14th Annual Meeting

May 9-10, 2019

University of Michigan North Campus Research Complex
Ann Arbor, Michigan
Maps

NCRC, 2800 Plymouth Road

- **[A1]** Plymouth and Nixon entrance
- **[A2]** Huron Parkway entrance
- Parking at Structure NC 100
- Building 18 entrance to go to Dining Hall

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GLMDMG Meeting (Dining Hall)
- Students/Postdocs Networking Lunch (South Atrium)
- GLMDMG Business Meeting (Room G064)
- Thursday Dinner (offsite at Holiday Inn)
Shuttle Bus (Golden Limousine, Inc.)

May 9
Pick up at the Holiday Inn and drop off at NCRC Building 18.
Continuous service from 9:00 AM until 11:00 AM.

May 10
Pick up at the Holiday Inn and drop off at NCRC Building 18.
Continuous service from 6:30 AM until 9:30 AM.

U-M Charter Bus Schedule
May 9

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<th>Departure Time</th>
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# Schedule

## Thursday, May 9, 2019

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<th>Time</th>
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<tr>
<td>9:00 AM</td>
<td>Registration</td>
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<td>Continental Breakfast</td>
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<td>Poster Setup &amp; Sponsor Booth Setup</td>
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<tr>
<td>11:00</td>
<td><strong>Welcome</strong>, Paul F. Hollenberg, Ph.D., Department of Pharmacology, University of Michigan, Ann Arbor, MI</td>
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<td>11:10</td>
<td>Moderator:  <strong>Paul F. Hollenberg, Ph.D</strong></td>
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<td><strong>Quo Vadis, Cytochrome P450? Successes, Advances, and Future Research</strong>, Frederick P Guengerich, Ph.D., Tadashi Inagami Professor of Biochemistry, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN</td>
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<td>11:50</td>
<td><strong>Hepatic P450 Degradation: Physiological, Pathological and Therapeutic Relevance</strong>, Maria Almira Correia, Ph.D., Professor of Pharmacology, Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA</td>
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<tr>
<td>12:30 PM</td>
<td>Lunch</td>
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<td>Students/Postdocs Networking Lunch with Industry Scientists (South Atrium)</td>
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<td>Poster Viewing and Sponsor Booths</td>
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<td>2:00</td>
<td>Moderator:  <strong>Stephen Hall, Ph.D., Senior Research Fellow, Eli Lilly &amp; Company</strong></td>
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<td><strong>Application Of Intestinal Organoid Cell Culture in Drug Metabolism and Toxicity Studies</strong>, Shujuan Chen, Ph.D., DABT, Assistant Professor, University of California, San Diego, La Jolla, CA</td>
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<td>2:40</td>
<td><strong>Challenges and Recent Progress Toward In Vitro-to-In Vivo Extrapolation of Transporter-Mediated Hepatic Clearance</strong>, Bridget Morse, Pharm.D., Ph.D., Senior Research Scientist, Eli Lilly, Indianapolis, IN</td>
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<td>3:20</td>
<td>Break</td>
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<tr>
<td>3:40</td>
<td>Moderator:  <strong>Rich Voorman, Ph.D. RMLV Partners</strong></td>
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<td><strong>Transporter Endogenous Biomarkers for Prediction of Clinical Drug-Drug Interactions</strong>, Kenta Yoshida, Ph.D., Scientist, Clinical Pharmacology, Genentech Research and Early Development, South San Francisco, CA</td>
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<td>4:20</td>
<td><strong>IVIVE-PBPK Modeling of Heterogeneous Drug Penetration into Human Brain Tumors</strong>, Jing Li, Ph.D., Associate Professor, Department of Oncology, Wayne State University School of Medicine, Detroit, MI</td>
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<td>5:00</td>
<td><strong>Poster Session (Author Available), Sponsor Booths, &amp; Mixer</strong></td>
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<td>7:30</td>
<td>Dinner (Holiday Inn Ann Arbor, 3600 Plymouth Road)</td>
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## Schedule

### Friday, May 10, 2019

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<tr>
<td>7:30 AM</td>
<td>GLDMDG Business Meeting (Room G064)</td>
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<td>7:30 AM</td>
<td>Continental Breakfast</td>
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<tr>
<td>9:00</td>
<td><strong>Moderator:</strong> Gary Jenkins, Ph.D., Director, AbbVie</td>
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| 9:00     | **Lessons from Targeted Covalent Bruton’s Tyrosine Kinase Inhibitors,**
|          | **Acalabrutinib and its Major Metabolite: Disposition of a Unique,**
|          | **Low Reactivity, High Selectivity Warhead,** Terry Podol, Ph.D.,
|          | Principal Consultant, Increased Value Pharmaceutical Outsourcing,**LLC,** Seattle, WA |
| 09:40    | **Discovery and Development of Venetoclax from the DMPK Perspective,**
|          | Dan Bow, Ph.D., Senior Principal Research Scientist, DMPK-BA Development Sciences, AbbVie, North Chicago, IL |
| 10:20    | Break                                                               |
| 10:40    | **Moderator:** Matthew Zaya, M.S., Zoetis                           |
| 10:40    | **Drug Metabolism by Gut Bacteria,**
|          | Hyun Young Jeong, Pharm.D., Ph.D., Professor of Pharmacy Practice,**College of Pharmacy,** University of Illinois at Chicago, Chicago, IL |
| 11:20    | **Contribution of Organic Cation Transporter 3 (OCT3) to Doxorubicin-**
|          | **Induced Cardiotoxicity,** Kevin M. Huang, B.S., Division of
|          | Pharmaceuticals and Pharmaceutical Chemistry,** College of Pharmacy
|          | and Comprehensive Cancer Center,** The Ohio State University,**
|          | Columbus, OH,** Student Abstract Award Winner **                    |
| 11:35    | **Insight of Cytochrome P450 24A1 and Adrenodoxin Interaction as**
|          | **Modulated by Carbon-25 Hydroxylation of Vitamin-D Ligand,**
|          | Amit Kumar, Ph.D., Department of Biochemistry,** State University
|          | of New York at Buffalo,** Buffalo, NY,** Postdoc Abstract Award Winner |
| 11:50    | **Closing Remarks,**
|          | Hyun Young Jeong, Pharm.D., Ph.D., Professor of Pharmacy Practice,**College of Pharmacy,** University of Illinois at Chicago, Chicago, IL |
Posters

1. Differential modulation of estrogenic activity in estrogen receptor (ER)α and β by flavonoids in women’s health botanicals.

Obinna C. Mbachu†, Caitlin Howell†, Huali Dong†, Charlotte Simmler†‡, Shao-Nong Chen†‡, Gonzalo R. Malca García†, Atieh Hajirahimkhani†, Dejan Nikolic†, Guido F. Pauli†‡, Birgit M. Dietz†, and Judy L. Bolton†*

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

The use of botanical dietary supplements (BDS) has increasingly become a preferred option in women’s health because of perceived safety and efficacy. Some compounds in these botanicals are estrogenic flavonoids and isoflavonoids. Studies suggest that estrogen receptor beta (ER)β could provide better safety profile because of its antiproliferative properties that counterbalance the hyperproliferative activity of ERα. A structure-activity relationship (SAR) study of (iso)flavonoids found in BDS was conducted to identify structures that demonstrate ERβ-selective estrogenic potency, efficacy, and affinity. Notable flavonoids tested were 8-prenylnaringenin (8-PN) present in Humulus lupulus (hops) extract, 8-prenylapigenin (8-PA) identified in Glycyrrhiza inflata extract (licorice species), icaritin and desmethylicaritin from Epimedium species (horny goat weed), genistein in Trifolium pratense (red clover) extract, 8-prenylgenistein known to be present in lupin bean. Alkaline phosphatase and ERE-luciferase assays were conducted using endometrial carcinoma cell lines (ERα+, Ishikawa) and breast carcinoma cells (ERβ+ transfected, MDA-MB-231:β41), respectively, as in vitro biological endpoints to determine ERα/β activity. Competitive binding assays were performed to quantify compound affinity for the respective ER subtypes. Results show flavonoids with A-ring prenylation at C8 position and C-ring unsaturation at C2-C3, which increases the molecular planarity, resulted in significant ERβ-preferential potency, efficacy, and affinity, while saturation at this position increases estrogenic activity in ERα. In contrast, prenylation on isoflavonoids or site-specific methoxy or hydroxyl groups on flavonols, significantly decreased overall estrogenicity. BDS containing such ERβ-selective flavonoids, e.g. G. inflata with 8-PA, could potentially lead to favorable biological outcomes in vivo when used for health purposes. Supported by NIH Grant P50AT000155 and 2T32AT007533-06.
2. Expansion of the Cytochrome P450 1A1 Active Site Enables a Diversified Substrate Profile

Aaron G. Bart¹ and Emily E. Scott²

Biophysics Program¹,² and Departments of Medicinal Chemistry² and Pharmacology² University of Michigan, Ann Arbor, MI 48109

Cytochrome P450 1A1 is a highly inducible human enzyme often responsible for activating procarcinogens and protoxins. Most CYP1A1 substrates are relatively compact and highly planar, exemplified by polycyclic aromatic hydrocarbons and flavonoids. However, there are CYP1A1 substrates that deviate from this profile in terms of size and geometry. This includes clinical tyrosine-kinase inhibitors, which CYP1A1 bioactivates into reactive metabolites linked to adverse responses associated with these drugs. These compounds deviate from the typical CYP1A1 substrate profile and do not fit into the compact and highly planar active site observed in the original CYP1A1 structure co-crystallized with alpha-napthoflavone. Thus, the CYP1A1 active site must be flexible, but without defining this flexibility it is difficult to use structure as a predictive tool for CYP1A1 bioactivation.

In order to obtain a more complete description of the conformational flexibility of CYP1A1, CYP1A1 was cocrystallized with larger and more structurally diverse substrates. Structures of CYP1A1 with the grapefruit juice furanocoumarin bergamottin and the tyrosine kinase inhibitor erlotinib were determined. In both structures these ligands are bound in orientations consistent with the respective inactive and reactive metabolites produced enzymatically. Both orientations preserved active site planarity and demonstrate conserved aromatic stacking with a phenylalanine. However, perturbation of specific residues that compose the active site roof led to an expansion of the CYP1A1 active site volume. With erlotinib this formed a channel from the active site to the protein surface, presenting a possible route for CYP1A1 accommodating larger substrates.

The new structures of CYP1A1 have advanced our understanding of the conformational landscape of this P450, and demonstrated that the active site topology of CYP1A1 is not always compact or strictly planar. This ensemble of CYP1A1 structures is currently being tested to assess whether they sufficiently capture a range of flexibility in the enzyme that would allow accurate predictions of diverse CYP1A1 substrates, with a specific focus on predicting poses of tyrosine-kinase inhibitors activated by CYP1A1 into reactive metabolites. In conclusion this structural study of CYP1A1 is expected to help in anticipating metabolic liabilities of new pharmaceutical agents.
3. **Structural Insights into Cytochromes P450 11B1 and 11B2 for Enhanced Treatment of Cushing’s Disease and Hypertension**

Simone Brixius-Anderko¹ and Emily E. Scott¹,²

¹Department of Medicinal Chemistry, University of Michigan, Ann Arbor, MI 48109
²Department of Pharmacology and Biophysics Program, University of Michigan, Ann Arbor, MI 48109

Human steroidogenic cytochrome P450 enzymes 11B1 (CYP11B1) and 11B2 (CYP11B2) generate cortisol and aldosterone, controlling stress/immune responses and blood pressure, respectively. Excessive cortisol production by CYP11B1 results in Cushing’s disease, while aldosterone overproduction by CYP11B2 causes hypertension and cardiac disease. Treatment of each disease by drugs inhibiting the respective enzyme has been impeded because CYP11B1 and CYP11B2 have 93% identical amino acid sequences with the active site 100% identical. No structures of CYP11B1 are available to identify structural differences that could be exploited for the directed design of more selective drugs. Here, we report the first X-ray protein structure of human CYP11B1 in complex with the inhibitor fadrozole, a breast cancer drug and non-selective inhibitor of both CYP11B enzymes. Comparison with the previously-available CYP11B2 structure with bound fadrozole reveals significant differences in the shape of the drug-binding cavity and orientation of the bound inhibitor. In fact, the two CYP11B enzymes bind two chemically distinct enantiomers of fadrozole, with CYP11B1 binding (S)-fadrozole and CYP11B2 binding (R)-fadrozole. Knowledge of these distinct active site architectures provides a structural basis for the design and improvement of drugs that inhibit only CYP11B1 or CYP11B2 to treat Cushing’s disease or hypertension, respectively, minimizing off-target effects during treatment.
4. **Epigenetic Regulation of OCTN1-mediated Cytarabine Transport in Acute Myeloid Leukemia**

   Jason T. Anderson¹, Daelynn R. Buelow¹, Christina Drenberg¹, Shuiying Hu¹, Alice A. Gibson¹, Alex Sparreboom¹, and Sharyn D. Baker¹

¹Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, OH, USA.

**BACKGROUND:** Cytarabine is used as first line therapy in acute myeloid leukemia (AML) and is reported to be transported into leukemic cells via OCTN1¹. One hallmark of AML is dysregulation at the genetic and epigenetic level. Currently, clinical trials are underway using methyltransferase inhibitors such as 5-azacytidine or decitabine to “epigenetically prime” AML patients and reverse these epigenetic dysregulations (NCT03164057). These clinical trials would benefit from a stronger understanding of the mechanism underlying improved patient outcomes. Here, we tested our hypothesis that these improved patient outcomes are due to epigenetic modifications impacting OCTN1 expression, intracellular accumulation of cytarabine and subsequent antileukemic effects in AML cells.

**METHODS:** Uptake and cytotoxicity studies were performed in AML cell lines with prior 3-day treatment with hypomethylating agents, 5-azacytidine or decitabine. Uptake was conducted by using radioactive substrates and quantified by scintillation counts. OCTN1 expression levels were compared between treated and untreated AML cells with normalization to GAPDH. Methylation profiles were determined using bisulfite modification and pyrosequencing (BS-Seq). Cytarabine cytotoxicity was determined via MTT and developed after 72 hours of treatment.

**RESULTS:** Cytarabine uptake (1 µM; 15 min) and cytotoxicity (72 hour) varied among the 10 AML cell lines studied and were used to categorize “high” and “low” uptake. AML cell lines that showed low uptake had consistently higher methylation patterns in CpG islands upstream of SLC22A4 (OCTN1) as determined by BS-Seq. Exposure to 500 nM of hypomethylating agent decitabine for 72 hours increased OCTN1 expression (5.0 fold), uptake of cytarabine (2.9 fold), and subsequent cytotoxicity (7.3 fold) in CHRF cells with low OCTN1 expression. In contrast, lower methylated cell line OCI-AML3 showed modest to no increases in OCTN1 (1.0 fold), uptake of cytarabine (1.5 fold) and cytotoxicity (2.8 fold) following decitabine treatment. These findings are consistent with the notion that methylation of OCTN1 was inversely related to cytarabine uptake.

**CONCLUSIONS:** These results identify SLC22A4 (OCTN1) methylation status as a contributor to the expression of OCTN1, cellular uptake and the efficacy of cytarabine; these results have broad implications for the future design of combination chemotherapy regimens.

**Source of Research Support:** 5R01CA138744-09

5. Targeting OCT3 (SLC22A3) to Ameliorate Doxorubicin Cardiotoxicity

Kevin M. Huang¹, Sherry Xia¹, Tarek Magdy², Alice A. Gibson¹, Ingrid Bonilla³, Cynthia Carnes³, Joanne Wang⁴, Paul W. Burridge², Shuiying Hu¹, and Alex Sparreboom¹

¹Division of Pharmaceutics and Pharmaceutical Chemistry, College of Pharmacy and Comprehensive Cancer Center, The Ohio State University, Columbus, OH, USA; ²Department of Pharmacology, Center for Pharmacogenomics, Northwestern University Feinberg School of Medicine, Chicago, IL, USA. ³Department of Pharmacology, College of Pharmacy, The Ohio State University, Columbus OH, USA. ⁴Department of Pharmaceutics, School of Pharmacy, University of Washington, Seattle, WA, USA.

OBJECTIVES:
We hypothesize that doxorubicin-induced cardiotoxicity (DIC) is dependent on a transporter-mediated mechanism. The present study aims to (i) identify the solute carrier that initiates excessive accumulation in the heart and (ii) determine its contribution to cardiotoxicity, and (iii) evaluate the feasibility of integrating transport inhibitors as a therapeutic intervention strategy.

METHODS:
Transporter gene expression studies were conducted on human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) from patients that did or did not experience DIC. The pharmacokinetics and disposition of doxorubicin in ex vivo isolated cardiomyocytes and in vivo whole heart homogenates were determined in wild-type and age-matched transporter-deficient animals. Cardiovascular performance was assessed by transthoracic echocardiography and measuring circulating cardiac troponin. The uptake and cytotoxicity of doxorubicin (DOX) in combination with transport inhibitors were evaluated using the NCI’s panel of breast cancer cell lines.

RESULTS & CONCLUSION:
Multiple solute carrier genes were overexpressed by ≥2-fold in hiPSC-CMs from patients experiencing DIC, including OCTN1, OCT1, and OCT3. Among these transporters, deficiency of OCT3 was associated with the most significant decrease in accumulation of DOX in isolated cardiomyocytes and disposition in the heart. Subsequent accumulation of DOX in cardiomyocytes of wild-type animals were diminished by the OCT3 inhibitors, the tyrosine kinase inhibitors (TKIs): dasatinib and nilotinib, through non-competitive mechanisms. Consistently, cardiovascular performance was preserved in OCT3-deficient animals, as measured by left ventricular ejection fraction (LVEF) and fractional shortening (FS), and reduced circulating levels of cardiac troponin (cTnI). Furthermore, combination with dasatinib or nilotinib do not antagonize the uptake or cytotoxicity of DOX in a panel of breast cancer cells.

SIGNIFICANCE:
We identified a previously unrecognized pathway of DIC that is initiated by an organic cation transporter system and highly sensitive to pharmacological inhibition. These findings shed further light on the etiology of DIC, and provide a rationale to exploit drug transporters as a novel targeted intervention strategy to mitigate this debilitating side effect.

ACKNOWLEDGEMENTS
The authors would like to gratefully acknowledge colleagues in Experimental Cancer Pharmacology Laboratory at The Ohio State University. The project was also supported in part by grants R01CA187176, R01CA215802, OSU Comprehensive Cancer Center using Pelotonia funds, and the Pelotonia Graduate Student Fellowship.
6. **Cell Model for Studying Nucleoside Transporters, a Key Component of the Blood-Testis Barrier**

Siennah R. Miller¹, Raymond K. Hau¹, Mark N. Morales², Nathan J. Cherrington¹ & Stephen H. Wright²

¹Department of Pharmacology & Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ, USA  
²Department of Physiology, College of Medicine, University of Arizona, Tucson, AZ, USA

Sertoli cells in the testis represent the principle element of the Blood-Testis Barrier (BTB). Equilibrative nucleoside transporters (ENTs) are responsible for the transport of nucleosides across rodent BTB and immunohistochemistry suggests that hENT1 is located on the basal membrane and hENT2 is located on the apicolateral membrane of Sertoli cells (Klein et al., 2013). Therefore, the ENTs are of particular interest in studying the disposition of nucleoside reverse transcriptase inhibitors (NRTIs) in the human male genital tract because of their similarity in chemical structure to nucleosides. This study characterized the transport of a specific nucleoside, uridine, to determine the relative roles of ENT1 and ENT2 in nucleoside transport in HeLa S3 cells, a potential model system for the study of ENT selectivity, using the ENT specific inhibitor S-(4-nitrobenzyl)-6-thioinosine (NBMPR). Uptake of 20 nM [³H]uridine was linear for 7 minutes and 100 nM NBMPR blocked 77% of this uptake, with the remaining uptake blocked by 100 µM NBMPR (to the same level produced by 5 mM unlabeled uridine). ENT1 and ENT2 are known to have different sensitivities for NBMPR and, consistent with this difference, inhibition of [³H]uridine uptake produced by increasing concentration of NBMPR proved to be biphasic, with IC₅₀s of 11.1 nM (ENT1) and 7.2 µM (ENT2). Consequently, uridine uptake measured in the presence of 100 nM NBMPR was taken to represent ENT2-mediated transport; subtracting that from total uptake represented ENT1-mediated transport. The kinetics of ENT1- and ENT2-mediated [³H]uridine uptake into HeLa S3 cells showed no difference in Jₘₐₓ (16.13 and 12.26 fmol cm⁻² min⁻¹) and a three-fold difference in Kₘ (13.28 and 35.18 µM). The resulting 3.5-fold difference in intrinsic clearance for uridine uptake (i.e., Jₘₐₓ/Kₘ) for hENT1 and hENT2 mediated transport (1.21 mL cm⁻² min⁻¹ and 0.348 mL cm⁻² min⁻¹, respectively), accounted for the observed inhibition of uridine transport produced by 100 nM NBMPR, indicating that at low concentrations, hENT1 is primarily responsible for uridine uptake in these cells. Together, these data suggest that HeLa S3 cells are an adequate model for studying the characteristics of nucleoside transporters present in the BTB. (Supported by R01GM123643).
7. **Label-free absolute protein quantification with data-independent acquisition**

*Bing He*<sup>a</sup>, Jian Shi<sup>a</sup>, Xinwen Wang<sup>a</sup>, Hui Jiang<sup>b</sup>, Hao-Jie Zhu<sup>a,⁎</sup>

<sup>a</sup> *Department of Clinical Pharmacy, University of Michigan, Ann Arbor, MI 48109, United States of America*

<sup>b</sup> *Department of Biostatistics, University of Michigan, Ann Arbor, MI 48109, United States of America*

Despite data-independent acquisition (DIA) has been increasingly used for relative protein quantification, DIA-based label-free absolute quantification method has not been fully established. Here we present a novel DIA method using the TPA algorithm (DIA-TPA) for the absolute quantification of protein expressions in human liver microsomal and S9 samples. To validate this method, both data-dependent acquisition (DDA) and DIA experiments were conducted on 36 individual human liver microsome and S9 samples. The MS2-based DIA-TPA was able to quantify approximately twice as many proteins as the MS1-based DDA-TPA method, whereas protein concentrations determined by the two approaches were comparable. To evaluate the accuracy of the DIA-TPA method, we absolutely quantified carboxylesterase 1 concentrations in human liver S9 fractions using an established SILAC internal standard-based proteomic assay; the SILAC results were consistent with those obtained from DIA-TPA analysis. Finally, we employed a unique algorithm in DIA-TPA to distribute the MS signals from shared peptides to individual proteins or isoforms and successfully applied the method to the absolute quantification of several drug-metabolizing enzymes in human liver microsomes. In sum, the DIA-TPA method not only can absolutely quantify entire proteomes and specific proteins, but also has the capability quantifying proteins with shared peptides.
8. **Prevalence of drug-drug interactions in oncology patients enrolled in two completed SWOG clinical trials**

Lauren A Marcath, PharmD1, Colin M Finley, BS1, Siu-Fun Wong, PharmD2, Daniel L Hertz, PharmD, PhD1

1University of Michigan, College of Pharmacy, Department of Clinical Pharmacy, Ann Arbor, MI, 48109; 2Chapman University School of Pharmacy, Irvine, CA, 92618

**Funding:** Supported in part by NIH/NCI grants CA180888 and CA180819. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**Background:** As the use of oral chemotherapy agents continues to rise, high rates of polypharmacy put oncology patients at risk of clinically relevant drug-drug interactions (DDIs) that may cause increased toxicity or decreased therapeutic efficacy. Oral chemotherapy agents are used long term and have more DDIs related to absorption and metabolism than conventional intravenous chemotherapy. DDIs in patients enrolled in clinical trials are especially concerning because they can adversely affect patient safety and lead to study drug overestimates of toxicity or underestimates of efficacy. At a single institution, 24.2% of National Clinical Trials Network subjects had moderate to major DDIs (Marcath, et. al., 2018). In a survey of SWOG sites, a National Clinical Trials Network group that is funded and supported by the National Cancer Institute, 51% of sites reported that DDI screening occurs during eligibility assessment only if a DDI is an exclusion criterion (Hertz, et. al., 2018), suggesting many DDI may be missed during screening, and as a result study patients and findings may be compromised. The objective of this study was to determine the prevalence of potential DDIs involving study drugs in patients enrolled in two SWOG clinical trials.

**Methods:** Completed trials were included if DDIs with the study drug were possible and medication lists were available from SWOG for retrospective use. Lexicomp was used to screen all medication lists for DDIs. All major and contraindicated DDIs (level D or X) involving the study agents were recorded. Interactions identified by Lexicomp were reviewed by a pharmacist and PharmD student for clinical relevance, defined as a potential DDI that would warrant a medication change at enrollment to ensure patient safety including both efficacy and toxicity concerns. Discordant decisions were discussed until reaching consensus.

**Results:** One hundred sixty-three patients who were enrolled in two completed SWOG trials (S0711 and S0528) were included. All patients received treatment with either dasatinib or everolimus/lapatinib. On average, patients were taking 6.8 concomitant medications (range: 1-29). Lexicomp detected one or more major/contraindicated DDI in 30.7% of patients (50/163) with a range of 1-3 DDIs. Sixteen percent of all patients had DDIs that were deemed clinically relevant (26/163, range: 1-2 clinically relevant interactions). Clinically relevant dasatinib interactions were caused by antacids (n=17) or proton pump inhibitors/histamine-2 receptor antagonists (n=9), all of which decrease absorption and could decrease treatment efficacy. Clinically relevant everolimus interactions were caused by moderate CYP3A4 inhibitors (n=2) that could increase everolimus concentrations and result in toxicity.

**Conclusions:** These findings confirm a high prevalence of potential DDIs involving study agents in patients enrolled on SWOG trials. The number of clinically relevant interactions was lower when excluding antacids, which may be safe to administer as long as patients are properly educated on separating their antacid dose from the study drugs by at least four hours. More stringent, systematic DDI screening is needed during enrollment of clinical trials to reduce DDI prevalence and ensure patient safety and study data integrity.
9. Genetic and pharmacological inhibition of OCT2 protects rats against oxaliplatin-induced peripheral neuropathy

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Background
Colorectal cancer (CRC) is the third leading cause of cancer-related mortalities in the United States. Oxaliplatin (OXPt) is widely used in the treatment of patients with CRC in both an adjuvant and metastatic setting; however, OXPt induces acute and chronic peripheral neuropathy (PN) in up to 90% of patients, characterized by paresthesia or sensory ataxia in the extremities. Previously, we identified organic cation transporter 2 (OCT2) as a contributor initiating OXPt-induced PN in mice, and that pre-treatment with the tyrosine kinase inhibitor, dasatinib, ameliorated acute and chronic forms of PN. Since the rat orthologue of OCT2 shares >90% sequence homology, we hypothesized that OCT2 inhibition can also afford protection in rats against OXPt-induced PN.

Methods
In vitro transport studies were conducted in stably-transfected HEK293 overexpressing rat OCT2. In vivo studies were conducted in age-matched wild-type and OCT2-deficient Sprague dawley rats receiving a single dose of OXPt (10mg/kg, i.p.), with or without pre-treatment with an OCT2 inhibitor (dasatinib, 15 mg/kg, p.o.). Mechanical-induced allodynia were recorded before and 24 hours after the treatment. Platinum exposure in plasma, dorsal root ganglia cells (DRGs), kidneys, and urinary excretion were analyzed by atomic absorption spectroscopy. Gene expression of OCT-related transporters in DRGs and kidneys were also analyzed by qPCR. The cytotoxicity of OXPt in the presence of dasatinib was evaluated in the NCI panel of colorectal cancer cells.

Results
Our in vitro studies showed that OXPt is also a substrate of rat OCT2 and that this transport process is sensitive to pharmacological inhibition by the tyrosine kinase inhibitor, dasatinib (IC₅₀ 25.8 nM). Treatment with OXPt significantly increased wild-type rats to mechanical allodynia sensitivity (40%), whereas OCT2-deficient rats were completely protected from OXPt-induced PN (p<0.05). Furthermore, pre-treatment with dasatinib in wild-type rats ameliorated OXPt-induced PN. Platinum accumulation in DRGs was 7.3-fold higher in wild-type rats compared to OCT2-deficient rats, while treatment with dasatinib significantly diminished accumulation in DRGs of wild-type rats. These results are corroborated by high expression of OCT2 in DRGs. In addition, plasma exposure and urinary excretion of total platinum was not significantly altered both wild-type and OCT2-deficient rats, treated with or without dasatinib. Lastly, in vitro screening of the NCI panel of CRCs demonstrated that concomitant treatment with dasatinib did not compromise the antitumor properties of OXPt.

Conclusion
Our study serves as a model that inhibition of OCT2 provides protection against OXPt-induced PN. As this debilitating side effect greatly diminishes quality of life, these type of preventative strategies addresses an unmet therapeutic need in colorectal cancer patients receiving oxaliplatin-based therapy.
10. **Zebrafish as a screening tool to evaluate organic cation transporter function**

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**Background**
The organic cation transporter OCT2 is expressed in renal tubular cells, dorsal root ganglia, brain, and outer hair cells, where it regulates uptake of endogenous and xenobiotic substrates such as cisplatin and oxaliplatin. We previously reported that various tyrosine kinase inhibitors (TKIs) with YES1-kinase inhibitory ability can inhibit OCT2 function (Sprowl et al, Nat Commun 2016). Against this background, we here explored the utility of zebrafish (*Danio rerio*) as an in vivo screening tool to evaluate the potential of OCT2 inhibitors to modulate the transport of substrates.

**Methods**
Sequence alignment was performed with MAFFT v7. *In vitro* experiments were performed with Hela cells expressing human OCT2 or the single zebrafish OCT (zfOct) with high genomic similarity to mammalian OCTs, or HEK293T cells for transfection of mutated zfOct. Uptake experiments were done with different substrates (ASP, cisplatin, oxaliplatin) with or without TKIs (10 µM). *In vivo* studies, uptake experiment and seeker response assay, were done in ABxLF zebrafish at 5dpf (days post-fertilization).

**Results**
The protein sequences of OCT2 and zfOct were 71% identical, and YES1-tyrosine phosphorylation sites were conserved. A comparative *in vitro* screen of OCT2 and zfOct using the prototypical fluorescent substrate ASP showed that both transporters could be inhibited by the same TKIs, including dasatinib (63.6% vs 66.5%) and nilotinib (53.6% vs 68.8%), with comparable IC50 values. Hela cells expressing OCT2 or zfOct accumulated cisplatin and oxaliplatin by >2-fold compared to control cells, and these processes were sensitive to inhibition by the same TKIs (inhibition from 40 to 100% of the uptake). Tyrosine in Phenylalanine mutagenesis in zfOct at previously confirmed YES1-regulated tyrosine phosphorylation sites in OCT2 was associated with impaired transport function, suggesting a similar regulatory mechanism for both transporters. *In vivo*, neuromasts, consisting of receptive hair cells, accumulated OCT2/zfOct specific substrates, and this process could be inhibited by pretreatment of zebrafish with cisplatin (100 µM, 6-h pre-incubation) or dasatinib (10 µM, 6 h). In addition, cisplatin treatment was associated with a disruption of neuromast-related control of hearing and balance.

**Conclusion**
These studies provide new insights into the characterization of zfOCT, and support the further exploration of zebrafish as a screening tool to study organic cation transporter function and its regulation.
11. **Mechanisms of Gemcitabine Oral Absorption as Determined by In S itu Intestinal Perfusions in Mice**

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**Purpose:**
Gemcitabine is a nucleoside analogue used to treat various solid tumors. Gemcitabine’s oral bioavailability (BA) is ≈10%, necessitating intravenous administration which increases toxicity concerns, patient discomfort, and cost of use. This work aims to elucidate the issues underlying gemcitabine’s low oral BA by systematically characterizing gemcitabine intestinal uptake in mice.

**Methods:**
Mouse *in situ* intestinal perfusions were performed to determine the intestinal permeability and transport kinetics of gemcitabine and to explore the role of concentrative (CNT) and equilibrative (ENT) nucleoside transporters in mediating uptake. Furthermore, perfusions with [14C]-gemcitabine were performed to validate the observed permeability values and explore the impact of ENT inhibition on gemcitabine intestinal and portal blood accumulation.

**Results:**
Gemcitabine exhibits concentration-dependent permeability, ranging from $1.7 \times 10^{-4}$ cm/s at 0.5 µM to $0.2 \times 10^{-4}$ cm/s at 2 mM, mediated by high-affinity ($K_m = 27$ µM, $V_{max} = 3.6$ pmol/cm²/sec) and low-affinity ($K_m = 700$ µM, $V_{max} = 35.9$ pmol/cm²/sec) transport systems. Co-perfusion with thymidine (CNT and ENT inhibitor), dilazep (ENT inhibitor), and perfusion in a Na⁺-free buffer (CNT inhibitor) reduced gemcitabine permeability by 95%, 50%, and 68%, respectively. Dilazep co-perfusion decreased the portal concentration of gemcitabine/gemcitabine metabolites by 68% but did not affect intestinal concentrations.

**Conclusion:**
Gemcitabine intestinal uptake is saturable and mediated almost exclusively via apically expressed CNTs and EN Ts. Efflux into portal blood is mediated, at least in part, via EN Ts. In contrast to published reports, gemcitabine displays high intestinal permeability, implying first-pass metabolism is mainly responsible for gemcitabine’s low oral BA.
12. **Energy Landscapes of Rolapitant Unbinding from CYP2D6 as determined by Molecular Dynamics**

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Rolapitant is an NK1 receptor antagonist used for chemotherapy-induced nausea and vomiting. In clinical studies, rolapitant was shown to inhibit the activity of CYP2D6 for at least a week after one dose. In the present study, rolapitant’s interaction with CYP2D6 was investigated *in silico* and *in vitro*. Rolapitant does not appear to be a mechanism-based inactivator or a tight-binding inhibitor of CYP2D6. Instead, rolapitant behaved as a reversible inhibitor. During the course of MD simulations, rolapitant was able to move into a position away from the heme iron and into a groove above Helix I but was unable to interact with the heme. During 1 μsec simulations, the escape of rolapitant from the enzyme followed the channel 2c pathway. B-factor analysis of the escape did not reveal consistent large protein structural movements with each escape. Finally, free energy landscapes for the MD simulations indicated that multiple low energy binding conformations for rolapitant exist in the active site area. (NIH 1F15-GM086767-03)
13. Evaluation of CRISPR-Cas9 and CRISPR-Cas13 in Modulating Drug Metabolizing Enzymes

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It is challenging to conduct in vitro to in vivo extrapolation for non-cytochrome P450s (CYP) drug metabolizing enzymes or transporters partly because selective drug inhibitors to discern the specific contribution to drug disposition are lacking. Clustered regularly interspaced short palindromic repeats (CRISPR) gene editing has recently gained rapid development to become a powerful technology with promising capabilities for targeted gene deletions. However, it is unknown how CRISPR may modulate drug metabolizing enzyme(s) activities in expanded primary hepatocytes (Upcyte) and primary hepatocyte co-culture (HµREL®) that maintain important metabolic and transport activities. As a result, the study objectives were to examine the efficiency and specificity/off-target effects of CRISPR in modulating expression and activities of drug metabolizing enzymes in these cell models at 1) DNA level, and 2) RNA level using CRISPR-Cas13 by comparing with siRNA technology. First, CRISPR-Cas9 was used to target the cells at the DNA level. Here, a SpCas9 and dual gRNA plasmid approach was utilized to conduct targeted deletion of UGT1A subfamily in Upcyte hepatocytes. Green fluorescent protein (GFP) was also co-transfected by nucleofection, which allowed fluorescence-activated cell sorting to collect GFP positive cells and eventually develop 120 visible single cell derived colonies. Among the four clones that were able to grow robustly, one was cultured and measured for enzyme activity and demonstrated approximately 90% lowered UGT1A1 metabolic activity as measured by estradiol glucuronide formation compared to the wild type. This clone had one deletion allele of UGT1A by PCR characterization of genomic DNA, and deep sequencing is ongoing to confirm its knockout status. Second, using CRISPR- Cas13 to target the cells at the RNA level, HµREL® HumanPool™ hepatic co-cultures were treated with pAAV-Cas13-gNT (non-targeting control) or AAV-Cas13- CYP3A4 for 7 days and then measured for enzyme activity by metabolite formation and mRNA expression levels by RT-qPCR. No cell deaths were observed in all infection conditions as measured by CellTiter-Glo® luminescent assay. The non-targeting control infection did not change CYP3A4 mRNA or activity. Furthermore, relative to the non-targeting control, CRISPR-Cas13-CYP3A4 AAV infection has led to approximately 30% reductions (p<0.05) in both CYP3A4 activity as measured by 1'-OH midazolam formation, and CYP3A4 mRNA levels in HµRel cells. Within all the other genes measured under the same conditions, i.e. CYP3A5, CYP2E1, CYP2B6, UGT1A1, UGT2B7 and AOX1, CRISPR-Cas13 has only led to significant alterations in gene expression for CYP2E1 and UGT2B7. By contrast, siRNA methodology, though showing more robust reduction of CYP3A4 mRNA, had more off-target effects as demonstrated by altered mRNA expression in the non-targeting siRNA transfection and significant changes in mRNA expressions of additional metabolizing enzymes following siRNA-CYP3A4 treatment. Overall, our data supports improved specificity by CRISPR/Cas-13 mediated knockdown. However, caution still needs to be taken to understand specificity and potential off-target issue when using CRISPR-generated cell models. With the continued improvement in its efficiency and appropriate cell model systems, CRISPR technology may become a useful tool in understanding roles of drug metabolizing enzymes and transporters in drug disposition.
14. Effect of Cytochrome b5 on the 17,20 Lyase Activity of Human Cytochrome P450 17A1

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Human cytochrome P450 17A1 is required for androgen biosynthesis and is the target of abiraterone, a drug widely used to treat advanced prostate cancer. P450 17A1 catalyzes both 17- hydroxylation and subsequent 17,20-lyase reactions with pregnenolone, progesterone, and allopregnanolone. The presence of cytochrome b5 (b5) markedly stimulates the 17,20-lyase reaction, with little effect on 17-hydroxylation; however, the mechanism of this b5 effect is not known. We determined the influence of b5 on coupling efficiency—defined as the ratio of product formation to NADPH consumption—in a reconstituted system using these 3 pairs of substrates for the reactions. Rates of NADPH consumption ranged from 4 to 13 nmol/min/nmol P450 with wild-type P450 17A1. Rates of NADPH consumption were similar for the 17-hydroxylase and corresponding 17,20-lyase reactions for each steroid series, and b5 only slightly increased NADPH consumption. For the 17,20-lyase reactions, b5 markedly increased product formation and coupling in parallel with all substrates, from 6% to 44% with the major substrate 17-hydroxypregnenolone. In order to investigate the mechanism of increased coupling in the presence of b5, we compared the rate of product formation of 17-hydroxylation and 17,20-lyase reaction under single turnover condition of P450 17A1 in the presence of either reductase or b5 using a Rapid Chemical Quench apparatus. Electron transfer from b5 and reductase to P450 17A1 in the presence of substrates was also investigated. Resonance Raman study of P450 on the ν(Fe-O) stretching modes with redox partners (reductase or b5) shows that reductase binding has no stretching mode change but b5 causes substantial changes (\~10 cm\textsuperscript{-1}). We conclude that b5 stimulation of the 17,20-lyase reaction primarily derives from more efficient use of NADPH for product formation rather than side products.
15. **Tryptophan-75 is a potential gating residue of Cytochrome P450 2D6**

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Cytochrome P450 2D6 is part of a superfamily of heme-containing proteins (CYPs) that catalyze many reactions involved in drug metabolism. The active site of CYP2D6 is buried, and both substrate access to the active site and subsequent metabolism are influenced by amino acid side chains, a phenomenon referred to as tunnel gating. This investigation hypothesizes that tunnel gating exists in CYP2D6, and that it is mediated in part by tryptophan-75 residue. Molecular Dynamics (MD) studies have shown that tryptophan-75 has the ability to swing out from the 2b channel to discharge a ligand. Also, visualization of 2D6 crystal structures by molecular imaging software showed the presence of several distinct conformations of the residue, some of which were observed to obstruct the opening of the 2b tunnel and thus inhibit access and egress of substrates from the active site. Based on the mobility of the residue in 3D space, we theorize that a gating mechanism of tryptophan-75 impacts ligand metabolism rates of CYP2D6. To test this effect, a tryptophan-75 to alanine mutant along with a reference *1 were expressed and purified in *E. coli*, and ligand binding and kinetic properties of each were examined with a bufuralol substrate. Spectral binding assays show similar spectral binding constants. Continued kinetic analyses will characterize each protein by its $v_{\text{max}}$, while continued MD studies aim to identify other potential gating residues of 2D6. We find this investigation to be important as tunnel gating and enzyme plasticity in CYPs is an area with many remaining questions and is of broad interest in the field of drug metabolism. (Support: NIH 2R15GM086767-03).
16. Modulation of CYP3A4 activity to increase the oral bioavailability of ibrutinib

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Objectives of the Study: Ibrutinib (Imbruvica; PCI-32765), an orally administered inhibitor of Bruton’s tyrosine kinase, is considered a breakthrough targeted therapy that is approved as frontline therapy in chronic lymphocytic leukemia (CLL). Ibrutinib has an average oral bioavailability in humans of <4% and exhibits substantial variability in exposure, which predisposes patients to unpredictable and potentially harmful adverse events such as bleeding and atrial fibrillation. As ibrutinib is subject to extensive first-pass metabolism by CYP3A4 following oral administration, inhibition of intestinal and/or hepatic CYP3A4 activity may be a promising strategy to decrease interindividual pharmacokinetic variability. The objective of this study was to evaluate the effect of the prototypical CYP3A4 inhibitor ketoconazole on the pharmacokinetics of ibrutinib and its main active metabolite dihydrodiol-ibrutinib (DiOH-ibrutinib) in mice.

Methods: To characterize the impact of ketoconazole on ibrutinib disposition, in vivo pharmacokinetic studies were performed in male and female FVB mice. Ketoconazole (50 mg/kg; p.o.) or vehicle control (PEG400; p.o.) were administered 30-min before a single oral dose of ibrutinib (10 mg/kg; formulated in DMSO/PEG/Tween). Concentrations of ibrutinib and DiOH-ibrutinib in plasma were determined by a validated method based on liquid chromatography-tandem mass spectrometry, and pharmacokinetic parameter estimates were calculated with the software package Phoenix WinNonlin (Version 8.1).

Results: Preliminary experiments revealed that the peak plasma concentration (Cmax) and the area under the curve (AUC) of ibrutinib were higher in female mice [mean (SD), Cmax 1040 (336) vs 403 (91.2) ng/mL; AUC, 744 (50.5) vs 429 (61.6) ng×h/mL], suggesting that the pharmacokinetic profile of ibrutinib exhibits sexual dimorphism. Administration of ketoconazole prior to ibrutinib resulted in a 10.8-fold (females) and 9.9-fold (males) increase in the AUC of ibrutinib, and the DiOH-ibrutinib to ibrutinib AUC ratio was substantially decreased by ketoconazole regardless of sex. The DiOH-ibrutinib to ibrutinib ratio with vehicle was 1.3 (females) and 0.9 (males), whereas the DiOH-ibrutinib to ibrutinib ratio with ketoconazole was 0.08 (females) and 0.1 (males). The plasma concentrations of DiOH-ibrutinib declined in parallel with the parent drug, indicating that the half-life of the metabolite is formation-rate limited. The apparent half-lives of both ibrutinib and the metabolite were increased by ketoconazole, suggesting that the modulation of ibrutinib pharmacokinetics by ketoconazole likely takes place at the level of intestinal (absorption) as well as hepatic metabolism (elimination). However, it cannot be excluded that ketoconazole affects the formation of one or more other ibrutinib metabolites that were not measured in this study or ketoconazole inhibits transporter-mediated uptake of ibrutinib in intestinal enterocytes and/or hepatocytes.

Conclusions: The current study demonstrates that mice provide a translationally useful model organism to evaluate the potential clinical impact of CYP3A4 inhibitors on the pharmacokinetics of ibrutinib. Previously obtained data with other kinase inhibitors indicate that patients with high CYP3A4 activity may benefit from a modified regimen (increased dose), to achieve drug concentrations required to interact with kinase targets, and a strategy by which enzyme activity is intentionally inhibited could achieve similar results. In view of the significant inverse correlation between decreasing absolute bioavailability and interindividual pharmacokinetic variability, the current data provide a rationale for the development of exploratory clinical studies aimed at decreasing the variability in ibrutinib exposure by concomitant administration of CYP3A4 inhibitors. Our ongoing studies utilize CYP3A4 knockout and humanized transgenic mice as well as alternative inhibitors such as cobicistat to refine combination regimens that can decrease inter-individual pharmacokinetic variability and ultimately result in more predictable, safe and effective treatment of CLL.
17. Role of OATP2B1 in Drug Absorption and Drug-Drug Interactions

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Background: The human organic anion-transporting polypeptide 2B1 (OATP2B1; SLC21A9) is localized to the basolateral membrane of hepatocytes and the brush-border membrane of small-intestinal enterocytes. Based on its distribution pattern and functional similarity to OATP1B-type transporters, it has been hypothesized that OATP2B1 might influence the absorption and disposition properties of a wide range of xenobiotic agents. Although several prescription drugs, including statins such as fluvastatin, have been identified as OATP2B1 substrates in vitro, the lack of a useful in vivo model system has limited our understanding of the pharmacological role of this transporter.

Experimental design: OATP2B1-overexpressing cells were generated by transfecting HEK293 cells with an empty vector (VC) or a construct containing human OATP2B1 or mouse OATP2B1 cDNA. Transporter function was evaluated in vitro by measuring the comparative cellular uptake of [3H]estrone-3-sulfate (ES) in VC cells and OATP2B1-overexpressing cells. Candidate substrates and inhibitors were identified based on the difference in intracellular accumulation of radioactivity. OATP2B1-knockout (KO) mice were obtained from the Slco2b1tm1a(KOMP)Wtsi clone, and derived chimera and subsequent progeny were mated to C57BL/6N for germline production and colony expansion. Mouse genotypes were confirmed by PCR, and OATP2B1 gene deletion was verified by qPCR on major organs. The pharmacokinetic profiles of fluvastatin, a known OATP2B1 substrate, and sorafenib, a known OATP2B1 inhibitor, were determined through serial blood collection and subsequent LC-MS/MS analysis. Noncompartmental pharmacokinetic parameters were calculated using WinNolin 6.2 software (Pharsight).

Results: An approximately four-fold increase in intracellular accumulation of radiolabeled ES (2.5 µM) was observed in the OATP2B1 overexpressing cells compared to VC cells (34.8 ± 2.45 pmol/mg vs 8.38 ± 0.341 pmol/mg, respectively). We then identified several drugs as candidate OATP2B1 substrates, including multiple tyrosine kinase inhibitors (TKIs) Knockout of OATP2B1 in organs from KO mice was confirmed at the gene level (P<0.01), and consistent with the intestinal localization of OATP2B1, we found that the absorption rate of fluvastatin was decreased compared with that in WT mice (Tmax: 0.5 h vs 1 h; Cmax: 1.05 µg/mL vs 2.19 µg/mL). No gender differences in mRNA expression levels were detected. Among 24 different agents tested, sorafenib was identified as one of the most potent OATP2B1 inhibitors in the class of TKIs. However, the pharmacokinetic properties of sorafenib and its main metabolites were not significantly affected by OATP2B1 deficiency.

Conclusion: Our studies have identified novel potent inhibitors of OATP2B1 among TKIs, a rapidly expanding class of drugs used in various therapeutic areas. The availability of a viable, well-characterized OATP2B1-deficient mouse model provides a previously unavailable opportunity to unequivocally determine the contribution of this transporter to the absorption and drug-drug interaction potential of drugs.
18. Evaluation of Small Interfering RNA Mediated Knockdown of Drug Metabolizing Enzymes as a Tool for Reaction Phenotyping

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Identification of the individual drug metabolizing enzymes responsible for the clearance of drug molecules is important in assessing the risk of drug-drug interactions. However, this is limited by paucity of tools available for the reaction phenotyping of less studied non-cytochrome P450 enzymes. Small interfering RNA (siRNA) has been shown to modulate enzyme activity through downregulation of mRNA level. This study aimed to explore the potency and specificity of siRNA knockdown of select drug metabolizing enzymes as a preliminary investigation into the use of siRNA as a tool for reaction phenotyping. Primary human hepatocyte co-culture (H₂rel) were treated with siRNAs targeting five representative drug metabolizing enzymes: CYP3A4, CYP2E1, AOX1, UGT1A1, and UGT2B7 and then the potency and specificity of gene knockdown was determined with enzyme activity assays and qPCR. Targeted knockdown of all the drug metabolizing enzymes was observed by a reduction in both the targeted enzyme’s activity and gene expression. In addition to the targeted knockdown, off-target effects were observed for all siRNA treatments at the activity level and some at the mRNA level. CYP2E1 and CYP3A4 were the most susceptible to off-target effects. With the lack of specificity of siRNA mediated gene knockdown, this technique appears to have limited utility for reaction phenotyping. Overall, these results suggest that more optimization, understanding, and elimination of the off-target effects would be needed for siRNA mediated gene knockdown to be a helpful technique in reaction phenotyping.
19. Activation of Heat Shock Protein 70 as a Potential Therapeutic Strategy for the Treatment of Protein Folding Disease

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The heat shock protein 90 and 70 (Hsp90/70) chaperone system provides an essential protein quality control mechanism for the cell. Heme containing enzymes, such as cytochrome P450s and neuronal nitric oxide synthase (nNOS), are susceptible to damage by xenobiotics and inactivation by specific environmental toxins or drugs leading to the selective degradation of these proteins. In the course of studies on the inactivation of nNOS, we discovered that covalent alteration of the heme binding cleft leads to misfolding of the cleft, which is selectively recognized by Hsp90/70. Hsp70 recruits the c-terminus of Hsp70 interacting protein (CHIP), an E3 ubiquitin ligase, leading to ubiquitination and proteasomal degradation of nNOS. In an analogous manner the Hsp90/70 chaperone system also regulates other client proteins that are prone to misfolding and aggregation, such as the polyglutamine-androgen receptor (polyQ-AR) a genetic mutant whose misfolding and aggregation leads to muscular- and neurodegeneration in Kennedy’s disease. We have demonstrated that genetic and pharmacological activation of Hsp70 increases the ubiquitination and degradation of misfolded nNOS and polyQ-AR in cells and alleviates neurotoxicity in a Drosophila model of Kennedy’s disease. To advance the development of Hsp70 activation as a therapeutic strategy for the treatment of neurodegenerative diseases, we have established a workflow to identify novel Hsp70 modulators that enhance degradation of misfolded Hsp90/70 client proteins, utilizing nNOS as a model. We have developed a thermal shift high-throughput screen to identify novel compounds that bind and thermostabilize Hsp70. Preliminary results suggest that this method can be utilized to identify compounds that increase nNOS ubiquitination in vitro and promote degradation of nNOS in HEK293 cells. We are currently working to develop high-throughput ELISA-based secondary assays for the quantification of nNOS ubiquitination in vitro and nNOS levels and ubiquitination in cells. This workflow has the potential to advance the development of therapeutics for the treatment of Kennedy’s disease and other neurodegenerative diseases such as Alzheimer’s, Huntington’s, and Parkinson’s disease which are caused by misfolding and aggregation of the Hsp90/70 client proteins tau, huntingtin, and α-synuclein.

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20. **Identification of Ubiquitination Sites on Nitric Oxide Synthase**

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Nitric oxide synthase (NOS) is an enzyme that catalyzes the synthesis of nitric oxide, an important cell signaling molecule. The three isoforms of NOS are neuronal (nNOS), endothelial (eNOS), and inducible (iNOS), which function in the nervous, cardiovascular, and immune systems, respectively. However, dysregulation of NO can contribute to diseases, including septic shock. Therefore, NO production is tightly controlled by many factors. For example, the Hsp90/Hsp70-based chaperone system regulates NOS ubiquitination and proteasomal degradation. Hsp90 stabilizes NOS and prevents ubiquitination, while Hsp70 promotes CHIP-dependent ubiquitination and subsequent proteasomal degradation. CHIP (C terminus of Hsp70 interacting protein) is an E3 ligase that, in conjunction with E1 activating and E2 conjugating enzymes, ubiquitinates proteins targeted by Hsp70. We plan to identify the ubiquitination site on all three NOS isoforms, starting with nNOS. Ubiquitinated nNOS was generated in an *in vitro* ubiquitination reaction containing nNOS, Hsp70, CHIP, E1, E2, and His-tagged ubiquitin. To isolate ubiquitinated nNOS, the reaction was filtered by size and then ubiquitinated nNOS was pulled down with nickel magnetic beads. The His-tagged ubiquitin nNOS conjugates were eluted with imidazole, digested with trypsin, and will be analyzed by nano-LC/MS/MS for the additional mass of two lysine amino acids that signify modification by ubiquitin. Determining the location of ubiquitination on NOS will be beneficial in understanding how the Hsp70/CHIP chaperone machinery impacts NOS recognition, ubiquitination, and degradation.

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21. The Potential Role of Drug Transporters in Resistance to Immunomodulatory Drugs (IMiDs) Therapy

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The immunomodulatory drugs (IMiDs), thalidomide, lenalidomide and pomalidomide, play a critical role in the treatment of multiple myeloma (MM). In combination with other agents, outcomes for MM patients have been greatly improved with IMiD therapy. Unfortunately, patients will ultimately develop resistance to these agents, though the mechanisms of resistance remain largely uncharacterized. It was previously reported that both lenalidomide and pomalidomide are substrates of p-glycoprotein (MDR1, ABCB1), though their clinical relevance remain unclear. Despite nearly identical chemical structures, the pharmacokinetics of lenalidomide and pomalidomide are dramatically different. Data from our lab showed significant difference between these two agents with respect to brain penetration in mice, apparent permeability (Papp) across MDCKII cell monolayers, and uptake in leukemic cells, independent of P-gp expression and despite similar Papp in a parallel artificial membrane permeability assay. Collectively, these data point to other, yet undetermined transporters that differentially modulate membrane permeability and which may be involved in development of resistance to these agents. To characterize differences in uptake in MM cell lines, we performed uptake studies for all three IMiDs in MM cell lines both sensitive and resistant to dexamethasone and lenalidomide. For each IMiD, we observed 2 to 3-fold differences in uptake among the cell lines and up to approximately 10-fold differences between the IMiDs within a given cell line. We are now proceeding both with targeted studies using established transporter models as well as a screen using endoribonuclease prepared siRNA to identify new transporters responsible for the observed differences in uptake and transport among the 3 IMiDs.

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Background Bupropion, an atypical antidepressant and smoking cessation aid, is associated with wide inter-subject variability in its efficacy and safety. Both pharmacokinetics (PK) and pharmacodynamics (PD) of bupropion and its metabolites are complex and reported to be stereoselective. As bupropion is thought to act on multiple central nervous system (CNS) targets, understanding CNS pharmacokinetics is critical to explain variability in bupropion’s therapeutic and toxic effects.

Hypothesis Relative exposures of bupropion enantiomers to each other and to their corresponding phase I diastereomeric pharmacologically active metabolites are different between blood and brain; these differences prevent reliance solely on systemic exposure measures to understand interpatient differences in response to racemic bupropion administration.

Objective of the proposed research is to characterize the exposure of bupropion-related substances in rat plasma and brain and develop rat Physiologically-based Pharmacokinetic (PBPK) models.

Methods Racemic bupropion (10 mg/kg) and S, S-hydroxybupropion (2 mg/kg) were administered subcutaneously to adult male Sprague Dawley rats (n = 24/compound). Brain and plasma were collected from rats (n = 3) at eight time points for up to 6 hours and analyzed for enantiomers and phase 1 diastereomer metabolites using a chiral LC-MS/MS method. Rat plasma protein and brain homogenate binding studies were conducted for all analytes to correct for unbound fraction using the equilibrium dialysis method. Noncompartmental analysis (NCA) was performed using Phoenix WinNonlin® (version 8, Certara). A full PBPK model was developed for R-bupropion, S-bupropion and preformed S, S-hydroxybupropion in Simcyp® (version 18, Certara).

Results The unbound brain to unbound plasma AUC0-6h ratio (Kp, uu) of R- and S-bupropion were 0.71 and 0.81 respectively. A 2 to 4-fold difference in Kp, uu of both oxidative (R, R-hydroxybupropion, preformed S, S-hydroxybupropion) and reductive metabolites (R, R- and S, S-threo-hydrobupropion; S, R- and R, S-erythro-hydrobupropion) was observed. PBPK model parameters (AUC0-6h, Cmax) for R-bupropion, S-bupropion and preformed S, S-hydroxybupropion were within 2-fold range of the observed data (as summarized in the table below). The Simcyp® rat model currently lacks a full PBPK module for formed metabolites. Accordingly, a full PBPK model comprising the two bupropion enantiomers and all the metabolites will be developed using mrgsolve in R. This model will support development of a rat-to-human translational PBPK model.

Conclusion A full PBPK model for bupropion enantiomers and pre-formed S, S-hydroxybupropion has been developed for rat.

References

23. **Insight of Cytochrome P450 24A1 and Adrenodoxin Interaction as Modulated by Carbon-25 Hydroxylation of Vitamin-D Ligand**

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The mitochondrial cytochrome P450 24A1 (CYP24A1) is responsible for side chain hydroxylation of vitamin D, the initial step in deactivation of the bioactive vitamin D. Mutations in CYP24A1 lead to incomplete clearance of active vitamin D and correlate with idiopathic infantile hypercalcemia, or elevated calcium in blood serum. The vitamin-D hydroxylation step requires reduction of heme iron in CYP24A1, derived from the iron-sulfur cluster of the redox partner protein Adrenodoxin (Adx). In non-mitochondrial P450 enzymes, conformational selection and long-range allostery have been demonstrated to occur in connection to substrate binding and redox partner recognition. However, such long-range allostery has not been demonstrated for a mitochondrial P450 enzyme, which relies on an electron donor distinct from enzymes in the endoplasmic reticulum.

In the current work we report a combined approach of site-directed mutagenesis, protein nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography and chemical cross-linking to evaluate the CYP24A1-adrenodoxin complex from rat (a carbon 24 hydroxylase). As expected, the interaction was primarily driven by the putative recognition domain of Adx (helix-3). However, the NMR data from titrations with CYP24A1 bound to substrate (1,25-dihydroxyvitamin D) versus the supplement (1α-hydroxyvitamin D) indicates that modulation of the redox-binding surface occurs in response to ligand binding in a way that confers or disrupts specificity of the redox complex. In order to characterize specific vitamin-D, active site interactions, we have also initiated co-crystallization of CYP24A1 with a set of azole-containing vitamin-D analogs.

These findings provide a structural basis for poor turnover of side-chain modified vitamin-D analogs like 1α-hydroxyvitamin D. More broadly, this work also represents high-resolution evidence in support of functional long-range and substrate driven allostery in a mitochondrial P450 enzyme.
24. **Investigation of the Interaction between Human CYP27B1 and its Redox Partner, Human Adrenodoxin**

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1α25(OH)2D3, the hormonally active form of Vitamin D3, plays an essential role in many physiological conditions, including Ca2+ homeostasis, regulation of the immune system, and cell differentiation. The activation of Vitamin D3 goes through two chemical modifications mediated by cytochrome P450 enzymes: the carbon 25 hydroxylation followed by 1α-hydroxylation. Mutations in the CYP27B1 gene abolish the production of 1,25(OH)2D3 and are the cause for vitamin D-dependent rickets type 1 (VDDR-1). To complete catalysis, CYP27B1 requires an interaction with its redox partner, adrenodoxin (Adx). In this study, we use site-directed mutagenesis, chemical cross-linking, and immunoblotting to investigate the interaction sites between CYP27B1 and Adx. Here, we find that mutation of proximal surface residues R153 and K371 of CYP27B1 disrupt the interaction with Adx, while mutation of R459 (a site that plays a role in development of VDDR-1) and K375 of CYP27B1 still enable the interaction. In order to incorporate these data into a preliminary model of the CYP27B1-Adx complex, we also utilized protein NMR to investigate the dimerization of human Adx. Our result suggests that sites important for Adx dimerization are near the iron-sulfur cluster, consistent with the crystal packing interface of human Adx. In summary, in this study, we determine specific sites on CYP27B1 that are important for interacting with Adx, as well as provide insight toward a biologically significant CYP27B1-Adx complex.

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Vitamin-D insufficiency is associated with rickets, osteomalacia, cardiovascular disease, and chronic kidney disease and affects nearly 50% of the population. In humans, vitamin D is metabolized by a series of cytochrome P450 enzymes, including inactivation by the mitochondrial enzyme CYP24A1. CYP24A1 in humans initially hydroxylates either the carbon-23 or carbon-24 position of 1,25-(OH)₂D₃ followed by a series of reactions to produce additional metabolites. In order to function, mitochondrial P450s require the reduction-oxidation protein partner adrenodoxin. The human isoform of CYP24A1 is not easily expressed and purified recombinantly, likely due to the labile nature of the enzyme. Therefore, most current structural studies are based on the rat isoform, which does not display carbon-23 regioselective hydroxylation. As a way to study the human isoform, we have initiated use of a fusion construct of human CYP24A1 and adrenodoxin (adx_hCYP24A1). The fusion contains a short linker sequence modeled after a bacterial P450-redox partner fusion. We have started to characterize the adx_hCYP24A1 fusion construct through substrate, analog, and styrylimidazole inhibitor binding. The ability of the fusion construct to bind ligand is measured by a change in the protein absorption spectrum. Notably, we've observed that the substrate for CYP24A1, 1,25-(OH)₂D₃, does not promote a change in spin state while the substrate analog and supplement 1α(OH)D₃ does promote a change to high spin, suggesting that 1) the binding mode for the analog is distinct from that of the substrate, and 2) the presence of adrenodoxin stabilizes a closed form of CYP24A1 that is specific to blocking access of 1,25(OH)₂D₃. For further characterization, we plan to complete additional ligand binding experiments, varying conditions, and X-ray crystallography studies in the presence and absence of ligand with the goal of understanding the adrenodoxin-CYP24A1 interaction and the structure of human CYP24A1 for use in developing potential therapeutic treatments of vitamin-D insufficiency.
26. Vitamin D 1α-Hydroxylase Purified Recombinanty as CYP27B1-Adrenodoxin Fusion

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Vitamin D metabolism consists of many reactions which are mediated by cytochrome P450 enzymes, including the production of vitamin D's most biologically active form. CYP27B1 hydroxylates 25(OH)D₃ at the 1α position to produce 1α,25(OH)₂D₃, which is able to interact with the vitamin D receptor and modulate the expression of target genes. Currently no structures of CYP27B1 exist, but there are several homology models created using the crystal structure of CYP24A1 from rat as a template. While these homology models have allowed for preliminary computational studies to be carried out in regards to structural aspects of CYP27B1, low amino acid sequence homology between these two enzymes at 32% illustrates a critical need for an empirical CYP27B1 structure. However, the recombinant production of membrane-associated CYP27B1 for in vitro structural studies has proven challenging, due in part to the difficulty of expressing and purifying sufficient quantities of the recombinant protein. In order to address this, we have employed a fusion construct of murine CYP27B1 linked to the C-terminus of its soluble redox partner, adrenodoxin, using a linker derived from a natural P450-ferrodoxin fusion. This construct has allowed for increased overall production of purified and stable CYP27B1. In order to further increase yield and stability of this protein, we have also incorporated the P450 inhibitor ketoconazole into the expression media. The incorporation of this broad-rangeazole inhibitor allows us to further increase yields. These preliminary results lay the foundation for continued structural characterization of this critical vitamin D metabolizing enzyme by X-ray crystallography, ¹⁹F NMR, and other biophysical methods.
27. **Conversion of tacrolimus to less active metabolites by gut bacteria**

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Tacrolimus exhibits low and variable drug exposure after oral dosing, but the contributing factors remain unclear. Based on our recent report showing a positive correlation between fecal abundance of *Faecalibacterium prausnitzii* and oral tacrolimus dose in kidney transplant patients, we tested whether *F. prausnitzii* and other gut abundant bacteria are capable of metabolizing tacrolimus. Incubation of *F. prausnitzii* with tacrolimus led to production of two compounds (the major one named M1), which were not observed upon tacrolimus incubation with hepatic microsomes. Isolation, purification, and structure elucidation using mass spectrometry and nuclear magnetic resonance spectroscopy indicated that M1 is a C-9 keto-reduction product of tacrolimus. Pharmacological testing using human peripheral blood mononuclear cells demonstrated that M1 is 15-fold less potent than tacrolimus as an immunosuppressant. Screening of 22 gut bacteria species revealed that most *Clostridiales* bacteria are extensive tacrolimus metabolizers. Tacrolimus conversion to M1 was verified in fresh stool samples from two healthy adults. M1 was also detected in the stool samples from kidney transplant recipients who had been taking tacrolimus orally. Prediction of M1 formation using *In vitro* model suggested extensive tacrolimus metabolism by intestinal gut bacteria. Together, this study presents gut bacteria metabolism as a previously unrecognized elimination route of tacrolimus, potentially contributing to the low and variable tacrolimus exposure after oral dosing.
28. **Gut bacteria convert etoposide to demethylated metabolite**

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Etoposide is a first-line chemotherapeutic agent used for lung, testicular and ovarian cancer. It is a narrow therapeutic index drug, and its bioavailability exhibits high inter- and intra-individual variability. The responsible factors remain unclear. The objective of this study was to examine the role of gut microbiota in etoposide disposition. Upon incubation of etoposide (100 µM) with mouse cecal contents or human stool samples, a rapid disappearance of etoposide was observed (50% loss in 3h), which was accompanied by the appearance of a new metabolite peak (M1) on HPLC-UV chromatogram. Boiling of cecal/stool samples or pre-treating the cecal contents with antibiotics abrogated M1 production, suggesting that the reaction is mediated by viable gut bacteria. Subsequent isolation, purification, and structure elucidation of M1 revealed that M1 is an O-demethylated product of etoposide (i.e., etoposide catechol). As compared to etoposide, M1 exhibited 3 to 5-fold lower cytotoxicity in MCF7 and Hela cells and similar permeability across Caco-2 monolayer. Among 48 human gut bacteria tested, only *Eubacterium limosum* was shown to metabolize etoposide into M1. These results suggest that gut microbiota-mediated metabolism may be a previously unrecognized elimination route of etoposide, potentially contributing to the variable etoposide exposure after oral dosing.
29. Mechanisms of Acquired and Intrinsic Resistance to CDK4/6 Inhibition in Estrogen Receptor Positive (ER+) Breast Cancer

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CDK4/6 inhibitors have improved rates of progression free survival in patients with metastatic, ER+ breast cancer, but resistance limits their clinical efficacy. Various mechanisms of resistance to CDK4/6 inhibitors have been reported, but a comprehensive understanding of this resistance remains elusive. We generated preclinical models of acquired (AR) and intrinsic (IR) resistance to CDK4/6 inhibitors using the ER+, estrogen-dependent MCF-7 and T47D breast cancer cell lines. Cells were cultured with either continuous high dose (500nM) or a dose-escalation (50nM to 500nM) of the CDK4/6 inhibitor palbociclib over three months. RNA expression and gene set enrichment analysis (GSEA) was used to nominate potential pathways associated with AR and IR palbociclib resistance. Reverse phase protein array (RPPA) and western blots were used to measure protein and phosphoprotein levels in the CDK4/6 inhibitor resistant cell lines to confirm gene upregulation at the protein level. Cellular proliferation assays were performed to calculate the half-maximal inhibitory concentration (IC50) with various inhibitors. Proliferation assays confirmed that MCF-7 AR and IR cells are resistant to palbociclib (IC50 = 0.808µM, 2.08µM, respectively) compared to parental cells (60nM); similar results were observed in the T47D cell lines. Cells resistant to either palbociclib, ribociclib, or abemaciclib demonstrated cross resistance to all three inhibitors. GSEA of transcriptomic data identified 579 genes (from MCF-7 AR cells) and 936 genes (from MCF-7 IR cells) that are differentially expressed between palbociclib-resistant MCF-7s and parental controls. From GSEA analysis, the interferon (JAK/STAT) signaling pathway was one of the most differentially expressed pathways identified between parental MCF-7s and MCF-7 AR cells, while hypoxia and HIF1α signaling was highly enriched in MCF-7 IR cells. A similar pattern was observed in MCF-7 cells harboring an activating ESR1 mutation (Y537S). Western blot analyses showed that baseline expression of phospho-STAT1 is significantly elevated in AR cells and HIFα is overexpressed in IR cells. In cellular proliferation assays, palbociclib-resistant MCF-7s and T47Ds retain sensitivity to JAK/STAT inhibitors such as the JAK2-selective compound AZ960. In conclusion, our data suggests that the intrinsic and acquired resistance to CDK4/6 inhibition in ER+ breast cancer may be controlled by different biological pathways, which may have implications for future patient selection and treatment.
30. **Dynamics of ternary redox complex influencing cytochrome P450 metabolon: an NMR study**

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Cytochrome P450s (cytP450s) are a ubiquitous superfamily of enzymes that are responsible for the catalysis of many different substrates including over 75% of the drugs on the market. In order to do this, each turn of the catalytic cycle requires two electrons which are donated by either Cytochrome P450 Reductase (CPR) or cytochrome b₅ (cyt b₅). We aim to understand how CPR and cyt b₅ compete for binding to cytP450 and to unravel the structural and dynamic basis for this competition and how the presence of a lipid membrane environment and various substrates impacts these interactions. Here we use NMR spectroscopy and other biophysical tools to characterize the interplay between this ternary complex of cytP450, CPR, and cyt b₅. We also use stopped-flow spectrophotometry to examine the effect of lipid nanodiscs on the metabolism of different substrates by the ternary complex. Our data reveals how substrates differently modulate the interaction between the two redox partners and cytP450 by enhancing the preference of cytP450 for one redox counterpart over the other. This work starts to define a complete picture of how this ternary complex works together to metabolize various substrates.

31. **Scaling Up a Drug-Drug Interaction Screen to a High Capacity and More Accurate Model using High Throughput Liquid Chromatography-Tandem Mass Spectrometry**

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Discrepancies in data between early stage fluorescent-based DDI screenings and a late stage LCMS based DDI assay has introduced a need for a more accurate early stage DDI screening. A high throughput mass spectrometer based assay is being developed using Apricot Design’s Dual Armed autosampler (ADDA) to bridge the gap. This technology allows for 13 second injections compared to the 114 second method used in the late stage DDI assay. Column and gradient selection was conducted based on quality of signal from a standard cocktail mix of the internal standards associated with the CYP2C9, CYP2D6, and CYP3A4 isoforms. This workflow will allow for an increase in the quality of data from the current fluorescent-based early stage assay while maintaining its high throughput nature.
32. A Novel Fluorophosphonate Probe for the Discovery of New Valacyclovir-Activating Enzymes

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Introduction: Prodrugs, inactive pharmaceuticals that require in vivo activation to take effect, represent an ever-increasing share of approved therapies. Most prodrugs are activated enzymatically, making identification of said enzymes important for understanding their pharmacokinetic/pharmacodynamic profiles and possible drug interactions. Typically, this is accomplished by activity-guided fractionation of the appropriate cell/tissue lysates.

Methods: Our lab is instead utilizing a modern chemoproteomic approach known as activity-based protein profiling for the discovery of novel pre-systemic activating enzymes of the antiherpetic ester prodrug valacyclovir (VACV). The only such enzyme currently known is a serine hydrolase (SH) known as BPHL. We synthesized a novel fluorophosphonate probe structurally similar to one of the only known inhibitors of BPHL which covalently labels the catalytic serine residue of several members of the SH family.

Results/Conclusions: Inhibition assays in Caco-2 cell homogenates revealed that our novel probes significantly inhibited VACV hydrolase activity compared to control and commercially available fluorophosphonate probes. We reasoned that competition between the probe and VACV would elucidate its binding partners and thus its potential activators. Thus, an assay was developed in which the biotinylated probe was used to label and enrich SHs with and without VACV competition, followed by on-bead trypsinization and bottom-up proteomic identification. Ultimately, four SHs were shown to have significantly reduced labeling in the presence of VACV. These enzymes are being tested for activity towards VACV with the ultimate goal of confirming the same activity in vivo.
33. Identifying Sites of Protein-Protein Interaction on Dimeric Neuronal Nitric Oxide Synthase P450 by Cross-Linking and Mass Spectrometry

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Neuronal nitric oxide synthase (nNOS) is a P450 enzyme responsible for the production of nitric oxide, an important neurotransmitter and signaling molecule in the brain. nNOS is tightly regulated through several mechanisms including the formation of a stable homodimer. While crystal structures have been successfully resolved for the heme-containing oxygenase and flavin-bound reductase domains of nNOS, the nature of interactions between full-length functional nNOS homodimers are not completely understood. Recent structural models from single particle EM studies demonstrate formation of a stable oxygenase domain dimer, however the interaction between domains in other regions of the nNOS homodimer have not been investigated in solution. In this study, we utilized a novel method to identify intermonomer interactions by CXL-MS. Specifically we separated intermonomer from intramonomer interactions by treatment with a MS-cleavable crosslinker then excising and analyzing both monomeric and dimeric nNOS from a single cross-linked sample. All seven intramonomer crosslinks present in monomeric nNOS were also found in dimeric sample, and 59 unique crosslinks were identified in the dimeric sample not present in the monomeric. These crosslinks agree with the existing model of holoenzyme domain movement, and identify numerous interdomain and intermonomer interactions not previously identified on nNOS. These data enhance our understanding of the nNOS holoenzyme’s structural arrangement in solution and provide useful insight into potential target sites for enzymatic regulation.

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34. **Associations Between Transient Receptor Potential Channel Polymorphisms and Aromatase Inhibitor Musculoskeletal Syndrome (AIMSS) in Breast Cancer Patients**

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**Introduction:** Aromatase Inhibitors (AIs) are used to treat post-menopausal women diagnosed with estrogen receptor positive (ER+) breast cancer. AIs inhibit CYP19 aromatase, the enzyme responsible for the peripheral conversion precursor androgens into estrogens. Approximately 30% of patients prescribed AIs discontinue their use before the recommended 5 years of treatment, in part, due to severe aromatase inhibitor-associated musculoskeletal syndrome (AIMSS) side effects. AIMSS are comprised of arthralgias and musculoskeletal symptoms, which often begin in distal joints of the hands and feet. The Transient Receptor Potential (TRP) channels are ion channels responsible for sensory functions including pain, taste, smell, and thermal and mechanical sensitivities. In murine models, both steroidal and non-steroidal AIs induce pain symptoms by interacting with the TRP Ankyrin 1 (TRPA1) channel or TRP vanilloid-1 (TRPV1) channel. AI-induced pain symptoms observed in mice are similar to AIMSS and include neurogenic inflammation, mechanical hypersensitivity, acute nociception, and reduced grip strength. It has been shown that the steroid hormone precursor androstenedione, also activates TRPA1 and TRPV1 and contributes to the pain response observed in mice. Inherited germline polymorphisms in TRPA1 and TRPV1 have been associated with differential pain and sensory responses to various stimuli within healthy populations and in patients with a variety of disease states.

**Methods:** We identified Single Nucleotide Polymorphisms (SNPs) in TRPA1 (rs920829, rs11988795, and rs13255063) and TRPV1 (rs8065080 and rs222747) that were previously associated with pain responses in non-cancer patients, and tested these for possible association with AIMSS. Taqman allelic discrimination assays were used to genotype patients enrolled in the Exemestane and Letrozole Pharmacogenetics (ELPh) trial, which included women with ER-positive early stage breast cancer randomly assigned to AIs, either adjuvant letrozole or exemestane. Tests for associations were run between SNPs and either AIMSS, Health Assessment Questionnaires (HAQ), or musculoskeletal-related treatment discontinuation.

**Results:** Of the 456 patients genotyped, there was a trend towards significance with the TRPV1 variant rs222747 and AIMSS observed at 12 months of AI treatment (Additive Model; p-value=0.053; OR=0.72; [95% CI 0.51-1.0]). In addition, there was a trend towards significance between the TRPA1 variant rs920829 and change in HAQ score (Additive Model; p-value=0.057; OR=0.66; [95% CI 0.43-1.01]).

**Conclusions:** This is the first study to test for associations between germline variants in TRPA1 and TRPV1 in AIMSS and our results suggest that genotypes may help predict women less likely to experience AIMSS. Currently, we are attempting to validate these results in additional larger patient cohorts of AI treated breast cancer patients.
35. Functional characterization of P450 17A1 mutations to probe a possible allosteric site

Neikelyn Burgos and Dr. Richard Auchus
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The enzyme cytochrome P450 17A1 is essential for all androgen biosynthesis in the adrenal and gonads. P450 17A1 catalyzes the 17-hydroxylase reaction, which is required for cortisol synthesis, and the 17,20-lyase reaction, which is a crucial step for 19-carbon androgen precursor synthesis. The 17,20-lyase activity – but not 17-hydroxylase activity – requires the cofactor protein cytochrome b5 (b5) to enhance the reaction. P450 17A1 is the target of abiraterone acetate, an approved treatment for castration-resistant prostate cancer. However, a major disadvantage of abiraterone acetate is that the drug inhibits both activities of P450 17A1, and 17-hydroxylase inhibition shifts steroid production from cortisol to mineralocorticoids. NMR studies demonstrate changes in a set of residues containing the F-G loop and part of the β-sheet region upon b5 binding to P450 17A1. We hypothesize that this region might be a druggable target to selectively inhibit 17,20-lyase activity. The objective of this study is to characterize the activity of P450 17A1 mutations in this site to determine if these perturbations affect 17,20-lyase activity but not 17-hydroxylase activity. The P450 17A1 variants were constructed using overlapping PCR in a pcDNA3 vector followed by, a restriction enzyme digest of the cDNA and ligated into a pCW bacterial expression vector. Sanger sequencing confirmed successful mutagenesis for the variants N52D and L217R. Screening in HEK293 cells showed mainly intact 17-hydroxylase activity; therefore, we expressed in bacteria and purified our P450 17A1 mutations. Reconstituted assays contained P450 17A1, 1 mol equivalent of P450-oxidoreductase in presence or absence of 1 mol equivalent of b5, 1 mM NADPH, and 20 μM substrate, either pregnenolone, 17-hydroxypregnenolone or progesterone. Preliminary results suggest that when compared to P450 17A1 wild-type these variants demonstrate a significant loss of both the 17-hydroxylase and the 17,20-lyase activity but that b5 is less effective in stimulating 17,20-lyase activity.
36. **Retrospective analysis of CYP3A4 mRNA induction in a 3-donor panel informs risk of misclassification after single donor screens**

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

Cytochrome P450 3A4 induction screening results leading up to candidate nomination are typically obtained in hepatocytes from a single donor. Expectations from project teams are that data from a single donor represent results obtained in a 3 donor study that is typical of regulatory agency submission. To that end, we have evaluated interdonor variability of multi-concentration CYP3A4 mRNA induction responses for 21 compounds (mix of internal and model compounds) in plated hepatocytes within a conventional 3-donor experiment. Data were from nine different donors, each prequalified by in-house criteria for suitability for induction testing. As EC50 and Emax values are not always obtainable, we interrogated data from 334 percent positive control (PC) values matched by compound and concentration across 3 donors. Values < 10% PC in a single donor screening study would typically be assigned a clear “non-inducer” whereas values ≥ 20% might be assigned as a probable inducer. Therefore, the likelihood that results from two additional donors would change classification from a non-inducer to probable inducer is of interest. Of 171 data points with < 10% PC, in only 5 cases (< 3%) were corresponding concentration matched data ≥ 20% PC in at least one of the other two donors. These data were 0.9% and 25% for AB-6, 5.6% and 26%, 5.6% and 27% for AB-7, 4.4 and 27% for rosiglitazone, and 5.6% and 44% for pleconaril. Conversely, of 134 data points of ≥ 20% PC, the same 5 cases (< 4%) of matched data had < 10% PC in at least one of the other two donors. Of 8 compounds exhibiting global means of ≤ 10% positive control at the highest concentration examined (median, 20 µM), representing 108 percent PC data points, no single value exceeded 10%. Overall, these data imply a small likelihood of gross misclassification from single concentration screening. We also examined statistical variability in concentration-matched percent PC data across 3 donors. As expected highest variability occurred with mean 0-10% positive control (CV = 1.0, N=43), followed by 10-20% (CV=0.62, N=9), 20-30% PC (CV = 0.5, N =7) and finally >30% (CV = 0.26, N = 34). Thus, the likelihood of compounds exhibiting percent PC categorization changes within these intervals, decreases as percent PC increase. These data should help in evaluating induction risk of single donor screens.
37. Applying IVIVE of Rat Liver Partitioning and Clearance to Improve Human Clearance Prediction

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It is imperative to understand the unbound tissue partitioning ($K_{P_u,u}$) and clearance in the liver for compounds whose disposition are determined by both transporters and metabolism. $K_{P_u,u}$ and clearance of substrates that are transported via the Organic Anionic Transporter Polypeptide 1B1 (OATP1B1) have been universally under-predicted. As a result, the addition of albumin to in vitro studies has been suggested in order to improve in vitro to in vivo extrapolation. In this study, we predicted $K_{P_u,u}$ and in-vivo clearance of compounds using rat and human hepatocytes. Rat liver $K_{P_u,u}$ and in vivo clearance was obtained via steady state infusion. Uptake in the presence and absence of transporter inhibitors was determined with cryopreserved hepatocytes in suspension with 4% bovine serum albumin (BSA). The unbound fraction was determined for both plasma and 4% BSA, and subsequently used to correct for free active and passive uptake clearance. Metabolic intrinsic clearance was measured via either oxidative or glucuronidation pathways using human and rat liver microsomes. Liver $K_{P_u,u}$ was calculated using various published methods. Using the extended clearance model (ECM) as well as total uptake, intrinsic clearance was calculated for all the substrates, and then scaled to in vivo clearance using the well-stirred model. From evaluation of uptake into human hepatocytes, the addition of albumin increased both the passive and active intrinsic clearance proportionally. However, addition of albumin effectively slowed the reaction sufficiently to enable accurate measurement of uptake. As a result, $K_{P_u,u}$ was under-predicted while incorporating the active and passive uptake clearances as well as the metabolism. Correspondingly, total uptake better predicted rat and human in vivo clearance when compared with the extended clearance method. Appropriate scaling factors were determined from rat in vitro and in vivo studies, and implemented to accurately scale human in vitro clearance to in vivo within two-fold of the observed value.
38. The Impact of Phenytoin Exposure on the Liver Proteome of Neonatal and Adult Mice Using DIA-MS Technology

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Phenytoin, a drug listed on the World Health Organization's Model List of Essential Medicines, has been widely used for the treatment of seizures and several other indications. However, it has a narrow therapeutic index and some severe side effects, such as drug-induced liver injury. In addition, phenytoin is a well-known inducer of several drug-metabolizing enzymes and transporters expressed in the liver, which could lead to severe drug-drug interactions. In the present study, an untargeted data-independent acquisition (DIA) proteomics approach was utilized to investigate 1) the impact of phenytoin exposure on the liver proteome of neonatal and adult mice, 2) the proteome differences between neonatal and adult mice livers, and 3) the differences between liver S9 fraction and microsome proteomes. About 2000 protein groups were identified in both liver S9 and microsomes samples prepared from neonatal and adult mice. Markedly different liver proteome profiles were observed between newborn (day 5) and adult mice (day 60). Intraperitoneal injection of phenytoin led to the alteration of multiple biological processes in the liver with a greater impact on the liver proteome in neonates than that in adults. However, the impact of phenytoin exposure on drug-metabolizing enzymes is smaller in neonatal mice than that in adult mice. In addition, we verified that most of the drug-metabolizing enzymes, along with many other membrane proteins, are enriched in the microsomes samples comparing to S9 fraction samples. Taken together, our study provides an insight into the impact of phenytoin treatment on the liver proteome of neonatal and adult mice, which might lead to a better understanding of the phenytoin-induced hepatotoxicity and drug-drug interactions.
39. Esterase Activities And Inhibition In Plasma Of Different Species

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Esterases are ubiquitous enzymes in animals and human. They catalyze hydrolysis of esters, thioesters and amides, and are widely present in tissues and body fluid. There are also species differences in esterase composition and expression. In this work, ketoprofen methylester (KME) was used as a substrate to evaluate esterase activity in cryopreserved and fresh plasma samples of mouse, rat, cat, dog, pig, sheep, cattle and horse. KME was incubated in the plasma samples and monitored at a series of time points by LC/MS. A variety of esterase inhibitors were evaluated to prevent hydrolysis of KME in rat, dog, pig and cattle plasma. The results indicated that hydrolytic activity to KME in the plasma of those species can be divided into three classes: strong hydrolysis (mouse, rat and horse), possible product inhibition (cat and dog), and weak hydrolysis (pig, sheep and cattle). The inhibition assay indicated that some esterase inhibitors were more effective than others in inhibition of esterase activity in the plasma but they were species dependent. This work provides useful information in product and assay design during veterinary drug discovery and development.

40. Lipid Bilayer Nanodiscs: A versatile tools for NMR Structural Characterization of Cytochrome P450 and its Redox Partners

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Abstract not available at time of printing
41. **Comparison of primary human hepatocyte spheroid generation and performance in different culture systems**

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As new development programs desire small molecule drugs with lower metabolic turnover, there is increasing need for hepatocyte models to maintain metabolic function for longer times in culture. Standard 2 dimensional (2D) primary hepatocyte cultures rapidly lose typical hepatocyte functionality, e.g. cytochrome P450 (CYP) activity, which can lead to underestimation of metabolism rates for lower turnover drugs and inability to create enough metabolite for subsequent identification. Primary human hepatocytes are capable of self-assembling into small spheroids when cultured in low-attachment plates. Compared with standard 2D culture, spheroids generated from PHH allow for more in-vivo-like cell organization, including extensive contact of adjacent cells. Thus, spheroid culture may support hepatocyte functionality for extended time. Many different methods are available for facilitating the formation of hepatocyte spheroids in culture. In this study, we compared and optimized the formation, culture and performance of cryopreserved primary human hepatocytes (PHH) in different spheroid culture systems and under various culture conditions and compared metabolic function and viability of the spheroids over 28 days in culture. Overall, all the different spheroid culture methods including ULA U-bottom and V-bottom plates as well as hanging drop culture and dedicated spheroid plates were suitable for PHH spheroid formation and long term culture. Little differences with respect to viability, functionality and assay suitability were observed. The formation of metabolites from CYP3A4, 1A2, and 2B6 activity was detectable for the full examination period of 4 weeks in most of the analyzed approaches. Hepatocyte donor-dependent differences in the persistence of metabolic activity were visible over time.
42. **Allosteric interactions in CYP3A4 probed by site directed mutations**

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Human CYP3A4 is involved in metabolism of more than 30% of drugs administered in clinical practice, and concomitantly represents the main locus for drug-drug interactions. In addition to a spacious and malleable substrate binding pocket which can accommodate two substrate molecules, CYP3A4 has a peripheral allosteric binding site formed at the protein – membrane interface between F-F’ and G-G’ loops and lipid head groups. Binding of effector molecules at this site changes the shape and size of the substrate binding pocket, and also modulates the conformational dynamics of the protein.

Using CYP3A4 incorporated in Nanodiscs, we compared the functional properties of several single point mutations at positions 211-215 (F-F’ loop), which are known to be involved in cooperativity and allosteric interactions. Combination of model substrates progesterone (PGS) and carbamazepine (CBZ) has been used as a well-established example of asymmetric heterotropic interactions [1,2]. Activation of CBZ epoxidation in the presence of PGS, which is observed in wild-type CYP3A4, was inhibited in most mutants. In addition, spectral titration experiments revealed substantial changes in Type I binding of both substrates in several mutants, most pronounced in L211H and L211W with PGS as a substrate.

Variations in substrate binding and metabolism, perturbations of cooperative properties and changes in the allosteric effect of PGS on the rate of CBZ epoxidation observed in CYP3A4 mutants indicate the presence of a highly sensitive regulatory mechanism in this important enzyme. Prediction of drug-drug interactions mediated by CYP3A4 requires better understanding of the specific details of allosteric interactions with various effectors at the protein-membrane interface.

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43. **LC-MS/MS Analysis of Cytochrome P450 Mediated Butylone Metabolism**

*Joshua Appel, Ping Lin, and Diane M. Calinski*

Synthetic cathinones (more commonly referred to as “bath salts”) have become popular drugs of abuse within the last decade. Users are attracted to many of the cocaine-mimicking physiological effects that synthetic cathinones provide, including euphoria and increased energy, alertness, and sociability. Unfortunately, synthetic cathinones also produce dangerous effects including seizures, cerebral edema, and heart attack. Due to such complications, synthetic cathinone exposure generated over 6,000 phone calls to poison control centers and nearly 23,000 emergency department visits in 2011. Despite the rise in the abuse of synthetic cathinones, a complete and through understanding of synthetic cathinone metabolism has yet to emerge. Identification of the metabolic pathways of synthetic cathinones may lead to improved patient treatment and to the potential development of more intelligently-designed overdose treatments. The goal of this study is to evaluate the metabolism of butylone, one synthetic cathinone known to be abused. Here, using liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS), we determined the phase I metabolites of butylone, and the cytochrome P450s that are responsible for the metabolism of butylone. The major metabolites were identified as Nor-butylone, N-hydroxyl butylone, dihydroxy-butylone, and dihydro-butylone. Of the four cytochrome P450s specifically investigated, CYP2D6 was determined to be the key enzyme for butylone metabolism. Chemicals that are structurally similar to butylone, such as 3’ 4’-methylenedioxyamphetamine (MDMA or Ecstasy) and methyline, are known to inhibit CYP2D6, which can complicate normally predictable metabolism of the drugs or co-administered drugs. Currently, we are investigating butylone for the inhibition of CYP2D6 and other cytochrome P450s.
44. The Effect of Flibanserin on Estrogen Receptor-Mediated Breast Cancer Growth

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Introduction: Breast cancer is the most commonly diagnosed malignancy in women, with an average lifetime risk being nearly 1 in 8. Approximately 70% of breast cancers express the estrogen receptor (ER) and women diagnosed with “ER-positive” breast cancer receive anti-estrogen therapy. One type of therapy, used in post-menopausal women, is suppression of endogenous estrogen production using aromatase inhibitors (AI) that block the conversion of precursor androgenic steroids into estrogens. In pre-menopausal women, selective estrogen receptor modulators (SERMs), such as tamoxifen, are standard of care. In order for anti-estrogens to be effective, patients should avoid exogenous sources of estrogenic compounds. One of the common side effects associated with menopause and anti-estrogen therapies is decreased libido. Flibanserin (Addyi) is a non-hormonal serotonin 1A receptor agonist approved for treatment of hypoactive sexual desire disorder (HSDD) in women. However, the extent to which flibanserin has estrogenic properties is currently unknown. We hypothesize that flibanserin can bind to ER and promote ER mediated breast cancer cell growth.

Methods: We assessed the ER agonist activity of flibanserin in the ER-positive, estrogen-dependent MCF-7 breast cancer cell line. Cell proliferation assays were performed by treating cells with flibanserin on a logarithmic scale, ranging from 1pM to 10μM, and stained with Crystal Violet dye to measure its relative effect on MCF-7 cell growth. Additionally, ER competitive binding assays were completed to determine flibanserin binding to the estrogen receptor.

Results: Preliminary computational models suggest that flibanserin can bind to ER with a binding energy comparable to 17 beta-estradiol (-9.03 kcal/mol and -10.94 kcal/mol, respectively). Cells treated with 10μM flibanserin exhibited a 30% decrease in growth compared to control treated cells. However, we were unable to determine a half-maximal inhibitory concentration (IC50), as a decrease in cell proliferation was only observed in the highest concentration of flibanserin tested (10μM). A preliminary ER competitive binding assay suggests binding of the estrogen receptor by flibanserin, although more studies are needed to confirm.

Conclusions: Flibanserin does not exhibit ER agonist activity but may have modest ER antagonist effects at high doses. These results are consistent with the computational modeling and preliminary binding assays suggesting that flibanserin can bind to ER.
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