GENETIC AND DIETARY REGULATION OF THE HUMAN PLACENTAL BCRP TRANSPORTER

By

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Glyburide is frequently used to treat gestational diabetes due to its low fetal accumulation resulting from placental efflux by the breast cancer resistance protein (BCRP/ABCG2) transporter. However, there is little understanding of the potential consequences of reduced placental BCRP function in pregnant women prescribed glyburide. Babies born to this population of women may be at an elevated risk of neonatal hypoglycemia potentially leading to low birth weight, increased morbidity, and impaired neurological development. The purpose of this dissertation research was to determine the influence of genetic and dietary factors on the placental expression of BCRP and its ability to transport glyburide. In 10 human term placentas, there were no differences in expression of BCRP mRNA or protein across the disc, supporting the use of a single sampling site to examine the interindividual expression of this transporter. In term placentas from 108 donors, ethnicity, transcription factor expression, and single nucleotide polymorphisms (SNPs) were associated with BCRP expression including the frequent C421A SNP, which correlated with lower BCRP protein expression. Further in vitro studies revealed that C421A-BCRP overexpressing cells had low BCRP protein expression at the cell surface and impaired transport of the BCRP substrate glyburide. Additionally, the dietary soy isoflavone genistein competitively inhibited the BCRP-mediated transport of glyburide in overexpressing cells (wild-type, WT-BCRP and C421A-BCRP) and human choriocarcinoma placental BeWo cells that endogenously
express BCRP. Importantly, genistein inhibited glyburide transport in placental BeWo cells at concentrations that are physiologically relevant (IC$_{50}$=0.18 ± 0.11 μM). Finally, estrogen receptor-mediated signaling stimulated by prolonged genistein treatment (48 h) down-regulated BCRP mRNA and protein expression in BeWo cells leading to reduced efflux of glyburide out of cells. Conversely, genistein (48 h) did not alter BCRP protein expression in term placental explants, suggesting that the regulation of placental BCRP expression by genistein may be limited to choriocarcinoma BeWo cells. Together, this research provides population and mechanistic data that demonstrate how a genetic variant (C421A) and diet (genistein) may alter the maternal-fetal disposition of glyburide as well as other chemicals that are BCRP substrates.
DEDICATION

This dissertation is dedicated to my parents and grandparents.
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CHAPTER 1: GENERAL INTRODUCTION

1.1. Human Placental Development and Function

During pregnancy, the placenta attaches the fetus to the maternal uterine wall and plays an important role in embryo-fetal nourishment, development, and growth. Current understanding of human placental structure and function has been described in detail in Williams Obstetrics (Cunningham et al., 2014). The following information has been adapted from this text unless otherwise noted.

1.1.1. Placental Development and Structure

Placental development begins at fertilization and occurs in parallel with the establishment of the embryo. By day 3-4 post-fertilization in humans, placental trophoblasts comprise the outer layer of cells of the hollow blastocyst (Figure 1.1). The inner cell mass of the blastocyst ultimately gives rise to other fetal tissues and cell types in the placenta. Upon rupture of the zona pellicuda, the trophoblasts interact directly with the uterine epithelium allowing for implantation. Beginning 5-8 days post-fertilization, the conceptus migrates through the endometrium, remodeling the uterine blood vessels and tissue to form the maternal decidua and lay the groundwork for placentation.

Trophoblasts. Distinct from the three embryonic germ layers (mesoderm, ectoderm, endoderm), trophoblast cells of the blastocyst give rise to cytotrophoblast stem cells that ultimately differentiate into two placental cell lineages: extravillous and villous trophoblasts (Figure 1.2). The extravillous trophoblasts (EVTs) further differentiate into either (1) interstitial EVTs that invade the decidua to the surface of the myometrium and ensure adequate anchoring of the placenta to the uterus or (2) endovascular EVTs, which remodel the uterine spiral arterioles to establish the maternal blood supply to the placenta. Replacement of the arteriole endothelium and smooth muscle with
endovascular EVT results in the formation of low-pressure lacunae, which remain plugged by EVT until remodeling is complete. By approximately the 10th week of pregnancy, placental blood flow is established with the loss of the EVT plugs such that the maternal blood circulates and interfaces with the villous trophoblasts. The villous cytotrophoblasts fuse to form multinucleated syncytiotrophoblasts which constitute the outermost layer of the villous tree structures in the placenta. The branched formation of the villi allows for increased surface area of the syncytiotrophoblasts for enhanced contact with the maternal blood. This direct interaction of the placental cells with the maternal circulation classifies the human placenta as hemochorial, which is similar to other mammals including high primates, rabbits, guinea pigs, mice, and rats. Once blood flow is established, the placenta will continue to develop; however the basic anatomical structures of the placenta including the chorionic plate, chorionic villi, spiral arteries, and basal plate (decidua) remain throughout pregnancy (Figure 1.3).

**Fetal Blood Vessels.** Blood flow between the fetus and the placenta occurs through the umbilical cord, which consists of two umbilical arteries and one umbilical vein. The chorionic plate houses the fetal blood vessels from the umbilical cord before they branch and form fetal capillary beds at the center of the placental villi. The direction of oxygenated fetal blood flow is different than the normal human circulation pattern. Oxygenated fetal blood flows from the placenta through the umbilical vein to the fetus, after which the blood becomes deoxygenated and flows back toward the placenta through the umbilical arteries. At the villous syncytiotrophoblast interface, fetal blood becomes reoxygenated via exchange with the uterine blood.
Fetal Membranes. Continuous with the umbilical cord, the fetal membranes (chorion and amnion) make up the chorionic plate and line the fetal side of the placenta and uterus. The amnion forms the top of the chorionic plate and is in direct contact with the amniotic fluid. The thin membrane is made up of a single-celled epithelium, on top of a layer of a fibrous mesoderm. Separating the amnion and the chorion is the zona spongiosa, which allows for movement of the amnion across the chorion and to increase tensile strength of the membranes. The chorionic membrane makes up the bottom of the chorionic plate and consists of fibroblastic mesenchyme and extravillous trophoblasts which anchor the membranes to the maternal decidua. Around 3 months of pregnancy, the amnion and chorion fuse to enhance membrane strength.

1.1.2 Human Placental Function

A unique organ that exists only for the duration of pregnancy, the placenta has a number of important roles including endocrine, immune, and chemical exchange functions that allow for communication between the maternal and fetal environments.

Endocrine Function of the Placenta. The placenta produces both peptide and steroid hormones, all which work in together to aid in the maintenance of pregnancy. The primary peptide hormone produced by the placenta, also called the “hormone of pregnancy”, human chorionic gonadotropin (hCG), is manufactured in the cytotrophoblasts and syncytiotrophoblasts. Following implantation, hCG levels in the maternal blood rapidly rise and reach maximum concentrations (~100,000-200,000 IU/L) near 10 weeks of pregnancy. Following this peak, maternal hCG blood concentrations plummet and remain lower for the remainder of pregnancy. While its major production site is the placenta, low levels of hCG (5 IU/L) can be observed in the serum of non-pregnant women (Snyder et al., 2005), suggesting that low amounts may be produced in
other endocrine tissues such as the pituitary gland. Similar in structure to luteinizing hormone (LH), hCG stimulates membrane LH/hCG receptors in various fetal and maternal tissues. The primary role of hCG is to initiate the secretion of progesterone from the corpus luteum which in turn promotes the maintenance of pregnancy.

Steadily rising throughout pregnancy, the peptide hormone human placental lactogen (hPL) reaches peak concentrations of nearly 10 µg/ml in the maternal serum at the end of gestation, near. Concentrations are low or undetectable in non-pregnant women as the hormone is only produced by placental syncytiotrophoblasts. The structural similarities of hPL with prolactin and growth hormone allow it to exert its physiological functions through the prolactin and growth hormone receptors expressed in various maternal tissues. hPL contributes to the altered maternal metabolic state of pregnancy to ensure nutritional and energy resources are available for both the mother and the fetus. This includes an increase in the availability of maternal free fatty acids (lipolysis), glucose (insulin resistance), in addition to protein synthesis.

By the 8th week of pregnancy, the placenta replaces the role of the corpus luteum in progesterone production, which steadily rises in the maternal blood until parturition (maximum concentration: 100-200 ng/ml). Blood progesterone concentrations vary in non-pregnant women based on the day of the menstrual cycle, however maximal concentrations do not exceed 25 ng/ml in non-gravid women. Playing a primary role in pregnancy maintenance, progesterone exerts the majority of its actions on the uterus through the progesterone receptors (nuclear PR-A and PR-B; membrane PR) by relaxing smooth muscle to inhibit its contraction, stimulating decidualization, and decreasing prostaglandin formation (reviewed in (Torrealday et al., 2000)).
Similar to progesterone, near the 8th week of pregnancy, the luteal-placental shift occurs and the placenta becomes the major source of estrogen hormone production for the remainder of pregnancy. Blood concentrations of estrogenic steroid hormones rise throughout pregnancy and reach maximal concentrations (17β-estradiol: 30 ng/ml, estriol: 15 ng/ml, estrone: 10 ng/ml) just prior to parturition. In non-pregnant women, 17β-estradiol (0.4 ng/ml), estriol (0.01 ng/ml), estrone (0.3 ng/ml) concentrations peak during the luteal phase of a normal menstrual cycle at levels much lower than the maximum pregnancy blood concentration (reviewed in (Torrealday et al., 2000)). Unlike placental progesterone production, placental estrogen production in the placenta relies on steroid hormone precursors produced primarily by the fetal adrenal glands and liver. Also contributing to pregnancy success, estrogens function through estrogen receptors (nuclear ERα and ERβ, membrane ER) to maintain vasodilation of the uterine blood vessels and ensure adequate blood flow to the placenta.

**Immune Function of the Human Placenta.** The human placenta offers the developing fetus tolerance from the maternal immune system in addition to providing immune protection. To the maternal immune system, the fetus and placenta represent a semi-allograft as both maternal and paternal antigens exist on fetal tissues. Proteins expressed (or not expressed) by placental trophoblasts allow the cells to remain in intimate contact with the maternal blood and uterine tissue without destruction by the immune system. For example, trophoblasts are void of most of the major histocompatibility complexes (MHC), human leukocyte antigen (HLA), which allows for limited recognition of the foreign cells by the maternal immune system. Those HLAs that are expressed in EVTs are HLA class 1 antigens (1a: C, 1b: E, G) that help regulate placental invasion by interaction with decidual natural killer cells. Other HLA class 1 proteins (A and B) and class 2 proteins are highly polymorphic, and if present on
placental trophoblasts would increase the risk for the detection of the placental cells as non-self and a cytotoxic response.

During the first trimester, 70% of the resident immune cells in the uterus and decidua are NK cells, suggesting an important role for these cells in the establishment of pregnancy. For example, decidual natural killer cells express inhibitory killer Ig-like receptors (KIR) that bind to HLA-Ia and b antigens expressed on EVTs. Upon binding, lytic properties of the decidual NK cells are inhibited, thereby allowing the EVTs to remain. Furthermore, cytokines and growth factors are produced by decidual NK cells including interleukin-8 (IL-8) and vascular endothelial growth factor (VEGF), respectively, which promote invasion and remodeling of the decidua and uterus.

In addition to escaping the maternal immune system, the fetus acquires maternal immune protection via the placental passage of IgG antibodies. This is particularly meaningful as no other antibodies are able to cross the placenta. Cell surface receptors that are specific for the Fc region of IgG antibodies are expressed on syncytiotrophoblasts such that upon binding, the receptor/antibody complex becomes endocytosed for ultimate transfer to the fetal circulation. Although IgG transfer begins around week 16 of gestation, the IgG antibody concentration is highest in the fetal/neonatal blood at term, providing passive immune protection for the neonate before maturation of the neonatal immune system.

**Chemical Exchange Function of the Placenta.** The placenta mediates the bidirectional chemical exchange between the maternal and fetal circulations. This includes the transfer of gases (oxygen and carbon dioxide), nutrients (glucose, vitamins, fatty acids, amino acids), waste products (uric acid and bilirubin), and xenobiotics (small molecule
pharmaceuticals and environmental chemicals). The physicochemical properties of the molecule ultimately determine the method of transfer: simple diffusion, facilitated diffusion, or active transport. Simple diffusion of gases and small (<500 Da), uncharged molecules is driven by a concentration gradient, such that no energy is required for the molecule to cross the lipid bilayer of the plasma membrane. Other molecules also transfer in a concentration gradient manner, but require a saturable facilitated diffusion system to successfully move chemicals across the cell membrane. For example, glucose uses specific transmembrane proteins called glucose transporters (GLUTs) to facilitate the diffusion of glucose across the cell membrane from a high to low concentration gradient. Chemicals that are transported against a concentration gradient utilize primary or secondary active transport for movement in and out of the trophoblast. Secondary active transporters transfer ions (i.e., Na\(^+\) or H\(^+\)) with their concentration gradients across the plasma membrane to produce the energy to transport other molecules against a concentration gradient (i.e., amino acid transporters). The majority of the facilitative transport proteins and secondary active transport proteins belong to the solute carrier (SLC) superfamily of transporters, including many of the drug transporters such as organic cation transporters (OCT), organic anion transporters (OAT), organic anion-transporting polypeptides (OATP), concentrative nucleoside transporters (CNT), equilibrative nucleoside transporters (ENT), multidrug and toxin extrusion protein (MATE), and carnitine transporters (CNT). Drug transporting SLCs are localized to both the apical and basolateral membrane of placental trophoblasts and play an important role in mediating the transplacental disposition of xenobiotics (Reviewed in Staud et al., 2012, *Figure 1.4*).

Primary active transporters in the placenta are efflux transporters that remove chemicals from cells and against concentration gradients with energy harnessed from ATP
hydrolysis. As members of the ATP-binding cassette (ABC) superfamily of transporters, the majority of the efflux transporters in the placenta are expressed on the apical membrane of placental syncytiotrophoblasts to promote the removal of potentially harmful endogenous byproducts (i.e., uric acid and bilirubin) and xenobiotics (pharmaceuticals and environmental chemicals) from the fetal-placental unit (Reviewed in (Staud et al., 2012), Figure 1.5). Multidrug resistance protein 1 (MDR1) or P-glycoprotein (Pgp) and the breast cancer resistance protein (BCRP) are abundantly expressed in the placenta and are the focus of placental pharmacology and toxicology research as the transporters play a critical role in regulating fetal exposure to potentially toxic compounds.

1.2. The Breast Cancer Resistance Protein (BCRP/ABCG2) Transporter

BCRP is an ATP-binding Cassette (ABC) transporter that interacts with a wide variety of chemicals including carcinogens, chemotherapy drugs, endogenous compounds, flavonoids, antibiotics, and diabetes medications that are substrates and/or inhibitors (Table 1.1). Different than other ABC transporters, BCRP is considered to be a “half” transporter as the translation of the ABCG2 transcript results in protein with 6 transmembrane domains and only one nucleotide binding domains as there are usually two. Interestingly, the BCRP protein must dimerize to properly localize and function within the plasma membrane. The transporter was discovered concurrently in three independent laboratories from which three different names were published in 1998. While two of the names referred to the function of the protein as a multidrug resistance transporter (“mitoxantrone resistance transporter” or MXR (Rabindran et al., 1998) and BCRP (Doyle et al., 1998), one name was derived from its abundant expression in human placenta tissue, terming it the “ABC transporter highly expressed in the placenta” or ABCP (Allikmets et al., 1998). The human gene nomenclature committee later
designated the transcript as the second member of the G subfamily of the ABC superfamily of transporters, *ABCG2* (Doyle and Ross, 2003); however, BCRP remains the most commonly used name when referencing the *ABCG2* protein.

Expressed in the epithelium of normal human tissue including the intestines, liver, and kidney (Maliepaard et al., 2001), BCRP plays an important role in the disposition and ultimately the excretion of chemicals that are substrates for BCRP. At blood-organ barriers including the blood-placenta, blood-brain, and blood-testis barriers (Maliepaard et al., 2001), BCRP functions to protect these tissues from chemical accumulation and subsequent toxicity. For example, there is evidence that fetuses of BCRP knockout mice accumulate more of the BCRP substrate and chemotherapeutic drug, topotecan than fetuses of wild-type mice (Jonker et al., 2002). This is particularly important as 81.2% of pregnant women in Europe, North America, South America, and Australia reported using at least one medication (over the counter or prescription) during pregnancy in 2012 (Lupattelli et al., 2014). Taken together with the wide substrate specificity of BCRP (Table 1.1), there is a particular risk of *in utero* exposure to drugs administered during pregnancy when placental BCRP function is compromised.

The focus of this thesis project is to understand mechanisms of BCRP regulation in the placenta. Areas of investigation for this project include genetic variants and dietary chemicals, such as soy isoflavones.

1.2.1. BCRP Genetic Variants

There are a number of single nucleotide polymorphisms (SNPs), or genetic variants that occur in the *ABCG2* gene (chromosome 4q22, *ABCG2*) (Imai et al., 2002a; Backstrom et al., 2003; Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005; Poonkuzhali
et al., 2008). The most well characterized genetic variants are localized to the coding region of the \textit{ABCG2} gene and result in amino acid changes in the subsequent BCRP protein (\textbf{Table 1.2, Figure 1.6}).

One of the most common genetic variants (C421A, rs2231142) has been associated with altered pharmacokinetics and pharmacodynamics of drugs in patients who express one or two variant alleles (\textbf{Table 1.3}). For example, lung cancer patients with one mutant allele (421CA) had a 3.7-fold greater risk of developing diarrhea, a toxic side effect of BCRP substrate gefitinib (Cusatis et al., 2006). Furthermore, healthy volunteers who were homozygous for the variant (421AA) exhibited significantly greater AUC and C\textsubscript{max} values for rosvustatin following a 20 mg oral dose (Keskitalo et al., 2009b). Due to this evidence, the International Transporter Consortium suggested that the polymorphism be considered in drug development and in making regulatory decisions (Giacomini et al., 2013).

While the effect of the C421A variant on BCRP function \textit{in vivo} is clear, the exact mechanism is still unknown. \textit{In vitro} and human tissue studies aimed to tease out this mechanism. Cells and membrane vesicles overexpressing the C421A variant confirmed the reduced function of C421A \textit{in vitro} as transport of various BCRP substrates including estrone-3-sulfate, topotecan, glyburide, and uric acid was compromised compared to that of the wild-type controls (Imai et al., 2002a; Kondo et al., 2004; Pollex et al., 2010; Woodward et al., 2013). While BCRP protein expression is also reduced in most of these model systems, the effect of the variant on the tissue expression has been less clear. The heterozygous variant genotype (421C/A) did not change BCRP protein expression in the intestine or placenta, however there was a significant decrease in BCRP protein expression in placentas of individuals homozygous for the SNP (421A/A) (Zamber et al.,
2003; Kobayashi et al., 2005; Urquhart et al., 2008). Furthermore, lower BCRP protein expression was correlated with the C421A genotype in human livers, however the data were expressed as both the 421CA and 421AA genotypes, making it difficult to differentiate the distinction between the two genotypes. Taken together, there may be differences in BCRP protein expression in cells or tissue based on cell-type and/or tissue.

Potential clinically-relevant genetic variants that occur in the non-coding region of the ABCG2 gene have begun to emerge. One study identified a number of SNPs in the 5′-untranslated region and various intronic regions of the ABCG2 gene and observed correlations with BCRP mRNA expression in intestine, liver, and lymphoblast (polymorphism discovery resource, PDR44) samples (Table 1.4) (Poonkuzhali et al., 2008). Since this report, three of these non-coding ABCG2 SNPs have been associated with altered pharmacodynamics of BCRP substrates. In the first intron, rs2622604 and rs3109823 were found to be associated with the development of severe myelosuppression as a side effect in the treatment of cancer with irinotecan (Cha et al., 2009). Also in the first intronic region, the rs3114020 variant genotype was associated with the altered pharmacokinetics of the epilepsy drug, lamotrigine, as there was a significantly higher blood concentration (/dose normalized by body weight) in subjects who were heterozygous or homozygous for the SNP (Zhou et al., 2015). Importantly, the effect of non-coding variants on BCRP expression and function in the placenta has been unexplored and warrants investigation.
1.2.2. Interaction of Isoflavones with the BCRP/ABCG2 Transporter

1.2.2.2 Introduction

Isoflavones

Isoflavones are secondary metabolites that occur naturally in plants of the *Leguminosae* family and include soybeans, red clover, peanuts, chickpeas, and alfalfa (2008). Isoflavones attract bacteria to the roots to aid in nodulation and nitrogen fixation (Rolfe, 1988). Collectively, isoflavones represent a subset of the larger class of chemicals, flavonoids, which are defined by a polyphenolic three ring structure (A, B, and C) ([Figure 1.7A](#)). The location of the B ring at position C3 rather than C2 separates the isoflavones from the remaining flavonoids ([Figure 1.7B](#)). This review article will focus on the most commonly studied isoflavones: genistein, daidzein, biochanin A, glycitein, and formononetin ([Figure 1.8](#)).

The most abundant source of isoflavones is soybeans, which contain primarily genistein (approximately 2.3 mg/g), daidzein, and glycitein (2008), in addition to trace amounts of formononetin and biochanin A (Burdette and Marcus, 2013). It is important to note that soybeans are used in the production of numerous manufactured dietary products including tofu, tempeh, soy infant formula, miso, soybean oil, cereal, and bacon bits (2008). Red clover is another naturally abundant source of isoflavones containing primarily formononetin, in addition to biochanin A, daidzein, and genistein (Burdette and Marcus, 2013). Within plants, isoflavones are present as two different forms: 1) glycoside ([Figure 1.9](#)) (i.e., genistin or genistein-7-O-β-D-glucoside) and 2) aglycone (i.e., genistein) (reviewed in (Barnes, 2010)). Glycosides occur at greater concentrations than aglycones in soybeans and other plants (Song et al., 1998); however fermented foods (i.e., miso, tempeh) contain a greater proportion of aglycones compared to
unfermented soy due to the hydrolysis of the glycosides to aglycones by microbial β-glucosidases during the fermentation process (reviewed in (Barnes, 2010)).

Following ingestion, isoflavone glycosides are converted to their aglycone form by epithelial and microbial β-glucosidases in the oral cavity (Walle et al., 2005) and small intestines (Day et al., 1998). This is important because it is primarily the aglycone form of isoflavones that pass across the intestinal epithelium (Izumi et al., 2000; Murota et al., 2002; Setchell et al., 2003) and possess biological activity (Akiyama et al., 1987; Morito et al., 2001). Approximately 70-90% of isoflavones are metabolized to glucuronide and sulfate conjugates by UDP-glucuronosyltransferases (UGTs; UGT1A1, 1A8, 1A9, 1A10) and sulfotransferases (SULTs; SULT1A1*2, 1E, 2E1), respectively, in the intestines and liver (Doerge et al., 2000; Pritchett et al., 2008). Conjugation occurs primarily at the C7 and C4' positions on the parent ring system (Figure 1.7B), to form monoconjugates (i.e., genistein-7-glucuronide, G-7-G; genistein-4'-glucuronide, G-4'-G; genistein-7-sulfate, G-7-S; genistein-4'-sulfate, G-4'-S) or diconjugates (i.e., genistein-7-glucuronide-4'-glucuronide; genistein-7-sulfate-4'-sulfate; genistein-7-glucuronide-4'-sulfate; genistein-7-sulfate-4'-glucuronide) (Shelnutt et al., 2002). The phase II metabolites are excreted into the intestinal lumen and bile duct and subsequently eliminated in the feces or deconjugated by gut microflora which results in reabsorption and enterohepatic recirculation of the isoflavones (Sfakianos et al., 1997). Though it is not the primary pathway of isoflavone metabolism, phase I oxidative metabolites of genistein, daidzein (Kulling et al., 2001), and formononetin (Tolleson et al., 2002) have been identified in the urine of healthy volunteers. Confirmed using human liver microsomes, the cytochrome P450 enzymes are responsible for the oxidation of isoflavones in the liver (Kulling et al., 2001; Tolleson et al., 2002). Additional phase I metabolism of these chemicals include oxidative demethylation, particularly for biochanin A and formononetin. Incubation of
biochanin A and formononetin with human liver microsomes resulted in the formation of genistein and daidzein, respectively (Tolleson et al., 2002). Another example of phase I metabolism of isoflavones is the formation of equol from daidzein by intestinal bacteria (Schroder et al., 2013). Equol is more effective in reducing prostate cancer growth \textit{in vitro} than daidzein (Hedlund et al., 2003) and its presence in urine has been associated with a decreased risk of breast cancer (Ingram et al., 1997). Evidence suggests that approximately 30-50\% of the population can produce equol from daidzein (Kelly et al., 1995; Rowland et al., 2000). This interindividual heterogeneity in the conversion of daidzein to equol may be a potential contributor to the inconclusive findings in clinical trials that examine the effects of a soy diet on various disease states.

Genistein is the most frequently studied isoflavone due to its presence in diverse dietary sources and an increasing number of reports that suggest genistein and other isoflavones have biological activity that may modulate disease progression (Wang et al., 2004a; Bitto et al., 2008; Wu et al., 2008; Yan and Spitznagel, 2009) (reviewed in (Banerjee et al., 2008)). Specifically, there is evidence that genistein and other isoflavones possess cancer preventative and/or therapeutic activity, and can improve cardiovascular, bone and post-menopausal health (Strom et al., 1999; Chen et al., 2003a; Hussain et al., 2003; Constantinou et al., 2005; Bitto et al., 2008; Clarkson et al., 2011; Squadrito et al., 2013). Much of this research has been supported by epidemiological findings that demonstrate health benefits conferred to Asian populations that consume a soy rich diet as compared to populations that consume a western diet (Wu et al., 2008; Yan and Spitznagel, 2009). Nevertheless, previous studies also suggest that genistein and other isoflavones may be adverse to human health by promoting cancer progression and/or interfering with reproductive development early in life (Allred et al., 2001; Ju et al., 2001; Newbold et al., 2001; Jefferson et al., 2002).
Additional work is needed to address the controversy as to whether these natural compounds have a negative impact on the health of humans clinically.

Many of the biological responses produced by isoflavones are believed to be mediated by cellular proteins including the estrogen receptor and tyrosine kinase receptors. In fact, due to the early discovery of isoflavones as estrogen receptor agonists (Martin et al., 1978) and modulators of reproductive function (Barrett et al., 1965), these compounds have been termed phytoestrogens. The phenolic ring structure of isoflavones, allow the compounds to bind to estrogen receptors (Martin et al., 1978) and initiate transcription of estrogen receptor-responsive genes. Typically, isoflavones bind with greater affinity to the estrogen receptor β than estrogen receptor α (Kuiper et al., 1998). Moreover, in 1987, Akiyama et al. reported that isoflavones can inhibit tyrosine kinase enzymes in A431 epidermoid cancer cells, including the epidermal growth factor receptor (genistein > prunetin >biochanin A >daidzein=genistin) (Akiyama et al., 1987). Since then, a number of reports have confirmed these findings in vitro as well as in vivo (Wegner et al., 1997; Aptel et al., 1999; Wang et al., 2004a; Sakla et al., 2007; Yan et al., 2010) and suggest that by targeting these molecular pathways often involved in cancer, isoflavones may possibly be combined with chemotherapeutic regimens to improve the efficacy of cancer treatment.

In addition to estrogen and tyrosine kinase receptors, isoflavones also interact with the breast cancer resistance protein (BCRP) efflux transporter, which is involved in the disposition of xenobiotics and endogenous compounds. These compounds are substrates of BCRP as well as inhibitors of its function. Further, many of the isoflavones modulate chemoresistance and in vivo pharmacokinetics of other BCRP substrates that are administered concomitantly.
**BCRP**

BCRP is a member of the ATP-binding cassette (ABC) superfamily of transmembrane transporters. Localized to the apical plasma membrane of cells, BCRP actively transports xenobiotic and endogenous substrates out of the cell with energy derived from the hydrolysis of ATP. In the 1990’s, BCRP was discovered in multiple laboratories using cell lines that conferred resistance to mitoxantrone, and other xenobiotics, and that lacked the previously characterized multidrug resistance proteins, multidrug resistance protein 1 (MDR1) and the multidrug resistance-associated protein 1 (MRP1) (Allikmets et al., 1998; Doyle et al., 1998; Rabindran et al., 1998). The discovery of the new transporter in a number of models including a breast cancer cell line (MCF-7/AdrVp) (Doyle et al., 1998), human placenta (Allikmets et al., 1998), and mitoxantrone-selected human colon carcinoma cells (S1-M1-80) (Rabindran et al., 1998), resulted in the concurrent publication of multiple names for the same transporter: BCRP, “ABC transporter highly expressed in the placenta” (ABCP), and the “mitoxantrone resistance (MXR) transporter”. Subsequently, the human gene nomenclature committee officially designated the transcript as the second member of the G subfamily of the ABC superfamily of transporters, ABCG2 (Doyle and Ross, 2003).

Since its discovery in cancer cells, BCRP has been found to play an important role in mediating the disposition of substrates in normal human tissues thereby preventing chemical accumulation and subsequent toxicity (Maliepaard et al., 2001). BCRP is expressed in the epithelium of tissues such as the intestines, liver, and kidney, which are involved in the pharmacokinetics of chemicals. Localization of BCRP to the apical or luminal surface favors active transport of substrates into the intestinal lumen, bile duct, and proximal tubule lumen, respectively (Maliepaard et al., 2001). In addition to playing a role in excretion, BCRP aids in the formation of blood-organ barriers as it is expressed in
syncytiotrophoblasts of the placenta, capillary endothelial cells of the blood-brain barrier, and luminal capillary endothelial cells of the blood-testis barrier (Maliepaard et al., 2001; Cooray et al., 2002; Bart et al., 2004). BCRP mRNA and protein are expressed most abundantly in the human placenta, earning its name as “the placental transporter” (Allikmets et al., 1998).

*In vitro, in vivo, and in situ* model systems have been employed to detect BCRP substrates and inhibitors including several drugs, dietary components, and endogenous compounds (*Table 1.1*). While previous review articles have summarized the interactions of flavonoids with ABC transporters (Morris and Zhang, 2006; Alvarez et al., 2010), this review article provides an up-to-date summary of the evidence for the interaction of isoflavones specifically with BCRP. This includes a discussion of isoflavones as BCRP substrates (*Table 1.5-1.7*), inhibitors of BCRP transport activity (*Table 1.8*), and regulators of BCRP expression (*Table 1.9*) with commentary on the implications of these interactions.

1.2.2.3 Evidence for Isoflavones as Substrates of BCRP

*In Vitro*

*Transport of Genistein.* Genistein was first described as a substrate for BCRP-mediated transport in 2004 (*Table 1.5*). Imai et al. demonstrated a significant decrease in the accumulation of $^3$H-genistein in human K562 myelogenous leukemia cells that overexpressed the human BCRP/ABCG2 gene (K562/BCRP cells) compared to the parent cells that lacked BCRP (K562 cells) (Imai et al., 2004). In addition, the transcellular transport of genistein was assessed using pig kidney epithelial cells (LLC-PK1 cells) that overexpressed the human BCRP/ABCG2 gene (LLC/BCRP cells). When
grown in monolayers, BCRP protein localizes to the apical membrane of LLC-PK1 cells (Perantoni and Berman, 1979; Imai et al., 2003). For these experiments, cells are grown on a thin filter in between two chambers that recapitulate the polarization of cells (apical and basolateral) typically seen in vivo. These studies revealed that genistein is selectively transported by BCRP in the basolateral-to-apical (BL-to-AP) direction as compared to the apical-to-basolateral (AP-to-BL) direction (Imai et al., 2004). The preference for BL-to-AP transport was abolished by the BCRP specific inhibitor, fumitremorgin C (FTC, 3 µM), further confirming a critical role for BCRP in the transcellular transfer of genistein (Imai et al., 2004). Since this initial report, a number of studies have characterized the bidirectional transport of genistein in other BCRP-overexpressing (i.e., Madin-Darby canine kidney II; MDCK/BCRP) and endogenously expressing (i.e., Caco-2) cell lines (Enokizono et al., 2007a; Mease et al., 2012; Kobayashi et al., 2013). Similar to the LLC/BCRP cells, the BL-to-AP transport of genistein (as quantified by LC/MS) was favored in the MDCK/BCRP cells with a efflux ratio that was 2-fold higher in the overexpressing cells as compared to the empty vector cells that lacked BCRP protein (Enokizono et al., 2007a). In such bidirectional permeability assays, an efflux ratio ≥ 2 indicates active efflux in the BL-to-AP direction as the value is calculated by dividing the apparent permeability ($P_{app}$) of a compound in the BL-to-AP direction by the $P_{app}$ of the compound in the AP-to-BL direction ($P_{app\ BL-AP}/P_{app\ AP-BL}$) (Polli et al., 2001; Mease et al., 2012). The human colorectal adenocarcinoma cell line, Caco-2 is an in vitro model of intestinal transport. In culture, Caco-2 cells form monolayers and the BCRP protein localizes to the apical, or microvillus membrane, similar to its known trafficking in tissues (Chantret et al., 1988; Xia et al., 2005; Englund et al., 2006). In Caco-2 monolayers, the BCRP specific inhibitor, FTC (1 µM) decreased the efflux ratio of genistein by 3-fold, confirming that genistein is actively transported by BCRP in the BL-to-AP direction (Mease et al., 2012). BCRP-mediated transport of
genistein in Caco-2 cells was confirmed in another study which demonstrated that the BCRP inhibitor, estrone-3-sulfate increased the permeability of genistein by 40% in these cells (Kobayashi et al., 2013).

Additional studies indicate that genistein is also transported by the mouse isoform of the Bcrp protein overexpressed in MDCK/Bcrp cells as the efflux ratio was approximately 2-fold higher than the cells harboring the control expression vector in two studies (Enokizono et al., 2007a; Kodaira et al., 2011). The similar ability of the mouse and human Bcrp/BCRP proteins to transport genistein in vitro, suggests that the mouse is an appropriate model for studying the in vivo transport of genistein by Bcrp/BCRP. This is in line with other compounds, such as cimetidine and aflatoxin B₁, which are transported by both the human and mouse BCRP/Bcrp orthologs (Pavek et al., 2005; van Herwaarden et al., 2006).

Transport of Daidzein. Similar to genistein, daidzein was first suggested to be a substrate of BCRP in 2004 using the ATPase assay (Cooray et al., 2004). Briefly, the ATPase assay uses plasma membranes isolated from cells or tissues that express BCRP and detects the ability of a compound to stimulate ATP hydrolysis as an indirect measure of substrate transport. In membranes isolated from the bacteria *L. Lactis* that overexpressed the human BCRP gene, daidzein (3-50 μM) stimulated the baseline ATP-hydrolysis relative to control membranes. Direct transport of daidzein by BCRP was verified in monolayers of MDCK/BCRP and Caco-2 cells (Enokizono et al., 2007a; Kobayashi et al., 2013). In the MDCK/BCRP cells, the efflux ratio was 3-fold greater than the empty vector cells (Enokizono et al., 2007a) while the BCRP inhibitor, estrone-3-sulfate increased the relative permeation (apical-to-basolateral transport) of daidzein by 80% compared to vehicle-treated control Caco-2 cells (Kobayashi et al., 2013). In
addition to interacting with human BCRP, daidzein was also transported in MDCK cells by mouse Bcrp (MDCK/Bcrp), which was evidenced by an approximately 2-fold greater efflux ratio than the empty vector cells in two separate studies (Enokizono et al., 2007a; Kodaira et al., 2011).

Transport of Isoflavone Conjugates. Approximately 70-90% of an orally administered dose of isoflavones undergo phase II conjugation by glucuronidation and sulfation in the intestines and liver (Doerge et al., 2000; Zhang et al., 2003b; Pritchett et al., 2008). Phase-II conjugates of genistein are also transported by BCRP in Caco-2 cells as demonstrated by the reduced BL-to-AP transport of G-7-G, G-4’-G, G-7-S, and G-4’-S in the presence of the BCRP inhibitor Ko143 (5 µM) (Yang et al., 2012). To better understand the transport of genistein-phase II metabolites by BCRP, human cervical carcinoma HeLa cells that endogenously expressed BCRP were genetically-engineered to overexpress the glucuronidation enzyme, UDP-glucuronosyltransferase (UGT) 1A9 (Jiang et al., 2012). Following a 2 h incubation with genistein (10 µM) and Ko143 (5 and 10 µM), these cells demonstrated reduced elimination of genistein-glucuronide and increased intracellular accumulation of the phase-II metabolite suggesting that genistein-glucuronide is transported by BCRP in the genetically-engineered HeLa cells (Jiang et al., 2012). Despite having a moderate and statistically significant effect on the overall glucuronidation rate in these cells, the authors still suggested that Ko143 did not reduce BCRP transport of genistein-glucuronide by decreasing the in situ formation of genistein-glucuronide (Jiang et al., 2012). To more thoroughly interpret these results, future experiments should address the ability of Ko143 to inhibit UGT function.

In addition to cells that overexpress the BCRP gene, there are alternative in vitro assays for identifying BCRP substrates that focus on plasma membrane transport. One common
model are membrane vesicles isolated from *Spodoptera frugiperda* (Sf9) insect cells that overexpress human BCRP and are configured in an inverted (or inside-out) orientation. Because of this orientation, one can measure intravesicular concentrations of BCRP substrates directly in the presence of ATP. Using Sf9-BCRP vesicles, van de Wetering and Sapthu (2012) demonstrated a high rate of ATP-dependent transport of genistein-sulfate and daidzein-sulfate collected from the urine of Bcrp -/- mice (FVB) (van de Wetering and Sapthu, 2012). The mice were not dosed with the isoflavones *per se*, but rather consumed a normal rodent diet (AM-II) which contained isoflavones at low levels. This experimental approach allowed for the *in vivo* generation of isoflavone metabolites at physiologically relevant concentrations. BCRP-dependent transport of six isoflavone secondary metabolites (genistein-sulfate I, genistein-sulfate II, daidzein-4'-sulfate, daidzein-7-sulfate, glycine-sulfate, and formononetin-sulfate) was confirmed using the inverted membrane vesicle system and 35S-labeled isoflavone metabolites (van de Wetering and Sapthu, 2012). These studies suggested that BCRP is particularly important in the transport of sulfated isoflavone conjugates.

An and Morris (2011) demonstrated that biochanin A-sulfate, not biochanin A, was selectively transported in the BL-to-AP direction in MDCK/Bcrp cells as compared to the AP-to-BL direction (An and Morris, 2011). The BCRP specific inhibitor, FTC did not increase the accumulation of biochanin A in MCF-7/MX100 cells overexpressing BCRP, further confirming that the aglycone, biochanin A was not a substrate for BCRP-mediated transport (An and Morris, 2011). In this same experiment, the contribution of human BCRP to the transport of biochanin A phase II metabolites could not be identified as low concentrations of phase II metabolites were detected intracellularly (An and Morris, 2011). The authors suspect this was due to the low expression of phase II metabolizing enzymes in the MCF-7 cells (An and Morris, 2011). Moreover, following the
addition of biochanin A to the apical and basolateral chambers, genistein was detected in the medium of MDCK/BCRP as a phase I metabolite (oxidative demethylation) of biochanin A (An and Morris, 2011). Similar to studies that added genistein to the medium, An and Morris (2011) confirmed that genistein is transported to a greater extent in the BL-to-AP direction compared to the empty vector cells. Another study found that after adding biochanin A to the apical chamber of a Caco-2 polarized monolayer, the AP-to-BL and BL-to-AP transport of biochanin A-glucuronide was reduced by the BCRP inhibitor dipyridamole (10 uM) (Wang et al., 2008b). The authors suggested that the abolished BL-to-AP transport is a result of reduced BCRP activity by dipyridamole (Wang et al., 2008b), however the authors acknowledged that dipyridamole is also an inhibitor of MDR1 function (Vaidya et al., 2009). Because MDR1 also localizes to the apical membrane of Caco-2 cells (Belliard et al., 2004), this transporter cannot be ruled out as playing a role in biochanin A-glucuronide transport. Furthermore, dipyridamole is an inhibitor of MRP1 that is localized to the basolateral membrane of Caco-2 cells (Prime-Chapman et al., 2004). This indicates that inhibition of MRP1 transport may be responsible for the reduced AP-to-BL transport of biochanin A-glucuronide in Caco-2 cells. Interestingly, formononetin-glucuronide transport was not altered with the addition of dipyridamole in a similar experiment (Wang et al., 2008b).

**In Vivo**

*Bioavailability of Individual Isoflavones.* Pharmacokinetic studies using Bcrp knockout (Bcrp -/-) mice have been conducted to assess the in vivo contribution of Bcrp to the disposition of various compounds including isoflavones and isoflavone metabolites. A number of studies have emphasized the ability of intestinal and hepatic Bcrp to transport isoflavones because the relevant route of exposure in the human population is oral ingestion. In two studies, oral (p.o.) administration of genistein (Enokizono et al., 2007a;
Yang et al., 2012) or daidzein (Enokizono et al., 2007a) resulted in significantly greater plasma concentrations of the respective isoflavone over time (or area-under-the-curve AUC) in Bcrp -/- mice on a FVB background strain as compared to wild-type mice. These data suggested that Bcrp was responsible for limiting the oral bioavailability of genistein and daidzein (Enokizono et al., 2007a; Yang et al., 2012). In a similar study performed by Alvarez et al. (50 mg/kg, 3 h) (Alvarez et al., 2011), the plasma AUC values for the aglycones, genistein and daidzein, alone in the Bcrp-/ mice were elevated by 1.5- and 2-fold, respectively, which was not significantly greater than the wild-type values, unlike the other two studies which noted statistical significance (Enokizono et al., 2007a; Yang et al., 2012). Alvarez et al. reported that the plasma AUC values for total genistein or daidzein (aglycone and metabolites) were significantly greater (up to 9-times) in Bcrp -/- mice compared to wild-type mice and proposed that the difference was due primarily to the increased concentration of phase II metabolites in the plasma of Bcrp -/- mice (Alvarez et al., 2011). For example, the plasma daidzein-sulfate concentration was significantly increased 6-fold in the Bcrp -/- mice compared to wild-type mice (Alvarez et al., 2011). Also, daidzein-glucuronide was detected in Bcrp -/- mice while the metabolite was undetectable in wild-types (Alvarez et al., 2011). In two different studies, systemic genistein-sulfate concentrations were elevated up to 26- and 7-fold in Bcrp -/- mice administered 20 mg/kg genistein (Yang et al., 2012) and 50 mg/kg genistein (Alvarez et al., 2011), respectively, as compared to wild-type mice. In the same studies, genistein-glucuronide concentrations were elevated up to 16- and 8-fold in Bcrp -/- mice following administration of 20 mg/kg (Yang et al., 2012) and 50 mg/kg genistein (Alvarez et al., 2011), respectively. This evidence is in line with an intestinal in situ perfusion study performed by Zhu et al. which demonstrated that the excretion of genistein-sulfate and genistein-glucuronide was significantly decreased in the small intestines of Bcrp -/- mice
compared to wild-type mice, suggesting that genistein conjugates are substrates for Bcrp-mediated transport (Zhu et al., 2010).

The exact mechanism by which isoflavones and their phase II metabolites occur at high concentrations in the plasma of Bcrp -/- mice is a topic of great controversy (Alvarez et al., 2012). Alvarez et al. offered an explanation for the increased plasma concentration of only the phase II metabolites by suggesting that decreased excretion of the aglycones due to the lack of intestinal Bcrp in Bcrp -/- mice increased exposure of the isoflavones to phase II metabolizing enzymes in the intestine and subsequently enhanced plasma concentrations of isoflavone metabolites (Alvarez et al., 2011). Enokizono et al. suggested that because glucuronidation is the primary metabolic pathway contributing to the elimination of genistein (Doerge et al., 2000; Zhang et al., 2003b), and there is no difference in the intestinal rate of glucuronidation between wild-type and Bcrp-/- mice (Enokizono et al., 2007b), that increased intestinal absorption of genistein is the primary cause of elevated plasma genistein concentrations (rather than altered glucuronide formation) in Bcrp-/- mice (Enokizono et al., 2007a). Yang et al. countered this assertion and demonstrated that there was no difference in the intestinal absorption of genistein using in situ perfusion of wild-type and Bcrp-/- mouse intestines and human Caco-2 cells in the presence and absence of Bcrp specific inhibitor, Ko143 (Yang et al., 2012). Instead, Yang et al. suggested that in Bcrp -/- mice, genistein conjugates were increasingly excreted into the blood rather than into the intestinal lumen and bile duct (due to loss of Bcrp function) and that any elevated genistein concentration in the plasma was likely due to the hydrolysis of genistein-glucuronides conjugates back to the aglycone form, in the blood (Yang et al., 2012). The multidrug resistance-associated protein 3 (MRP3) is expressed on the basolateral membrane of the rat intestine (Rost et al., 2002) and Yang et al. suggested that this transporter may be a candidate for the
active transport of genistein conjugates into the portal blood (van de Wetering et al., 2009), providing a possible mechanistic explanation for increased plasma concentrations of isoflavone phase II metabolites in Bcrp -/- mice (Yang et al., 2012). It is important to note that a number of experimental methods varied between studies and may have contributed to the differences in the isoflavone (aglycone and metabolite) plasma profiles; these included the time period (i.e., 3 h vs 4 h vs 24 h) and/or isoflavone dose (i.e., 50 mg/kg vs 8.1 mg/kg vs 20 mg/kg).

In another study, Bcrp-/- knockout mice consumed a normal chow diet containing trace amounts of isoflavones, and exhibited significantly greater concentrations of daidzein-sulfate and genistein-sulfate in the plasma of male and female mice, respectively, compared to the wild-types (van de Wetering and Sapthu, 2012). A study of this nature suggests that BCRP/Bcrp may play a role in the disposition of isoflavones at a wide range of concentrations, however these findings must be interpreted with caution as the exact dose of isoflavone that each mouse received was unknown.

Bioavailability of Isoflavone Mixtures. To recapitulate a more relevant dietary exposure to isoflavones mixtures, Alvarez et al. also examined the plasma profiles of the individual compounds following the intragastric co-administration of genistein (25 mg/kg) and daidzein (25 mg/kg) (Alvarez et al., 2011). Interestingly, the findings of this experiment were similar to the results of studies in which mice were treated with individual isoflavones (Alvarez et al., 2011). Notably, the AUC values for both aglycones, genistein and daidzein, were increased 1.6- and 2-fold in the Bcrp-/- mice but still not significantly different from wild-type mice (Alvarez et al., 2011). Further, the total isoflavones and phase II metabolites were significantly greater in Bcrp-/- than wild-type mice (Alvarez et al., 2011). In the animals dosed with a mixture of isoflavones, the phase II metabolite
concentrations were approximately equal to the concentrations of the same metabolites in animals treated with individual isoflavones (Alvarez et al., 2011).

*Intestinal Metabolism and Transport of Isoflavones.* To better understand the role of intestinal Bcrp in the disposition of isoflavones, Enokizono et al. (Enokizono et al., 2007a) and Yang et al. (Yang et al., 2012) examined plasma profiles of isoflavones following alternate routes of exposure. Following a continuous intravenous (i.v.) infusion of genistein (i.v., 1.4 mg/kg/h, 2h) in the right jugular vein, or an intraperitoneal (i.p.) administration (20 mg/kg, 24 h), there was no difference in the plasma concentration of genistein between the Bcrp−/− and wild-type mice suggesting that Bcrp in the intestine plays a primary role in altering plasma genistein concentrations (Enokizono et al., 2007a; Yang et al., 2012). The plasma profiles of the genistein secondary metabolites in the i.p. dosing study were similar to the oral dosing studies in that the AUC values of G-7-G, G-4′-S, and G-7-S were significantly greater in Bcrp−/− mice compared to wild-type mice (Yang et al., 2012). Interestingly, in the study performed by Enokizono et al., i.v. administration of daidzein (1.4 mg/kg/h, 2 h) resulted in a significantly increased plasma concentration of the aglycone in the Bcrp−/− mice as compared to the wild-type mice, but to a lesser extent than the p.o. dose (Enokizono et al., 2007a). The authors note that the increased plasma concentration of daidzein following i.v. infusion was unexpected, but suggest that “impaired urinary excretion” of daidzein may be a contributing factor since about 10% of daidzein is eliminated via the urine in rats administered an oral dose of daidzein (100 mg/kg) (Bayer et al., 2001; Enokizono et al., 2007a).

*Biliary Transport of Isoflavones and Conjugates.* In the study performed by Yang et al., genistein-sulfate (G-7-S and G-4′-S) bile levels were significantly reduced by 93% compared to wild-type mice following a 2.5 h *in situ* intestinal perfusion experiment
(Yang et al., 2012). Interestingly, there were no changes in the parent or genistein-glucuronide concentrations between genotypes suggesting that hepatic Bcrp selectively transports genistein-sulfate metabolites. Another study examined the role of Bcrp in the hepatic excretion of daidzein-sulfate in Bcrp-/- mice fed a normal rodent diet with trace amounts of isoflavones (van de Wetering and Sapthu, 2012). Daidzein-sulfate was detected in the bile of male and female Bcrp-/- mice at significantly lower concentrations (~60%) than the wild-types.

**Distribution of Isoflavones.** The transport of isoflavones by Bcrp in other organs has also been investigated in mice. Tissues with low basal expression of Bcrp, such as the stomach, have preferential isoflavone accumulation further supporting the contention that Bcrp limits isoflavone accumulation in an organ-specific manner (Enokizono et al., 2007a; Zhou et al., 2008b; Kodaira et al., 2011). In wild-type mice, the Bcrp protein is localized to the capillary endothelial cells of the blood brain barrier (Cisternino et al., 2004), endothelial cells within the testis (Enokizono et al., 2007a; Dankers et al., 2012), and the epithelial (head) and endothelial cells (body) of the epididymis. Two studies examined the brain concentrations of genistein and daidzein following a 120 minute i.v. infusion of genistein or daidzein into the right jugular vein of adult FVB wild-type and Bcrp -/- mice (Enokizono et al., 2007a; Kodaira et al., 2011). Both studies found that the brain concentrations of the two isoflavones were increased significantly in Bcrp-/- mice compared to wild-type mice, demonstrating that Bcrp limits the distribution of genistein and daidzein to the brain. In the same study by Enokizono et al. (Enokizono et al., 2007a), testis concentrations of genistein and daidzein were significantly increased by 200% and 400%, respectively, in Bcrp-/- mice compared to wild-type mice suggesting that Bcrp limits accumulation of isoflavones in this reproductive organ. Further, epididymal concentrations of genistein were increased 150% in Bcrp-/- mice compared
to wild-type mice. No difference was observed in ovarian concentrations of genistein between Bcrp-/- and wild-type mice (Enokizono et al., 2007a) even though Bcrp mRNA is detectable in mouse ovaries (Tanaka et al., 2005; Dankers et al., 2012). Bcrp protein, however is not consistently expressed throughout the ovary but when present, it is localized to the small capillaries of the ovary endothelium (Dankers et al., 2012). Dankers et al. suggested that the varying protein expression throughout the organ may be a result of “the dynamic environment of the estrous cycle, where there is a constant regeneration and degradation of capillaries” (Dankers et al., 2012). Constantly changing protein expression may result in unclear findings in the tissue distribution of Bcrp substrates. Future studies should control for rodent estrous cycle stage to examine the accumulation of isoflavones in the ovary.

In the placenta, the BCRP protein localizes to the apical membrane of syncytiotrophoblasts which are in direct contact with the maternal circulation (Maliepaard et al., 2001). Transporter function and localization in this important organ of pregnancy suggest that BCRP is responsible for protecting the fetus from exposure to harmful chemicals that may be present in the maternal circulation. The mouse orthologue, Bcrp, in the placenta limited fetal exposure to Bcrp substrates including topotecan (Jonker et al., 2002) and is therefore a useful model for studying the in vivo function of placental Bcrp/BCRP. To evaluate the role of placental Bcrp in the in utero distribution of genistein, pregnant FVB wild-type and Bcrp-/- mice were infused via the right jugular vein with genistein (1.25 mM) for 120 min and there was no difference in the plasma concentration between the wild-type and Bcrp-/- dams (Enokizono et al., 2007a). Interestingly, Bcrp -/- fetuses accumulated significantly more (1.6-fold) genistein than the wild-type fetuses (Enokizono et al., 2007a). Further, significantly greater concentrations of genistein were detected in the brains of the Bcrp-/- fetuses (Enokizono et al., 2007a).
than the wild-type fetal brains, demonstrating the importance of Bcrp in protecting against genistein penetration of the developing blood-brain barrier. Of note, there is evidence that isoflavones such as genistein alter the epigenome of developing mice (Dolinoy et al., 2006; Vanhees et al., 2011). Taken together, future research should focus on risk factors that may reduce BCRP activity in the placenta and impair the development of offspring following in utero exposure to isoflavones.

1.2.2.4 Evidence for Isoflavones as Inhibitors of BCRP Function

Recent research has begun to explore the ability of isoflavones to alter the pharmacokinetics or pharmacodynamics of chemicals that are transported by BCRP/Bcrp. This may be beneficial in situations where isoflavones enhance the efficacy of a particular treatment, such as a chemotherapeutic drug. However, negative outcomes such as an increase in the toxicity or a reduction in the efficacy of the drug may also occur as a result of BCRP inhibition by isoflavones. To date, a variety of in vitro and in vivo experimental models have been used to detect isoflavone-inhibitors of BCRP/Bcrp function and to better understand the potential implications of concomitantly consuming isoflavones (i.e., a soy rich diet) with drugs that are transported by BCRP.

*Altered Chemoresistance.* Many chemotherapy drugs, including mitoxantrone, SN-38 (the active metabolite of irinotecan), topotecan, and methotrexate are substrates of BCRP (Table 1.1). These drugs are routinely used to screen for potential inhibitors of BCRP function indirectly by assessing the cytotoxic effect of the chemotherapy drug on cell growth in the presence of the potential inhibitor. In the presence of BCRP inhibitors, cells that express the BCRP protein cannot effectively remove the cytotoxic chemical which leads to a potentiation of the chemotherapeutic response. Human breast cancer and lung cancer cells expressing BCRP, MCF-7/MX100 and NCI-H460/MX20,
respectively, are commonly used as models for measuring BCRP activity. MCF-7 and NCI-H460 cells that express BCRP at high levels are selected for using 100 nM or 20 nM mitoxantrone, respectively, prior to experiments (Zhang et al., 2004b). Forty-eight hour exposure with genistein (10-50 μM) or biochanin A (5-50 μM) reversed mitoxantrone resistance in MCF-7/MX100 cells in a concentration-dependent manner using the sulforhodamine B assay to assess cell density (Zhang et al., 2004a; Zhang et al., 2004b). Furthermore, genistein inhibited the cell growth of human leukemia cells that overexpressed the BCRP transporter (K562/BCRP) in the presence of SN-38 compared to cells cultured without isoflavones (Imai et al., 2004; Katayama et al., 2007). Daidzein inhibited the cell growth of K562/BCRP cells in the presence of SN-38 but to a lesser extent than genistein (Katayama et al., 2007). Genistein (10 μM) alone did not reduce the cell viability of MCF-7 breast cancer cells following 72 h incubation (Pick et al., 2011), whereas 48 h incubation of K562 leukemia cells with genistein (10-100 μM) modestly reduced cell viability by about 15% (Zhang et al., 2012). The inconsistency of these findings may arise from innate differences in the protective mechanisms of each cell type. For example, moderate levels of ABC transporters (MDR1 and BCRP) (Pick et al., 2011) are expressed in the MCF-7 cells and may participate in the removal of genistein or other toxic metabolites thereby protecting the cells from potential toxic effects of the compounds. Additionally, two different methods were used to detect cell viability. Zhang et al. utilized a more sensitive and direct measure of cell viability, trypan blue, which quantified the number of viable cells (Zhang et al., 2012), while Pick et al. drew conclusions based on the ATP assay which indirectly indicates the amount of ATP generated which was positively correlated with cell viability (Pick et al., 2011).

**Probe Substrates.** Well-characterized and easily detected BCRP substrates (i.e., fluorescent and radiolabeled compounds) are commonly used as probe substrates for
detecting inhibitors of BCRP-mediated transport. Using whole cell model systems, BCRP function is assessed by determining the intracellular accumulation of a probe substrate in the absence and presence of an inhibitor. Membrane-based assays including inverted plasma membrane vesicles and ATPase assays are isolated subcellular systems that are used to better characterize transporter kinetics and stimulation of transporter ATPase activity by a substrate in the presence of an inhibitor.

*Cell-Based Assays.* In addition to being a chemotherapy drug, mitoxantrone also has fluorescent properties and intracellular accumulation of the drug can be quantified by flow cytometry. A 30-minute co-incubation of mitoxantrone with genistein, daidzein, or biochanin A in MCF-7/MX100 and NCI-H460/MX20 cells, significantly increased the accumulation of mitoxantrone up to 4-fold compared to the control cells treated with mitoxantrone alone (Zhang et al., 2004a; Zhang et al., 2004b). In addition, intracellular topotecan fluorescence increased in K562/BCRP cells in the presence of genistein (30 and 100 μM) relative to the parent K562 cells (Imai et al., 2004). Pick et al. described genistein as an enhancer of fluorescent Hoechst 33342 accumulation in MDCK/BCRP and MCF-7/MX cells with IC$_{50}$ values for BCRP efflux of 6.9 μM and 8.8 μM, respectively, in each cell type (Pick et al., 2011). Daidzein significantly increased the accumulation of mitoxantrone in MCF-7/MX cells and K562/BCRP cells up to 3-fold which was similar to the fluorescence intensity detected in the respective parental cells (Cooray et al., 2004). In the same cell types, daidzein increased the accumulation of the fluorescent derivative of the BCRP substrate and blood pressure medication, prazosin (BODIPY-prazosin) to levels comparable to that in the parental cell line (Cooray et al., 2004).
Similar to their interaction with human BCRP, the mouse Bcrp protein is also sensitive to isoflavone-mediated inhibition. Biochanin A (2.5-25 µM) significantly increased the intracellular fluorescence of mitoxantrone up to 4 and 2.5-fold in MDCK cells that expressed mouse or human Bcrp/BCRP, respectively (An and Morris, 2010). In addition, the BL-to-AP transport of mitoxantrone was attenuated by biochanin A (5 µM) in MDCK/Bcrp cells (An and Morris, 2010). Together, genistein and daidzein (100 µM each) reduced the BL-to-AP transport of BCRP substrates, nitrofurantoin and danofloxacin, in MDCK/BCRP and MDCK/Bcrp cells (Merino et al., 2010; Perez et al., 2013), respectively. Even further, a combination of multiple flavonoids including biochanin A and genistein (apigenin, biochanin A, chrysos, genistein kaempferol, 1 µM total flavonoid) increased the accumulation of mitoxantrone in MCF-7/MX100 cells to a greater extent (EC$_{50}$: 0.23 ± 0.08 µM) than each individual flavonoid (i.e., genistein EC$_{50}$: 14.9 ± 2.69 µM). These data suggest that low concentrations of various isoflavones or flavonoids may have an additive or synergistic effect on the inhibition of BCRP transport (Zhang et al., 2004a).

**Membrane-Based Assays.** Inhibitors of BCRP function can be detected using BCRP inverted plasma membrane vesicles. In this assay, inhibitors are recognized by a significant decrease in the vesicular accumulation of a BCRP probe substrate compared to vesicles without the inhibitor. In BCRP vesicles, there was a trend for aglycones (genistein> biochanin A> glycine> formononetin> daidzein) to reduce the ATP-dependent transport of the BCRP substrate, $^3$H-methotrexate (100 µM) to a greater extent than their respective glucosides (biochanin A-glucoside, sissotrin> genistin> formononetin-glucoside, ononin> glycitin>daidzin) as detected by liquid scintillation counting (Tamaki et al., 2010). In general, because the glucosides are quickly converted to the aglycone forms in vivo, the former finding holds more bearing on the potential for
BCRP inhibition *in vivo*. Importantly, the concentration of genistein that inhibits BCRP function in inverted vesicles, is within the range of plasma concentrations of genistein (1-3 μM, n=12 healthy, human volunteers) that may be found circulating following dietary consumption of soy products (Gardner et al., 2009). Interestingly, soybean extract (1 mg/ml) reduced the uptake of $^3$H-methotrexate in BCRP-overexpressing vesicles to the same extent as BCRP inhibitor, FTC (5 µM) suggesting that the multiple compounds present in a soy diet, including genistein, daidzein, glycitein and their glucosides, collectively reduce BCRP function (Tamaki et al., 2010).

Another plasma membrane-based method used to detect functional inhibitors of BCRP-mediated transport is the ATPase assay. ATP hydrolysis is measured as an indirect indicator of substrate transport and is reduced in the presence of a BCRP-specific functional inhibitor (Glavinas et al., 2007). In the mammary gland, BCRP is expressed on the apical membrane of alveolar epithelial cells and actively concentrates substrates in the milk of lactating animals (mouse and cow) and humans (Jonker et al., 2005). In plasma membranes isolated from the mammary gland of a lactating cow, genistein (10 μM) reduced the Bcrp ATPase activity stimulated by mitoxantrone (Pulido et al., 2006), suggesting that genistein may alter the transport of drugs into the milk of lactating animals and potentially humans.

*Altered Pharmacokinetics of Other Drugs.* The ability of dietary constituents, including isoflavones, to alter the *in vivo* pharmacokinetics and pharmacodynamics of a drug is important to consider since their sale and consumption are unregulated. This is especially of concern when a drug that is a BCRP substrate is administered during lactation. Inhibition of chemical transfer into the milk may provide benefits or deficits to the offspring resulting in protection of the infant from the toxic effects of the transferred...
drug, or reduced transfer of antiviral medications intended to limit the infant’s exposure to HIV, respectively. Enrofloxacin is an antibacterial drug that has been used in veterinary medicine to treat gram-negative and gram-positive bacterial infections in ruminants such as cows and sheep. Its metabolite, ciprofloxacin, is also efficacious in vivo and is used in humans for the treatment of bacterial infections. Importantly, ciprofloxacin is recognized by the American Academy of Pediatrics as a maternal medication usually compatible with breastfeeding (2001). Both the parent enrofloxacin compound and its metabolite ciprofloxacin are recognized substrates of BCRP (Pulido et al., 2006; Haslam et al., 2011). To understand the effect of genistein on the milk concentrations of concomitantly administered enrofloxacin in sheep, 12 lactating sheep were infused with enrofloxacin either with or without genistein via the left jugular vein (Pulido et al., 2006). There was no change in the plasma concentration of enrofloxacin (area under the curve, AUC or maximal concentration, C_{max}) between the sheep that received genistein and those that did not. However, genistein decreased the enrofloxacin milk content which was demonstrated by a 1.5-fold reduction in the enrofloxacin milk AUC value (Pulido et al., 2006). These data suggest that in dairy animals, genistein may reduce milk concentrations of enrofloxacin and shorten the time in which the animals are withdrawn from producing usable milk. Further, in humans genistein may protect the developing infant from unnecessary exposure to ciprofloxacin.

Because genistein and daidzein are often found together in nature, Perez et al. (Perez et al., 2009; Perez et al., 2013) examined the effect of a combined isoflavone exposure on the distribution of BCRP substrates danofloxacin and nitrofurantoin into the milk of lactating sheep. Danofloxacin is an antibiotic used in veterinary medicine to treat a broad range of bacterial infections. Nitrofurantoin is an antibiotic that is often prescribed for the treatment of urinary tract infections in humans and animals. In particular, nitrofurantoin is
prescribed to pregnant (2013) and lactating women as it is recognized by the American Academy of Pediatrics as a maternal medication typically compatible with breast feeding (2001). The compounds concentrate in the milk of lactating animals (danofloxacin and nitrofurantoin) (Merino et al., 2005; Escudero et al., 2007) and/or women (nitrofurantoin) (Gerk et al., 2001). In the nitrofurantoin experiment, sheep exposed to genistein and daidzein via the standard diet or exogenous dosing with the isoflavones (10 mg/kg genistein + 10 mg/kg daidzein) by oral gavage, experienced a significant decrease in the milk AUC of nitrofurantoin (Perez et al., 2009). Additionally, the nitrofurantoin-treated control animals experienced higher plasma AUC and $C_{\text{max}}$ nitrofurantoin as compared to those that were exposed to isoflavones (Perez et al., 2009). Because this is not in line with the Bcrp-inhibition paradigm, the authors suggest that the isoflavone treated sheep may have experienced a reduced plasma concentration of nitrofurantoin due to decreased absorption or enhanced elimination of nitrofurantoin. Unfortunately, they do not offer mechanistic evidence to explain the reduced systemic exposure to the drug. In the danofloxacin experiment, lactating sheep received a 15-day soy supplemented diet that significantly reduced the milk AUC and $C_{\text{max}}$ of danofloxacin compared to the sheep that received regular forage without isoflavones (Perez et al., 2013). Interestingly, the sheep that received an oral dose of genistein and daidzein (10 mg/kg genistein + 10 mg/kg daidzein) did not demonstrate a similar decrease in milk concentrations of danofloxacin suggesting that the form in which the compounds are administered (i.e., liquid vs. solid, aglycone vs. glycone) or presence of other compounds in the diet may determine its effect on the pharmacokinetics of other chemicals (Perez et al., 2013). The authors list the various constituents of the soy diet but did not mention the amount of genistein, daidzein, or other isoflavones present in each diet (Perez et al., 2013).
Bcrp/- mice can be used to elucidate the functional role of BCRP/Bcrp in mediating potential nutrient-drug interactions in an in vivo situation. In the absence of isoflavones, nitrofurantoin was confirmed as a BCRP substrate due to increased plasma AUC of nitrofurantoin in Bcrp/- mice compared to wild-type mice (Merino et al., 2005),(Merino et al., 2010). While treatment of wild-type mice with genistein and daidzein had no effect on the plasma AUC of nitrofurantoin over a 2 hr period compared to vehicle-treated controls, higher plasma nitrofurantoin levels were in fact observed at 30 min (Merino et al., 2010). At that same time point, genistein and daidzein significantly reduced the nitrofurantoin concentrations in the milk and bile of wild-type mice as compared to the vehicle-treated mice (Merino et al., 2010). This study suggests that examination of early time points may be necessary to observe changes in drug pharmacokinetics brought on by isoflavones. These data combined with those in the sheep indicate that a soy diet may reduce the potential for infant exposure to nitrofurantoin in milk.

Only one study has investigated whether biochanin A can alter the pharmacokinetics of other BCRP/Bcrp substrates (An and Morris, 2010). Male wild-type ND4 Swiss Webster mice were administered an intravenous dose of mitoxantrone (5 mg/kg) 5 minutes following an intravenous dose of biochanin A (10 mg/kg) (An and Morris, 2010). Biochanin A actually enhanced the mitoxantrone elimination from the kidney and spleen as there was a trend for those organs to accumulate less mitoxantrone than the vehicle-treated mice, though this was not statistically significant (An and Morris, 2010). These findings were inconsistent with the in vitro results of this same study that found biochanin A to be an inhibitor of the Bcrp-mediated transport of mitoxantrone, indicating that there may be some disconnect between in vitro and in vivo models for measuring inhibition of BCRP function. Future research should examine the effects of isoflavones on the pharmacodynamics of a drug, such as measuring the contribution of the isoflavones to
the exacerbation of a toxic side effect. Such evidence may provide the impetus for improving the current guidelines for prescribing drugs that are BCRP substrates.

1.2.2.5 The Regulation of BCRP by Isoflavones

Genistein interacts with a variety of cellular proteins that modulate gene expression and/or protein localization such as the estrogen receptor, aryl hydrocarbon receptor, and the epidermal growth factor receptor (Martin et al., 1978; Akiyama et al., 1987; Kuiper et al., 1998; Zhang et al., 2003a; Yan et al., 2010). Because BCRP expression is regulated by the aryl hydrocarbon receptor (Zhang et al., 2003a), Ebert et al. examined the ability of genistein (1-50 μM) to alter BCRP mRNA expression in Caco-2 cells and described no effect compared to the vehicle-treated cells following a 48 h incubation (Ebert et al., 2007). This finding was confirmed when Arias et al. reported no change in BCRP protein expression in Caco-2 cells following 48 h exposure to genistein (0.1-10 μM) (Arias et al., 2014). Further, a 5-day exposure to genistein (3 and 10 μM) did not alter the BCRP protein expression in K562 cells that expressed BCRP (Imai et al., 2004). Conversely, genistein (15 μM, 24 h) reduced the mRNA expression of BCRP by approximately 70% in gastric cancer (MGC-803) cells (Huang et al., 2014). Taken together, the effect of genistein and possibly other isoflavones on BCRP regulation is likely tissue-specific. Further investigation of the effect of isoflavones on BCRP expression in other cell lines and tissue types that rely on BCRP function for protection against xenobiotic accumulation, such as the placenta and kidneys, is required to fully understand the role of isoflavones in the regulation of BCRP expression and ultimately function.
1.2.2.6 Conclusions

Isoflavones interact with the BCRP efflux transporter through a variety of ways including direct transport, chemical antagonism, and altered transporter expression. Evidence for the selective removal of isoflavones from target organs which express BCRP/Bcrp (Enokizono et al., 2007a; Kodaira et al., 2011; Yang et al., 2012), suggests that the transporter plays an important role in the in vivo disposition of isoflavones. Therefore the potential effects of isoflavones on human health may be influenced by the efficiency of BCRP function. In addition to transporting parent isoflavones, BCRP also transports sulfated and glucuronidated isoflavone metabolites (Alvarez et al., 2011; Jiang et al., 2012; van de Wetering and Sapthu, 2012; Yang et al., 2012) which, too, possess some biological activity (Zhang et al., 1999). Furthermore, sulfatase and glucuronidase deconjugating enzymes are present in the liver, intestines, blood, and fetus (Sfakianos et al., 1997; O'Leary et al., 2001; Nishikawa et al., 2010; Yang et al., 2012) and may convert the metabolites back to the parent compound. Because of these hydrolytic reactions, the ability of BCRP to transport both parent isoflavones and their metabolites is important. Due to the potential for isoflavones to disrupt the endocrine system, future research should focus on understanding the role of BCRP/Bcrp in reproductive organs and how disruption of transport may lead to inappropriate exposure of isoflavones to target organs and developing tissues.

As a result of being directly transported by BCRP, isoflavones may competitively inhibit the transport of other chemicals including pharmaceuticals which depend on BCRP for appropriate distribution, metabolism, and elimination. In addition to competitive inhibition, evidence suggests that isoflavones may also non-competitively inhibit BCRP-mediated transport as there are more isoflavone and flavonoid compounds that inhibit BCRP function than those that are directly transported (Imai et al., 2004; Katayama et al., 2007;
Further examination of the molecular interactions of isoflavones with ABC transporters and other proteins that contain a nucleotide binding domain, reveal that the compounds may disrupt BCRP function through interference with ATP hydrolysis via association with the BCRP nucleotide binding domain (Randak et al., 1999) (reviewed in (Di Pietro et al., 2002)). It is important to note that affinity of a compound for a particular site within the BCRP protein will determine the nature and extent of the chemical-transporter interaction. A high-resolution 3D structure of the human BCRP protein is not yet available, though working towards completing this task will help to elucidate the complexities of BCRP-isoflavone interactions.

According to self-reported surveys, the average consumption of total isoflavones ranges between 2 and 50 mg/day and is vastly different depending upon ethnicity and geographic location (Uehar et al., 2000; Frankenfeld et al., 2003; Messina et al., 2006). Consumption of a soy food diet in this range resulted in total plasma isoflavone concentrations of approximately 19-500 nM. However, in a pharmacokinetic study which enlisted 12 healthy volunteers to consume soy food (96 mg/day) with 3 meals every day for 7 days, led to a total plasma isoflavone concentration of about 5 µM on day 7 (Gardner et al., 2009). These data suggest that the isoflavone concentrations which inhibited BCRP function in vitro (low µM range (Zhang et al., 2004b; An and Morris, 2010; Tamaki et al., 2010; Pick et al., 2011)) may be applicable to a population that consumes soy regularly. To recapitulate a soy diet, a few investigations examined the effect of multiple isoflavones or flavonoids on BCRP function (Zhang et al., 2004a; Tamaki et al., 2010; Alvarez et al., 2011). These preliminary studies revealed the compounds may have additive or synergistic effects, supporting the need for future studies to address the effect of isoflavone mixtures on BCRP function. This is of particular importance because the population which consumes soy is rapidly increasing,
particularly in the United States. Since 1996, soy food sales in the United States dramatically increased from 1 billion to about 5.2 billion dollars spent per year by 2011 according to the Soy Foods Association of North America (2011c), which is likely a result of multiple reports suggesting the health benefits of soy consumption. Furthermore, there has been a steady increase in the overall prescription of drugs in the United States over the past 10 years (Gu et al., 2010). Taken together with the findings that suggest that isoflavones alter the pharmacokinetics of drugs which are substrates for BCRP, the risk for a nutrient-drug interaction mediated by BCRP is conceivable and warrants further investigation to help improve the guidance for the prescription of drugs that are BCRP substrates.
1.3. Gestational Diabetes

Affecting 5-10% of pregnancies in the United States (2011b; DeSisto et al., 2014), gestational diabetes (GD) is one of the most common complications of pregnancy (Dabelea et al., 2005; Ferrara, 2007). Specific ethnic groups including Native American, Asian, Hispanic, and African-American individuals are at a greater risk for GD (reviewed in (Ferrara, 2007)). The generally accepted definition of GD is hyperglycemia with onset or first recognition during pregnancy. While clinical parameters vary across country, and even between obstetric clinics, The American College of Obstetricians and Gynecologists (ACOG) guidelines recommend that all pregnant women be screened for GD using a two-step approach: (1) 50 g oral glucose solution with one hour venous glucose determination. Those meeting or exceeding threshold values (135-140 mg/dl) undergo a (2) 100 g oral glucose tolerance test for three hours with venous glucose determination at fasting, one, two, and three hours. The threshold for each time point is 95, 180, 155, and 140 mg/dl respectively, such that two abnormal values are indicative of GD (ACOG, 2013). In pregnant women, GD is a state of insulin resistance which occurs due to the interplay between glucose metabolism, fatty acid metabolism, hormones produced by the placenta and adipokines produced by adipose tissue. More specifically, the increased or decreased circulating levels of human placental lactogen or adiponectin, respectively, disrupt signaling downstream of the insulin receptor, thereby interfering with glucose uptake (reviewed in (Al-Noaemi and Shalayel, 2011)).

Approximately 30-40% of patients with GD do not achieve sufficient glycemic control through diet and exercise and must rely on pharmacotherapy to reduce blood glucose levels (Durnwald and Landon 2005). Effective management of GD avoids adverse outcomes associated with maternal hyperglycemia including large birth weights (macrosomia, odds ratio (OR) 3.7, 95% CI 2.2 to 6.9), shoulder dystocia (OR 4.07, 95%
Cl 1.63-10.16), premature delivery (OR 1.10, 95% CI 0.53-2.27), cesarean delivery (OR 1.88, CI 1.45-2.43), and development of type II diabetes in both mother (OR 3.91, CI 1.81-8.42) and baby (OR 5.7, 95% CI 2.4-13.4) later in life (Dabelea and Pettitt, 2001; Cheung and Byth, 2003; Langer et al., 2005; Vambergue et al., 2008). Subcutaneous insulin is the only form of therapy approved by the U.S. Food and Drug Administration (FDA) for the pharmacological treatment of GD. However patient discomfort, reduced compliance, and the high cost of insulin has led to the use of oral diabetes medications as alternatives for the management of GD. Furthermore, the similar pathogenesis of GD and type II diabetes (i.e., insulin resistance) supports the probable efficacy of type II diabetes oral medications in controlling GD. It is important to note that previously, small molecule hypoglycemic medications were not recommended for use in pregnancy due to the potential for teratogenic effects and the possibility of causing hyperinsulinemia and/or hypoglycemia in the developing fetus (Nitowsky et al., 1966; Zucker and Simon, 1968; Kemball et al., 1970). Most of these adverse findings were observed with the use of first generation sulfonylurea oral diabetes medications. Subsequent lines of therapy have since been developed and may offer a safer alternative to GD treatment with an oral diabetes medication.

1.3.1. Glyburide in the Treatment of Gestational Diabetes

Often used in the treatment of type II diabetes, glyburide (INN: glibenclamide) is a second-generation sulfonylurea drug. Blood glucose is reduced in these patients by increased insulin secretion, as glyburide blocks the potassium efflux in pancreatic-β cells via inhibition of the ATP-dependent sulfonylurea receptor (SUR1, ABCC8) (Aguilar-Bryan et al., 1995). In vitro, glyburide inhibits SUR1 in the low nanomolar range (K_d=0.7-2 nM) (Gaines et al., 1988; Gopalakrishnan et al., 2000). The average clinical dose (1.25-20 mg) results in a C_{max} of 30-300 nM in patients that have been prescribed
glyburide for the treatment of type II diabetes or GD (Langer et al., 2000; Hebert et al., 2009).

Human placental perfusion ex vivo studies performed in the early 1990’s revealed that there was limited maternal-to-fetal transfer of glyburide (Elliott et al., 1991; Elliott et al., 1994). With this supporting evidence, Langer et al. performed a prospective randomized clinical trial testing the efficacy and safety of glyburide (n=201) in the treatment of GD as compared to insulin (n=203) (Langer et al., 2000). The study concluded that glyburide was as effective as insulin in maintaining normal blood glucose and glycosylated hemoglobin levels in pregnant women with GD. While glyburide was present in the maternal circulation, the medication was undetectable in the cord serum of respective neonates, which was supported by no difference in neonatal outcome between groups (limit of detection < 10 ng/ml, 20 nM). Additional researchers confirmed the comparable efficacy of glyburide and insulin in managing GD (Bertini et al., 2005; Anjalakshi et al., 2007). However, there are conflicting data with regards to neonatal outcome following glyburide treatment. Results from a retrospective study indicate that women prescribed glyburide for GD were at a significantly greater risk for the development of preeclampsia as compared to those treated with insulin (glyburide, n=236; insulin, n=286) (Jacobson et al., 2005). Conversely, a randomized controlled clinical trial indicated there was no difference in preeclampsia incidence between a glyburide and insulin regimen (glyburide, n=201; insulin, n=203) (Langer et al., 2000). The same randomized controlled clinical trial described no differences in neonatal outcome (i.e., neonatal hypoglycemia, however hypoglycemia occurred more frequently in the offspring of women treated with glyburide as compared to those treated with insulin in another randomized controlled clinical trial with a lesser sample size (glyburide, n=48; insulin, n=49) (Ogunyemi et al., 2007). Another more recent study revealed that neonates taking glyburide for gestational
diabetes are at a greater risk for developing hypoglycemia (relative risk, RR=1.4; 95% CI, 1.00-1.95) as well as respiratory distress (RR= 1.63; 95% CI, 1.23-2.15), birth injury (RR=1.35; 95% CI, 1.00-1.82), and being large for gestational age (RR=1.43; 95% CI, 1.16-1.76) (Camelo Castillo et al., 2015). In a study performed in 2015, glyburide was detected in the cord blood of neonates at lower concentrations (1.37-65.5 nM) than was observed in the maternal blood (1.88-143 nM); however, there was a significant inverse relationship between cord glyburide concentration and neonatal blood-glucose concentration (Schwartz et al., 2015).

Despite the inconsistencies in these findings, many obstetricians now prescribe glyburide as a first line of therapy in GD management. Results from the above investigations should be considered with caution as few of the studies were adequately powered to detect statistically significant differences in all of the outcomes measured. Further, particular ethnic groups were represented more frequently than others indicating the need for future studies which separate outcome based on ethnicity.

1.3.2. Placental BCRP-Mediated Transport of Glyburide

There is limited transfer of glyburide across the placenta (Elliott et al., 1994; Sivan et al., 1995; Langer et al., 2000) lending to the desirability for its use in pregnancy. Many of the physicochemical and pharmacokinetic properties of glyburide contribute to the reduced placental transfer of the drug. This includes the molecular weight (494 g/mol), lipophilicity (LogP=4.5 (Wei et al., 2008)), pKa (5.3 (Lobenberg et al., 2000)), high plasma protein binding (99.8% (Nanovskaya et al., 2008)), short elimination half-life (4-6 h (Donahue et al., 2002)), and the low volume of distribution (7.44 L (Rydberg et al., 1995)). Most importantly, glyburide is actively transported from the fetal to the maternal circulation (Kraemer et al., 2006) by the BCRP efflux transporter as demonstrated by the
disruption of BCRP transport by specific inhibitors such as novobiocin, nicardipine, fumitremorgin C (FTC), and Ko143 in a variety of model systems (Table 1.10). For example, FTC abolished the selective basolateral-to-apical transport of glyburide across MDCK/BCRP and MDCKII/Bcrp cell monolayers indicating that both the human and mouse BCRP/Bcrp proteins transport glyburide (Zhou et al., 2008a). Furthermore, ex vivo perfusion of the human term placenta revealed that the BCRP inhibitor nicardipine significantly reduced the fetal-to-maternal concentration ratio of glyburide at 180 min (Pollex et al., 2008). While some reports suggest other ABC transporters contribute to glyburide transport (Gedeon et al., 2006; Hemauer et al., 2010; Tournier et al., 2013), the majority of the available evidence agree that MDR1 and multidrug resistance-associated proteins (MRPs) are not responsible for glyburide transport in the placenta as inhibitors specific for these transporters (i.e., MDR1: verapamil; MRPs: indomethacin) did not affect glyburide transport in transporter overexpressing cells (Gedeon et al., 2006), MDCKII cells (Zhou et al., 2008a), human placental vesicles (Gedeon et al., 2008b) and human placental perfusion (Gedeon et al., 2008a).

1.4. Research Objective and Hypothesis

Taking all of this information together, an important research hypothesis emerged: Dietary concentrations of genistein impair the ability of BCRP to efflux glyburide by 1) directly inhibiting transporter function and 2) reducing its protein expression in placental trophoblasts. Three specific aims of this thesis were employed to evaluate this hypothesis:

1) Characterize the intra- and interindividual expression of BCRP in human term placentas from healthy pregnancies.

2) Determine the impact of genetic and dietary factors on the in vitro placental transport of glyburide by BCRP.
3) Determine whether the dietary constituent, genistein reduces the mRNA and protein expression of placental BCRP.

The findings of this research will provide population data regarding the interindividual variability in placental BCRP expression as well as mechanistic data regarding the disposition of glyburide during pregnancy in the presence of genistein that may be applicable to other medications that are also transported by placental BCRP. Significant knowledge gaps will also be filled in the fields of placental transport and developmental toxicology, which may lead to future clinical studies that improve the individualized prescribing of glyburide for women with GD based on environmental (dietary) and genetic risk factors.
Figure 1.1. Blastocyst structure (Adapted from (Cunningham et al., 2014)).
Figure 1.2. Cytotrophoblast lineages: Villous and extravillous trophoblasts
(Adapted from (Moffett and Loke, 2006)).
Figure 1.3. Structure of human term placenta (Adapted from (Cunningham et al., 2014))
Figure 1.4. Localization of SLC drug transporters in the human placenta (Staud et al., 2012).
Figure 1.5. Localization of ABC drug transporters in the human placenta (Staud et al., 2012).
Figure 1.6. Localization of amino acid changes in the BCRP protein due to SNPs in the ABCG2 gene (generated using (Omasits et al., 2014)).
Figure 1.7. The parent ring structures of A) flavonoids and B) isoflavones.
Figure 1.8. Chemical structures of the most commonly studied isoflavones: genistein, daidzein, biochanin A, glycitein, and formononetin.
Figure 1.9. Basic chemical structure of an isoflavone-glycoside.
Table 1.1. Substrates and inhibitors of BCRP/Bcrp.

<table>
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<tr>
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<td>(Burger et al., 2004)</td>
</tr>
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<td>Mitoxantrone</td>
<td>(Allikmets et al., 1998; Doyle et al., 1998; Rabindran et al., 1998)</td>
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<td>(Brangi et al., 1999)</td>
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</tr>
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<tr>
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<td>Hoechst 33342</td>
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Table 1.2. Summary of SNPs in the coding region of the \textit{ABCG2} gene.

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² AC084732 (1st nucleotide of gene = 1)

³ Translational start site, ATG= +1

* Excluding intron nucleotides
N.R.: Not reported

PDR44: Polymorphism Discovery Resource 44

¹(Kobayashi et al., 2005), ²(Poonkuzhali et al., 2008), ³(Zamber et al., 2003) (% individuals with at least one variant allele), ⁴(Imai et al., 2002a), ⁵(Mizuarai et al., 2004), ⁶(Bosch et al., 2005), ⁷(Backstrom et al., 2003), ⁸(Urquhart et al., 2008), ⁹(Prasad et al., 2013), ¹⁰(de Jong et al., 2004), ¹¹(Cunningham et al., 2015)
Table 1.3. Summary of the effects of the C421A variant on the pharmacokinetics or pharmacodynamics of drugs that are BCRP substrates.

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<tr>
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<th>Effect</th>
<th>Patient Demographics</th>
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<th>Reference</th>
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<td>Diflomotecan (Intravenous)</td>
<td>CC (n=15) CA (n=5)</td>
<td>↑ AUC 3-fold ↑ C(_{\text{max}}) 2.7-fold</td>
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<td>No effect observed with oral dosing</td>
<td>(Sparreboom et al., 2004)</td>
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<td>Healthy, volunteers</td>
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<td>Fluvastatin (Oral)</td>
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Table 1.4. Summary of SNPs in the non-coding regions of the \textit{ABCG2} gene.

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\(^a\) AC084732 (1st nucleotide of gene = 1)
\(^b\) Translational start site, ATG= +1
N.R.: Not reported
PDR44: Polymorphism Discovery Resource 44
\(^1\) (Kobayashi et al., 2005), \(^2\) (Poonkuzhali et al., 2008), \(^3\) (Cunningham et al., 2015)
Table 1.5. Summary of *in vitro* and *in vivo* experiments describing genistein as a substrate of BCRP/Bcrp.

<table>
<thead>
<tr>
<th>Experiment type</th>
<th>Model</th>
<th>Genistein concentration</th>
<th>Detection method</th>
<th>Time</th>
<th>BCRP inhibitor</th>
<th>References</th>
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<tr>
<td>Cellular uptake</td>
<td>K562/BCRP cells</td>
<td>30 nM (3H)</td>
<td>LSC</td>
<td>4 h</td>
<td>-</td>
<td>(Imai et al., 2004)</td>
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<tr>
<td>Trancellular transport</td>
<td>LLC-PK1/BCRP cells</td>
<td>30 nM (3H)</td>
<td>LSC</td>
<td>4 h</td>
<td>3 μM FTC</td>
<td>(Imai et al., 2004)</td>
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<tr>
<td></td>
<td>MDCK/BCRP cells</td>
<td>3 μM</td>
<td>LC/MS</td>
<td>4 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
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<tr>
<td></td>
<td>Caco-2 cells</td>
<td>100 nM (14C)</td>
<td>LSC</td>
<td>1 h</td>
<td>1 μM FTC</td>
<td>(Mease et al., 2012)</td>
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<td>Caco-2 cells</td>
<td>50 μM</td>
<td>HPLC-ECD</td>
<td>50 min</td>
<td>Estrone-3-sulfate</td>
<td>(Kobayashi et al., 2013)</td>
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<td>MDCK/Bcrp</td>
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<td>LC/MS</td>
<td>4 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
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<td></td>
<td>MDCK/Bcrp</td>
<td>1-3 μM</td>
<td>LC/MS</td>
<td>3 h</td>
<td>-</td>
<td>(Kodaira et al., 2011)</td>
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<tr>
<td>Oral (p.o.)</td>
<td>FVB Bcrp +/- mice</td>
<td>8.1 mg/kg</td>
<td>LC/MS</td>
<td>4 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
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<td>FVB Bcrp +/- mice</td>
<td>2-20 mg/kg</td>
<td>UPLC/MS/MS</td>
<td>24 h</td>
<td>-</td>
<td>(Yang et al., 2012)</td>
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<td>Intravenous (i.v.) infusion</td>
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<tr>
<td>Brain</td>
<td>FVB Bcrp +/- mice</td>
<td>1.4 mg/kg/h</td>
<td>LC/MS</td>
<td>2 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
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<td>FVB Bcrp +/- mice</td>
<td>0.8 mg/kg initial, 4.3 mg/kg/h</td>
<td>LC-MS/MS</td>
<td>2 h</td>
<td>-</td>
<td>(Kodaira et al., 2011)</td>
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<td>Testis</td>
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<td>1.4 mg/kg/h</td>
<td>LC/MS</td>
<td>2 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
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<tr>
<td>Epididymis</td>
<td>FVB Bcrp +/- mice</td>
<td>1.4 mg/kg/h</td>
<td>LC/MS</td>
<td>2 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
</tr>
<tr>
<td>Placenta</td>
<td>Pregnant FVB Bcrp +/- mice</td>
<td>1.4 mg/kg/h</td>
<td>LC/MS</td>
<td>2 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
</tr>
<tr>
<td>Fetal Brain</td>
<td>Pregnant FVB Bcrp +/- mice</td>
<td>1.4 mg/kg/h</td>
<td>LC/MS</td>
<td>2 h</td>
<td>-</td>
<td>(Enokizono et al., 2007a)</td>
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Table 1.6. Summary of *in vitro*, *in situ*, and *in vivo* experiments describing genistein-sulfate as a substrate of BCRP/Bcrp.

<table>
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<tr>
<th>Experiment type</th>
<th>Model</th>
<th>Genistein concentration</th>
<th>Detection method</th>
<th>Time</th>
<th>BCRP inhibitor</th>
<th>References</th>
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<tr>
<td>Transcellular transport</td>
<td>Caco-2</td>
<td>2-10 μM</td>
<td>UPLC-MS/MS</td>
<td>4 h</td>
<td>5 μM Ko143</td>
<td>(Yang et al., 2012)</td>
</tr>
<tr>
<td>Vesicle uptake</td>
<td>Sf9-BCRP vesicles</td>
<td>Chow (Amount unknown; mouse urine)</td>
<td>HPLC/MS</td>
<td>40 s</td>
<td>-</td>
<td>(van de Wetering and Sapthu, 2012)</td>
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<td></td>
<td>Sf9-BCRP vesicles</td>
<td>35S-genistein-sulfate</td>
<td>LSC</td>
<td>40 s</td>
<td>-</td>
<td>(van de Wetering and Sapthu, 2012)</td>
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<td><em>In situ</em> Intestinal perfusion</td>
<td>FVB Bcrp-/- mice</td>
<td>10 μM</td>
<td>UPLC-DAD-MS/MS</td>
<td>120 min</td>
<td>-</td>
<td>(Zhu et al., 2010)</td>
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<tr>
<td>Biliary excretion</td>
<td>FVB Bcrp-/- mice</td>
<td>10 μM</td>
<td>UPLC-MS/MS</td>
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<tr>
<td>Plasma</td>
<td>FVB Bcrp-/- mice</td>
<td>50 mg/kg</td>
<td>HPLC-DAD-MS/MS</td>
<td>3 h</td>
<td>-</td>
<td>(Alvarez et al., 2011)</td>
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<tr>
<td></td>
<td>FVB Bcrp-/- mice</td>
<td>2-20 mg/kg</td>
<td>UPLC-MS/MS</td>
<td>24 h</td>
<td>-</td>
<td>(Yang et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>FVB Bcrp-/- mice</td>
<td>Chow (Amount unknown)</td>
<td>HPLC/MS</td>
<td>-</td>
<td>-</td>
<td>(van de Wetering and Sapthu, 2012)</td>
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<td>Intraperitoneal (i.p.)</td>
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<td>Plasma</td>
<td>FVB Bcrp-/- mice</td>
<td>20 mg/kg</td>
<td>UPLC/MS/MS</td>
<td>24 h</td>
<td>-</td>
<td>(Yang et al., 2012)</td>
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Table 1.7. Summary of *in vitro*, *in situ*, and *in vivo* experiments describing genistein-glucuronide as a substrate of BCRP/Bcrp.

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<th>Time</th>
<th>BCRP inhibitor</th>
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<td>120 min</td>
<td>5-10 μM Ko143</td>
<td>(Jiang et al., 2012)</td>
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<tr>
<td>Transcellular transport</td>
<td>Caco-2</td>
<td>2-10 μM</td>
<td>UPLC-MS/MS</td>
<td>4 h</td>
<td>5 μM Ko143</td>
<td>(Yang et al., 2012)</td>
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<tr>
<td><em>In situ</em></td>
<td>Intestinal perfusion</td>
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<tr>
<td>SI Excretion</td>
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<td>10 μM</td>
<td>UPLC-DAD-MS/MS</td>
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<td>(Zhu et al., 2010)</td>
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<td><em>In vivo</em></td>
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<td>HPLC-DAD-MS/MS</td>
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<td>-</td>
<td>(Alvarez et al., 2011)</td>
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<td>2-20 mg/kg</td>
<td>UPLC/MS/MS</td>
<td>24 h</td>
<td>-</td>
<td>(Yang et al., 2012)</td>
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<td>Intraperitoneal (i.p.)</td>
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<td>24 h</td>
<td>-</td>
<td>(Yang et al., 2012)</td>
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Table 1.8. Summary of *in vitro*, *in situ*, and *in vivo* experiments describing genistein as an inhibitor of BCRP/Bcrp function.

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<th>BCRP substrate</th>
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<td>10-50 μM</td>
<td>Sulforhodamine B assay</td>
<td>48 h</td>
<td>0-1000 μM Mitoxantrone</td>
<td>(Zhang et al., 2004a; Zhang et al., 2004b)</td>
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<td>K562/BCRP cells</td>
<td>3 μM</td>
<td>Cell counter</td>
<td>5 days</td>
<td>SN-38</td>
<td>(Imai et al., 2004)</td>
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<td>Cell counter</td>
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<td>SN-38</td>
<td>(Katayama et al., 2007)</td>
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<td>50 μM</td>
<td>Flow cytometry</td>
<td>30 min</td>
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<td>(Zhang et al., 2004a; Zhang et al., 2004b)</td>
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<td>NCI-H460/MX20 cells</td>
<td>50 μM</td>
<td>Flow cytometry</td>
<td>30 min</td>
<td>3 μM Mitoxantrone</td>
<td>(Zhang et al., 2004b)</td>
</tr>
<tr>
<td>Vesicle uptake</td>
<td>Sf9-BCRP vesicles</td>
<td>10-100 μM</td>
<td>LSC</td>
<td>2 min</td>
<td>100 μM ³H-Mitoxantrone</td>
<td>(Tamaki et al., 2010)</td>
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<td>ATPase activity</td>
<td>Lactating cow mammary gland plasma membranes</td>
<td>10 μM</td>
<td>Colorimetric detection of P&lt;sub&gt;i&lt;/sub&gt;</td>
<td>40 min</td>
<td>50 μM Mitoxantrone</td>
<td>(Pulido et al., 2006)</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral (p.o.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>Lactating sheep</td>
<td>0.8 mg/kg</td>
<td>HPLC</td>
<td>24 h</td>
<td>Enrofloxacin</td>
<td>(Pulido et al., 2006)</td>
</tr>
</tbody>
</table>
Table 1.9. Summary of experiments describing the regulation of BCRP by genistein.

<table>
<thead>
<tr>
<th>Model</th>
<th>Genistein Concentration</th>
<th>Treatment Duration</th>
<th>Effect on BCRP mRNA</th>
<th>Effect on BCRP protein</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2 cells</td>
<td>1-50 μM</td>
<td>48 h</td>
<td>↔</td>
<td>N.D.</td>
<td>(Ebert et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>0.1-10 μM</td>
<td>48 h</td>
<td>N.D.</td>
<td>↔</td>
<td>(Arias et al., 2014)</td>
</tr>
<tr>
<td>K562/BCRP cells</td>
<td>3-10 μM</td>
<td>5 days</td>
<td>N.D.</td>
<td>↔</td>
<td>(Imai et al., 2004)</td>
</tr>
<tr>
<td>MGC-803 cells</td>
<td>15 μM</td>
<td>24 h</td>
<td>↓</td>
<td>↔</td>
<td>(Huang et al., 2014)</td>
</tr>
</tbody>
</table>
Table 1.10. Summary of *in vitro, in vivo, ex vivo, and in situ* experiments describing glyburide as a substrate of BCRP/Bcrp.

<table>
<thead>
<tr>
<th>Model</th>
<th>Glyburide concentration</th>
<th>Time (min)</th>
<th>Inhibitor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF7/MX-BCRP Overexpressing Cells</td>
<td>200 µM (^3)H-glyburide</td>
<td>5-60</td>
<td>200 µM Novobiocin</td>
<td>(Gedeon et al., 2006)</td>
</tr>
<tr>
<td>MDCKII-BCRP</td>
<td>0.5 and 5 nM (^3)H-glyburide</td>
<td>60-240</td>
<td>10 µM FTC</td>
<td>(Zhou et al., 2008a)</td>
</tr>
<tr>
<td></td>
<td>10 nM (^3)H-glyburide</td>
<td>60</td>
<td>5-50 µM</td>
<td>(Hofman et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>3.7 kBq/ml (^3)H-glyburide</td>
<td>60</td>
<td>10 µM FTC</td>
<td>(Tournier et al., 2013)</td>
</tr>
<tr>
<td>MDCKII-Bcrp</td>
<td>0.5 and 5 nM (^3)H-glyburide</td>
<td>60-240</td>
<td>10 µM FTC</td>
<td>(Zhou et al., 2008a)</td>
</tr>
<tr>
<td>Human Placental Brush Boarder Membrane</td>
<td>400 mM glyburide + 200 µM</td>
<td>0.5-20</td>
<td>200 mM Novobiocin</td>
<td>(Gedeon et al., 2008b)</td>
</tr>
<tr>
<td>Vesicles</td>
<td>(^3)H-glyburide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>100 nM (^3)H-glyburide</td>
<td>1</td>
<td>25 nM Ko143</td>
<td>(Hemauer et al., 2010)</td>
</tr>
<tr>
<td>HEK-BCRP (wild-type and C421A)</td>
<td>0.2-100 µM (^3)H-glyburide</td>
<td>20-60</td>
<td>10 µM FTC</td>
<td>(Pollex et al., 2010)</td>
</tr>
<tr>
<td><strong>In vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant Bcrp (-/-) Mice</td>
<td>0.5 mg/ml glyburide (retro-orbital injection)</td>
<td>0.5 – 240</td>
<td>N/A</td>
<td>(Zhou et al., 2008a)</td>
</tr>
<tr>
<td>Pregnant Rat</td>
<td>0.2 µM glyburide</td>
<td>10-60</td>
<td>2 µM GF120918</td>
<td>(Cygalova et al., 2009)</td>
</tr>
<tr>
<td><strong>Ex vivo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Placental Perfusion</td>
<td>200 ng/ml</td>
<td>180</td>
<td>20 µM Nicardipine</td>
<td>(Pollex et al., 2008)</td>
</tr>
<tr>
<td><strong>In situ</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat Placenta Perfusion</td>
<td>200 nM</td>
<td>10-60</td>
<td>2 µM FTC</td>
<td>(Hofman et al., 2012)</td>
</tr>
</tbody>
</table>

(-/-): knockout
CHAPTER 2: REGIONAL EXPRESSION OF THE BCRP/ABCG2 TRANSPORTER IN TERM HUMAN PLACENTAS

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* These two authors contributed equally as first authors.
2.1 Abstract
The breast cancer resistance protein (BCRP, ABCG2) is an efflux transporter that removes xenobiotics that cross the placenta back to the maternal circulation, thereby limiting exposure of the fetus to drugs and chemicals. Currently, variability of BCRP expression within the placenta is not known. Ten placentas were collected from healthy women undergoing elective Cesarean sections at term. Villous samples were dissected in defined regions (medial, intermediate, and peripheral) and BCRP mRNA and protein were quantified. There were no regional differences in mRNA expression of housekeeping genes (GAPDH, RPL13a, PRL, 18S). GAPDH had the lowest correlation with BCRP Ct values and was used for BCRP mRNA normalization. No differences in placental BCRP mRNA and protein were observed among the sample sites (<20% variability). Sampling site does not affect the expression of BCRP, supporting the utility of single site sampling protocols to assess the interindividual regulation of this transporter in human placentas.

Keywords
BCRP, ABCG2, Transporter, Placenta, Housekeeping gene
Abbreviations

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; cDNA, complementary DNA; Ct, threshold cycle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; PRL, prolactin; RPL13a, ribosomal protein L13a; 18S, 18S ribosomal RNA
2.2 Introduction

The breast cancer resistance protein (BCRP) is an ATP-binding cassette (ABC) transporter that is expressed on the apical surface of placental syncytiotrophoblasts (Maliepaard et al., 2001). This transporter extrudes a wide range of xenobiotics that cross the placenta back into the maternal circulation, thereby protecting the fetus from drug and chemical accumulation and toxicity. The BCRP gene (ABCG2) is expressed in the human placenta approximately 100 times greater than that of any other organ (Allikmets et al., 1998; Doyle et al., 1998), suggesting that BCRP plays a critical fetoprotective role. Studies of placental cell lines and placental primary cultures (Ceckova et al., 2006; Evseenko et al., 2007b), mutant/knockout mice (Jonker et al., 2002; Enokizono et al., 2007a; Zhang et al., 2007; Zhou et al., 2008a) and a more recent study of an ex vivo human placental perfusion model (Pollex et al., 2008) have all documented the importance of the BCRP transporter in determining fetal exposure to a number of drugs and environmental chemicals, including antineoplastic agents (mitoxantrone, topotecan), antidiabetic medications (glyburide), antibiotics (nitrofurantoin), and phytoestrogens (genistein).

When studying the expression of specific transcripts and proteins in the placenta, it is important to consider sampling methodologies and to control for intraplacental variability. Studies have shown that placental structure and blood flow patterns across the human placental disc are not uniform (reviewed in (Kliman, 2000)). Histological studies have also shown that placental tissue near the chorionic surface and in the placental margins may have more syncytial knots and villous fibrin, which is typically characteristic of underperfused villi (Fox, 1997; Benirschke K, 2000; Wyatt et al., 2005). In fact, studies have suggested that the mRNA expression of hypoxia-related transcripts, such as vascular endothelial growth factor and connective tissue growth factor, is dependent
upon sampling site within the placental disc, with up-regulation noted in the presumed hypoxic lateral-chorionic sites as compared to medio-basal sites (Wyatt et al., 2005). It is unknown whether BCRP exhibits such regional differences in mRNA and protein expression in term human placentas. Therefore, the purpose of this study was to examine the mRNA and protein expression of BCRP in defined regions of healthy term human placentas. Knowledge of the intraindividual expression of BCRP in the placenta may provide insight into its regulation and is important for future studies assessing the interindivdual expression of this transporter.
2.3 Materials and Methods

Patient Selection

Ten placentas were obtained after written informed consent from healthy women with uncomplicated pregnancies following term delivery (as defined by standard clinical criteria) by scheduled Cesarean section. Inclusion criteria included healthy women between the ages of 18-40 years, term gestation (≥ 36 weeks), and scheduled Cesarean section without labor. Table 1 includes patient demographic data (Table format modified from (Nelson and Burton, 2011)). Exclusion criteria included chronic medical conditions (e.g. hypertension, diabetes, autoimmune disorders), pregnancy-induced medical conditions (e.g. pregnancy-induced hypertension, preeclampsia, gestational diabetes), maternal infection, clinical chorioamnionitis, medication use (with the exception of prenatal vitamins), maternal smoking, alcohol or drug abuse, multiple pregnancies, and known fetal chromosomal abnormalities. The study was approved by the Institutional Review Boards of Robert Wood Johnson Medical School (Protocol #0220100258) and Rutgers University (Protocol #E12-024).

Sample Collection

All placentas were obtained within ten minutes of delivery and processing was completed within one hour. Each placenta was rinsed thoroughly with phosphate-buffered saline (PBS) three times. Placentas were carefully inspected for any visible abnormalities and location of the umbilical cord. Only placentas with central or eccentric cord insertion were used. The maternal decidua and the chorionic plate along with overlying membranes were removed. Using a scalpel, six tissue samples were collected along the long axis per placenta according to the scheme in Figure 2.1. ‘M1’ and ‘M2’ samples were categorized as “medial” or from the area closest to the umbilical cord insertion. These samples were obtained approximately 1 cm distal to the cord insertion.
site. ‘I₁’ and ‘I₂’ samples were categorized as “intermediate”, and were mid-distance from cord insertion and periphery. ‘P₁’ and ‘P₂’ samples were classified as “peripheral” and were obtained approximately 2 cm away from the lateral placental margin (to avoid fibrin-rich areas). Each sample was approximately 2 cm x 1 cm x 0.5 cm in dimension.

Individual samples were dissected into three sub-segments of equal size. The first sub-segment was placed in a PAXgene Tissue Container (Qiagen, Germantown, MD) in the PAXgene Tissue Fix for 4 h, moved to PAXgene Tissue Stabilizer, and stored at -20°C for histologic and RNA analysis. The second and third sub-segments were frozen and stored at -80°C for protein analysis.

**Histology**

The first sub-segment of each placenta sample was embedded in paraffin and sectioned into 5 µm thick sections. Placenta sections were stained with hematoxylin and eosin, examined, and imaged by light microscopy (VS120-S5, Olympus, Center Valley, PA).

**RNA Isolation and Real-Time Quantitative PCR**

Total RNA was isolated using a Tissue miRNA Kit (Qiagen, Germantown, MD) according to the manufacturer’s instruction. The concentration of total RNA in each sample was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and purity was confirmed with a Bioanalyzer Instrument (Agilent, Santa Clara, CA). Complementary DNA (cDNA) was generated using the Ovation Pico WTA System (NuGEN, San Carlos, CA) in combination with the Biomek FXP Laboratory Automation Workstation (Beckman Coulter, Inc., Brea, CA). Quantitative analysis of mRNA was performed with 500 ng cDNA, commercially-available primers for each gene, and Taqman probes (Applied Biosystems, Foster City, CA) using a ViiA7 RT-PCR system
(Applied Biosystems) in the Bionomics Research and Technology Center at Rutgers University. For the comparison of threshold cycle (Ct) values across the 3 placenta regions, the mean of two sample sites per placenta were used (for example, M₁ and M₂). Ct values for BCRP mRNA were first converted to delta Ct values by comparing to the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and then to delta delta Ct values by designating the average delta Ct values for medial samples from each placenta as the control group. **Supplemental Table 2.1** includes the gene name and assay ID information.

**Western Blot Analysis**

Placentas were homogenized in sucrose (250 mM)-Tris base (10 mM) buffer (pH 7.5) containing protease inhibitors (Sigma Aldrich, St. Louis, MO). Homogenates were centrifuged (100,000 x g) for 1 hr at 4°C and the pellets, or crude membrane fractions, were resuspended in sucrose-Tris base buffer with protease inhibitors. Protein concentrations were determined by a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed by loading 10 µg placenta membrane protein and 5 µg human embryonic kidney-293 cells overexpressing an empty vector or wild-type BCRP in polyacrylamide 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA) that were resolved by electrophoresis. Gels were transblotted onto polyvinylidene fluoride membrane in a 7-minute transfer apparatus (Life Technologies, Carlsbad, CA). Membranes were then blocked in 5% non-fat dairy milk in PBS with 0.5% Tween-20 for 1 h. BCRP (BXP-53, Abcam, Cambridge, MA) and β-actin (ab8227, Abcam, Cambridge, MA) primary antibodies were diluted in 2% non-fat dairy milk in PBS with 0.5% Tween-20 and incubated with the membranes at dilutions of 1:5000 and 1:2000, respectively. Primary antibodies were probed using species-specific HRP-conjugated secondary antibodies (Sigma Aldrich, St. Louis, MO) and the
SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL). Detection and semiquantitation of protein bands was performed with a FluorChem imager (ProteinSimple, Santa Clara, CA).

Statistical Analysis
Intraclass correlation coefficients were calculated using SAS (SAS Institute, Cary, NC). Linear regression analysis and one-way analysis of variance followed by a Newman-Keuls multiple comparison post hoc test were performed using GraphPad Prism v5 (GraphPad Software, La Jolla, CA). Significance was set at p < 0.05.
2.4 Results

Histologic Evaluation of Human Placentas

The health and sampling quality of term human placentas were assessed by histologic examination (n=10). Eight of the placentas consisted of mostly normal tissue (Figure 2.2), while two placentas displayed minor areas of patchy villous edema or avascular villi (not shown). Small areas of maternal decidua were noted in three of the placentas (not shown). All placenta samples were included in subsequent data analysis.

Assessment of Placental Housekeeping Genes

Total RNA was isolated from term human placentas (n=10) and four housekeeping genes were evaluated to determine the appropriate placental housekeeping gene for the expression of BCRP mRNA levels. There were no differences in the regional (medial, intermediate, peripheral) Ct values of the four housekeeping genes: GAPDH, ribosomal protein L13a (RPL13a), prolactin (PRL), and 18S ribosomal RNA (18S) (Figure 2.3A). Between placentas, GAPDH, RPL13a, and 18S Ct values were not significantly different. However, interplacental PRL Ct values varied greatly. Using linear regression analysis, BCRP Ct values correlated least with GAPDH Ct values ($R^2=0.003$) (Figure 2.3B). In addition, intraclass correlation of BCRP and GAPDH was 0.06 compared to 0.46, -0.45, and 0.16 for 18S, PRL, and RPL13a, respectively. In line with the recommendation of Adibi et al. 2009 (Adibi et al., 2009), the low intraclass correlation of BCRP with GAPDH was used as a criterion for selecting GAPDH as standard for normalizing BCRP mRNA levels. Furthermore, intraclass correlation analysis of BCRP mRNA normalized to each of the tested housekeeping genes were 0.70, 0.32, 0.65, and 0.35 yielding $p$ values of 0.61, 0.54, 0.33, and 0.056 for GAPDH, 18S, RPL13a, and PRL, respectively. These data further support the conclusion that expression of BCRP mRNA is not related to GAPDH mRNA and therefore, GAPDH is a suitable gene for normalization.
BCRP mRNA and Protein Expression in Term Human Placentas

BCRP mRNA and protein expression levels were compared between three regions (six sub-regions) of the placenta. BCRP mRNA expression was normalized to GAPDH and compared to the medial regions of each placenta. As displayed in Figure 2.4, there was up to a 20% difference in mean mRNA expression level between the medial, intermediate, and peripheral sub-regions of the placenta that was not statistically significant. Likewise, the protein expression of BCRP, normalized to β-actin loading control, was similar in the three different regions of the placenta, with a statistically insignificant difference (up to 20% variability between sub-region means) (Figure 2.5). It should be noted that the raw blot is only shown for one placenta (Figure 2.5A), however all ten placentas were used for graphical representation (Figure 2.5B).
2.5 Discussion

In this study, we investigated the intraplacental variability in mRNA and protein expression of the BCRP efflux transporter in term human placentas. Regional variation in the expression of several genes and proteins within healthy, term human placentas has previously been reported in a number of studies (Wyatt et al., 2005; Avila et al., 2010; Brameld et al., 2011). Though many different factors contribute to this site-to-site variability, it is speculated that the physical location of cells and their relative exposure to oxygen is responsible for the variation seen in several hypoxia-related transcripts.

Hypoxia has been shown to alter BCRP expression in different experimental models, and depending on the tissue sample, hypoxic conditions can either up-regulate or down-regulate BCRP expression. For instance, BCRP mRNA expression is increased under hypoxic conditions in hematopoietic stem cells, and this increase is associated with up-regulation of hypoxia-inducible factor 1 (Krishnamurthy et al., 2004). In contrast, BCRP mRNA expression is decreased in placentas from growth-restricted infants exposed to hypoxia in utero, when compared to placentas of healthy infants (Evseenko et al., 2007a). In the present study, we found that expression of BCRP was not dependent upon location within the placental disc. Our findings suggest that differences in maternal blood flow and oxygen tension across the placenta do not affect BCRP mRNA and protein expression. However, it should be noted that local oxygen levels were not quantified in these 10 placentas, and our findings were not correlated with histopathological changes consistent with hypoperfusion.

The lack of intraplacental variability in levels of BCRP mRNA and protein suggests that a small number of samples from each placenta may be sufficient when comparing BCRP expression between placentas. However, our results must be generalized with caution.
Firstly, three of the ten placentas studied were contaminated with small amounts of maternal decidua, thereby potentially confounding the above results as the samples may have contained maternal mRNA and protein. Although, it should be noted that BCRP mRNA expression in human uterus is lower than levels observed in the placenta (reviewed in (Klaassen and Aleksunes, 2010)). Secondly, only healthy women who were not in labor and who had reached term gestation were included in this study. Based on previous studies showing that BCRP expression does not change with labor or mode of delivery (Cesarean or vaginal) (Yeboah et al., 2006; Mason et al., 2011), we speculate that our findings of negligible sampling site variability can be generalized regardless of the absence or presence of labor and the type of delivery. However, our findings cannot be generalized to the study of placentas with underlying pathologic conditions, such as preeclampsia, diabetes, or fetal growth restriction. Studies have shown that there may be intraplacental variation in overall gene expression in diseased states, such as fetal growth restriction (Tzschoppe et al., 2010). Therefore, sampling from a single site in the placenta may be sufficient to detect differences in BCRP expression between healthy placentas, whereas further studies are needed to assess site-to-site variability for BCRP expression in the setting of pathological conditions of pregnancy.

In addition to analyzing intraplacental BCRP expression, the expression of the four potential internal controls, GAPDH, RPL13a, PRL, and 18S, was also evaluated. Several studies have previously demonstrated that GAPDH and 18S are stably expressed in healthy placentas and that they can reliably be used as housekeeping genes (Patel et al., 2002; Murthi et al., 2008). The present study confirms minimal intraplacental variability in expression of these two genes. RPL13a was recently proposed as a superior reference gene in bone-marrow and placental-derived human mesenchymal stromal cells (Studer et al., 2012). Although there is no literature on the use RPL13a as
an endogenous control in placental tissue, our results demonstrate minimal site-to-site variability in its expression. On the basis of this observation, it can be suggested that RPL13a can also be used as a reference gene for placental studies. PRL expression across the placental disc was also analyzed. A previous study has shown that there is insignificant intraplacental variability in its expression (Pidoux et al., 2004). Although our results confirmed the lack of intraplacental variability, an extensive amount of interplacental variability in the expression of PRL was noted, thereby limiting its utility as a reference gene. Therefore, it can be concluded that GAPDH, RPL13a, and 18S are all suitable as internal controls for normalization in placental tissues, with GAPDH being superior to both RPL13a and 18S in this sample set.

2.6 Conclusion

To our knowledge, this is the first study that has evaluated the intraplacental variability in BCRP mRNA and protein expression in healthy, term human placentas. Our results demonstrate that BCRP mRNA and protein levels are similar across the placental disc and sampling site does not contribute to variation in its expression.
**Figure 2.1. Regional sampling of term human placentas.** Placentas were collected and processed within one hour of birth. Six regionally-defined samples (medial: $M_1$ and $M_2$, intermediate: $I_1$ and $I_2$, peripheral: $P_1$ and $P_2$) measuring 2 cm x 1 cm x 0.5 cm in dimension were collected along the long axis of the placenta. Each sample was split into three sub-segments and processed for histologic, mRNA, and protein analysis.
Figure 2.2. Histology of the medial region of term human placentas. Placenta sub-
segments were fixed in PAXgene Tissue Fix for (4 h) and then placed in PAXgene
Tissue Stabilizer and stored at -20°C prior to routine tissue processing and paraffin
embedding. Sections (5 µM) of placentas were stained with hematoxylin and eosin and
imaged by light microscopy for histopathologic examination. All placentas displayed
mostly normal histologic features of the placenta. Three representative images of the M
medial region from three placentas are shown above (A-C) (x 4 magnification). Insets
demonstrate the villous structure at a higher magnification (x 40 magnification).
Figure 2.3. Messenger RNA expression of housekeeping genes in term human placenta. Regional Ct values of four housekeeping genes (GAPDH, RPL13a, Prolactin, 18S) and BCRP in human term placenta was determined by qPCR. (A) Data
are presented as box and whisker plots with median, mean and range of the	housekeeping gene Ct values for each sampling location (medial, intermediate,
peripheral) (n=10). The average Ct value for two sites was used for each region of the
placenta (for example, average of M₁ and M₂ sites for 'Medial'). The overall median is
marked by a line and the mean is marked by a plus sign (+). The box represents 50% of
the data while the whiskers represent those data that fall in the 25th and 75th quartiles.
(B) Correlation between housekeeping gene and BCRP Ct values was determined by
linear regression.
Figure 2.4. Regional BCRP mRNA expression in term human placentas. Placental mRNA expression of BCRP was quantified by qPCR. Ct values for BCRP mRNA were first converted to delta Ct values by comparing to the reference gene, GAPDH, and then to delta delta Ct values by designating the average delta Ct values for medial samples from each placenta as the control group. Data are presented as a box and whisker plot with median, mean and range of values (n=10), relative to the medial mRNA expression (set to 1.0). The median is marked by a line and the mean is marked by a plus sign (+). The box represents 50% of the data while the whiskers represent those data that fall in the 25th and 75th quartiles.
Figure 2.5. Regional BCRP protein expression in term human placentas. Protein expression of BCRP was quantified by western blot (10 µg membrane fraction protein/lane) from placentas. β-actin was used as a loading control. The western blot data are presented as (A) a representative western blot from one placenta including human embryonic kidney-293 cells overexpressing an empty vector and wild-type BCRP, or the negative and positive control, respectively, and (B) semi-quantification of protein band density for each region normalized to the medial BCRP protein expression. Data are presented as mean ± standard error (n=10).
Table 2.1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Clinical Characteristics of Pregnancies for Placentas Studied</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gestational age</strong> (weeks)</td>
<td>Mean = 39</td>
</tr>
<tr>
<td><strong>Maternal age</strong> (years)</td>
<td>Mean = 29.3</td>
</tr>
<tr>
<td><strong>Maternal Ethnicity</strong></td>
<td>Caucasian = 4, African American = 1, Asian = 1, Hispanic = 4, Other = 0</td>
</tr>
<tr>
<td><strong>Paternal Ethnicity</strong></td>
<td>Caucasian = 3, African American = 2, Asian = 1, Hispanic = 3, Other = 1</td>
</tr>
<tr>
<td><strong>Birth weight</strong> (grams)</td>
<td>Mean = 3400</td>
</tr>
<tr>
<td><strong>Placental weight</strong> (grams)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mean = 663</td>
</tr>
<tr>
<td><strong>Baby’s sex</strong></td>
<td>Female = 4</td>
</tr>
<tr>
<td><strong>Delivery to processing</strong> (mins)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mean = 40</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results are presented as means ± standard deviation (SD)

<sup>b</sup> Wet, untrimmed weight

<sup>c</sup> Time from delivery to placement of specimen in PAXgene FIX in a PAXgene container
Supplemental Table 2.1. Real-time qPCR Taqman Gene Expression Assays.¹

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Abbreviation</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
<td>GAPDH</td>
<td>Hs02758991_g1</td>
</tr>
<tr>
<td>ribosomal protein L13a</td>
<td>RPL13a</td>
<td>Hs04194366_g1</td>
</tr>
<tr>
<td>prolactin</td>
<td>PRL</td>
<td>Hs00168730_m1</td>
</tr>
<tr>
<td>eukaryotic 18S rRNA</td>
<td>18S</td>
<td>Hs03003631_g1</td>
</tr>
<tr>
<td>ATP-binding cassette, subfamily G (WHITE),</td>
<td>ABCG2</td>
<td>Hs01053790_m1</td>
</tr>
<tr>
<td>member 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹ All primer probe assays were purchased from Applied Biosystems (Foster City, CA).
CHAPTER 3: INTERINDIVIDUAL REGULATION OF THE BCRP/ABCG2 TRANSPORTER IN TERM HUMAN PLACENTAS

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3.1 Abstract

Introduction. The “blood-placenta barrier” contains efflux transporters including the breast cancer resistance protein (BCRP/ABCG2) that limits the placental passage of potentially toxic chemicals. Regulators of BCRP expression in term human placentas including transcription factors and non-coding ABCG2 genetic variants are unknown.

Methods. To investigate the interindividual variation in the placental expression of BCRP, 108 term placentas were collected from mothers with uncomplicated pregnancies. Placental mRNA expression of BCRP and transcription factors that regulate BCRP expression was measured using multiplex branched DNA analysis. BCRP protein expression and ABCG2 genotypes were determined using western blot and Fluidigm Biomark genetic analysis, respectively. Results. Placentas were obtained from an ethnically diverse population including Caucasian (32%), African American (16%), Asian (16%), Hispanic (16%) and mixed backgrounds (20%). No relationship between infant gender and BCRP mRNA expression was observed. BCRP mRNA expression correlated with two known transcription factors NRF2 ($r^2 = 0.29$) and AhR ($r^2 = 0.26$). Furthermore, single nucleotide polymorphisms in the 5'UTR (A61562C) or second intron region (A96567G) of the ABCG2 gene were associated with a 50% reduction in BCRP mRNA expression in placentas from Asian women. Interestingly, the ABCG2 nonsynonymous polymorphism (C421A, Q141K) was not associated with altered BCRP mRNA levels, but did correspond with a 50-60% decrease in BCRP protein in placentas carrying one or two copies of the variant.

Discussion. Results from this study advance our understanding of the constitutive regulation of placental BCRP expression and may help to identify infants at risk for increased fetal exposure to chemicals that are BCRP substrates.

Keywords: BCRP, ABCG2, SNPs, placenta, transporter, Q141K
**Abbreviations**

5’ UTR, 5’ untranslated region; *ABCG2*, ATP-binding cassette subfamily G member 2; AF, allele frequency; AhR, aryl hydrocarbon receptor; BCRP, breast cancer resistance protein; CYP, cytochrome P450; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF1α, hypoxia inducible factor alpha; NRF2, nuclear factor (erythroid-derived 2)-like 2; PPARγ, peroxisome proliferator-activated receptor gamma; PBS/T, PBS with 0.5% Tween-20; PR, progesterone receptor; RPL13A, ribosomal protein L13A; SNP, single nucleotide polymorphism; SP1, SP1 transcription factor; SP3, SP3 transcription factor
3.2 Introduction

In the placenta, the breast cancer resistance protein (BCRP/ABCG2) efflux transporter plays an important role in limiting fetal exposure to chemicals including carcinogens, chemotherapy drugs, endogenous compounds, flavonoids, antibiotics, and antidiabetic drugs (Burger et al., 2004; Imai et al., 2004; Merino et al., 2005; Gedeon et al., 2006; van Herwaarden et al., 2006; Nakayama et al., 2011). BCRP is localized to the apical membrane of syncytiotrophoblasts and actively transfers compounds out of the placenta and back to the maternal circulation (Maliepaard et al., 2001; Jonker et al., 2002). Due to the critical role for BCRP in protecting the fetus from exposure to xenobiotics, it is important to identify regulators of constitutive BCRP expression and function in placenta including transcription factors and single nucleotide polymorphisms.

The promoter region of the ABCG2 gene contains response elements for various transcription factors, nuclear receptors, and steroid hormone receptors (Ee et al., 2004; Krishnamurthy et al., 2004; Tompkins et al., 2010) (Figure 3.1), suggesting that BCRP is transcriptionally regulated by these receptors. For example, polycyclic aromatic ligands of the aryl hydrocarbon receptor (AhR) enhance BCRP mRNA expression in colon adenocarcinoma cells by stimulating binding of the AhR/arylhydrocarbon receptor nuclear translocator heterodimer to the xenobiotic response elements upstream of the ABCG2 transcriptional start site (Tompkins et al., 2010). Furthermore, AhR protein expression in the human term placenta is detectable and varies between individuals (Jiang et al., 2010).

A number of single nucleotide polymorphisms (SNPs) occur in the ABCG2 gene (chromosome 4q22, ABCG2, Figure 3.2) (Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005; Poonkuzhali et al., 2008). The most well-characterized genetic
variants are localized to the coding region of the \textit{ABCG2} gene and result in amino acid changes in the subsequent BCRP protein (i.e., G34A\textrightarrow{}V12M and C421A \textrightarrow{}Q141K) (Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005). The C421A variant occurs frequently in Asian (allele frequency, AF: 0.35) and Caucasian (AF: 0.10) populations (Zamber et al., 2003; Kobayashi et al., 2005). Importantly, C421A has been associated with altered pharmacokinetics and pharmacodynamics of drugs in patients that express one or two variant alleles, likely due to reduced BCRP function in the intestine, liver and/or kidneys. For example, lung cancer patients with one mutant allele (421CA) had a 3.7-fold greater risk of developing diarrhea, a toxic side effect of the BCRP substrate gefitinib (Cusatis et al., 2006). Furthermore, healthy volunteers that were homozygous for the variant (421AA) exhibited significantly greater AUC and C\textsubscript{max} values of rosvustatin following a 20 mg oral dose (Keskitalo et al., 2009b). Due to this evidence, the International Transporter Consortium suggested that the C421A polymorphism be considered in during drug development and regulatory decision making (Giacomini et al., 2013).

Potential clinically-relevant genetic variants that occur in the non-coding region of the \textit{ABCG2} gene have begun to emerge. SNPs in the 5'-untranslated region and various intronic regions of the \textit{ABCG2} gene have been identified and associated with the variation in mRNA expression of BCRP in intestine, liver, and lymphoblast samples (polymorphism discovery resource, PDR44) (Poonkuzhali et al., 2008). Importantly, the effect of non-coding variants on BCRP expression and function in the placenta has been unexplored and warrants investigation. The purpose of this study was to identify factors that contribute to the interindividual expression of placental BCRP mRNA and protein expression including gender, transcription factor expression, ethnicity/race, and genetics.
3.3 Methods

Patient Selection

One hundred and eight placentas were obtained with written informed consent from healthy women with uncomplicated pregnancies. Criteria for inclusion in this study included healthy women, ages 18-40, term gestation, and scheduled Cesarean section without labor. Exclusion criteria were pregnancy-induced medical conditions (i.e., pregnancy-induced hypertension, preeclampsia, gestational diabetes), chronic medical conditions (i.e., hypertension, diabetes, autoimmune disorders), maternal infection, clinical chorioamnionitis, medication use (except for prenatal vitamins), maternal alcohol or drug use, and known fetal chromosomal abnormalities. Patient demographic information is listed in Table 3.1. Ethnicity and race were self-reported. Where possible, infant ethnicity and race were obtained from both maternal and paternal reporting. In the absence of paternal information, the ethnicity and race of the mother were used. This study was approved by the Institutional Review Boards of Robert Wood Johnson Medical School (RWJMS) (Protocol #0220100258) and Rutgers University (Protocol #E12-024).

Sample Collection

Placentas were collected within ten minutes of delivery for processing within one hour. Visible abnormalities and location of umbilical cord was assessed and only normal placentas with central or eccentric cord insertions were used. A small piece of umbilical cord close to the placenta was placed in a PAXgene Tissue Container (Qiagen, Germantown, MD) in the PAXgene Tissue Fix for 4 h at 4°C, moved to PAXgene Tissue Stabilizer and stored at -80°C for ABCG2 SNP genotyping. To sample placental tissue, overlying membranes, maternal decidua, and chorionic plate were removed. From the “maternal” side of the placenta, two pieces of chorionic tissue were collected along the
long axis approximately 1 cm distal to the cord insertion site as previously described (Memon et al., 2014). The dimensions of each sample were approximately 2 cm x 1 cm x 0.5 cm. After rinsing three times with PBS to remove maternal blood, each sample was further dissected into two sub-segments of equal size for RNA and protein analysis. Tissue was placed in a PAXgene Tissue Container and processed as described above. Samples were stored at -80°C until RNA analysis. For assessment of protein expression, samples were frozen in liquid nitrogen and stored at -80°C.

**Single Nucleotide Polymorphism Genotyping**

Umbilical cord samples were homogenized and total DNA was isolated with the PAXgene Tissue DNA Kit (Qiagen). A DropSense96 UV/Vis droplet reader was used to quantify total DNA and confirm the integrity of the DNA (Trinean, Gentbrugge, Belgium). The Fluidigm BioMark Genetic Analysis system was used to genotype 20 SNPs (Figure 3.2) in the *ABCG2* gene in the Bionomics Research and Technology Center at Rutgers University. Primer sequences used for *ABCG2* SNP analysis are provided in Tables 3.2-3.5.

**RNA Isolation and Multiplex Branched DNA Assay**

PAXgene stabilized placental tissue was homogenized using a TissueLyser (2 min, 50 Hz; Qiagen) in the TR1 buffer provided in the PAXgene Tissue RNA Kit (Qiagen) plus 1% β-mercaptoethanol. Total RNA was isolated with the PAXgene Tissue RNA Kit (Qiagen) according to the manufacturer’s instruction. Concentration of total RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and integrity was confirmed using a 2100 Bioanalyzer instrument (Agilent, Santa Clara, CA). A multiplex branched DNA signal amplification assay (QuantiGene Assay, Affymetrix, Santa Clara, CA) and a Bio-Plex MAGPIX multiplex reader (Bio-Rad,
Hercules, CA) were used to quantify mRNA expression of BCRP, aryl hydrocarbon receptor (AhR), estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), hypoxia inducible factor alpha (HIF1α), nuclear factor (erythroid-derived 2)-like 2 (NRF2), peroxisome proliferator-activated receptor gamma (PPARγ), progesterone receptor (PR), SP1 transcription factor (SP1), and SP3 transcription factor (SP3). Data were normalized to the geometric mean of two reference genes that were previously determined to have low correlation with placental BCRP mRNA expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L13A (RPL13A) (Memon et al., 2013). Probe information is provided in Supplemental Table 3.1.

**Western Blot**

Samples were processed for western blot analysis as previously described (Memon et al., 2013). Briefly, frozen placenta samples were homogenized in a sucrose (250 mM)-Tris base (10 mM) buffer (pH 7.5) with protease inhibitors (1%, Sigma Aldrich, St. Louis, MO). Following centrifugation (100,000 x g) for 1 h at 4 C, pellets containing crude membrane fractions were resuspended in sucrose-Tris base buffer with protease inhibitors. Protein concentrations were measured using by a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed by adding 10 μg total protein to polyacrylamide 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA) that were resolved by electrophoresis. Gels were carefully trimmed as previously described (Kiyatkin and Aksamitiene 2009) and the same size proteins were transferred to polyvinylidene fluoride membranes in overnight transfer apparatuses (BCRP: 100-60 kDa, β-Actin: 60-30 kDa; Bio-Rad Criterion Blotter, Bio-Rad, Hercules, CA). Membranes were blocked in 5% non-fat dairy milk in PBS with 0.5% Tween-20 (PBS/T) for 1 h. Primary antibodies (BCRP, BXP-53, 1:5000, Enzo Life Scientific, Famingdale, NY; β-
Actin, Ab8227, 1:2000, Abcam, Cambridge, MA) were diluted in 2% non-fat milk in PBS/T and incubated with the membranes overnight at 4°C. After washing with PBS/T, HRP-conjugated secondary antibodies were added to the blots for 1 h. Supersignal West Dura Extended Duration Substrate (Pierce Biotechnology) was used for chemiluminescent detection of proteins using a Fluorchem Imager (ProteinSimple, Sanata Clara, CA). Band densities were semiquantitated using the AlphaView Software (ProteinSimple). β–Actin was used as a loading control.

Statistical Analysis

Graphpad Prism V5 (Graphpad Software Inc., La Jolla, CA) was used to perform linear regression analysis, Mann-Whitney test (non-parametric), or Kruskal-Wallis test (nonparametric) according to the number of comparisons and variables. Statistical significance was set to p<0.05.
3.4 Results

Interindividual Expression of BCRP in Term Human Placentas

Between all individuals, placental BCRP mRNA and protein expression varied up to 6-fold (n=108) and 23-fold (n=68), respectively (Figure 3.3A and B). There were no significant differences in the BCRP mRNA or protein expression of term placentas between female and male infants (Figure 3.3C and D).

Correlation of Transcription Factor Expression with BCRP Expression

The mRNA expression of 9 transcription factors known to regulate BCRP expression (AhR, ERα, ERβ, HIF1α, NRF2, PPARγ, PR, SP1, SP3) was quantified in human term placentas (n=108). Linear regression analysis demonstrated that BCRP mRNA correlated most closely to the expression of AhR ($r^2$=0.26) and NRF2 ($r^2$=0.29) mRNAs (Figure 3.4).

Assessment of Infant Ethnicity/Race and ABCG2 Genetic Variants as Contributors to Variation in Placental BCRP Expression

While there were no significant differences in the mRNA or protein expression of BCRP between ethnicities/races, there was up to a 25% difference in the medians of BCRP protein expression between placentas of African American and Asian infants (Figure 3.5).

Twenty SNPs in the non-coding and coding regions of the ABCG2 gene were assessed in 108 human term placentas (Figure 3.2). While allele frequencies varied by SNP, intronic SNPs occurred more frequently than those in the exonic regions (Table 3.6). Overall, individuals heterozygous for the A61562C variant in the 5’ untranslated region (5’UTR) had reduced BCRP mRNA expression (data not shown), which was even more
pronounced in placentas from Asian infants (Figure 3.6). No other associations between SNPs and overall BCRP mRNA expression across all samples were observed. When separated by ethnicity/race however, significant differences were revealed. Placentas from infants of Asian descent expressing one variant allele at the 96567-gene position (Intron 2, A/G) had 50% lower placental BCRP mRNA expression as compared to a wild-type background (G/G). Interestingly, there were no individuals that were homozygous for the A61562C and A96567G genotypes in the entire sample population (n=108). Term placentas from infants of mixed ethnicity that expressed a variant allele in the 5’UTR (C46847T or C72144T) exhibited significantly decreased or increased mRNA expression of BCRP as compared to their respective wild-type counterparts (Figure 3.6). Heterozygous expression of one SNP in the 5’UTR of the ABCG2 gene (G59582A) was associated with 40% increased BCRP mRNA expression in placentas of Hispanic infants (Figure 3.6). Interestingly, no changes in BCRP protein were associated with the occurrence of ABCG2 non-coding SNPs in the sample population examined (n=68; data not shown).

Genetic variants located in the coding region of the ABCG2 gene were not associated with altered BCRP mRNA expression including the C421A variant in the 5th exon of the gene (Figure 3.7). However, at the protein level the 421A/A genotype was associated with 60% lower BCRP expression in placentas as compared to the wild-type genotype (421C/C) which was statistically significant. Of note, there was a trend for heterozygous individuals (421C/A) to have 50% lower BCRP protein expression. Interestingly, there was no correlation between BCRP mRNA and protein expression ($r^2=0.065$; Figure 3.8).
3.5 Discussion

This study characterized interindividual differences in the expression of the BCRP efflux transporter in term placentas from healthy women of ethnically and racially diverse populations. While there were no differences in BCRP mRNA or protein expression according to infant gender nor ethnicities/races, mRNA expression of BCRP correlated to the greatest extent with the mRNA expression of two transcription factors, NRF2 \( (r^2 = 0.29) \) and AhR \( (r^2 = 0.26) \). Furthermore, SNPs in the non-coding regions of the \textit{ABCG2} gene were associated with increased or decreased BCRP mRNA expression based on ethnicity. There was up to 60% reduced BCRP protein expression in term placentas expressing a common SNP located in the 5\textsuperscript{th} exon (C421A). Taken together, these data suggest that the C421A genetic variant has the greatest implications for influencing placental BCRP expression and thereby fetal exposure to potentially toxic chemicals.

We observed no significant differences in BCRP mRNA or protein expression between infant genders (\textbf{Figure 3.3C and D}). In contrast, others demonstrated that BCRP expression is more highly expressed in the intestines and livers of adult females than those of adult males (Zamber et al., 2003; Prasad et al., 2013). Differences may be due to variations in expression of regulators of BCRP expression based on developmental age including the immature secretion of sex steroid hormones (i.e., testosterone).

The promoter region of the \textit{ABCG2} gene is well-characterized and contains response elements for transcriptional regulators including AhR (Tompkins et al., 2010), ER\( \alpha \) and \( \beta \) (Ee et al., 2004), HIF1\( \alpha \) (Krishnamurthy et al., 2004), NRF2 (Singh et al., 2010), PPAR\( \gamma \) (Szatmari et al., 2006a), PR (Wang et al., 2008a), SP1 (Bailey-Dell et al., 2001), and SP3 (Yang et al., 2013). Linear regression analysis revealed that AhR and NRF2 mRNA expression exhibited the greatest correlation with BCRP mRNA expression of the
transcription factors assessed (Figure 3.6). Both transcription factors act at the promoter regions of genes involved in detoxification pathways including cytochrome P450 1A1 (CYP1A1, AhR) and NAD(P)H quinone oxidoreductase 1 (NRF2) (Kobayashi et al., 1996; Dhakshinamoorthy and Jaiswal, 2001). While placental AhR protein expression varies across individuals (Jiang et al., 2010), the interindividual expression of NRF2 in human term placentas has yet to be explored.

In a prior study, non-coding SNPs in the ABCG2 gene were first correlated with high (A61562C, G94112A) or low (G46932C, C61785T, C78551T) BCRP mRNA expression in liver, intestine, and lymphoblasts (Poonkuzhali et al., 2008). The underlying mechanisms for alterations of mRNA expression include a change in transcription factor binding (promoter region, 5'UTR) and/or disruption of gene splicing (introns) (Boccia et al., 1996; Wang and Sadee, 2015). Since the initial investigation by Poonkuzhali et al., three of these non-coding ABCG2 SNPs were linked to altered pharmacodynamics of BCRP substrates (Poonkuzhali et al., 2008). In the first intron, C78551T and A92873G were associated with the development of severe myelosuppression as a side effect in the treatment of cancer with irinotecan (Cha et al., 2009). Also in the first intronic region, the A92894C variant genotype was associated with the altered pharmacokinetics of the epilepsy drug, lamotrigine, as there was a significantly higher blood concentration in subjects that were heterozygous or homozygous for the SNP (Zhou et al., 2015).

This is the first report investigating the association of placental BCRP expression with genetic variants in the non-coding region of the ABCG2 gene. While only one SNP in the 5'UTR (A61562C) was associated with lower BCRP expression than the wild-type genotype of all individuals studied, segregation of mRNA expression data by ethnicity and genotype revealed additional associations. There was significantly lower BCRP
mRNA expression in placentas from Asian donors that were heterozygous for the A61562C genotype as well as the A96567G genotype, a novel SNP located in the 2\textsuperscript{nd} intronic region of $ABCG2$ (Figure 3.7). These data are different than those reported by Poonkuzhali et al., who associated lower (G46932C, C61785T, C78551T) or higher (A61562C, G94112A) BCRP mRNA expression with $ABCG2$ SNP genotype (2008), which may be explained by tissue differences in BCRP gene transcription. This tissue-specific effect of intronic SNPs has been reported for an intronic SNP occurring in the CYP3A4 gene as human livers had lower mRNA and expression of the intronic variant CYP3A4*22, which was not observed in the intestine (Wang and Sadee, 2015).

We also examined SNPs occurring in the coding region of the $ABCG2$ gene. Regardless of ethnicity/race, those that were homozygous for the coding region variant (C421A) had 60% less BCRP protein than the wild-type controls, with no differences in BCRP mRNA. This is in agreement with a study performed in 2005 which examined BCRP expression in placentas from healthy Japanese women (Kobayashi et al., 2005). Importantly, we did not observe a correlation between BCRP mRNA and protein expression (Figure 3.8). This is in line with other studies that found no correlation between the gene and protein expression in other normal tissues including intestine (n=42) and liver (n=65) (Zamber et al., 2003; Prasad et al., 2013). This discordance suggests that other regulatory mechanisms including micro RNA (Pillai et al., 2005) and post-transcriptional modifications (Imai et al., 2005) may play a more critical role than transcription in the regulation of BCRP protein expression and function in the placentas from women with uncomplicated pregnancies.

In summary, these data demonstrate the interindividual expression of placental BCRP in term placentas and reveal associations of BCRP with genetics as well as transcription
factor. These findings improve our understanding of the regulation of placental BCRP expression in normal pregnancy and provide the foundation for identifying individuals that may be at risk for reduced BCRP expression and function.
**ABCG2 Promoter Region**

![Diagram of ABCG2 promoter region]

**Figure 3.1.** Map of transcription factor and nuclear receptor response elements in the promoter region of the *ABCG2* gene. Location of response elements numbered with respect to the transcriptional start site (TSS, +1) and the first nucleotide of the gene (*AC084732*) (Adapted from (Basseville et al., 2014)).
**ABCG2 Gene**

Figure 3.2. Map of single nucleotide polymorphisms in the non-coding and coding regions of the *ABCG2* gene. Non-coding SNPs are numbered according to the 1st nucleotide of the gene (+1; AC084732) and coding SNPs are numbered according to the translational start site (+1) and excluding intronic nucleotides. When applicable, *ABCG2* SNPs that result in amino acid changes in the BCRP protein are denoted following the arrow (→).
Figure 3.3. Interindividual BCRP mRNA and protein expression in term human placentas. (A and C) Messenger RNA expression of BCRP was assessed in 108 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Data are presented as a (A) scatter dot plot and (C) box and whisker plot with median and range of values (n= 47 females, 58 males). (B and D) Protein expression of BCRP was assessed by western blot analysis in 68 term placentas. Data are represented as a (B) scatter dot plot and (D) box and whisker plot with median and range of BCRP protein band density normalized to the β-Actin loading control band density (n=36 females, 29 males). The box represents 50% of the data while the whiskers represent those data that fall in the 25th and 75th quartiles.
Figure 3.4. Correlation of BCRP mRNA expression with transcription factor mRNA expression. Messenger RNA expression was assessed in 108 term placentas using a multiplex branched DNA signal amplification assay. mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Correlation between BCRP mRNA expression and transcription factor expression was determined by linear regression.
Figure 3.5

A  mRNA

B  Protein

n= 18  n= 17  n= 35  n= 16  n= 22

n= 10  n= 9  n= 25  n= 11  n= 13

BCRP mRNA Expression

BCRP Protein Expression
Figure 3.5. Evaluation of placental BCRP expression by infant ethnicity/race. (A) Messenger RNA expression of BCRP was assessed in 108 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Data are presented as a box and whisker plot with median and range of values and a bar graph (n=18 African American (AA), 17 Asian (A), 35 Caucasian (C), 16 Hispanic (H), 22 Mixed Ethnicity/Race (ME/R)). (B) Protein expression of BCRP was assessed in 68 term placentas by western blot analysis. Data are represented as a box and whisker plot with median and range of BCRP protein band density normalized to the β-Actin loading control band density (n=10 African American (AA), 9 Asian (A), 25 Caucasian (C), 11 Hispanic (H), 13 Mixed Ethnicity/Race (ME/R)). The box represents 50% of the data while the whiskers represent those data that fall in the 25th and 75th quartiles. The bars represent individual placental BCRP expression.
Figure 3.6. Association of ABCG2 SNPs with BCRP mRNA expression by infant ethnicity. Messenger RNA expression of BCRP was assessed in 108 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Data are presented as a box and whisker plot with median and range of values. Sample sizes are listed underneath each genotype. Stacked genotypes listed on the X-axis represent a combination of placentas that were heterozygous and homozygous for the SNP of interest. Asterisks represent statistically significant differences (p<0.05) compared to wild-types.
Figure 3.7. Association of the C421A genotype with placental BCRP expression.

(A) Messenger RNA expression of BCRP was assessed in 108 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Data are presented as a box and whisker plot with median and range of values (n= 88 C/C, 13 C/A, 5 A/A). (B) Protein expression of BCRP was assessed in term placentas by western blot analysis. Data are represented as a western blot and box and whisker plot with median and range of BCRP protein band density normalized to the β-Actin loading control band density (n= 5 C/C, 5 C/A, 5 A/A). The box represents 50% of the data while the whiskers present those data that fall in the 25th and 75th quartiles. Asterisks represent statistically significant differences (p<0.05) compared to wild-types (C/C).
Figure 3.8. Correlation between BCRP mRNA and protein expression. Messenger RNA expression of BCRP was assessed in 68 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Protein expression of BCRP was assessed in term placentas by western blot analysis. Correlation between BCRP mRNA and protein expression was determined by linear regression.
Table 3.1

<table>
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<tr>
<th>Parameter</th>
<th>Clinical Characteristics of Pregnancies for Placentas Studied</th>
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<tr>
<td><strong>Gestational age (weeks)</strong></td>
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<td><strong>Maternal age (years)</strong></td>
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<td><strong>Birth weight (grams)</strong></td>
<td>Mean = 3298.5 SD = 671.0</td>
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<tr>
<td><strong>Placental weight (grams)</strong></td>
<td>Mean = 670.3 157.4</td>
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<td><strong>Infant gender</strong></td>
<td>Female = 47 Male = 58</td>
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</table>

*Wet, untrimmed weight

*Gender was unknown for 3 infants
Table 3.2. ABCG2 SNP allele specific primer 1 (forward) sequences used for genotyping.

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<th>ABCG2 SNP ID</th>
<th>Nucleotide Change</th>
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<tr>
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<td>A141618G</td>
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* AC084732 (1st nucleotide of gene = 1)

*Excluding intronic nucleotide
Table 3.3. **ABCG2 SNP allele specific primer 2 (forward) sequences used for genotyping.**

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<th>ABCG2 SNP ID</th>
<th>Nucleotide Change</th>
<th>Allele Specific Primer 2 (Forward)</th>
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<td>G59582A</td>
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<td>A61562C</td>
<td>AAAAAACATCAAAAAAGCAATTGCAACC</td>
</tr>
<tr>
<td>rs55930652</td>
<td>C61785T</td>
<td>GGCCACATAGTATTACAGACATGCA</td>
</tr>
<tr>
<td>rs2725226</td>
<td>C72144T</td>
<td>GCGGGTTCAAGCGATTCTACTA</td>
</tr>
<tr>
<td>rs3114020</td>
<td>T73809C</td>
<td>TGATTTACACTGCCTCCCAA</td>
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<td>rs2622604</td>
<td>C78551T</td>
<td>AAAAAACATCTCTTCTTTACTCTCTGGT</td>
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<td>A92873G</td>
<td>ACCTCACAAGGTGTCTTTAAAATTTCG</td>
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<td>rs3114018</td>
<td>A92894C</td>
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<td>G94112A</td>
<td>CTGATTCTGTCTAAATCCATTTTGC</td>
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<td>ACAACTGTTCTAATGAAACAAACAAAGAAT</td>
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<td>A141618G</td>
<td>CAGGGCAAGGTAAACAGTTTTAAATTTTC</td>
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</table>

* AC084732 (1st nucleotide of gene = 1)

*Excluding intronic nucleotides
Table 3.4. ABCG2 SNP locus specific primer (reverse) sequences used for genotyping.

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<td>G59582A</td>
<td>GACTGGTTCCTGAGCTCTGGA</td>
</tr>
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<td>rs141093520</td>
<td>A61562C</td>
<td>ACCATGCCAGCTGATGATGATG</td>
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<td>rs55930652</td>
<td>C61785T</td>
<td>CATGGAGAAACCTCTGTCTGTCTCT</td>
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<td>T73809C</td>
<td>TGATGGTTTCTATTTATAGGTCGTTTATTTGCTAACT</td>
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<td>C78551T</td>
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<td>A92873G</td>
<td>AGACTTCTTAAGAGTATCAAATAACTTATTGCTTGAATT</td>
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<td>rs3114018</td>
<td>A92894C</td>
<td>GTGGAAACCTCACAAGAGTGCTTTAAAATTC</td>
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<td>T93490C</td>
<td>CAATCTCTGCTCCTCGGCT</td>
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<td>CCTCGAATTCTGCCCTCAGT</td>
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<td>G94112A</td>
<td>ACAGGCAACCTGAGAGAGAG</td>
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<td>rs72552713</td>
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<td>rs2231142</td>
<td>C421A*</td>
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<td>A141618G</td>
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*AC084732 (1st nucleotide of gene = 1)

*Excluding intronic nucleotides
Table 3.5. *ABCG2* SNP specific target amplification primer sequences used for genotyping.

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<td>A61562C</td>
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<td>rs55930652</td>
<td>C61785T</td>
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<td>TTATTTGATTTCCAACCTGCTCC</td>
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<td>A92873G</td>
<td>TTGTTGCTTTTATTGTGGGAAACC</td>
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<td>A92894C</td>
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*a AC084732 (1st nucleotide of gene = 1)  
*Excluding intronic nucleotides
Table 3.6. Allele frequencies of SNPs separated by ethnicity/race.

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a AC084732 (1st nucleotide of gene = 1)

*Starting at translational start site (+1) and excluding intron nucleotides
### Supplemental Table 3.1. QuantiGene multiplex branched DNA assay probe information.

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<th>Sequence Length</th>
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CHAPTER 4: GENETIC AND DIETARY REGULATION OF GLYBURIDE EFFLUX
BY THE HUMAN PLACENTAL BCRP TRANSPORTER

Kristin M Bircsak, Vivek Gupta, Poi Yu Sofia Yuen, Barry I Weinberger,
Anna M Vetrano, Lauren M Aleksunes

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Jersey, Ernest Mario School of Pharmacy, Piscataway, NJ, USA (K.M.B., L.M.A.),
Department of Obstetrics and Gynecology, Rutgers University Robert Wood Johnson
Medical School, New Brunswick, NJ, USA (V.G.), Department of Pediatrics, Rutgers
University Robert Wood Johnson Medical School, New Brunswick, NJ, USA (P.Y.S.Y.,
A.M.V.), Hofstra North Shore-LIJ School of Medicine, Cohen Children's Medical Center
of New York, New Hyde Park, NY, USA (B.I.W.), Environmental and Occupational
Health Sciences Institute, Rutgers, The State University of New Jersey, Piscataway, NJ,
USA (L.M.A.)
4.1 Abstract

Glyburide is frequently used to treat gestational diabetes due to its low fetal accumulation resulting from placental efflux by the BCRP/ABCG2 transporter. Here we sought to determine how exposure to the dietary phytoestrogen genistein and expression of a loss-of-function polymorphism in the ABCG2 gene (C421A) impacted the transport of glyburide by BCRP using stably-transfected human embryonic kidney 293 (HEK) cells, human placental choriocarcinoma BeWo cells and human placental explants. Genistein competitively inhibited the BCRP-mediated transport of $^3$H-glyburide in both wild-type (WT) and C421A-BCRP HEK-expressing cells, with greater accumulation of $^3$H-glyburide in cells expressing the C421A variant. In BeWo cells, exposure to genistein for 60 min increased the accumulation of $^3$H-glyburide 30-70% at concentrations relevant to dietary exposure ($IC_{50}$ ~180 nM). Continuous exposure of BeWo cells to genistein for 48 h reduced the expression of BCRP mRNA and protein by up to 40%, which impaired BCRP transport activity. Pharmacological antagonism of the estrogen receptor attenuated the genistein-mediated downregulation of BCRP expression, suggesting that phytoestrogens may reduce BCRP levels through this hormone receptor pathway in BeWo cells. Interestingly, genistein treatment for 48 h did not alter BCRP protein expression in explants dissected from healthy term placentas. These data suggest that while genistein can act as a competitive inhibitor of BCRP-mediated transport, its ability to down-regulate placental BCRP expression may only occur in choriocarcinoma cells. Overall, this research provides important mechanistic data regarding how the environment (dietary genistein) and a frequent genetic variant (ABCG2, C421A) may alter the maternal-fetal disposition of glyburide.
Abbreviations (alphabetical order):

ABC, ATP-binding cassette; BCA, bicinchoninic acid; BSA, bovine serum albumin; BCRP, breast cancer resistance protein; cDNA, complementary DNA; DMSO, dimethylsulfoxide; EV, empty vector; ER, estrogen receptor; GD, gestational diabetes; hCG, human chorionic gonadotropin; HEK, human embryonic kidney 293; IC$_{50}$, half maximal inhibitory concentration; INN, international nonproprietary name; J$_{max}$, maximum accumulation rate; K$_m$, substrate concentration of half the maximum accumulation rate; LDH, lactate dehydrogenase; MDR1, multidrug resistance protein 1; MRP, multidrug resistance-associated protein; qPCR, quantitative polymerase chain reaction; RPL13A, ribosomal protein L13A; WT, wild-type
4.2 Introduction

Gestational diabetes (GD) is on the rise worldwide (Dabelea et al., 2005; Ferrara, 2007) and affects 5-10% of pregnant women in the United States (DeSisto et al., 2014). Glyburide (INN: glibenclamide) is a second-generation sulfonylurea drug used in the treatment of type II diabetes and more recently GD. In 2000, Langer et al. performed a prospective randomized clinical trial that demonstrated the efficacy and safety of glyburide in the treatment of GD as compared to insulin (Langer et al., 2000). Furthermore, glyburide was undetectable in cord serum (limit of detection < 10 ng/ml), suggesting limited fetal exposure. These findings launched additional investigations (Bertini et al., 2005; Jacobson et al., 2005; Anjalakshi et al., 2007; Ogunyemi et al., 2007) and ultimately a major shift in the management of GD. By 2011, over 50% of U.S. obstetricians were prescribing glyburide as first line pharmacotherapy for GD (Camelo Castillo et al., 2014).

Kraemer et al. (2006) were the first to demonstrate that glyburide is actively removed from the fetal to the maternal circulation, suggesting that transport plays a major role in the transplacental disposition of glyburide (Kraemer et al., 2006). While multiple transporters in the placenta interact with glyburide (Gedeon et al., 2006; Gedeon et al., 2008a; Gedeon et al., 2008b; Hemauer et al., 2010), there is significant evidence that points to the breast cancer resistance protein (BCRP/ABCG2) as the major transporter responsible for the active extrusion of glyburide from the placenta. This has been demonstrated using BCRP-overexpressing cells, Bcrp knockout mice, pregnant rats, human placentas and human placental vesicles (Pollex et al., 2008; Zhou et al., 2008a; Pollex et al., 2010; Feinshtein et al., 2013).
BCRP is a transmembrane protein that moves xenobiotics and endogenous chemicals out of cells. In the intestine, liver and kidney, BCRP is expressed on the apical membrane of epithelial cells and promotes the excretion of drugs and chemicals from the body (Maliepaard et al., 2001). In the placenta, BCRP is primarily localized to the apical membrane of syncytiotrophoblasts where it transports substrates back to the maternal circulation, such that fetal exposure to various chemicals including glyburide is restricted (Maliepaard et al., 2001). Although placental BCRP plays a critical role in protecting the fetus from exposure to glyburide, there is limited understanding of the potential consequences of reduced placental BCRP function in pregnant women prescribed glyburide for GD. Babies born to this population of women may be at an elevated risk of neonatal hypoglycemia as a result of fetal glyburide exposure, potentially leading to low birth weight, increased morbidity and impaired neurological development (reviewed in (Williams, 1997)). BCRP function may be compromised by genetic single nucleotide polymorphisms, as well as environmental factors, including dietary constituents.

A single nucleotide polymorphism occurring at nucleotide 421 (C>A) in the ABCG2 gene leads to an amino acid change from glutamine to lysine (Q141K). In vitro, the C421A genotype is associated with reduced BCRP function (Kondo et al., 2004; Pollex et al., 2010). Importantly, the C421A genotype occurs frequently in Asian (C/A: 30%, A/A: 10%) and Caucasian (C/A: 15%, A/A: 1%) populations (Imai et al., 2002a; Zamber et al., 2003; Kobayashi et al., 2005).

Genistein is a soy isoflavone that is found naturally in plants of the *Leguminosae* family and occurs abundantly in soybeans (2008; 2014). Considering the increasing consumption of soy-containing products (i.e., tofu, soy formula, dietary supplements)
over the last 10 years (2014), it is important to recognize that components of soy, including genistein, may interfere with the disposition of prescribed drugs. Genistein alone and in combination with other isoflavones has been shown to directly interfere with the BCRP-mediated disposition of drugs in vivo including BCRP substrates enrofloxacin and nitrofurantoin (Pulido et al., 2006; Merino et al., 2010). Genistein also interacts with proteins that may regulate placental BCRP expression, including the estrogen receptor and the epidermal growth factor receptor (Kuiper et al., 1998; Traxler et al., 1999). Considering this evidence, genistein may reduce placental BCRP function in two distinct manners, by 1) direct inhibition of BCRP activity and 2) altered transcriptional regulation of BCRP.

Due to the increased use of glyburide in GD treatment and the sensitivity of the developing fetus, it is critical to consider separately and together the influence of genetic and environmental factors on the placental BCRP-mediated disposition of glyburide. The purpose of this study was to use complementary in vitro and ex vivo model systems to characterize the molecular mechanisms by which dietary-relevant concentrations of genistein impair the transport of glyburide by BCRP in placental trophoblasts.
4.3 Materials and Methods

**Chemicals**

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell Culture**

Human embryonic kidney-293 (HEK) cells were stably transfected with an empty vector (EV, pcDNA 3.1), the human wild-type (WT) BCRP/ABCG2 gene or the human C421A BCRP/ABCG2 variant (kindly provided by Dr. Robert Robey, National Cancer Institute, National Institutes of Health, Bethesda, MD) (Morisaki et al., 2005). Cells were maintained in Dulbecco’s Modified Eagle Medium (Life Technologies, Carlsbad, CA) with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 1% penicillin-streptomycin (Life Technologies) and 0.2 mg/ml geneticin (Life Technologies) to select for transfected cells. Human placental choriocarcinoma BeWo cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in phenol-red free Dulbecco’s Modified Eagle Medium: F-12 (Life Technologies) with 10% charcoal-stripped and dextran-treated fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Life Technologies). All cells were maintained at 37°C with 5% CO₂ and used in experiments at 80-90% confluence. For the regulation studies, BeWo cells were incubated with genistein (0-10 μM) or ICI 182,780 (0-1 μM) for 48 h, after which they were processed for mRNA, protein, or functional analysis. All chemicals were dissolved in dimethyl sulfoxide (DMSO) such that the final percentage (% v/v) of DMSO did not exceed 0.1% of the treatment media.
Flow Cytometry

HEK cells overexpressing the EV, WT-BCRP or C421A-BCRP gene were suspended in cold isotonic PBS with 0.5% bovine serum albumin (BSA), centrifuged (500 g, 5 min) and washed three times in PBS with 0.5% BSA. Cells in suspension were blocked with human IgG (2 μg/500,000 cells in PBS/0.5% BSA) for 15 min at room temperature. Cells were then incubated for 45 min at 4°C with the monoclonal phycoerythrin-labeled anti-BCRP antibody (5D3) or the phycoerythrin-labeled negative control IgG antibody (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol. Cells were washed three times (5 min with PBS/0.5% BSA) and resuspended in 2% paraformaldehyde/PBS for flow cytometry analysis using a Gallios/FC500 Cytometer with 488-nm wavelength laser excitation (Beckman Coulter, Indianapolis, IN) in the Flow Cytometry/Cell Sorting Core Facility at Rutgers University.

Hoechst 33342 Accumulation Assay

The BCRP-specific fluorescent substrate, Hoechst 33342, was used to quantify BCRP function in HEK and BeWo cells as previously described (Bircsak et al., 2013). Briefly, HEK and BeWo cells were trypsinized and added to a 96-well plate. Following centrifugation (500 g, 5 min, 5°C) and removal of the media, cells were loaded with Hoechst 33342 (7-15 μM) in the presence or absence of the BCRP-specific inhibitor, Ko143 (1-3000 nM), or the test compound, genistein (0.1-100 μM) for 30 min at 37°C and 5% CO₂ (uptake phase). Cells were washed, centrifuged, and resuspended in substrate-free media with or without inhibitor for 1 h (efflux phase). During all phases, inhibitor compounds were initially dissolved in DMSO and did not exceed 1% (v/v) of the final treatment media. Following the efflux phase, cells were centrifuged, washed, and resuspended in cold PBS for quantification of intracellular fluorescence using the Cellometer Vision automated cell counter (Nexcelom Bioscience, Lawrence, MA). Cell
suspension (20 µl) was added to the cell counting chamber and each sample was analyzed using bright-field images for cell size and cell number. A VB-450-302 filter (excitation/emission: 375/450) allowed for intracellular fluorescence detection of Hoechst 33342. The total number of cells analyzed for each sample ranged from 200 to 2000. In BeWo cells, raw fluorescence intensity for each cell was normalized to cell size.

\[ ^3 \text{H-Glyburide Accumulation Assay} \]

HEK and BeWo cells were trypsinized and added to a 96-well plate. Following centrifugation (500 g, 5 min, 5°C) and removal of the media, cells were loaded with \(^3\text{H-glyburide (0.1-10 \mu M; Specific Activity: 40 \mu Ci; Perkin Elmer, Waltham, MA) in the presence and absence of the BCRP-specific inhibitor, Ko143 (1-3000 nM), or the test compound, genistein (0.01-20 \mu M) for 1 h at 37 °C and 5% CO}_2. Inhibitors were dissolved in DMSO such that the final concentration of DMSO did not exceed 1% (v/v) in the final treatment media. Following the 1 h incubation, cells were centrifuged, washed, and lysed using 1 M NaOH. Lysates were then neutralized using 1 M HCl and added to 4 ml ScintiSafe Econo 1 liquid scintillation fluid (Fisher Scientific, Waltham, MA) in 7 ml glass liquid scintillation vials (Perkin Elmer). Radioactivity was detected using a TriCarb 2100TR Liquid Scintillation Analyzer (Perkin Elmer-Packard, Waltham, MA). A standard curve was used to extrapolate glyburide concentrations. Accumulation of glyburide was normalized to the total protein concentration of cell lysates from representative wells using the bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL).

\[ \text{Western Blot} \]

HEK and BeWo cells were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and 1% protease inhibitor cocktail). Protein concentrations of cell lysates and placental explant homogenates were determined by the BCA assay.
Five to ten μg total protein were added to SDS-polyacrylamide 4-12% Bis-Tris gels (Life Technologies) which were resolved by electrophoresis. Transfer of proteins onto polyvinylidene fluoride membranes was completed using an overnight transfer apparatus (Biorad Criterion Blotter, Biorad, Hercules, CA). Membranes were blocked using 5% non-fat milk in 0.5% Tween-20-PBS, then incubated with primary antibodies in 2% non-fat milk in 0.5% Tween-20-PBS for 2 h. Primary antibodies detected proteins including BCRP (BXP-53, 1:5000, Enzo Life Sciences, Farmingdale, NY), β-Actin (Ab8227, 1:2000, Abcam, Cambridge, MA) and Na⁺/K⁺ ATPase (Ab76020, 1:20000, Abcam). The membranes were washed and species-specific HRP-conjugated secondary antibodies were added to the blots for 1 h. Supersignal West Dura Extended Duration Substrate (Pierce Biotechnology) was used for chemiluminescent detection of proteins with a Fluorchem Imager (ProteinSimple, Santa Clara, CA). Semi-quantitation of bands was performed using the AlphaView Software (ProteinSimple). β-Actin or Na⁺/K⁺ ATPase were used as loading controls where appropriate.

**RNA Isolation and Real-Time Quantitative PCR**

BeWo cells were collected in Buffer RLT provided in the RNeasy Mini Kit (Qiagen, Germantown, MD), plus 1% β-mercaptoethanol. Cells were lysed using QIAshredder columns (Qiagen), and total RNA was isolated with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Total RNA concentration and purity (260/280) were determined using a Nanodrop spectrophotometer (Fisher Scientific). Complementary DNA (cDNA) was generated from total RNA (500 ng) using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies) and a MultiGene OptiMax Thermal Cycler (Labnet International Inc., Edison, NJ). Quantitative PCR (qPCR) was performed with specific forward and reverse primers (Integrated DNA Technologies, Inc., Coralville, IA), cDNA, Sybr Green dye (Life Technologies) and a ViiA7 RT-PCR System.
(Life Technologies) in the Bionomics Research and Technology Center at Rutgers University. Ct values were converted to delta delta Ct values by comparison to ribosomal protein 13A (RPL13A) as a reference gene and the DMSO-treated control cells. Primer sequences for BCRP and RPL13A are provided in Supplemental Table 4.1.

**Patient Selection**

Written informed consent was obtained and placentas were collected from five healthy women with uncomplicated pregnancies following term delivery by scheduled cesarean section. Inclusion criteria were healthy women, ages 18-40, term gestation (≥ 36 weeks) and scheduled cesarean section without labor. Exclusion criteria included chronic medical conditions (i.e., hypertension, diabetes, autoimmune disorders), pregnancy-induced medical conditions (i.e., pregnancy-induced hypertension, preeclampsia, gestational diabetes), maternal infection, clinical chorioamnionitis, medication use (with the exception of prenatal vitamins), maternal smoking, alcohol or drug abuse and known fetal chromosomal abnormalities. All placentas expressed two wild-type alleles (C/C) at the 421 nucleotide position in the ABG2 gene as determined by a Fluidigm Biomark Genetic Analysis system in the Bionomics Research and Technology Center at Rutgers University. Other patient demographic information is provided in Supplemental Table 4.2. The Institutional Review Boards of Robert Wood Johnson Medical School (RWJMS) (Protocol #0220100258) and Rutgers University (Protocol #E12-024) approved this study.

**Placental Explants**

All placentas were obtained within 10 min of delivery and processed within 2 h. Placentas were carefully inspected for any visible abnormalities and location of umbilical cord. Only placentas with central or eccentric cord insertion were used. The maternal
decidua and the chorionic plate along the overlying membranes were removed and sections of villous tissue were washed in PBS three times to remove maternal blood before dissection into 2 x 2 x 2 mm (8 mm³) pieces of tissue. In a 24-well dish, two pieces of 8 mm³ villous tissue were cultured in DMEM:F12 media without phenol red (Life Technologies) and with 10% charcoal-stripped and dextran-treated fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Life Technologies). To allow for complete degeneration and regeneration of the syncytium as described by other laboratories (Siman et al., 2001), explants were maintained in culture for 5 days, with the media changed and collected every 24 h. On day 5, genistein (0-10 μM) treatment began for 48 h (media not changed during 48 h treatment). Genistein was dissolved in DMSO and the final DMSO percentage was 0.1% (v/v) in the treatment media. Following the 48 h treatment, explants were washed in PBS and frozen in liquid nitrogen before homogenization in sucrose (250 mM)-Tris (10 mM) buffer (pH 7.4) with protease inhibitors (1%, v/v), using the TissueLyser LT (Qiagen) according to the manufacturer’s protocol.

**hCG ELISA and LDH Assay**

Each day of explant culture, media was collected for assessment of syncytiotrophoblast function and overall tissue integrity by quantification of human chorionic gonadotropin (hCG) and lactate dehydrogenase (LDH), respectively. The hCG ELISA was completed using the manufacturer’s protocol (Calbiotech, Spring Valley, CA). Activity of LDH in the media was determined using the manufacturer’s protocol.

**Statistical Analysis**

Data are presented as mean ± SE of multiple independent experiments (n=3-5) and analyzed using Graphpad Prism 5.0 (Graphpad Software Inc., La Jolla, CA). Two-way
ANOVA with Bonferroni post-test, One-way ANOVA with Newman-Keuls post-test, or a two-tailed student's t-test were used to assess statistical significance (p<0.05) according to the number of comparisons and variables. Nonlinear regression analysis (dose-response: [log] inhibitor vs response—Variable slope (four parameters) or Michaelis-Menten) was used to determine kinetic parameters (IC\textsubscript{50}, K\textsubscript{m}, J\textsubscript{max}).
4.4 Results

Functional Inhibition of BCRP by Genistein

WT- and C421A-BCRP Overexpressing HEK Cells. Whole cell and cell surface protein expression of BCRP were assessed by western blot and flow cytometry, respectively. Three independent experiments revealed that BCRP protein expression in whole cell lysates was reduced by about 30% in C421A-BCRP expressing cells compared to WT-BCRP expressing cells (p=0.172) (Figure 4.1A). At the cell surface, BCRP protein expression in the C421A-BCRP cells was significantly decreased by 50% compared to the WT cells (Figure 4.1B). Both methods confirmed the absence of BCRP protein in HEK cells expressing the EV (data not shown).

Two substrate accumulation assays (Hoechst 33342 and ³H-glyburide) were employed to examine modulation of BCRP transport activity by pharmacological inhibition and genetic variation. Initial experiments determined the optimal substrate concentrations for use in the two accumulation assays (Hoechst 33342, 7 µM and ³H-glyburide, 10 µM unlabeled glyburide and 0.1 µM ³H-glyburide; Supplemental Fig 4.1). In the absence of inhibitor, there was a trend for the cells overexpressing the variant BCRP (C421A) to accumulate 50-100% more Hoechst 33342 or ³H-glyburide than the respective WT-BCRP cells (Figure 4.2). The BCRP-specific inhibitor Ko143 increased the accumulation of Hoechst 33342 and ³H-glyburide in a concentration-dependent manner in both BCRP genotypes (Figures 4.2A and 4.2C), demonstrating the ability of the assays to detect inhibition of BCRP activity. Similar to Ko143, genistein increased the accumulation of Hoechst 33342 and ³H-glyburide in WT- and C421A-BCRP expressing cells (Figures 4.2B and 4.2D). Confirming the involvement of the BCRP transporter in the genistein-induced substrate accumulation, EV cells lacking BCRP protein accumulated both substrates which was not altered by genistein (data not shown). It should be noted that
Ko143 significantly increased the accumulation of both BCRP substrates in the C421A-BCRP cells at lower concentrations than in WT-BCRP cells. Further, the half-maximal inhibitory concentration (IC<sub>50</sub>) value for the inhibition of <sup>3</sup>H-glyburide transport in the C421A-BCRP (10.1 ± 1.79 nM) cells by Ko143 was 50% lower than the WT-BCRP cells (21.5 ± 1.04 nM) (Table 4.1). For genistein, the IC<sub>50</sub> values for the inhibition of <sup>3</sup>H-glyburide transport in both BCRP genotypes were comparable (WT: 4.56 ± 0.15, C421A: 3.92 ± 0.73) (Table 4.1).

To determine the mechanism by which genistein inhibited the BCRP-mediated transport of glyburide, accumulation experiments were performed using varying concentrations of both glyburide and genistein. Genistein significantly decreased the K<sub>m</sub> value for glyburide transport by 80% without changing the J<sub>max</sub> in either BCRP genotypes (Figure 4.3; Table 4.2), suggesting that genistein competitively inhibited the BCRP-mediated efflux of glyburide.

**Placental BeWo Cells.** Expression and function of endogenous BCRP protein in placental BeWo cells was confirmed by western blot (Figure 4.4A) and substrate accumulation assays (Hoechst 33342 and <sup>3</sup>H-glyburide) (Supplemental Fig 4.2), respectively. Ko143 and genistein significantly increased the accumulation of both Hoechst 33342 and <sup>3</sup>H-glyburide by 30-100% in placental BeWo cells (Figure 4.4B-E). Notably, concentrations as low as 0.01 µM genistein inhibited <sup>3</sup>H-glyburide transport (IC<sub>50</sub>= 0.18 ± 0.11 µM). Prototypical inhibitors of other efflux transporters (MDR1: PSC833, 2 µM; MRPs: 25 µM MK571) did not alter the accumulation of <sup>3</sup>H-glyburide in the BeWo cells (data not shown), supporting the specific involvement of BCRP in the inhibition of glyburide transport by genistein.
Regulation of Placental BCRP Expression by Genistein

BeWo Cells. To determine whether genistein could alter the transcriptional regulation of BCRP expression, placental BeWo cells were incubated with genistein for 48 h. Compared to control cells, genistein significantly decreased the BCRP mRNA and protein expression up to 40% (Figure 4.5A and B). Furthermore, 48 h exposure to genistein (5 μM) increased the accumulation of \( ^3 \)H-glyburide by 30% in placental BeWo cells, which was comparable to the pharmacological inhibition of glyburide transport by Ko143 (100 nM) (Figure 4.5C). Notably, there was no effect of genistein on mRNA and protein expression of other efflux transporters including the multidrug resistance-associated protein 1 (MRP1) (data not shown). Because genistein is a phytoestrogen, the mechanism by which it downregulated BCRP mRNA and protein expression in BeWo cells was investigated using the estrogen receptor α antagonist ICI 182,780. Exposure to ICI 182,780 (1 μM) for 48 h did not alter BCRP protein expression; however, the combination of genistein and ICI 182,780 together abolished the down-regulation of BCRP protein expression caused by genistein alone (Figure 4.6).

Placental Explants. Additional experiments aimed to determine whether genistein-mediated down-regulation of BCRP expression could occur in native placental tissue. Explants were obtained from healthy human term placentas and allowed to undergo shedding and syncytialization for 5 days prior to treatment with genistein on days 5-7. Viability of the explants was confirmed by a decrease in LDH activity in the media over seven days (Supplemental Figure 4.3A; Siman et al., 2001). The degree of syncytialization was verified by detection of hCG in the media and by histologic analysis (Supplemental Figure 4.3B; (Siman et al., 2001)). Genistein treatment did not affect LDH activity or hCG secretion. Furthermore, BCRP protein expression was not altered by genistein (1-10 μM, 48 h) in human term placental explants (Figure 4.7).
4.5 Discussion

The present study demonstrated the influence of genetic and dietary factors on the BCRP-mediated transport of glyburide using complementary in vitro and ex vivo placenta model systems. The C421A-BCRP genotype alone reduced the transport of $^3$H-glyburide compared to the WT-BCRP control cells, while genistein competitively inhibited $^3$H-glyburide transport by BCRP to a similar extent in both genotypes. Confirming the potential for a placenta-specific glyburide accumulation, the BCRP-mediated transport of $^3$H-glyburide was inhibited by a short-term exposure (1 h) to genistein in placental BeWo cells. Moreover, BCRP mRNA, protein and function were reduced in BeWo cells exposed to genistein for 48 h; however, the same genistein exposure did not alter BCRP protein expression in normal placental explant tissues. Results from the present study aim to direct future investigations to consider genetic and environmental (dietary genistein) influences when optimizing glyburide treatment in GD patients.

In this study, we confirmed the reduced capability of the C421A-BCRP protein to transport $^3$H-glyburide in HEK cells that overexpress the mutated BCRP protein (Q141K), as compared to WT-BCRP overexpressing cells (Figure 4.2, Table 4.2). While Pollex et al. (2010) described BCRP protein cell surface expression to be equal between genotypes, we observed less total (30%) and cell surface (50%, p<0.05) BCRP protein expression in the C421A-BCRP HEK cells (Figure 4.1). Considering these findings, altered BCRP function in our experiments may be due to reduced BCRP protein trafficking to the cell surface. This is in line with other studies reporting decreased BCRP protein expression and function in C421A-BCRP overexpressing cells (Imai et al., 2002a; Kondo et al., 2004; Tamura et al., 2007; Furukawa et al., 2009; Woodward et al., 2013). In human tissue, the heterozygous variant genotype (421C/A) did not change BCRP protein expression in the intestine or placenta, but there was a significant
decrease in BCRP protein expression in placentas of individuals homozygous for the SNP (421A/A) (Zamber et al., 2003; Kobayashi et al., 2005; Urquhart et al., 2008). Taken together, there may be differences in BCRP protein expression in cells or tissue based on the copy number of the C421A gene. Furthermore, there may be a cell-type and/or tissue specific effect of C421A on BCRP protein expression. Most importantly, patients expressing one or two variant alleles exhibit increased blood concentrations (sulfasalazine, diflomotecan, rosuvastatin) and enhanced side effects (gefitinib) of drugs that are substrates for BCRP (Sparreboom et al., 2004; Cusatis et al., 2006; Urquhart et al., 2008; Keskitalo et al., 2009b). Due to these clinical findings and the frequent occurrence of the SNP, the International Transporter Consortium named the C421A BCRP genetic variant as a clinically relevant transporter polymorphism that should be evaluated in drug development studies (Giacomini et al., 2013).

Over the past 10 years there has been an increase in the sale and consumption of soy in the United States, likely due to the growing number of reports which suggest that a soy diet provides health benefits (Strom et al., 1999; Chen et al., 2003a; Hussain et al., 2003; Constantinou et al., 2005; Bitto et al., 2008; Clarkson et al., 2011; Squadrito et al., 2013). Taken together with the increase in the prescribing of glyburide for GD (Camelo Castillo et al., 2014), there is a need to investigate the impact of soy on glyburide transport. Genistein and its conjugated metabolites (genistein-sulfate and genistein-glucuronide) are substrates for BCRP, suggesting that they competitively inhibit BCRP transport (Imai et al., 2004; Enokizono et al., 2007a; Alvarez et al., 2011; Mease et al., 2012). However, others propose that genistein can non-competitively inhibit BCRP transport via disruption of ATP hydrolysis as well (Randak et al., 1999; Di Pietro et al., 2002). In both the WT-BCRP and the C421A-BCRP overexpressing cells, genistein competitively inhibited the BCRP-mediated transport of $^3$H-glyburide to similar degrees
While various studies demonstrated genistein to interfere with the transport of other BCRP substrates (i.e., mitoxantrone, enrofloxacin) (Zhang et al., 2004b; Pulido et al., 2006), this is the first report implicating genistein in the inhibition of \(^3\)H-glyburide transport by BCRP. It is important to note that Ko143 significantly reduced \(^3\)H-glyburide transport in the C421A-BCRP overexpressing cells at a lower concentration than the WT-BCRP overexpressing cells, while genistein equally inhibited \(^3\)H-glyburide transport between cell types (Table 4.1). This suggests that individuals with the C421A-BCRP genotype may be at a greater risk for chemically induced BCRP inhibition depending on the inhibitor compound.

BeWo cells are commonly used as a model of placental transport because they endogenously express functional BCRP protein and produce the placenta-specific hormone, hCG (Takeuchi et al., 1990). In placental BeWo cells, genistein increased the accumulation of Hoechst 33342 and \(^3\)H-glyburide (Figure 4.4). Interestingly, the concentrations of genistein which inhibited \(^3\)H-glyburide transport out of the BeWo cells were quite low and within the range of those observed in the plasma or serum of people consuming a soy diet (0.01- 3 µM) (Arai et al., 2000; Uehar et al., 2000; Frankenfeld et al., 2003; Gardner et al., 2009). These data provide plausibility to the assertion that genistein may be able to inhibit BCRP transport of substrates in vivo. The BCRP-specific inhibitor, Ko143, was used as a positive control to confirm the involvement of BCRP in the genistein-mediated cellular accumulation of Hoechst 33342 and \(^3\)H-glyburide.

Placental BCRP function may also be affected by chemical-mediated changes in mRNA and/or protein expression. Following 48 h exposure to genistein, BCRP mRNA and protein expression was decreased in BeWo cells compared to the vehicle treated cells (Fig 5A and B). In turn, this caused significant accumulation of \(^3\)H-glyburide in cells that
were exposed to genistein (5 μM, 48 h) (Fig 5C). Only one other study has reported a significant decrease in ABCG2 mRNA expression following an in vitro genistein exposure (15 μM, 24 h, gastric cancer cells, MGC803) (Huang et al., 2014), while many others detected no change in BCRP mRNA and/or protein expression in other cell types (Imai et al., 2004; Ebert et al., 2007; Arias et al., 2014).

In the 1960’s, genistein was recognized as a phytoestrogen due to its ability to alter reproductive function in sheep (Barrett et al., 1965) and agonize both ERα and β with a greater affinity for ERβ (Martin et al., 1978; Kuiper et al., 1998). Importantly, an estrogen response element has been identified in the promoter region of the ABCG2 gene (Ee et al., 2004). Furthermore, estradiol down-regulated BCRP protein expression in various cells and tissues including placental BeWo cells (Imai et al., 2005; Wang et al., 2006; Mahringer and Fricker, 2010), which express both ERα and ERβ (Gambino et al., 2012). To explore the involvement of the ER signaling pathway in the genistein-mediated downregulation of BCRP expression in placental BeWo cells, the ERα antagonist ICI 182,780 was used (Van Den Bemd et al., 1999; Peekhaus et al., 2004). ICI 182,780 (1 μM) alone did not alter BCRP expression but was able to prevent the down-regulation of BCRP by genistein (Figure 4.6). These findings suggest that ER signaling participates in the repression of BCRP expression in BeWo cells.

Placental explants are an ex vivo model of the human term placenta as they retain the morphology of the human placental villi, contain multiple cell types, and are frequently used in examining transporter regulation in normal human placenta (Atkinson et al., 2006; Javam et al., 2014). Genistein did not alter BCRP protein expression in human term placental explants (Figure 4.7). While these findings contrast those observed in placental BeWo cells, it is important to note that extrapolation of gene regulation findings
observed in choriocarcinoma cells to normal placenta must be made with caution. DNA methylation patterns vary between normal primary trophoblasts and choriocarcinoma cells, contributing to the overall differential gene expression and regulation profiles between the two types of cells (Novakovic et al., 2011). Altogether, results from this study indicate that the direct pharmacological inhibition of BCRP by genistein may have greater implications for BCRP-mediated transport of glyburide rather than modulation of transcription and/or translation.

The prescription of glyburide for management of GD has reached an all-time high as most initial reports found no differences in neonatal hypoglycemia rates between insulin and glyburide managed GD (Langer et al., 2000; Jacobson et al., 2005; Anjalakshi et al., 2007). More recently, Schartz et al., described a weak, but significant negative correlation between neonatal blood glucose concentration and umbilical cord blood glyburide concentration (Schwartz et al., 2015). There were interindividual differences in the umbilical cord blood concentration to maternal blood concentration ratio of glyburide for which the authors named genetic differences in the BCRP/ABCG2 gene as a likely contributor. Differences in diets, including those that are rich in soy may also contribute to this variability.

In summary, our data demonstrate that genistein inhibits the BCRP-mediated efflux of glyburide in vitro by direct inhibition and reduced protein expression in placental BeWo cells but not placental explants. To better optimize the individualized prescribing of glyburide for GD, future research should address the contribution of the C421A-BCRP genotype and genistein in the diet to the transplacental passage of glyburide.
Figure 4.1. Characterization of BCRP protein in WT- and C421A-BCRP overexpressing HEK cells. (A) BCRP protein expression in HEK whole cell lysates was determined by western blot (10 µg protein homogenate/lane). Na⁺/K⁺ ATPase was used as a loading control. Western blot data are presented as a representative western blot from one experiment. The bar graph shows the semiquantitation of band densities and represents the mean ± SE from three independent experiments. (B) BCRP protein expression on the cell surface of HEK cells was determined by flow cytometry. Cells were incubated for 45 min with the phycoerythrin-labeled anti-BCRP antibody (5D3) or the phycoerythrin-labeled negative control IgG antibody (green: WT-BCRP 5D3 stained cells, blue: C421A-BCRP 5D3 stained cells, red: WT-BCRP IgG control). Flow cytometry histogram data are presented as one representative experiment. Bar graph represents
mean ± SE fluorescence intensity of individual cells from 3 independent experiments. Daggers (†) represent statistically significant differences (p<0.05) compared to WT-BCRP.
Figure 4.2

**Hoechst 33342**

**3H-Glyburide**

**Figure 4.2**
Figure 4.2. Inhibition of BCRP transport in WT- and C421A-BCRP overexpressing HEK cells. BCRP function was assessed by measuring the cellular accumulation of (A and B) Hoechst 33342 (7 µM) or (C and D) $^3$H-glyburide (10 µM unlabeled glyburide, 0.1 µM $^3$H-glyburide) in the presence of increasing concentrations of the BCRP-specific inhibitor, Ko143 or genistein. Intracellular fluorescence and radioactivity were quantified by a Cellometer Vision or a liquid scintillation counter, respectively. Bar graphs represent mean ± SE (n=3 independent experiments). Asterisks (*) represent statistically significant differences (p<0.05) compared to 0 µM genotype control. Daggers (†) represent statistically significant differences (p<0.05) compared to WT-BCRP control.
Figure 4.3. Characterization of the genistein-glyburide interaction mediated by BCRP. BCRP function was assessed in WT- and C421A-BCRP HEK-expressing cells by measuring the cellular accumulation of $^3$H-glyburide (0-500 μM unlabeled glyburide, 0.1 μM $^3$H-glyburide) in the presence of increasing concentrations of genistein (0-10 μM) and was quantified using a liquid scintillation counter. Data represent mean ± SE (n=3 independent experiments). Nonlinear regression analysis (Michaelis-Menten) was used for curve-fitting analysis.
Figure 4.4. Inhibition of BCRP transport in placental BeWo cells. (A) BCRP protein expression in placental BeWo whole cell lysates was determined by western blot (10 µg protein homogenate/lane). β-Actin was used as a loading control. (B-E) BCRP function was assessed in the presence of increasing concentrations of Ko143 (B and D) or genistein (C and E), by the accumulation of (B and C) Hoechst 33342 (15 µM) or (D and E) ³H-glyburide (0.1 µM). Intracellular fluorescence or radioactivity was quantified using a Nexcelom Cellometer Vision or a liquid scintillation counter, respectively. Bar graphs represent mean ± SE (n=3-4 independent experiments). Asterisks (*) represent statistically significant differences (p<0.05) compared to the 0 µM control.
Figure 4.5. Regulation of BCRP transporter expression and function in placental BeWo cells. Following a 48 h exposure to genistein (0-10 µM), (A) qPCR was used to quantify BCRP and housekeeping gene, ribosomal protein L13A (RPL13A), mRNA expression in placental BeWo cells. (B) BCRP protein expression in placental BeWo whole cell lysates was determined by western blot (10 µg protein homogenate/lane). β-Actin was used as a loading control. Western blot data are presented as a representative western blot from one experiment. (C) BCRP function was assessed by the cellular accumulation of $^3$H-glyburide (0.1 µM) which was quantified using a liquid scintillation counter. All bar graphs represent mean ± SE (n=3-4 independent experiments). Asterisks (*) represent statistically significant differences (p<0.05) compared to the 0 µM genistein control.
Figure 4.6. Estrogen receptor-mediated regulation of the BCRP transporter in placental BeWo cells. BCRP protein expression in placental BeWo whole cell lysates was determined by western blot (10 µg protein homogenate/lane) following a 48 h exposure to the estrogen receptor antagonist, ICI 182,780 in the presence and absence of genistein. β-Actin was used as a loading control. All western blot data are presented as a representative western blot from one experiment. The bar graphs are the semiquantitation of band density and represent the mean ± SE from three independent experiments. Asterisks (*) represent statistically significant differences (p<0.05) compared to the 0 µM control.
Figure 4.7. Regulation of BCRP transporter expression in human term placental explants. Five healthy human term placentas were collected and processed within 2 h of delivery. Explants were cultured for 5 days with the media replaced every day, before treatment with genistein (1-10 µM) on day 5 for 48 h. BCRP protein expression in total explant lysates was determined by western blot (5 µg protein homogenate/lane). β-Actin was used as a loading control. Western blot data are presented as a representative western blot from one placenta. The scatter dot plot is the semiquantitation of band density and represent five individual placentas with mean ± SE.
Supplemental Figure 4.1. Characterization of Hoechst 33342 and $^3$H-glyburide accumulation assays in HEK cells. Intracellular accumulation of (A) Hoechst 33342 or (B) $^3$H-glyburide was determined in HEK cells overexpressing an EV or the WT-BCRP using a Cellometer Vision or liquid scintillation counter, respectively. Data are presented as mean ± SE.
Supplemental Figure 4.2. Characterization of Hoechst 33342 and $^3$H-glyburide accumulation assays in placental BeWo cells. Intracellular accumulation of (A) Hoechst 33342 or (B) $^3$H-glyburide was determined in placental BeWo cells using a Cellometer Vision or liquid scintillation counter, respectively. Data are presented as mean ± SE.
Supplemental Figure 4.3. Characterization of human term placental explant viability and trophoblast function. (A) LDH activity in explant media was monitored over 7 days to confirm placental explant viability. Degree of syncytialization was assessed by (B) detection of hCG into the media and (C) histologic analysis (20x magnification). Data are presented as mean ± SE from five individual placentas. The image is from one representative placenta.
Supplemental Figure 4.4. Visual abstract.
Table 4.1. Inhibition of $^3$H-glyburide transport in BCRP-overexpressing cells and placental BeWo cells.

<table>
<thead>
<tr>
<th>Transport IC$_{50}^{a}$</th>
<th>HEK WT-BCRP</th>
<th>HEK C421A-BCRP</th>
<th>BeWo</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ko143 (nM)</strong></td>
<td>21.5 ± 1.04</td>
<td>10.1 ± 1.79$^b$</td>
<td>3.62 ± 2.0</td>
</tr>
<tr>
<td><strong>Genistein (µM)</strong></td>
<td>4.65 ± 0.15</td>
<td>3.92 ± 0.73</td>
<td>0.18 ± 0.11</td>
</tr>
</tbody>
</table>

$^a$IC$_{50}$ value calculated using Graphpad nonlinear regression analysis (dose-response: [log] inhibitor vs response—Variable slope (four parameters))

$^b$p<0.05 compared to WT-BCRP control
Table 4.2. Kinetic parameters of glyburide transport in HEK cells overexpressing BCRP protein (WT or C421A).a

<table>
<thead>
<tr>
<th>Genistein (µM)</th>
<th>0</th>
<th>2</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT-BCRP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(K_m) (µM)</td>
<td>70.5 ± 9.8</td>
<td>12.1 ± 3.80(^b)</td>
<td>1.0 ± 0.11(^b)</td>
</tr>
<tr>
<td>(J_{\text{max}}) (pmol (^3)H-glyburide/mg protein)</td>
<td>0.69 ± 0.10</td>
<td>0.68 ± 0.07</td>
<td>0.89 ± 0.13</td>
</tr>
<tr>
<td><strong>C421A-BCRP</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(K_m) (µM)</td>
<td>14.5 ± 1.05(^c)</td>
<td>2.52 ± 1.09(^b)</td>
<td>0.61 ± 0.13(^b)</td>
</tr>
<tr>
<td>(J_{\text{max}}) (pmol (^3)H-glyburide/mg protein)</td>
<td>0.73 ± 0.12</td>
<td>0.79 ± 0.06</td>
<td>0.82 ± 0.08</td>
</tr>
</tbody>
</table>

\(a\)\(K_m\) and \(J_{\text{max}}\) values calculated using Graphpad nonlinear regression analysis (Michaelis-Menten)

\(^b\)\(p<0.05\) compared to 0 µM genotype control

\(^c\)\(p<0.05\) compared to 0 µM WT-BCRP control
Supplemental Table 4.1. Primer sequences for human BCRP/ABCG2 and RPL13A genes (5’ to 3’).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer Sequence</th>
<th>Reverse Primer Sequence</th>
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</thead>
<tbody>
<tr>
<td>ABCG2</td>
<td>ATCAGCTGGTTATCACTGTGAGGCC</td>
<td>AGTGGTTATCCTGCTTGAAGGC</td>
</tr>
<tr>
<td>RPL13A</td>
<td>GGTGCAGGTCCTGGTGCTTGA</td>
<td>GGCCTCGGGAAGGTTGGTG</td>
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Supplemental Table 4.2. Patient demographic information.

<table>
<thead>
<tr>
<th>Patient Demographic Information</th>
<th>(37-39^a)</th>
<th>(34-39^a)</th>
<th>Caucasian = 3</th>
<th>African American = 2</th>
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<tbody>
<tr>
<td>Gestational Age (weeks)</td>
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<tr>
<td>Maternal Age (years)</td>
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<tr>
<td>Maternal Race</td>
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<td>African American = 2</td>
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<tr>
<td>Paternal Ethnicity</td>
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<td>Caucasian = 3</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>African American = 2</td>
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</tr>
<tr>
<td>Birth Weight (grams)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Placental Weight (grams)</td>
<td>(552-771^a)</td>
<td></td>
<td></td>
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</tr>
<tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Male = 3</td>
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</table>

\(^a\) Results are presented as a range

\(^b\) Wet, untrimmed weight
CHAPTER 5: OVERALL DISCUSSION AND CONCLUSIONS

Summary

The overall objective of this dissertation research was to assess the contribution of genetic and dietary (genistein) factors to placental BCRP/ABCG2 expression and function. Disruption of BCRP function by genetic and/or dietary factors may enhance fetal exposure to chemicals that are substrates for placental BCRP including the gestational diabetes (GD) drug glyburide. The increase in the occurrence of GD in the United States (Ferrara, 2007) coupled with the major transition in the field of obstetric pharmacology away from insulin to glyburide for the management of GD (Camelo Castillo et al., 2014), enhances the potential for glyburide-induced hypoglycemia in fetuses and neonates.

The findings presented in the previous chapters of this dissertation support the hypothesis that dietary concentrations of genistein impair the ability of BCRP to efflux glyburide by 1) directly inhibiting transporter function and 2) reducing its protein expression in placental trophoblasts. Three specific aims were developed to address this hypothesis: 1) Characterize the intra- and interindividual expression of BCRP in human term placentas from healthy pregnancies (CHAPTERS 2 and 3), 2) Determine the impact of genetic and dietary factors on the in vitro placental transport of glyburide by BCRP (CHAPTER 4), 3) Determine whether the dietary constituent, genistein reduces the mRNA and protein expression of placental BCRP (CHAPTER 4).

The first aim characterized the intra- and interindividual expression of placental BCRP in term placentas of uncomplicated pregnancies. An initial pilot study found that BCRP mRNA and protein expression were not regionally dependent in term placentas (n=10), supporting the use of a single sampling site (medial) for assessing interindividual
regulation of placental BCRP. The medial sampling site was then used to determine contributors to the inter-individual regulation of placental BCRP expression across ethnically and racially diverse backgrounds (n=108). Analysis revealed that transcription factor mRNA expression (AhR and NRF2), ethnicity, and SNPs occurring in the non-coding and coding regions of the ABCG2 gene were associated with placental BCRP expression and thereby were contributors to the observed 6- and 23-fold BCRP differences in mRNA and protein expression, respectively. Most importantly, the SNP located in the 5th exon of the gene (C421A) occurred frequently in Asian and Hispanic populations and was associated with significantly lower BCRP protein expression in term placenta. These findings suggest that individuals expressing one or two alleles of the C421A variant have low placental BCRP protein expression which in turn may impact the function.

The second aim utilized in vitro model systems to examine the effect of the C421A genotype and the dietary soy component genistein on the BCRP-mediated transport of glyburide. Overexpression of the C421A-BCRP variant resulted in lower BCRP protein at the cell surface and a greater accumulation of $^3$H-glyburide than the WT-BCRP overexpressing cells. The soy isoflavone genistein competitively inhibited the BCRP-mediated transport of $^3$H-glyburide in both BCRP overexpressing cell lines (WT and C421A). In placental BeWo cells, genistein significantly increased the accumulation of glyburide at concentrations relevant to dietary exposure (IC$_{50}$ ~180 nM). Together, genetics and/or environment (dietary genistein) may decrease the BCRP-mediated efflux of glyburide out of the placenta, thereby increasing fetal exposure to the GD drug. The third aim determined the regulation of placental BCRP by genistein in choriocarcinoma BeWo cells and explants dissected from term placentas. A 48 h exposure to genistein downregulated BCRP mRNA and protein expression in BeWo cells which translated to a
functional deficit as the genistein-exposed cells accumulated significantly more $^{3}$H-glyburide than the vehicle-treated cells. Pharmacological antagonism of ERα mitigated the genistein-induced reduction of BCRP protein in BeWo cells. Results from the explant studies revealed that genistein (48 h) did not downregulate BCRP protein expression in normal placental tissue. These findings suggest that the ability of genistein to regulate the transcription and/or translation of placental BCRP may be restricted to choriocarcinoma cells.

**Genetics**

Of the twenty $ABCG2$ SNPs examined, only one coding variant (C421A) was associated with reduced BCRP protein expression in term placentas (Figure 3.6). Not only does the C421A variant influence the pharmacokinetics and pharmacodynamics of BCRP (Table 1.3), but it was also linked to pathologic conditions including hyperuricemia and gout (Woodward et al., 2009; Kottgen et al., 2013). This is likely due to the disrupted secretion of urate by BCRP in the brush-border membrane of the kidney proximal tubule cells (Woodward et al., 2009). The influence of the C421A variant on urate transport in the placenta has yet to be established, however maternal hyperuricemia was associated with preeclampsia and can be used to predict maternal and perinatal outcomes in preeclamptic pregnancies (Slemons and Bogert, 1917; Livingston et al., 2014).

We observed reduced protein expression and function of BCRP in HEK cells overexpressing the C421A SNP as compared to the WT-BCRP-expressing cells (Figure 4.1 and 4.2). Although we did not determine the effect of the variant on BCRP mRNA expression in HEK overexpressing cells, others have reported no difference in the BCRP mRNA expression in cells that overexpress the C421A variant versus the WT-BCRP (Furukawa et al., 2009). This is supported by our work (Figure 3.6) and others in native
placental tissue (Kobayashi et al., 2005); demonstrating significantly lower BCRP protein in placentas carrying two variant alleles and no differences in BCRP mRNA expression between genotypes. A possible explanation for low BCRP protein expression with no difference in mRNA, is the location of the non-synonymous amino acid change (Q141K) within the nucleotide binding domain (NBD) (Figure 1.6). In HEK-BCRP overexpressing cells, the C421A variant disrupted the stability of the BCRP protein and targeted it for proteasomal degradation (Furukawa et al., 2009; Woodward et al., 2013). Upon understanding the mechanism of reduced BCRP expression and function in these cells, Woodward et al., were able to use small molecules to rescue the genetic-induced downregulation of BCRP expression and function in overexpressing cells by enhancing the stability (VRT-325) and dimerization (4-phenylbutyrate) of the BCRP protein (Woodward et al., 2013). These findings suggest a potential therapeutic pathway for managing a C421A-induced functional deficit in the BCRP protein, which may be particularly beneficial to a patient population with the C421A genotype and suffering from gout.

Future research should assess the function of BCRP in placentas with the C421A genotype (421CC vs 421CA vs 421AA) using primary trophoblasts isolated from placentas and/or by placental perfusion. To determine whether this functional deficit is relevant in vivo, in patients taking glyburide to manage GD, maternal and neonatal blood could be collected at delivery to determine glyburide concentration. These findings could be correlated with C421A genotype and placental BCRP protein expression. Even further, maternal concentration of genistein can be determined and correlated with glyburide concentration.
**Genistein**

*Direct Inhibition.* This is the first report demonstrating inhibition of the BCRP-transport of glyburide by the parent isoflavone, genistein using *in vitro* models of both the C421A- and WT-BCRP genotypes ([Figures 4.2, 4.3, 4.4]). Due to the baseline deficit in BCRP function in the C421A-BCRP-expressing cells, we suspected that genistein would inhibit the BCRP-mediated transport of glyburide in these cells to a greater extent than the WT-BCRP-expressing cells. This hypothesis was supported by another study which observed significantly greater inhibition of BCRP function by the hypertensive drug telmisartan in cells overexpressing the F489L-BCRP (T1465C, exon 12) variant as compared to the WT-BCRP overexpressing control cells (Deppe et al., 2014). In our *in vitro* BCRP-overexpression system, we did not observe a similar effect with the C421A variant; instead the BCRP-mediated transport of glyburide by WT- and C421A- BCRP was equally inhibited by genistein ([Figure 4.2, Table 4.1]). This can be explained by differences in substrates and/or inhibitors used in the two studies as the wide variety of chemicals that interact with BCRP may interact with distinct regions of the protein (i.e., substrate binding domain, NBD). Additionally, the nonsynonymous change in the amino acid at position 489 occurs in the substrate binding domain within the plasma membrane, while the C421A→Q414K occurs in the NBD ([Figure 1.6]). While the Q414K amino acid change in the NBD enhances the degradation of the BCRP protein in overexpressing cells (Woodward et al., 2013), this amino acid change may also disrupt the function of the transporter. For instance, the amino acid change may directly affect the ATPase activity of BCRP by reducing the efficiency with which ATP binds to the transporter.

It is important to note that genistein is rapidly metabolized by phase II metabolizing enzymes to genistein-sulfate and genistein-glucuronide (Doerge et al., 2000; Pritchett et
which are also substrates for BCRP as demonstrated by various \textit{in vitro} and \textit{in vivo} experiments \cite{Alvarez_2011, Jiang_2012, van_de_Wetering_2012, Yang_2012}. This suggests that \textit{in vivo}, glyburide transport in the placenta is likely to be inhibited by a combination of the parent compound and the phase II metabolites, genistein-sulfate and genistein-glucuronide. Even further, soy food contains other isoflavones (i.e., daidzein, glycine) \cite{USDA_2008} that interact with BCRP \cite{Enokizono_2007a, Kobayashi_2013}, providing the possibility for a inhibition of BCRP transport by a combination of isoflavones and their phase II metabolites and is an important topic for future investigation.

Genistein (0.18 ± 0.11 μM) also inhibited the BCRP-mediated transport of glyburide in placental choriocarcinoma BeWo cells that endogenously express BCRP (Figure 4-4). The IC$_{50}$ value was within the range of concentrations observed in the plasma of individuals consuming a soy diet \cite{Arai_2000, Frankenfeld_2003, Gardner_2009}; however, the BCRP-mediated interaction between genstein and glyburide remains to be explored in a normal placental environment. BeWo cells are an adequate model of placental transport; however limitations include their representation of a 1$^{\text{st}}$ trimester model trophoblast and carcinoma phenotype \cite{Pattillo_1968}. Gestational diabetes is typically diagnosed in the 2$^{\text{nd}}$ and 3$^{\text{rd}}$ trimesters due to the timing of the glucose tolerance tests as recommended by the ACOG (24-28 weeks), suggesting that glyburide use and placental BCRP function is of particular concern during the 2$^{\text{nd}}$ and 3$^{\text{rd}}$ trimesters \cite{ACOG_2013}. Although BCRP expression across gestation is unclear \cite{Mathias_A_2005, Meyer_zu_Schwabedissen_2006, Yeboah_2006}, first trimester trophoblasts express different levels of other proteins including hCG and MDR1 compared to second and third trimester placentas \cite{Kato_1990, Mathias_A_2005}. Cancer cells are different than normal cell types in a variety of ways such that...
cancer cells are able to evade the many mechanisms of immune protection in vivo (reviewed in (Pleasance et al., 2010; Hanahan and Weinberg, 2011)). Many of these characteristics including changes in cell-to-cell interactions which may influence plasma membrane structure and thereby function of the various membrane-associated proteins and lipids (reviewed in (Kojima, 1993)), including BCRP. A placental perfusion experiment would help to better identify the effect of genistein on the fetal-to-maternal transfer of glyburide in a normal placental environment.

Because both genistein and glyburide are administered orally, it is important to address the possibility of a genistein-glyburide interaction in the intestine and liver. In the intestine, genistein (primarily the parent compound) may inhibit the transfer of glyburide out of the intestine which in turn may increase the absorption of glyburide into the portal-system and may lead to higher than normal concentrations of the drug in the liver. In the liver, genistein (and/or its conjugated metabolites) may further inhibit the excretion of the drug into the bile thereby leading to the absorption of glyburide into the circulation at higher than normal concentrations. This increased blood concentration of glyburide, still together with the genistein metabolites may reach the placenta and interact with BCRP. While genistein-inhibition of glyburide transport may equally affect maternal (i.e., intestines and liver) and fetal (i.e., placenta) tissues, occurrence of the C421A variant may be limited to the fetal tissues (mother: 421CC, father: 421AA) suggesting that disruption of glyburide transport by BCRP may occur preferentially in the placental under these circumstances of fetal-specific genetic-predisposition. In turn, if the maternal genotype is homozygous (421AA), then it is plausible to consider that reduced BCRP function in the intestines and liver would lead to increased systemic concentrations of glyburide thereby enhancing the potential for fetal exposure.
According to the biopharmaceutical drug disposition classification system (BDDCS), glyburide is a class II drug which is a class of drugs that is defined by the low solubility and high permeability properties of the drugs. The high permeability characteristics of class II drugs allow the compounds to diffuse freely into intestinal cells, however the low solubility limits intracellular concentrations at the intestinal cells (Benet, 2013). Due to the low levels of intracellular drug, efflux transporter function does not become saturated thereby leading to adequate functioning of the transporter. As a result, the efflux transporters become the driver of the overall oral bioavailability of the drug. This suggests that glyburide absorption in the intestine is driven by BCRP such that if BCRP is not functioning appropriately in the intestine, more glyburide would be absorbed into the portal circulation and potentially into the systemic circulation.

*Regulation of BCRP Expression.* In placental BeWo cells, there was a significant decrease in the mRNA and protein expression of BCRP following a 48 h exposure to genistein (Figure 4-5A and B). Even further, this resulted in impaired removal of \(^{3}\text{H}\)-glyburide from the BeWo cells (Figure 4-5C) suggesting that the transcriptional regulation of BCRP by genistein disrupts the transporter function. Due to the number of cellular proteins with which genistein interacts (i.e., estrogen receptors (nM), tyrosine kinase receptors (EGFR, IC\(_{50}\): 12 μM), AhR (10 μM, cell type specific), PPAR\(\gamma\) (5-50 μM 24 h in KS483 cells); (Akiyama et al., 1987; Kuiper et al., 1998; Dang et al., 2003; Zhang et al., 2003a), the chemical’s biological functions range from estrogenic, tyrosine kinase inhibitory, to antioxidant (Reviewed in (Polkowski and Mazurek, 2000)). Considering this and our novel evidence of genistein-mediated transcriptional regulation of BCRP function, identifying the exact mechanism which contributed to the BCRP downregulation in BeWo cells was challenging. Treatment of BeWo cells with agonists and antagonists of various signaling pathways resulted in conflicting data that were not consistent
between experiments (data not shown). The ERα antagonist, ICI,182,780 consistently rescued BCRP protein expression in BeWo cells treated with genistein for 48 h (Figure 4-6) suggesting the involvement of the ERα pathway. While genistein preferentially interacts with ERβ (IC<sub>50</sub>: 8.4 nM) as opposed to ERα (IC<sub>50</sub>: 145 nM) (Kuiper et al., 1998), the concentrations used in this experiment (1-10 µM) suggest that genistein may interact with both ERα and β.

In previous studies, the ER antagonist ICI,182,780 (1 µM) had specific and opposing effects on ERα and ERβ expression. The anti-estrogen changed the conformation of both estrogen receptors leading to increased degradation and stabilization of the ERα and ERβ receptors, respectively (Van Den Bemd et al., 1999; Peekhaus et al., 2004). Protein expression of ERs was not determined in the BeWo regulation experiments, however confirmation of the ERα degradation and ERβ stabilization in this BeWo treatment paradigm would provide credence to the hypothesis that genistein decreased BCRP expression through the estrogen receptor pathway in BeWo cells. Assessment of ER target gene expression including Cyclin-D1 and may provide additional support, however ER-responsive gene expression is tissue specific and has not yet been described in placenta. Knockdown of individual ERs using silencing RNA will verify the roles of the ER in BCRP expression in BeWo cells.

Human placentas and BeWo cells express both ERα and β (Bukovsky et al., 2003; Gambino et al., 2012) and the mRNA expression of both receptors peak in the second trimester of human placentas with a decline into the third trimester (Fujimoto et al., 2005). The authors of this paper speculate that the high circulating concentrations of estradiol in the third trimester negatively regulate ER expression in the placenta (Fujimoto et al., 2005). By IHC and western blot, ERα was preferentially expressed in the
cytotrophoblasts and associated with a lesser differentiated state, while ERβ was localized to the syncytiotrophoblasts and associated with a greater differentiated state in term placenta (Bukovsky et al., 2003). This phenomenon has not been reported in BeWo cells, however because BeWo cells represent a model of the cytotrophoblasts it is plausible that the cells express higher protein levels of ERα than ERβ. We did not determine protein expression of ERα or ERβ in the BeWo cells, however the mRNA expression of both was narrowly explored. The Ct values determined by qPCR were 28.27 (ERα) and 31.94 (ERβ) (data not shown), indicating that the mRNA expression of ERα is greater than ERβ in placental BeWo cells.

Long term (48 h) incubation of term placental explants with genistein did not alter BCRP expression (Figure 4-7). It is important to note that the effects of genistein varied between individuals suggesting that genistein differentially regulates BCRP expression depending on the individual. Major differences in the BeWo and explant model systems including epigenomic regulation of gene and protein expression (Novakovic et al., 2011) may explain the differences in the effect of genistein on BCRP expression between model systems. Additionally, explants offer a more complex system than trophoblasts alone. Capillary endothelial cells also express BCRP (Maliepaard et al., 2001) and may confound the results from the explant studies as the western blot results represent BCRP expression in the whole tissue homogenate and not the trophoblasts alone. Future research should address the regulation of BCRP expression in explants using IHC to localize BCRP expression or by using isolated primary trophoblasts. If additional studies reveal the genistein-mediated decrease in BCRP expression is specific to choriocarcinoma cells, this finding may still be important. A portion of patients with choriocarcinoma exhibit resistance to chemotherapy drugs (i.e., methotrexate) and often require multiple chemotherapeutics to effectively treat the cancer (McGrath et al., 2010).
Importantly, methotrexate is a BCRP substrate, suggesting that multidrug resistance in these patients may be aided by the addition of genistein to the combination therapy. More mechanistic studies are required, however one can speculate that genistein may selectively downregulate BCRP function in the cancer cells and may have less off-target toxic side-effects as compared to other combination therapies (methotrexate-etopside-actinomycin D).

**Overall Conclusions and Implications**

The findings from this research demonstrate the interindividual variability in placental BCRP expression as well as mechanistic data regarding the disposition of glyburide in pregnancy in the presence of genistein. While direct extrapolation to a human population must be considered with caution, this research together with those studies recommended for future investigation will help to direct clinical trials. Understanding the role for genetics and diet in the disposition of drugs during pregnancy as predisposing factors for increased fetal exposure to drugs that are BCRP substrates helps to push forward the frontiers of personalized medicine and helps to ensure the safety of mothers and their unborn children.
CURRICULUM VITAE

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Doctoral Research: The Joint Graduate Program in Toxicology, Rutgers University, Piscataway, NJ, 2010-present (research advisor: Dr. Lauren Aleksunes)

- Perform in vitro screen of pesticide interaction with efflux transporters BCRP and MDR1.
- Examine the effects of a genetic and/or chemically induced reduction in BCRP transport activity and expression in the placenta with respect to fetal exposure and accumulation of BCRP substrates.
- Evaluate regional expression of the BCRP transporter in human placenta.
- Characterize the contribution of transcription factors and SNPs to the interindividual expression of placental BCRP in term placentas.

Undergraduate Senior Capstone Experience with Honors in Chemistry: Departments of Biology and Chemistry, Washington College, Chestertown, MD, 2009-2010 (research advisors: Dr. Mindy Reynolds and Dr. Leslie Sherman)

- Determine levels and speciation of cadmium in river sediments of the Chester River in Chestertown, MD by flame atomic absorption spectroscopy.
- Expose adult and larval zebrafish to environmentally relevant concentrations of cadmium and measure behavioral and developmental changes, respectively.
**Undergraduate Research Internship:** Department of Biology, Washington College, Chestertown, MD, 2009  
(research advisor: Dr. Aaron Krochmal)  
- Surgically implant radio transmitters into *P. obsoleta* (black rat snake) and *T. sirtalis* (common gartersnake) to examine individual use of an agricultural habitat by radiotelemetry.

**Undergraduate Research Internship:** Department of Chemistry, Washington College, Chestertown, MD, 2008  
(research advisor: Dr. Rick Locker)  
- Characterize the enzyme kinetics of the oxidation of *o*-phenylenediamine (OPD) to daminophenazine (DAP) by hydrogen peroxide using a stopped-flow kinetics apparatus.

**PUBLICATIONS:**


POSTERS AND ABSTRACTS:


22. **Bircsak K**, Wang Q, Aleksunes L (2013) In Vitro Characterization of a Nutrient-Drug Interaction Mediated by the Placental BCRP Transporter. American Association for Pharmaceutical Scientists Workshop on Drug Transporters, Bethesda, MD; Pharmacy Research Day, Rutgers University, Piscataway, NJ; Delaware Valley Drug Metabolism Spring Discussion Group, Langhorne, PA


**HONORS AND AWARDS:**

2015 Most Outstanding Poster Award, Mid-Atlantic Society of Toxicology Regional Chapter, Edison, NJ

2015 American Society for Pharmaceutical and Experimental Therapeutics Graduate Student Travel Award, Boston, MA
2015  Women in Toxicology Graduate Student Achievement Award, Society of Toxicology, San Diego, CA

2014  Geoffrey Hogan Memorial Award, Mid-Atlantic Society of Toxicology Regional Chapter

2014  1st Place Reproductive and Developmental Toxicology Specialty Section Graduate Student Poster Award, Society of Toxicology, Phoenix, AZ

2012  Society of Toxicology Graduate Student Travel Award, San Francisco, CA

CONTINUING EDUCATION:

2014  Human Placenta Workshop, Queen’s University, Kingston, Ontario, Canada
2013  Course in Translational Science in Perinatal Biology, University of Texas Health Science Center, San Antonio, TX
2013  Research and Publication Ethics, Elsevier Online Webinar
2012  US FDA Workshop on DART Testing- From In Vivo to In Vitro, US FDA, Silver Spring, MD
2012  MicroRNAs in Biology and Toxicology, Society of Toxicology Annual Meeting, San Francisco, CA

MENTORSHIP:

2015  Summer Undergraduate Research Fellow (SURF) Program: Roundtable Discussion
2013-2015  Maternal-Fetal Medicine Fellow (Lissa Francois)
2013-present  Pharm.D. Research Student (Yixin Lin)
2013-2014  Toxicology, Health, and Environmental Disease (THED) High School Summer Program Instructor
2013  Pharm.D. Research Student (Ary Park)
2012-2014  Pharm.D. Honors Research Student (Qi Wang)
2012-2013  Community Outreach and Education Core (COEC) Scientific/Center for Environmental Exposures and Disease (CEED): Scientific Roundtable Discussion with Middle School Students

PROFESSIONAL AFFILIATIONS AND MEMBERSHIPS:

2014-present  American Society for Pharmaceutical and Experimental Therapeutics, Graduate Student Member
2013  American Association of Pharmaceutical Scientists, Graduate Student Member
2011-present  Society of Toxicology, Graduate Student Member
2014-2016, Graduate Student Representative for the Reproductive and Developmental Toxicology Specialty Section
2011-present  Mid-Atlantic Society of Toxicology, Student Member
2014-2016, Graduate Student Representative for MASOT
2011  National Chemistry Honor Society: Gamma Sigma Epsilon,
Honorary Member

2010-present Rutgers Association of Toxicology Students
   2014-2015, Speaker Committee Co-Chairperson
   2013-2014, President
   2012-2013, Treasurer and Graduate Student Association Representative

2009-2013 Scientific Research Society: Sigma Xi, Associate Member
2008-2013 American Chemical Society, Graduate Student Member
2008-2010 National Biological Honor Society: Beta Beta Beta, Full Member
APPENDIX 1: RECRUITMENT SCRIPT, CONSENT FORM, AND QUESTIONNAIRE

RECRUITMENT SCRIPT FOR PARTICIPATION IN A RESEARCH STUDY
PLACENTAL TISSUE SPECIMEN COLLECTION

TITLE OF STUDY: Metabolic and Transport Genes in Human Placenta and Fetal Membranes

NAME OF PRINCIPAL INVESTIGATOR: Anna M. Vetrano, Ph.D.

The following recruitment script will be spoken by the study coordinator (the principal investigator) or another member of the study team (a Co-investigator) to patients admitted for a scheduled cesarean section that are not in labor.

"Doctors at Robert Wood Johnson Medical School and Robert Wood Johnson University Hospital are conducting a research study. The title of the study is 'Metabolic and Transport Genes in Human Placenta and Fetal Membranes'. The purpose of this research is to determine how chemicals and medications are passed from mom to baby through the placenta and the surrounding tissues. This study will involve less than 30 minutes to complete a consent form and questionnaire and for the tissues to be collected by the investigators at the end of the delivery.

For this study, we are recruiting women between 18 and 40 years of age who are at least 36 weeks pregnant. Patients in this study should have had uncomplicated pregnancies. In addition, patients cannot have diabetes, high blood pressure, a fever, a condition known as preeclampsia, HIV infection, or a history of smoking, drug, or alcohol abuse.

Would you be interested and eligible to participate in this research study?"
CONSENT TO TAKE PART IN A RESEARCH STUDY
PLACENTAL TISSUE SPECIMEN COLLECTION

TITLE OF STUDY: Metabolic and Transport Genes in Human Placenta and Fetal Membranes

NAME OF PRINCIPAL INVESTIGATOR: Anna M. Vetrano, Ph.D.

This consent form is part of an informed consent process for a research study and it will give you information that will help you to decide whether you wish to volunteer for this research study. It will help you to understand what the study is about and what will happen in the course of the study.

If you have questions at any time during the research study, you should feel free to ask them and should expect to be given answers that you completely understand.

After all of your questions have been answered, if you still wish to take part in the study, you will be asked to sign this informed consent form.

The study coordinator (the principal investigator) or another member of the study team (a co-investigator) will also be asked to sign this informed consent. You will be given a copy of the signed consent form to keep.

You understand that you are not giving up any of your legal rights by volunteering for this research study or by signing this consent form.

Why is this study being done?
The purpose of this research is to determine how chemicals and medications are passed from mom to baby through the placenta and the surrounding tissues (fetal membranes including amnion, chorion, decidua).

Why have you been asked to take part in this study?
In order to test our idea, we need to recruit 300 pregnant women who will authorize the collection of placental and surrounding tissue after they have given birth.

Who may take part in this study?
Pregnant women 18-40 years old may participate in this study. Women should be healthy with uncomplicated pregnancies (no diabetes, preeclampsia, high blood pressure, infection, fever) following term (≥ 36 weeks) delivery by caesarean section and should not have a history of smoking, drug, or alcohol abuse while pregnant.
**How long will the study last and how many subjects will take part in it?**
This research will continue for 10 years. During this time we will recruit 300 pregnant women to volunteer for this study at Robert Wood Johnson University Hospital. We ask you to permit us to collect placenta and surrounding tissue after you have given birth.

**What will you be asked to do if you take part in this research study?**
The investigator will cut sections of tissue from the placenta and surrounding tissues after they have been removed from you following the birth of your baby. This will allow us to determine how the placenta’s genetic information affects the passage of chemicals between you and your baby. There is no sampling of tissue from either you or your baby. Samples are either tested immediately or frozen for analysis at a later date.

**What is the time involved in participating in this study?**
The time required for participating in this study includes 5-10 minutes to review this consent form, 15 minutes to answer the questionnaire form, and 10 minutes at the end of the delivery when the investigators will collect the tissues. The total time commitment will be less than 30 minutes.

**What are the risks and/or discomforts you might experience if you take part in this study?**
Taking tissue from the placenta and surrounding tissues after the delivery of your baby does not involve any risk or discomfort to you or your baby.

**Psychological or Social Risks Associated with Loss of Privacy**
Your privacy is very important to us and we will use many safety measures to protect your privacy. However, in spite of all the safety measures that we will use, we cannot guarantee that your identity will never become known.
- While the databases developed for this project will be coded and will not contain information that is traditionally used to identify you, such as your name, address, telephone number, or medical record number, people may develop ways in the future that would allow someone to link your medical information in our protected databases back to you. It is also possible that there could be violations to the security of the computer systems used to store the codes linking your medical information to you.
- Although your genetic information is unique to you, you do share some genetic information with your children, parents, brothers, sisters, and other blood relatives. Consequently, it may be possible that genetic information from them could be used to identify you. Similarly, it may be possible that genetic information from you could be used to help identify them. Further patterns of genetic variation also can be used by law enforcement agencies to identify a person or his/her blood relatives (for example, to establish paternity).
- There also may be other privacy risks that we have not foreseen.

**Economic Risks of Harm**
Since some genetic variations can help to predict the future health problems of you and your relatives, this information might be of interest to health providers, life insurance companies, and others. Therefore, your genetic information potentially could be used in ways that could cause you or your family economic distress.
"There are state and federal laws that protect against genetic discrimination. There is a federal law called the Genetic Information Nondiscrimination Act (GINA). In general, this law makes it illegal for health insurance companies, group health plans, and most employers to discriminate against you based on your genetic information. This law generally will protect you in the following ways: 1. Health insurance companies and group health plans may not request your genetic information that we get from this research; 2. Health insurance companies and group health plans may not use your genetic information when making decisions regarding your eligibility or premiums; and 3. Employers with 15 or more employees may not use your genetic information that we get from this research when making a decision to hire, promote, or fire you or when setting the terms of your employment. However, it does not protect you against discrimination by companies that sell life insurance, disability insurance, or long-term care insurance".

**Are there any benefits for you if you choose to take part in this research study?**
You will receive no direct benefits or payment from participating in this study. Results of your tests will not be provided to you because the information learned will be no longer of value in your prenatal care since your baby will already be delivered.

**What are your alternatives if you don’t want to take part in this study?**
Your only choice is not to take part in this study.

**Will genetic testing be performed on your placenta?**
The investigators will test the DNA for a limited number of genes involved in metabolizing and moving chemicals between you and your baby. Results of genetic tests will not be provided to you, since due to the preliminary nature of the findings, the information will not be of clinical use.

**Who will be allowed to look at your research records from this study?**
In addition to key members of the research team, the following people will be allowed to inspect parts of your research records related to this study:

- The Institutional Review Board (a committee that reviews research studies to protect people participating in research.)
- Rutgers, The State University of New Jersey.
- Department of Health and Human Services-government agency that oversees and funds research involving human beings.
- Office for Human Research Protections (OHRP) (regulatory agency that oversees human subject research).

**How will information about you be kept private or confidential?**
All efforts will be made to keep your personal information in your research record confidential, but total confidentiality cannot be guaranteed. Upon enrollment in the study, you will have a code number assigned to you and your actual name will not be used. Dr. Anna Vetrano (Principal Investigator) will keep the file linking your name to the code number for no more than 10 years after the study is closed. At that time, the linking information will be destroyed.
Your data may be used in scientific publications or presented at a professional conference. If the findings from the study are published, you will not be identified by name. Your identity will be kept confidential.

To help us protect your privacy, we have obtained a Certificate of Confidentiality from the National Institutes of Health. With this Certificate, the researchers cannot be forced to disclose information that may identify you, even by a court subpoena, in any federal, state, or local civil, criminal, administrative, legislative, or other proceedings. The researchers will use the Certificate to resist any demands for information that would identify you, except as explained below.

The Certificate cannot be used to resist a demand for information from personnel of the United States Government that is used for auditing or evaluation of Federally funded projects or for information that must be disclosed in order to meet the requirements of the federal Food and Drug Administration (FDA).

You should understand that a Certificate of Confidentiality does not prevent you or a member of your family from voluntarily releasing information about yourself or your involvement in this research. If an insurer, employer, or other person obtains your written consent to receive research information, then the researchers may not use the Certificate to withhold that information.

**Will there be any cost to you to take part in this study?**
If you agree to take part in this study, there will be no cost to you to retrieve the tissue samples for this research project.

**Will you be paid to take part in this study?**
If you agree to take part in this study, you will not be paid to participate. Your blood, tissue and health information will be used only for research purposes and will not be sold for profit. It is possible that some of the research conducted using your samples and health information may lead to the development of new medical tests and techniques, new drugs or other commercial products. Should this occur, there is no plan to provide you with any part of the profits generated from such products.

**What will happen if you do not wish to take part in the study or if you later decide not to stay in the study?**
You may choose not to be in the study. If you do choose to take part it is voluntary. You may refuse to take part or may change your mind at any time.

If you do not want to enter the study or decide to pull out of the study, your relationship with the study staff will not change, and you may do so without penalty. If you do not want to participate in the study, none of your personal information will be recorded. If you decide to participate and later change your mind, Dr. Anna Vetrano (Principal Investigator) will destroy the file linking your personal information to the samples collected. All patient identifying information and the tissue samples will be destroyed by Dr. Anna Vetrano at the end of the study (10 years).

You may also withdraw your consent for the use of your data, but you must do this in writing to Dr. Anna Vetrano. 1 Robert Wood Johnson Place, Room MEB 396, New Brunswick, NJ 08903, Phone 732-235-5697, Fax: 732-235-6609.
Who can you call if you have any questions?
If you have any questions about taking part in this study, you can call the study coordinator (principal investigator): Anna M. Vetrano, Ph.D., Division of Neonatology, Robert Wood Johnson Medical School, 1 Robert Wood Johnson Place, Room MEB 396, New Brunswick, NJ  08903, Phone 732-235-5697, Fax: 732-235-6609.

If you have any questions about your rights as a research subject, you can call: IRB Director, Institutional Review Board, 390 George St., Suite 506, New Brunswick, NJ 08901, Phone: 732-235-9806, Fax: 732-235-9810.

You will receive a copy of this consent form if you agree to participate in the research study.

What are your rights if you decide to take part in this research study?
You understand that you have the right to ask questions about any part of the study at any time. You understand that you should not sign this form unless you have had a chance to ask questions and have been given answers to all of your questions.

You have read this entire form, or it has been read to you, and you believe that you understand what has been discussed. All of your questions about this form and this study have been answered.

Subject Name:__________________________________________________________

Subject Signature:____________________________________ Date:____________

Signature of Co-Investigator:

To the best of your ability, you have explained and discussed the full contents of the study, including all of the information contained in this consent form.

Co-Investigator/Person Obtaining Consent:____________________________________

Signature:____________________________________ Date:____________
QUESTIONNAIRE FOR PARTICIPATION IN A RESEARCH STUDY
PLACENTAL TISSUE SPECIMEN COLLECTION

TITLE OF STUDY: Metabolic and Transport Genes in Human Placenta and Fetal Membranes

NAME OF PRINCIPAL INVESTIGATOR: Anna M. Vetrano, Ph.D.

This questionnaire form will ask questions about yourself and the father of your baby that will assist in our studies. Please answer questions to the best of your ability.

If you have questions at any time while completing this questionnaire, you should feel free to ask them and should expect to be given answers that you completely understand.

Please hand this form to the study coordinator (the principal investigator) or another member of the study team (a Co-investigator) after it has been completed.

How old are you? __________________________

How many weeks pregnant are you? _________________________

What ethnicity do you describe yourself as?
A) Hispanic or Latino
B) Not Hispanic or Latino

What race do you describe yourself as?
A) American Indian/Alaska Native
B) Asian
C) Black or African American
D) Native Hawaiian or Other Pacific Islander
E) Caucasian

What ethnicity does the baby’s father describe himself as?
C) Hispanic or Latino
D) Not Hispanic or Latino
E) Unknown
What race does the baby’s father describe himself as?

F) American Indian/Alaska Native
G) Asian
H) Black or African American
I) Native Hawaiian or Other Pacific Islander
J) Caucasian
K) Unknown

What medications, supplements, or herbal products are you currently taking? ___

Have you been diagnosed with, or do you have a history of any of the following?
A) Diabetes
B) Preeclampsia
C) Hypertension
D) Febrile Illness
E) HIV infection
F) Former or current smoker
G) Drug or alcohol abuse
APPENDIX 2: INHIBITION OF HUMAN MDR1 AND BCRP TRANSPORTER ATPASE ACTIVITY BY ORGANOCHLORINE AND PYRETHROID INSECTICIDES

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\textsuperscript{4}Department of Environmental and Occupational Medicine, University of Medicine and Dentistry of New Jersey, Piscataway, NJ, USA

* These two authors contributed equally as corresponding authors.
Non-Standard Abbreviations:

ATP-binding cassette (ABC); breast cancer resistance protein (BCRP);
dichlorodiphenyldichloroethane (DDD); dichlorodiphenyldichloroethylene (DDE);
dichlorodiphenyltrichloroethane (DDT); multidrug resistance protein (MDR); multidrug resistance-associated protein (MRP); P-glycoprotein (P-gp)
A-2.1 Abstract

Despite the growing evidence suggesting that pesticides contribute to chronic diseases, there is a limited understanding of how these chemicals are removed from cells and whether pesticides can alter the disposition of drugs. The present study examined the effects of two classes of insecticides (organochlorine and pyrethroid) on the ATPase activity of the human multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (BCRP) efflux transporters. Using plasma membranes from cells over-expressing MDR1 and BCRP, it was demonstrated that the organochlorine pesticide DDT (\(o,p'\)-DDT and \(p,p'\)-DDT isomers), as well as its metabolite (\(p,p'\)-DDD), inhibit both MDR1 and BCRP ATPase activity. In addition, \(p,p'\)-DDE, and two pyrethroid pesticides inhibited BCRP ATPase activity between 4 and 7 \(\mu M\). Additional research is necessary to further characterize the functional inhibition of MDR1 and BCRP activity and determine whether pesticides alter the transporter-mediated disposition of other chemicals.

Keywords: insecticides, transporter, brain, BCRP, MDR1, pesticides
A-2.2 Introduction

Each year approximately 5.2 billion pounds of pesticides are used worldwide in agricultural and residential settings (Grube et al., 2011). Human exposure to pesticides results from the environmental persistence of some chemicals including organochlorine insecticides such as dichlorodiphenyltrichloroethane (DDT) and the increasingly widespread use of newer agents such as synthetic pyrethroids (2002; Le Grand et al., 2012). Chronic exposure and elevated body burdens of pesticides has been linked to negative human health effects such as diabetes, cancer, and neurodegenerative diseases including Parkinson’s and Alzheimer’s diseases (Richardson Jr and et al., 2009; Hayden et al., 2010; Airaksinen et al., 2011; Brauner et al., 2012). Because of the high potential for exposure to pesticides in the environment, it is important to examine the cellular mechanisms that regulate the disposition of pesticides and the potential for target organ toxicity.

The ATP-binding cassette (ABC) transporters are a superfamily of transmembrane proteins that protect vulnerable organs from the accumulation of toxic compounds by actively removing them from the cell (reviewed in (Klaassen and Aleksunes, 2010)). ABC transporters move substrates out of the cell against concentration gradients using energy derived from the hydrolysis of ATP. The multidrug resistance protein 1, or P-glycoprotein (MDR1/P-gp/ABCB1), was initially found to be over-expressed in cancer cells, but was later detected in normal human tissues as well (Juliano and Ling, 1976; Thiebaut et al., 1987). MDR1 protein is localized to the apical membrane of proximal tubule cells, placental trophoblasts, hepatocytes, and capillary endothelial cells that constitute the blood-brain barrier (reviewed in (Klaassen and Aleksunes, 2010)). Substrates of MDR1 include several structurally diverse compounds such as colchicine,
doxorubicin, digoxin, imatinib, prazosin, and verapamil (Ueda et al., 1987; Tanigawara et al., 1992; Shapiro et al., 1999; Mahon et al., 2003). Similar to MDR1, the breast cancer resistance protein (BCRP/ABCG2) is strongly expressed on the apical membranes of cells in the placenta, liver, mammary glands, kidneys, and brain (reviewed in (Klaassen and Aleksunes, 2010)). BCRP transports chemicals such as mitoxantrone, glyburide, cimetidine, and sulfasalazine (Doyle et al., 1998; van der Heijden et al., 2004; Pavek et al., 2005; Gedeon et al., 2006). MDR1 and BCRP also exhibit an overlap in substrate specificity, including the anticancer drug imatinib and the antihypertensive drug prazosin (Burger et al., 2004; Enokizono et al., 2008).

A number of pesticides have been reported to interact with MDR1 as substrates and/or inhibitors. Incubation of human HepG2 hepatoma cells with the organochlorine pesticide \( p,p' \)-DDT resulted in the cellular retention of the MDR1 substrate, rhodamine 123, suggesting that \( p,p' \)-DDT inhibits MDR1 activity (Shabbir et al., 2005). Interestingly, the DDT metabolite, \( p,p' \)-dichlorodiphenyldichloroethylene (\( p,p' \)-DDE) did not alter the retention of rhodamine 123 in HepG2 cells (Shabbir et al., 2005). Inhibition of MDR1 function by micromolar concentrations of another organochlorine pesticide endosulfan as well as the pyrethroid pesticide permethrin has been demonstrated in mouse melanoma cells (B16/F10) over-expressing the human MDR1 transporter (Bain and Leblanc, 1996). In addition, ATPase activity can be stimulated by endosulfan and another pyrethroid pesticide cypermethrin in plasma membranes from the Chinese hamster ovary cells that contain the MDR1 transporter, indirectly suggesting that these pesticides are substrates for MDR1. Furthermore, in reconstituted proteoliposomes containing the hamster MDR1 protein, endosulfan (50 μM) and cypermethrin (70 μM) each inhibited ATPase activation stimulated by the MDR1 substrate, tetramethylrosamine (Sreeramulu et al., 2007). In
comparison to MDR1, little is known about whether pesticides can act as substrates and inhibitors of BCRP.

The purpose of this study was to determine whether organochlorine and pyrethroid pesticides and/or metabolites can activate and/or inhibit human MDR1 and BCRP ATPase activity in plasma membrane fractions (Figure A-2.1). Knowledge of the \textit{in vitro} interactions of pesticides with MDR1 and BCRP will provide guidance on how transporters may contribute to the overall disposition and toxicity of pesticides.
A-2.3 Materials and Methods

Chemicals

All pesticides were purchased from Chem Service (West Chester, PA). Unless specified, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

ATPase Assay

The human MDR1 and BCRP PREDEASY ATPase Kits were purchased from Xenotech (Lenexa, KS) and used to determine the activation and inhibition of transporter-dependent ATPase activity by pesticides. The manufacturer provided data sheets for each ATPase Kit validating the basal (MDR1: 8-16 nmol Pi/min/mg protein; BCRP: 7-20 nmol Pi/min/mg protein) and stimulated (MDR1: 30-60 nmol Pi/min/mg protein; BCRP: 25-50 nmol Pi/min/mg protein) ATPase activity Activation. To determine activation, plasma membranes from Spodoptera frugiperda (Sf9) cells transfected with human MDR1 or BCRP were incubated (37°C) in 96-well plates with assay medium, 2 mM ATP, and pesticides (0.03-100 µM) in the presence and absence of 1.2 mM sodium orthovanadate for 30 minutes. Sodium orthovanadate inhibits ABC transporter ATPase activity and is used to calculate vanadate-sensitive activity by subtraction from the total ATPase activity. Inhibition. Inhibition was analyzed in a similar manner, with the addition of specific MDR1 and BCRP activators (MDR1 40 µM verapamil; BCRP 10 µM sulfasalazine) to the assay medium. Following incubation (37°C) of the membranes with a colorimetric reagent, liberation of inorganic phosphate was detected by absorption at 610 nm using a Spectramax spectrophotometer (Sunnyvale, CA). Concentration-response experiments for the activation and inhibition studies of MDR1 (activation: verapamil, inhibition: cyclosporin A) and BCRP (activation: sulfasalazine, inhibition: Hoechst 33342) were also performed. DMSO was used to prepare pesticide stock solutions (5 mM) and its concentration in the final reaction mixture did not exceed 2%. 
All incubations were performed in triplicate. The basal (MDR1: 8-16 nmol P/min/mg protein; BCRP: 7-20 nmol P/min/mg protein) and stimulated (MDR1: 30-60 nmol P/min/mg protein; BCRP: 25-50 nmol P/min/mg protein) ATPase activity was consistent with the manufacturer data sheets for each ATPase kit. In this study data for the basal activity were subtracted from the stimulated transporter ATPase activity.

**Data Analysis**

Data are presented as mean ± standard error of three determinations from one experiment. Non-linear regression analysis was performed for activation and inhibition experiments using the GraphPad Prism V5 software program (GraphPad, La Jolla, CA). Data generated from compounds that activated ATPase activity were fit to a curve using Michaelis-Menten kinetics for the calculation of $V_{\text{max}}$ and $K_m$, the maximum velocity and concentration at half of $V_{\text{max}}$, respectively. Compounds that inhibited ATPase activity were analyzed by fitting the data to a concentration-response-inhibition curve to generate IC$_{50}$ values, or the concentration of half the maximal inhibition. $R^2$ values were calculated to determine the goodness of fit.
A-2.4 Results

Characterization of ATPase assays

Prototypical activators and inhibitors of MDR1 and BCRP ATPase activity were used to confirm specific transporter activity. The MDR1 substrate, verapamil, activated MDR1 ATPase activity at concentrations over 0.5 μM with maximal activation observed at 10 μM (Figure A-2.2A, $V_{\text{max}}$: 17.3 nmol P$_i$ released/min/mg protein, $K_m$: 1.9 μM). Activation of MDR1 by 40 μM verapamil was inhibited by cyclosporin A at concentrations as low as 0.3 μM (Figure A-2.2C, IC$_{50}$: 0.7 μM). Likewise, the BCRP substrate, sulfasalazine, stimulated BCRP ATPase activity (Figure A-2.2B, $V_{\text{max}}$: 24.0 nmol P$_i$ released/min/mg protein, $K_m$: 0.40 μM) which was inhibited by co-incubation with Hoechst 33342 (Figure A-2.2D, IC$_{50}$: 3.1 μM).

Effect of pesticides on basal and stimulated MDR1 and BCRP ATPase activity

Of the ten pesticides screened for interaction with MDR1 and BCRP, none of the compounds stimulated baseline ATPase activity (data not shown). Four organochlorine pesticides and/or metabolites (o,p'-DDT, p,p'-DDT, p,p'-DDD, and endosulfan) inhibited MDR1 ATPase activity (Figure A-2.3). All four compounds yielded IC$_{50}$ values ranging from 3.8 μM (p,p'-DDT) to 33.6 μM (endosulfan) when analyzed by nonlinear regression (Table 2.1). These same four compounds along with p,p'-DDE, and two pyrethroids (permethrin and resmethrin) inhibited BCRP-mediated ATP hydrolysis (Figure A-2.4). Of these seven pesticides, the inhibitory potency, or IC$_{50}$ values, ranged from 3.0 μM to 10.4 μM (Table 2.1). Cypermethrin, deltamethrin, and dieldrin did not inhibit stimulated MDR1 or BCRP ATPase activity (data not shown).
**A-1.5 Discussion**

The current study investigated the interactions of ten insecticides and/or metabolites with human MDR1 and BCRP using isolated plasma membrane fractions. Overall, seven of the chemicals inhibited MDR1 and BCRP ATPase activity suggesting that they reduce the transport of MDR1 and/or BCRP substrates. This is important because reduced activity of ABC transporters by chemicals has been found to alter the disposition and subsequently toxicity of substrates in vivo. For example, the antiparasitic drug metabolite, trichlabendazole sulfoxide, increased the plasma concentration of BCRP substrate, sulfasalazine, in wild-type mice as compared to BCRP knockout mice (Barrera et al.). Similarly, a chemical interaction (insecticide Spinosad® and antiparasitic ivermectin) mediated by MDR1 resulted in elevated brain levels of ivermectin in dogs, which has been associated with neurotoxicity (Marques-Santos et al., 1999; Dunn et al., 2011). Such findings highlight the importance of evaluating chemical interactions mediated by transporters.

Our data support previous research demonstrating $p,p'$-DDT and endosulfan as inhibitors of human MDR1 transport function (Shabbir et al., 2005; Pivčević and Žaja, 2006; Sreeramulu et al., 2007). Also in accordance with previous work, we found limited interaction of $p,p'$-DDE with human MDR1 (Bain and Leblanc, 1996; Shabbir et al., 2005). Despite the structural similarities between $p,p'$-DDT and $p,p'$-DDE, differences in the interactions with MDR1 may be due to the double bond present between the two main chain carbons of $p,p'$-DDE. This is supported by the inhibition of MDR1 ATPase activity by two structurally similar $p,p'$-DDT analogues, $o,p'$-DDT and $p,p'$-DDD, that do not contain the double bond. In the present study, neither permethrin nor cypermethrin altered MDR1 ATPase activity stimulated by verapamil. This observation is in contrast to previous reports that suggested both pyrethroids inhibit or stimulate MDR1 function.
These divergent results may be due to the use of different substrates (doxorubicin and tetramethylrosamine) and/or species (hamster) of MDR1 in the model system (Bain and Leblanc, 1996; Sreeramulu et al., 2007).

BCRP ATPase activity was inhibited by the same four organochlorine pesticides and metabolites that inhibited MDR1 ATPase activity, along with \( p,p' \)-DDE and two of the pyrethroid pesticides, permethrin and resmethrin. Despite the overall inhibitory effect of the organochlorine and pyrethroid pesticides on BCRP, the neonicotinoid pesticide imidacloprid did not interact with BCRP, demonstrating specificity of the assay (data not shown). In addition, we tested the ability of \( o,p' \)-DDT, \( p,p' \)-DDT, \( p,p' \)-DDD, and endosulfan to inhibit the activation of the human multidrug resistance-associated protein 2 (MRP2) transporter. All four chemicals produced only modest inhibition of MRP2 suggesting that the broad overlap in organochlorine-transporter interaction is specific to MDR1 and BCRP and is not a result of global ATPase inhibition (data not shown).

None of the pesticides activated ATPase activity alone indicating that the compounds are likely not substrates for either transporter. However, our laboratory has observed false negative results in this assay. Specifically, we have observed activation of MDR1 by the antipsychotic drug, risperidone but not its metabolite paliperidone (9-hydroxyrisperidone) (data not shown), despite the fact that both chemicals are MDR1 substrates (Wang et al., 2004b). Further works is necessary to interpret IC50 values from transporter ATPase assays to concentrations achievable from occupational and environmental exposures to pesticides. Considering the shortcomings of subcellular assays, the ATPase assay should be used as a screening method to investigate potential interactions of chemicals with ABC transporters. Quantification of substrate accumulation in cell over-expression systems and \textit{in vivo} pharmacokinetic studies
should be conducted following screening with the ATPase assay to directly identify substrates of ABC transporters and the \textit{in vivo} implications of chemical interactions mediated by transporters. Future attention should also be placed on how combinations of pesticides can additively or synergistically inhibit transport activity.

Further work is necessary to interpret IC$_{50}$ values from transporter ATPase assays to concentrations achievable from occupational and environmental exposures to pesticides. Considering the shortcomings of subcellular assays, the ATPase assay should be used as a screening method to investigate potential interactions of chemicals with ABC transporters. Quantification of substrate accumulation in cell over-expression systems and \textit{in vivo} pharmacokinetic studies should be conducted following screening with the ATPase assay to directly identify substrates of ABC transporters and the \textit{in vivo} implications of chemical interactions mediated by transporters. Future attention should also be placed on how combinations of pesticides can additively or synergistically inhibit transporter activity.

In conclusion, a number of the insecticides tested inhibited MDR1 and BCRP ATPase activity revealing the potential role of pesticides in altering the disposition and accumulation of other chemicals. Future research should further characterize the interaction of the seven pesticides of interest with MDR1 and BCRP to better understand the influence of pesticides on xenobiotic disposition.
Figure A-2.1. Structure of organochlorine and pyrethroid insecticides or metabolites.
Figure A-2.2. Prototypical activation and inhibition of MDR1 and BCRP ATPase activity. (A, B) Membranes were incubated with ATP and varying concentrations of activator (MDR1: verapamil, BCRP: sulfasalazine) in the presence and absence of sodium orthovanadate for 30 minutes. (C, D) Membranes were incubated with ATP, activator (verapamil 40 μM; sulfasalazine 10 μM), and varying concentrations of inhibitor (MDR1: cyclosporin A, BCRP: Hoechst 33342) in the presence and absence of sodium orthovanadate for 30 minutes. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Data are presented as the vanadate-sensitive ATPase activity of three replicates (mean ± SE) from one experiment. $R^2$ values demonstrate the goodness of fit of the curves generated by non-linear regression analysis.
Figure A-2.3. Inhibition of MDR1 ATPase activity by insecticides. MDR1 membranes were incubated with ATP, verapamil (40 μM), and varying concentrations of test insecticide, in the presence and absence of sodium orthovanadate for 30 minutes. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Data are presented as the vanadate-sensitive ATPase activity of three replicates (mean ± SE) from one experiment. R² values demonstrate the goodness of fit of the curves generated by non-linear regression analysis.
Figure A-2.4. Inhibition of BCRP ATPase activity by insecticides. BCRP membranes were incubated with ATP, sulfasalazine (10 μM), and varying concentrations of test...
organochlorine (A) and pyrethroid (B) insecticides, in the presence and absence of sodium orthovanadate for 30 minutes. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Data are presented as the vanadate-sensitive ATPase activity of three replicates (mean ± SE) from one experiment. $R^2$ values demonstrate the goodness of fit of the curves generated by non-linear regression analysis.
Table A-2.1. Inhibition of MDR1 and BCRP ATPase activity by insecticides\(^a\).

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>IC(_{50}) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDR1</td>
</tr>
<tr>
<td>(o,p')-DDT</td>
<td>7.8</td>
</tr>
<tr>
<td>(p,p')-DDT</td>
<td>3.8</td>
</tr>
<tr>
<td>(p,p')-DDD</td>
<td>10.7</td>
</tr>
<tr>
<td>(p,p')-DDE</td>
<td>-</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>-</td>
</tr>
<tr>
<td>Endosulfan</td>
<td>33.6</td>
</tr>
<tr>
<td>Cypermethrin</td>
<td>-</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>-</td>
</tr>
<tr>
<td>Permethrin</td>
<td>-</td>
</tr>
<tr>
<td>Resmethrin</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Data are presented as IC\(_{50}\) values calculated by non-linear regression analysis (n=3) from one experiment. Dashes (-) indicate that an IC\(_{50}\) value was unable to be generated for the insecticide-transporter interaction.
APPENDIX 3: ASSESSMENT OF DRUG TRANSPORTER FUNCTION USING FLUORESCENT CELL IMAGING

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A-3.1 Abstract

ATP-binding cassette (ABC) proteins, including the breast cancer resistance protein (BCRP) and the multidrug resistance proteins (MDRs), actively transport structurally diverse chemicals from a number of tissues. Moreover, transporters are being increasingly cited as mediators of clinically relevant drug-drug interactions. The potential outcomes of concomitantly administering two drugs that interact at the same transporter include altered disposition and toxicity and/or efficacy of one or both of the drugs. Research demonstrating the role of transporters in clinical pharmacokinetics has shed light on the need for in vitro screening methods that detect drug-transporter interactions during preclinical development. This paper describes a cell-based model for the detection of functional inhibitors of BCRP and MDR1 by measuring fluorescent substrate accumulation in suspended cells that overexpress or endogenously express these proteins using an automated cell counter. An alternate protocol is provided describing the use of a spectrophotometer with fluorescence detection capabilities to identify functional inhibitors of BCRP and MDR1 in transporter overexpressing cells. While a spectrophotometer is available in most laboratories, an automatic cell counter offers convenience, sensitivity, and speed in measuring the cellular accumulation of fluorescent substrates and identification of novel inhibitors.

Keywords: ABC transporter, MDR1, BCRP, ABCB1, ABCG2
**A-2.2 Introduction**

The role of ATP-binding cassette (ABC) efflux transporters as mediators of drug-drug interactions is currently a topic of great interest due to the potential for altered drug disposition, therapeutic efficacy, and toxicity. ABC transporters, such as the multidrug resistance protein 1 (MDR1/ABCB1) and the breast cancer resistance protein (BCRP/ABCG2), are transmembrane proteins that transport chemicals out of the cell with energy derived from the hydrolysis of ATP. Expressed in a number of tissues, transporters contribute to the excretion of chemicals (such as in the intestine, liver, kidneys) as well as to the protection of sensitive organs (such as in the brain and placenta) (reviewed in (Klaassen and Aleksunes, 2010)). The chemicals that interact with ABC transporters as substrates and/or inhibitors include a wide variety of xenobiotics such as pharmaceuticals and environmental chemicals ((Tanigawara et al., 1992; Gedeon et al., 2006; van Herwaarden et al., 2006)). Chemotherapeutic agents such as doxorubicin ((Shen et al., 2008)) and vinblastine ((Hammond et al., 1989)), as well as the cardiac glycoside, digoxin ((Tanigawara et al., 1992)), interact with MDR1. Examples of BCRP specific substrates and/or inhibitors include the antibiotic drug nitrofurantoin (Wright et al., 2011)), hypoglycemic drug glyburide (Gedeon et al., 2008b)) and dietary carcinogens, such as heterocyclic amines and aflatoxin B1 ((van Herwaarden et al., 2006)). Many drugs exhibit substrate specificity for only MDR1 or BCRP, however there are some chemicals that interact with both transporters such as the blood pressure medication prazosin and the chemotherapy drug topotecan ((de Vries et al., 2007; Zhou et al., 2009)). The significant effect of transporters on the pharmacokinetics of new chemical entities *in vivo* has encouraged the publication of a report by the International Transporter Consortium that describes the importance of screening for drug-transporter interactions and provides initial guidelines for evaluating transporter function during drug development testing (Giacomini et al., 2010)).
Chemicals that are functional inhibitors of ABC transporters can interfere with the transport of substrates by competitive or non-competitive inhibition (Giacomini et al., 2010). The functional inhibition of transporters can be determined by measuring the accumulation of a fluorescent substrate in cells that overexpress the ABC transporter of interest in the presence and absence of the test chemical. Detection of fluorescent substrates presents advantages over radioactive and analytical (i.e., mass spectrometry) methods including the sensitive detection of fluorescent substrates, relatively low cost, and convenience. Visualization of fluorescent substrate retention may be performed using a fluorescence microscope which does not provide a quantitative measure. A spectrophotometer with fluorescence detection capabilities has been used as a quantitative measure of fluorescent substrate accumulation (Ozvegy-Laczka et al., 2004; Barthomeuf et al., 2005), however the procedure utilizes cell lysates rather than whole cells and the overall sensitivity of detection is lower. A more sensitive method, flow cytometry, has been used previously to detect and quantify the intracellular accumulation of fluorescent substrates in the presence of ABC transporter inhibitors (García-Escarp et al., 2004; Ivnitski-Steele et al., 2008; Kim et al., 2012). While flow cytometry is able to measure the fluorescence intensity of individual cells with optimal sensitivity, the high cost, and required access to a Core Facility emphasize the need for additional simple and user-friendly methods for the identification of functional inhibitors of ABC transporters.

This unit describes methods for detecting the effect of test chemicals on the function of ABC transporters using fluorescent dyes in MDR1- and BCRP-overexpressing cell lines as well as cell lines endogenously expressing both transporters. A fluorescence detection method that utilizes an automated cell counter, the Cellometer® Vision (Nexcelom Bioscience, Lawrence, MA), was demonstrated to be similarly effective at
identifying ABC transporter inhibitors as flow cytometry ((Robey et al., 2011)). The Cellometer® Vision offers sensitivity, rapid detection of intracellular fluorescence intensity, convenience of use, and is cost effective. The Basic Protocol includes a step-by-step procedure of the method introduced by Robey et al. (2011) for quantifying transporter function in cells overexpressing an ABC transporter by measurement of intracellular fluorescent substrate retention the Cellometer® Vision. Alternate Protocol 1 describes a similar method for quantifying transporter function in cells that endogenously express ABC transporters. For laboratories without access to the Cellometer® Vision, alternate instructions for fluorescence detection in lysates of cells overexpressing an ABC transporter using a 96-well plate format and a microplate spectrophotometer are also provided.

NOTE: All protocols using human-derived cells must follow appropriate blood-borne pathogen procedures approved by an Institution.
A-3.3 BASIC PROTOCOL: Measurement of Transporter Function in ABC Transporter-Overexpressing Cells using an Automated Fluorescent Cell Counter

This protocol provides a detailed account of the steps involved in the quantification of ABC transporter function in suspended cells using an automated cell counter, the Cellometer® Vision. The Cellometer® Vision is able to detect the effect of specific ABC transporter inhibitors on the accumulation of a fluorescent substrate quickly and with great sensitivity. Because the Vision has interchangeable fluorescence optic modules, a wide variety of chemicals that fluoresce (excitation/emission) at 375/450 nm, 475/535 nm, 525/595 nm, and other wavelengths can be used. Fluorescent substrates and positive control inhibitors including recommended concentrations for this procedure are listed in Table 1 for the MDR1 and BCRP transporters. This basic protocol focuses on analysis of chemical transport by a single transporter over-expressed in a cell line that exhibits low basal expression and activity of other transporters. A cell line transfected with an empty vector plasmid can be used as a control. This approach allows an investigator to focus on a single transporter protein and investigate novel chemicals as potential inhibitors. Because this method uses overexpressing cells, one can expect significant differences in substrate fluorescence intensity between the active and the inhibited transporter.

Materials

Appropriate transporter-overexpressing cell lines:

- *Laboratory-Generated Cell Lines*: Cells can be transfected with plasmids containing various ABC efflux transporters using Lipofectamine™ and other transfection reagents. Plasmids for the various ABC efflux
transporters as well as empty vector controls can be purchased at Origene Technologies (Rockville, MD) as well as other companies.

- **Commercially-Available Cell Lines:** Companies have already prepared cells that overexpress various ABC transporters which are licensed for use. Examples include the SB MDCKII BCRP transfected cell line, SB MDCKII MDR1 transfected cell line, SB HL60 MRP1 selected cell line, and SB K562 MRP1 selected cell line from Solvo Biotechnology (Boston, MA).

Complete cell culture medium

Cell dissociation medium (i.e., 0.25% Trypsin)

Dimethylsulfoxide (DMSO)

Fluorescent substrates:

- Rhodamine 123 (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO, 10 mM stock
- Hoechst 33342 (Sigma-Aldrich, St. Louis, MO), dissolved in deionized water, 10 mM stock

Positive control inhibitors:

- PSC833 (Xenotech, Lenexa, KS), dissolved in DMSO, 1mM stock
- Ko143 (Sigma-Aldrich, St. Louis, MO), dissolved in DMSO, 1 mM stock

Test inhibitors, dissolved in DMSO, 1 mM and 10 mM stock solutions for each chemical

Two clear 96-well round bottom microtiter plates with lids (one plate to balance the centrifuge rotor)

Microtiter plate centrifuge set to 5ºC, 500 x g for 5 min.
Tubes (amber-colored if available)
  - One 15 ml tube
  - Eleven 1.5 ml tubes
  - Ten 2 ml tubes

Cell culture incubator (37°C, 5% CO₂)

Automatic serological pipettor

Multichannel (8) automatic pipettor (100-1000 μl)

Multichannel (8) manual pipettor (5-50 μl)

Chilled phosphate-buffered saline (PBS)

Ice

Aluminum foil to cover plate

Paper towels

Cellometer® Vision and computer software

Cellometer® counting chamber slides

Cellometer® Vision fluorescence optics modules
  - VB-450-302 (Ex/Em: 375/450 nm for Hoechst 33342) and/or
  - VB-595-502 (Ex/Em: 525/595 nm for Rhodamine 123)

Prepare chemicals

1. Prepare stock solutions of a fluorescent substrate (FS) and positive control inhibitor (I) (Table A-3.1). Selection of the appropriate FS and I depends on the transporter of interest.

2. Choose two test inhibitors and prepare two stock solutions for each inhibitor in DMSO at concentrations of 1 mM and 10 mM.
3. Dilute FS in 7 ml complete cell culture medium to the desired final concentration in a 15 ml tube (Table A-3.1). 

   Remember to limit the light exposure of the fluorescent substrates until the end of the experiment by keeping the lights dim, using amber-colored tubes, or covering the tubes and/or plate with aluminum foil.

   a. Aliquot the volumes of FS solution into 1.5 ml tubes labeled 1-10 (Figure A-3.1, Table A-3.2). NOTICE that tube 11 is a negative control, which should receive the indicated volume of complete cell culture medium without FS.

4. Add test inhibitors (or I) and DMSO to the tubes (1-11) from step 3a (Table A-2.2). The final concentration of DMSO will be 1%. When complete, place solutions to the side of the cell culture hood and out of the light.

**Prepare cells**

5. Add 2 ml of cell dissociation medium to confluent cells overexpressing empty vector or the transporter of interest in T75 cell culture flasks for 3-4 min or until cells begin to detach from the flask.

6. Add 6 ml of complete cell culture medium to each flask, pipette cells and medium into 15 ml tubes and centrifuge at 400 x g for 4 min at room temperature.

7. Remove supernatant (containing cell dissociation medium) from the tube without disturbing the cell pellet. Then resuspend cells in 10 ml of fresh medium.
8. Determine the concentration of each cell type (transporter overexpressing and empty vector control) using the cell count function on the Cellometer® Vision and dilute to 500,000 cells/ml.

*Ideally, you will need at least 6 ml of cell suspension (500,000 cells/ml) for each cell type in order to plate the appropriate number of cells. It is possible to use lower concentrations of cells (>100,000 cells/ml), however, this will result in fewer cells for imaging. Keep in mind that cells will be lost during the experiment. A pilot experiment varying cell concentration may target the optimal concentration of cells for a specific cell line.*

9. Add 200 μl of diluted cells to the appropriate wells of a clear 96-well round bottom plate according to Figure 2.

10. Centrifuge the plate at 500 x g for 5 min at 5°C. *Do not forget to balance the centrifuge rotor with a second plate.* 5°C was chosen to ensure the transporters are inactive in between incubations.

11. Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove excess medium from the surface of the plate *(Video A-3.1).*
**Loading phase**

12. Add 100 µl of previously prepared solutions (Table A-3.2) to the appropriate wells (Figure 2) (4 wells/treatment).

13. Cover plate with lid and incubate in cell culture incubator for 30 min at 37ºC and 5% CO₂ (keep dark).

*Most cell types will remain in suspension during incubation. If the cells attach to the walls of the 96-well plate during incubation this will not alter the course of the experiment, however care should be taken at the end of the experiment to adequately resuspend the cells in PBS (see Step 28). During the incubation, chill the medium in a 4ºC refrigerator or on ice.*

**Prepare for efflux phase**

14. Label ten 2 ml tubes A-J (Table A-3.3).
   a. Add 500 µl chilled medium to tubes A-H.
   b. Add 1000 µl chilled medium to tube I.
   c. Add 2000 µl chilled medium to tube J.

15. Prepare tubes (A-J) with and without inhibitors and DMSO (Table A-3.3). The final concentration of DMSO will be 1%.

16. After the 30 min incubation has ended, centrifuge the plate at 500 x g for 5 min at 5ºC.
If the centrifuge is in a different room than the cell culture hood, keep plate on ice and covered with aluminum foil when walking between laboratories. The low temperature and foil will assure that the transporters remain inactive during this time and that the fluorescent substrates are not quenched by light.

17. Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove medium from the surface of the plate (Video A-3.1).

18. Add 100 μl chilled medium to each well using a multichannel (8) automatic pipettor. This will wash the cells of residual substrate and/or inhibitor.

   The chilled medium ensures that the transporters will not begin to efflux substrates prior to the designated efflux phase. If a multichannel automatic pipettor is unavailable, keep the plate on ice while adding chilled medium.

19. Centrifuge the plate at 500 x g for 5 min at 5°C.

20. Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove medium from the surface of the plate (Video A-3.1). Keep the plate on ice until 60 min incubation at 37°C (Step 22).
**Efflux phase**

21. Add 100 μl of previously prepared inhibitor solutions (Table A-3.3) to appropriate wells of the 96-well plate (Figure A-3.2). *Note: FS (row A) and No FS (row G) receive Tube J, medium with no inhibitor.*

22. Cover plate with lid and incubate in cell culture incubator for 60 min at 37°C and 5% CO₂ (keep dark). *During incubation, chill PBS in 4°C refrigerator or on ice.*

**Prepare to measure intracellular fluorescence**

23. After the 60 min efflux incubation, centrifuge the plate at 500 x g for 5 min at 5°C.

24. Throw the medium into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove medium from the surface of the plate (Video A-3.1).

25. Place the plate on ice and rinse cells by adding 100 μl chilled PBS to each well using a multichannel (8) automatic pipettor.

26. Centrifuge the plate at 500 x g for 5 min at 5°C.

27. Throw the PBS into a biohazard waste container with a forceful flick. Blot the 96-well plate with a paper towel to remove PBS from the surface of the plate (Video A-3.1).

28. Place the plate on ice for the remainder of the experiment and resuspend the cells in up to 50 μl of PBS by pipetting up and down with a multichannel manual
pipettor to mix cells and lift from the bottom of the well. If cells attach to the plate, use extra force to remove the cells from the bottom of the wells by individually pipetting each well to ensure the cells are in suspension. After resuspended in PBS, the cells should no longer attach to the plate because the plate will be on ice for the remainder of the experiment.

**Measure intracellular fluorescence using the Cellometer® Vision**

29. Turn on the Cellometer® Vision and open the Cellometer® Vision computer software.

30. From the dropdown menu choose the appropriate ABC transporter assay (Hoechst 33342 or Rhodamine 123) and make sure the appropriate fluorescence optics module is installed in the instrument (Hoechst 33342: VB-450-302; Rhodamine 123: VB-595-502).

31. The parameters for both assays should be:

a. Cell type: Choose cell type (will not distinguish between overexpressing and empty vector cell types) from dropdown menu. Common cell types available with the Cellometer® Vision software include: Human embryonic kidney 293 (HEK) cells, Madin-Darby canine kidney epithelial (MDCK) cells, Human promyelocytic leukemia cells (HL60), and Human erythromyeloblastoid leukemia cells (K562). If the desired cell type is not listed use ‘Initial cell type’ which uses a wide range of parameters that fit many different types of cells. However, if greater specificity is desired, it is possible to add the new cell type with the appropriate parameters by
taking an image of your cells with the Cellometer® Vision and
electronically sending it to specialists at Nexcelom Bioscience. After
characterization, the cell type (and parameters) can be remotely added to
the cell type dropdown menu on your computer. Be sure to send the
image a few days before performing the experiment as it may take some
time for Nexcelom to generate the parameters and add the cell type to the
computer program.

b. Imaging Mode: Brightfield and Fluorescence

c. Dilution: 1.0

d. Brightfield exposure: 10.9 ms.

e. Fluorescence exposure: 2.0 s. Alter fluorescence exposure time based on
signal observed when previewing the F1 image (see Step 35). The range
should not be less than 90%.

32. Load 20 μl of cell suspension from one well of the 96-well plate (on ice) into one
chamber of a Cellometer® counting chamber slide (Figure A-3.3).

33. Insert loaded chamber slide into the Cellometer® Vision and preview brightfield
image. Focus the brightfield image with the knob located on the right side of the
Cellometer® Vision.

34. Stop preview brightfield image.
35. Preview F1 image and if range is greater than 90%, click count.

36. After counting, an excel file is generated containing size and fluorescence intensity data for every individually recognized cell that fits the parameters of the chosen cell type. The excel file along with brightfield and fluorescent images of the cells can be exported to save.

37. Repeat steps 32-36 for the remaining wells taking care to cover the plate from the light in between sample retrieval.
A-2.4 ALTERNATE PROTOCOL 1: Measurement of Transporter Function in Cells that Endogenously Express ABC Transporters using an Automated Fluorescent Cell Counter

This alternate protocol describes the use of the Cellometer® Vision to analyze fluorescent chemical transport in cells that endogenously express ABC transporters. Use of cells that normally express ABC transporters, rather than cells that are genetically manipulated to express the proteins at high levels may bring one a step closer to understanding how a specific ABC transporter functions in the presence of other normal cellular activities occurring in that cell type (i.e., other transporters and drug metabolizing enzymes). In order to delineate the specific role of one ABC transporter on the transport of a substrate, it is important to first characterize the expression and function of all of the transporters that are present. When choosing an appropriate fluorescent substrate for functional assays, co-incubation of the cells with a specific inhibitor for each ABC transporter that is present is necessary to understand the contribution of individual transporters to the transport of the fluorescent substrate. Once a substrate is identified, a dose-response experiment should be performed to determine the concentration of substrate that elicits the greatest difference in fluorescence intensity in the presence and absence of the specific transporter inhibitor. After the substrate concentration is optimized, steps from the Basic Protocol can be followed to measure the effect of two test inhibitors on the transport of a fluorescent substrate using the Cellometer® Vision automated cell counter. Keep in mind that you will not need wells for empty vector cells, eliminating half of the wells on the 96-well plate and half of the solutions that are required. If your chemical requires a different fluorescence optics module (Ex/Em: 475/535 nm; i.e., Calcein AM), be sure to install the correct one prior to using the Cellometer® Vision.
A-2.5 ALTERNATE PROTOCOL 2: Measurement of Transporter Function in ABC Transporter-Overexpressing Cells using a 96-well Plate Fluorescence Reader

Without access to an automated cell counter, measuring intracellular fluorescence intensity of suspended cells that overexpress an ABC transporter using a 96-well plate fluorescence reader may similarly identify potential inhibitors of ABC transporter function. Disadvantages of this method include lower sensitivity as well as a lack of data that reports cell size and the frequency distribution of cell fluorescence intensity that is provided by the Cellometer® Vision. However, since most laboratories have access to a 96-well plate reader with fluorescence detection, this method may provide sufficient results. Additionally, previous work has identified tyrosine kinase inhibitors and the coumarin cnidiadin, as inhibitors of BCRP and MDR1 function, respectively, using a multiplate reader to measure the intracellular accumulation of fluorescent substrates in cell overexpression systems (Ozvegy-Laczka et al., 2004; Barthomeuf et al., 2005).

This alternate protocol provides modifications to the Basic Protocol to measure intracellular fluorescent substrate accumulation using a 96-well plate fluorescence reader.

The concentration of fluorescent substrate used should be similar to the recommendations in Basic Protocol 1 (Table A-3.1), however an initial dose-response experiment should be performed to determine the concentration of fluorescent substrate that can be altered by a prototypical inhibitor (MDR1: PSC-833, BCRP: Ko143) with the greatest degree of transporter inhibition. The materials and steps described in Basic Protocol 1 may otherwise be followed with a few alterations: (1) two 96-well flat bottom plates (one for each test inhibitor) must be used instead of round bottom plates; (2) a different plate set-up providing a greater sample size for each cell type and treatment
due to the decreased sensitivity of the instrument and variability within groups. An additional row of cells should be plated to determine the final concentration of cells within each well in order to properly report the data (i.e., fluorescence intensity/10^5 cells); (3) after step 27, lyse the cells in 50 μl of 50% methanol rather than resuspending in PBS; (4) read the plate at the appropriate excitation and emission wavelengths for each substrate (Hoechst 33342: 355/465 nm; Rhodamine 123: 511/534 nm) using a 96-well plate reader with fluorescence detection such as a SpectraMax Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA). Of note, the cells should be washed very well before lysis.
A-3.6 Commentary

Background Information

ABC efflux transporters influence the pharmacokinetics of many structurally diverse chemicals. As their name implies, these transporters require ATP to remove chemicals from the tissues in which they are located including the intestines, liver, kidneys, brain, and placenta (reviewed in (Klaassen and Aleksunes, 2010)). There is increasing evidence for the role of efflux transporters in the disposition and subsequently efficacy and/or toxicity of xenobiotics in vivo. In particular, there is a need for thorough investigations of substrate-transporter interactions particularly with new chemical entities (Giacomini et al., 2010)). Currently, clinical transporter-drug interactions are beginning to be listed on the package inserts for drugs such as the immunosuppressant Sandimmune® (cyclosporine). The package insert indicates that up to 35 drugs, each when taken concomitantly with cyclosporine, may alter the plasma concentration of cyclosporine as a result of interacting with MDR1 and/or the drug metabolizing enzyme, cytochrome P450 3A4 (2012)). Additionally listed are the effects of cyclosporine on the toxicity of other drugs that are transported by MDR1 such as digoxin. Digoxin is a cardiac glycoside with a narrow therapeutic index. Impaired clearance of digoxin because of MDR1 inhibition may increase the risk for cardiac toxicities including ventricular tachycardia and atrioventricular block (Dorian et al., 1988)). Cyclosporine has also been observed to increase the exposure of patients taking the chemotherapy drug Hycamtin® (topotecan) by 2- to 3-fold when the two drugs are taken together (2011a)). The BCRP transporter has similarly been recognized as a potential mediator of clinically relevant drug-drug interactions considering the overlap in the chemicals that interact with the transporter. The growing list of drugs that interact with MDR1 and
BCRP emphasizes the particular relevance of screening for drug-transporter interactions during preclinical development.

This paper describes cell-based methods for determining the effect of test inhibitors on ABC transporter (BCRP and MDR1) function. This can be accomplished by measuring fluorescent substrate accumulation within the cells that have been incubated with and without the test inhibitors. **Table A-3.4** lists additional fluorescent substrates and positive control inhibitors that are often used in measuring ABC transporter function by flow cytometry including chemicals that interact with another class of transporters, the multidrug resistance-associated proteins (MRPs). If a particular substrate of interest is not available as a fluorescent molecule, radiolabeled substrates may be used in a similar cell-based model to measure the intracellular accumulation of the radioactive chemical in the presence of a test inhibitor. Drawbacks of the model include the high cost of working with radioactivity, the need for custom synthesis of a substrate radioisotope if the substrate is not commercially available, and the potential for isotope binding to the filter plate and/or cellular constituents which may confound the results. An alternate *in vitro* model for studying chemical-transporter interactions includes the inverted membrane vesicles that are isolated plasma membranes from cells that overexpress a particular ABC transporter. The vesicle model system may not be ideal for measuring certain chemical interactions, as it is not a cell-based model and therefore cannot indicate if a metabolite may interact with the transporter. Flow cytometry utilizes cells in suspension and fluorescent substrates to detect chemical-transporter interactions, however this method can often require access to a Core Facility. The automated cell counter, the Cellometer® Vision (Nexcelom Bioscience, Lawrence, MA) is able to rapidly measure the intracellular substrate fluorescence within cells that overexpress or endogenously express ABC transporters. Unlike a 96-well plate fluorescence reader, the Cellometer®
Vision is a more sensitive measure of fluorescence intensity within cells, which ensures that the measure is a reflection of intracellular substrate accumulation. Experiments performed using the Cellometer® Vision exhibit between 15.5-28.5% standard deviation for fluorescent substrates commonly used in our laboratory using human embryonic kidney 293 (HEK) cells overexpressing BCRP, demonstrating the repeatability of the procedure (Table 5). For a modest cost, laboratories can purchase their own Cellometer® Vision which may be an affordable and convenient alternative to flow cytometry. The Cellometer® Vision has also been used to examine how transcriptional regulation may increase or decrease the functional activity of ABC transporters in brain microglia cells (Gibson et al., 2012). With the ability of the Cellometer® Vision to measure fluorescence intensity of individual cells, another potential application of the automated cell counter may include the detection of cell surface expression of ABC transporters to determine transcription efficiency using fluorescently-labeled antibodies. Similarly, if transfection plasmids include the gene that codes for green fluorescent protein, transfection efficiency may be monitored with the Cellometer® Vision using the appropriate fluorescence optics module (VB 535-402).

**Critical Parameters and Troubleshooting**

Recommended substrate concentrations are provided for the Basic Protocol using the ABC transporter-overexpressing cells. Keep in mind that it may be necessary to adjust the concentration of the fluorescent substrate based on the cell type used to achieve the greatest difference in fluorescence intensity in the presence and absence of inhibitors. If you choose to use a different cell type or fluorescent substrate, an initial concentration-response experiment with and without the positive control inhibitor will aid in optimizing the fluorescent substrate concentration. The speed of the centrifugation is also critical to troubleshoot to ensure that cells are not lost when the medium is thrown from the 96-well
plate. The speed recommended in this protocol (500 x g) is based upon experiments using a microcentrifuge with a radius of 32 cm. Be sure to consider centrifuge speed if your centrifuge has a radius larger than 32 cm.

Because some cell types may exhibit a range of cell sizes, you should consider using the “normalize fluorescence to cell size” option ((Gibson et al., 2012)). It should also be noted that data can be re-analyzed with different parameters by using the original images acquired rather than repeating the experiment.

The purpose of the loading phase is to equally load all of the cells with the fluorescent substrate before the efflux phase. To ensure that there is equal loading of cell types and that the test inhibitors do not interfere with the uptake of a substrate, baseline fluorescence should also be quantified in each cell type and treatment following the uptake phase.

This assay may generate false-negative results as it is possible that your cell line may not express the appropriate uptake transporters needed for the test inhibitor to gain entry to the cell for access to the intracellular portion of the transporter.

**Anticipated Results**

Results generated by the Cellometer® Vision allow you to present the data as a frequency distribution, as is commonly seen with flow cytometry, as well as mean fluorescence intensity. The range of fluorescence intensity will vary based upon the substrate and cell type. No matter what the substrate or cell type is, the cells co-incubated with positive control inhibitor (and the empty vector cells) should generate a frequency distribution that is shifted to the right as compared to the ABC transporter
overexpressing or endogenously expressing cells incubated without inhibitor ((Robey et al., 2011); Figure A-3.4A). This reflects greater fluorescence retention in cells where MDR1 or BCRP has been inhibited (i.e., no efflux of substrate). Moreover, the mean fluorescence intensity of the cells plus positive control inhibitor and empty vector cells should be much greater than the transporter overexpressing cells without the inhibitor (Figure A-3.4B). If the test chemicals inhibit the transport of a specific ABC transporter substrate, then one would expect both a graded shift of the frequency distribution to the right and increasing average fluorescence intensity, with increasing concentration of test inhibitor. After finding a chemical that inhibits transporter efflux with the initial screen, it may be desirable to increase the number of wells per treatment to obtain a higher sample size in order to perform statistics. Furthermore, an extended concentration-response experiment with various substrate and the test inhibitor concentrations can help to characterize the nature of the chemical-transporter interaction.

**Time Considerations**

Approximately six to seven hours should be allotted to conduct the cell uptake and efflux steps and to individually pipette and analyze each well from the 96-well plate using the Cellometer® Vision.
Figure A-3.1. Sample preparation. Distribution of fluorescent substrate (FS) solution and medium in preparation for the addition of positive control inhibitor and various concentrations of test inhibitors in subsequent steps.
### Figure A-3.2. Plate layout.

The layout for a 96-well round bottom plate demonstrates the placement of cell types and prepared solutions (100 μl). The following abbreviations are used: fluorescent substrate (FS), fluorescent substrate plus various concentrations of test inhibitors (FS + test inhibitor), fluorescent substrate plus positive control inhibitor (FS + I), and no fluorescent substrate (No FS).

<table>
<thead>
<tr>
<th></th>
<th>Test Inhibitor 1</th>
<th>Test Inhibitor 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Overexpressing cells**
- **Empty vector cells**
Figure A-3.3. **Cell loading procedure for imaging.** This image demonstrates the addition of suspended cells (20 μl) into a counting chamber slide. It should be noted that a blue dye was used to visualize loading. During a typical experiment, the solution will be colorless.
Figure A-3.4. Example results with fluorescent substrates. Human embryonic kidney 293 cells stably-transfected with human BCRP or MDR1 genes or empty vector plasmids were incubated with fluorescent substrates (BCRP: Hoechst 33342 and MDR1: Rhodamine 123). Positive inhibitors (BCRP: Ko143 and MDR1: PSC833) were used to block efflux of fluorescent substrates. (A) Line graphs represent the distribution of individual cell fluorescence. Each point represents the mean percent of cells ± SE exhibiting a quantity of fluorescence. (B) Data (bar graphs) are presented as mean relative fluorescence ± SE normalized to cell size.
Table A-3.1. **Recommended substrates and inhibitors.** Recommended stock and final concentrations of fluorescent substrate (FS) and positive control inhibitor (I) for each efflux transporter.

<table>
<thead>
<tr>
<th>Efflux Transporter</th>
<th>Fluorescent Substrate (FS)</th>
<th>Positive Control Inhibitor (I)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCRP</strong></td>
<td>Hoechst 33342</td>
<td>Ko143</td>
</tr>
<tr>
<td>Diluent</td>
<td>Deionized water</td>
<td>DMSO</td>
</tr>
<tr>
<td>Stock</td>
<td>10 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Final</td>
<td>7 µM</td>
<td>1 µM</td>
</tr>
<tr>
<td><strong>MDR1</strong></td>
<td>Rhodamine 123</td>
<td>PSC833</td>
</tr>
<tr>
<td>Diluent</td>
<td>DMSO</td>
<td>DMSO</td>
</tr>
<tr>
<td>Stock</td>
<td>10 mM</td>
<td>1 mM</td>
</tr>
<tr>
<td>Final</td>
<td>5 µM</td>
<td>1 µM</td>
</tr>
</tbody>
</table>
**Table A-3.2. Preparation of inhibitor solutions for the loading phase.** Preparation of control [fluorescent substrate (FS), fluorescent substrate plus positive control inhibitor (FS + I), no fluorescent substrate (No FS)] and test inhibitor (FS + test inhibitor) solutions. \(^1\) The FS volumes are from Figure 1. \(^2\) Tube 11 has medium without FS.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Solution</th>
<th>FS (μl)(^1)</th>
<th>Stock test inhibitor 1 (μl)</th>
<th>Stock test inhibitor 2 (μl)</th>
<th>Stock I (μl)</th>
<th>DMSO (μl)</th>
<th>Total Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FS</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>1010</td>
</tr>
<tr>
<td>2</td>
<td>FS + 1 μM test inhibitor 1</td>
<td>500</td>
<td>0.5 (1 mM stock)</td>
<td>-</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>3</td>
<td>FS + 10 μM test inhibitor 1</td>
<td>500</td>
<td>0.5 (10 mM stock)</td>
<td>-</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>4</td>
<td>FS + 50 μM test inhibitor 1</td>
<td>500</td>
<td>2.5 (10 mM stock)</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>505</td>
</tr>
<tr>
<td>5</td>
<td>FS + 100 μM test inhibitor 1</td>
<td>500</td>
<td>5 (10 mM stock)</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>505</td>
</tr>
<tr>
<td>6</td>
<td>FS + 1 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>0.5 (1 mM stock)</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>7</td>
<td>FS + 10 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>0.5 (10 mM stock)</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>8</td>
<td>FS + 50 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>2.5 (10 mM stock)</td>
<td>-</td>
<td>2.5</td>
<td>505</td>
</tr>
<tr>
<td>9</td>
<td>FS + 100 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>5 (10 mM stock)</td>
<td>-</td>
<td>0</td>
<td>505</td>
</tr>
<tr>
<td>10</td>
<td>FS + I</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>9</td>
<td>1010</td>
</tr>
<tr>
<td>11</td>
<td>No FS</td>
<td>1000(^2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>1010</td>
</tr>
</tbody>
</table>
Table A-3.3. Preparation of inhibitor solutions for the efflux phase. \(^1\)Medium chilled in 4°C refrigerator or on ice.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Solution</th>
<th>Medium (μl)(^1)</th>
<th>Stock test inhibitor 1 (μl)</th>
<th>Stock test inhibitor 2 (μl)</th>
<th>Stock I (μl)</th>
<th>DMSO (μl)</th>
<th>Total Volume (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1 μM test inhibitor 1</td>
<td>500</td>
<td>0.5 (1 mM stock)</td>
<td>-</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>B</td>
<td>10 μM test inhibitor 1</td>
<td>500</td>
<td>0.5 (10 mM stock)</td>
<td>-</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>C</td>
<td>50 μM test inhibitor 1</td>
<td>500</td>
<td>2.5 (10 mM stock)</td>
<td>-</td>
<td>-</td>
<td>2.5</td>
<td>505</td>
</tr>
<tr>
<td>D</td>
<td>100 μM test inhibitor 1</td>
<td>500</td>
<td>5 (10 mM stock)</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>505</td>
</tr>
<tr>
<td>E</td>
<td>1 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>0.5 (1 mM stock)</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>F</td>
<td>10 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>0.5 (10 mM stock)</td>
<td>-</td>
<td>4.5</td>
<td>505</td>
</tr>
<tr>
<td>G</td>
<td>50 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>2.5 (10 mM stock)</td>
<td>-</td>
<td>2.5</td>
<td>505</td>
</tr>
<tr>
<td>H</td>
<td>100 μM test inhibitor 2</td>
<td>500</td>
<td>-</td>
<td>5 (10 mM stock)</td>
<td>-</td>
<td>0</td>
<td>505</td>
</tr>
<tr>
<td>I</td>
<td>I</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>9</td>
<td>1010</td>
</tr>
<tr>
<td>J</td>
<td>No inhibitor</td>
<td>2000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20</td>
<td>2020</td>
</tr>
</tbody>
</table>
Table A-3.4. Fluorescent substrates and inhibitors frequently used for the quantification of ABC transporter function.

<table>
<thead>
<tr>
<th>Fluorescent Substrates</th>
<th>ABC Transporter</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bodipy-prazosin</td>
<td>BCRP, MDR1</td>
<td>(Kimchi-Sarfaty et al., 2002; Robey et al., 2011)</td>
</tr>
<tr>
<td>Pheophorbide A</td>
<td>BCRP</td>
<td>(Robey et al., 2004; Dohse et al., 2010)</td>
</tr>
<tr>
<td>Bodipy-paclitaxel</td>
<td>MDR1</td>
<td>(Kimchi-Sarfaty et al., 2002; Gow et al., 2008)</td>
</tr>
<tr>
<td>Hoechst 33342</td>
<td>BCRP, MDR1</td>
<td>(Shapiro and Ling, 1997; Kim et al., 2002)</td>
</tr>
<tr>
<td>Calcein AM</td>
<td>MDR1, MRPs</td>
<td>(Homolya et al., 1996; Olson et al., 2001; Roy et al., 2009)</td>
</tr>
</tbody>
</table>

**Inhibitors**

- Elacridar: BCRP, MDR1 (de Bruin et al., 1999) (Woodahl et al., 2004)
- Verapamil: MDR1, MRPs (Konya et al., 2006) (Vellonen et al., 2004)
- Probenecid: MRP1 (Dogan et al., 2004)
- MK571: MRPs, BCRP, MDR1 (Matsson et al., 2009)
Table A-3.5. Repeatability of transporter function using fluorescent cell imaging.

Human embryonic kidney 293 cells stably transfected with the BCRP gene or empty vector plasmid were incubated with fluorescent substrate (Hoechst 33342 or BODIPY-glyburide). Fluorescence intensity was measured by the Cellometer® Vision. Data represent percent (%) standard deviation of absolute fluorescence intensity values measured in five independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Percent (%) Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hoechst 33342</td>
</tr>
<tr>
<td><strong>BCRP Overexpressing</strong></td>
<td>15.5</td>
</tr>
<tr>
<td><strong>Empty Vector</strong></td>
<td>19.3</td>
</tr>
</tbody>
</table>
Video A-3.1 Procedure for discarding medium.

Link to video: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3920305/
APPENDIX 4: HEPATIC AND RENAL BCRP TRANSPORTER EXPRESSION IN MICE TREATED WITH PERFLUOROOCTANOIC ACID

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**Abbreviations**

Abc, ATP-binding cassette; Bcrp, breast cancer resistance protein; bDNA, branched DNA; Cyp4a14, cytochrome P450 4a14; Oat, organic anion transporter; Oatp, organic anion transporting polypeptide; PPARα, peroxisome proliferator-activated receptor alpha; PFOA, perfluorooctanoic acid, SE, standard error
A-3.1 Abstract

The breast cancer resistance protein (Bcrp) is an efflux transporter that participates in the biliary and renal excretion of drugs and environmental chemicals. Recent evidence suggests that activation of the peroxisome proliferator activated receptor alpha (PPARα) can up-regulate the expression of Bcrp. The current study investigated the regulation of hepatic and renal Bcrp mRNA and protein in mice treated with the PPARα agonist perfluorooctanoic acid (PFOA) and the ability of PFOA to alter human BCRP function in vitro. Bcrp mRNA and protein expression were quantified in the livers and kidneys of male C57BL/6 mice treated with vehicle or PFOA (1 or 3 mg/kg/d po gavage) for 7 days. PFOA treatment increased liver weights as well as the hepatic mRNA and protein expression of the PPARα target gene, cytochrome P450 4a14. Compared to vehicle-treated control mice, PFOA increased hepatic Bcrp mRNA and protein between 1.5- and 3-fold. Immunofluorescent staining confirmed enhanced canalicular Bcrp staining in liver sections from PFOA-treated mice. The kidney expression of cytochrome P450 4a14 mRNA, but not Bcrp, was increased in mice treated with PFOA. Micromolar concentrations of PFOA decreased human BCRP ATPase activity and inhibited BCRP-mediated transport in inverted membrane vesicles. Together, these studies demonstrate that PFOA induces hepatic Bcrp expression in mice and may inhibit human BCRP transporter function at concentrations that exceed levels observed in humans.

Keywords: ABCG2, BCRP, PFOA, PPARα, transporter
A-3.2 Introduction

During the last century, perfluorooctanoic acid (PFOA) became a frequent intermediate in the production of non-stick cookware, lubricants, cosmetics, fire fighting foam, hydraulic fluid, carpets, upholstery, and other commercial products. PFOA is a synthetic fluorinated compound with an 8 carbon backbone. Because carbon-fluorine bonds are very stable, PFOA resists degradation and metabolism, which has led to its bioaccumulation and persistence in the environment. Moreover, PFOA is negligibly cleared in humans, which leads to a long serum half-life of 2.3 to 3.5 years (Olsen et al., 2007; Bartell et al., 2010). A number of adverse health effects, including hyperuricemia (Steenland et al., 2010) and hypercholesterolemia (Costa et al., 2009; Steenland et al., 2009; Frisbee et al., 2010), have been reported in workers occupationally exposed to PFOA as well as individuals who lived or worked in districts with water contaminated by PFOA from a chemical plant. Because of the environmental persistence, limited excretion in humans, and the potential adverse effects of PFOA, the U.S. Environmental Protection Agency has called for a voluntary phase-out by eight major manufacturers (reviewed in (Yacovino et al., 2013)). Despite this effort, continued human exposure is expected due to the use of existing PFOA-containing products and the potential for other domestic and international companies to continue production (reviewed in (Post et al., 2012)).

PFOA is an agonist of the rodent and human nuclear receptor peroxisome proliferator activated receptor alpha (PPARα) (Maloney and Waxman, 1999). Activation of PPARα in rodents is associated with increased liver weight, proliferation of peroxisomes, and enhanced catalase activity (Ikeda et al., 1985; Uy-Yu et al., 1990). Likewise, hepatic activation of PPARα signaling by PFOA is often monitored by up-regulation of cytochrome (Cyp) P450 4A genes (Sohlenius et al., 1992; Diaz et al., 1994). While
humans do not exhibit hepatic peroxisome proliferation nor tumorigenesis following PPARα activation (reviewed in (Reddy and Lalwai, 1983), mouse and human PPARα do share a number of target genes involved in metabolism and transport following activation by PFOA (Nakamura et al., 2009; Bjork et al., 2011).

Breast cancer resistance protein (Bcrp) is an efflux transporter encoded by the ATP binding cassette G2 subfamily isoform 2 (Abcg2) gene. The rodent transporter is denoted by lowercase letters (Bcrp) and the human isoform is written in capitalized letters (BCRP). Bcrp/BCRP is widely expressed on the apical surface of excretory tissues such as the liver, kidney, and intestines as well as in sensitive organs such as the placenta, testes, and brain (reviewed in (Klaassen and Aleksunes, 2010)). BCRP transports a wide array of endogenous substrates, including urate and folate, as well as anticancer drugs, sulfasalazine, and glyburide (reviewed in (Klaassen and Aleksunes, 2010)). In the liver and kidneys, Bcrp secretes chemicals into bile and urine, respectively. PFOA is readily excreted in the urine of rats (Vanden Heuvel et al., 1991; Vanden Heuvel et al., 1992). Additionally, prior studies point to a role for organic anion transporters (OATs) in the renal secretion and reabsorption of PFOA (Weaver et al., 2010; Yang et al., 2010; Han et al., 2012). However, it is unknown whether efflux transporters, such as Bcrp, can contribute to its renal secretion.

Recent evidence suggests that activation of the peroxisome proliferator activated receptor alpha (PPARα) can up-regulate the expression of Bcrp. Little is known about the ability of PFOA to regulate Bcrp expression and function. Therefore, the purpose of the present study was to 1) determine whether PFOA treatment alters the mRNA and protein expression of Bcrp in the liver and kidneys of mice and 2) assess the ability of PFOA to activate and inhibit human BCRP ATPase activity and transport in vitro.
A-4.3 Materials and Methods

Chemicals
Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animal Treatment
Adult male C57BL/6 mice (027 strain) were purchased from Charles River Laboratories (Wilmington, MA). Mice (n=6) were dosed daily with vehicle (deionized water, 10 ml/kg), 1, or 3 mg/kg/day of perfluorooctanoic acid ammonium salt (77262, Sigma-Aldrich, St. Louis, MO) for seven days by oral gavage. PFOA doses and dosing regimen were previously used to investigate regulation of hepatic nuclear receptor pathways in mice (Rosen et al., 2008). Terminal weights were recorded and mice were sacrificed by decapitation 24 hours following the final dose. Organs and tissues were collected and weighed, snap frozen in liquid nitrogen, and stored at -80°C until use for protein and RNA analyses. The Rutgers University Institutional Animal Care and Use Committee approved these studies.

RNA Isolation
Total RNA was isolated using RNABee (Tel-Test, Friendswood, TX) and the RNeasy Midi Kit according to the manufacturers’ recommendations (Qiagen, Valencia, CA). RNA integrity was confirmed using an Amersco Formaldehyde-Free RNA Gel Kit (ISC Bioexpress, Kaysville, UT). Total RNA concentrations were quantified at 260 nm using a NanoDrop Spectrophotometer (Thermo Scientific, Rockford, IL).
Branched DNA (bDNA) Analysis

The mRNA expression of mouse Cyp4a14 and Bcrp were quantified using the bDNA signal amplification assay (Panomics QuantiGene, High Volume bDNA Signal Amplification Kit 1.0; Affymetrix, Santa Clara, CA). Multiple oligonucleotide probe sets (containing capture, label, and blocker probes) specific to mouse mRNA transcripts were designed using ProbeDesigner software (version 1.0; Bayer Corp., Diagnostics Div., Tarrytown, NY). Probe sequences for Bcrp and Cyp4a14 were published previously (Cheng et al., 2005; Tanaka et al., 2005). Luminescence was read on a Spectramax luminometer (Molecular Devices, Sunnyville, CA).

Western Blot Analysis

Livers and kidneys were homogenized in sucrose-Tris buffer (10 mM Tris base, 250 mM sucrose) containing 1% protease inhibitor cocktail (Sigma P8340). Protein concentrations were measured using a bicinchoninic acid assay (Peirce Biotechnology, Rockford, IL). Fifty µg of protein homogenate were loaded per well onto an 8% SDS-PAGE resolving gel (Invitrogen: XCell SureLock Midi Gel System; Carlsbad, CA) and run at 100 volts at room temperature for 3 hours. Proteins were transblotted onto a nitrocellulose membrane in a 7-minute transfer (iBlot, Life Technologies). Membranes were then blocked with 5% non-fat dry milk in 0.5% PBS/Tween-20 overnight at 4°C. Primary antibodies were hybridized overnight at 4°C. The following primary antibodies were used: Cyp4a14 (sc-46087 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), Bcrp (Bxp-53 1:5000 dilution, Enzo Life Sciences, Farmingdale, NY) and beta actin (ab8227 1:2000 dilution, Abcam, Cambridge, MA). Following incubation with species-appropriate secondary antibodies, membranes were washed and subsequently incubated with 2 ml of SuperSignal West Dura Extended Duration Substrate for 2
minutes (Thermo Scientific, Rockford, IL). Protein band intensity was semi-quantified using a FluorChem Imager (ProteinSimple, Santa Clara, California).

**Indirect Immunofluorescence**

Slides were prepared from frozen tissue sections. Cryosections (5 μm) were allowed to warm to room temperature. Slides were fixed for 5 minutes in a coplin jar with 4% paraformaldehyde and then rinsed with PBS for 5 minutes. Sections were placed in a coverplate chamber system using PBS and then rinsed for 5 minutes with PBS. Goat serum (5%) in 0.1% triton X in PBS/T was used to block slides for 60 minutes. Primary antibody (Bxp-53 1:100 in 5% goat serum) was added and incubated overnight at 4°C. Slides were rinsed with PBS and then incubated in secondary antibody (Antirat AlexiFluor 488 IgG, 1:100 in 5% goat serum/PBS-T) for 60 minutes (Invitrogen, Carlsbad, CA). Sections were air dried and mounted in Prolong Gold with 4’,6-diamidino-2-phenylindole (Invitrogen Corp.). Images were acquired on a Zeiss Observer D1 microscope with an x-cite series 120Q fluorescent illuminator (Zeiss Inc., Thornwood, NY) and a Jenoptik camera with ProgRes CapturePro 2.8 software (Jenoptik, Easthampton, MA). All sections were both stained and imaged under uniform conditions for each antibody. Negative controls without primary antibody were included to ensure minimal non-specific staining (data not shown).

**Human BCRP ATPase Assay**

BCRP-expressing membranes were purchased from Xenotech (Lenexa, KS). The ATPase assay was used to determine the activation and inhibition of transporter-dependent ATPase activity by PFOA according to the manufacturer's protocol. **Activation.** To determine activation, plasma membranes from *Spodoptera frugiperda* (Sf9) cells transfected with human BCRP were incubated at 37°C in 96-well plates with
assay medium, 2 mM ATP, and PFOA (0.03-100 µM) in the presence and absence of 1.2 mM sodium orthovanadate for 30 minutes. Sodium orthovanadate inhibits ABC transporter ATPase function and was used to calculate vanadate-sensitive activity. 

Inhibition. Inhibition was analyzed in a similar manner, with the addition of a specific BCRP activator (sulfasalazine, 10 µM) to the assay medium. Hoechst 33342 (0.1 mM) was used as a positive control for inhibitory activity. Following incubation of membranes at 37°C with a colorimetric reagent, liberation of inorganic phosphate was detected by absorption at 610 nm.

**Human BCRP Membrane Vesicles.**

Control and human BCRP membrane vesicles (5 mg/ml, 500 µl) were purchased from Cellz Direct (Durham, NC). Vesicles were generated from the plasma membranes of Sf9 insect cells infected with a baculovirus expressing the human BCRP transproter. ATP-dependent transport of Lucifer yellow (50 µM) over a 10 min period at 37°C was used to quantify BCRP activity in membrane vesicles (20 µg) according to the manufacturer's recommendation. Control vesicles and MgAMP (no MgATP) were used as negative controls to account for background diffusion of Lucifer yellow into vesicles. Reactions were terminated with ice-cold stopping buffer (40 mM MOPS-Tris, 70 mM KCl, 7.5 mM MgCl₂) and vesicles were washed in 96-well filter plates using vacuum filtration (Millipore, Billerica, MA). Vesicles were solubilized by addition of 50% methanol for 15 minutes at room temperature and vesicle contents were filtered through to a new 96-well plate. Lucifer yellow fluorescence was read at an excitation wavelength of 430 nm and emission wavelength of 538 nm.
**Statistical Analysis**

Data are presented as mean ± standard error (SE). GraphPad Prism® version 5 software (GraphPad Software, La Jolla, CA) was used for statistical analysis. Differences among groups were determined by a one-way analysis of variance with a Newman Keul’s posthoc test for multiple comparisons. Differences were considered statistically significant at p<0.05.
A-4.4 Results

Hepatic and kidney weights in PFOA-treated mice

Treatment of mice with 1 and 3 mg/kg PFOA daily for 7 days increased liver weights by 38% and 91%, respectively (data not shown). Likewise, the liver-to-body weight ratios increased in a dose-dependent manner in PFOA-treated mice (Figure A-4.1). Conversely, PFOA did not alter the absolute nor the relative kidney weights compared to vehicle-treated mice.

Hepatic and renal expression of Cyp4a14 and Bcrp mRNA in PFOA-treated mice

Compared to vehicle-treated mice, Cyp4a14 mRNA was induced more than 100-fold in the liver at both PFOA doses. A 10-fold up-regulation was observed only in kidneys of mice treated with 3 mg PFOA/kg (Figure A-4.2). Bcrp mRNA was increased 1.9- and 3.2-fold in the livers of mice treated with PFOA 1 mg/kg and 3 mg/kg, respectively. No change in kidney Bcrp mRNA was observed in PFOA-treated mice.

Hepatic and renal expression of Cyp4a14 and Bcrp protein in PFOA-treated mice

Similar to mRNA expression, levels of Cyp4a14 protein were increased up to 15-fold in livers of PFOA-treated mice (Figure A-4.3). Likewise, Cyp4a14 protein was elevated up to 4-fold in the kidneys, however, it was not statistically significant. Bcrp protein was detected in the liver and kidneys with a molecular weight estimated to be 72-74 kDa (FIG), which is consistent with the published size in the kidneys and livers of wild-type, but not Bcrp-null mice (Jonker et al., 2002). In the kidneys, an additional faint band was also observed at 33-34 kDa (data not shown). Consistent with Cyp4a14, Bcrp protein expression in livers was increased up to 2.3-fold in response to PFOA; no change was observed in the kidneys. Immunofluorescent staining revealed apical localization of BCRP on hepatocytes and proximal tubule epithelial cells, with greater basal expression...
in the kidneys of vehicle-treated mice (Figure A-4.4). PFOA treatment increased BCRP staining intensity in the livers of mice; no change was apparent in the kidneys. It is presumed that the staining in the kidneys is primarily due to the ~72-74 kDa protein detected on western blot. In the liver, the extent of Bcrp staining was similar between centrilobular and periportal regions suggesting that the induction following PFOA treatment was not limited to discrete regions of the liver.

**PFOA inhibition of human BCRP ATPase activity and transport in inverted membrane vesicles**

Plasma membranes expressing human BCRP were used to indirectly test whether PFOA is a substrate by quantifying the liberation of inorganic phosphate following ATP hydrolysis. Increasing concentrations of PFOA did not alter the rate of ATP hydrolysis in BCRP-expressing membranes (Figure A-4.5A). At high concentrations of PFOA (30 and 100 μM), the constitutive activity of BCRP was reduced below baseline. Additional experiments aimed to determine whether PFOA can inhibit BCRP activity. For this purpose, BCRP-expressing membranes were activated with the substrate sulfasalazine (Figure A-4.5A). High concentrations of PFOA (30 and 100 μM) decreased BCRP hydrolysis of ATP in a manner similar to the inhibitor Hoescht (Ht) 33342. In addition, PFOA inhibited BCRP-mediated transport of Lucifer yellow in inverted vesicles from BCRP-expressing cells at concentrations ranging between 5 and 50 μM (Figure A-4.5B).
**A-4.5 Discussion**

The present study demonstrated that daily treatment of mice with PFOA for 7 days increased Bcrp mRNA and protein in liver, but not in kidneys. Elevated hepatic Bcrp expression was associated with markers of peroxisome proliferation including up-regulation of Cyp4a14 mRNA and protein, as well as hepatomegaly; this suggests PPARα-mediated signaling may regulate Bcrp in livers of PFOA-treated mice. Little activation of PPARα was observed in kidneys with Cyp4a14 mRNA increases only seen at the highest PFOA dose. Using *in vitro* models of human BCRP transport, it was also demonstrated that PFOA can inhibit BCRP-mediated ATP hydrolysis and efflux at high micromolar concentrations. The mean serum concentration of PFOA in Ohio and West Virginia residents has been reported as 69.2 - 80.3 ng/ml following high exposure to PFOA (Steenland et al., 2009; Frisbee et al., 2010). As a result, it is unlikely that sufficiently high PFOA concentrations can be achieved in humans to significantly inhibit BCRP-mediated transport.

Evidence suggests that PPARα plays a role in mediating Bcrp basal and inducible expression in the livers of mice (Moffit et al., 2006). Exposure of CD-1 mice to clofibrate, a PPARα agonist, significantly up-regulates hepatic Bcrp mRNA and protein. Additionally, mice lacking PPARα exhibited decreased constitutive Bcrp protein and impaired induction of Bcrp mRNA in response to clofibrate treatment (Moffit et al., 2006). Similarly, Bcrp mRNA is induced by other PPARα agonists (Wy 1463 and GW7647) in the livers of wild-type mice, but not PPARα-null mice (Hirai et al., 2007). However, no change in Bcrp expression was observed in the intestines of either genotype. This suggests tissue-specific PPARα-mediated Bcrp regulation. Similarly, the current study demonstrated Bcrp up-regulation in the liver, but not the kidneys, of PFOA-treated mice. More recently, the ability of human PPARα to regulate BCRP mRNA and protein
expression was demonstrated in hCMEC/D3 endothelial cells, which serve as a model of the human blood-brain barrier (Hoque et al., 2012). Down-regulation of PPARα using small interfering RNAs reduced BCRP expression and function. It was also observed that PPARα binding to the BCRP/ABCG2 promoter region increased when hCMEC/D3 cells were exposed to clofibrate (Hoque et al., 2012). Results from this study provided direct evidence of PPARα regulation of BCRP in human brain microvessel endothelial cells. In addition to PPARα, recent evidence suggests that ligand-activated PPARγ can also up-regulate BCRP mRNA in dendritic cells by directly binding to conserved enhancer region in the promoter of the human BCRP/ABCG2 gene (Szatmari et al., 2006b). Based on the ability of PPARα and PPARγ to transactivate the human BCRP/ABCG2 gene, future studies should be aimed at determining whether PFOA can upregulate BCRP in human hepatocytes at environmentally-relevant concentrations. Furthermore, PFOA can activate signaling through the constitutive androstane receptor and the pregnane x receptor (Cheng and Klassen, 2008; Rosen et al., 2008a; Bjork et al., 2011). Additional studies are needed to determine whether these transcription factors can up-regulate the expression of Bcrp mRNA in the liver.

Using *in vitro* expression systems, it has been demonstrated that OATs and organic anion transporting polypeptides (OATPs) can transport PFOA and other perfluorinated carboxylates of various carbon backbone lengths (Weaver et al., 2010). The basolateral Oat1/OAT1, Oat3/OAT3, and Oatp1a1 and apical Oat2 and urate (URAT) transporters increase the *in vitro* uptake of PFOA (Nakagawa et al., 2008; Weaver et al., 2010). Similarly, human OAT4 and URAT proteins can influx PFOA into cells which may contribute to the long half-life in humans (Yang et al., 2010). It is currently unknown whether apical efflux transporters participate in the renal disposition of PFOA. Based on initial data obtained from the ATPase assay (*Figure A-4.5A*), PFOA does not appear to
be a substrate of BCRP; however direct transport measures are needed to confirm these findings. Likewise, the multidrug resistance-associated protein 2 is not thought to secrete PFOA into urine (Katakura et al., 2007). Thus, further investigation is needed to identify the efflux transporter(s) for the renal secretion of PFOA.

In conclusion, PFOA up-regulates Bcrp mRNA and protein only in the livers of mice with no change observed in the kidneys. While it is unknown whether the doses of PFOA used can similarly up-regulate BCRP following environmental or occupational exposure, these findings add to our mechanistic understanding of PFOA target genes. Functional in vitro studies suggest that PFOA may inhibit BCRP transporter at concentrations that exceed levels observed in humans.
Figure A-4.1. Liver and kidney weights in PFOA-treated mice. Adult male C57BL/6 mice were treated with vehicle or PFOA daily for 7 days by p.o. gavage and tissues were collected 24 hours after the final dose. Terminal body and organ weights were recorded during necropsy. Organ weights are presented as ratios relative to body weight (n=6). Data are shown as mean ± SE. Asterisks (*) represent statistically significant differences (p < 0.05) compared to 0 mg/kg control mice.
Figure A-4.2. Messenger RNA expression of Cyp4a14 and Bcrp in livers and kidneys of PFOA-treated mice. Quantification of mRNA in livers and kidneys was determined after 7-day exposure to PFOA using the bDNA assay. Induction of Cyp4a14 mRNA was used as positive control for PPARα activation. Data are presented as mean ± SE (n=5). Asterisks (*) represent statistically significant differences (p < 0.05) compared to 0 mg/kg control mice.
Figure A-4.3. Protein expression of Cyp4a14 and Bcrp in livers and kidneys of PFOA-treated mice. Semi-quantification of protein expression was determined after 7-day exposure to PFOA using western blot analysis. Induction of Cyp4a14 protein was used as positive control for PPARα activation in PFOA-treated mice. β-actin was used as a loading control. The western blot data are presented as individual blots and mean relative protein expression (normalized to β-actin). Data are presented as mean ± SE (n=4). Asterisks (*) represent statistically significant differences (p < 0.05) compared to 0 mg/kg control mice.
Figure A-4.4. Immunofluorescent staining of Bcrp in livers and kidneys of PFOA-treated mice. Indirect immunofluorescence against apical Bcrp transporter (green) was conducted on liver cryosections after 7-day exposure to PFOA. Representative regions are shown. Magnification, x100. Images were cropped, enlarged, and provided as insets.
Figure A-4.5. Interaction of PFOA with human BCRP transporter. (A) BCRP membranes were incubated with ATP and varying concentrations of PFOA in the presence and absence of sulfasalazine (10 μM) for 30 minutes. Additional reactions included sodium orthovanadate in order to determine the ATPase assay attributed to transport. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Hoescht 33342 (Ht) was used as a positive control inhibitor of BCRP activity. Data are presented as vanadate-sensitive ATPase activity expressed as means ± SE (n=3-6). Asterisks (*) represent statistically significant differences (p < 0.05) compared to 0 μM PFOA in the absence of sulfasalazine. Plus signs (+) represent statistically significant differences (p < 0.05) compared to 0 μM PFOA in the presence of sulfasalazine. (B) Inverted BCRP-
expressing vesicles (20 µg) were incubated with Lucifer yellow (50 µM) for 10 minutes in
the presence and absence of ATP and increasing concentrations of PFOA (n=3). Data
are presented as mean ± SE normalized to maximal ATP-dependent BCRP activity (no
PFOA). Asterisks (*) represent statistically significant differences (p < 0.05) compared to
0 µM PFOA.
APPENDIX 5: IN VITRO SCREEN OF ENVIRONMENTAL CHEMICALS IDENTIFIES ZEARALENONE AS A NOVEL SUBSTRATE OF THE PLACENTAL BCRP/ABCG2 TRANSPORTER

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A-5.1 Abstract

The BCRP (ABCG2) transporter is responsible for the efflux of chemicals from the placenta to the maternal circulation. Inhibition of BCRP activity could enhance exposure of offspring to environmental chemicals leading to altered reproductive, endocrine, and metabolic development. The purpose of this study was to characterize environmental chemicals as potential substrates and inhibitors of the human placental BCRP transporter. The interaction of BCRP with a panel of environmental chemicals was assessed using the ATPase and inverted plasma membrane vesicle assays as well as a cell-based fluorescent substrate competition assay. HEK cells transfected with wild-type BCRP and the Q141K genetic variant, as well as BeWo choriocarcinoma placental cells that endogenously express BCRP were used to further test inhibitor and substrate interactions. To varying degrees, the 11 chemicals inhibited BCRP activity at micromolar concentrations in activated ATPase membranes and inverted membrane vesicles. Genistein, zearalenone, and tributyltin increased the retention of the fluorescent BCRP substrate, Hoechst 33342, between 50-100% from BeWo cells. Additional experiments characterized the mycotoxin and environmental estrogen, zearalenone, as a novel substrate of BCRP in HEK-BCRP and BeWo cells. Interestingly, the BCRP genetic variant Q141K exhibited reduced efflux of zearalenone compared to the wild-type protein. Environmental chemicals inhibited BCRP function. However, transport activity was reduced at concentrations that may not be environmentally relevant. Nonetheless, screening assays and direct quantification experiments identified zearalenone as a novel human BCRP substrate. Additional studies are needed to determine whether placental BCRP prevents fetal exposure to zearalenone in vivo.
Non-Standard Abbreviations:

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; EV, empty vector;
HEK, human embryonic kidney; MRP, multidrug resistance-associated protein; Sf9, *Spodoptera frugiperda* 9; WT, wild-type
A-5.2 Introduction

The breast cancer resistance protein (BCRP) is an efflux transporter highly expressed on the maternal surface of the placenta and is responsible for the fetal-to-maternal movement of chemicals such as bile acids and steroids (Suzuki et al., 2003; Grube et al., 2007; Blazquez et al., 2012) and drugs including glyburide and nitrofurantoin (Zhang et al., 2007; Gedeon et al., 2008b; Zhou et al., 2008a). BCRP resides in the plasma membrane of placental syncytiotrophoblasts (Yeboah et al., 2006) and translocates xenobiotics to the maternal circulation using energy generated from the hydrolysis of ATP. Interference of BCRP efflux activity in the placenta increases fetal xenobiotic accumulation (Zhou et al., 2008a) and may enhance the risk of developmental adverse effects.

There is some evidence that dietary and environmental chemicals are substrates for BCRP. For example, the dietary soy isoflavone genistein that possess estrogenic activity (Kuiper et al., 1998) have been identified as a substrate of BCRP (Enokizono et al., 2007a). The plasticizer bisphenol A and its glucuronide conjugate interact with BCRP as substrates and/or inhibitors (Mazur et al., 2009; Dankers et al., 2013). The ability of BCRP to transport these chemicals may be important in understanding developmental toxicities that result from exposure during pregnancy. Prenatal administration of genistein or the mycotoxin zearalenone to pregnant mice alters mammary gland and reproductive development in female offspring (Alonso-Magdalena et al., 2010). In addition, recent data suggest that in utero exposure of mice to bisphenol A has been linked to glucose intolerance, insulin resistance, and ovarian cysts, in male or female offspring (Vandenberg et al., 2007; Newbold et al., 2009; Alonso-Magdalena et al., 2010). Taken together, these data suggest that BCRP-mediated transport in the
placenta may be a protective mechanism that attempts to limit fetal exposure to environmental chemicals and prevent developmental toxicity.

There are a number of experimental approaches used to assess the ability of chemicals to interact with the BCRP transporter as substrates and/or inhibitors. Screening assays include the ATPase activity assay and inverted plasma membrane vesicles, which can be conducted in multi-well plate formats (Elsby et al., 2011; Bircsak et al., 2013; Tang et al., 2013; Gallus et al., 2014). In both assays, the human BCRP gene (ABCG2) is overexpressed in Spodoptera frugiperda (Sf9) cells and the plasma membrane is isolated as fragments (for the ATPase assay) or inverted into membrane vesicles. Cell-based screening models include cultured cells which overexpress transfected BCRP or endogenously express BCRP. These assays employ the use of fluorescent BCRP substrates that can be monitored with flow cytometers or fluorescence cell counters (Robey et al., 2011; Bircsak et al., 2013). One advantage of BCRP-overexpressing cell lines is the ability to compare transport between the wild-type protein and the single nucleotide polymorphism Q141K (c.421C>A, rs2231142) in the absence of other drug transporters (Morisaki et al., 2005). The Q141K variant of BCRP exhibits reduced efflux of the diabetes drug glyburide (Pollex et al., 2010) and the cancer drug topotecan (Morisaki et al., 2005). Because the Q141K allele can be observed frequently in Caucasians (7.4-11.1%) and East Asians (26.6-35.0%), there is the potential for significant populations to have a reduced capacity to efflux BCRP substrates (Giacomini et al., 2013). Clinically, individuals bearing the Q141K polymorphism exhibit elevated plasma concentrations of the hypolipidemic drug rosuvastatin (Tomlinson et al., 2010), the cancer drug gefitinib (Cusatis et al., 2006) and chemicals such as uric acid (Matsuo et al., 2011; Zhang et al., 2013); the latter of which leads to an increased risk of gout (Matsuo et al., 2011; Zhang et al., 2013). Whereas BCRP-overexpressing cell lines offer
the advantage of investigating BCRP function in the absence of other drug transporters, it is equally important to utilize cells that endogenously express the complement of transporters normally found in a tissue. In particular, human choriocarcinoma cell lines, such as BeWo cells, recapitulate first trimester syncytiotrophoblasts and express the BCRP protein as well as other uptake and efflux transporters (Ceckova et al., 2006). As a result, BeWo cells are routinely used to assess placental transport (Ceckova et al., 2006). Although BeWo cells are a cancer cell line, they exhibit a number of features of placental trophoblasts including the ability to syncytialize into multinucleated cells and secrete hormones such as human chorionic gonadotropin (Prouillac et al., 2009).

In order to further characterize the ability of placental BCRP to reduce fetal concentrations of environmental chemicals, a combination of in vitro screening and cell efflux studies are needed. Therefore, the purpose of this study was to 1) screen eleven environmental chemicals as potential BCRP substrates and/or inhibitors and 2) quantify the direct transport of the mycotoxin zearalenone as a novel BCRP substrate in BeWo placental cells and cells overexpressing the wild-type or the Q141K variant BCRP gene. The eleven chemicals screened included the phytoestrogen genistein, mycotoxin zearalenone, plasticizer bisphenol A, insecticide methoxychlor, biocide tributyltin, arcaricide propargite, and the fungicides myclobutanil, prochloraz, propiconazole, tebucanzole, as well as epoxiconazole. A number of these xenobiotics have been reported to adversely impact the endocrine, reproductive, and neurological development of animals (Table A-5.1). Therefore, the current evaluation of their interactions with the placental BCRP transporter aimed to advance our understanding of mechanisms that may regulate fetal exposure to environmental chemicals.
A-5.3 Materials and Methods

Chemicals

Unless specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Human BCRP ATPase Assay

BCRP-expressing plasma membranes were purchased from Xenotech (Lenexa, KS). The ATPase assay was used to quantify the activation and inhibition of transporter-dependent ATPase activity by environmental chemicals according to the manufacturer's protocol. Activation. To determine activation, plasma membranes (4 µg) from *Spodoptera frugiperda* (Sf9) cells transfected with human BCRP were incubated at 37°C in 96-well plates with assay medium, 2 mM ATP, and environmental chemicals (0.01-100 µM) in the presence and absence of 500 µM sodium orthovanadate for 30 min. Sodium orthovanadate inhibits the ATPase function of ATP-binding cassette (ABC) transporters and was used to calculate vanadate-sensitive activity by subtraction from the total ATPase activity. Inhibition. Inhibition was analyzed in a similar manner, with the addition of a specific activator of BCRP activity (sulfasalazine, 10 µM) to the assay medium. The BCRP inhibitor Ko143 (0.001-50 µM) was used as a positive control for antagonist activity. Following incubation of membranes at 37°C with a colorimetric reagent, liberation of inorganic phosphate was quantified by absorption at 610 nm. Samples were run in triplicate.

Human BCRP Membrane Vesicles

Control and human BCRP plasma membrane vesicles (5 mg/ml, 500 µl) were purchased from Life Technologies (Carlsbad, CA). Vesicles were generated from the plasma membranes of *Sf9* insect cells infected with a baculovirus expressing the human
BCRP gene, *ABCG2*. ATP-dependent transport of Lucifer yellow (50 μM) over a 2-minute period at 37°C was used to quantify BCRP activity of membrane vesicles (20 μg) in the presence of environmental chemicals (0.01-100 μM) or Ko143 (0.01-10 μM) in reaction buffer (50 mM MOPS-Tris, 70 mM KCl, 7.5 mM MgCl₂, pH 7.0) according to the manufacturer’s protocol. Control vesicles (no transporter) and no MgATP incubations were used as negative controls to account for background diffusion of Lucifer yellow into vesicles. Reactions were terminated with ice-cold stopping buffer (40 mM MOPS-Tris, 70 mM KCl, pH 7.0) and vesicles were washed in a 96-well filter plate using vacuum filtration (Millipore, Billerica, MA). Vesicles were solubilized by addition of 50% methanol for 15 minutes at room temperature and vesicle contents were filtered through to a new 96-well plate. Lucifer yellow fluorescence was read at an excitation wavelength of 430 nm and emission wavelength of 538 nm. Samples were run in triplicate or quadruplicate.

**Cell Culture**

Human embryonic kidney-293 (HEK) cells expressing empty vector (EV), human wild-type (WT) BCRP, or the Q141K BCRP variant were kindly provided by Dr. Robert Robey (National Cancer Institute, National Institutes of Health, Bethesda, MD). Cells were grown in Dulbecco's Modified Eagle Medium (Life Technologies) with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 1% penicillin-streptomycin (Life Technologies). Human BeWo choriocarcinoma placenta cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco's Modified Eagle Medium F-12 (ATCC) with 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Life Technologies). Cells were incubated at 37°C with 5% CO₂ and used in experiments at 80 to 90% confluence.
Western Blot

BeWo and HEK cells were lysed and protein concentrations were determined by the bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed by using 5 μg protein homogenate on SDS-polyacrylamide 4–12% Bis–Tris gels (Life Technologies) that were resolved by electrophoresis. Proteins were transferred from gels onto polyvinylidene fluoride membranes using a 7-min iBlot (Life Technologies). Membranes were blocked in 5% non-fat dairy milk in phosphate-buffered saline (PBS) with 0.5% Tween-20 overnight. BCRP (BXP-53, Abcam, Cambridge, MA), GAPDH (ab9485, Abcam), and β-Actin (ab8227, Abcam) primary antibodies were diluted in 2% non-fat dairy milk in PBS with 0.5% Tween-20 and incubated with the membranes at dilutions of 1:5000 and 1:2000 (GAPDH and β-Actin), respectively. Blots were further probed using species-specific HRP-conjugated secondary antibodies (Sigma Aldrich, St. Louis, MO): antirat IgG (BCRP) and antirabbit IgG (GAPDH and β-Actin) at dilutions of 1:2000. Following which SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Rockford, IL) was added to the blots for 2 minutes. Detection and semiquantitation of protein bands were performed with a FluorChem imager (ProteinSimple, Santa Clara, CA). The density of bands were assessed by the Alpha Viewer (ProteinSimple) and normalized to β-Actin or GAPDH levels.

Fluorescent Substrate Transport Assay

Transporter function was quantitated using a fluorescent cell counter and the fluorescent substrate Hoechst 33342 as previously described (Gibson et al., 2012; Bircsak et al., 2013). Environmental chemicals were dissolved in dimethyl sulfoxide (final concentration was less than 0.5%). Cells were then centrifuged at 500 x g for 5 min at 5°C, resuspended, and loaded with the fluorescent BCRP substrate Hoechst 33342 (HEK cells: 7 μM, BeWo cells: 15 μM) with or without the BCRP inhibitor Ko143 (1 μM).
or select environmental chemicals (genistein, zearalenone, tributylin, methoxychlor, and bisphenol A, 1-100 µM) for 30 min at 37°C and 5% CO$_2$ (uptake period). Cells were then washed with cold medium, centrifuged, resuspended, and incubated in substrate-free medium in the presence and absence of test chemicals or Ko143 inhibitor for 1 h (efflux period). Cells were then washed, resuspended in cold PBS, and fluorescence was quantified using the Nexclom Cellometer Vision (Nexcelom Bioscience LLC., Lawrence, MA). Twenty microliters of cell suspension was applied to the cell counting chamber, and each sample was analyzed using the bright-field images for cell size and cell number. The fluorescent intensity of each cell was subsequently analyzed with the appropriate filter VB-450-302 (Hoechst 33342; excitation/emission: 375/450 nm). For experiments using BeWo cells, raw fluorescence intensity for each cell was normalized to cell size to account for differences in cell size. The average fluorescence for each sample was determined and this experiment was performed in triplicate or quadruplicate.

**Alamar Blue Assay**

The Alamar Blue assay was used to assess cell viability based on the reduction potential of metabolically active cells (Life Technologies). Cells were seeded in black clear-bottom 96-well plates and exposed to different concentrations of zearalenone (2-100 µM) or the known BCRP substrate mitoxantrone (0.1-0 µM, Sigma Aldrich) for 72 h. After exposure, 100 µL of AlamarBlue® reagent (Invitrogen Carlsbad, CA) was added to each well and incubated for 3 h at 37°C. The fluorescence was measured at 570 nm excitation and 585 nm emission wavelengths. Samples were run in quadruplicate.

**ELISA Assay**

BeWo and HEK cells were cultured in 6-well plates and remained adherent during zearalenone (10 or 50 µM) uptake (30 min) and efflux (1 h) phases. Cells were washed
with PBS between phases as well as after the efflux phase and subsequently lysed with lysis buffer (20mM Tris-Hcl, 150mM NaCl, 5mM EDTA, 1% Triton X-100 and 1% protease inhibitor cocktail). Zearalenone concentrations in cell lysates were quantified using an ELISA kit (Abnova, Taiwan). Zearalenone standards or sample were added to a 96-well plate coated with zearalenone antibody for 40 min. Then, the zearalenone-alkaline phosphatase conjugate was added to compete for binding with zearalenone. After incubation and washing, the p-nitrophenyl phosphate substrate was added for 20 min followed by 3 N NaOH, which was used as a stop solution. Absorbance was measured at 450 nm. Intracellular zearalenone concentrations were extrapolated from the standard curve and normalized to protein concentration. This experiment was performed in triplicate.

**Statistical Analysis**

Data are expressed as mean ± SE and analyzed using Prism 5.0 (GraphPad SoftWare, Inc., San Diego, CA). LC$_{50}$ and IC$_{50}$ values were calculated using non-linear regression curve fitting analysis (log (inhibitor) vs. response - variable slope with four parameters). Data were analyzed by one-way ANOVA with Newman-Keuls post-hoc test comparison of 3 or more groups or a paired Student’s t-test for comparison of 2 groups. Statistical significance was set at p<0.05.
A-5.4 Results

Inhibition and Activation of BCRP ATPase Activity

Plasma membranes expressing human BCRP were used to indirectly test whether environmental chemicals can interact with BCRP by quantifying the liberation of inorganic phosphate following ATP hydrolysis. Sulfasalazine and Ko143 were used as a prototypical activator and inhibitor of BCRP ATPase activity, respectively. Ko143 inhibited sulfasalazine-mediated BCRP activation with an IC_{50} value of 1 μM. Ten of the eleven chemicals decreased BCRP-mediated ATPase activity, while no change in ATPase activity was detected in the presence of genistein. Based on inhibition of ATPase activity, the most potent inhibitors of BCRP were tributyltin, propargite, and methoxychlor followed by zearalenone, prochloraz, and propiconazole (Figure A-5.1 and Table A-5.2). Of the eleven environmental chemicals screened for interaction with BCRP, only genistein stimulated baseline ATPase activity at concentrations of 0.01 μM and higher (Figure A-5.1B).

Inhibition of BCRP Transport Activity in Membrane Vesicles

A second in vitro screening approach was used to test the inhibition of BCRP transport of Lucifer yellow in inverted plasma membrane vesicles generated from BCRP-overexpressing cells. Ko143 inhibited BCRP transport with an IC_{50} value of 0.04 μM (Figure A-5.2). All environmental chemicals reduced BCRP activity in inverted vesicles. The most potent inhibitors of BCRP (IC_{50} value less than 5 μM) were genistein and zearalenone (Figure A-5.2 and Table A-5.2).

BCRP-Mediated Transport in BeWo Cells

Human BeWo cells express BCRP protein (Figure A-5.3A). Incubation of BeWo cells with Ko143 increased the accumulation of Hoechst 33342 as demonstrated by enhanced
staining (Figure A-5.3B) and a greater percentage of cells with elevated fluorescence (Figure A-5.3C and D). Even the lowest concentration of Ko143 tested (0.03 µM) increased Hoechst 33342 levels in BeWo cells.

**Inhibition of BCRP Transport Activity in BeWo Cell**

Based on the initial ATPase activity and membrane vesicle screens, five environmental chemicals (genistein, bisphenol A, zearalenone, methoxychlor, and tributyltin) were selected for additional characterization by Hoechst accumulation in BeWo cells. None of the chemicals increased intracellular Hoechst fluorescence intensity at the lowest concentration tested (1 µM) (Figure A-5.4). Genistein, zearalenone, and tributyltin increased Hoechst fluorescence at 10 µM and above, whereas higher concentrations of bisphenol A (50 µM) and methoxychlor (100 µM) were needed to inhibit BCRP (Figure A-5.4).

**BCRP Expression, Function and Inhibition in Overexpressing Cells**

Additional studies aimed to further examine the interaction of BCRP with an environmental chemical. Zearalenone was selected based upon its inhibitory activity in the ATPase, inverted vesicle, and BeWo assays. For this purpose, HEK cells transfected with EV, WT BCRP and the genetic BCRP variant Q141K were used. Expression of total BCRP protein in cell lysates was similar between WT and Q141K BCRP (Figure A-5.5A). However, compared to cells transfected with EV, WT BCRP was able to reduce Hoechst accumulation by 80% whereas the Q141K BCRP variant lowered fluorescence by only 50% (Figure A-5.5B). The ability of BCRP to protect against the cytotoxicity of xenobiotics is well known (Abbott, 2006). Cell viability was assessed in the three cell lines treated with the known BCRP substrate mitoxantrone or zearalenone (Figure A-5.5C). Mitoxantrone decreased cell viability in HEK-EV cells with an LC_{50} value of 0.34
μM. WT BCRP, and to some extent the Q141K variant, protected against mitoxantrone toxicity as evidenced by higher LC₅₀ values (3.19 μM and 1.14 μM, respectively). Similarly, WT BCRP but not the Q141K variant conferred resistance to zearalenone toxicity in overexpressing cells relative to HEK-EV cells (Figure A-5.5C) and as evidenced by LC₅₀ values (EV: 45.6 μM, WT: 171.2 μM, and Q141K: 40.6 μM). Similar to BeWo cells, zearalenone increased Hoechst accumulation in WT BCRP cells at concentrations between 10-100 μM similar to Ko143 (Figure A-5.5D), confirming directly that zearalenone can inhibit BCRP activity.

Transport of Zearalenone by BCRP

To directly test whether zearalenone is a substrate of BCRP, an ELISA was used to quantify zearalenone concentrations. Compared to cells expressing the EV and the Q141K variant, zearalenone levels were reduced 25% in cells transfected with WT BCRP (Figure A-5.6A). Similarly, inhibition of BCRP activity in BeWo cells with Ko143 increased zearalenone levels by 2.7-fold (Figure A-5.6B). It should be noted that exposure of BeWo cells to zearalenone did not alter BCRP protein expression (Figure A-5.6C).
A-4.5 Discussion

The current manuscript utilized a series of transporter screening assays to investigate the interaction of eleven environmental chemicals with the human BCRP transporter. Each of the subcellular and cellular assays provided complementary data to categorize the chemicals as potential BCRP substrates and/or inhibitors. Based on the ATPase and vesicle inhibition results, five chemicals (genistein, tributyltin, propargite, methoxychlor, and zearalenone) appeared to be the most potent inhibitors identified by IC_{50} values in at least one of the assays. Subsequent experiments in human BeWo placental cells, which endogenously express BCRP, revealed inhibition of Hoechst 33342 efflux by genistein, zearalenone and tributyltin. Bisphenol A was also included in the BeWo inhibition studies for comparison since it was a relatively weak inhibitor in the ATPase and vesicle assays (Table A-5.2). Using BeWo cells, as well as HEK cells expressing wild-type BCRP and the Q141K variant, zearalenone was confirmed as a novel substrate of BCRP. Collectively, these studies provide a complement of transporter screens to prioritize environmental chemicals as potential transporter substrates.

The BCRP transporter was identified in 1998 by multiple groups (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). Initial studies demonstrated that human BCRP mRNA was most prominently expressed in placenta. Interestingly, one of the laboratories coined the name “ABC transporter of the placenta or ABCP”, however, this abbreviation did not hold (Allikmets et al., 1998). Nonetheless, subsequent studies have localized BCRP specifically to placental trophoblasts and fetal capillary endothelial cells (Jonker et al., 2000; Maliepaard et al., 2001). It should be noted that Bcrp-null mice are fertile (Jonker et al., 2002), suggesting that this transporter is not necessary for reproduction or development. Rather, the BCRP transporter is responsible for removing chemicals from the placental villi back to the maternal blood (reviewed in (Ni and Mao,
Because of this ‘barrier’ function, there is increasing interest in identifying drugs and chemicals that are substrates. Most importantly, mechanisms that reduce or inhibit BCRP function may increase exposure of the fetus to potentially toxic substrates. For example, the protein expression of the Q141K genetic variant is reduced in human placentas from a Japanese population (Kobayashi et al., 2005), suggesting that BCRP function may be reduced in placentas that express this variant. Further clinical studies are necessary to determine whether pregnant women with Q141K homozygous placentas do in fact have higher fetal exposure to drugs and adverse developmental outcomes.

The current study identified zearalenone as a novel substrate of placental BCRP (Fig 6). Pilot studies in our laboratory using Bcrp-null mice have demonstrated ~10-fold higher plasma zearalenone levels after oral dosing compared to wild-types (data not shown). This likely results from the lack of BCRP protein in the intestinal tract of the null mice. In addition to BCRP, other ABC transporters including multidrug resistance-associated protein (MRP) 1 and MRP2 can transport zearalenone in in vitro systems (Videmann et al., 2009). Similar to BCRP, MRP2 is expressed on the apical (maternal-facing) surface of syncytiotrophoblasts whereas MRP1 localizes to the basolateral (fetal-facing) surface (St-Pierre et al., 2000). The relative contribution of each of these transporters, as well as any potential passive transfer of zearalenone to the fetus, is not known. Nonetheless, zearalenone has been detected in fetal tissues from pregnant rats albeit at much lower levels than found in placenta or maternal liver (Bernhoft et al., 2001). This is important because of reports suggesting adverse developmental consequences of in utero exposure to zearalenone in multiple species. For example, maternal exposure to zearalenone (10 mg/kg/d) from gestation days 15 to 19, altered reproductive and mammary gland development in female offspring (Nikaido et al., 2004). In another
study, treatment of pregnant mice with zearalenone throughout pregnancy increased the number of apoptotic cells in the testes of male offspring (Lopez-Casas et al., 2012).

While the Q141K overexpressing cells had similar total BCRP protein as the wild-type cells (Figure A-5.5), trafficking of the protein to the plasma membrane is reduced ((Woodward et al., 2013) and data not shown). Thus, reduced efflux of zearalenone and impaired protection against mitoxantrone and zearalenone cytotoxicity by Q141K compared to wild-type BCRP cells is more likely due to the cell surface expression of BCRP protein rather than the intrinsic transport activity.

Each of the various assays employed in this study have advantages and limitations. For example, the ATPase and inverted vesicles provide direct access of test compounds to the transporter intracellular surface and do not require active uptake or passive permeability that are required in cellular systems such as BeWo cells. Based on the data shown in this study, it is clear that the ATPase assay can fail to detect substrates. Only genistein activated ATP hydrolysis in the BCRP-expressing plasma membranes (Figure A-5.1B), which is consistent with the known ability of BCRP to transport genistein (Enokizono et al., 2007a). However, bisphenol A and zearalenone did not stimulate ATP hydrolysis despite being substrates ((Dankers et al., 2013) and Fig. 6). Both the ATPase membranes and inverted vesicles demonstrated interactions of the environmental chemicals with the BCRP transporter, albeit at high concentrations in some cases. It is not clear whether the inhibition of BCRP is competitive or non-competitive. Additional experimentation would be needed to more thoroughly characterize the inhibition characteristics. Prior work confirms the utility of BeWo cells as a model of placental BCRP transport (Evseenko et al., 2006). In the current study, Hoechst 33342 was used as a fluorescent substrate of BCRP, which allows for
quantification by a fluorescent cell counter (Figures A-5.3 and 4). Interestingly, the chemicals that yielded more potent inhibition in the ATPase and vesicle assays, including genistein and zearalenone, showed similar results in the BeWo cell experiments. Additional experimental models including Bcrp-null mice as well as perfused human placentas are needed to more thoroughly characterize the in vivo and ex vivo disposition of zearalenone by BCRP.

Using a complement of in vitro assays, this study characterized the interaction of eleven environmental chemicals with the BCRP transporter. The chemicals inhibited BCRP to varying degrees, however, transport activity was only reduced at micromolar concentrations that may not be environmentally relevant. Nonetheless, screening assays prioritized zearalenone as a probable substrate that was confirmed using transport assays in which zearalenone was directly quantified. Future studies are needed to determine whether BCRP in the placenta reduces fetal exposure to zearalenone in vivo and whether impairment of BCRP activity due to genetic variants such as Q141K increases the developmental toxicity of zearalenone.
Figure A-5.1
**Figure A-5.1. Inhibition and activation of human BCRP ATPase by environmental chemicals.** BCRP membranes were incubated with ATP and increasing concentrations of environmental chemicals in the presence (A) and absence (B) of sulfasalazine (10 μM) for 30 min at 37°C. Additional reactions included sodium orthovanadate in order to determine the ATPase activity attributed to transport. The amount of inorganic phosphate released was determined by spectrophotometry following addition of a colorimetric reagent. Data are presented as the vanadate-sensitive mean ATPase activity ± SE of 3 replicates from one experiment. Following non-linear regression analysis, $R^2$ values ranged between 0.87-0.99 with the exception of genistein inhibition ($R^2 = 0.13$).
Figure A-5.2
Figure A-5.2. Inhibition of human BCRP transport in inverted vesicles by environmental chemicals. Inverted BCRP-expressing plasma membrane vesicles (20 μg) were incubated with Lucifer yellow (50 μM) for 2 min in the presence and absence of ATP and increasing concentrations of test chemical at 37°C. Data are presented as mean ATP-dependent BCRP activity ± SE of 3-4 replicates from one experiment. Non-linear regression analysis yielded R² values between 0.98-0.99.

Figure A-5.3. Characterization of BCRP expression and function in human BeWo placental cells. (A) Western blot of BCRP protein (~72 kDa) in BeWo cell lysates. GAPDH was used as a loading control. (B) Representative images of Hoechst 33342 accumulation (blue) in BeWo cells in the absence and presence of Ko143 (1 μM) collected using the Nexcelom Cellometer Vision. (C) Line graphs represent the distribution of individual cell Hoechst 33342 fluorescence. Each point represents the
mean percentage of cells ± SE (n = 4) exhibiting a quantity of fluorescence. (D) Bar graphs represent the mean relative fluorescence ± SE (n = 4) adjusted for cell size and normalized to control (no Ko143). Asterisks (*) represent statistically significant differences (p < 0.05) compared with control.
A.  

Fluorescence (\% of Control)

B.  

Concentration (\mu M)

Figure A-5.4
Figure A-5.4. Inhibition of BCRP transport by environmental chemicals in human BeWo placental cells. (A) Line graphs represent the distribution of individual cell Hoechst 33342 fluorescence in the absence and presence of the various environmental chemicals (50 μM). Each point represents the mean percentage of cells ± SE (n = 4) exhibiting a quantity of fluorescence. (B) Bar graphs represent the mean relative fluorescence ± SE (samples run in duplicate from four independent experiments). Data were adjusted for cell size and normalized to control (0 μM). Asterisks (*) represent statistically significant differences (p < 0.05) compared with control.
Figure A-5.5. Interaction of human BCRP transporter with zearalenone in BCRP-overexpressing cells.  (A) Western blot of BCRP protein (~72 kDa) in cell lysates from HEK cells overexpressing empty vector (EV), wild-type BCRP (WT) and the Q141K BCRP variant (Q141K). GAPDH was used as a loading control. BCRP protein expression was semi-quantified and presented as a bar graph.  (B) Accumulation of Hoechst 33342 (7 μM) was quantified using the Nexcelom Cellometer Vision. Bar graphs represent the mean relative fluorescence ± SE (n = 4) adjusted for cell size (C) Cell viability was assessed using the Alamar Blue Assay following exposure to mitoxantrone (5 μM) and zearalenone (100 μM) for 72 h. Data are normalized to untreated control cells and presented as mean ± SE. (D) Accumulation of Hoechst 33342 (7 μM) was quantified in WT cells exposed to increasing concentrations of zearalenone using the Nexcelom Cellometer Vision. Bar graphs represent the mean relative fluorescence (normalized to 0 μM) ± SE (n = 4) adjusted for cell size. Asterisks (*) represent statistically significant differences (p < 0.05) compared with control (0 μM or EV). Daggers (†) represent statistically significant differences (p < 0.05) compared with WT.
Figure A-5.6. Transport of zearalenone by the human BCRP transporter. (A) HEK cells overexpressing empty vector (EV), wild-type BCRP (WT) and the Q141K BCRP variant (Q141K) were treated with zearalenone (50 μM) for 60 min (uptake period), washed, and then incubated in fresh culture media for 30 min (efflux period). (B) Human BeWo placental cells were treated with zearalenone (10 μM) in the presence of absence of the BCRP inhibitor, Ko143 (1 μM) for 60 min (uptake period), washed, and then incubated in fresh culture media with or without Ko143 (1 μM) for 30 min (efflux period). Intracellular zearalenone accumulation was quantified by ELISA and normalized to protein concentrations of the cellular lysates. Data are presented as mean ± SE (n = 12-15). (C) Western blot of BCRP protein (~72 kDa) in cell lysates from BeWo cells treated with 0, 1 or 10 μM zearalenone. B-actin was used as a loading control. BCRP protein expression was semi-quantified and presented as a bar graph. Asterisks (*) represent statistically significant differences (p < 0.05) compared to EV cells. Daggers (†) represent statistically significant differences (p < 0.05) compared to no Ko143.
Table A-5.1. Examples of adverse developmental effects of environmental chemicals.

<table>
<thead>
<tr>
<th>Environmental chemical</th>
<th>Organ system</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>Mammary gland and reproductive system</td>
<td>Rats</td>
<td>Hilakivi-Clarke et al., 1998</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>Mammary gland and reproductive system</td>
<td>Mice</td>
<td>Newbold et al., 2009; Vandenberg et al., 2007; Nikaido et al., 2004</td>
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<tr>
<td>Zearalenone</td>
<td>Mammary gland and reproductive system</td>
<td>Mice, rats</td>
<td>Hilakivi-Clarke et al., 1998; Nikaido et al., 2004</td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>Neurological and reproductive systems</td>
<td>Mice, rats</td>
<td>Palanza et al., 1999; Armenti et al., 2008</td>
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<td>Myclobutanil</td>
<td>Reproductive system</td>
<td>Rats</td>
<td>Rockett et al., 2006; Goetz et al., 2007</td>
</tr>
<tr>
<td>Prochloraz</td>
<td>Mammary gland and reproductive system</td>
<td>Rats</td>
<td>Jacobsen et al., 2012; Noriega et al., 2005</td>
</tr>
<tr>
<td>Tributyltin</td>
<td>Adiposity and reproductive system</td>
<td>Mice</td>
<td>Chamorro-Garcia et al., 2013; Si et al., 2012</td>
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<tr>
<td>Propiconzaole</td>
<td>Reproductive system</td>
<td>Rats</td>
<td>Rocket et al., 2006; Goetz et al., 2007</td>
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<tr>
<td>Tebuconazole</td>
<td>Mammary gland and reproductive system</td>
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<td>Jacobsen et al., 2012</td>
</tr>
<tr>
<td>Epoxiconazole</td>
<td>Mammary gland and reproductive system</td>
<td>Rats</td>
<td>Jacobsen et al., 2012</td>
</tr>
</tbody>
</table>

*The timing of developmental exposure (such as prenatal, perinatal, etc.) varied between studies. In addition, some studies were conducted as mixtures. It should be noted that there has been little investigation of adverse effects in offspring following exposure to propargite during development.*
Table A-5.2. *In vitro* inhibition of human BCRP transporter by environmental chemicals using the ATPase and inverted vesicle assays.\(^1\)

<table>
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<tr>
<th>Chemical</th>
<th>ATPase Assay</th>
<th>Vesicle Assay</th>
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</thead>
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<tr>
<td>Genistein</td>
<td>N.D.</td>
<td>1.9</td>
</tr>
<tr>
<td>Tributyltin</td>
<td>1.2</td>
<td>30.5</td>
</tr>
<tr>
<td>Propargite</td>
<td>2.5</td>
<td>31.8</td>
</tr>
<tr>
<td>Methoxychlor</td>
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<td>38.0</td>
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<tr>
<td>Zearalenone</td>
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<td>4.8</td>
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<tr>
<td>Prochloraz</td>
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<td>18.7</td>
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<tr>
<td>Propiconazole</td>
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<td>41.1</td>
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<td>Epoxiconazole</td>
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<td>64.7</td>
</tr>
<tr>
<td>Bisphenol A</td>
<td>&gt;100.0</td>
<td>55.5</td>
</tr>
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</table>

\(^1\) The ability of environmental chemicals to inhibit BCRP-mediated ATPase activity in sulfasalazine (10 μM)-stimulated membranes and Lucifer yellow (50 μM) transport in inverted plasma membrane vesicles overexpressing human BCRP was tested. IC\(_{50}\) values were calculated in Prism 5.0 using non-linear regression analysis. In the ATPase assay, IC\(_{50}\) values for tebuconazole, epoxiconazole, and bisphenol A were estimated to be greater than the concentrations tested. N.D.: not detected.
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