes in QTc across pregnancy trimesters, with and without preeclampsia.

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**38. Structures of Cytochrome P450 Reductase FMN Domain Mutants That Allosterically Alter Cytochrome P450 Catalysis**

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Cytochrome P450 enzymes are a superfamily of heme-containing monooxygenases critical for human health. These enzymes use their heme prosthetic group to activate molecular oxygen for monooxygenation of drugs or endogenous homeostatic molecules such as hormones and vitamins. To accomplish this, the heme requires two single-electron reductions. For most P450 enzymes, electrons are delivered by the redox partner protein cytochrome P450 reductase, which is a highly dynamic protein composed of two flavin containing domains. The FMN-containing domain of reductase directly binds twice to the P450 to transfer single electrons to the heme each time.

Both clinical and experimental literature suggest that reductase affects P450 enzyme function differently depending on the P450 isoform. Indeed, different mutations in the reductase can result in P450-dependent increases or decreases in catalytic activity (1), resulting in severe disease states such as Antley-Bixler syndrome (2). This implies that the reductase FMN domain has different interactions with individual P450 enzymes. Most recently it was reported that some FMN domain mutations differentially alter the regiospecificity of caffeine metabolism by CYP1A2 (3). Therefore, current studies aim to determine X-ray crystal structures of reductase FMN domain of five such mutants. The goal of this work is to determine what effect these mutations might be having on the structure of the FMN domain and the resulting implication of those structural changes on P450 structure and activity. Highly purified FMN domain mutant proteins were generated and crystallized in conditions similar to that of the wild-type reductase FMN domain (4). Mutants diffracted to 1.1-1.5 Å resolution. These structures are aiding in a better understanding of the differential functional effects that have been widely documented in experimental and clinical literature.

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**39. Optimization of inhibitor concentration to better understand fm3A4 and fm2D6 Using HUREL® Human Pool™ Liver Hepatocyte Model**

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During drug discovery and development, it is often desirable to advance compounds that are slowly metabolized, but this can present some unique challenges in determining other properties downstream. Lack of understanding of clearance can often lead to overestimation of dose for compounds going to first in human studies and misunderstanding fraction metabolized (fm) by enzymes to quantify drug-drug interaction (DDI) risk can lead to costly clinical DDI studies, resulting in a delayed understanding of true clearance and DDI risk. Heretofore, conventional test systems commonly employed to assess metabolic depletion (human liver microsomes, human hepatocyte suspensions, etc) have been unable to reliably answer these questions. Over the past few years, various methods have been evaluated to measure intrinsic clearance ($CL_{int}$) of low clearance compounds, including the hepatocyte relay method, spheroids, micropatterned co-culture models (e.g., HepatoPac®) and random co-culture models (e.g., HμREL®). These systems allow drug candidate incubations to be sampled over several days enabled by sustained metabolic competency over that time period. Many of these systems have also been investigated for use in reaction phenotyping – which is challenging since the choice of inhibitor must be enzyme-selective, not exhibit excessive metabolic depletion or cause CYP induction. The HμREL® system has remained understudied for reaction phenotyping. Therefore, our aim in this study was to use HUREL® Human Pool™ liver hepatocyte model to optimize inhibitor concentrations to better understand fm3A4 and fm2D6, using model compounds alprazolam and dextromethorphan, respectively. The inhibitor concentrations selected in our initial studies demonstrated a reduction in metabolic depletion from 72-95% that was maintained over the 7-day course of the incubation. These findings demonstrate the possible utility of this approach for assessing fraction metabolism in this long-term co-culture system.

**40. Physiologically-Based Pharmacokinetic Modeling in Patients with Fontan-Associated Liver Disease**

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Fontan procedure is the final surgery that children with single ventricle congenital heart disease (SVCHD) often undergo. The procedure helps reduce cardiac workload and volume overload. This procedure significantly increases the survival rate and prolongs the lives of patients with SVCHD. However, it also creates a passive circulatory system that causes elevated central venous pressure and reduced cardiac output, leading to end-organ damage, specifically liver congestion and fibrosis referred to as Fontan-Associated Liver Disease (FALD). Adults with nonalcoholic fatty liver disease are known to have decreased expression of drug-metabolizing enzymes and transporters, but it is unknown whether the expression and activity of these enzymes change in patients with Fontan physiology, and if so, to what extent these changes occur in patients with different FALD severities. We expect that demographic parameters, such as age, weight, height, ethnicity, and sex distribution in patients with Fontan circulation will also be different from the general population. These changes could have an impact on drug pharmacokinetics (PK). The uncertainties in PK in this population render selection of safe and effective drug doses difficult.

**41. Multi-Omic Drivers of Hepatic Drug Metabolism in African Americans**

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**Background:** Variation in drug metabolizing enzymes (DMEs), their regulatory regions, or within regulatory proteins can contribute to interindividual variability of drug response and adverse drug reactions (ADRs). Multi-omic data can complement genetic data, giving regulatory context to genomic regions of otherwise unknown function. Current multi-omic studies underrepresent minority populations, making results both difficult to interpret and limiting their predictive value in clinic. This study aims to identify genetic variation and epigenetic changes associated to Pgx genes using primary human hepatocytes in an African American (AA) cohort.

**Method:** Hepatocytes were extracted from 75 AA cadaveric livers, followed by genome-wide genotyping, mRNA sequencing, and DNA methylation profiling. Quantitative trait mapping (eQTL, eQTM, mQTL) was conducted with age, sex, and genomic principal components 1-2 as covariates. In addition to these covariates, probabilistic estimation of expression residuals (PEER) factors were calculated from the gene expression data to account for unmeasured confounding variables in transcriptome and methylation data. Significant results were prioritized based off overlap between quantitative trait mapping results as well as PharmGKB annotation.

**Results:** eQTLs, mQTLs, and eQTMs were identified, some with previous links to drug metabolism and differential allele frequency between AA populations and European. One such variant, rs1332018, is associated with increased *GSTM3* expression (eQTL), decreased methylation at 5 different methylation sites (mQTL), and all five of those CpG sites are associated with *GSTM3* expression (eQTM). Rs1332018 is a 5'UTR variant with a MAF of 0.81 in ASW and 0.55 in EUR.

**Conclusion:** A sizable portion of genetic variants that associate to gene expression (eQTL) also associate to DNA methylation proportion (mQTL). CpG sites under genetic regulation (mQTL) largely do not overlap with CpG sites which regulate gene expression (eQTM). Rs1332018, which overlaps eQTL, mQTL, and eQTM results, is associated with the known phase II DME GSTM3. These results will be integrated within statistical finemapping methods to help prioritize results from a drug metabolism GWAS within the same cohort. This will result in a comprehensive surveillance of genomic, transcriptomic and DNA methylation landscapes for association to variation in drug metabolism, a first-of-its-kind study.

**42. Strain- and Route-Dependent Plasma Pharmacokinetics and DRG Accumulation of Paclitaxel**

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**Background:** Paclitaxel is an antineoplastic agent that is highly effective in treating a variety of cancers, but its use is limited by the development of adverse events, notably paclitaxel-induced peripheral neuropathy (PIPN). Preclinical models have been used to characterize the mechanisms underlying paclitaxel-induced toxicities. However, the potential impact of route- and strain-dependent pharmacokinetics (PK) and organ accumulation has largely been ignored, despite the importance of paclitaxel accumulation in the dorsal root ganglion (DRG) to development of PIPN. Based on dose-dependence of paclitaxel toxicities and observed differences in susceptibility to PIPN between strains, we hypothesized that paclitaxel PK and organ accumulation is strain- and route-dependent and that this results in variable toxicity phenotypes.

**Methods:** We performed PK studies to evaluate paclitaxel plasma PK and organ accumulation. We administered 10 mg/kg paclitaxel to seven strains of mice (C57BL/6NTac, 129S6/SvEvTac, FVB, NSG, BALBc, CD2F1, and DBA) either IP or IV. Strains were selected based on their use in preclinical models, existing bioavailability data, or both. Concentrations of paclitaxel in plasma, DRG, and brain were determined by a validated LC-MS/MS method and analyzed using Phoenix WinNonlin. Assessment of transcriptional levels of transporters and enzymes relevant to the distribution and metabolism of paclitaxel in tissues of interest is ongoing. *In vitro* and *ex vivo* methods were used to determine whether paclitaxel is directly toxic to neurons.

**Results:** Paclitaxel PK parameters differed significantly between strain and route of administration. AUC variation between strains was 1.7-fold for IV dosing and 2.0-fold for IP. Both brain and DRG levels showed significant strain-dependent differences after administration, with 3.4-fold variation observed in brain and 2.0-fold variation observed in DRG. *Ex vivo* experiments demonstrated a concentration-dependent decrease in neuronal viability with paclitaxel treatment.

**Discussion:** We show that paclitaxel plasma PK, DRG, and brain accumulation are route- and strain-dependent. We also show that paclitaxel exhibits concentration-dependent cytotoxicity to primary neurons at clinically achievable concentrations. These observations provide rationale to further investigate (i) if strain- and route-dependent paclitaxel PK variation are related to variation in toxicity phenotypes, (ii) if paclitaxel toxicities are a direct effect of cellular accumulation, and (iii) if paclitaxel toxicities are dependent on accumulation in specific cell types.

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

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The accumulation of misfolded proteins can result in various diseases, including several neurodegenerative disorders. Critical proteins that are associated with the onset of some of these disorders are client proteins of the heat shock protein (Hsp) 90 and Hsp70 chaperone system that selectively regulates their stability and degradation. Previously, we've determined the opposing roles Hsp90 and Hsp70 have on client protein stability using neuronal nitric oxide synthase (nNOS), a well-established client protein. Hsp90 stabilizes misfolded client proteins and prevents their ubiquitination, whereas Hsp70 enhances their ubiquitination and degradation. Thus, the Hsp90/70 chaperone system is 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 may target functional client proteins in near native states, we hypothesize that targeting Hsp70 may be more selective for misfolded proteins. 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 the known Hsp70 modulator, YM-01, as well as a novel agent discovered through screening efforts at the Center for Chemical Genomics. 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. The effect of these Hsp70 modulators on C331A nNOS was prevented in cells co-treated with the proteasome inhibitor, lactacystin. The Hsp90 inhibitor, radicicol, was not as selective as loss of both C331A nNOS and wild type nNOS were observed. These results suggest that Hsp70 modulation selectively removes the misfolded client protein while sparing the natively folded or near native nNOS proteins. 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.

**44. Utilizing RNA-seq to Guide Cell Painting Drug Repurposing Assay to Overcome Palbociclib Resistance in Invasive Lobular Carcinoma Breast Cancer Cell Line Model**

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**Background:** We have produced two cell lines of MDA-MB-134 cells that either acquired resistance (AR) or were intrinsically resistant (IR) to palbociclib. Transcriptional profiling was performed for these and the non-resistant cells. The RNA-seq data informed the selection of biomarkers to enable high-content screening and the selection of candidates for apathway-focused drug repurposing screen.

**Methods:** AR and IR cells express phospholipase-A1 (PLA1) at a rate 10-fold lower than non-resistant cell lines. PLA1 affects lipid accumulation in the cell by producing free fatty acids and lysophospholipids; we probed for this difference and visualized it using a lipid stain, HCS LipidTox Red. We also visualized differences in downstream actin reorganization in the cytoskeleton using phalloidin to label F-actin. We also wanted to observe cell and nuclear morphology changes between lines; Hoechst 3342 was used to visualize the nuclei, and HCS Cell Mask Orange was used to visualize the whole cell. Cells were plated in 384-well plates at 10,000 cells per well and then imaged on a Yokogawa CV8000 at 20x. Images were analyzed using Cellpose and Cellprofiler, and XGboost machine learning regression models were trained and validated against controls.

**Results:** Significant phenotypic differences, including lipid content and actin organization, were observed between AR/IR and parental cells. AR cells also have a decreased cell size and are morphologically distinct from the parental line. The IR line resembles the parental line morphology more than the AR. Still, there are subtle differences that a machine learning model can distinguish to differentiate between them reliably. AR/IR cells have significantly increased androgen receptor expression. We performed a pathway-focused drug repurposing screen targeting androgen modulators, lipid modulators, and others.

**Conclusions:** In the initial high-throughput screen, we identified that androgen-targeted drugs and androgen-degrading PROTACs show the best efficacy. Comparative potency data for AR/IR vs. parental cells will be shown. We can also conclude that the resistant cell lines have a distinct morphology from that of the parental cell line, with lipid accumulation being the largest difference.

## 45. Androgen Receptor is Upregulated in Invasive Lobular Breast Cancer Cells that have become Resistant to CDK4/6 Inhibitors

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**Background:** Approximately 10% of breast cancer types are classified as invasive lobular carcinoma (ILC) and are estrogen receptor (ER) – positive (+) with a loss of E-cadherin. Cyclin-dependent kinase 4/6 (CDK4/6) inhibitors have become standard of care for the treatment of patients with metastatic, ER+ breast cancer, including patients with ER+ ILC. The MDA-MB-134 breast cancer cell line is one of the most used immortalized cell line models for studying ILC. Long-term treatment of these cells with CDK4/6 inhibitors and selection in estrogen-free growth media identified Androgen Receptor (AR) as one of the most significantly upregulated genes. We hypothesize that AR confers resistance to CDK4/6 inhibitors and can be targeted with anti-androgens.

**Methods**: We generated MDA-MB-134 cells resistant to CDK4/6 inhibitors and endocrine therapy conditions by culturing under different treatments for a period of greater than 6-months. Cellular growth assays were used to determine CDK4/6 inhibitor resistance and estrogen-independent growth. RNA-Seq was performed using NovaSeq-6000 platform (Illumina) to identify pathways associated with resistance. AR upregulation was identified by RNA-Seq and confirmed at the protein level by western blot. AR inhibitors and degraders were tested for growth changes.

**Results**: The ILC cell line, MDA-MB-134, treated with either palbociclib or endocrine therapy conditions resulted in an upregulation of androgen receptor (AR) over parental cells (Mean $Log_2FC$ 7.94, SD 0.17, p=1.00E-06). Drug treatment with proteolysis targeting chimera (PROTAC) AR degraders (ARD) over 24 hours resulted in dose dependent decreased AR protein expression (Vehicle vs. 10nM, 100nM, 1000nM). Dose response assays with ARD69 showed a greater growth inhibitory response in CDK4/6 resistant cells vs. Parental cells (Maximum Growth inhibition: Selected Cells Mean 46.1%, SEM 1.9 vs. Parental Mean 14.5%, SEM 1.4). Combination treatment PROTAC ARD and CDK4/6 inhibitors did not result in resensitization of the resistant cells.

**Conclusions**: Human ILC breast cancer cell line models resistant to CDK4/6 inhibitors or endocrine therapies were characterized using RNA-Seq and dose response assays. We discovered an upregulation of AR in our resistant cells, which is a novel finding in ILC cell lines treated with CDK4/6 inhibitors. Our results provide evidence that AR upregulation is a mechanism of resistance to CDK4/6 inhibition that can be targeted by anti-androgen therapies.

## 46. A Universal Method to Analyze Cellular Internalization of Receptor-Mediated Endocytosis of Large Molecules

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*AbbVie*

Endocytosis is a crucial cellular process which allows targeted macromolecules, that cannot directly penetrate the plasma membrane, to enter target cells. Thus, endocytosis cannot be overlooked when designing new and effective drug delivery targets, especially for biopharmaceuticals. While bound receptors sites or cellular binding partially explain targeted delivery, accurate cellular internalization methods must be established to fully describe and evaluate efficiency of drug delivery, and potential to inform TMDD or tumor penetration.

**Purpose:** Assess the internalization potential of ligand-target endocytic pathways and quantitatively measure the internalized signal and endocytic rate.

**Method:** Antibodies of interest were labeled with Alexa Fluor 488 tetrafluorophenyl ester (Invitrogen). aiming for approximately 1-2 fluorophores per antibody. The labeled antibody was purified to remove any unincorporated dye using size exclusion purification columns. Degree of labeling were measured by a Nanodrop 2000 (Thermo Scientific).

Single cell suspension was incubated with AF488-labeled antibody to saturate all the cell surface receptors. Incubation at 37C was either performed in a continuous or in a pulse-chase method. Cells were sampled at select timepoints (ranging 0-24hrs), rapidly chilled on ice, washed with cold PBS to remove excess unbound antibody, and divided into quench/non-quench samples. Quench cell samples were incubated on ice with 100nM anti-AF488 quenching antibody (Thermo A11094) which quenched surface bound signal while preserving intracellular signal. DAPI (2uM) was added to distinguish live population before analyzing on imaging flow cytometer (ImageStream MKII, CYTEK). Images and fluorescence emission were collected (40X: AF488:488nm; DAPI:405nm).

**IDEAS Data analysis software:** Raw median fluorescence intensity of AF488 in live cell population was corrected for background signal (unstained control), quenching efficiency (non-quench controls) and normalized to degree of labeling (DOL). Surface and Internalized signal were calculated, and rate of endocytosis was obtained by plotting fraction internalized antibody over time.

**Results:** Cellular internalization of known biologics in different formats (HER2, CD71) was studied to validate this method above.

1. Internalization profiles of monospecific antibodies against two separate antigens were compared to that of a dual variable domain-antibody (DVD-IgG) targeting both antigens in the same molecule. Each monospecific antibodies had a unique internalization profile (one showed slower rate and lower percentage of internalization and other showed faster rate and higher percentage of internalization) whereas DVD-IgG showed combined properties of the two monospecific antibodies.

2. The net internalization profiles for the monoclonal antibody and the monoclonal antibody conjugated to a drug-linker were similar.

3. A quantitative method to evaluate ligand-receptor processing was established.

**47. Monitoring Drug Metabolic Pathways through Extracellular Vesicles in Mouse Plasma**

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The ability to monitor the response of metabolic enzymes to drug exposure in individuals is highly appealing and critical to personalized medicine. Although pharmacogenomics assesses genotypic differences, it does not report changes in metabolic enzyme activities due to environmental factors such as drug interactions. Here, we report a quantitative proteomics strategy to monitor drug metabolic pathways by profiling metabolic enzymes in circulating extracellular vesicles (EVs) upon drug exposure. Mass spectrometry (MS)-based measurement revealed that changes in metabolic enzyme abundance in EVs paralleled those in hepatic cells isolated from liver tissue. Coupling with multiplexed isotopic labeling, we temporally quantified 34 proteins involved in drug absorption, distribution, metabolism, and excretion (ADME) pathways. Out of 44 known ADME proteins in plasma EVs, previously annotated mouse cytochrome P450 3A11 (Cyp3a11), homolog to human CYP3A4, and uridine 5'-diphospho (UDP) glucuronosyltransferase 2A3 (Ugt2a3), increased upon daily rifampicin dosage. Dasatinib, a tyrosine kinase inhibitor to treat leukemia, also elevated Cyp3a11 levels in plasma EVs, but to a lesser extent. Altogether, this study demonstrates that measuring drug enzymes in circulating EVs as an effective surrogate is highly feasible and may transform today's drug discovery and development for personalized medicine.

**48. An Improved Method to Express Heterodimeric nNOS for Biochemical and Structural Characterization**

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Nitric Oxide Synthase (NOS) is a self-sufficient heme-containing oxidoreductase enzyme that catalyzes the formation of the signaling molecule, NO, using L-arginine and molecular oxygen as its substrates. All three NOS isoforms require cofactors and homodimerization to be catalytically active because the electrons flow from one monomer into the opposite in a “trans” fashion. Crystal structures of the individual domains have been reported, but the entire NOS enzyme structure has not been solved, in part due to the highly dynamic nature of the reductase domain. We hypothesized that removing the reductase domain of one monomer would stabilize NOS and make it amenable to structural elucidation. To test this, we coexpressed a catalytically inactive FLAG-tagged monomer ($nNOS_{E592A}$-FLAG) and a His-tagged oxygenase domain ($nNOS_{1-720}$-His) of neuronal NOS (nNOS) such that upon assembly in insect cells, only the heterodimer, but not homodimers, would have activity. This new process yielded approximately 1/3 of heterodimers out of total dimers, assessed by catalytic activity. This was followed by purification and subsequent stabilization of the heterodimer using a sucrose-glutaraldehyde gradient fixation (Grafix) method. The quality of the preparation was assessed by single-particle negative stain transmission electron microscopy (TEM) where there was good particle distribution and adequate particle size. Implementing the efficient heterodimer expression system and the Grafix method allows us to obtain low-resolution structures of heterodimeric nNOS that are amenable for further structural characterization such as cryogenic electron microscopy (CryoEM). This approach could potentially aid in understanding inter-domain electron shuttling from the reductase domain to the oxygenase domain which has remained elusive.

**49. Beyond Rule of Five and PROTACs in Modern Drug Discovery: Polarity Reducers, Chameleonicity, and the Evolving Physicochemical Landscape**

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Developing orally bioavailable drugs demands an understanding of absorption in early drug development. Traditional methods and physicochemical properties optimize absorption for rule of five (Ro5) compounds; beyond rule of five (bRo5) drugs necessitate advanced tools like the experimental measure of exposed polarity (EPSA) and the AbbVie multiparametric score (AB-MPS). Analyzing AB-MPS and EPSA against ∼1000 compounds with human absorption data and ∼10,000 AbbVie tool compounds (∼1000 proteolysis targeting chimeras or PROTACs, ∼7000 Ro5s, and ∼2000 bRo5s) revealed new patterns of physicochemical trends. We introduced a high-throughput "polarity reduction" descriptor: ETR, the EPSA-to-topological polar surface area (TPSA) ratio, highlights unique bRo5 and PROTAC subsets for specialized drug design strategies for effective absorption. Our methods and guidelines refine drug design by providing innovative in vitro approaches, enhancing physicochemical property optimization, and enabling accurate predictions of intestinal absorption in the complex bRo5 domain.

**50. Diphyllin Metabolism Elucidation and Optimization**

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Diphyllin is a naturally occurring compound with a variety of biological activities, including antiviral activity. The core of its antiviral activity comes from its function as a vacuolar-ATPase inhibitor, a host protein that helps facilitate viral entry. To maximize diphyllin's utility as a viral entry inhibitor, work has been done to increase potency through derivatization. Although derivatization has significantly improved potency, most diphyllin derivatives undergo rapid metabolism. To maximize diphyllin derivatives' effect as antivirals, their metabolic stability must be improved to ensure there is a high amount of drug available for as long as possible. Metabolically labile sites on the diphyllin scaffold were predicted computationally to provide a starting point for optimization. Deuterium and fluorine substitutions were employed to rule out stable sites and identify which parts of diphyllin contribute most to metabolism. Deuterium substitution ruled out four hypothesized sites of metabolism. Fluorine substitution on the scaffold successfully stabilizes the diphyllin. This fluorinated diphyllin was further derivatized, resulting in potent compounds with improved metabolic stability. To further investigate the major site of metabolism, we have begun to synthesize the hypothesized major metabolite. After validation, we will have a clearer target to further optimize our compounds.

## 51. OATP2B1 Deficiency Ameliorates Irinotecan-induced Gastrointestinal Toxicity

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**Background**
The clinical use of prodrug irinotecan (CPT-11) in the treatment of various solid tumors, including colorectal cancer, is associated with severe side effects including diarrhea observed in up to 82% of patients. It has been suggested that high local exposure to its active metabolite SN-38 within the intestinal lumen, following biliary excretion of SN-38 and microbial deconjugation of SN-38G, is causally linked with the incidence of diarrhea. However, the mechanism by which SN-38 is taken up into intestinal enterocytes remains unknown. Here, we hypothesized that intestinal transport mechanism of SN-38 is dependent on the organic anion transporting polypeptide OATP2B1, and we explored this thesis in a novel, clinically relevant mouse model.

**Methods**
Age- and sex-matched wild-type (WT) mice and OATP2B1-deficient [OATP2B1(-/-)] mice receiving a purified diet (AIN-93G) to increase susceptibility to treatment-related diarrhea, were treated with CPT-11 (90-mg/kg, i.p.) for 5 consecutive days. Diarrhea and body weight were scored daily for 7 days. At the end of the experiment mice were sacrificed and their intestine length was measured. To evaluate pharmacokinetic parameters, serial plasma samples were collected after CPT-11 administration and analyzed for the presence of CPT-11 and its metabolites by LC-MS/MS. Pharmacokinetic parameters were derived from non-compartmental analysis using Phoenix WinNonlin.

**Results**
Although treatment with CPT-11 was not associated with reduced body weight loss in OATP2B1(-/-) mice than WT mice in comparison to pretreatment body weight, OATP2B1 deficiency resulted in significantly lower overall diarrhea scores and longer total intestine length in OATP2B1(-/-) mice ($p<0.05$). These observations were further confirmed by histological examination indicating more severe damage to enterocytes in WT mice. These phenotypic alterations in OATP2B1(-/-) mice occurred without substantial changes in the systemic exposure to CPT-11 or its main metabolites in OATP2B1 (-/-) vs WT mice respectively.

**Conclusion**
Here, we demonstrated that OATP2B1 deficiency provides partial protection against CPT-11 mediated diarrhea, a potentially lethal dose-limiting toxicity, based on the observed changes in body weight, diarrhea scores, and histological analysis. The notion that the plasma pharmacokinetic parameters of CPT-11 and its metabolites were not influenced by OATP2B1 deficiency supports the thesis that treatment-related gastrointestinal toxicity is at least in part dependent on OATP2B1-mediated uptake of SN-38 in intestinal enterocytes. Collectively, our study indicates that plasma concentrations of SN-38 are a poorly predictive biomarker of diarrhea and provides an incentive for the future development of interventional strategies aimed at increasing the tolerance with the use of OATP2B1 inhibitors.

**52. Phenylmethylsulfonyl Fluoride (PMSF) Proves Effective in Stabilizing Cyanamides for Bioanalysis**

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Cyanamides are essential in drug discovery and development both as versatile building blocks in chemical synthesis and as pharmacological tools. Cyanamides electrophilic nature makes them highly reactive to nucleophiles including biological ones, such as the amino acids cysteine and lysine. As a result, certain cyanamides lack stability in biological matrices, posing a challenge for their bioanalysis. Trichlorfon, an acetylcholinesterase inhibitor, has been tentatively utilized to stabilize cyanamides during sample preparation for pharmacokinetic studies. However, trichlorfon being an insecticide is highly toxic. To find a substitute stabilizer that is less detrimental to the environment and operators, we investigated the stability of three cyanamide analogs in blood and plasma from different species. We also studied the effect of two commonly used stabilizers, phenylmethylsulfonyl fluoride (PMSF) and phosphoric acid, in comparison to trichlorfon. A benchtop stability assay revealed that the reactivity of cyanamides is compound and matrix dependent. PMSF emerged as the most effective among the three stabilizers evaluated, efficiently stabilizing tested cyanamides in both whole blood and plasma at room temperature for up to two hours. In conclusion, PMSF can serve as a safe alternative stabilizer for cyanamides bioanalysis.

**53. Development of Alkaline Phosphatase Prodrug Bioconversion Assays to Support Drug Discovery**

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Phosphate prodrug strategies can be used to overcome liabilities such as poor solubility or crystallinity, and excess aggregation of antibody drug conjugate payloads. Phosphate prodrugs require enzymatic conversion to active molecules. Therefore, assessing the likelihood of bioconversion in vivo by in vitro experiments should help prioritize candidate synthesis as well as pharmacokinetic studies. Ultimately, these in vitro assays should enable in vitro in vivo extrapolation and the data generation needed to build models predictive of bioconversion. Using human as well as preclinical species, subcellular fractions, serum and plasma, we have developed in vitro bioconversion assays measuring substrate (pro-drug) depletion corroborated by monitoring or quantifying active drug formation. Assay conditions were optimized towards achieving maximum catalytic activity. For example, EDTA, a common anti-coagulant was found to be inhibitory to plasma phosphatases and subcellular fraction incubations were carried out in Tris-Cl buffer rather than phosphate buffer which may inhibit bioconversion. Bioanalytical conditions were also optimized to enable analysis of prodrug and active drug (with wide difference in polarity) in the same run. Sodium orthovanadate was confirmed as an effective inhibitor of phosphatases upon coincubation and we have used this agent to elucidate the role of phosphatase mediated bioconversion in various biomatrices ($K_i$ value, 9.8 µM; competitive inhibition model). Test compounds as substrates for phosphatase included model prodrugs, fosamprenavir, fosphenytoin and prednisolone phosphate. In general, intestinal S9 exhibited conversion rates higher than liver S9 or serum, and significant catalytic activity was observed only with some model compounds in serum from rodents, but not other species. We anticipate these assays to find utility in candidate nomination and selection by confirming bioconversion and/or prediction extent of conversion in vivo.

**54. Evaluation of TL-895 Pharmacokinetic Properties and Drug-Drug Interaction Potential**

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TL-895 (M7583) is an investigational, highly selective, orally active, and potent inhibitor of bone marrow tyrosine kinase on chromosome X (BMX) and bruton's tyrosine kinase (BTK) for the treatment of acute myeloid leukemia (AML) and myelofibrosis (MF). To support the preclinical evaluation of TL-895, we sought to (i) characterize the *in vitro* kinase selectivity of TL-895, (ii) determine substrate and inhibitory properties toward OATP1B-type transporters and CYP3A4, common determinants of elimination pathways of kinase inhibitors, (iii) define the pharmacokinetic properties of TL-895 in mice with a newly developed and validated analytical method, and (iv) functionally investigate the drug-drug interaction (DDI) potential of TL-895 for combination studies.

Utilizing kinase assays, we found that TL-895 is highly selective for BMX and BTK with an EC50 of 0.30 and 2.1 ng/mL for BMX and BTK, respectively, and a S(35)-score of 0.015. *In vitro* cellular accumulation assays indicated that TL-895 modestly inhibits the function of OATP1B1, OATP1B3, and the murine orthologue OATP1B2, suggesting limited potential for DDIs with OATP1B substrates. Utilizing competitive-counterflow (CCF) assays with tritiated estradiol-glucuronide (EbG) in HEK293 cells overexpressing OATP1B1 or OATP1B3, TL-895 was identified as a putative OATP1B1 substrate. Studies with CYP3A4 microsomes confirmed that TL-895 is a CYP3A4 substrate, a finding that was verified *in vivo* in CYP3A-deficient mice. Ensuing pharmacokinetic experiments in male and female mice on C57BL/6, NSG, or FVB background strains identified doses of TL-895 that provide measures of systemic exposure that are comparable to those observed in humans receiving the recommended Phase II dose (300 mg). Consistent with the lack of OATP1B inhibitory properties, murine studies with the OATP1B1 substrate gilteritinib and the endogenous biomarker CDCA-24G revealed that TL-895 is unlikely to cause clinically relevant DDIs.

Our study indicates that TL-895 is a highly selective inhibitor of the BMX and BTK kinases. Since TL-895 demonstrates a modest propensity to inhibit the hepatic uptake transporters OATP1B1, OATP1B3, and OATP1B2, the predicted DDI potential is low, and this is the focus of further ongoing validation studies. Collectively, our *in vivo* pharmacokinetic studies suggest that mice are a translationally predictive model organism to evaluate the absorption, disposition, and DDI liabilities of TL-895 and provide a foundation for subsequent investigation aimed at refining the dose and schedule of this promising BMX/BTK inhibitor to be used in future combinatorial regimens.

## 55. Association of Germline rs17130142 Genotype on Paclitaxel Pharmacokinetics

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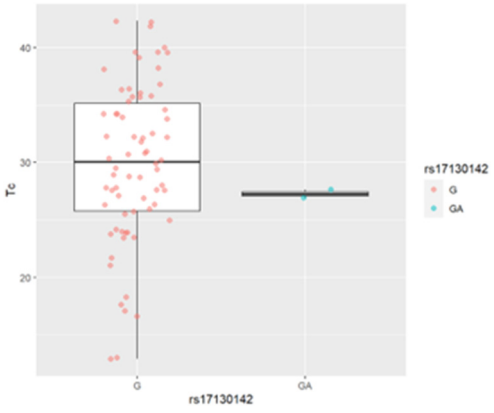
**Objective:** Over 60% of patients who receive paclitaxel treatment are affected by paclitaxel-induced peripheral neuropathy (PIPN) that negatively affects functional ability and quality of life. Paclitaxel exposure, estimated by the amount of time the systemic concentration remains above the threshold of 0.05 µM ($T_{c>0.05}$), is predictive of PIPN and the CEPAC-TDM trial demonstrated that dose reduction in patients with supratherapeutic $T_{c>0.05}$ reduces PIPN (M Joerger. Ann Oncol. 2016). A previous Genome-Wide Association Study (GWAS) reported higher $T_{c>0.05}$ in patients carrying the germline rs17130142 genetic variant (B Gao. Sci Rep. 2018). The objective of this analysis was to validate this association in an independent patient cohort to determine its potential use to personalize paclitaxel dosing.

**Method:** CEPAC-TDM enrolled 365 patients with non-small cell lung receiving paclitaxel with carboplatin or cisplatin and randomized them 1:1 to paclitaxel therapeutic drug monitoring (TDM) or standard dosing. A blood sample was collected 18-30 hours after infusion from patients on the TDM arm. Paclitaxel plasma concentration was determined using LC-UV and to $T_{c>0.05}$ was estimated using NONMEM. Germline DNA was genotyped for rs17130142 using a TaqMan allelic discrimination assay. The association between rs17130142 genotype and $T_{c>0.05}$ was tested by two-sample t-test and Wilcoxon test.

**Results:** 72 participants on the TDM arm were genotyped for rs17130142 and assayed for paclitaxel $T_{c>0.05}$. The rs17130142 heterozygous allele carriers (n=2) had lower $T_{c>0.05}$ compared to wild-type participants (n=70) (27.25h vs. 29.93h, p=0.005, Figure). This association was not significant when analyzed via Wilcoxon test (p=0.42).

**Conclusion:** In our analysis, patients carrying a rs17130142 allele had shorter paclitaxel $T_{c>0.05}$, which is in the opposite direction of the previous GWAS study. The association in the opposite direction strongly suggests that there is no robust association between this variant and paclitaxel exposure, and this variant should not be used to inform paclitaxel dosing.



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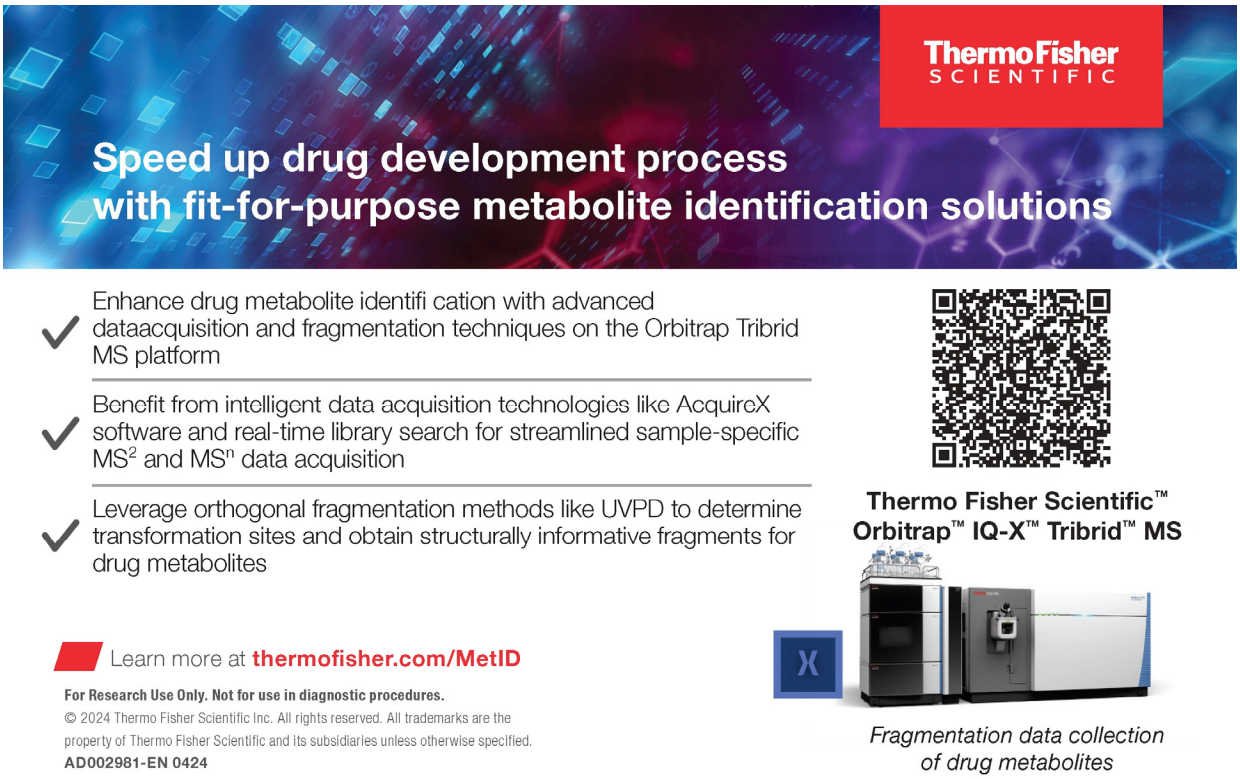
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**Cyclic Ion Mobility - High Resolution Mass Spectrometry for the Rapid Profiling of Drug Metabolites in Biofluids,** Iggy Kass, Ph.D., HRMS Business Development Executive, Waters Corporation


**Plateable Hepatocytes in Non-Small Molecule Drug Development**, Christopher Bohl, Ph.D., Senior Manager Technical Support, BioIVT