

Efflux transporters in drug disposition during pregnancy

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ABBREVIATIONS

1 α ,25-(OH)₂D₃, 1 α ,25-dihydroxyvitamin D₃; 25OHD₃, 25-hydroxyvitamin D₃; 25OHD₃-G, 25OHD₃-3-O-glucuronide; 25OHD₃-S, 25OHD₃-3-O-sulfate; ABC, ATP-binding cassette; AhR, aryl hydrocarbon receptor; AUC, area under concentration-time curve; BBB, blood-brain barrier; BCRP/Bcrp, breast cancer resistance protein; BPB, blood-placenta barrier; BUP, buprenorphine; Cryo-EM, cryogenic electron microscopy; CV, conventional; CYP, cytochrome P450; GD, gestational day; GF, germ-free; GLB, glyburide, GMR, geometric mean ratio; HEK, human embryonic kidney; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Mate, multidrug and toxin extrusion protein; MDCK, Madin-Darby canine kidney; MRP, multidrug resistance protein; MSD, membrane spanning domain; NBD, nucleotide binding domain; NBUP, norbuprenorphine; NBUP-G, norbuprenorphine-glucuronide; NFT, nitrofurantoin; Ntcp, sodium taurocholate cotransporting polypeptide; OATP/Oatp, organic-anion-transporting polypeptides; OCT/Oct, organic cation transporter; Octn, organic cation/carnitine transporter; P-gp, P-glycoprotein; R-MET, (R)-methadone; S-MET, (S)-methadone; THC, tetrahydrocannabinol; TM, transmembrane; UGT/Ugt, UDP-glucuronosyltransferase; WT, wild-type.

SIGNIFICANCE STATEMENT

Dr. Qingcheng Mao and his team have made significant contributions to the investigation of the role of efflux transporters, especially P-glycoprotein and breast cancer resistance protein, in maternal-fetal exposure to many xenobiotics: nitrofurantoin, glyburide, buprenorphine, bupropion, tetrahydrocannabinol and their metabolites. Studies of individual compounds and the expression of transporters during gestation and pregnancy have improved the understanding of maternal-fetal pharmacokinetics.

ABSTRACT

Evidence-based dose selection of drugs in pregnant women has been lacking due to challenges in studying maternal-fetal pharmacokinetics. Hence, many drugs are administered off-label during pregnancy based on data obtained from non-pregnant women. During pregnancy, drug transporters play an important role in drug disposition along with known gestational age-dependent changes in physiology and drug-metabolizing enzymes. In this review, as Dr. Qingcheng Mao's former and current lab members, we summarize the collective contributions of Dr. Mao, who lost his life to cancer, focusing on the role of drug transporters in drug disposition during pregnancy. Dr. Mao and his team initiated their research by characterizing the structure of Breast Cancer Resistance Protein [BCRP, ATP-Binding Cassette (ABC) G2]. Subsequently, they have made significant contributions to the understanding of the role of BCRP and other transporters, particularly P-glycoprotein (P-gp/ABCB1), in the exposure of pregnant women and their fetuses to various drugs, including nitrofurantoin, glyburide, buprenorphine, bupropion, tetrahydrocannabinol, and their metabolites. This review also highlights the gestation- and pregnancy-dependent transporter expression at the blood-brain and blood-placenta barriers in mice.

BODY OF MANUSCRIPT

Introduction

Pregnant women are a unique patient population from the perspective of drug disposition because maternal exposure to drugs also means simultaneous exposure of the fetus, which poses potential risks in fetal development. Given the scarcity of data on maternal-fetal pharmacokinetics and the safety and efficacy of drugs in this particular population, many medications are administered off-label to pregnant women without appropriate evidence-based guidance in dosing regimens. This knowledge gap is of great concern to both mother and fetus, as well as their healthcare providers. Pregnancy is associated with a wide range of physiological changes, including but not limited to increases in blood volume, cardiac output, and glomerular filtration rate, as well as alterations in the expression and activity of drug-metabolizing enzymes (Pinheiro and Stika, 2020), which may in turn alter the pharmacokinetics, safety, and efficacy profile of drugs administered during pregnancy.

Breast Cancer Resistance Protein (BCRP) and P-glycoprotein (P-gp) are members of the ATP-binding cassette (ABC) efflux transporter family encoded in humans by their respective genes *ABCG2* (Allikmets *et al.*, 1998; Austin Doyle *et al.*, 1998; Miyake *et al.*, 1999) and *ABCB1* (Ueda *et al.*, 1987). In rodents, Bcrp is encoded by *Abcg2* (Allen *et al.*, 1999) and two isoforms of P-gp are encoded by *Abcb1a* and *Abcb1b* (Borst and Schinkel, 2013). Like all ABC transporters, BCRP and P-gp bind and hydrolyze ATP via their highly conserved nucleotide binding domains (NBDs). P-gp has two tandemly repeated membrane spanning domains (MSDs), each of which consists of six transmembrane (TM) segments followed by an NBD. In comparison, BCRP is considered a “half transporter” with only one MSD, where the six TMs are preceded by an NBD, and it requires dimerization to be functional.

Both BCRP and P-gp are highly expressed in various physiological barriers, such as the blood-brain barrier (BBB), the placental syncytiotrophoblast, the intestinal epithelium, the liver bile canaliculi, and kidney proximal tubular epithelium (Maliepaard *et al.*, 2001; Aronica *et al.*, 2005; Fetsch *et al.*, 2006; Hodges, 2011). Both transporters have broad substrate specificities, including many structurally and

chemically unrelated hydrophilic and hydrophobic compounds (Saidijam *et al.*, 2017; Han, Gao, and Mao, 2018). The broad substrate specificity and high expression of BCRP and P-gp in organs important for drug disposition affords them critical roles in determining drug pharmacokinetics, efficacy, and toxicity (Safar *et al.*, 2019).

During pregnancy, P-gp and BCRP are primarily expressed at the apical membrane of the syncytiotrophoblast and play important roles in transplacental drug disposition and fetal exposure to xenobiotics (Rocchi *et al.*, 2000). The levels of BCRP protein in the term human placenta show large inter-individual variability up to 14-fold (Bircsak *et al.*, 2018). The expression in placental cells can be regulated by many factors, including but not limited to progesterone (Wang, Lee, Zhou, *et al.*, 2008), 17 β -estradiol (Wang, Zhou, *et al.*, 2006), prostaglandin E2 (Mason *et al.*, 2014), hypoxia signaling (Francois *et al.*, 2017), emotional distress (Mina *et al.*, 2015), and bacterial infection (Petrovic *et al.*, 2015). Nuclear receptors, such as Peroxisome Proliferator-Activated Receptor gamma (Lin *et al.*, 2017), constitutive androstane receptor, pregnane X receptor, and aryl hydrocarbon receptor (AhR), are highly expressed in the syncytiotrophoblast of the first and third-trimester placenta, and can also regulate BCRP expression in response to drugs and xenobiotics exposure when activated by their corresponding ligands. (Jiang *et al.*, 2010; Tan *et al.*, 2010; Tompkins *et al.*, 2010; Pavsek and Smutny, 2014).

This review written by Dr. Mao's former graduate students and postdoctoral researchers summarizes first, the research conducted by Dr. Mao in his early career and subsequently, his work as a principal investigator carried out in his laboratory at the University of Washington (2002-2023, Washington, USA). Together, these efforts have substantially advanced our understanding of the structure, function, and regulation of BCRP and P-gp in governing drug disposition, especially during pregnancy. In his all too brief but brilliant career, Dr. Mao passionately explored the role of transporters in pharmacology and trained numerous students and postdoctoral fellows who now wish to honor his memory.

Dr. Mao's early exploration of transporters

Dr. Mao's life-long research interest in membrane transporters started with his Ph.D. thesis research at the University of Bern (1991-1995, Switzerland), where he developed a method for purifying the mannose transporter in phospholipid vesicles from *Escherichia coli* that enabled measurement of both vectorial transport and phosphorylation activity (Mao *et al.*, 1995). During his postdoctoral training at the University of North Carolina (1995-1997, North Carolina, USA), Dr. Mao developed methods to produce and purify functional human P-gp using a *Saccharomyces cerevisiae* expression system. These methods facilitated greater production of active P-gp and allowed sufficient protein production for biochemical and biophysical studies (Mao and Scarborough, 1997). During his work at Queen's University (1997-2002, Canada), Dr. Mao carried out biochemical and structural investigations of another ABC transporter, multidrug resistance protein (MRP1/ABCC1). These studies included photoaffinity and mass spectrometry experiments to identify MRP1 substrate binding sites (Mao *et al.*, 2002; Wu *et al.*, 2005) as well as characterization of inhibitory monoclonal antibodies against MRP1 (Hipfner *et al.*, 1999). Most importantly, he developed a protocol for purifying MRP1 into proteoliposomes (Mao *et al.*, 1999, 2000) using four monoclonal antibodies (QCRL-2, -3, -4 and -6) to study MRP1. This work represented the first functional reconstitution of substrate transport in a lipid vesicle containing MRP1 (Mao *et al.*, 2000) and enabled subsequent structural studies using both X-ray crystallography (Rosenberg *et al.*, 2001) and Cryogenic Electron Microscopy (Cryo-EM) (Rosenberg, Oleschuk, *et al.*, 2010). Studies using these proteoliposomes proved to be a breakthrough by giving comparable kinetic parameters to those obtained in plasma membrane vesicle studies. As a result of his work, a crystal structure determination of MRP1 was achieved, which suggested that MRP1 functions as a dimer (Rosenberg *et al.*, 2001). These early experiences with transporters laid the foundation for his independent research career focused on P-gp and BCRP as a Principal Investigator at the University of Washington beginning in 2002 and only ended with his untimely death in 2023.

Structure and function of BCRP

After joining the University of Washington School of Pharmacy in 2002, Dr. Mao and his team began their investigations of the structure and function of BCRP and determined the membrane topology of BCRP by using a hemagglutinin tag insertion approach (Wang, Lee, Cai, *et al.*, 2008). Dr. Mao also continued his collaborative structural work with Dr. Mark F. Rosenberg (University of Manchester, United Kingdom) which led to the structural characterization of BCRP, purified from *Pichia pastoris*, by Cryo-EM of 2-dimensional crystals, where conformational changes were observed (Rosenberg, Bikadi *et al.*, 2010). Dr. Mao also used Fluorescence Resonance Energy Transfer microscopy to demonstrate that BCRP forms a homodimer or homooligomer *in vivo* in intact cells (Ni, Mark, *et al.*, 2010). Further biochemical investigations identified several basic residues within or near the TM2 helix and some polar residues within or near TM1 and TM6 of BCRP that have critical roles in determining the substrate selectivity and structural conformation of the transporter (Cai *et al.*, 2010; Ni, Bikadi, *et al.*, 2010). Dr. Mao also determined that two helix-disrupting proline residues proximal to TM1 and in TM3 are important for BCRP activity and substrate selectivity. His homology modeling further suggested the potential role of these prolines in facilitating communication between the MSD and NBD, or as flexible hinges that may be essential for BCRP's broad substrate specificity (Ni *et al.*, 2011).

Early efforts on BCRP substrate and inhibitor identifications

Dr. Mao's lab had many ongoing projects aimed at identifying new substrates and inhibitors of BCRP. These efforts involved various *in vitro* cell lines, experimental methods, and commonly prescribed drugs useful in diverse therapeutic areas. One example was the discovery of the inhibitory effects of dipyridamole, an antiplatelet medicine, and various calcium channel blockers on BCRP-mediated mitoxantrone efflux in BCRP-over-expressing human embryonic kidney (HEK) cells (Zhang *et al.*, 2005). Furthermore, Dr. Mao and his team used radiolabeled compounds in transporter assays to determine substrates of BCRP, and found that dipyridamole is transported by BCRP in both HEK cells and Madin-Darby canine kidney (MDCK) cells stably expressing the transporter. This finding was corroborated by the complete abolition of dipyridamole transport by fumitremorgin C, a known BCRP inhibitor

(Rabindran *et al.*, 2000). The development of these functional assay methods using these two cell lines, the application of flow cytometry, and substrate transport assays set the stage for the continued investigation of the impact of BCRP on drug disposition *in vitro*.

To further assess the effect of BCRP on the fetal distribution of its substrates *in vivo*, Dr. Mao and his team used nitrofurantoin (NFT), a model BCRP substrate often prescribed to pregnant women to treat urinary tract infections, as a probe (Zhang *et al.*, 2007). Pregnant wild-type (WT) mice and mice deficient in Bcrp (*Bcrp*^{-/-}) were used as the animal models in this study. It was found that the fetal area under the concentration-time curve (AUC) of intravenously administered NFT in the *Bcrp*^{-/-} mice was approximately 5 times greater than that in the WT mice while the maternal plasma AUC was comparable between the two groups of mice. These results demonstrated that Bcrp significantly limits fetal distribution of NFT in pregnant mice. However, the observed minor effect of Bcrp on maternal systemic exposure to NFT in the dam indicated a complex interplay among Bcrp expression levels in different tissues, pregnancy, and potential alterations of other drug- metabolizing enzymes and/or transporters during pregnancy.

These unexpected unexplained observations motivated Dr. Mao and his team to take further steps to evaluate the effect of pregnancy on the maternal pharmacokinetics of NFT and to elucidate the role of BCRP in pregnancy-related pharmacokinetic changes, if any, using WT and *Bcrp*^{-/-} mice (Zhang *et al.*, 2009). While pharmacokinetic parameters remain unchanged in pregnant WT or *Bcrp*^{-/-} mice after intravenous administration of NFT versus non-pregnant mice, for orally administered NFT, pregnancy led to a 70% decrease in dose-normalized AUC in *Bcrp*^{-/-} mice, but not in WT mice. These observations suggested that Bcrp plays a minor role in the systemic clearance of NFT in pregnant mice, but also that pregnancy can affect the expression and activity of certain intestinal efflux transporters and/or metabolic enzymes in *Bcrp*^{-/-} mice, resulting in a drastic decrease in drug exposure to oral but not intravenous administration of NFT in pregnant *Bcrp*^{-/-} mice. These studies illustrate the need to carefully dissect the potentially multiple and diverse impacts of pregnancy on transporter expression across different tissues *in vivo*.

Role of BCRP on drug disposition during pregnancy

Given the insights gained from their early findings, Dr. Mao and his team expanded their investigation into the role of BCRP in mediating pregnancy-induced changes in the disposition of additional therapeutic agents deployed in obstetric practice.

1) Glyburide

The NFT studies encouraged Dr. Mao and his lab to investigate another drug commonly prescribed in pregnancy: glyburide (GLB). GLB is a standard treatment for gestational diabetes and is known to have restricted placental transfer (Langer *et al.*, 2000) and increased clearance during pregnancy (Hebert *et al.*, 2009). Therefore, to ensure the safety and efficacy of GLB in pregnant women, it was important to understand the potential impact of BCRP and pregnancy-associated alterations in its expression as determinants of maternal-fetal pharmacokinetics of GLB.

Dr. Mao and his team demonstrated that GLB is transported by BCRP/Bcrp using *in vitro* transport assays. Consistent with the observations with NFT, the maternal plasma AUC of GLB in *Bcrp*^{-/-} mice was comparable to that in WT mice after intravenous administration, but the fetal tissue/maternal plasma AUC ratio in *Bcrp*^{-/-} mice was significantly greater (2.5-fold) than that in WT mice. These data suggest that Bcrp plays a minor role in the maternal clearance of GLB during pregnancy, but significantly impedes GLB penetration across the placental barrier (Zhou *et al.*, 2008).

To help explain the increased clearance during pregnancy, Dr. Mao and his team first showed that cytochrome P450 3A (CYP3A) plays a major role in the metabolism of GLB *in vitro* (Zhou, Narahariseti, *et al.*, 2010). Next, the hypothesis that the increased hepatic CYP3A activity during pregnancy contributes to the increase in systemic clearance of GLB was explored. Thus, the systemic clearance of GLB in pregnant mice was shown to be increased by approximately 2-fold ($p < 0.01$) compared with nonpregnant mice, a magnitude of change similar to that observed in a clinical study (Hebert *et al.*, 2009). Plasma protein binding of GLB in mice was not altered by pregnancy but the half-life of GLB depletion by hepatic S9 (microsomal) fractions prepared from pregnant mice was

significantly shorter than that of nonpregnant mice. Moreover, GLB depletion by hepatic S9 fractions was drastically inhibited by the CYP3A/Cyp3a inhibitor ketoconazole (Zhou, Zhang, *et al.*, 2010). These data suggest that the increased systemic clearance of GLB in pregnant mice is likely caused by an increase in hepatic Cyp3a activity during pregnancy. These findings provide a basis for further understanding of pregnancy-induced changes in drug disposition, particularly drugs metabolized by CYP3A/Cyp3a.

2) **Narcotic opioids**

The rising rates in incidences of drug abuse, associated hospitalizations, and drug abuse cessation treatments in the United States prompted Dr. Mao and his team to focus on abused drugs with potentially deleterious outcomes in pregnancy, such as opioids (Tobon *et al.*, 2019). Earlier studies had reported quantifiable levels of buprenorphine (BUP), norbuprenorphine (NBUP), and isomers of methadone (R-MET, and S-MET) in maternal and umbilical cord plasma as well as meconium (Jones *et al.*, 2005; Gordon *et al.*, 2010; Debelak *et al.*, 2013; Marin and McMillin, 2016), indicating that these drugs can cross the blood-placenta barrier (BPB) and expose the fetus. To further explore this phenomenon, Dr. Mao and his team investigated the pharmacological properties of these opioids on the placenta using *in vitro* human cell models: BeWo, JEG3 and primary human villous trophoblast cells. It was demonstrated that these drugs, at clinically relevant concentrations, significantly induced BCRP mRNA expression (Neradugomma *et al.*, 2017). Subsequently, using small molecule inhibitors like CH223191 {1-methyl-N-[2-methyl-4-[2-(2-methylphenyl)diazanyl]phenyl]-1H-pyrazole-5-carboxamide}, model ligands like 3-methylcholanthrene (Li *et al.*, 1998) and the BCRP promoter reporter plasmid X₄-4.2 as tools, Dr. Mao and his team demonstrated that BUP, NBUP, R-MET, and S-MET can activate AhR which in turn is actively recruited onto the BCRP promoter thus inducing its expression. This work from Dr. Mao and his team was the first to demonstrate that drugs of abuse and medications for cessation treatments (for example, R-MET, S-MET, BUP, and NBUP) can induce BCRP expression in human placental trophoblasts by activating the AhR signaling cascade, a finding which has important implications in understanding the pharmacology of prescription medications like MET and BUP.

Dr. Mao and his team further investigated the role of BCRP on the disposition of BUP and its metabolites, NBUP and norbuprenorphine-glucuronide (NBUP-G) using animal models. NBUP may cause significant respiratory depression as a side effect of BUP (Athanasos *et al.*, 2019). Understanding the impact of NBUP on fetal development and identifying the factors controlling fetal exposure may help mitigate potential fetal toxicity. In this research, a 2.5-fold increase in the systemic clearance of NBUP in pregnant mice compared to nonpregnant mice was observed, which can be explained by the induction of hepatic UDP-glucuronosyltransferase (Ugt) expression during pregnancy. The explanation was also supported by the increased intrinsic clearance of NBUP observed in mouse liver microsome metabolite formation assays (Liao, Gao, Phillips, *et al.*, 2018). Additional studies demonstrated that fetal exposure to NBUP and NBUP-G in pregnant mice represents approximately 60% and 700% of maternal plasma exposure, respectively. Interestingly, significantly different impacts of P-gp on fetal and maternal brain exposure to NBUP were observed, with P-gp playing a more pronounced role in restricting NBUP distribution across BBB compared to the BPB. This discrepancy can be attributed, at least in part, to the differential expression and fraction of P-gp transport in the respective tissue barriers, with a higher abundance of P-gp in the BBB than in the BPB (Liao *et al.*, 2017).

This work by Dr. Mao and his team unveiled the complex interaction of opioids and two major ABC transporters during pregnancy using proof-of-concept animal models. These insights have high translational value in furthering the clinical studies of pregnancy-induced changes in the disposition of opioids.

3) **Bupropion**

As a part of ongoing research collaborations, Dr. Mao and his team also devoted some efforts towards evaluating the role of drug transporters in bupropion disposition during pregnancy. Bupropion is used during pregnancy for the treatment of depression and smoking cessation (Csef, 1995; Slemmer *et al.*, 2000). The parent drug undergoes extensive metabolism producing three active metabolites: erythrohydrobupropion, hydroxybupropion, and threo hydrobupropion (Findlay *et al.*, 1981). Based on

previously published drug-drug interaction studies, it was found that inhibition of the principle enzymes alone did not fully explain the fetal exposure of bupropion and its active metabolites (Turpeinen *et al.*, 2005). Thus, it was hypothesized that transporters may also contribute to the disposition of bupropion. An *in vitro* study was conducted utilizing Chinese Hamster Ovary and HEK 293 cell lines, and plasma membrane vesicles prepared from cells overexpressing OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), OATP2B1 (SLCO2B1), OATP4A1 (SLCO4A1), OCT1 (SLC22A1), BCRP, MRP2 (ABCC2), or P-gp (Han, Gao, Zhang, *et al.*, 2018). Transport of bupropion and its three active metabolites was quantified using a LC-MS/MS assay. These transporters were selected for this exploratory study in view of their recognized role in hepatic transport of xenobiotics. OATP4A1 was explicitly selected for its potential role in placental apical membrane transport during pregnancy (Roth *et al.*, 2012). Both the transport kinetics and inhibition of cellular uptake of bupropion and its active metabolites were assessed by determining the effects of established inhibitors on the activity of each transporter. The data suggested that bupropion and its active metabolites are not substrates of the major transporters evaluated, implying that the transporters probably play a minor role in the overall disposition of bupropion. This study definitively ruled out a growing suspicion of the possible role of transporters in bupropion pharmacokinetics, and served to redirect attention back to drug-metabolizing enzymes as being more likely determinants of maternal and fetal bupropion exposure.

4) Tetrahydrocannabinol and its metabolites

In 2019, Dr. Mao and his team started to investigate the interaction between cannabinoids and efflux transporters. Cannabis use in the pregnant population has been on a steady rise since its legalization in many states beginning in 2014 (Carliner *et al.*, 2017). Δ^9 -tetrahydrocannabinol (THC) is the primary psychoactive compound in cannabis. The mildly psychoactive 11-OH-THC and non-psychoactive THC-COOH, are the main circulating metabolites of THC in humans. Previous studies suggested that THC could potentially be a P-gp and/or BCRP substrate in gene knockout mouse models (Bonhomme-Faivre *et*

al., 2008; Spiro *et al.*, 2012). However, whether THC and its metabolites are substrates and/or inhibitors of the human orthologs of BCRP and P-gp remained uninvestigated.

Using Transwell and cellular accumulation assays with MDCKII cells and membrane vesicles over-expressing human P-gp or BCRP along with specific substrates and inhibitors as probes for these transporters, Dr. Mao and his team demonstrated that THC-COOH alone is a weak substrate and inhibitor of BCRP; but that neither THC nor 11-OH-THC are substrates of P-gp or BCRP (Chen *et al.*, 2021).

To further confirm these *in vitro* observations and to investigate the roles of P-gp and/or BCRP in the maternal-fetal disposition of THC and its metabolites, Dr. Mao and his team selected WT, *P-gp*^{-/-} (*Abcb1a*^{-/-}/*Abcb1b*^{-/-}), *Bcrp*^{-/-} (*Abcg2*^{-/-}), or *P-gp*^{-/-}/*Bcrp*^{-/-} (*Abcb1a*^{-/-}/*Abcb1b*^{-/-}/*Abcg2*^{-/-}) pregnant FVB mice as the *in vivo* models. On gestational day (GD) 18, after administering 3 mg/kg THC to each pregnant mouse via retro-orbital injection, plasma and various tissues (maternal brains, placentas, fetuses) were collected upon sacrificing the mice at different timepoints. Using a pooled data bootstrap approach, the dose-normalized AUCs and tissue/maternal plasma AUC geometric mean ratios (GMRs) were estimated. Surprisingly, the tissue-to-maternal plasma AUC GMRs of THC and its major metabolites in the maternal brains, placentas, and fetuses of *P-gp*^{-/-}, *Bcrp*^{-/-}, or *P-gp*^{-/-}/*Bcrp*^{-/-} pregnant FVB mice were lower to differing extents (28-78%) than those in WT mice. In particular, the maternal brain/maternal plasma AUC GMR for THC was only 28% lower in *P-gp*^{-/-} pregnant mice compared to WT pregnant mice. The unbound fraction of THC and its metabolites in maternal plasma and investigated tissues were not found to be significantly different among all genotypes (Chen *et al.*, 2023). Also, none of the transporters known to be at the BBB of mice exhibited different expression levels in *P-gp* and/or *Bcrp* deficient mice compared to WT. While these observations in pregnant mice still lack satisfactory explanations, they do point to the fact that there is still a lot yet to be understood in regard to drug transport across the BBB. Importantly, Dr. Mao and his team were able to eliminate P-gp or BCRP as being involved in drug-cannabinoid interactions in humans.

Gestational-dependent transporter mRNA, protein expression, and activity in mice

Dr. Mao's ground breaking *in vivo* studies with NFT and GLB showed that pregnancy induces significant changes in the expression and/or activity of drug-metabolizing enzymes and transporters; however, the outcomes due to the combined changes in these key processes of drug disposition turn out to be complex and not easily predictable as suggested by the unchanging maternal plasma AUC levels. Furthermore, there is much that is not known regarding the expression of other transporters in different stages of pregnancy. This led Dr. Mao and his team to undertake a microarray analysis of the mRNA expression of selected key drug transporter genes such as *Abc* and solute carrier (*Slc*, *Slco*) transporters in the maternal liver, kidney, placenta, and small intestine of pregnant mice on GD 10, 15 and 19 compared to GD 0 (Shuster *et al.*, 2013).

Dr. Mao and his team found that *Abc* transporter mRNA expression in maternal tissues appeared relatively unaffected by pregnancy compared to nonpregnant controls (GD 0) in mice, except for *Abcc3* (*Mrp3*) and *Abcb1a/1b* (*P-gp*). In the liver, the expression of *Abcc3* and *Abcb1a* was decreased by ~70% and 40%, respectively, on GD 15. In the kidney, *Abcb1a* expression was consistently decreased by 30-40% throughout mid to late gestation. *Abcc4/Mrp4* expression in the kidney was also decreased by 20-30% on GD 10 and 15 and returned to nonpregnant levels by term (GD 19). Changes in transporter expression in the small intestine were minimal compared to the liver and kidney. However, there were many changes in placental *Abc* transporters. Specifically, *Abcb1a* and *Abcb1b* were 70% and 80% lower, respectively, on GD 10 compared to near term, GD 19. *Abcc5* (*Mrp5*) expression was 3-fold higher on GD 10 compared to GD 19. It appeared that most *Abc* transporters in the placenta tended to be suppressed during mid to late gestation (GD 10 and 15) but returned to baseline by GD 19.

Dr. Mao and his team also found the mRNA expression of *Slc* and *Slco* transporters in liver and kidney changed throughout gestation. In the liver, *Slco1b2* [*organic-anion-transporting polypeptides (Oatp) 1b2*, ortholog of human OATP1B3], *Slc10a1* (*Sodium taurocholate cotransporting polypeptide, Ntcp*), and *Slc47a1* (*Multidrug and toxin extrusion protein 1, Mate1*), were down-regulated by ~25% on GD 15 and GD 19. In the kidney, many *Slc* and *Slco* transporters exhibited only modest changes in expression. *Slco4c1* (*Oatp4c1*), though, showed markedly decreased expression levels (~60-70%) across gestation. In

the placenta, *Slc22a3* (*Organic cation transporter 3, Oct3*) expression was 80% lower on GD 10 compared to GD 19. In contrast, *Slc22a5* (*Organic cation/carnitine transporter 2, Octn2*) and *Slc6a2* (*Norepinephrine transporter*) were both up-regulated more than 2.5-fold on GD 10. Overall, relatively few *Slc* and *Slco* genes involved in drug transport were affected during pregnancy. Changes in expression of *Slco1b2* in the liver, *Slco4c1* in the kidney, and *Slc22a3* in the placenta, however, may affect the pharmacokinetics of certain drugs during pregnancy. Extrapolation of these findings to human pregnancy is not straightforward because of the interspecies variability in substrate specificity and relative activities of the transporters, and the uncertain correlation between gene expression and protein levels (Schwanhäusser *et al.*, 2011). Nonetheless, given the fact that the pregnant mouse is a commonly used animal model for gestational research, the detailed mRNA expression data will serve as important background information for future investigations.

Given the observed alterations in mRNA expression, Dr. Mao's lab went on to quantify the corresponding changes in protein abundance of transporters and Ugt enzymes in liver, kidney, and brain of both pregnant and nonpregnant mice by mass spectrometry-based proteomics. The protein abundances of P-gp (*Abcb1a/1b*), Bcrp (*Abcg2*), Mrp2 (*Abcc2*), Bsep (*Abcb11*), Mrp4 (*Abcc4*), Oatp1a1 (*Slco1a1*), and Oatp1b2 (*Slco1b2*) in liver were approximately 50-100% higher in pregnant mice while the protein levels of *Abcc3* (*Mrp3*) and *Slco1a4* (*Oatp1a4*) were approximately 40% lower compared to nonpregnant mice. Transporter protein abundance in the kidney was not significantly changed during pregnancy when scaled to the whole organ. Different than the mRNA expression levels, none of the transporters examined showed significant changes in protein levels in the brain due to pregnancy (Liao, Gao, Bhatt, *et al.*, 2018). The differential protein expression changes in various organs during pregnancy further motivated the team to investigate how pregnancy could impact the maternal disposition of other commonly prescribed medications. To further examine the consequences of gestational-dependent changes in transporter expression, Dr. Mao and his team again chose GLB as an *in vivo* probe. By evaluating the maternal-fetal disposition of GLB on GD 10, 15 and 19 in the pregnant mice, it was observed that maternal GLB clearance steadily increased over gestation and approximately doubled by mid-late gestation, which is

consistent with increases in GLB depletion rates in the mouse liver microsomes prepared from livers at varying gestational ages. Fetal exposure to GLB was <5% of maternal exposure, but doubled on GD 19 versus GD 15 (Shuster *et al.*, 2014). These observations are consistent with known changes in murine placental Bcrp mRNA expression (2-3 times greater on GD 15 compared to GD 19) (Wang, Wu, *et al.*, 2006). P-gp could also contribute to the gestational age-dependent changes in fetal exposure to GLB because both human and mouse P-gp expression in the placenta decreases as gestation progresses (Mathias *et al.*, 2005; Aleksunes *et al.*, 2008; Zhang *et al.*, 2008). The data obtained in this animal study correlates with a clinical trial which showed a decreased maternal systemic GLB exposure throughout gestation (Hebert *et al.*, 2009). However, increased GLB dose during pregnancy may increase fetal exposure in late gestation. Any dose adjustments will need to take into account the benefit-risk considerations for both the mother and the fetus.

The role of transporters on the enterohepatic circulation of 25-hydroxyvitamin D₃ (25OHD₃) and its metabolites

In addition to investigating the role of transporters in governing pharmacokinetics in pregnant women, Dr. Mao and his team collaborated with other labs in other areas of drug transporter research. The most interesting example is the work on transporter involvement in the enterohepatic circulation of metabolites of vitamin D₃. In humans, 25-hydroxyvitamin D₃, (25OHD₃), the most abundant circulating metabolite of vitamin D₃, is further transformed to the biologically active metabolite 1 α ,25-dihydroxyvitamin D₃ (1 α ,25-(OH)₂D₃) by CYP27B1 in the kidney and extrarenal tissues, and to inactive metabolites by other cytochrome P450 enzymes (Hewison *et al.*, 2007; Feldman *et al.*, 2014). In addition, 25OHD₃ undergoes sulfation and glucuronidation in the liver, forming two major conjugated metabolites, 25OHD₃-3-O-sulfate (25OHD₃-S) and 25OHD₃-3-O-glucuronide (25OHD₃-G), both of which are detected in human blood and bile (Gao *et al.*, 2017). The conjugates may undergo biliary excretion and be deconjugated to 25OHD₃ and then converted to 1 α ,25-(OH)₂D₃ in the intestinal lumen, which is likely to exert local pharmacological effects on the intestine. It seemed important to characterize the enterohepatic transport

mechanisms of 25OHD₃-S and 25OHD₃-G, which may be determinants of interindividual variability in mineral homeostasis.

Based on a series of plasma membrane vesicle and cell-based transport studies carried out by Dr. Mao and his team, 25OHD₃-G was shown to be a substrate of human MRP2, MRP3, OATP1B1, and OATP1B3, and that 25OHD₃-S is probably a substrate of BCRP, OATP2B1, and OATP1B3. Sinusoidal and canalicular efflux of both conjugates was also demonstrated using sandwich-cultured human hepatocytes (Gao *et al.*, 2018). Given the substantial expression of these transporters in hepatocytes and intestinal enterocytes, these findings demonstrated for the first time that transporters could play important roles in the enterohepatic circulation of 25OHD₃ conjugates, providing an alternative pathway of 25OHD₃ delivery to the intestinal tract, which could be critical for vitamin D receptor-dependent gene regulation in enterocytes.

Additional investigations on the interaction between gut microbiome and drug disposition during pregnancy

Dr. Mao and his team were intrigued by the emerging understanding of the role of the gut microbiome on human health, and began to explore the role of gut microbiome on drug disposition during pregnancy. To elucidate the impact of pregnancy on gut microbiome and expression of key hepatic drug metabolic enzymes, transporters, and metabolic pathways, a multiomics-based approach was taken using 8-week-old C57BL/6J, pregnant (GD 15) and nonpregnant, conventional (CV) and germ-free (GF) mice (Barriot and Riou, 1988; Han *et al.*, 2020).

GF mice versus CV mice exhibited differential regulation of some key metabolic enzymes as reflected in the mRNA expression of Cyp3a, Cyp3a11, Cyp3a16 and Cyp3a41. Some of these findings were consistent with previously reported changes in Cyp expression during pregnancy in rodent models, but other findings were inconsistent with previous reports, possibly due to sex- and strain-dependent differences (Selwyn *et al.*, 2015, 2016). The study also showed a discordance between mRNA levels and protein levels for many metabolizing enzymes and transporters, which may be attributed to post-

transcriptional or epigenetic regulation that may be influenced by the gut microbiome (Woo and Alenghat, 2022). Pathway analyses also revealed that shifts in microbiome composition during pregnancy have a noticeable effect on the metabolism of retinol, linoleic acid and arachidonic acid, as well as steroid hormone biosynthesis pathways when comparing GF pregnant to CV pregnant mice.

Although this study is incomplete (for instance, it did not evaluate term-pregnancy or fetal development), it is the first study to examine the effects of pregnancy-gut microbiome interplay on drug disposition, and provides the motivation for further in-depth studies to elucidate the impact of microbiome-related metabolic pathway shifts during pregnancy.

SUMMARY

Transporters play an important role in drug disposition due to their broad substrate spectrum and rich expression in all the crucial physiological barriers, including but not limited to the BBB, BPB, hepatocytes, and kidney proximal tubules (Giacomini *et al.*, 2010). Given the profound physiological changes that occur in pregnancy (Pinheiro and Stika, 2020), in retrospect we should not have been surprised to learn over the past two decades that the expression and activities of drug transporters, especially the efflux transporters and changes during gestation impact both maternal and fetal exposure to various xenobiotics. However, many drugs commonly prescribed during pregnancy remain poorly understood with respect to their pharmacokinetics and, more importantly, their relevance to drug efficacy and safety in the mother and fetus. Dr. Mao dedicated his research work to address these knowledge gaps in transporter biology and pharmacology during pregnancy.

Dr. Mao made significant contributions to the field of drug transporters throughout his scientific career. Following the start of his own lab at the University of Washington, Dr. Mao shifted his research to a more translational path by focusing on the functional roles of membrane transporters and metabolic enzymes in the disposition of a number of therapeutically important agents in pregnancy, namely NFT, BUP, methadone, bupropion, 25OHD₃, and THC. Through his meticulous work, Dr. Mao demonstrated the utility and value of pregnant mouse as a surrogate in vivo model (WT and transgenic) that overcomes the

ethical challenges of obtaining and using human tissue, while still offering translatability to clinical relevance in human pregnancy. Dr. Mao's research has revealed that transporters like P-gp and BCRP usually have differential impacts on the plasma, fetal, and maternal brain exposure to xenobiotics including drugs. Equally important, the *in vitro* and *in vivo* models and assays developed in his lab and utilized in his work serve as a guide enabling other labs and young investigators to set up reproducible methodologies for conducting transporter research. Dr. Mao also wrote several critical and comprehensive reviews to summarize the knowledge gaps and better assist other researchers to pursue further research in this field (Mao and Unadkat, 2015; Han, Gao, and Mao, 2018; Mao and Chen, 2022).

Dr. Mao was an avid, enthusiastic, and devoted researcher with an incredible work ethic. His kindness, patience, and collaborative spirit remain as an inspiration to each of us. Dr. Mao had an enormous impact in the drug transporter field as well as being a distinguished member of the faculty at the University of Washington, School of Pharmacy. His untimely passing leaves a large void behind. Rest in peace Professor Qingcheng Mao, our inspiring mentor and dear friend.

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Contributed to the writing of the manuscript: All authors contributed equally to the writing of the manuscript and are listed alphabetically based on last names.

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