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Placental P-glycoprotein: Role in Disposition of Medications Administered During Pregnancy

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Placental P-glycoprotein: Role in Disposition of Medications Administered During Pregnancy

by

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Placental P-glycoprotein: Role in Disposition of Medications Administered During Pregnancy

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Abstract: The placenta supports fetal growth and regulates the bio-distribution of substances between the maternal and fetal circulation. Active efflux transporters in the placental apical membrane are involved in placental elimination of compounds and thus fetal protection. The goal of this investigation was to evaluate developmental and genetic influences on expression and activity of the placental efflux transporter P-glycoprotein (P-gp) and its role in regulating the placental distribution of medications used during pregnancy. P-gp expression and transport activity were defined in brush border membrane vesicles prepared from human placenta of various gestational ages. The expression and transport activity of P-gp declined progressively throughout gestation. There was a lack of correlation between P-gp expression and activity in individual samples, which may be influenced by three polymorphisms in the Multidrug Resistance 1 (*MDR1*) gene encoding P-gp associated with decreased protein expression yet increased transport activity in these patients. P-gp was found to transport medications used in the treatment of conditions during pregnancy: namely opiate dependence, cigarette smoking, and gestational diabetes. Considerations of P-gp involvement in placental distribution must therefore be taken into account when planning therapy of these conditions during pregnancy. Finally, the need for an *in vivo* animal model for studying placental P-gp transport of investigational drugs during pregnancy led to our identification of the baboon as a comparable model to human placental P-gp expression and activity. Future studies should investigate gene-wide analysis of functional *MDR1* variants and the *in vivo* role of placental P-gp in the disposition of medications used during pregnancy.

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Chapter 1: Introduction

Pregnant patients represent a challenging population for outlining and implementing medical treatment. A variety of conditions ranging from primary care to chronic disease—either related or unrelated to the pregnancy—may occur and require pharmacologic intervention for some or all of the gestational period. Maternal physiologic changes accompanying the onset and progression of pregnancy, as well as the vulnerability of the developing fetus, complicate decisions on the dosing and administration of pharmacotherapy. A thorough understanding of the effects of pregnancy on drug distribution and elimination are essential for both researchers in drug development and prescribing physicians. The placenta provides the interface between maternal and fetal circulatory systems, and has a crucial role in regulating fetal exposure to components of maternal blood. Therefore, investigation and characterization of the role of the placenta in fetal protection is essential for medical decision making whether treating the mother or fetus.

Chapter 2: Background and Literature Review

PLACENTAL ANATOMY AND PHYSIOLOGY

The placenta is the organ connecting the fetus to the maternal uterus, and is essential to fetal survival, development and growth. It forms early (second week) in pregnancy and continues to grow and provide crucial support to the fetus throughout gestation until delivery. The primary functions of the placenta include transfer of nutrients and oxygen from the maternal circulation to the fetus and removal of waste products from the fetus to the mother. In addition to the exchange of nutrients and waste, the placenta also mediates anabolic and catabolic pathways, such as steroid and hormone synthesis and metabolic activation and detoxification reactions. The fetus and the placenta act in concert to produce steroid hormones (androgens, estrogens and progestins) controlling intrauterine growth, organ maturation, and parturition. In addition to the steroids, the placenta produces polypeptide hormones human chorionic gonadotropin (hCG), human placental lactogen (hPL), and human chorionic corticotrophin (hCC) which are released into the maternal circulation and affect maternal physiology to optimize conditions for the development of the fetoplacental unit (DeCherney et al., 2007).

The human placenta connects to the fetus by the umbilical cord which inserts into the placental chorionic plate (Illustration 1). Vessels branch out over the surface of the placenta and further divide to form a vascular network covered by a thin layer of cells. This vascular network forms villous tree structures, which are grouped into lobules facing the endometrium. Maternal blood enters the intervillous space through spiral arteries,

which are branches of the uterine arteries. Maternal blood bathes the chorionic villi and flows to a collecting sinus, which leads to uterine veins. Nutrients, oxygen, and antibodies pass into fetal blood of the villi; metabolic waste products pass from fetal blood into the mother's blood. Normally, there is no contact or mixture of fetal and maternal blood.

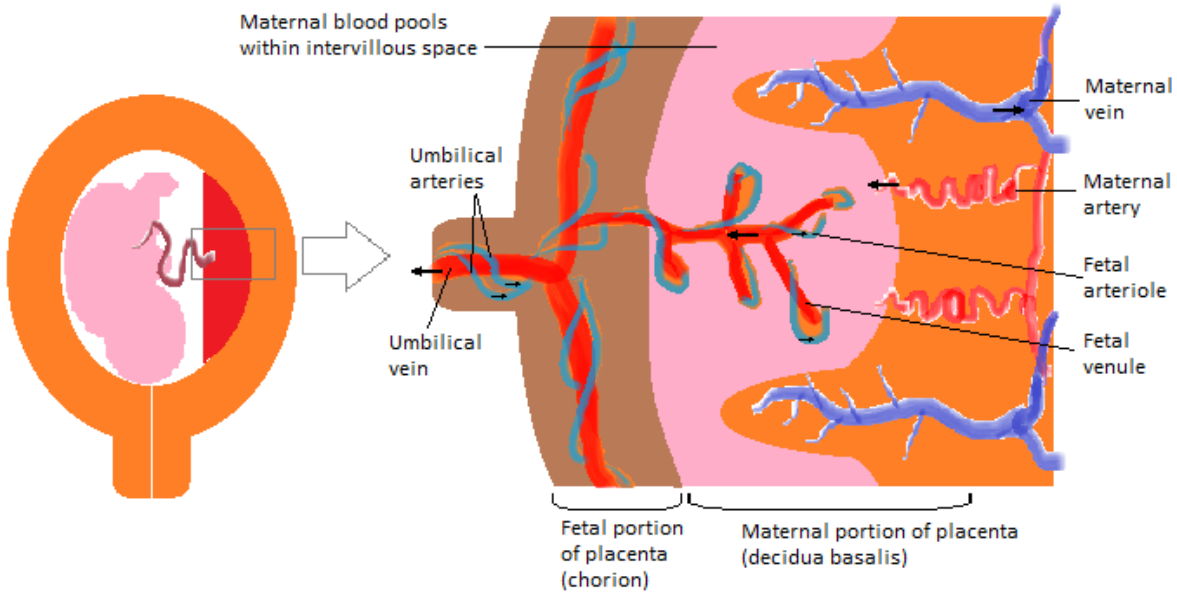


Illustration 1. Diagram of the blood circulation in the mature placenta.
Fetal vessels comprise the chorionic villous tissue of the placenta, covered by a thin layer of cells. Maternal blood enters the intervillous space through spiral arteries, bathes the chorionic villi and flows to a collecting sinus and uterine veins.

The placental tissue, known as the trophoblast, is divided into layers: the underlying cytotrophoblast layer and overlying syncytiotrophoblast layer (Illustration 2). The syncytiotrophoblast is a multinucleate epithelial layer which covers the surface of the placenta, forming what is referred to as the “barrier” of the placenta. It forms as a result of differentiation and fusion of the underlying cytotrophoblast cells, a process which

continues throughout placental development. Near term, the syncytiotrophoblast represents a single cell layer separating the maternal blood space from the fetal capillary.

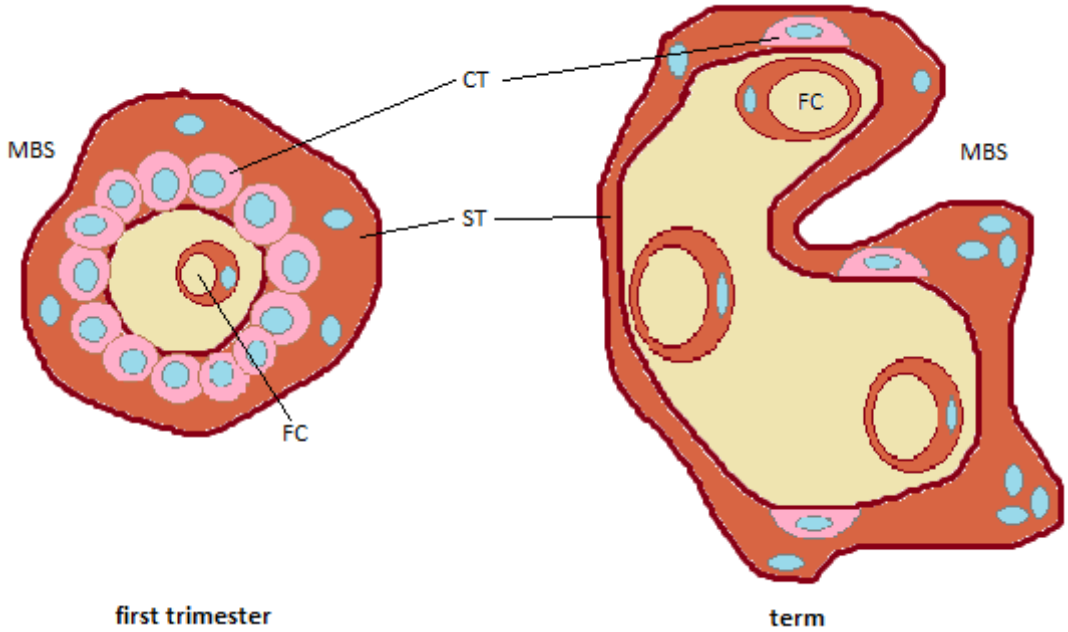


Illustration 2. Cross section of trophoblast tissue.
Comparison first trimester and term cytotrophoblast (CT), syncytiotrophoblast (ST) cells separating the maternal blood space (MBS) from the fetal capillary (FC). First trimester placental tissue is comprised of several layers of cytotrophoblast cells, which progressively fuse through advancing gestation to form the single multinucleate syncytiotrophoblast cell layer.

Placental Functional Barrier

The placenta had classically been viewed as an impermeable barrier to drugs and environmental chemicals exposed to the maternal circulation. However, over the past 50 years evidence has accumulated that many substances cross the placenta and exposure to them is associated with distinct adverse outcomes in newborns. In the 1950s-60s, thalidomide was prescribed worldwide to pregnant women as anti-emetic and anti-anxiety therapy, and was associated with documented skeletal birth defects in thousands

of exposed newborns (Lenz, 1988; McBride, 1961; Smithells, 1962). In the 1970s, it was demonstrated that prenatal alcohol exposure is associated with cognitive deficit, facial malformations, and pre/post natal growth retardation (Riley et al., 2005; West et al., 2005). It has since been recognized that a wide variety of drugs, environmental chemicals, and endogenous substances are able to cross the placenta and potentially induce birth defects.

Concern over the uncertainty regarding safety and potential adverse effects of medications on the fetus may lead physicians to under-prescribe or discontinue the use of therapeutics to treat maternal conditions. Therefore, many treatment modalities are avoided during pregnancy, despite the fact that they may improve maternal outcome and there are no reported adverse fetal effects. However, conditions associated with high risk pregnancy, such as spontaneous preterm labor, gestational diabetes, opiate abuse, and cigarette smoking, may pose a greater risk to the fetus if left untreated than the alternative pharmacologic intervention. Therefore, in order to assess the risk of fetal exposure to drugs during pregnancy, an important step is to determine the potential for the drug to cross the placenta and access the fetal circulation. Better understanding of the parameters that determine fetal exposure may enhance physician confidence in assessing the risks and benefits of prescribing medications during pregnancy.

Drugs administered during pregnancy may have direct and/or indirect effects on the fetus. A drug may reach the fetal circulation at a concentration in which directly interferes with fetal development and growth, or the drug can affect the same physiologic function within the fetus as within the mother. Additionally, a drug may indirectly

adversely affect the fetus by interfering with placental functions necessary for normal fetal growth and development. Therefore, the effects of a drug on the fetus depend largely on its biodisposition (metabolism, transfer) by placental tissue.

The ability of a drug present in the maternal circulation to access the fetus depends on many factors, including chemical properties of the drug (ionization, molecular weight, protein binding); placental structure/anatomy (capillary exchange area, basement membrane thickness, blood flow); and the placental functional barrier, which is composed of metabolic enzymes and active transporters (Syme et al., 2004). The role of enzymes and transporters in regulating placental transfer, and thus potential fetal exposure, remains unknown for many therapeutics currently prescribed or in preclinical studies for consideration during pregnancy. Additionally, pregnancy-induced changes in maternal physiology may affect the expression and activity of placental and non-placental enzymes and transporters and thus bioavailability of medications. Therefore, the expression and activity of proteins involved in regulating placental biodisposition of medications used during pregnancy is the subject of ongoing investigations.

Placental Metabolic Enzymes

The placenta expresses both phase I (oxidation, reduction, hydrolysis) and phase II (conjugation reactions) enzymes (Collier et al., 2002; Meigs et al., 1968). Cytochrome P450 (CYP) isoforms, which function in steroidogenesis, metabolism of vitamins, fatty acids, a range of medications and xenobiotics, vary in their expression and activity throughout gestation in human placenta (Pasanen, 1999). Uridine diphosphate glucoronosyltransferases (UGTs), involved in phase II metabolic conjugation of

glucuronic acid to xenobiotics, are expressed in human placenta throughout gestation (Collier et al., 2002; Collier et al., 2002). Glutathione S-transferases catalyze the conjugation of glutathione to biologically active electrophiles, and are highly active in human placenta throughout pregnancy (Pacifici et al., 1981). Epoxide hydrolases, responsible for conversion of epoxides into trans-glycols or trans-dihydrodiols, are expressed in human placenta after 8-9 weeks gestation (Pacifici et al., 1983), although their importance in fetal development or protection is unclear. Sulfotransferases biotransform steroids, catecholamines, and some medications via sulfate conjugation, and isoforms are expressed and active in human placental tissue (Sodha et al., 1983). Additionally, carbonyl-reducing enzymes expressed in human placenta include medium-chain dehydrogenases/reductases, aldo-keto reductases, short-chain dehydrogenases/reductases, and quinone reductases (Nishimura et al., 2006). The 11beta-hydroxysteroid dehydrogenase enzyme is a short-chain dehydrogenase responsible recently identified as being highly active in the term human placenta (Wang et al., 2010).

Although the total placental metabolite formation represents a fraction of hepatic metabolites (Pasanen, 1999), the placenta represents a distinct metabolic pathway from that of the liver (Wang et al., 2010; Yan, 2008). Therefore, the function of placental enzymes in biotransformation of endogenous compounds and xenobiotics, along with the close proximity to the fetal circulation, indicate the importance of placental metabolism on fetal safety and development. Additionally, the substrates/ products of biotransformation from the above enzymes can be substrates of active transporters of the

placenta, and the interrelationship between metabolism and active transport may provide synergistic barrier function in fetal protection.

Placental Active Transporters

Active transporters have important roles throughout the body, including: barrier to absorption (efflux across GI epithelia); determinant of tissue distribution (influx into and efflux out of target cells, blood brain barrier & blood testes barrier, placental barrier); modulation of metabolism (influx into and efflux out of hepatocytes); role in elimination (biliary and urinary excretion, breast milk). In recent years, a large number of active transporters have been found to be expressed in the placental syncytiotrophoblast tissue (Ganapathy et al., 2000; Syme et al., 2004). These include ATP-binding cassette (ABC) transporters: P-glycoprotein (P-gp), Multidrug resistance proteins (MRPs), Breast Cancer Resistance Protein (BCRP); as well as transporters from other families such as Serotonin transporter (SERT), Norepinephrine transporter (NET), Organic Cation Transporters (OCTs), Mono- and Di-carboxylate Transporters (MCT and NaDC3), and Sodium/multivitamin transporter (SMVT) (Syme et al., 2004). Perhaps the most widely recognized are the ABC transporter family, which appear to have a major role in the placental barrier.

ABC Transporters

ATP Binding Cassette (ABC) transporters represent the largest protein superfamily with over 49 isoforms identified in humans to date (Dean et al., 2005). First identified in tumors where overexpression conferred resistance to chemotherapy drugs,

their expression has since been detected in the plasma membrane of many tissues, including kidney, liver, brain, small intestine, placenta, and more. They influence the bioavailability and excretion of their widely varying and structurally-unrelated substrates: xenobiotics, steroids, nuceotide analogs, other endogenous compounds. Most of the transporters are membrane-associated with repeats of six membrane-spanning regions, and are characterized by the presence of nucleotide binding domains (NBD), also referred to as ATP-binding cassettes (Illustration 3) (Kuo, 2007). To date, there is not a clear consensus on the structural localization of the substrate binding sites. A common feature of the ABC transporter isoforms is that they couple ATP binding/hydrolysis to the efflux of their substrates across the cell membrane to the extracellular space (Dean et al., 2005). Three ABC subfamilies have been recognized as playing an important role in drug efflux from cells, namely P-glycoprotein (P-gp), Multidrug Resistance Protein (MRP), and Breast Cancer Resistance Protein (BCRP). MRP and BCRP will be introduced briefly, while the main focus of this investigation will be to elaborate the role of P-gp in the placental functional barrier.

Multidrug Resistance Protein (MRP)

The Multidrug Resistance Proteins (MRPs) are a family of 9 ABC transporters which efflux unconjugated amphiphilic anions, as well as lipophilic compounds as glutathione, glucuronate and sulfate conjugates (Borst et al., 2000; Suzuki et al., 1998). The MRP isoforms are distributed widely throughout the body, and at least 3 members have been detected in placental apical membrane: MRP1, MRP2, and MRP3 (Flens et al., 1996; St-Pierre et al., 2000; Sugawara et al., 1997). Direct measurement of transport by

MRP isoforms expressed in placental apical membrane has been the subject of limited investigation (Gedeon et al., 2008), and the role of MRP efflux in placental apical membrane is not clearly established. Studies in nonplacental tissues have revealed that MRP3 transports conjugated organic anions in bile canaliculi (Bodo et al., 2003), and also confers resistance to chemotherapeutic agent methotrexate (Zeng et al., 2000). MRP1 and MRP2-mediated transport of glutathione conjugates suggests their role in cellular defense (Cui et al., 1999; Leier et al., 1994), although the physiological role of these MRP isoforms, as well as MRP3, in the human placenta remains speculative.

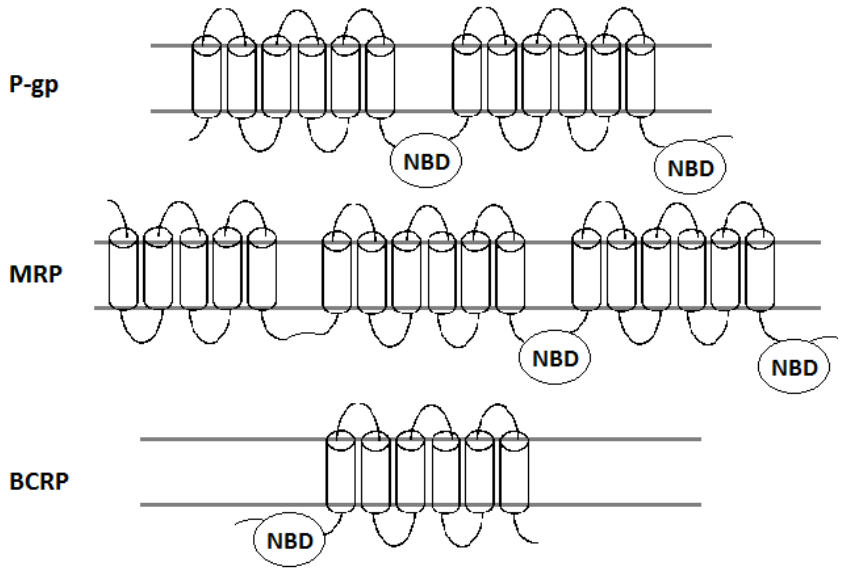


Illustration 3. Secondary structures of three ABC family transporters: P-glycoprotein (P-gp), Multidrug Resistance Protein (MRP1), and Breast Cancer Resistance Protein (BCRP). The transporters are membrane-associated with repeats of six membrane-spanning regions, and are characterized by the presence of cytoplasmic nucleotide binding domains (NBD)

Breast Cancer Resistance Protein (BCRP)

Breast Cancer Resistance Protein (BCRP) was identified in the 1990s following reports of non-P-gp and non-MRP mediated drug resistance to mitoxantrone (You et al., 2007). BCRP consists of one transmembrane domain and one nucleotide binding domain (in contrast to the other ABC transporters which contain at least 2 of each, Figure 3); most likely functioning as a dimer (Xu et al., 2004). It is expressed in liver, testes, intestines, blood-brain barrier, mammary ductal epithelium, and is highly expressed in placental syncytiotrophoblast (Allikmets et al., 1998; Doyle et al., 1998). Expression of BCRP in breast cancer cells first allowed identification of chemotherapeutic drugs as BCRP substrates (Chen et al., 1990). Many other BCRP substrates have since been identified, including some also transported by P-gp (Polli et al., 2004). Recent evidence of BCRP efflux of glucuronide, glutathione, and sulfate conjugates as well as endogenous steroids (You et al., 2007), suggests its role in fetal protection and/or secretion of steroids produced by the feto-placental unit.

P-glycoprotein (P-gp)

One of the most well known members of the ABC transporter family is P-gp, the product of the *MDR1* gene. P-gp is known to actively efflux a wide variety of substances at the blood-brain barrier, intestinal brush border, pancreatic, hepatic, and mammary ducts, adrenal gland, cochlea and vestibule of the inner ear, hematopoietic cells, and placental apical membrane (Nakamura et al., 1997; Sparreboom et al., 1997; You et al., 2007). P-gp interacts with hundreds of diverse and structurally-unrelated substrates including fluorescent dyes, antibiotics, chemotherapeutic agents,

immunosuppressants, β-blockers, opioids, steroids, lipids, cytokines, and a variety of other compounds (Linardi et al., 2006). Due to its extensive tissue distribution and transport of many substrates currently used in clinical therapy, P-gp has become increasingly recognized as a key regulator on the absorption and disposition of drugs.

Consistent with the role of P-gp in protecting the tissues from harmful exogenous or endogenous substances, placental P-gp transports it substrate from the fetal-to-maternal direction in order to limit transplacental transfer and fetal exposure/accumulation. Possibly more so than any other ABC transporter, P-gp has been highlighted in reports demonstrating its role in regulating the placental disposition of medications. Dual perfusion of the human placental lobule revealed that inhibition of P-gp significantly increases the maternal-to-fetal transfer of its substrates across the placenta (Nekhayeva et al., 2006).

Much remains unknown regarding how factors affecting P-gp expression and activity, including drug-drug interactions, may influence placental P-gp function and thus its role in fetal protection. P-gp efflux activity is subject to competitive and allosteric modulation. Evidence suggests that P-gp contains multiple binding sites (Martin et al., 2000), in which compounds may act as either 1) substrates, transported by P-gp or 2) modulators, which alter P-gp function but are not transported. Both transport and regulatory sites are able to switch between high- and low-affinity conformations and are subject to allosteric modulation from substrate binding at either site. Additionally, two drugs which are both substrates transported by P-gp could cause competitive inhibition for the same transport site. The importance of P-gp in drug–drug interactions has been

identified in USFDA guidelines (Huang et al, 2007), and it is increasingly accepted that co-administration of P-gp substrates can result in competition for efflux which could affect their pharmacokinetics and pharmacodynamics (Zhou, 2008).

Due to the central role of P-gp in regulating the disposition of many endogenous compounds and xenobiotics, yet its poorly-understood response to developmental, genetic, and environmental regulators, it serves as the main focus of this investigation in placenta.

EFFECT OF DEVELOPMENTAL CHANGES ON THE PLACENTAL BARRIER

The placenta undergoes marked changes with advancing gestation as part of the normal maturation process and in order to accommodate the increasing metabolic demands of the growing fetus (de Swiet et al, 1992). A combination of anatomical changes may either enhance or inhibit the passive diffusion of substances across the placenta. Changes that are likely to increase transfer of xenobiotics are: a reduction in proportion of cytotrophoblast cells; an increase in blood flow and number of capillaries; and thinning of capillary endothelium (Huppertz, 2007). The average exchange area of the placental villous tissue expands from 3.4 m^2 at 28 weeks gestation to 12.6 m^2 at term (Aherne et al., 1966; Aherne et al., 1966; de Sweit et al., 1992), while the diffusion distance between the maternal blood space and fetal capillary decreases from 50-100 μm at the second month to 4-5 μm at term (Kaufmann et al., 1992). Furthermore, the rate of blood flow supplying the blood space surrounding the placental villous tissue increases from approximately 50 mL/min at 10 weeks to 600 mL/min at term (Kaufmann et al., 1992). Conversely, an increase in deposition of fibrinoid tissue and increased thickness of

basement membrane are likely to impede the passive diffusion of substances (Huppertz, 2007).

In addition to the above changes in the anatomical barrier of the placenta, changes in the functional barrier of the placenta (activity or amount of transporters) may also impact fetal exposure. The expression of placental P-gp is suspected to change with advancing gestation (MacFarland et al., 1994; Sun et al., 2005), supported by evidence that P-gp mRNA expression decreased dramatically throughout advancing gestation (Mathias et al., 2005). However, the physiologic implications of this decline in expression (effect on P-gp transport activity and transplacental transfer of its substrates) remain unknown. Dual perfusion of the human placental lobule revealed that suspected P-gp substrate, methadone, is transferred to a significantly greater extent in term placentas than in preterm (Nanovskaya et al., 2008), providing indirect evidence that P-gp may be more active in preventing placental transfer in earlier gestation. However, the direct measurement of placental P-gp transport activity, in relation to gestational age, has not been investigated to date. Therefore, the **first aim** of this investigation was to examine the correlation between human placental P-gp protein expression and transport activity at various gestational ages.

EFFECT OF GENETICS ON THE PLACENTAL BARRIER

The rate of certain pregnancy complications differs significantly between ethnic groups, suggesting a role of genetic variation in the development of high risk pregnancy. For example, non-Caucasian ethnicity is associated with significantly increased risk for development of Gestational Diabetes Mellitus (GDM) when controlling for age,

pregravid body mass index (BMI), and other GDM risk factors (Solomon et al., 1997). African American ethnicity is also an independent risk factor for predicting spontaneous preterm delivery (Goldenberg et al., 2008) as well as the development of preeclampsia (hypertension during pregnancy) (Eskanazi et al., 1991). Furthermore, the progression to severe preeclampsia is more likely among women of African American and Hispanic descent (Stone et al., 1994). The risk of delivering a small for gestational age (SGA) neonate, a finding commonly associated with smoking tobacco during pregnancy, varies by ethnic background of pregnant smokers (Okah et al., 2007). Neonatal withdrawal symptoms in mothers treated with methadone for substance abuse during pregnancy are more severe in African American neonates (Hagopian et al., 1996) yet are unrelated to maternal methadone dose across ethnicities (Bakstad et al., 2009).

Although other factors such as lifestyle, socioeconomic status, and access to healthcare (which vary by ethnicity) cannot be ruled out, a genetic basis for ethnic disparities in pregnancy complications warrants further investigation. Our working hypothesis is: distinct from the role of maternal genotype in posing disease risk during pregnancy, the placental genotype (derived from the fetal genome) plays a significant role in risk of pregnancy complications by influencing the biodisposition of endogenous substrates and xenobiotics.

Of the active proteins involved in the placental barrier, P-gp displays perhaps the most well-documented genetic variability between populations (Ambudkar et al., 2003). An important factor that could affect the activity of P-gp is the presence of single nucleotide polymorphisms (SNPs) in the DNA of its gene. In some cases, SNPs in the

gene encoding an enzyme result in altered protein expression or activity of the variant enzyme in comparison to the wild type. The altered activity of the enzyme/transporter could affect the pharmacokinetics of the medication and thus be associated with a sub-type of the population referred to as "poor" or "fast" metabolizers/transporters. Accordingly, patients with polymorphisms making placental P-gp more highly expressed or active may respond differently to drug substrates of P-gp, which may in turn affect fetal exposure (Hitzl et al., 2004). However, the association between genetic variants in the *MDR1* gene with P-gp expression or activity, and thus the effect on biodistribution of P-gp substrates, remains controversial.

Polymorphisms in the *MDR1* Gene

The human *MDR1* gene encoding P-gp exhibits considerable genetic variability with specific single nucleotide polymorphisms (SNPs) and haplotypes occurring at high frequencies in certain populations (Ambudkar et al., 2003). More than 50 SNPs in the *MDR1* gene have been reported (Saito et al., 2002), and among the most common associated with altered function are represented by nucleotide substitutions: C1236T, C3435T, and G2677T/A. C1236T is a synonymous (no amino acid change) polymorphism occurring in exon 12, which corresponds with Glutamate 412 in the first ATP binding domain of P-gp (Illustration 4) (Wang et al., 2006). C3435T, also synonymous, occurs in exon 26 and corresponds to the Isoleucine 1145 in the second ATP binding domain of P-gp (Illustration 4). The G2677T/A SNP, found in exon 21, replaces Alanine at position 893 with Serine and less commonly Threonine in a cytoplasmic loop of P-gp (Illustration 4). Despite the fact that two of the above SNPs

are associated with no change in amino acid sequence, all three have been demonstrated to independently decrease P-gp protein transport function *in vitro* when introduced to stable recombinant epithelial cells (Salama et al., 2006).

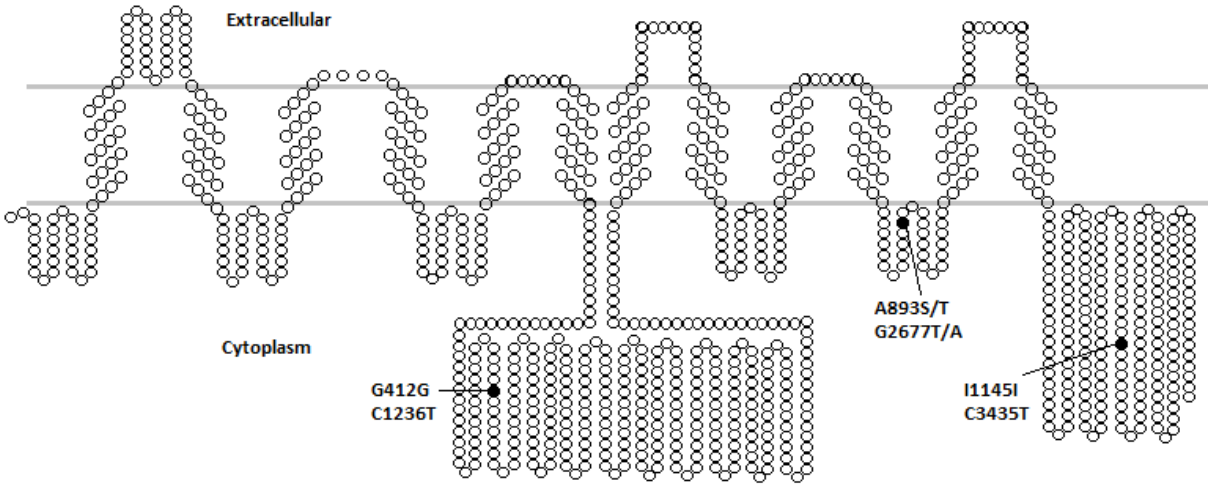


Illustration 4. Protein Structure of P-gp. Arrows indicate location and change (if any) in amino acid sequence within associated with each polymorphism. G2677T/A results in an amino acid change from Alanine to Serine or Threonine, while C1236T and C3435T cause no amino acid change.

The C1236T, C3435T, and G2677T/A variants are found together in up to 49% of the population in ethnic groups such as Chinese, Malay and Indian, 45-55% of Caucasians, and 5-10% of African Americans (Pauli-Magnus et al., 2004). Information on the relationship between these variants and the *in vivo* protein expression and activity of P-gp in humans remains unclear. In Japanese women, the G2677T/A polymorphisms were associated with reduced placental P-gp expression (Tanabe et al., 2001), similarly a study of German mothers of Caucasian ethnicity reported significantly lower P-gp expression in placentas carrying the G2677T and C3435T variants (Hitzl et al., 2004). Carriers homozygous for the C3435T polymorphism (TT) have both reduced P-gp expression and efflux activity in non-placental human tissues such as intestine and

leukocytes (Hitzl et al., 2001; Hoffmeyer et al., 2000). On the other hand, the presence of C3435T and G2677T/A polymorphisms did not appear to affect placental P-gp activity as evidenced by the transplacental transfer of its substrate Saquinavir (Molsa et al., 2005). Furthermore, several reports have associated these polymorphisms with increased P-gp activity in human non-placental tissues *in vivo* (Green et al., 2009; Kwan et al., 2009; Taubert et al., 2006).

The conflicting reports on C3435T and G2677T/A, to date, warrant further investigation the relationship between *MDR1* SNPs and placental P-gp protein expression and activity. Furthermore, despite the fact that the C1236T SNP has been implicated in P-gp protein function *in vitro*, the effect of the C1236T polymorphism on placental P-gp expression and function *in vivo* remains unknown. Therefore, the **second aim** of this investigation was to examine the relationship between *MDR1* polymorphisms and human placental P-gp expression and transport activity, and to identify potential differences in genotype-phenotype relationship among African American, Caucasian and Hispanic ethnicities.

ROLE OF P-GP IN REGULATING THE PLACENTAL BIODISPOSITION OF MEDICATIONS USED TO TREAT CONDITIONS DURING PREGNANCY

In some conditions affecting the pregnant patient, treatment requires the administration of a medication for most of the gestation period. Preclinical investigations used to evaluate the safety of such medications for use during pregnancy involve studies of the placental disposition and potential for fetal exposure. The primary focus of our investigations involves three main conditions: opiate addiction, tobacco smoking, and

gestational diabetes. Each of these patient populations—if left untreated—face health risk to the mother and/or fetus, and benefits offered by pharmacotherapy may outweigh the potential harm.

Opiate Addiction

An estimated 4.3% of women aged 15- 44 reported illicit drug use during pregnancy (Substance Abuse and Mental Health Services Administration, 2005), with up to 15-30% use during pregnancy reported in high-risk, urban populations (Schempf, 2007). Estimates of the prevalence of opiate abuse during pregnancy range from 1-2% to as high as 21% of pregnant patients (Brown et al., 1998). Opiate use during pregnancy is associated with low birth weight and increased risk of preterm delivery (Hulse et al., 1997), as well as the development of neonatal abstinence syndrome (NAS) (Fajemirokun et al., 2006). Newborns suffering from NAS may experience fever, irritability, hyperactive reflexes, dehydration, poor weight gain, and in severe cases, seizure or death (Kaltenbach et al., 1998).

Maintenance therapy of pregnant opiate abusers aims to provide a steady concentration of opiate in the maternal blood to minimize the effects of repeated withdrawals on the fetus. Maintenance therapy also enhances maternal compliance with obstetric care, and is associated with improved neonatal outcomes such as increased birth weight and less severe withdrawal (Fajemirokun et al., 2006; Kaltenbach et al., 1998). Benefits may also include reduced maternal drug seeking behaviors, including intravenous injection and commercial sex, both of which may expose the woman to infectious diseases such as HIV and hepatitis (Minozzi et al., 2008). The synthetic opiate

methadone has become the gold standard treatment for the management of pregnant heroin users (NIH 1998; CSAT 2005). Buprenorphine, a partial μ-opioid agonist, is also used for maintenance of the pregnant opiate addict, and limited studies have reported that the administration of buprenorphine is associated with milder adverse effects on the neonate than methadone (Fisher et al., 2000; Johnson et al., 2001; Jones et al., 2005).

Despite demonstrated improvement in maternal and neonatal outcome, both methadone and buprenorphine are also associated with the development of NAS. However, the incidence and severity of NAS do not correlate with dose of methadone or buprenorphine administered to the mother (Bakstad et al., 2009; Lejeune et al., 2006). The lack of dose response between maternal concentration and neonatal withdrawal indicate that the concentration of opiate in the fetal circulation may not be proportional to that in the maternal circulation. Therefore, the drug may not be crossing the placenta via passive diffusion alone; instead there may be carrier-mediated or active transport affecting its transfer. Furthermore, the difference in neonatal outcomes between the two opiates indicates that they each could cross to the fetal circulation to different extent. Taken together, it is apparent that the role of the placenta in fetal exposure to opiates warrants further investigation.

The transplacental transfer of methadone and buprenorphine was previously investigated in using the technique of dual perfusion of human placental lobule. Both opiates crossed the placenta, and the rate of methadone transfer from the maternal to fetal circuit was higher (29.4 ± 4.6%) than that for buprenorphine (11.6 ± 2.5%) (Nanovskaya et al., 2002; Nekhayeva et al., 2005). The concentration of buprenorphine retained in the

placental tissue compared to that in the maternal or fetal circuits was significantly greater than for methadone. This indicates that placental distribution, i.e., extent of transfer and retention/accumulation in the tissue, is different between the two opiates.

Evidence suggests that P-gp may have a role in the efflux of opiates from the placental tissue. The addition of a P-gp inhibitor during dual perfusion of the placental lobule resulted in increased transfer of methadone, but not buprenorphine, in the fetal circuit (Nanovskaya et al., 2005; Nekhayeva et al., 2006). *In vitro*, buprenorphine, methadone, and morphine each stimulated ATP hydrolysis in human cDNA-P-gp membrane preparations (Nanovskaya et al., 2005). Therefore, it appears that methadone and buprenorphine both interact with P-gp (as judged by stimulation of ATP hydrolysis). Furthermore, indirect evidence suggests that methadone—but not buprenorphine—is transported by P-gp from the feto-placental unit. However, a direct determination of opiate interaction with human placental P-gp and the kinetics of its transfer of these potential substrates has not been attempted to date.

Gestational Diabetes Mellitus

Normal pregnancy is associated with metabolic changes leading to decreased insulin sensitivity and reduced glucose tolerance, a physiologic change which ensures glucose supply to the growing fetus. However 3- 5% of pregnant women proceed to develop insulin resistance and are diagnosed with gestational diabetes mellitus (GDM) (Gabbe et al., 1977). GDM is associated with risks to the fetus including macrosomia (excess fetal growth), neonatal hypoglycemia, and increased risk for insulin resistance in adult life. Furthermore, GDM patients face up to a 50% chance of developing overt

diabetes mellitus in the decade after pregnancy without effective and early treatment (ADA; National Institute of Diabetes and Digestive and Kidney Diseases. National Institutes of Health, 2005).

First line management of GDM includes dietary regulation, exercise, and blood glucose monitoring. When lifestyle modifications fail to achieve glycemic control, additional treatment modalities such as insulin therapy or oral hypoglycemic agents must be considered. Oral hypoglycemic agents metformin, rosiglitazone and glyburide are effective in non-pregnant patients with diabetes mellitus, and may offer therapeutic potential for pregnant patients with GDM as well.

Meformin is a biguanide hypoglycemic agent used as first line pharmacotherapy in patients with type 2 DM. Metformin reduces blood glucose levels by inhibiting hepatic gluconeogenesis, although the molecular mechanism of this action is not well understood. Limited clinical evidence has demonstrated that GDM patients treated with metformin had less weight gain and improved neonatal outcomes compared with those treated with insulin (Balani et al., 2009; Rowan et al., 2003). *In vivo* (Hague et al., 2003; Vanky et al., 2005) and *ex vivo* (Nanovskaya et al., 2006) investigations reported that metformin is transferred across the placenta from the maternal to fetal direction. Dual perfusion of placental lobule revealed that metformin is transferred in the fetal-to-maternal direction to a greater extent than maternal-to-fetal, suggesting a role of efflux transporters in its distribution (Tertti et al., 2010). However, metformin has not clearly been identified as a substrate of placental efflux transporters to date.

Rosiglitazone is a thiazoladinedione used for the treatment of type 2 diabetes mellitus and insulin resistance in polycystic ovary syndrome (PCOS) (Belli et al., 2004; Malinowski et al., 2000; Rautio et al., 2006; Sepilian et al., 2005). Thiazoladinediones act by binding to nuclear peroxisome proliferator-activated receptors (PPARs), inducing multiple downstream effects including increased insulin sensitivity. Rosiglitazone is not approved by the FDA for use during pregnancy, however nonpregnant women treated with rosiglitazone for type 2 diabetes or PCOS could become pregnant and continue use during pregnancy (Demissie et al., 2006). Despite reports of adverse effects of rosiglitazone in nonpregnant patients with cardiovascular disease (Komajda et al., 2010), limited data have reported that rosiglitazone use during pregnancy is relatively safe and is associated with delivery of term healthy newborns (Demissie et al., 2006; Kalyoncu et al., 2005; Yaris et al., 2004). Rosiglitazone enters the fetal circulation, as was detected in fetal tissue following first trimester abortions (Chan et al., 2005), however the transfer of rosiglitazone across the perfused term placenta is variable (Holmes et al., 2006; Nanovskaya et al., 2008). *In vitro* evidence suggests that rosiglitazone interacts with P-gp (Festuccia et al., 2008; Weiss et al., 2009), however a potential role of P-gp in mediating the placental transfer of rosiglitazone remains unknown.

Glyburide (glibenclamide) is a second generation sulfonylurea drug used for treatment of type 2 diabetes mellitus. Glyburide inhibits pancreatic beta cell K^+ channels, leading to increased intracellular calcium and stimulation of insulin release (Proks et al., 2002). Glyburide is efficacious in treatment of pregnant patients with GDM and is not associated with adverse effects on maternal and neonatal outcome (Jacobson et al., 2005;

Langer et al., 2000). Investigation of the placental transfer of glyburide (Nanovskaya et al., 2006) revealed the transfer of glyburide from the fetal-to-maternal direction was higher than in the reverse direction, i.e., maternal-to-fetal. The asymmetric transfer of glyburide could be explained, in part, by the activity of placental efflux transporters. Recent investigations have provided evidence that glyburide could be a substrate of placental ABC transporters (Gedeon et al., 2006; Gedeon et al., 2008), however the direct measurement of its ATP-dependent transport kinetics in placenta is lacking.

In general, evidence supports that oral hypoglycemic agents could be beneficial in the treatment of GDM when used as monotherapy. The reported asymmetric placental transfer of hypoglycemic drugs favoring fetal-to-maternal transport suggests the involvement of efflux transporters in their distribution. However, it remains unclear whether glyburide, rosiglitazone, and metformin are transported by one or more of the same placental efflux transporters.

Cigarette Smoking

Tobacco smoking during pregnancy has been associated with spontaneous abortion, placental pathology, preterm labor, low birth weight, stillbirth, sudden infant death syndrome, and childhood developmental problems (Rogers., 2009). Approximately 13% of mothers report smoking during the last 3 months of pregnancy (England et al., 2007), and many find it hard to stop or reduce smoking without the aid of medication. The benefits of smoking cessation during pregnancy include significant increase in birth weight and reduction in risk of preterm birth, as well as long term health benefits to both mother and child (Lumley et al., 2004).

Bupropion is an antidepressant which has had success as an alternative to nicotine replacement therapy for smoking cessation in the non-pregnant patient. Bupropion may also offer therapeutic benefit for smoking cessation in the pregnant patient, although its safety for use during pregnancy is not well established. Bupropion is labeled Category C by the US Food and Drug Administration (FDA), indicating animal studies revealed adverse effect and there are not adequate studies in humans, but potential benefits may warrant its use during pregnancy despite potential risks (www.fda.gov).

Preclinical studies investigating bupropion biodisposition by human placenta revealed that bupropion crosses from the maternal to fetal circulation (Earhart et al., 2010), and is metabolized by placental tissue (Wang et al., 2010). Placental transfer of bupropion to the fetal circulation was approximately 20% (Earhart et al., 2010), in comparison to 30-40% transfer of nicotine which diffuses freely across the placental tissue (Nekhayeva et al., 2005; Pastrakuljic et al., 1998; Sastry et al., 1987). On one hand, the lipophilic nature of bupropion may promote binding to placental tissue and thus decrease its transfer to the fetal circulation. On the other hand, its incomplete placental transfer could also be explained, in part, by the activity of placental efflux transporters. Bupropion stimulated ATP hydrolysis by P-gp expressing membranes (Wang et al., 2008), indicating that it interacts with P-gp. However, the direct measurement of its transport by P-gp expressed in human placenta has not been reported to date.

Drug-drug interactions

The co-administration of multiple pharmacologic substances introduces the potential for drug-drug interactions which may potentiate unexpected adverse outcomes

for mother or fetus. For example, many illicit drug abusers use more than one substance, and around 6% of pregnant women reported using multiple substances during pregnancy (Havens et al., 2009). The efficacy of treatment could be decreased in the case of polypharmacy, as evidenced by increased severity of neonatal abstinence syndrome in methadone treated patients who smoke versus nonsmokers (Winklbaur et al., 2009). Additionally, dual perfusion of placental lobule revealed that co-administration of opiates can increase placental permeability and thus maternal-to-fetal transfer of chemicals (Malek et al., 2009).

Aside from illicit drug abusers, patients with GDM may also be affected by co-administration of multiple medications. Clinical evidence suggests that combination therapy may be effective in treating patients with uncontrolled DM. Glucovance, a combination of metformin and glyburide, is considered safe for use during pregnancy. Furthermore, in nonpregnant patients with inadequate glycemic control despite established glyburide/metformin therapy, the addition of rosiglitazone improves glycemic control (Dailey et al., 2004). However, the placental distribution of these investigational medications may differ depending on their use as mono or combination therapy.

Co-administration of multiple drugs which are substrates of placental efflux transporters could introduce competition for a single efflux transporter, thus increasing their transfer to the fetal circulation. A drug interaction could also occur when the transporter responsible for efflux is also responsible for regulating the feto-placental elimination of an endogenous substrate. In this case, the administered drug could act as an inhibitor of transport of the endogenous substrate. Therefore, the potential interaction

between multiple drugs and co-administered medications must be considered. The importance of P-gp in drug–drug interactions has been identified in USFDA guidelines (Huang et al., 2007), and it is increasingly accepted that co-administration of P-gp substrates can result in competition for efflux which could affect their pharmacokinetics and pharmacodynamics (Zhou, 2008). Therefore, the **third aim** of this investigation is to determine the role of P-gp in regulating the placental biodisposition of medications used to treat conditions during pregnancy, namely opiate dependence, smoking cessation, and gestational diabetes mellitus.

Although the other aims of this investigation focus on characterizing placental P-gp, the study of drugs which have unknown transporter specificity requires the consideration of other ABC transporters. Specifically, BCRP is highly expressed in placenta and has overlapping transport of some P-gp substrates (Polli et al., 2004). Therefore, the interaction of BCRP with the investigational drugs in this study was also screened.

BABOON AS AN ANIMAL MODEL FOR INVESTIGATING PLACENTAL P-GP *IN VIVO*

Due to ethical and safety concerns for mother and fetus, *in vivo* studies of the placental biodisposition of potentially teratogenic drugs are not conducted in the pregnant human. However, this information is crucial in predicting in the distribution of potentially harmful xenobiotics between the maternal and fetal circulation/tissues. Therefore, other models must be employed for preclinical study of investigational drug safety and the role of transporters in regulating placental drug disposition. The following

techniques could be considered as models to investigate the role of P-gp in placental distribution of medications; however each technique inherently has associated limitations and difficulties.

In Vitro Assays

P-gp Reconstituted Proteoliposomes:

The incorporation of purified P-gp into proteoliposomes, a plasma membrane structural model, has been used in analysis of transporter function. However, important structural and functional differences have been noticed between native P-pg and that reconstituted into proteoliposomes. A notable increase of the phosphorylation level occurs in P-gp proteoliposomes when compared to P-gp from native plasma membrane vesicles (Lelong-Rebel et al., 2005). It is unknown whether this altered phosphorylation status has an effect on P-gp function. Additionally, numerous effects have been attributed to the presence of detergent, such as the activation of P-gp ATPase by low concentrations of octylglucoside (Jones, 1999) as well as other altered function and properties of the reconstituted proteoliposomes by detergent (Naito et al., 1995). Therefore, the use of P-gp in reconstituted liposomes may not offer reliable predictions of the role of P-gp expressed in placental apical membrane throughout gestation.

MDRI Gene Transfected Cells:

Difficulty has prevented widespread use of cells transfected with the *MDR1* gene encoding P-gp. For example, the use of E. coli for host cell expression leads to misfolding of the protein (Linton et al., 2002). Recombinant baculovirus-infected insect

cells expressing human P-gp are available, however several parameters, including the composition of the culture medium, baculovirus titer, and post-infection incubation time, alter the expression and glycosylation properties of P-pg in these cells (Kodan et al., 2009). Madin Darby canine kidney (MDCK) cells transfected with the *MDR1* gene have been used, however it has been demonstrated that in culture these cells lose contact inhibition and cell polarization (Braun et al., 2000). This loss of organization and polarization would make it difficult to quantify unidirectional efflux by active transporters, and limits the applicability of this model to the polarized syncytiotrophoblast membrane.

Drug-selected Cell Lines:

Multidrug resistant cancer cell lines may be established *in vitro* through continual drug selection. Although they offer a sufficient means for investigating the regulation of ABC transporter expression and function, rarely do these continual drug selections emulate what is found in the clinical setting. In fact, chemotherapy resistant cell lines often display acquired resistance due to new mutations in the *MDR1* gene (Mickley et al., 1997). Drug selection with products that act by targeting DNA or microtubules leads to DNA damage, nonhomologous recombination, and acquired drug resistance. Therefore, the P-gp expressed in cancer cells which have been selected based on chemotherapy resistance often represent the product of hybrid mRNA which does not exactly represent parent cell P-gp mRNA or protein. Furthermore, the tumor types used for the development of drug-selected cell lines may produce distinctly different *MDR1* transcript. For example, the mRNA produced in P-gp expressing colon cancer cell lines is

shorter in its 5' end that the *MDR1* mRNA produced in the MCF-7/Adr (human breast carcinoma) and that of K562/Adr (human erythroleukemia) P-gp expressing cell lines (Gómez-Martínez et al., 2007).

The Caco-2 cell line consists of human epithelial colorectal adenocarcinoma cells classically used as a monolayer to predict intestinal permeability of orally administered drugs. The Caco-2 monolayer represents a close estimation of absorptive epithelium in that a polarized cell monolayer consisting of metabolic enzymes between two membrane barriers with distinctly different transporters. However, the Caco-2 monolayer forms weaker tight junctions, lower expression of uptake transporters, and overexpression of efflux transporters, which limit the ability to extrapolate information gained from these studies to *in vivo* membrane (Press et al., 2008). Additionally, since Caco-2 cells represent a heterogenous cell population, they are subject to genetic drift or selective growth, which contributes to wide variability in data obtained from different investigations (Herold et al., 1994).

Overall, the limitation of the above three models, namely proteoliposomes, transfected cells, and multidrug selected cell lines, is that they do not represent physiologically expressed protein in its native cell membrane. These limitations may cause the permeation of some compounds (such as efflux substrates) to be underestimated or overestimated relative to *in vivo*, leading to poor correlation of *in vitro*/*in vivo* data. Therefore, they do not give direct information about human placental P-gp and its activity in regulating placental bio-distribution of compounds.

Placental Membrane Vesicles:

Membrane vesicles prepared from human placental tissue allow the direct determination of the transport kinetics of potential P-gp substrates. P-gp is localized in the brush border membrane of placental syncytiotrophoblast (Nakamura et al., 1997), a polarized epithelium expressing different transporters/proteins on the brush border (apical) and basal membranes. The asymmetry of the syncytiotrophoblast membrane allows the separation and isolation of the brush border membranes. Vesicles formed during the preparation of brush border membranes assume two configurations: inside-out (IOV) and right-side out (ROV). A preparation enriched in IOVs can be used to investigate the transport of radiolabeled substrate of P-gp which, following active transport, becomes 'trapped' inside the vesicle. The IOV preparation can be utilized to determine the *in vitro* time-dependent, P-gp mediated accumulation of a radioisotope of the substrate, as well the kinetics of transport.

Vesicular transport assay offers a valuable tool for directly measuring the transport kinetics of P-gp substrates across the cell membrane. However, this technique has limited applicability to *in vivo* conditions such as investigation of the extent of transfer of substances from the maternal to fetal circulation or metabolism of compounds by the placental tissue.

Ex Vivo Perfusion

The technique of dual perfusion of placental lobule (DPPL) utilizes human placenta obtained immediately after delivery in which a test drug is perfused in through a simulated maternal or fetal circulation and detected in samples taken from both circuits

during the experimental period. DPPL retains the anatomical and functional integrity of placental tissue and has been proven as the best available *ex vivo* method to determine the transfer of drugs from the maternal to the fetal circuit and to evaluate potential toxicity of the drugs on placental functions (Schneider et al., 1985). This technique has been extensively utilized and validated for determining the bidirectional transplacental transfer, distribution, and metabolism of numerous drugs in human placenta. Plus, the addition of a P-gp inhibitor during perfusion may provide indirect evidence of P-gp involvement if the inhibitor alters the bi-directional transfer of a substrate.

Although DPPL allows determination of placental transfer and toxicity of test compounds, it does not allow the direct measurement of efflux of P-gp substrates. Additionally, this model system has limited applicability to *in vivo* pharmacokinetics, since the models lacks excretion or metabolism that would normally eliminate a drug from the maternal circulation.

In Vivo Animal Models

In vivo studies provide an invaluable tool for studying placental physiology with maternal and fetal pharmacokinetic mechanisms (metabolism, elimination, distribution) intact. However, due to ethical and safety concerns for the mother and fetus, this population in humans is excluded from most experimentation and substituted with animal models of pregnancy. Many mammals have been used to model the human placenta in structure, development, and function. However, each animal model represents a unique set of challenges when extrapolating data to humans due to crucial differences between human and other mammal placentation which must be taken into consideration.

Placental experimentation using a small animal model, such as rodent, has distinct advantages due to rapid generation time, lower expense, vast amounts of genetic information, and the ability to genetically overexpress or ablate genes. However, crucial differences in placental development, structure, and function exist between rodent and human, which limits their ability to model physiologic mechanisms in human placenta (Table 1).

Implantation refers to the process by which the embryo adheres to the wall of the uterus. Implantation has been classified into three broad categories: centric, eccentric and interstitial (Wimsatt et al., 1975). Centric implantation occurs when the blastocyst fuses with the luminal epithelium of the uterus without penetrating through it. Rabbits, dogs, cows, pigs, sheep, and many marsupials have centric implantation. In eccentric implantation, the uterine luminal epithelium forms an invagination to surround the trophoblast. Mice, rats and hamsters have eccentric implantation. The third type of implantation is interstitial, in which the trophoblast passes through the luminal epithelium to invade the endometrial stroma and become imbedded into the wall of the uterus. Implantation in humans and other primates have been classified into this category (Wimsatt et al., 1975).

The interstitial pattern of implantation also equates to more extensive invasion of the trophoblast into the blood supply comprised of uterine spiral arteries (Lee et al., 2004). It is not surprising that differences in the extent to which the feto-placental unit invade the maternal uterus may translate to differences in the maternal-placental-fetal distribution of compounds between species.

Table 1: Comparison in placental characteristics between mouse, human, and baboon

Placentation in Mouse, Human, and Baboon			
	Mouse	Human	Baboon
Implantation	Eccentric	Interstitial	Interstitial
Invasion of uterine arteries	Shallow	Deep	Deep
Tissue Architecture	Labyrinthine	Villous	Villous
Cell Barrier	Three trophoblast layers	Single syncytiotrophoblast layer	Single syncytiotrophoblast layer
Placental Hormones	Placental lactogen	Chorionic gonadotropin, somatomammotropin, growth hormona, progesterone, lactogen	Chorionic gonadotropin, somatomammotropin, growth hormone, progesterone, lactogen
Gestation	3 weeks	40 weeks	26 weeks

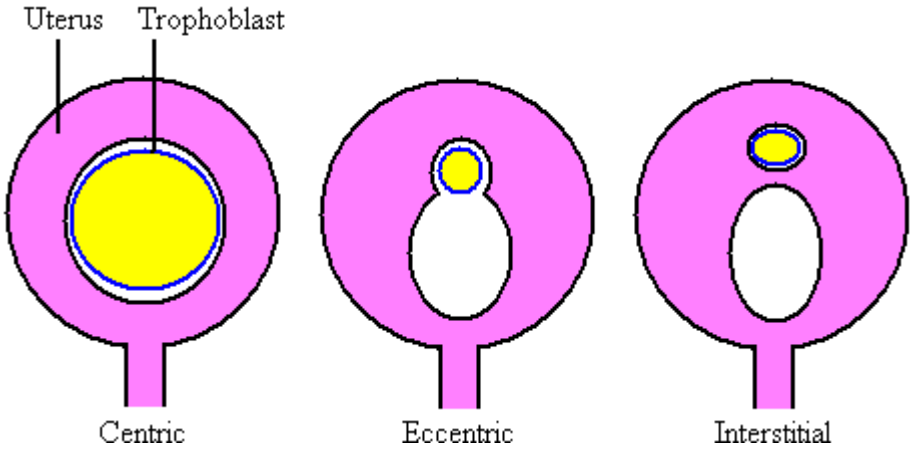


Illustration 5. Patterns of Implantation.
Centric implantation (left) is characteristic of rabbit, dog, cow, pig, sheep, and marsupial placenta. Eccentric implantation (middle) is characteristic of rodent placenta. Interstitial implantation (right) is characteristic of human and other primate placenta (baboon).

The tissue architecture represents the structure of the vascular exchange area between maternal and fetal blood compartments. The labyrinthine structure in rodents

represents a network of trophoblast cells invading the uterine stroma which occurs early in mouse and human placenta development. However, as the human placenta matures, the placental trophoblast undergoes extensive branching to form a villous tree (Rossant et al., 2001). This structure alters the exchange area between the maternal blood space and the placental (fetal) capillary network, and such extensive branching does not occur with mouse placental development (Illustration 6). Furthermore, rodents have three trophoblast cell layers (trichorial) — two syncytial layers and a single 'mononuclear' cell type of unknown function — whereas humans have a single syncytial layer plus an underlying trophoblast stem cell layer (Rossant et al., 2001).

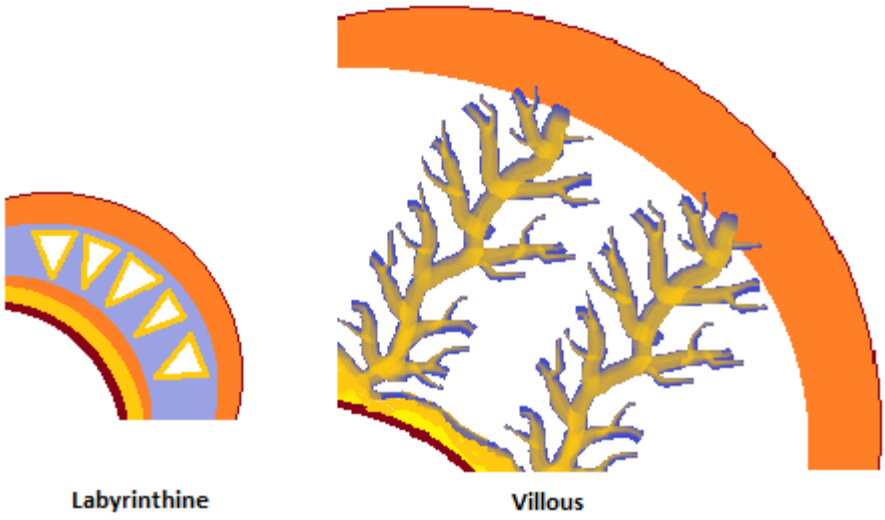


Illustration 6. Labyrintine and Villous Placental Tissue Architecture
Labyrinthine placental structure (left) is found in rodent placenta, while the highly branched villous structure (right) represents human and primate placenta.

The variability in placental tissue architecture equates to wide interspecies differences in the placental permeability of molecules, complicating the interpretation of transplacental transfer studies (Schneider, 1991). The sheep placenta does not allow diffusion of hydrophilic molecules with a molecular weight > 400 daltons, although the

guinea pig placenta allows diffusion of some molecules with molecular weight up to > 5000 daltons (Schneider, 1991). Digoxin crosses the placenta in humans and rodents, while the ovine placenta is relatively impermeable to it (Nau, 1986). Similarly, the extent of placental transfer of gentamycin differs between human and goat models (Nau, 1986).

In addition to small molecule permeability, other pharmacokinetic factors affecting the transplacental passage of drugs in whole animal models show interspecies diversity (e.g. protein binding, drug-metabolizing enzymes and fetal renal drug clearance) and affect the transfer of drugs into the fetus (Mihaly et al., 1983; Nau, 1986). Therefore, extrapolation of animal data to the human model is difficult.

Placental endocrine function can be characterized by its hormone production, and mouse and human placental endocrine functions are very different. Ovarian progesterone production is required throughout pregnancy in the mouse. At first the corpus luteum is maintained by pituitary prolactin, later placental lactogen from the trophoblastic giant cells takes over this function (Carter et al., 2007). In human pregnancy, maintenance of the corpus luteum depends on human chorionic gonadotropin (hCG) produced by the trophoblast. However, from about eight weeks of gestation, progesterone production by the syncytiotrophoblast is sufficient to maintain pregnancy. Chorionic gonadotropin, although produced by various primates, is not found in mice or other rodents. Human and other primate placental endocrine function is also characterized by secretion of chorionic somatomammotrophic hormone and placental growth hormone (Carter et al., 2007). In addition to endocrine function, other similarities in placental functions – specifically the metabolism of medications – have been identified between human and baboons

(Zharikova et al., 2007).

Functional differences between mouse and human placenta may, in part, be a reflection of the great difference in gestation length. Pregnancy lasts for just three weeks in mice compared to 40 weeks in humans. Consequently, many of the developmental processes that occur in humans during intrauterine life represent postnatal (after birth) events in rats and mice (Carter et al., 2007). In contrast, humans and other primates have relatively long gestation periods and typically give birth to single, well-developed offspring.

The baboon placenta and fetus share developmental milestones with human (Enders et al., 1997), and relative stage of gestation can be compared to human. The gestational age of 40-55 days in baboons correspond to 8-12 weeks of gestation in humans; 94-108 days corresponds to 22-24 weeks in humans; 142-156 days in baboons corresponds to 32-34 weeks in humans; and 185 days gestation is comparable to term (40 weeks) in human. Throughout gestation, baboon (*Papio cynocephalus*) placental structure and function are very similar to that of human placenta (Houston, 1969). Previous work in our laboratory has demonstrated that the baboon placenta is an effective model for comparing the effects of cytochrome P450 enzymes and esterases to human placenta. Due to the fact that the ATP-binding cassette transporters, including P-gp, are highly conserved among primates, it is likely that P-gp plays a similar role in human and baboon placental barrier (Dean et al., 2005).

Characterizing the placental expression and activity of P-gp in the baboon would provide a valuable tool for understanding in vivo P-gp activity and role in fetal protection

from xenobiotics. To date, there is little to no information on the role of P-gp in baboon placental physiology. Therefore, the **fourth aim** of this project was to investigate the baboon as an animal model for studying placental P-gp *in vivo*.

Chapter 3: Experimental Design/Methods

CLINICAL MATERIAL

Human placenta:

A staff of trained research nurses was responsible for transporting the placentas immediately after delivery to our laboratory according to a protocol approved by the Institutional Review Board of UTMB. Along with placenta, the nurses provide a data sheet that includes maternal age, race, health conditions, medications, gestational age, type of delivery, and gender, weight, and APGAR score of the newborn. The APGAR score is determined by evaluating the newborn on five criteria (activity, pulse, grimace, appearance, respiration) on a scale from zero to two and summing up the five values obtained. The resulting APGAR score ranges from 0 to 10.

Exclusion criteria:

Placentas obtained from pregnancies with documented drug abuse during pregnancy or infections with HIV or hepatitis were excluded from the study. Additionally, placentas collected without all of the information included in the data sheet were excluded from analysis.

Inclusion criteria:

Term human placentas (38-41 weeks) obtained from uncomplicated pregnancies and preterm placentas (<37 weeks) of various etiology were included in the study.

PREPARATION AND INVESTIGATION OF HUMAN PLACENTAL BRUSH BORDER MEMBRANE VESICLES

P-glycoprotein is localized to the brush border membrane of placental syncytiotrophoblast cells. The syncytiotrophoblast is a polarized epithelium, expressing different transporters/proteins on the brush border (apical) and basal membranes. The asymmetry of the syncytiotrophoblast membrane allows separation and isolation of the brush border membrane and study of its trans-membrane/membrane-associated proteins. Vesicles formed from the brush border membrane naturally assume two configurations: inside-out (IOV) and right-side out (ROV). IOVs containing P-gp have the cytoplasmic ATP-binding domain exposed and transport substrate from the outside to the inside of the vesicle (Illustration 7).

The isolation of IOVs can be used to study transport of radio-labeled P-gp substrate which, following active transport, becomes 'trapped' inside the vesicle. This technique can be utilized to determine *in vitro* time-dependent, P-gp mediated accumulation of radioligand, as well as the pharmacokinetic parameters of Kt and Vmax for a given substrate.

Placental brush border membrane vesicles were prepared using a protocol modified from Ushigome et al. (Ushigome et al., 2003). Tissue was cut from the maternal side, washed two times in 0.9% NaCl. All of the following steps were carried out at 4°C. The cut tissue was transferred to sucrose-HEPES-Tris (SHT) buffer (250 mM sucrose, 10mM HEPES-Tris, pH 7.4), and stirred for one hour to disrupt brush border membrane. The tissue plus buffer was filtered through two layers of woven cotton gauze,

and tissue was discarded. The filtrate was centrifuged at 800 x g for 10 min, to remove blood and cell debris. The supernatant was combined with 20 mM $MgCl_2$ (1:1 ratio) and stirred for 10 minutes. The solution was centrifuged at 10,500 x g, 10 minutes. The pellet was discarded and the supernatant was centrifuged at 20,000 x g, 20 minutes. The pellet, containing brush border membranes, was re-suspended in SHT buffer with a 26-gauge needle.

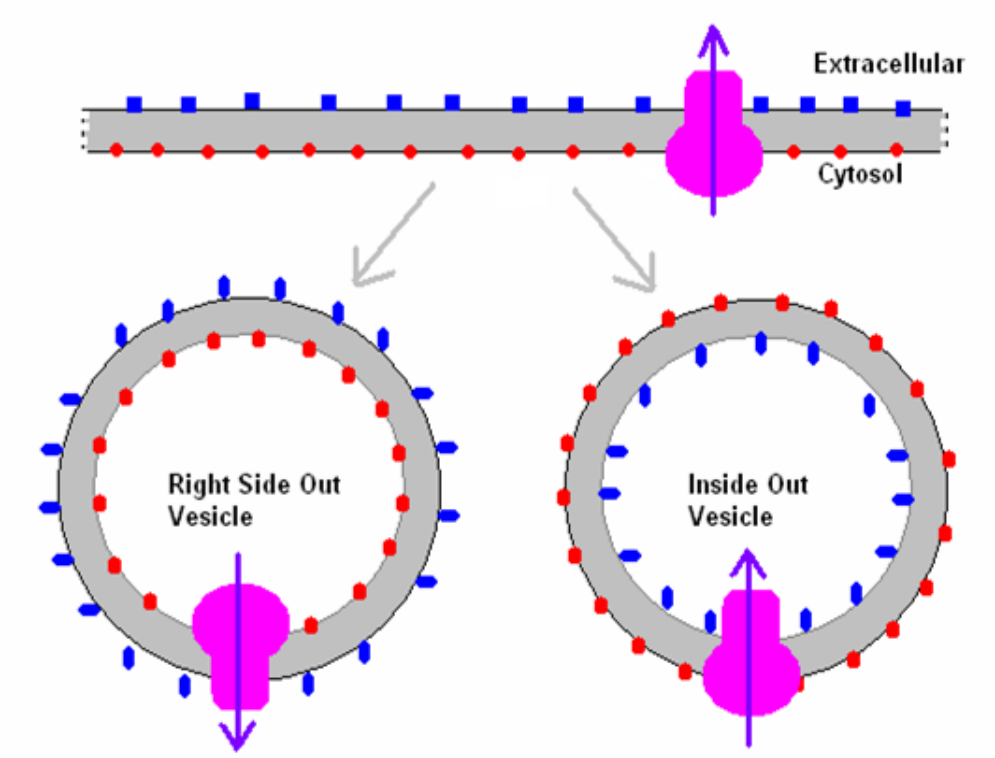


Illustration 7. Orientation of Membrane Vesicles
Disruption of the plasma membrane allows spontaneous formation of membrane vesicles. Membrane-associated proteins maintain their orientation to the membrane which can form Right Side Out (left) or Inside Out (right) vesicles.

To maximize the proportion of IOVs, affinity chromatography was used to separate ROVs (Awasthi et al., 1994). Lectin immobilized on an agarose column binds the N-acetyl-glucosaminyl residues of glycolipids and glycoproteins (extracellular) on the

ROV, and retains the ROVs in the column. The lectin has no access to the N-acetyl-glucosaminyl residues in the IOV, and they pass through the column in the elutant void volume. 50 mM methyl-α-D-Mannopyranoside was used to displace the retained ROVs from the lectin affinity column following IOV elution. Vesicles enriched in the inside out configuration were used for all membrane vesicle transport experiments.

Vesicles were aliquoted and immediately stored at -80ºC until use. ATP-dependent transport activity was verified an aliquot from each placental preparation, and those with low or no detectible ATP-dependent transport after thawing were excluded from the study. For experiments using a pool of multiple samples, the pool was prepared using membrane preparations of 60 placentas obtained from uncomplicated term pregnancies. The large pool size reduces the confounding variable of inter-individual variation in the activity of transporters, and provides multiple and long-term use of the same lot of membranes.

Electron Microscopy

The brush border membrane vesicle pellet was fixed in 2.5% formaldehyde and 0.03% $CaCl_2$. The pellet was then washed in 0.1M cacodylate buffer and fixed in 1% OsO_4 in 0.1M cacodylate buffer. The pellet was *en bloc* stained with 2% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Poly/Bed 812 (epoxy). Ultra thin sections were then cut on a Leica Ultracut S ultramicrotome, stained with lead citrate and examined in a Philips 201 electron microscope at 60 kV.

Alkaline Phosphatase Activity

To confirm purity of brush border membrane, alkaline phosphatase was assayed as an enzymatic marker of brush border (Hulstaert et al., 1973; Smith et al., 1977). Upon dephosphorylation by phosphatases, the substrate pNPP (p-Nitrophenyl Phosphate) turns yellow and can be detected at absorbance of 405 nm. AnaSpec Sensolyte pNPP Alkaline Phosphatase Colorimetric Assay Kit was utilized (AnaSpec, Inc., Fremont, CA). Briefly, 50 μL of sample containing 300 μg brush border membrane protein was prepared in Triton X-100 lysis buffer, in triplicates. The reaction was initiation by the addition of 50 μL of pNPP substrate mixture and incubated at 37°C for 15 min. Absorbance was read at 405 nm using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA). Alkaline phosphatase from calf intestine was used as a standard.

The enrichment of alkaline phosphatase activity in the final membrane preparation was compared to activity in initial syncytiotrophoblast cell lysate. An enrichment of at least 10-fold was established as a lower limit based on a previous report (Smith et al., 1977), and any preparations not achieving this level were excluded from the study.

Acetylcholinesterase Activity

Acetylcholine is a neurotransmitter released into the extracellular (synaptic) space. Acetylcholinesterase (ACE) breaks down acetylcholine, therefore must have access to the extracellular space, rendering ACE a marker of the extracellular side of the phospholipid bilayer. The level of ACE can indicate the proportion of inside out vesicles.

To estimate the orientation of vesicles (i.e., the proportion of inside out vesicles) following affinity chromatography, acetylcholinesterase activity was assayed.

Control vesicles were lysed in hypotonic buffer to represent 100% of acetylcholinesterase activity. Intact or lysed vesicles (5 mg/mL) were suspended in 0.1 M phosphate buffer containing 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) and 0.1M acetylthiocholine chloride and incubated at room temperature for 3 minutes. Absorbance was read at 412 nm using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA). The acetylcholinesterase activity was determined as moles of substrate hydrolyzed/min*mg protein using the formula: $R = ((1.22 \times 10^{(-3)})*\Delta A) / C$ (*ΔA=change in absorbance, C=mg/ml protein*). The percentage of inside out vesicles was calculated as $R_{lysed} - R_{intact}$ = % inside out (R_{lysed} : activity in lysed vesicles, R_{intact} : activity in intact vesicle preparation). All assays were performed in triplicate.

Total Protein Determination

The total protein concentration in all samples was determined by detergent-compatible Bradford protein assay. Briefly, Bradford Reagent (Bio-Rad Laboratories, Hercules, CA, USA) was diluted 4:1 (v/v) with water. Samples of brush border membranes (10 μL) were added to the reagent (5 mL) and determined in triplicate using a Genesys 20 spectrophotometer (Thermo Scientific, Waltham, MA) at 595 nm. Total protein concentration in vesicle preparations was determined using bovine serum albumin (10 to 100 μg total protein) as a standard.

AIM 1: HUMAN PLACENTAL P-GP PROTEIN EXPRESSION AND TRANSPORT ACTIVITY AT VARIOUS GESTATIONAL AGES.

Approach

The objective of this aim was to characterize the relationship between protein expression and transport activity of human placental P-gp at various gestational ages. It was hypothesized that P-gp transport activity would decline in parallel to its protein expression throughout gestation. To achieve this goal, P-pg protein level was determined using Western Blot analysis of placental brush border membranes prepared from placentas of various gestational ages. The transport activity of P-gp was determined as the uptake of P-gp prototypic substrate, paclitaxel, into preparations of placental IOVs.

Determination of P-gp Protein Levels by Western Blot

Western blot quantification of P-gp protein expression was carried out using 7.5% SDS/polyacrylaminde gel electrophoresis. The amount of total placental apical membrane protein loaded on each well was 10 μg. At the end of electrophoresis, the gel was electroblotted on nitrocellulose membranes overnight at 4 ºC and a constant potential of 25 V. Blots were probed with anti-P-gp murine monoclonal antibodies (mAb C219) diluted 1:200 (v/v) and secondary goat anti-mouse horseradish peroxidase-conjugated antibodies diluted 1:1000 (v/v). Detection of the protein bands was carried out by spot densitometry and digital imaging of the enhanced chemiluminescence spots. The amount of expressed β-actin was used to normalize the amount of P-gp in each loaded sample on the gel. A positive control consisted of human P-gp membranes (Gentest Corporation)

Characterization of P-gp Transport Activity of Substrate Paclitaxel

Transport activity was determined using a protocol modified from Ushigome, et. al, (Ushigome et al., 2003) which measured uptake of radiolabeled P-gp substrate, [^{3}H]-paclitaxel (38 Ci/mmole, 83dpm/fmole), in placental BBMVs. Each reaction was carried out in SHT buffer (10 mM HEPES-Tris, 250 mM sucrose) containing 4mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine phosphokinase, either 2 mM ATP or 3 mM NaCl, and placental BBMVs at a concentration of 0.05 μg/μL (7 μg total protein), in duplicates. The reaction was initiated by the addition of [^{3}H]-paclitaxel, at a final concentration ~ 200 nM unless otherwise indicated. The reaction was terminated after 1 minute by the addition of 1 mL ice cold buffer, and vesicles were isolated using rapid filtration by a Brandel Cell Harvester using Whatman glass fiber filter strips (pore size 0.7 μM). The amount of [^{3}H]-paclitaxel retained was measured using liquid scintillation analysis, active transport was calculated as the difference in [^{3}H] paclitaxel in the presence and absence of ATP and expressed as pmol/mg protein*min. P-gp-specific transport of paclitaxel was confirmed by the addition of the P-gp inhibitor verapamil and monoclonal antibody C219.

Calculation of Kt and Vmax

Uptake of radiolabeled substrate by membrane vesicles was measured by incubating with different initial concentrations of substrate from 0.02 to 2 μM. Kt and V_{max} were determined by the least-squares fit of the data to the Michaelis-Menten equation. The reciprocal of radioligand uptake (1/uptake) was plotted vs the reciprocal of radioligand concentration in the incubation medium (1/c) for each experiment. The Kt

and V_{max} values for each experiment were obtained from the X and Y-intercepts, respectively, and pooled together to calculate the mean ± SEM. Statistical significance was analyzed by using students T-test with a limit of significance of 0.05.

Calculation of Ki

The concentration of inhibitor that reduced [^{3}H]-paclitaxel transport velocity by half was identified as the IC_{50}. The Ki was calculated using the Kt for paclitaxel transport identified experimentally and the equation of Cheng and Prusoff: $Ki = (IC_{50})/ (1 + [paclitaxel]/Kt_{paclitaxel})$ (Cheng et al., 1973).

AIM 2: GENETIC ANALYSIS OF P-GP POLYMORPHISMS

Approach

The objective of this aim was to examine the relationship between common polymorphisms in the *MDR1* gene encoding P-gp with its placental expression and activity. It was hypothesized that the functional variants C1236T, C3435T, and G2677T/A would be associated with altered P-gp expression and activity in human placenta. To achieve this goal, DNA was extracted from human placental nuclear fractions, analyzed for the presence of the three *MDR1* polymorphisms, and correlated with placental P-gp expression and transport activity in these patients.

PCR-RFLP-based Genotyping

DNA was extracted from placental nuclear fraction (n =120) using the PureGene Genomic DNA purification Kit (Gentra Systems, Minneapolis, MN). The PCR-RFLP-base genotyping assay (Tanabe et al., 2001) was used for the determination of the G2677T/A polymorphism. Briefly, the forward primer FP 5'-

TACCCATCATTGCAATAGCAG -3', and the reverse primer RP 5'-TTTAGTTTGACTCACCTTGCTAG-3', were used to generate a 107 base-pair fragment. The PCR reaction mixture (50 µL) consisted of ~50 ng of genomic DNA, 200 µM dNTPs, 1x-PCR buffer solution, 1.0 mM $MgCl_2$, 5 pmol of each primer, and 1 U of Taq DNA polymerase (Promega, Madison, WI). The PCR conditions consisted of an initial melting step of 94ºC for 5 min, followed by 35 cycles of melting at 94ºC for 30 sec, annealing at 50ºC for 45 sec, and extension at 72ºC for 60 sec. A final extension step at 72ºC for 5 min terminated the process. A 20 µL amplicon was digested at 37ºC overnight with 2 U of NheI restriction enzyme, which recognizes the wild-type G allele. The digested product was run on a 2% agarose gel at 85V for 1 h and the genotypes were identified according to the banding pattern observed. For quality control, representative samples of both the reference and the variant alleles were confirmed by direct sequencing. The G allele was classified as wild-type (WT), and the A and T minor alleles were classified together as variant (V) genotype.

TaqMan Genotyping

TaqMan real-time PCR utilizes a probe comprised of a single-stranded oligonucleotide complementary to a segment of 20-60 nucleotides flanking the SNP of interest. A fluorescent reporter (ex: 6-carboxyfluorescein, FAM) and a quencher (ex: minor groove binder, MGB) are covalently attached to the 5'- and 3'-ends of the probe, respectively. There are two reporter probes, one complementary to each SNP allele. The close proximity between fluorophore and quencher inhibits fluorescence from the fluorophore, however during PCR, the 5' to 3' exonuclease activity of Taq polymerase

degrades the part of the probe that has annealed to the template. Degradation of the probe releases the fluorophore and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence. Fluorescence detected in the real-time PCR thermal cycler represents one or both of the fluorophores and thus the presence of wild type allele, variant allele, or both.

Placental nuclear fractions (n =200) were isolated using subcellular fractionation. DNA was extracted from the placental nuclear fraction using the Qiagen DNeasy Blood & Tissue kit (Qiagan, Inc., Valencia, CA). Genotyping of the SNPs at position 1236 and 3435 (rs1128503 and rs1045642, respectively) was performed using TaqMan® Drug Metabolism Genotyping Assays. The triallelic SNP at position 2677 of *MDR1* (rs2032582) was genotyped using 2 different Custom TaqMan® SNP Gynotyping Assays (ABI): one for G/A genotyping, and one for G/T genotyping (Morita et al., 2006). The reactions consisted of 2x Taqman Universal Master Mix, 20x or 40x Genotyping Assay Mix, DNase-free water, and at least 10ng of genomic DNA in a final volume of 10 μL per reaction. The PCR amplification was performed under the following conditions: 10 minutes at 95ºC followed by 40 cycles at 92ºC for 15 seconds and 60 ºC for 1 minute. Allelic discrimination was determined after the amplification by performing and end-point read.

The operator interpreting the P-gp expression and activity results was blinded to the genotype of the subject. Samples from wild-type and variant were run together in mixed batches, and 10% of the samples were randomly selected and subjected to repeat analysis.

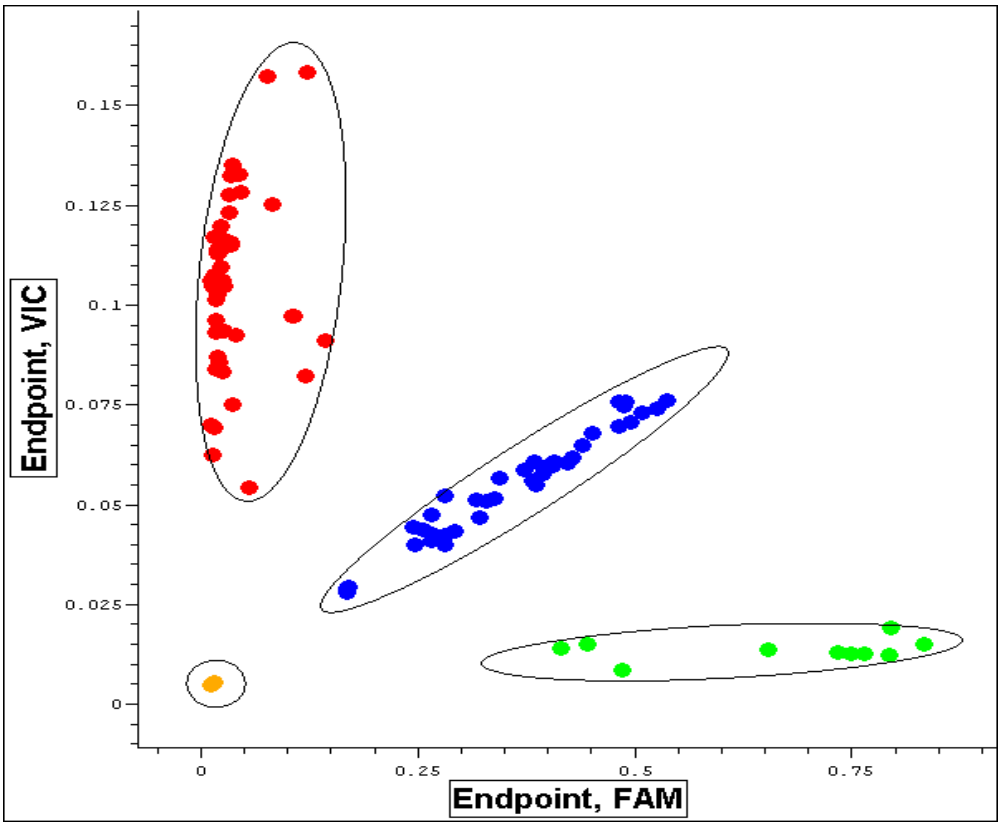


Illustration 8. Determination of *MDR1* allele using TaqMan Genotyping.
The output of the SNP genotyping assay is analyzed by the fluorescence of either the VIC fluorophore shown in red (the probe complimentary to the wild type allele); or FAM fluorophore shown in green (the probe complimentary to the variant allele). Fluorescence of both VIC and FAM, shown in blue, represents a heterozygote.

AIM 3: ROLE OF P-GP IN REGULATING THE PLACENTAL BIODISPOSITION OF MEDICATIONS USED TO TREAT CONDITIONS DURING PREGNANCY

Approach

The objective of this aim was to examine the role of P-gp in the efflux of medications used to treat opiate dependence (synthetic opiates), smoking cessation (bupropion), and gestational diabetes mellitus (oral hypoglycemic agents). Based on

previous indirect evidence in placental studies, it was hypothesized that placental P-gp effluxes the medications of interest in the fetal-to-maternal direction. To achieve this goal, the P-gp mediated transport of radiolabeled medications was measuring in preparations of placental IOVs. The involvement of other ABC transporters expressed in the placenta was also screened.

Opiate Maintenance Therapy: Buprenorphine, Methadone, and Morphine

The interaction of hypoglycemic agents with ABC transporters P-gp and BCRP was investigated by stimulation of ATP hydrolysis in membranes expressing P-gp (Gentest) and BCRP (Solvo). Reactions were carried out in low-binding 96-well plates (Corning Costar, NY, U.S.A.). The reaction mixtures, in a final volume of 60 μl, contained 50mM Tris–Mes buffer (pH 6.8), 40μg P-gp membranes, test drug and 4mM Mg-ATP. After pre-warming at 37 °C for 3 min, the reactions were initiated by the addition of Mg-ATP. Verapamil and sulfasalazine served as positive control for stimulation of P-gp and BCRP ATPase, respectively. For each reaction, identical incubations containing 100m M orthovanadate, an inhibitor of ATP hydrolysis by ABC transporters, served as control for baseline ATPase activity. ATPase activity was quantified by determining the increase in P*i* concentration that was subtracted from the activity generated in the presence of orthovanadate from the activity generated without orthovanadate to yield vanadate-sensitive ATPase activity. All incubations at each condition (with and without orthovanadate) were performed in duplicate. An 8 point standard curve of 0—150 nM P*i* was included in duplicate in each plate prior to incubation. After incubation of the reaction mixtures at 37 °C for 40—60 min, 30 μl of

10% sodium dodecylsulfate with 0.1% Antifoam A was added to terminate the reaction. Then, to each incubation well, 200 µl of 35mM ammonium molybdate in freshly prepared 15mM Zinc Acetate: 10% ascorbic acid pH 5.0 in a 1:4 proportion was added and incubated at 37 °C for 20 min. The P*i* release was measured by a µQuant microplate reader (Bio-Tek Instruments, Winooski, VT) at 620 nm.

Placental P-gp interaction with the opiates was determined using varying concentrations of methadone, buprenorphine, or morphine as determined by their inhibition of [^{3}H]-paclitaxel uptake by placental IOVs. The concentration of opiate that reduced [^{3}H]-paclitaxel transport velocity by half was identified as the IC_{50}. The Ki was calculated using the Kt for paclitaxel transport identified experimentally and the equation of Cheng and Prusoff: $Ki = (IC_{50})/ (1 + [paclitaxel]/Kt_{paclitaxel})$ (Cheng et al., 1973).

The direct measurement of opiate transport was determined by measuring ATP-dependent [^{3}H]-methadone, [^{3}H]-buprenorphine, and [^{3}H]-morphine uptake individually in human placental IOVs. P-gp-mediated transport ($T_{P\text{-}gp}$) was determined by the addition of P-gp inhibitor, verapamil (600 µM) (Tsuruo et al., 1981). Kt and V_{max} were determined by the least-squares fit of the data to the Michaelis-Menten equation. The reciprocal of radioligand uptake (1/uptake) was plotted vs the reciprocal of radioligand concentration in the incubation medium (1/c) for each experiment.

Gestational Diabetes Mellitus: Oral Hypoglycemic Agents

The interaction of hypoglycemic agents with ABC transporters P-gp and BCRP was investigated by stimulation of ATP hydrolysis in membranes expressing P-gp (Gentest) and BCRP (Solvo). The ATPase assay procedure is same as above, using 100

nM glyburide, rosiglitazone, and metformin as substrates. The concentration of 100nM was chosen based on placental tissue concentration of glyburide and rosiglitazone determined from perfusion experiments (Nanovskaya et al., 2006(a); Nanovskaya et al., 2006(b) Nanovskaya et al., 2008). The concentration of metformin was selected in the nanomolar range because organic cation transporters of the placenta are known to transport metformin in the micromolar range and we were aiming to minimize interference from these transporters (Bourdet et al., 2005).

The direct measurement of hypoglycemic drug transport was determined by measuring ATP dependent [^{3}H]-glyburide, [^{3}H]-rosiglitazone, and [^{14}C]-metformin uptake individually in human placental IOVs. The effect of metformin on rosiglitazone transport by P-gp was determined using varying concentrations of cold metformin measuring its inhibition of [^{3}H]-rosiglitazone uptake by placental IOVs. P-gp-mediated transport ($T_{P\text{-}gp}$) was determined by the addition of P-gp inhibitor, verapamil (600 μM) (Tsuruo et al., 1981). BCRP-mediated transport (T_{BCRP}) was determined by the addition of BCRP-selective inhibitor, 25 nM KO143 (Allen et al., 2002). MRP1-mediated transport (T_{MRP1}) was determined by the addition of MRP1 inhibitor, indomethacin (100 μM) (Benyahia et al., 2004). Total ABC protein-mediated transport (T_{ABC}) was determined for P-gp, BCRP, and MRP1 using 1 μM KO143

Kt and V_{max} were determined by the least-squares fit of the data to the Michaelis-Menten equation. The reciprocal of radioligand uptake (1/uptake) was plotted vs the reciprocal of radioligand concentration in the incubation medium (1/c) for each experiment.

Smoking Cessation: Bupropion

The interaction of bupropion with ABC transporters P-gp and BCRP was investigated by stimulation of ATP hydrolysis in membranes expressing P-gp (Gentest) and BCRP (Solvo). The ATPase assay procedure is same as above, and concentration dependence of bupropion was assessed at 0, 1, 10, 50, 100, 250, 500, and/or 750 and 1000 uM bupropion.

The direct measurement of bupropion transport was determined by measuring [^{3}H]-bupropion uptake in human placental IOVs. The protocol for the uptake assay is based on that for P-gp substrate, paclitaxel, described above. P-gp-mediated transport ($T_{P\text{-}gp}$) was determined by the addition of P-gp inhibitor, verapamil (600 μM) (Tsuruo et al., 1981). BCRP-mediated transport (T_{BCRP}) was determined by the addition of BCRP-selective inhibitor, 25 nM KO143 (Benyahia et al., 2004). Kt and V_{max} were determined by the least-squares fit of the data to the Michaelis-Menten equation. The reciprocal of radioligand uptake (1/uptake) was plotted vs the reciprocal of radioligand concentration in the incubation medium (1/c) for each experiment.

Correlation between P-gp and BCRP protein expression in human placental apical membrane was determined using Western Blot Analysis (described in Aim 1). Blots were probed with murine anti-P-gp monoclonal antibodies (mAb C219) or anti-BCRP monoclonal antibodies (mAb BXP-21).

AIM 4: BABOON PLACENTA P-GP PROTEIN EXPRESSION AND ACTIVITY

Approach

The goal of this aim was to evaluate the baboon placenta as a model for studying human placental P-gp activity. Due to other similarities between baboon and human placental structure and function, it was hypothesized that baboon P-gp expression and activity would be similar to human placenta of relative gestational age. To achieve this goal, brush border membrane vesicles were prepared from baboon placenta. P-gp protein expression, as determined by Western Blot analysis, and transport activity, as determined by uptake of P-gp prototypic substrate paclitaxel, were correlated with that of human placenta.

Clinical Material

Placentas were obtained by trained veterinarian by cesarean section, according to a protocol approved by the Institutional Animal Care and Use Committee of the Southwest National Primate Research Center, San Antonio, TX. Immediately following placenta collection laboratory space at the Southwest National Primate Research Center was utilized to prepare brush border membrane vesicles (see method above) from baboon placenta. Vesicle preparations were packaged on dry ice immediately following preparation and transported to UTMB.

Baboon Placental P-gp Protein Expression

Western blot quantification of P-gp protein expression in baboon BBMVs was carried out using 7.5% SDS/polyacrylaminde gel electrophoresis. The amount of total placental apical membrane protein loaded on each well was 10 μg. At the end of

electrophoresis, the gel was electroblotted on nitrocellulose membranes overnight at 4 ºC and a constant potential of 25 V. Blots were probed with anti-P-gp murine monoclonal antibodies (mAb C219) diluted 1:200 (v/v) and secondary goat anti-mouse horseradish peroxidase-conjugated antibodies diluted 1:1000 (v/v). Detection of the protein bands was carried out by spot densitometry and digital imaging of the enhanced chemiluminescence spots. The amount of expressed β-actin was used to normalize the amount of P-gp in each loaded sample on the gel. Positive controls consisted of human P-gp membranes (Gentest Corporation) and human placental BBMVs with known level of P-gp expression.

Baboon Placental P-gp Transport Activity

Transport activity was determined using the same protocol as human placental membrane vesicle samples, which measured uptake of radiolabeled P-gp substrate, [^{3}H]-paclitaxel (38 Ci/mmole, 83dpm/fmole). Each reaction was carried out in SHT buffer (10 mM HEPES-Tris, 250 mM sucrose) containing 4mM $MgCl_2$ 10 mM creatine phosphate, 100 μg/ml creatine phosphokinase, either 2 mM ATP or 3 mM NaCl, and placental BBMVs at a concentration of 0.05 μg/μL (7 μg total protein). The reaction was initiated by the addition of [^{3}H]-paclitaxel, at a final concentration ~ 200 nM unless otherwise indicated. The reaction was terminated after 1 minute by the addition of 1 mL ice cold buffer, and vesicles were isolated using rapid filtration by a Brandel Cell Harvester using Whatman #32 glass fiber filter strips (pore size 0.7 μM). The amount of [^{3}H]-paclitaxel retained was measured using liquid scintillation analysis, active transport was calculated as the difference in [^{3}H]-paclitaxel in the presence and absence of ATP and expressed as

pmol/mg protein*min. P-gp-specific transport of paclitaxel was confirmed by the addition of the P-gp inhibitor verapamil and monoclonal antibody C219.

Statistical Analysis

For genetic analysis, Hardy-Weinberg equilibrium of determined allele frequencies was assessed using the χ^2-test. For comparison of protein expression and uptake studies between comparison groups, statistical significance was determined using a paired student's t-test. A probability of $p < 0.05$ was considered to indicate statistical significance.

Chapter 4: Results and Discussion

INVESTIGATION OF HUMAN PLACENTAL BRUSH BORDER MEMBRANE VESICLES

Electron microscopy of vesicle preparations

Figure 1 shows an electron micrograph of human placental brush border membrane vesicles at low magnification and high magnification. Micrographs revealed single bilayer vesicles averaging 0.5 to 1 μM in diameter.

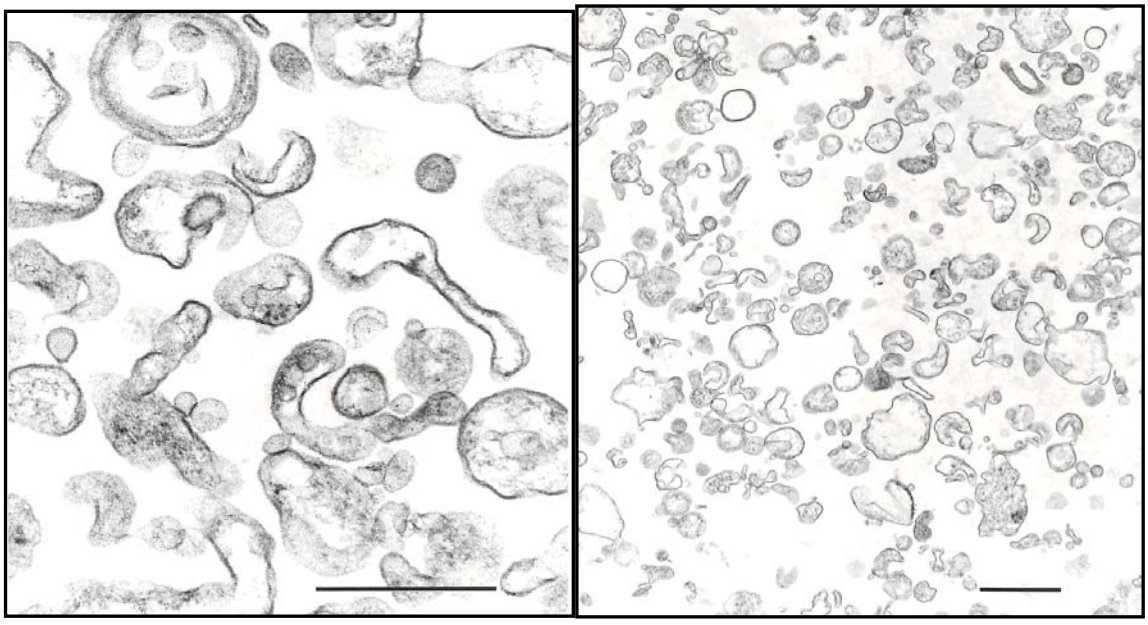


Figure 1. Electron micrograph of brush border membrane vesicles. Scale bar = 1 μm. Left: high magnification (x 14,100, scale bar 1 μm), Right: low magnification (x 62,700, scale bar 1 μm). (Hemauer et al., 2009)

Vesicle Composition

Alkaline phosphatase is an enzymatic marker for the brush border membrane of placental syncytiotrophoblast. The preparations of membrane vesicles (n = 14) averaged 13 ± 4 fold enrichment of alkaline phosphatase activity over syncytiotrophoblast cell

lysate, indicating that the procedure for preparing the vesicles enriched the purity of brush border membrane.

Vesicle Orientation

Affinity chromatography was utilized to separate vesicles oriented inside-out. Acetylcholinesterase assay revealed that the proportion of inside out vesicles (IOVs) following affinity chromatography was 78 ± 15%. The enrichment in activity of both acetycholinesterase and P-gp substrate accumulation were compared between initial membrane suspensions before affinity chromatography and IOVs. Together, acetylcholinesterase activity and ATP-dependent uptake of [^{3}H]-paclitaxel demonstrated 1.9 and 2.5-fold enrichment, respectively, in the proportion of IOVs. This data indicated that the affinity chromatography column doubled the proportion of IOVs in the preparations used in this investigation.

Interpretation: Evaluation of Placental BBMVs

The purpose of this section was to evaluate whether 1) the membrane preparation formed vesicles composed of a single (bilayer) cell membrane without substantial contamination from other cellular debris, 2) the cell fraction prepared consisted of brush border membrane, and 3) the enriched preparation contained a consistent fraction of inside-out oriented vesicles.

Electron microscopic observations showed membranous structures forming single bilayer membrane vesicles. It was estimated from the electron micrographs that vesicles were on average 0.5-1 μM diameter, while some appeared significantly smaller. The pore size of the glass filter used to isolate vesicles following rapid filtration was 0.7 μM,

therefore, it is questionable whether smaller vesicles were retained on the filter. However, determination of protein concentration in the filtrate revealed that it was below the detection limit, indicating that the majority of membranes were retained by the filter. Thus, the composition, size, and structure of the membrane vesicle preparations were concluded to be adequate for use in this project.

The alkaline phosphatase assay, an enzymatic marker of brush border membrane, allowed the conclusion that the vesicle preparation contained and enrichment of brush border membrane. The fold-increase in alkaline phosphatase activity over initial syncytiotrophoblast cell lysate is an indicator of the purity of the brush border membrane in the final preparation. A previous report established approximately 14-fold enrichment as a reference for concluding that the final preparation consisted of placental brush border (Smith et al., 1977). A mean of 17-fold enrichment was reported from multiple investigators (summarized by Jimenez et al., 2004), however this represented enrichment over placental homogenate (which contains other cell types) instead of syncytiotrophoblast cell lysate used in our preparation. It is likely that the higher enrichment reported was the result of lower baseline alkaline phosphatase activity in placental homogenate due to contamination of other cell types. Therefore, the enrichment of approximately 13-fold used in the preparations for this study was deemed acceptable.

This system used in to represent efflux activity in this investigation was uptake into inside out vesicles. To optimize reaction conditions, the proportion of vesicles oriented inside out was enriched using affinity chromatography. Acetycholinesterase

assay, an enzymatic marker of the extracellular-facing membrane, allowed the conclusion that the proportion of IOVs was consistent (~78%) across preparations.

In conclusion, a method for the isolation of placental brush border membrane vesicles, and the enrichment of vesicles oriented inside-out, has been established and validated for use in studying placental efflux transporter activity.

AIM1: P-GP EXPRESSION AND ACTIVITY

P-glycoprotein expression in IOVs

P-gp protein expression was determined in 200 individual samples of human placental brush border membrane. Western blot analysis of 4 selected term placentas is shown as an example in Figure 2. The expression of P-gp ranged between 0.006 – 0.14 µg P-gp/µg total. Term human placenta (>37 weeks gestation) displayed significantly lower Pgp protein expression (0.04 µg P-gp/µg total protein) than preterm (0.06 µg P-gp/µg total protein) (p=.0007). P-gp expression declined progressively with gestational age (p = .0001) (Figure 3). Comparison of P-gp expression between term placentas delivered during "active labor" versus "no labor" revealed significantly decreased P-gp expression in active labor (.03 µg P-gp/µg total protein, vs .05 µg P-gp/µg total protein, respectively, $p < 0.001$).

P-gp mediated transport of paclitaxel

Due to the reversed orientation of the transporters within the inside-out vesicles, transporter uptake of a drug into inverted vesicles represents its efflux activity. ATP dependent uptake of [^{3}H]-paclitaxel into IOVs was determined in 200 individual placentas. In the presence of and absence of an ATP-regenerating system (creatine

phosphate and creatine phosphokinase), the uptake of [^{3}H]-paclitaxel into placental IOVs was rapid and peaked at 10 seconds. Overall, the maximum uptake of paclitaxel ranged between 1-140 with a mean of 23 pmol*mg protein^{-1}min^{-1}. The time course of paclitaxel uptake by IOVs indicates that it reaches equilibrium, with opposing diffusion out of the vesicles, within minutes.

The ATP-dependent uptake of [^{3}H]-paclitaxel by P-gp in the IOVs exhibited classical saturation kinetics with V_{max} of 20 ± 3 pmol*mg protein^{-1}min^{-1} and an apparent Kt of 66 ± 38 nM (Figure 5).

In parallel with P-gp protein expression, transport activity of the probe substrate paclitaxel decreased with gestation, although this trend did not reach statistical significance (Figure 6).

Confirmation of P-gp Mediated Transport

Transport of [^{3}H]-paclitaxel was inhibited by C219 (monoclonal antibody to P-gp) and by verapamil (P-gp selective inhibitor), 85 ± 5% and 99 ± 9% respectively, indicating paclitaxel transport is mediated specifically by P-gp (Figure 7.A). The Ki for verapamil inhibition of paciltaxel transport was approximately 300 μM (Figure 7.B).

Correlation between P-gp Protein Expression and Transport Activity

No correlation was observed between P-gp protein expression and transport activity (R^2 = 0.00001) (Figure 8).

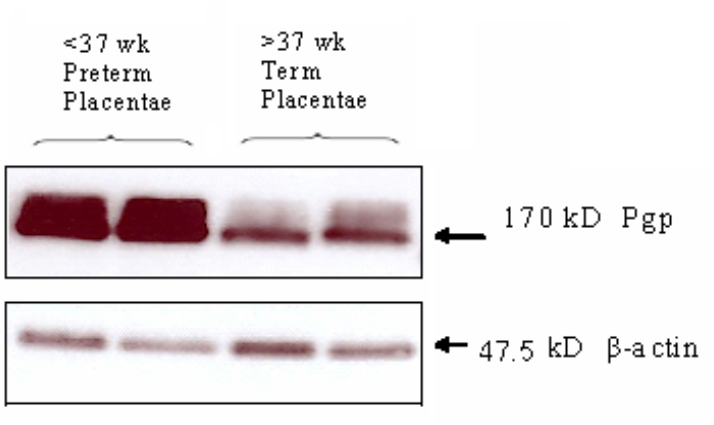


Figure 2. P-gp protein expression in human placental brush border membranes in 4 representative term placentas (10 μg/lane). The immunoblot was probed with the monoclonal antibody C219. Immunoreactive protein bands detected by immunoblotting were analyzed by densitometry. P-gp expression was determined as a proportion of the total amount of β-actin present per lane. Preterm placentas (≤37 weeks gestational age) displayed significantly greater P-gp protein expression than term placentas (>37 weeks gestational age, p=.0007)

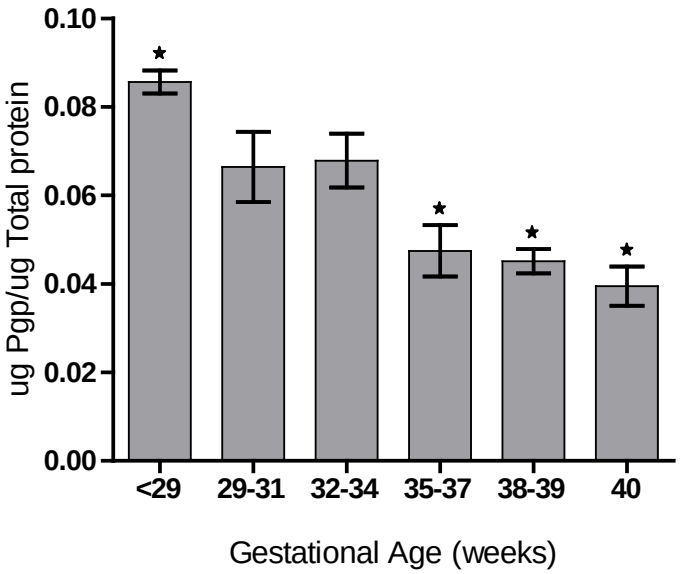


Figure 3. P-gp protein expression decreases with gestational age.
P-gp protein expression as determined by Western Blot. Expression was analyzed in 200 placentas and grouped according to gestational age intervals (<29 weeks, 29-31 weeks, 32-34 weeks, 35-37 weeks, 38-39 weeks, and ≥ 40 weeks) . Placental P-gp protein expression declines significantly with advancing gestational age (p = .0001) Bars represent mean ± SD.

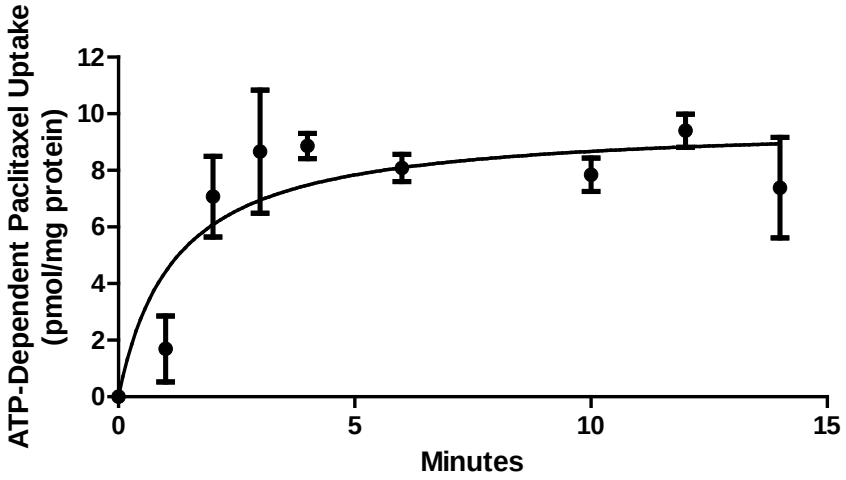


Figure 4. Time dependent P-gp transport activity was measured in human term placental brush border membrane vesicles (n = 4, error bars represent mean ± SEM). Vesicles (0.05 mg/mL) were incubated for 1-14 minutes with 70 nM [^{3}H]-paclitaxel in the presence or absence of an ATP regenerating system and collected by rapid filtration using a cell harvester. [^{3}H]-paclitaxel retention was detected using liquid scintillation counting. (Hemauer et al., 2009)

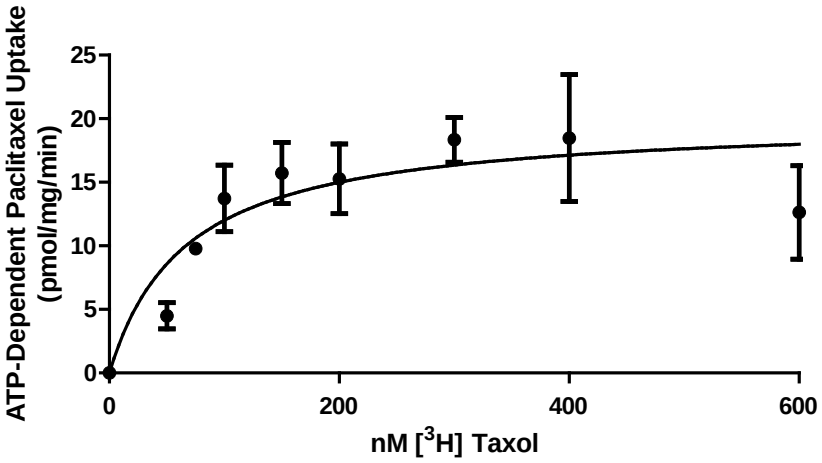


Figure 5. Concentration dependent P-gp transport activity. Saturation curve of ATP dependent uptake of paclitaxel by term placental IOVs (n = 10, error bars represent mean ± SEM). Vesicles (0.05 mg/mL) were incubated with 20-500 nM [^{3}H] paclitaxel in the presence or absence of an ATP regenerating system and collected by rapid filtration using a cell harvester. [^{3}H]-paclitaxel retention was detected using liquid scintillation counting. (Hemauer et al., 2009)

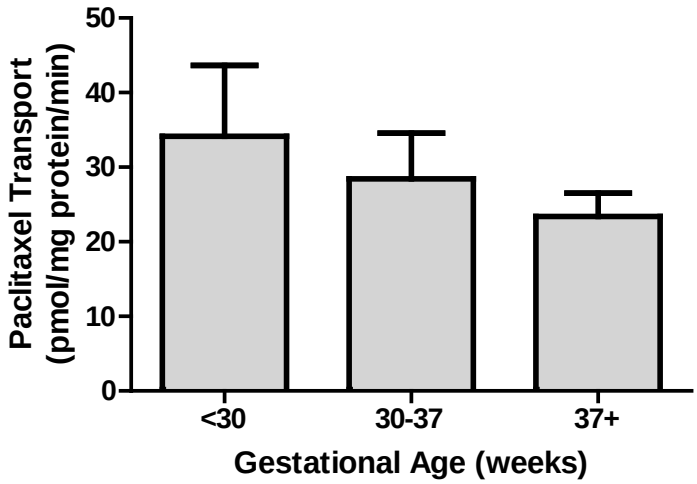


Figure 6. P-gp transport activity decreases with gestational age.

P-gp transport activity as determined by [^{3}H]-paclitaxel transport by IOVs was analyzed in 200 placentas and grouped according to gestational age. Bars represent mean ± SD ($p = 0.20$).

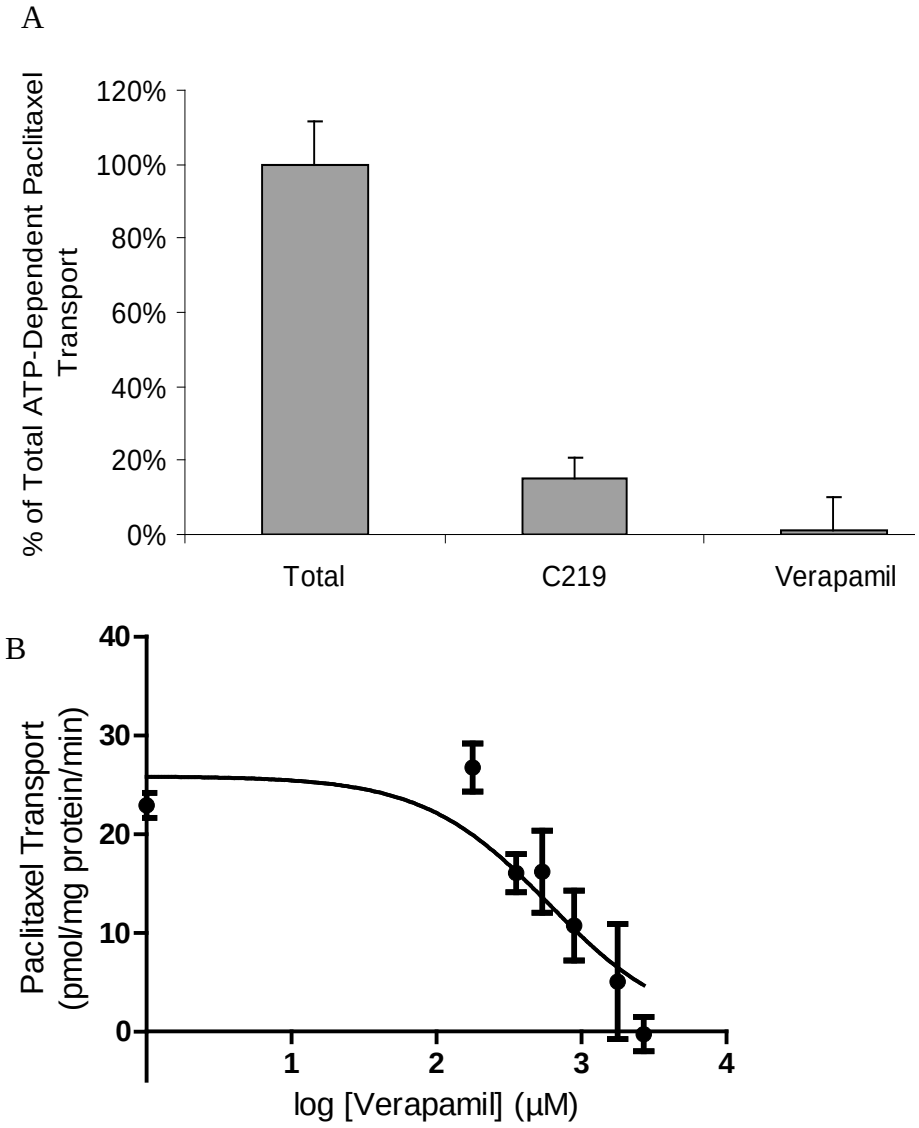


Figure 7. Confirmation of P-gp specific transport.
Inhibition of paclitaxel transport by monoclonal antibody C219 and P-gp-selective inhibitor verapamil (n = 4, error bars represent mean ± SEM). (A) Term placental vesicles (0.05 mg/mL) were incubated with C219 (0.05 mg/mL) or verapamil (2 mM). (B) Verapamil inhibited ATP-dependent transport of [^{3}H]-paclitaxel (70 nM) with a K_i of approximately 300 µM. (Hemauer et al., 2009)

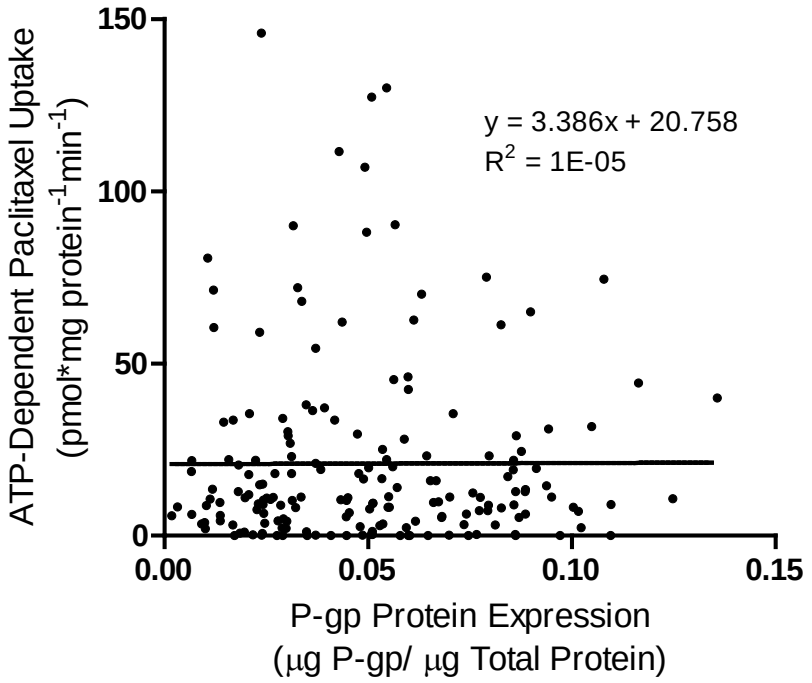


Figure 8. Correlation between P-gp protein expression and transport activity. P-gp expression (μg P-gp/ μg Total Protein) and ATP-dependent [^{3}H]-paclitaxel uptake in placental IOVs (n = 200). No correlation was observed between P-gp protein expression and transport activity (R^2 = 0.00001). (Hemauer et al., 2009)

Interpretation: Placental P-gp Protein Expression and Transport Activity

The objective of this aim was to evaluate the protein expression and transport activity of placental P-gp through advancing gestation. It was determined that, in 200 human placentas, placental P-gp expression declined significantly with advancing gestation. This supports the view that P-gp likely has a greater presence in the placental barrier and may be more active in extruding its substrates (including potentially teratogenic substances) during early gestation. In light of this finding, it is also important to note that placental permeability of P-gp substrates (including medications used during labor) may be significantly greater than expected near the time of delivery. In fact, stratification of patients delivering term placentas into “active labor” versus “non-active

labor" groups revealed that P-gp protein expression was significantly lower in placentas delivered as the result of active labor.

Although it has been previously reported in mice and a small number of human samples that the placental expression of P-gp declines throughout gestation (Mathias et al., 2005; Zhang et al., 2008), it remained unclear whether P-gp transport activity in fact correlated with its expression in placental tissue. Therefore, this study examined functional activity of P-gp and its corresponding expression in 200 individual placental preparations of IOVs. The wide range of P-gp expression between individual placentas observed in this investigation is in agreement with previous reports (Nanovskaya et al., 2005). In addition to the variability in P-gp protein expression, there was no correlation between placental P-gp protein expression and its activity (coefficient r^2 = 0.00001), regardless of gestational age. Similarly, a lack of correlation between P-gp expression and its activity has been reported in human lymphocytes (Vasquez et al., 2005). Therefore, it can be concluded that the expression of P-gp protein in placental syncytiotrophoblast cannot be used as a predictor of its activity in the efflux of a drug/medication from the feto-placental unit to the maternal circulation.

Despite the lack of correlation between expression and activity in individual samples, the mean expression and activity of placental P-gp both declined with advancing gestation. Therefore, it is plausible that P-gp plays more of a role in limiting fetal exposure to xenobiotics during the earlier vulnerable period of organogenesis. The implications for this finding are that medications administered during pregnancy which are also P-gp substrates may have greater placental permeability near term. For

conditions such as opiate maintenance or smoking cessation which require treatment throughout gestation, as well as gestational diabetes which is diagnosed/treated beyond 28 weeks gestation, fetal exposure near term must be taken into consideration.

This investigation confirmed that inside out vesicles composed of placental brush border membrane can be used to measure P-gp activity by the efflux of its prototypic substrate paclitaxel, as well as the kinetics (K_t and V_{max}) of transport. Time-dependent accumulation of [^{3}H]-paclitaxel in the IOVs was displayed using an ATP-regenerating system. The drug accumulation reached a steady state within minutes, which represents the result of two competing processes: active transport of the drug by P-gp into the vesicle lumen (against its concentration gradient), and passive diffusion of the drug out of the vesicle (down its concentration gradient). The time course of drug accumulation demonstrates that studies of initial transport velocity should be conducted within the first minute to be located on the linear portion of the time-concentration curve.

Paclitaxel transport by placental P-gp exhibited saturation kinetics and was ATP-dependent. To date, there had been no previous investigations reporting either the direct transport of paclitaxel by placental P-gp or on the kinetics of its transfer. An investigation of the kinetics of paclitaxel transport by non-placental P-gp (expressed in CEM leukemia cells) reported a K_t of 144 ± 56 nM in parental cells. In the same cell line, after several passages in cultures containing vinblastine (conferring multidrug resistance), the resulting cells overexpressed P-gp and revealed their transport of paclitaxel with an apparent K_t of 63 ± 46 nM (Lin et al., 2001). Accordingly, the apparent K_t of 66 nM determined for placental IOVs in this study is in agreement with that reported for the leukemia cell line

selected for multidrug resistance. The difference in transport kinetics of P-gp substrate paclitaxel between placenta and leukemia parental cells (before drug selection) further highlights that the properties of P-gp mediated transport may differ between experimental systems.

Efflux transporters, including P-gp, have overlapping substrate specificity with other members of the ABC family of transporters as well as those in other families. Accordingly, it was important to determine whether our "probe substrate" paclitaxel was extruded by P-gp only or by more than one of the transporters localized in the apical membranes of human placenta. For example, BCRP is co-localized to the placental apical membrane (Yeboah et al., 2006) and has overlapping substrate specificity with P-gp (Polli et al., 2004). Paclitaxel was chosen for this study as a P-gp specific substrate based on previous reports that paclitaxel is preferentially transported by P-gp but not by BCRP or the other ABC transporters (You et al., 2007). The inhibition of paclitaxel transport by verapamil and P-gp monoclonal antibody C219 confirm that the ATP-dependent paclitaxel transport observed in our system of inside out vesicles is mediated specifically by P-gp.

In conclusion, the placental brush border membrane vesicle preparations characterized in this investigation provide an effective method for the identification of the drugs/medications that are substrates of P-gp as well as their interaction (inhibition) of the transport of its prototypic substrate, paclitaxel.

AIM 2: P-GP POLYMORPHISMS AND EFFECT ON EXPRESSION AND ACTIVITY

P-gp protein expression and transport activity by Maternal Ethnicity

P-glycoprotein expression was determined by western blot analysis in 199 individual placentas. Of these, maternal ethnicity consisted of 59 Caucasian, 58 African American, and 82 Hispanic. P-gp protein expression averaged 0.03 ± 0.004, 0.04 ± 0.004, and 0.05 ± 0.004 μg P-gp/ μg total protein in Caucasian, African American, and Hispanic patients, respectively, with significantly higher expression in placentas delivered from Hispanic mothers than those delivered from Caucasian ($p = 0.002$) (Figure 9.A).

P-gp transport activity of its prototypic substrate, paclitaxel, was 9.7 ± 1.4, 11.6 ± 1.6, and 32.1 ± 5.7 pmol*mg protein^{-1}min^{-1} in placentas delivered from Caucasian, African American, and Hispanic mothers, respectively (Figure 9.B). A three-fold increase in transport activity in Hispanic patients over Caucasian and African American placentas was found to be significant ($p < 0.001$).

Genotype distribution and allele frequency

Placentas from 199 term deliveries were analyzed for the C1236T, C3435T and G2677T/A tri-allelic polymorphism using TaqMan® Genotyping Assay and PCR-RFLP-base genotyping assay. The frequency of variant allele of the C1236T and G2677T/A SNPs differed significantly between Caucasian, African American, and Hispanic mothers. The frequency of the variant allele for the C3435T polymorphism was similar in Caucasian and Hispanic mothers, yet was significantly lower in African American

mothers than in either Hispanic or Caucasian. All allele frequencies were found to be in Hardy Weinberg equilibrium, (p values of 0.79, 0.23, and 0.29 for the 1236, 3435, and 2677 SNPs, respectively). Allele frequencies are listed in Table 2.

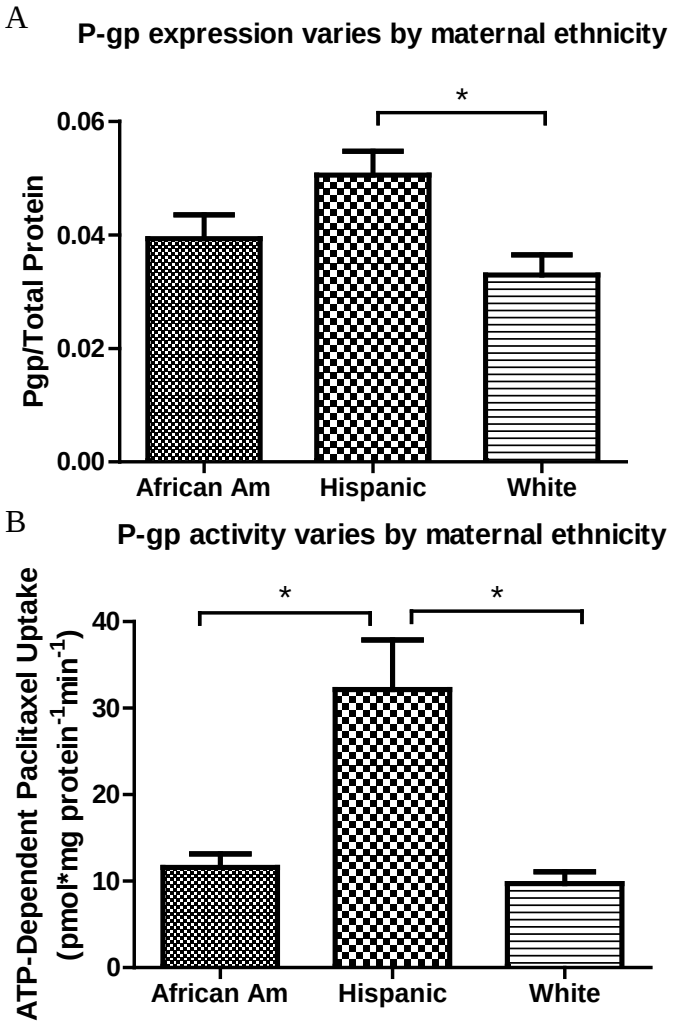


Figure 9. P-gp expression and transport activity varies by maternal ethnicity.

(A) P-gp protein expression was significantly higher in term placentas deliverd from mothers of Hispanic ethnicity than from Caucasian mothers (p= 0.002). (B) P-gp transport activity of paclitaxel in human placental IOVs was significantly higher in term placentas delivered from mothers of Hispanic ethnicity than from both African American and Caucasian mothers ($p < .001$).

Table 2. Allele frequencies of *MDR1* polymorphisms at positions 1236, 3435, and 2677 using TaqMan® Genotyping Assay (all SNPs) and RFLP analysis (G2677T/A only).

	1236			3435			2677		
Ethnicity	wt		var	wt		var	wt		var
Caucasian	0.58		0.42	0.46		0.54	0.76		0.24
African American	0.77		0.23	0.74		0.26	0.86		0.14
Hispanic	0.39		0.61	0.48		0.52	0.5		0.5
Comparison	A	B	C	A	B	C	A	B	C
p value	<0.01	<0.01	<0.01	<0.01	<0.01	0.69	0.02	<0.01	<0.01

A – Caucasian v. African American; B – African American v. Hispanic; C – Caucasian v. Hispanic

Effect of *MDR1* Polymorphisms on Placental P-gp Expression

P-gp protein expression was quantified using Western Blot analysis of brush border membrane isolated from human placenta (n = 199), as represented in Figure 10. The effect of *MDR1* polymorphisms on levels of P-gp protein expression in the placenta was evaluated using a dominant model. Placentas with the homozygous and heterozygous variant alleles were grouped together and compared to the referent homozygous wild type group.

The C1236T variant was associated with 11% lower P-gp protein expression than the CC wild type (0.039 ±0.002 vs. 0.045 ±0.002 μg P-gp/ μg total protein) (Figure 11.A) The C3435T and G2677T/A variants each were associated with a significant 16% reduction in placental P-gp protein expression compared with wild type (0.039 ±0.002 vs. 0.046 ±0.003 μg P-gp/ μg total protein, $p < 0.05$) (Figure 11.B, 11.C). For each of the three polymorphisms examined, P-gp protein expression in heterozygotes was not

different from homozygous variants.

When stratified by maternal ethnicity, only in placentas delivered from Hispanic mothers was P-gp protein expression significantly reduced in variants for the 1236T and 2677T/A polymorphisms (Figure 12).

Effect of *MDR1* Polymorphisms P-gp transport activity

The effect of the *MDR1* polymorphisms C1236T, C3435T, and G2677T/A, as determined by TaqMan Genotyping Assay, on P-gp transport activity was determined in 105 term placentas. P-gp transport activity was determined by measuring the uptake of its prototypic substrate [^{3}H]-paclitaxel, in placental brush border membrane vesicles.

P-gp-mediated active transport of paclitaxel was significantly greater in homozygous variant 1236T/T genotype (24 ± 5 pmol*mg protein^{-1}min^{-1}) than in homozygous wild-type (C/C) (14 ± 2 pmol*mg protein^{-1}min^{-1}, p = 0.04) (Figure 13.A). Similarly, homozygous variants of the 3435T/T genotype displayed significantly greater P-gp transport activity than homozygous wild-type (C/C) (27 ± 6 vs. 14 ± 2 pmol*mg protein^{-1}min^{-1}, p = 0.02) (Figure 13.B). The G2677T/A variants (A/A, A/T, or T/T) exhibited a trend toward increased transport activity over homozygous wild type (14 ± 2 vs. 21 ± 7 pmol*mg protein^{-1}min^{-1}), but did not reach statistical significance (p = 0.20) (Figure 13.C). With all three SNPs examined, there was a gene-dose effect, where heterozygous placentas (wild type/variant) had intermediate level of P-gp transport activity compared to placentas with the wild type (lowest activity) and homozygous variants (highest activity).

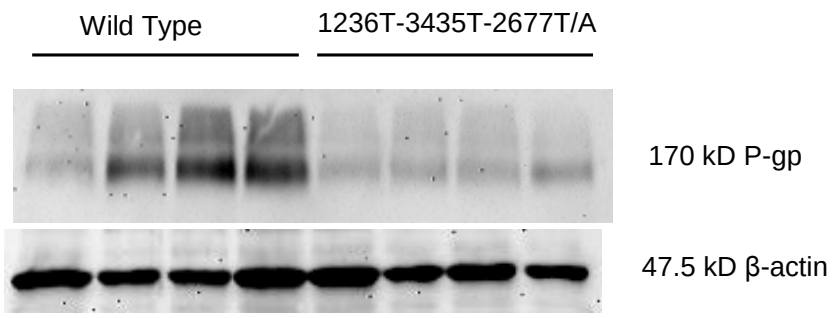


Figure 10. Western blot of P-gp protein expression in term human placental brush border membranes in representative wild type and variants representing 1236T-3435T-2677T/A genotype (10 μg/lane). The immunoblot was probed with the P-gp monoclonal antibody C219. Immunoreactive protein bands detected by immunoblotting were analyzed by densitometry. P-gp expression was determined as a proportion of the total amount of β-actin present per lane. (Hemauer et al., 2010)

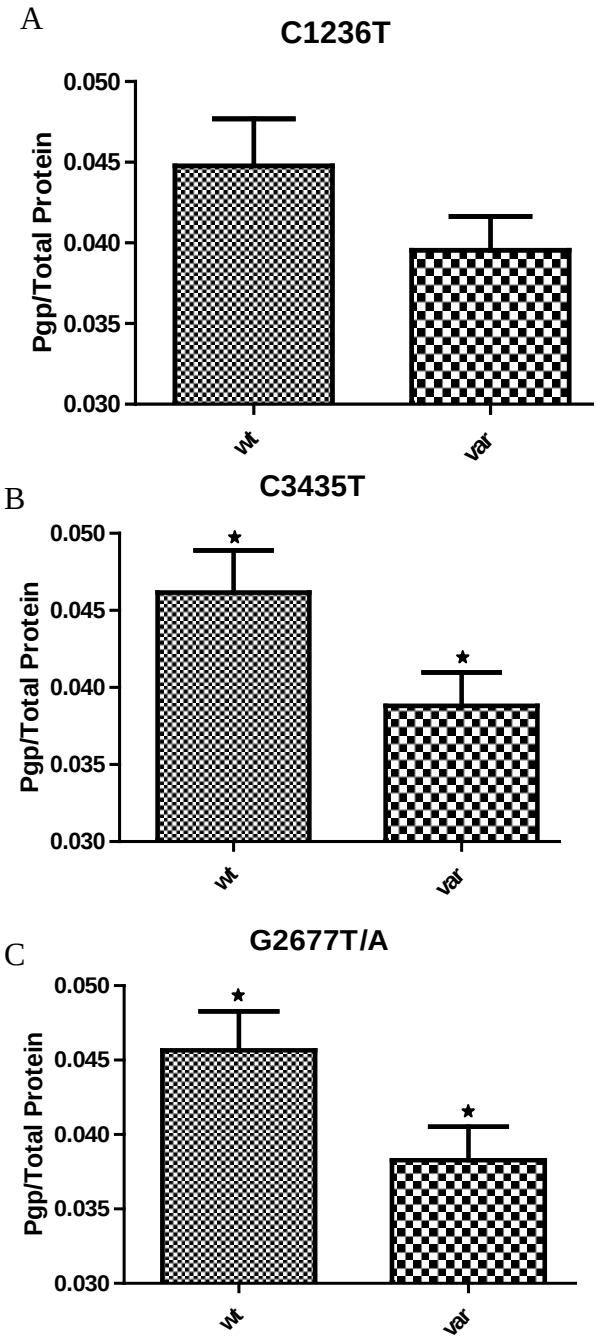


Figure 11. P-gp protein expression by *MDR1* genotype in term placental brush border membrane samples from wild type (wt/wt) and variant (wt/var, var/var) genotypes (mean ± SEM of 199 individual samples). (A) C1236T Variants had 11% lower P-gp protein expression than wild type. (B) C3435T and (C) G2677T/A variants each had a significant 16% reduction in placental P-gp protein expression compared with wild type ($p < 0.05$). (Hemauer et al., 2010)

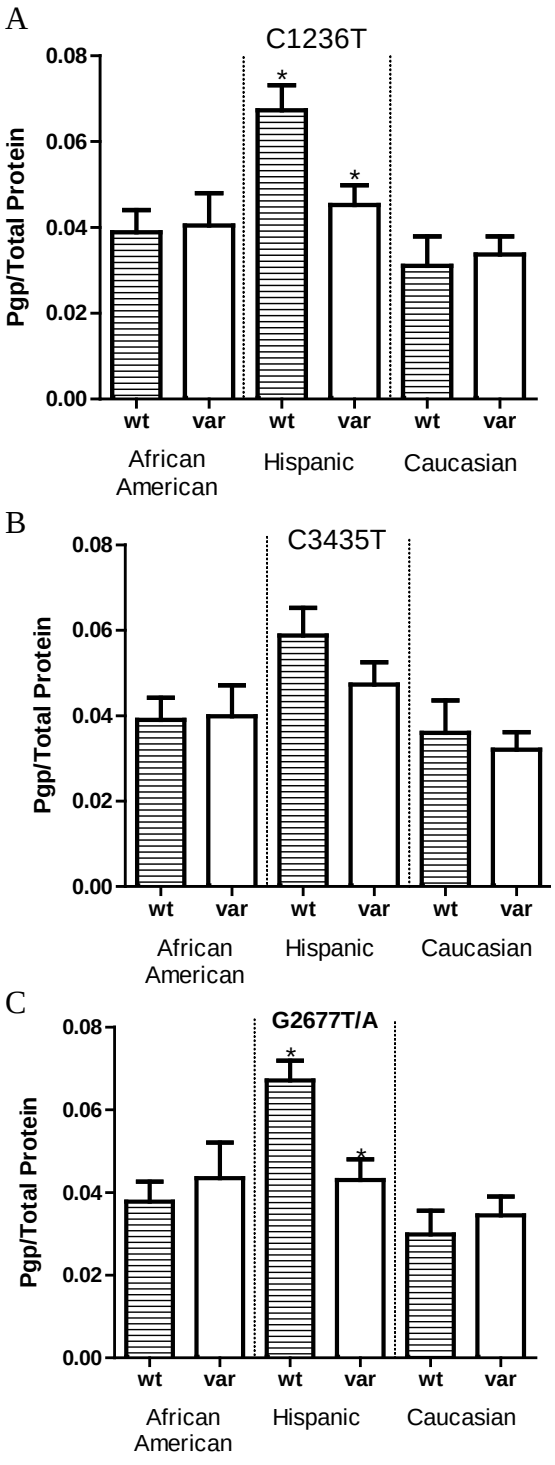


Figure 12. P-gp protein expression by *MDR1* genotype stratified for maternal ethnicity. Placentas delivered from mothers of Hispanic ethnicity displayed significantly decreased protein expression in variants for the (A) C1236T and (C) G2677T/A polymorphisms. (B) C3435T was not associated with a significant difference in protein expression.

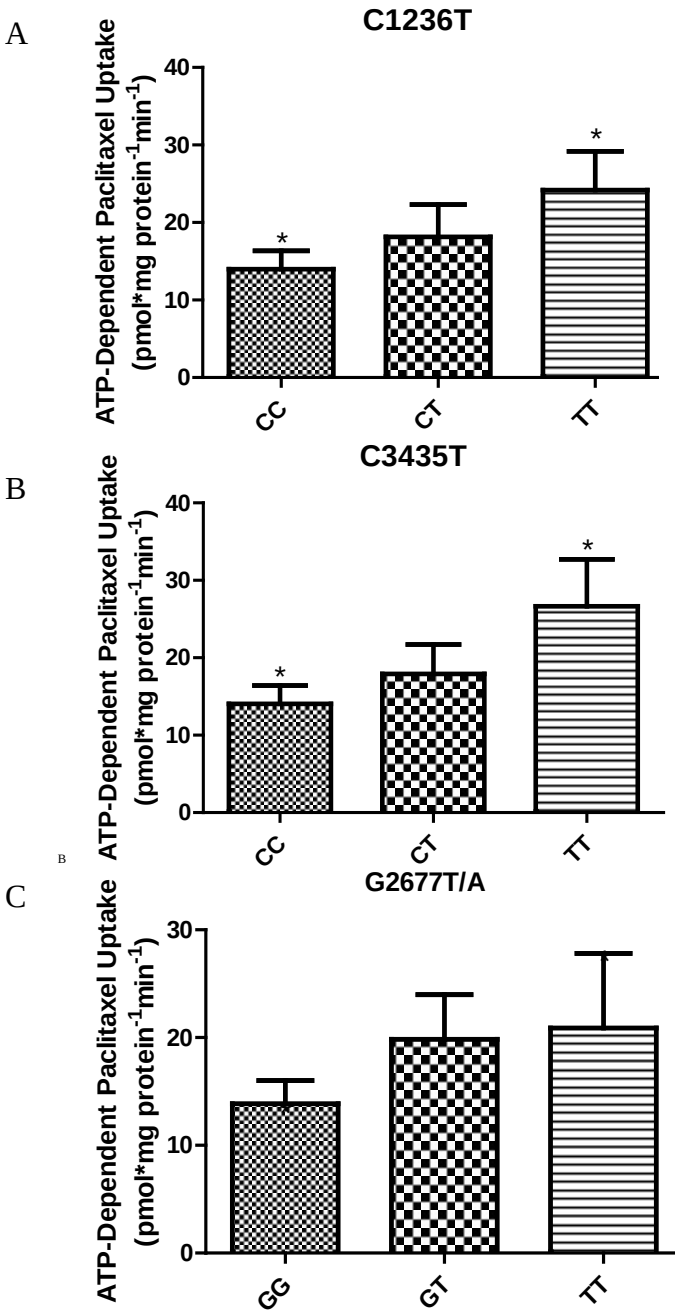


Figure 13. P-gp transport activity by *MDR1* genotype
(A) P-gp-mediated active transport of paclitaxel was significantly greater in homozygotes for the variant 1236T/T genotype than in wild-type (C/C) homozygotes ($p = 0.04$). (B) Homozygous variants of the 3435T/T genotype displayed significantly greater P-gp transport activity than wild-type (C/C) homozygotes ($p = 0.02$). (C) G2677T/A variants (A/A, A/T, or T/T) had a trend toward increased P-gp transport activity over wild type homozygotes. (mean ± SEM of 105 individual samples). (Hemauer et al., 2010)

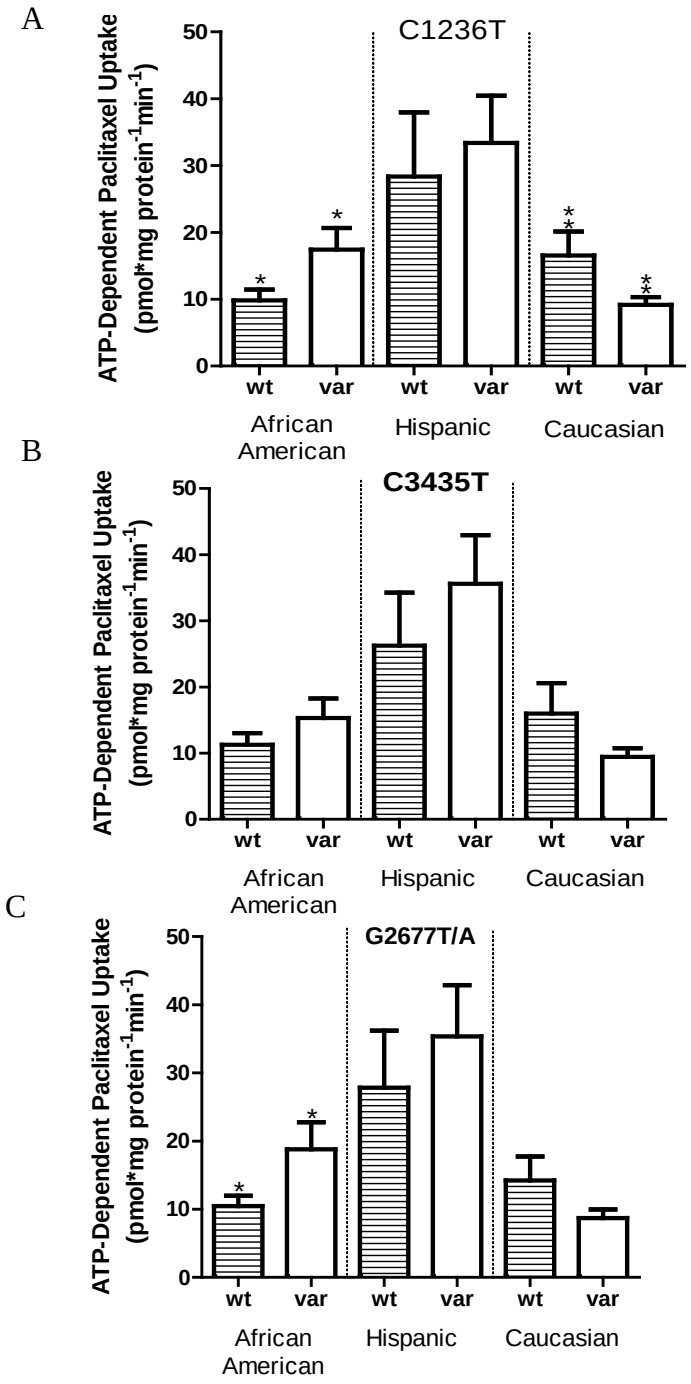


Figure 14. P-gp transport activity by *MDR1* genotype stratified for maternal ethnicity. Placentas delivered from mothers of African American ethnicity had significantly increased transport activity in (A) C1236T and (C) G2677T/A variants ($p < 0.05$). Conversely, P-gp activity in placentas delivered from Caucasian mothers was significantly decreased in variants for the C1236T polymorphism ($p < 0.05$). (B) C3435T was not associated with a significant difference in P-gp transport activity. (mean ± SEM of 105 individual samples).

When stratified by maternal ethnicity, the observed increase in P-gp transport activity occurred in placentas delivered from both African American and Hispanic mothers although it was only statistically significant in placentas delivered from African Americans with the C1236T and G2677T/A variants ($p<0.05$). Contrastingly, placentas delivered from Caucasian mothers displayed decreased P-gp transport activity in variants for all three SNPs (Figure 14) with significant reduction associated with C1236T variants ($p<0.05$). For both genotypes (wild type and variant), placentas delivered from Hispanic mothers displayed approximately two to three- fold increased P-gp transport activity over placentas from African American and Caucasian mothers.

Interpretation: Relationship between Maternal Ethnicity, *MDR1* Genotype, and P-gp expression and activity

The objective of this aim was to examine the relationship between *MDR1* polymorphisms, maternal ethnicity, and placental P-gp expression and activity. The relationships between ethnicity, genotype, and P-gp expression and activity were analyzed in three ways: first by maternal ethnicity, second by placental genotype, and third following stratification for both maternal ethnicity and placental genotype.

When analyzed by maternal ethnicity alone, the data indicate that P-gp protein expression is significantly greater in term healthy placentas delivered from Hispanic mothers than in placentas delivered from either African American or Caucasians. Furthermore, average P-gp transport activity of its prototypic substrate paclitaxel was three-fold higher in placentas delivered from Hispanic mothers. The implications of this

finding are that populations associated with decreased placental P-gp expression and activity (African American and Caucasian) may have greater risk of fetal exposure to xenobiotics in the maternal circulation. The link between ethnic background and genetic susceptibility of disease risk has long been recognized in clinical practice, where the rates of pregnancy complications are greater in certain ethnic groups (Eskanazi et al., 1991; Goldenberg et al., 2008; Hagopian et al., 1996; Okah et al., 2007; Solomon et al., 1997; Stone et al., 1994).

In order to elucidate the genetic factors contributing to the above finding, we investigated three common polymorphisms in the *MDR1* gene for their effect on placental P-gp protein expression and its activity in the transport of its prototypic substrate, paclitaxel. The single nucleotide polymorphisms examined were C1236T, C3435T, and G2677T/A.

MDR1 is a highly polymorphic gene, with distinct allele frequencies noted between ethnic groups and populations. Reports on the C1236T, C3435T, and G2677T/A SNPs reveal significant differences in allele frequencies between Caucasian, African Americans, and Hispanic Americans (Kroetz et al., 2003; Pauli-Magnus et al., 2004). Similarly, in the patients delivering at our site, the allele frequencies for the three polymorphisms differed significantly between these maternal ethnic groups and are in agreement with those frequencies reported separately in Caucasian, Hispanic, and African Americans (Sherry et al., 2001). The finding that the allele frequencies in our samples agree with the allele frequencies for these ethnic groups in other populations supports that

our samples adequately represent the Caucasian, Hispanic, and African American populations.

The three SNPs are associated with altered P-gp expression and function in other tissue types, yet a clear consensus on their role in the placental barrier had not been established to date. The results of this study indicate the C3435T and G2677T/A alleles were associated with significantly reduced placental P-gp protein expression. Additionally, the C1236T variant was associated with a trend toward decreased placental P-gp expression. This finding is in agreement with other reports in which variants for the above alleles were associated with decreased P-gp protein expression in placental and non-placental tissues (Hitzl, 2001 et al.; Hitzl et al., 2004; Hoffmeyer et al., 2000; Tanabe et al., 2001). Similar association between variant allele and reduced placental protein expression has also been reported in ABC transporter Breast Cancer Resistance Protein (BCRP) (Kobayashi et al., 2005). Interestingly, the reduction in P-gp observed with each variant allele was associated with an increase in its transport of the prototypic substrate paclitaxel. This observation is consistent with reports demonstrating that variant alleles could be associated with increased P-gp activity *in vivo* (Gréen et al., 2009; Kwan et al., 2009; Taubert et al., 2006). On the other hand, this is the first investigation that compared genotype and protein expression with direct measurement of P-gp transport activity using membrane vesicles prepared from human placentas.

The synonymous SNPs (associated with no change in amino acid sequence) C1236T and C3435T were associated with altered functional activity of P-gp. There are several plausible explanations for this finding. First, the finding could be due to C1236T

and C3435T occurring in linkage disequilibrium with the functional non-synonymous G2677T/A polymorphism. Alternatively, the synonymous SNPs have been hypothesized to introduce a rare codon, which affects the timing of cotranslational folding and insertion of P-gp into the membrane, thereby altering the structure of substrate and inhibitor interaction sites (Kimchi-Sarfaty et al., 2007). In fact, the synonymous polymorphisms result in P-gp with altered conformation, drug and inhibitor interactions despite similar mRNA and protein levels (Kimchi-Sarfaty et al., 2007). Finally, it is possible that both linkage to the functional G2677T/A polymorphism and the introduction of a rare codon with altered conformation and substrate interaction contribute to the relationship between synonymous SNPs and P-gp function.

All three variant alleles examined, namely C1236T, C3435T, and G2677T/A were associated with decreased protein expression and an increase in the transport activity of placental P-gp. The observed inverse relationship between protein expression and activity with the variant alleles could—in addition to inter-individual variability observed between placentas—contribute to the lack of correlation between P-gp protein expression and transport activity reported in vesicle preparations of human placenta.

The mechanism by which protein expression is reduced yet activity is increased in variants remains unknown. However, it should be noted that there are several substrates of P-gp that are transported and are also inducers of *MDR1* gene expression (Wang et al., 2009). It is plausible that in variants with increased P-gp-mediated efflux of a substrate/inducer, there is lower intracellular concentration and thus less induction of *MDR1* gene expression by that particular substrate/inducer leading to lower P-gp protein

expression. Therefore, it is possible that the decrease in P-gp protein expression (decreased *MDR1* gene induction) is secondary to an increase in its transport activity in variants.

A second explanation for the lack of positive correlation between protein expression and activity in variants is that the total P-gp protein expression versus the amount of P-gp active at the placental brush border membrane may be regulated independently. A previous report from our laboratory and others have demonstrated that P-gp protein expression declines with advancing gestation (Gil et al., 2005; Nanovskaya et al., 2008; Sun et al., 2006). However, there was no significant decrease in P-gp transport activity associated with increasing gestational age (unpublished, experiments performed by S Hemauer June 2008-March 2009). Therefore, there could be regulation of an "inactive pool" of expressed P-gp protein in placental tissue that is yet to be elucidated. Furthermore, subcellular localization of P-gp within organelles as demonstrated in nonplacental tissue (Maraldi et al., 1999; Molinari et al., 2002) may also be present in placenta and contribute to protein expression disproportionate with activity. A role of the C1236T, C3435T, and G2677T/A polymorphisms in the subcellular localization and/or membrane insertion of expressed P-gp could contribute to total P-gp efflux activity and cannot be ruled out at this time.

A third possible contributing factor to the decreased expression yet increased activity in variants could be unidentified genetic influences. Stratification by maternal race revealed that the decreased P-gp expression in variants was only apparent in placentas delivered by Hispanic mothers. Furthermore, the increased P-gp activity in

variant alleles occurred in placentas from African American and Hispanic but not Caucasian mothers. The contrasting results between ethic groups provide evidence that other genetic factors are likely involved. Furthermore, the 3-fold higher P-gp transport activity in Hispanic ethnicity over either African American or Caucasian cannot be explained by the C1236T, C3435T, and G2677T/A polymorphisms alone, since each conferred less than a 2-fold increase in activity.

It should be noted that analysis of placental genotype and phenotype by maternal ethnicity must be interpreted with caution. Since the placenta is of embryonic origin, the placental DNA analyzed in this study is that of the fetus (maternal and paternal DNA), not the mother. Therefore, the data which are segregated by maternal ethnicity are based on the assumption that maternal ethnicity is the same as fetal, which is unknown because information on paternal ethnicity was not available in these patients.

In summary, variants for the C1236T, C3435T, and G2677T/A alleles in the *MDR1* gene encoding P-gp are associated with reduced protein expression and increased transport of P-gp selective substrate, paclitaxel, in human term placenta. Furthermore, maternal ethnicity appears to be related to P-gp expression, activity, and genotype-phenotype relationship. The implication of these findings is that populations associated with decreased placental P-gp efflux activity may be at greater risk of potential fetal exposure to xenobiotics in the maternal circulation. Future studies should include gene-wide analysis of functional *MDR1* polymorphisms. Additionally, the effects of chronic exposure to P-gp substrates on the regulation of its protein expression and activity should be pursued.

AIM 3: ROLE OF P-GP IN REGULATING THE PLACENTAL BIODISPOSITION OF MEDICATIONS USED TO TREAT CONDITIONS DURING PREGNANCY

Opiate Maintenance Therapy

Stimulation of ATP Hydrolysis by P-gp and BCRP

Buprenorphine, methadone, and morphine stimulated ATP hydrolysis by membranes expressing P-gp (2.2 ± 1.7, 5.0 ± 0.03, and 3.6 ± 2.3 nmoles Pi/mg protein*min, respectively) (Figure 15.A). However, buprenorphine, methadone and morphine did not stimulate ATP hydrolysis by BCRP -1.9 ± 0.7, -1.5 ± 1.8, and 0.6 ± 12.8 nmoles Pi/mg protein*min, respectively) (Figure 15.B), indicating that the opiates did not interact with BCRP in the system used in the investigation. Therefore, additional investigations did not focus on BCRP as a mediator of opiate transport.

Inhibition of Paclitaxel Transport by Opiates

Buprenorphine inhibited 80% of ATP-dependent transport of paclitaxel with a Ki of approximately 44 ± 5 μM (Figure 16.A). Methadone inhibited 60% of ATP-dependent transport of paclitaxel with a Ki of approximately 18 ± 3 μM (Figure 16.B). Morphine inhibited 100% of ATP-dependent transport of paclitaxel with a Ki of approximately 90 ± 3 μM (Figure 16.C).

Opiate Transport by P-gp

Selective chemical inhibition of P-gp by verapamil in a pool (n = 60) of vesicle preparations revealed the following Kt and Vmas for each opiate: [^{3}H]-methadone 300 ± 100 nM and 4.3 ± 0.6 pmol/mg protein min; [^{3}H]-morphine 226 ± 200 nM, and 3.6 ± 2.4

pmol/mg protein min (Figure 17.A, 17.B). Verapamil did not inhibit uptake of [^{3}H]-buprenorphine by inside-out vesicles, indicating that P-gp is not involved in buprenorphine transport (Figure 17.C). Taken together, the data demonstrate that methadone and morphine are substrates of and transported by placental P-gp. Conversely, buprenorphine is not transported by P-gp, yet its inhibition of paclitaxel transport supports its role as a P-gp inhibitor.

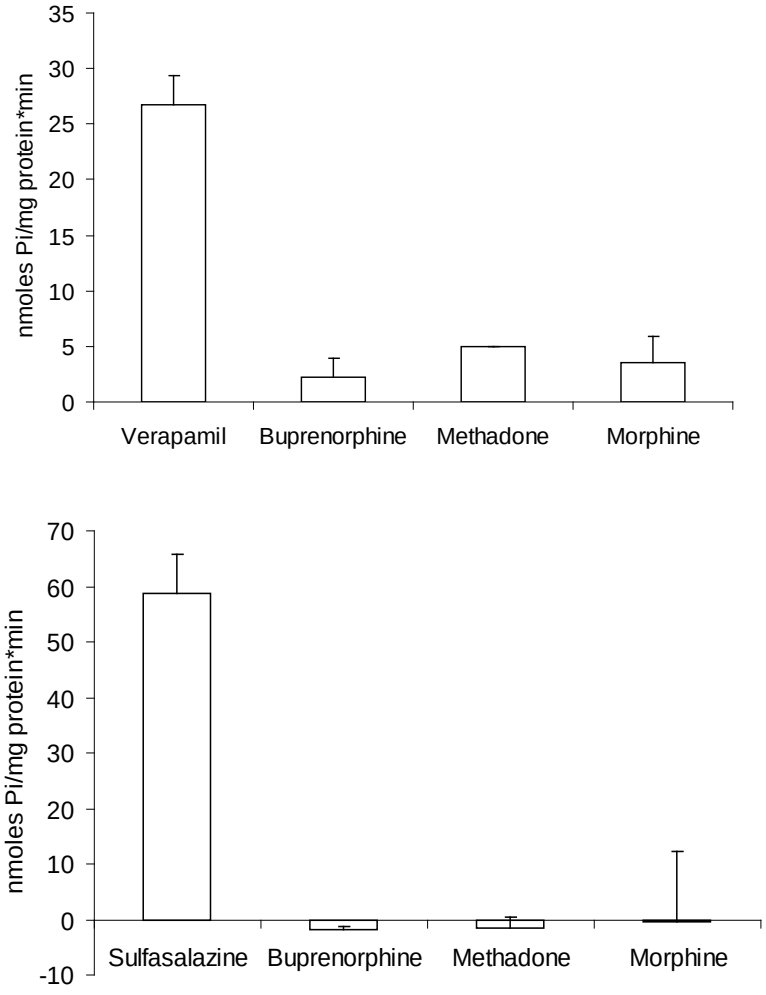


Figure 15. Stimulation of ATP Hydrolysis by P-gp and BCRP
Buprenorphine, methadone, and morphine each stimulated ATP hydrolysis by P-gp, in comparison to positive control verapamil. In contrast none of the three opiates stimulated ATP hydrolysis by BCRP, in comparison to positive control sulfasalazine.

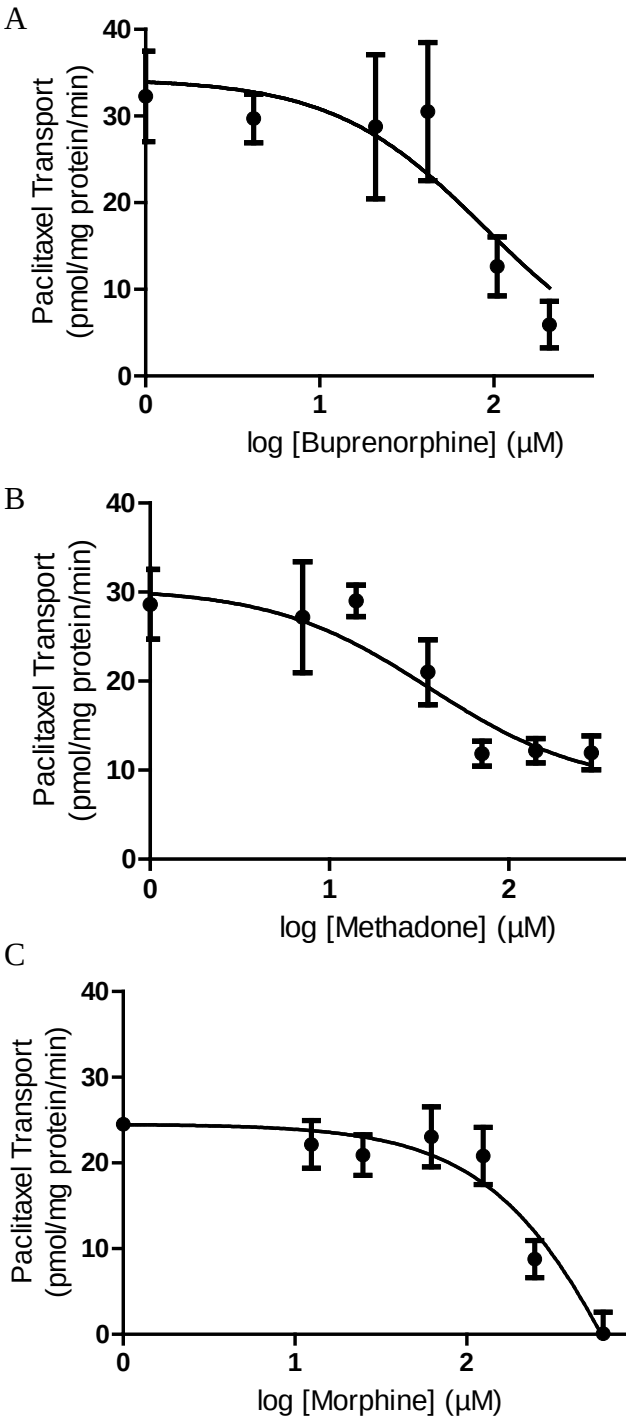
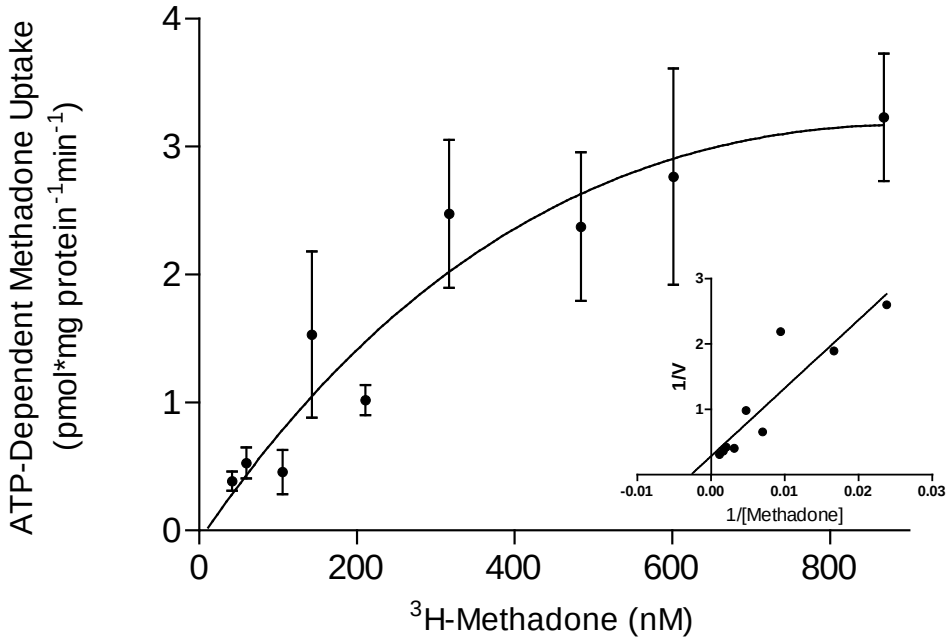


Figure 16. Opiate inhibition of P-gp-mediated paclitaxel transport
(A) Methadone (B) Buprenorphine, and (C) Morphine inhibited ATP-dependent transport of paclitaxel (70 nM) with a Ki of ~18 μM, 44 μM, and 90 μM, respectively (8 experiments in a pool of 60 placentas, error bars represent mean ± SEM). (Hemauer et al., 2009)

A


ATP-Dependent Methadone Uptake
(pmol*mg protein-1min-1)
4
3
2
1
0
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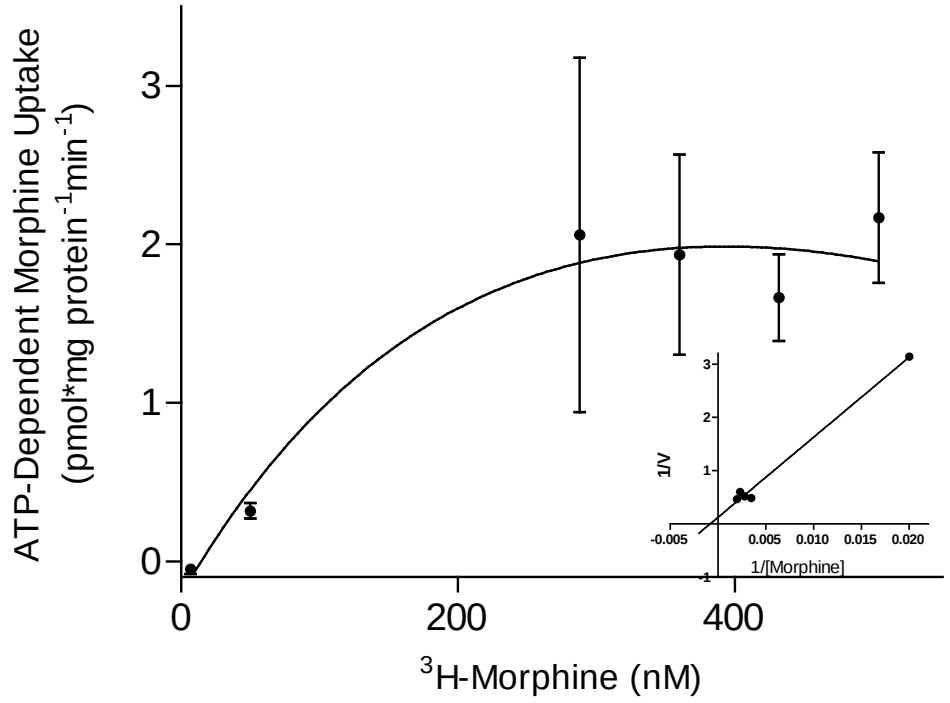
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(pmol*mg protein-1min-1)
3
2
1
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3H-Morphine (nM)
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2
1
-1
-0.005
0.005
0.010
0.015
0.020
1/[Morphine]

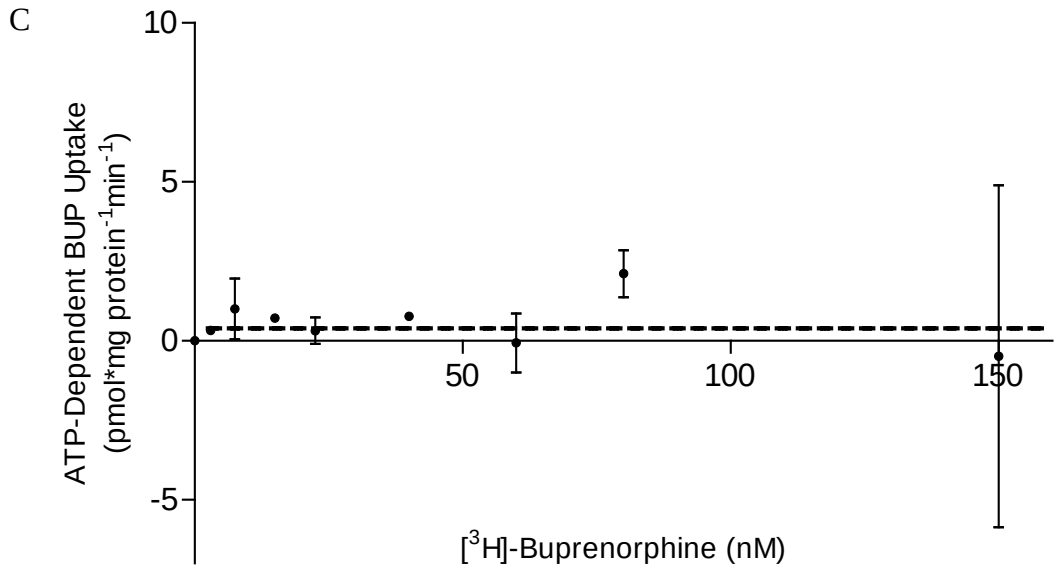


Figure 17. Opiate transport by P-gp in term placental vesicles.
Concentration dependent transport activity of methadone, morphine, and buprenorphine by P-gp in term placental IOVs (pool of 60, error bars represent mean ± SEM). Vesicles were incubated with radiolabeled (A) methadone, (B) morphine, or (C) buprenorphine in the presence or absence of P-gp selective inhibitor verapamil. Methadone and morphine displayed ATP-dependent, saturable transport which could be inhibited by verapamil. In contrast, buprenorphine did not display ATP-dependent transport by P-gp.

Hypoglycemic Agents

Stimulation of ATP Hydrolysis by P-gp and BCRP

Glyburide, rosiglitazone, and metformin each stimulated ATP hydrolysis by membranes expressing P-gp and BCRP (Figure 18.A, 18.B). Therefore, the involvement of both P-gp and BCRP in the transport of the hypoglycemic agents was investigated.

Glyburide Transport

The total ATP-dependent uptake of [^{3}H]-glyburide, at its concentration of 100 nM, by placental IOVs was 3.2 ± 0.3 pmol/mg protein*min. Inhibition of P-gp by verapamil decreased [^{3}H]-glyburide uptake by 9 ± 5%. Inhibition of BCRP by 25 nM KO143 decreased [^{3}H]-glyburide uptake by 25 ± 5%.

Since inhibition of P-gp and BCRP only accounted for less than 40% of ATP-dependent glyburide transport, involvement of placental MRP1 transporter was examined. Inhibition of MRP1 by indomethacin decreased [^{3}H]-glyburide uptake by 43 ± 4%. Total inhibition of P-gp, BCRP, and MRP1 using 1 μM KO143 decreased [^{3}H]-glyburide uptake by 78 ± 4 %. Thus, the contributions of each transporter to the total efflux were MRP1>BCRP> P-gp (Figure 19.A).

The activity of MRP1, the major transporter responsible for the efflux of glyburide in this study, exhibited ATP-dependent transport with an apparent K_t of 358 ± 195 nM and V_{max} of 3.6 ± 0.9 pmol/mg protein*min (Figure 19.B).

Rosiglitazone Transport

The total ATP-dependent uptake of [^{3}H]-rosiglitazone, at its concentration of 100 nM, by placental IOVs was 1.2 ± 0.2 pmol/mg protein*min. Inhibition of P-gp by

verapamil decreased [^{3}H]-rosiglitazone uptake by 71 ± 26%. Inhibition of BCRP by 25 nM KO143 and inhibition of MRP1 by indomethacin had no effect on rosiglitazone uptake. The inhibition of P-gp, BCRP, and MRP using 1 μM KO143 decreased rosiglitazone uptake by 72 ± 36 %. Taken together, P-gp was responsible for the majority of ATP-dependent rosiglitazone efflux in this system, with negligible contributions by BCRP and MRP1 (Figure 20.A).

The activity of P-gp, the major transporter responsible for rosiglitazone efflux in this study, exhibited ATP-dependent transport with an apparent K_t of 84 ± 47 nM and V_{max} of 1.7 ± 0.3 pmol/mg protein*min (Figure 20.B).

Metformin Transport

The total ATP-dependent uptake of [^{14}C]-metformin, at a concentration of 100 nM, by placental IOVs was 35 pmol/mg protein*min. Inhibition of P-gp by verapamil decreased [^{14}C]-metformin uptake by 58 ± 10%. Inhibition of BCRP by 25 nM KO143 decreased [^{14}C]-metformin uptake by 25 ± 8%. Inhibition of MRP1 using indomethacin had no effect on [^{14}C]-metformin uptake. The inhibition of P-gp, BCRP, and MRP using 1 μM KO143 decreased [^{14}C]-metformin uptake by 89 ± 6 %. Therefore, the majority of ATP-dependent [^{14}C]-metformin effux was achieved by P-gp, followed by BCRP (Figure 21.A).

The activity of P-gp, the major transporter responsible for metformin efflux in this study, exhibited ATP-dependent transport with an apparent K_t of 100 ± 85 nM and V_{max} of 33 ± 10 pmol/mg protein*min (Figure 21.B).

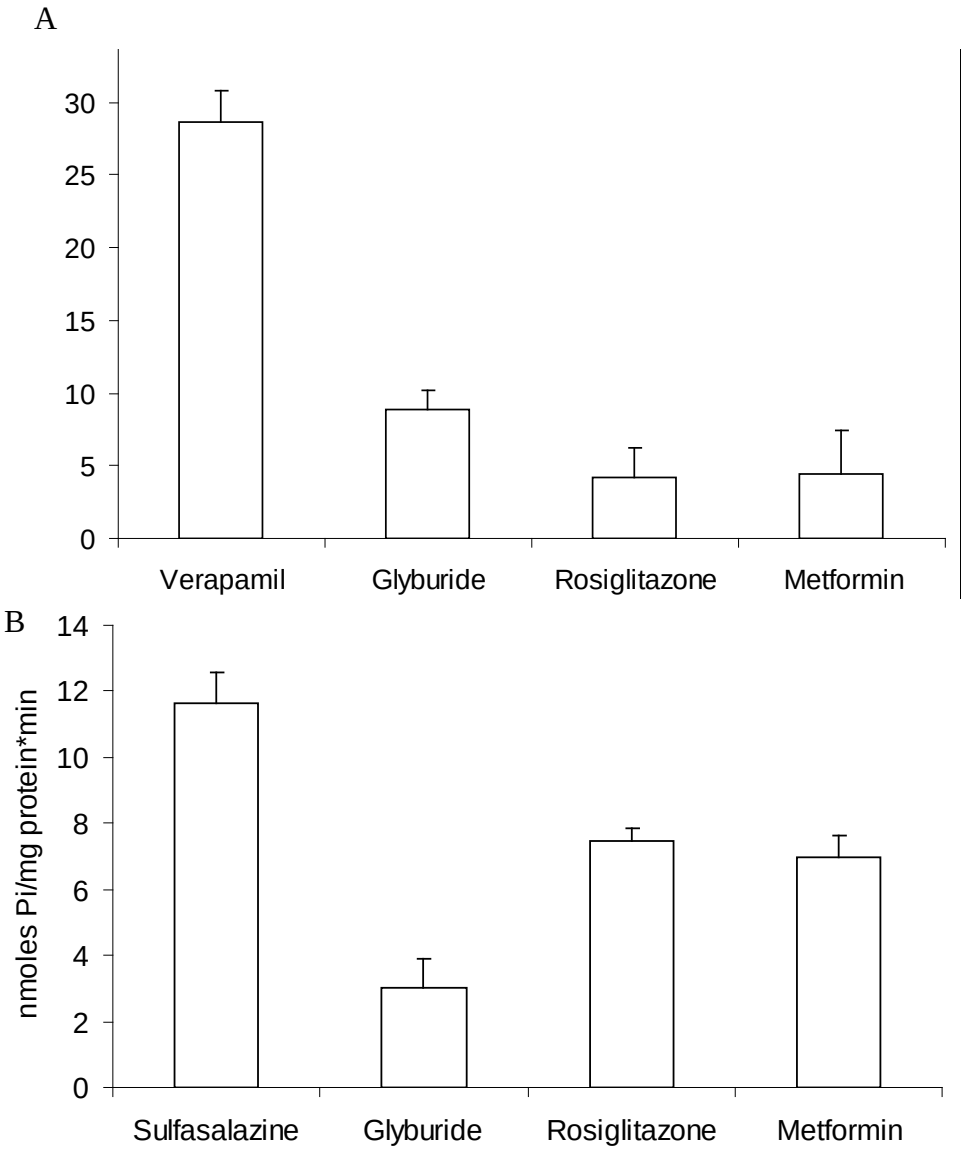
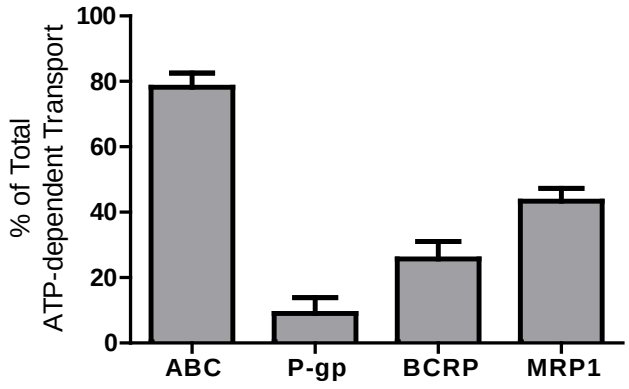


Figure 18. Hypoglycemic drug stimulation of ATP hydrolysis by P-gp and BCRP
Glyburide, rosiglitazone, and metformin each stimulated ATP hydrolysis by (A) P-gp and (B) BCRP, in comparison to positive controls verapamil and sulfasalazine, respectively.

A



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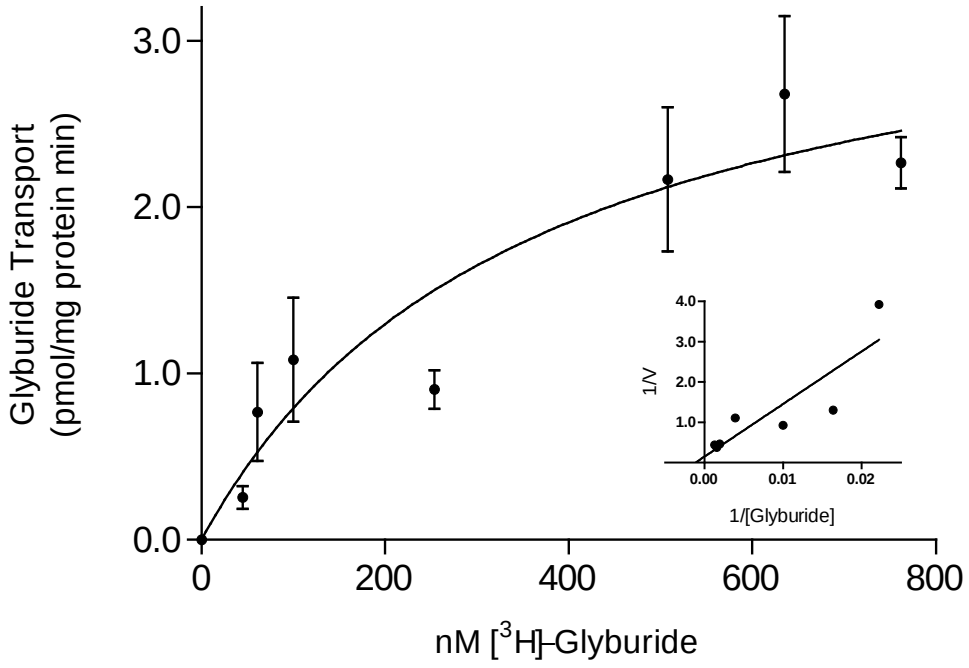


Figure 19. Glyburide efflux by placental ABC Transporters
(A) The relative contributions of P-gp, BCRP, and MRP1 to glyburide efflux were determined using chemical inhibition of ATP-dependent transport of 100nM [^{3}H]-glyburide in term placental IOVs (pool of 60 preparations). Uptake was measured for 1 minute in placental IOVs (protein concentration of 0.05 μg/μL) in the presence/absence of inhibitor selective for P-gp (600 μM verapamil), BCRP (25 nM KO143), MRP1 (100 μM indomethacin), or total P-gp + BCRP + MRP (1 μM KO143). (B) MRP1 displayed apparent K_t of 360 ± 195 nM glyburide and V_{max} = 3.6 ± 0.9 pmol/mg protein*min for ATP-dependent glyburide transport, with corresponding Lineweaver-Burk plot (inset). Data are presented as mean ± SEM of 4-8 experiments. (Hemauer et al., 2010).

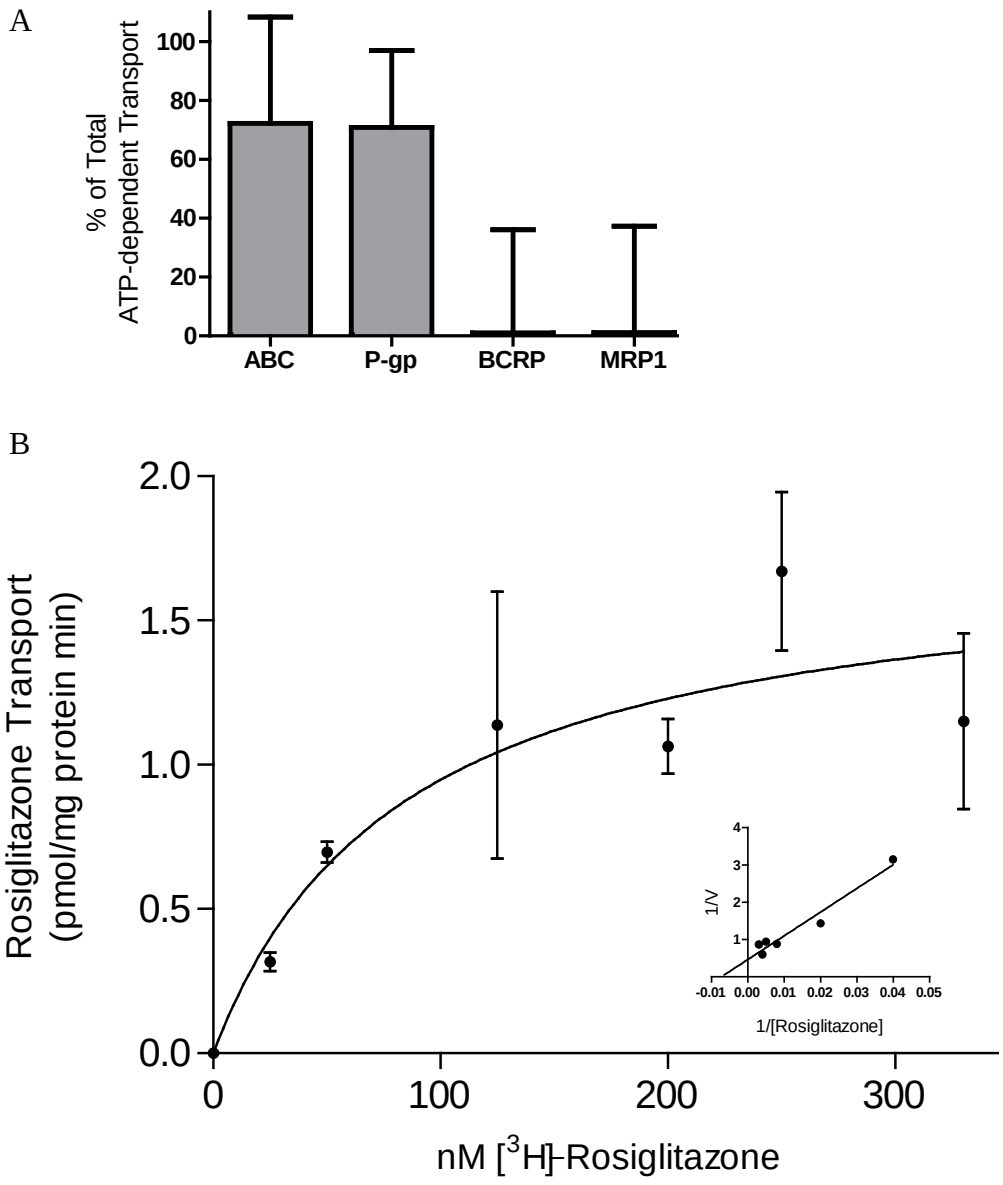


Figure 20. Rosiglitazone efflux by placental ABC Transporters
(A) The relative contributions of P-gp, BCRP, and MRP1 to rosiglitazone efflux were determined using chemical inhibition of ATP-dependent transport of 100nM [^{3}H]-rosiglitazone in term placental IOVs (pool of 60 preparations). Uptake was measured for 1 minute in placental IOVs (protein concentration of 0.05 μg/μL) in the presence/absence of inhibitor selective for P-gp (600 μM verapamil), BCRP (25 nM KO143), MRP1 (100 μM indomethacin), or total P-gp + BCRP + MRP (1 μM KO143). (B) P-gp displayed apparent K_t of 84 ± 47 nM rosiglitazone and V_{max} = 1.7 ± 0.3 pmol/mg protein*min for ATP-dependent rosiglitazone transport, with corresponding Lineweaver-Burk plot (inset). Data are presented as mean ± SEM of 4-8 experiments. (Hemauer et al., 2010).

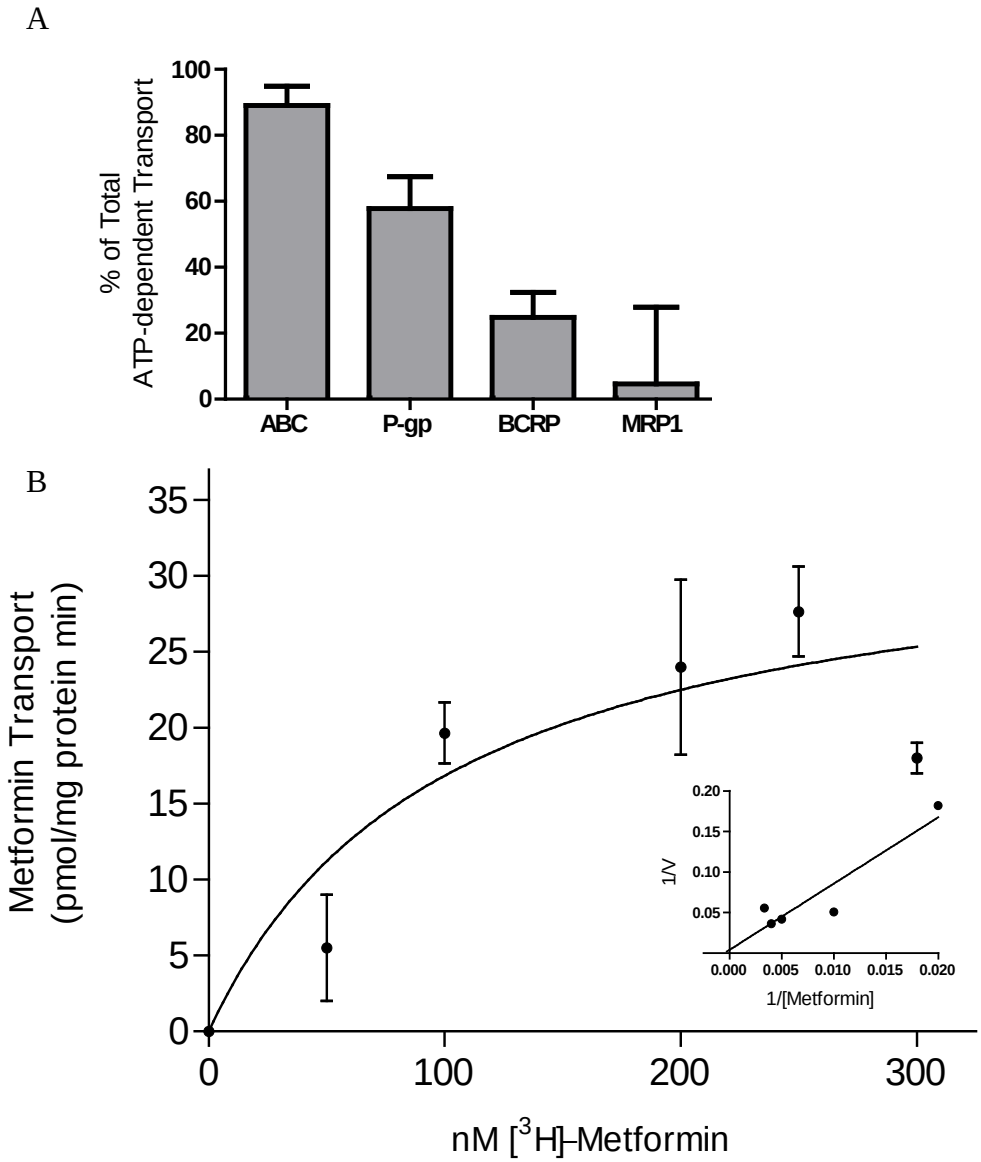


Figure 21. Metformin efflux by placental ABC Transporters
(A) The relative contributions of P-gp, BCRP, and MRP1 to metformin efflux were determined using chemical inhibition of ATP-dependent transport of 100nM [^{14}C]-metformin in term placental IOVs (pool of 60 preparations). Uptake was measured for 1 minute in placental IOVs (protein concentration of 0.05 μg/μL) in the presence/absence of inhibitor selective for P-gp (600 μM verapamil), BCRP (25 nM KO143), MRP1 (100 μM indomethacin), or total P-gp + BCRP + MRP (1 μM KO143). (B) P-gp displayed apparent K_t of 100 ± 85 nM metformin and V_{max} = 34 ± 10 pmol/mg protein*min for ATP-dependent metformin transport, with corresponding Lineweaver-Burk plot (inset). Data are presented as mean ± SEM of 4-8 experiments. (Hemauer et al., 2010).

Inhibition of Rosiglitazone Transport by Metformin

Since P-gp was responsible for the majority of transport of both metformin and rosiglitazone, the potential for drug interactions between the two agents was investigated. The effect of metformin on inhibiting rosiglitazone transport by P-gp, was examined. Metformin inhibited ATP-dependent transport of [^{3}H]-rosiglitazone by 69 ± 6 % with an apparent IC_{50} of approximately 600 nM (Figure 22).

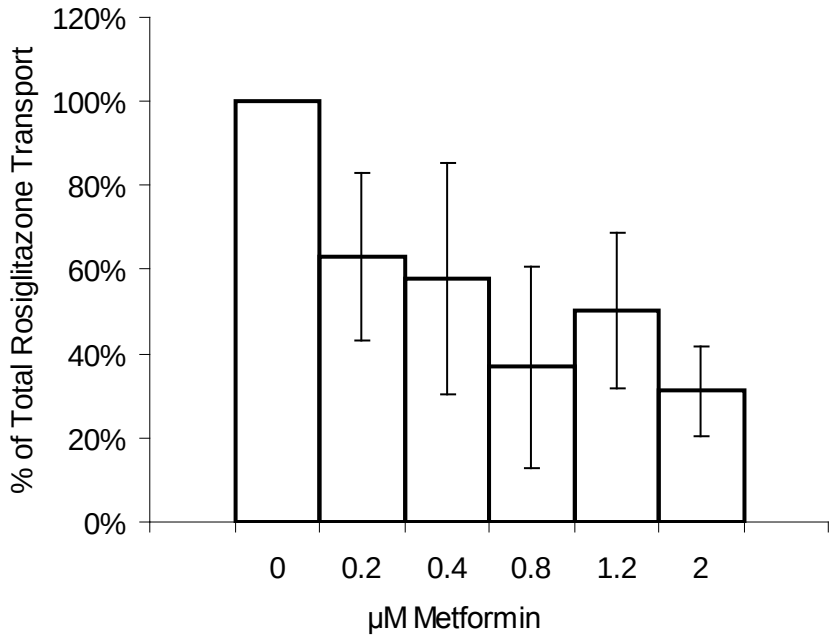


Figure 22. Metformin-rosiglitazone drug interactions.
Inhibition of P-gp- mediated [^{3}H]-rosiglitazone transport in term placental brush border membrane vesicles (0.5 mg/mL) by metformin.

Smoking Cessation Agent Bupropion

Stimulation of ATP Hydrolysis by P-gp and BCRP

Bupropion stimulated ATP hydrolysis in both P-gp and BCRP in a concentration-dependent manner (Figure 23.A, 23.B). Bupropion stimulation of P-gp ATPase activity displayed a K_m of 23 ± 19 μM and V_{max} of 9.5 ± 1.6 nmoles/mg protein*min. Bupropion stimulation of BCRP ATPase activity displayed a K_m of 0.6 ± 0.4 μM and V_{max} of 6 ± 0.8 nmoles/mg protein*min. Since bupropion interacts with both transporters, as evidenced by stimulation of ATPase activity, the involvement of both P-gp and BCRP in the transport of bupropion was investigated.

Bupropion transport by P-gp and BCRP

Bupropion was transported by P-gp (K_t 0.5 ± 0.2 μM, V_{max} 6 ± 0.7 pmol/mg protein*min) and BCRP (K_t 3 ± 2 μM, V_{max} 30 ± 13 pmol/mg protein/min) (Figure 24.A, 24.B). Thus, bupropion has higher affinity for transport by P-gp (as evidenced by lower K_t), while BCRP exhibits greater total transport activity (V_{max}). As displayed in Figure 25, a positive correlation was determined between P-gp and BCRP protein expression in BBMVs ($n = 200$, $p < 0.05$).

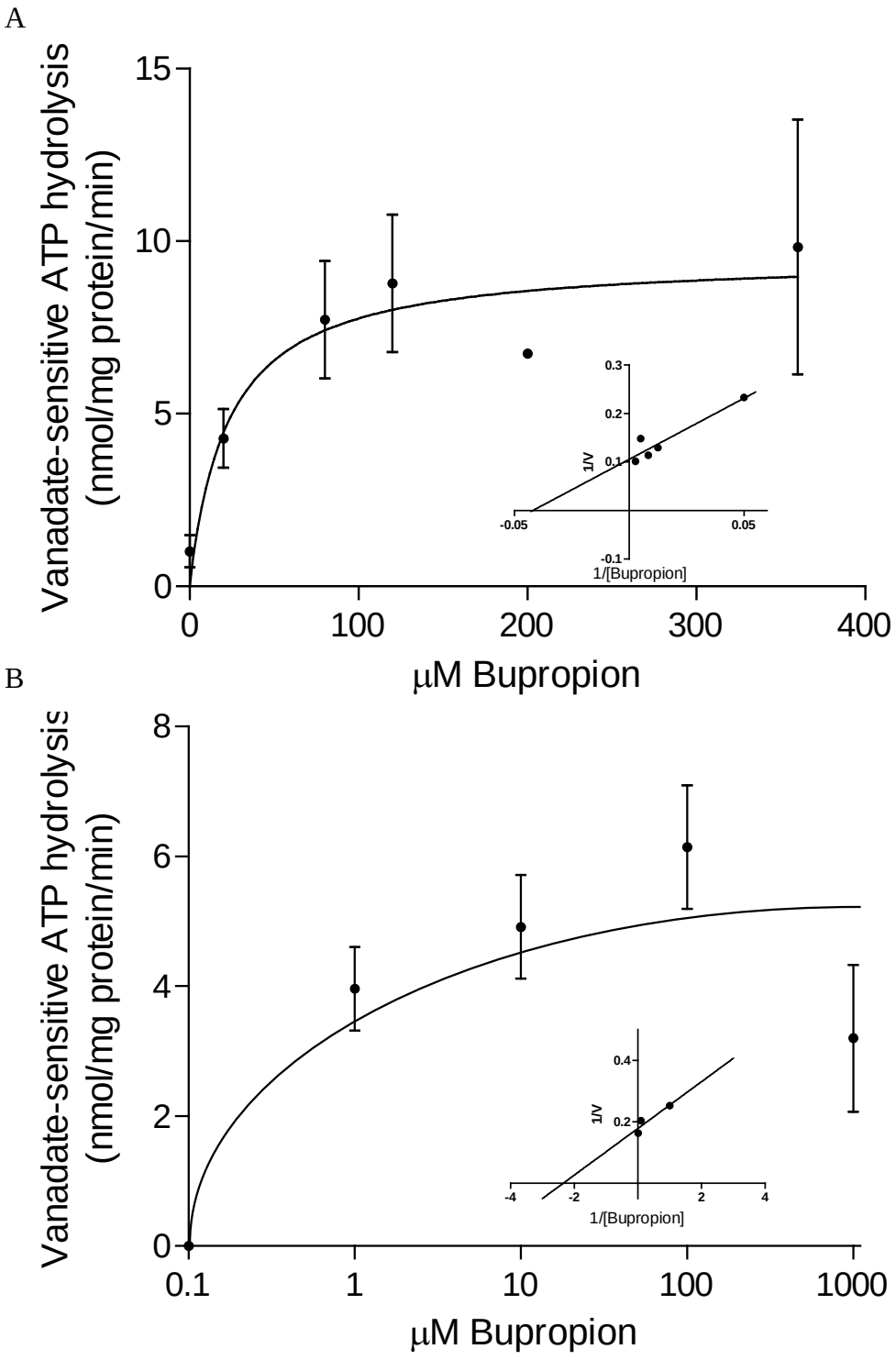
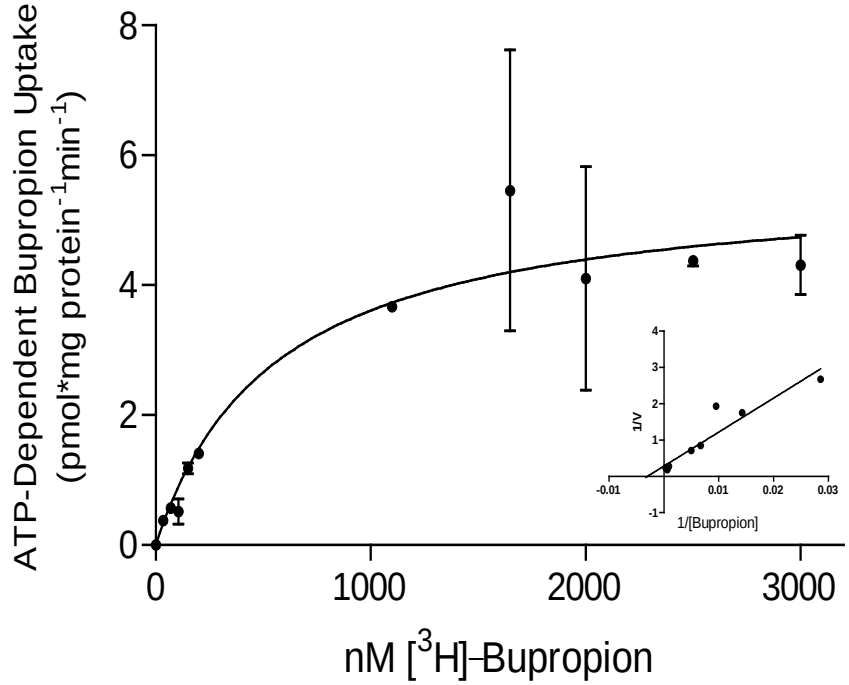


Figure 23. Bupropion stimulation of ATP hydrolysis by P-gp and BCRP
Stimulation of ATPase activity, as measured by inorganic phosphate release, was determined in commercially available membranes expressing P-gp and BCRP.

A



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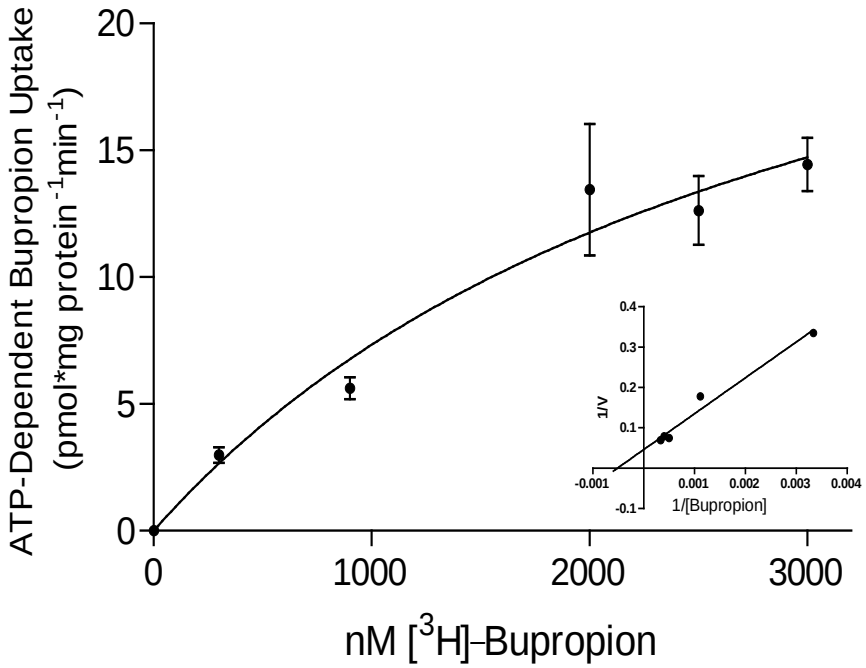


Figure 24. Bupropion transport by P-gp and BCRP.
(A) P-gp displayed apparent K_t of 0.5 ± 0.2 μM and V_{max} = 6 ± 0.7 pmol/mg protein*min for bupropion transport (B) BCRP displayed apparent K_t of 3 ± 2 μM and V_{max} = 30 ± 13 pmol/mg protein*min for bupropion transport, with corresponding Lineweaver-Burk plot (inset). Data are presented as mean ± SEM of 4-8 experiments in a pool of 60 placentas.

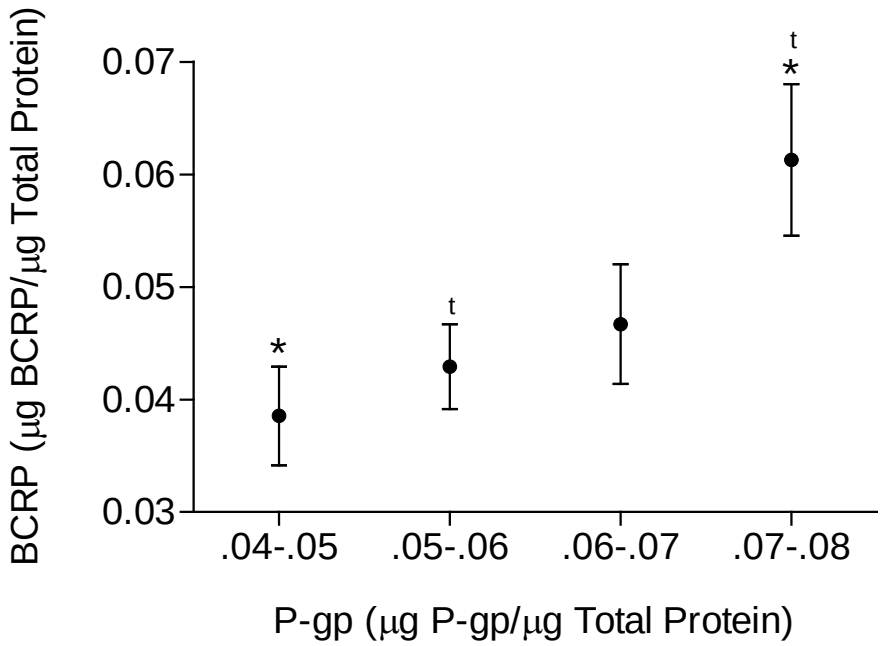


Figure 25. Correlation between P-gp and BCRP protein expression in human placenta. Placentas (n = 200) of varying gestational age were analyzed for P-gp and BCRP expression using Western Blot analysis. Across gestational ages, there was a positive correlation between protein expression of P-gp and BCRP.

Interpretation: Therapeutics Used During Pregnancy

Opiates

The three opiates investigated stimulated ATP hydrolysis by P-gp membranes, however there was no stimulation of ATP hydrolysis detectable by BCRP expressing membranes. Therefore, the role of BCRP in transporting these opiates was not pursued in this investigation. Indeed, a report of flow cytometric assay with human *MDR1*- and BCRP-transfected HEK293 (human embryonic kidney) cells supports that BCRP is not involved in the transport any of the tested compounds (buprenorphine, methadone, or morphine) (Tournier et al., 2009).

The interaction of opiates with P-gp expressed in placenta can be reflected by opiate inhibition of P-gp mediated transport of paclitaxel. In our system, methadone, buprenorphine, and morphine inhibited the transfer of paclitaxel by placental P-gp with an apparent K_i of approximately 18, 44, and 90 μM, respectively. The apparent K_i for inhibiting paclitaxel transport determined for the three opiates used in this study were lower (higher affinity) than that determined for the known inhibitor of P-gp, verapamil (apparent K_i, 300 μM). Therefore, methadone, buprenorphine, and morphine are more potent than verapamil as inhibitors of paclitaxel transport by human placental P-gp. Interestingly, methadone caused partial (60%) inhibition of P-gp mediated transport of paclitaxel (Figure 16.A), which could suggest the involvement of more than one binding site affecting the transport of paclitaxel and methadone. It is recognized that there are multiple drug binding sites on P-gp capable of allosteric interactions (Martin et al., 2000), and further investigation of the substrate specificity of the binding sites is needed.

The direct measurement of transport of [^{3}H]-methadone, [^{3}H]-buprenorphine, and [^{3}H]-morphine uptake by placental P-gp indicate that P-gp transports methadone and morphine, yet is not involved in buprenorphine transport. In a previous report from our laboratory, utilizing the *ex vivo* model of dual perfusion of placental lobule (DPPL), we indirectly identified methadone as a P-gp substrate by using a P-gp inhibitor to increase methadone placental transfer (Nanovskaya et al., 2005). However, placental transfer of buprenorphine was unaffected by inhibition of P-gp during perfusion. Taken together, the data in this study demonstrate that methadone and morphine are substrates of and

transported by placental P-gp. Conversely, buprenorphine is not transported by P-gp, yet its inhibition of paclitaxel transport supports its role as a P-gp inhibitor.

P-gp transports a variety of xenobiotics, endogenous compounds, and possibly metabolic waste products from the fetoplacental unit to the maternal circulation. Consequently, the extent to which a compound may interfere with P-gp (drug interactions) at the maternal-fetal interface could affect the ability of the placenta to serve as a functional barrier in limiting fetal exposure to administered medications. Drug-drug interaction represents one of many factors which may influence biodisposition of therapeutics at the maternal-fetal interface. An earlier report of placental aromatase inhibition by methadone and buprenorphine demonstrated that the human placenta is a target for drug interactions in pregnant women under treatment with these medications (Zharikova et al., 2006). However, opiate interactions with P-gp, as they relate to the development and severity of NAS, require future *in vivo* studies during pregnancy.

Genetic variants in *MDR1* have been associated with differing methadone plasma levels and maintenance dose in opiate-dependent patients (Coller et al., 2006; Crettol et al., 2006; Levran et al., 2008), however there is no clear consensus on the role of *MDR1* genotype in the transport of methadone or interaction with other opioids. Since three functional variants were associated with altered P-gp activity in the transport of its substrate, paclitaxel, future studies should examine the relationship between these polymorphisms, methadone transport, and the risk of neonatal abstinence symptoms in patients treated with methadone maintenance therapy during pregnancy.

Hypoglycemic Drugs

The hypoglycemic drugs each stimulated ATP hydrolysis by membranes expressing P-gp to different extent. Additionally, each hypoglycemic drug displayed stimulation of ATP hydrolysis by BCRP. Therefore, the role of placental BCRP was also investigated in the transport of the three drugs. Furthermore, previous reports of the MRP isoforms involvement in glyburide transport led to the investigation of its involvement in the efflux of these drugs as well.

The activity of placental ABC transporters in the efflux of glyburide, rosiglitazone, and metformin was determined utilizing placental membrane inside out vesicle. Each of the transporters, MRP1, BCRP, and P-gp, transported glyburide in our preparation of brush border membrane IOVs, indicating efflux across the syncytiotrophoblast cell membrane in the fetal-to-maternal direction. Glyburide efflux in IOVs supports previous evidence of its asymmetric transfer reported from perfusion experiments in our laboratory and by others. Specifically, it was demonstrated that the fetal-to-maternal transfer of glyburide was greater than maternal-to-fetal transfer during placental perfusion (Nanovskaya et al., 2006), and significant transfer of glyburide occurs against a concentration gradient from the fetal to the maternal circulation (Kraemer et al., 2006). Interestingly, verapamil (an inhibitor of P-gp) did not affect glyburide transfer during its perfusion in a placental lobule (Kraemer et al., 2006) suggesting that only a minor amount of glyburide was transported by P-gp. In this investigation, we confirmed that P-gp is responsible for a small fraction of glyburide efflux, namely ~9%.

Data sited in this report demonstrated that placental MRP1 was involved in the efflux of glyburide and its activity was greater than that of BCRP or P-gp. In most tissues, MRP1 is localized to the basolateral membrane of epithelial cells (Borst et al., 1999; Borst et al., 2000), however the placenta provides an exception because MRP1 is also detectable in the apical membrane of syncytiotrophoblast tissue (Flens et al., 1996; St-Pierre et al., 2000). The data in this investigation suggest that MRP1 could also have a role in the efflux of compounds in the fetal-to-maternal direction. MRP1 similarly shares efflux function in the blood-brain barrier with P-gp and BCRP, where the three are co-localized in the luminal membrane of brain capillary endothelial cells (De Boer et al., 2003; Kusch-Poddar et al., 2005). Thus, it can be concluded that—similar to the blood brain barrier—MRP1, BCRP, and P-gp work in concert to efflux glyburide across the placental apical membrane in the fetal-to-maternal direction.

The data obtained in this investigation indicate that P-gp and BCRP are both involved in the efflux of metformin across placental apical membranes, which could explain its asymmetric fetal-to-maternal transfer and limited placental tissue retention observed in perfusion studies (Kovo et al., 2008; Nanovskaya et al., 2006). To our knowledge, this is the first report to identify P-gp as the major placental efflux transporter for metformin. Previously metformin was not identified as a P-gp substrate in *in vivo* investigations of rat intestine (Song et al., 2006), or in Caco-2 monolayers composed of human colonic adenocarcinoma (Proctor et al., 2008). However, interspecies and inter-tissue differences exist in the tissue permeability of P-gp substrates and the selectivity/specificity of the inhibitors used (Suzuyama et al., 2007; Terasaki et al., 1984).

Moreover, our preparations of human placental brush border membranes allowed the direct measurement of metformin efflux by physiologically-expressed membrane transporters, a determination that is difficult to quantify *in vivo* or in drug-selected cancer cell lines. The total transport of metformin (at a final concentration of 100 nM) by the ABC transporters examined equaled 35 pmol/mg protein*min, which represents 10- and 30-fold greater transport than that of glyburide and rosiglitazone transport, respectively.

The apparent K_t and V_{max} of P-gp -mediated metformin transport (100 ± 85 nM and 34 ± 10 pmol/mg protein*min) are similar to that of the prototypic P-gp substrate, paclitaxel (K_t of 66 ± 38 nM and V_{max} of 20 ± 3 pmol/mg protein*min) determined in Aim 1, indicating that metformin is a high affinity substrate of placental P-gp. The distribution of metformin in non-placental tissues is significantly influenced by transporters such as Organic Cation Transporter 2 (OCT2) and genetic variation in OCT2 alters metformin biodisposition (Choi et al., 2008). Since P-gp is the major placental ABC transporter responsible for metformin efflux, and functional polymorphisms in the *MDR1* gene encoding P-gp affect its placental expression and transport activity (as demonstrated in Aim 2), it is plausible that *MDR1* genetic variation could significantly influence placental disposition of metformin.

P-gp was the major placental transporter responsible for rosiglitazone efflux in apical membrane preparations from syncytiotrophoblast. Evidence from human brain microvascular endothelial cells (HBMECs) supports that P-gp is also the major brain-to-blood transporter of rosiglitazone (Festuccia et al., 2008). In our system, the rate of rosiglitazone transport by P-gp was much lower than metformin (1.7 ± 0.3 vs. 36 ± 15

pmol/mg protein*min), however the two drugs had similar affinity for P-gp (apparent K_t of 84 ± 47 and 100 ± 85 nM, for rosiglitazone and metformin respectively). Furthermore, metformin was able to inhibit rosiglitazone by P-gp in a dose-dependent manner. This presents a potential drug interaction which has physiologic relevance because metformin plasma concentrations exceed 2 μM in pregnant patients, a concentration which was able to "out-compete" rosiglitazone for transport by P-gp. Thus, at therapeutic concentrations, coadministration of both of these drugs is likely to render competition for P-gp mediated efflus and potentially increased rosiglitazone passage to the fetal circulation.

Bupropion

Bupropion stimulated ATP hydrolysis by both P-gp and BCRP expressing membranes. Therefore, both transporters were investigated in the direct measurement of bupropion efflux by placental inside out vesicles.

The P-gp and BCRP mediated transport of bupropion were determined using placental brush border IOVs. Bupropion displayed ATP-dependent transport by both P-gp and BCRP which was inhibited by P-gp selective inhibitor, verapamil, and BCRP selective inhibitor KO143, respectively. In our system, Bupropion appears to display higher affinity for transport by P-gp (as evidenced by lower K_t), while BCRP exhibits greater total transport activity (V_{max}). This is in contrast to stimulation of ATPase activity, in which bupropion displayed higher affinity for BCRP (lower K_m). However, ATPase activity was determined in commercially available recombinant baculovirus-infected insect cells expressing human P-gp and BCRP, which may reflect different transporter-substrate interaction properties than human placental membrane vesicles.

The data obtained from the placental IOVs indicate that P-gp and BCRP may work in parallel to extrude bupropion from the placenta, but with distinct roles. P-gp may be the first line of defense in transporting bupropion from the fetal-to-maternal direction (its high affinity represents transport occurs at low bupropion concentrations), with BCRP serving as a second line of defense when bupropion concentrations are elevated in the placental tissue. The positive correlation between P-gp and BCRP placental expression across gestational ages indicates that, in addition to having similar transport function, the regulation of expression of the two transporters may be influenced similarly.

Consideration for using pharmacotherapy for the pregnant patient seeking smoking cessation involves weighing the risk to benefit of bupropion. Although its safety for use during pregnancy is not well established, the exposure to bupropion may pose less risk than the components of cigarette smoke which contain thousands of compounds including carcinogens. Additionally, nicotine contained in cigarette smoke crosses the placenta freely, and is not extruded in the maternal direction by placental efflux transporters. The transplacental transfer of nicotine during ex vivo perfusion of the placenta revealed its transfer the fetal circulation (up to 40% of the initial concentration in the maternal circulation) (Nekhayeva et al., 2005; Pastrakuljic et al., 1998; Sastry et al., 1987) was greater than that of bupropion (20% transfer to the fetal circulation) (Earhart et al., 2010). Thus when comparing bupropion versus nicotine alone (aside from the numerous other chemicals in cigarette smoke), it appears that the placental permeability of nicotine, and thus fetal exposure, is greater than bupropion.

Smokers on methadone maintenance therapy during pregnancy are associated with greater prevalence and severity of neonatal abstinence syndrome in their newborns (Winklbaur et al., 2009). Therefore, future treatment regimens may incorporate smoking cessation therapy into methadone maintenance programs in these patients. Since methadone and bupropion are both effluxed from the fetal to maternal direction by P-gp, concern should be raised over whether competition will increase the fetal exposure to one or both of these medications.

In conclusion, this investigation has demonstrated that P-gp, in addition to other ABC transporters of the placenta, is involved in the fetal-to-maternal efflux of medications used to treat opiate dependence, gestational diabetes, and smoking cessation during pregnancy. The declining activity of P-gp throughout gestation, as well as genetic variability in P-gp function, may influence fetal exposure to these medications.

AIM 4: BABOON PLACENTAL P-GP PROTEIN EXPRESSION AND TRANSPORT ACTIVITY

P-gp Protein Expression

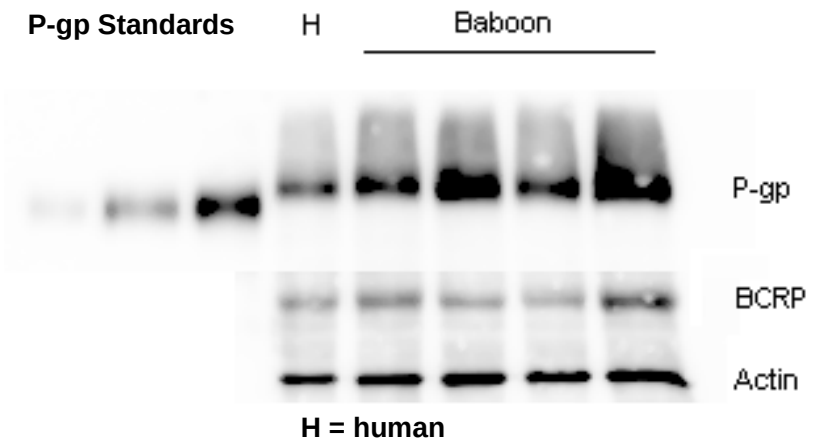
P-gp protein expression was determined in the baboon placenta (n = 9) using Western Blot analysis (Figure 25.A), and compared to data obtained from human placentas (n = 200) of equivalent gestational stage. P-gp protein expression in baboon placenta (0.058 ± 0.02 µg P-gp/µg total protein) was similar to that of human placenta (0.042 ± .02 µg P-gp/µg total protein) with no statistical difference between the two groups (Figure 25.B).

The presence of BCRP protein expression was also confirmed in baboon placental brush border membrane. However, the selectivity of the BXP-21 antibody used for BCRP detection for baboon BCRP is unknown and quantification of BCRP protein levels was not determined.

P-gp Transport Activity

P-gp transport activity was measured by transport of prototypic substrate [^{3}H]-paclitaxel in placental brush border membrane vesicles from baboon (n = 9) and compared to human P-gp transport activity of equivalent gestational stage (n = 200). Transport of 150 nM [^{3}H]-paclitaxel in baboon placentas (20 ± 5 pmol*mg protein^{-1}min^{-1}) was similar to that determined in representative human placenta (22 ± 9 pmol*mg protein^{-1}min^{-1}) and commercially available membranes expressing P-gp (21 ± 2 pmol*mg protein^{-1}min^{-1}) (Figure 26). However, no statistical difference was found among the three groups.

A



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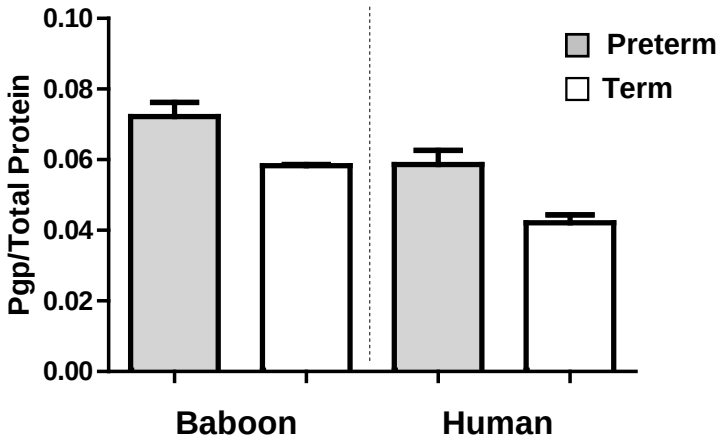
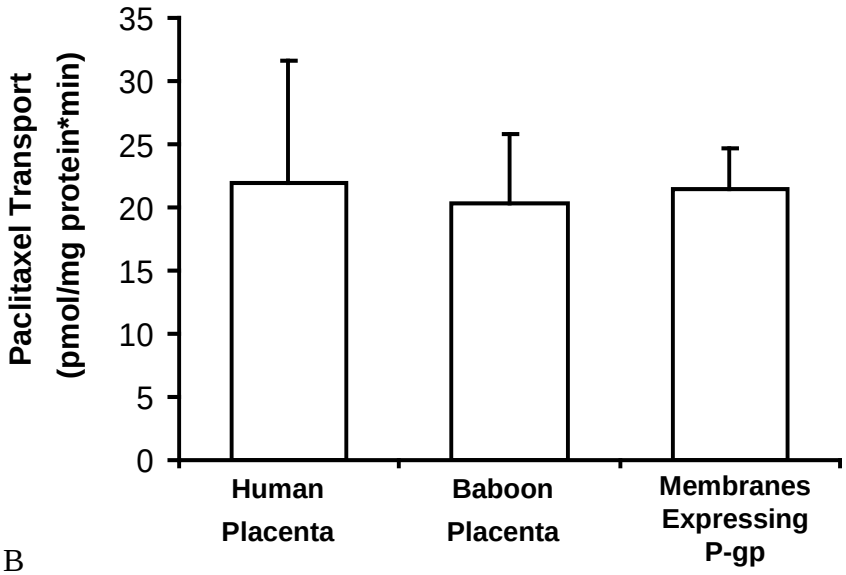


Figure 26. P-gp protein expression in baboon placenta
Western blot of baboon placental brush border membranes (10 μg/lane). P-gp Standards represent commercially available membranes expressing P-gp (Gentest, Co.). A representative human placenta (H) was included as a positive control. The immunoblot was probed with the monoclonal antibody C219 for P-gp, and monoclonal antibody BXP-21 forBCRP. Expression was determined as a proportion of the total amount of β-actin present per lane. (B) Mean ± SD were calculated in 200 human and 9 baboon placentas.

A



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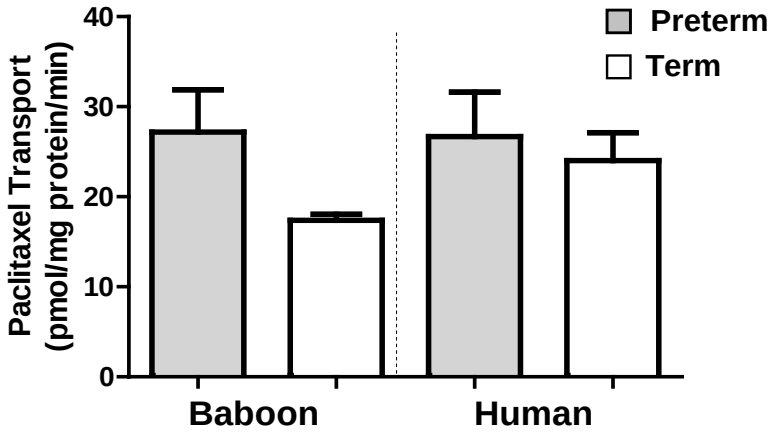

Figure 27. Baboon placental P-gp transport activity.
ATP dependent uptake of paclitaxel by human placental IOVs, baboon placental IOVs, and commercially available membranes expressing P-gp (n = 10, error bars represent mean ± SEM). Vesicles (0.05 mg/mL) were incubated with 150 nM [^{3}H] paclitaxel in the presence or absence of an ATP regenerating system and isolated by rapid filtration using a cell harvester. [^{3}H]-paclitaxel retention was detected using liquid scintillation counting.

Interpretation: Baboon as an Animal Model

The goal of this aim was to evaluate the baboon as an experimental model for investigating human placental P-gp activity *in vivo*. The initial step in evaluating baboon placenta as a model necessitates the confirmation that P-gp expression and activity are present in baboon placenta and comparable to that found in human placenta of equivalent gestational age. Therefore, the interpretation of this aim is straightforward in that the data have confirmed the presence, activity, and similarity of baboon placental P-gp to that found in human placenta.

P-gp protein expression in baboon placenta was similar to that of human placenta in both preterm and term groups. Similarly, P-gp transport activity as measured by transport of prototypic substrate [^{3}H]-paclitaxel in baboon placentas was similar to that determined in human placenta, with the exception of a trend towards lower P-gp transport activity in the term baboon placenta. Previous investigations in our laboratory have included extensive characterization of baboon placenta as a model of human placenta metabolic enzymes (Ravindran et al., 2006; Yan et al., 2008; Zharikova et al., 2007). The addition of information on baboon placental P-gp expression and activity adds dimension to our understanding of the role of baboon placenta in fetal protection, and supports the use of baboon placenta as a model of placental metabolic function.

In conclusion, the similarities found in quantification of P-gp expression and activity in placentas between species indicate that the baboon may serve as an effective model for predicting *in vivo* placental P-gp function in pregnant humans. Future studies may develop from this model in which a P-gp substrate and a modulator

(inhibitor/enhancer) of P-gp are co-administered to the pregnant baboon and evaluated for fetal exposure to the P-gp substrate. Such investigations could help to outline treatment strategies when the pharmacotherapeutic regimen targets specifically the mother (placental passage should be avoided) versus fetus (placental passage should be maximized).

Chapter 5: Conclusions

The goals of this investigation were:

- Investigation of placental brush border membrane vesicles;
- Characterization of human placental P-gp transport activity in correlation with its placental protein expression and gestational age
- Investigation of the relationships between, ethinicity, *MDR1* genetic polymorphisms and placental P-gp expression and activity
- Determination of the role of placental P-gp in regulating the maternal-fetal disposition of pharmacotherapeutics used during pregnancy
- Characterization of baboon placental P-gp protein expression and transport activity as a potential model for predicting human placental P-gp function *in vivo*

Placental P-gp protein expression and transport activity were defined in brush border membrane vesicles prepared from human placenta of various gestational ages. The protein expression and transport activity of P-gp, as characterized for prototypic substrate paclitaxel, declined progressively throughout gestation. However, due to a lack of correlation between protein expression and transport activity in individual samples, it can be concluded that P-gp protein expression should not be used to predict its transport activity in human placenta. Additionally, the declining efflux activity of P-gp with advancing gestation may equate to greater fetal exposure to medications administered in late gestation and near delivery.

Three polymorphisms in the *MDR1* gene encoding P-gp, namely C1236T, C3435T, and G2677T/A, were associated with decreased protein expression yet increased transport activity of human placental P-gp. This inverse relationship between decreased protein expression and increased functional activity in variants may explain, in part, the lack of correlation between expression and activity in our patients. Additionally, the genotype-phenotype relationship in the *MDR1* variants examined was different between African American, Caucasian, and Hispanic ethnicities, indicating there is likely involvement of additional genetic factors between these ethnic groups that are yet to be elucidated.

P-gp was found to transport medications used in the treatment of conditions during pregnancy: opiate dependence (synthetic opiods), cigarette smoking (bupropion), and gestational diabetes (oral hypoglycemic agents). Considerations of P-gp involvement in placental distribution of these medications must therefore be taken into account when planning therapy of these conditions during pregnancy. Additionally, drug-drug interactions resulting from co-administration of P-gp substrates may introduce competition for transport which alters placental permeability and fetal exposure to those compounds.

Finally, the need for an *in vivo* animal model for studying placental P-gp transport of investigational drugs during pregnancy led to our identification of the baboon as a comparable model to human placental P-gp expression and activity.

The value of establishing a system to study direct transporter-mediated efflux across the placental membrane, as well as the characterization of an animal model of

human placental P-gp, is two-fold. First, compounds can be tested as P-gp substrates in human placental IOVs, followed by *in vivo* study of their placental transfer and fetal accumulation/toxicity in the pregnant baboon. Second, the ability to assess *in vivo* activity of P-gp in baboon placenta, plus the method of isolating placental brush border membrane for *in vitro* study in the same placenta, will allow the evaluation of how closely placental membrane vesicles can emulate *in vivo* conditions in the same animal. If P-gp activity in the transport of its substrates is determined to be similar in both baboon placental vesicles and in *in vivo* studies of pregnant baboon, it would provide positive evidence that human placental P-gp activity in membrane vesicles is also predictive of *in vivo* conditions in the pregnant human.

Future studies should investigate the effects of long term exposure to P-gp substrates on placental P-gp expression and activity in human and baboon. Additional future study should include gene-wide analysis of functional *MDR1* variants and the role of genotype in the disposition of medications used to treat conditions of pregnancy. Finally, the validation of the baboon as an animal model to investigate the *in vivo* role of placental P-gp in drug disposition could aid in the future outlining of pharmacotherapeutic strategies for the treatment of the mother and/or the fetus during pregnancy.

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