ROLES OF SULFOTRANSFERASE ENZYMES IN TRANS-PLACENTAL DISPOSITION

By

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This dissertation is dedicated to my family.

ABSTRACT

The trophoblast cell layer constitutes the rate-determining barrier for trans-placental transfer. Several isoforms of the sulfotransferase enzymes are functional in placenta but there is only limited information available on the utility of cultured trophoblast cells for studying placental sulfation. We examined the expression and activities of four sulfotransferase isoforms (SULT1A1, SULT1A3, SULT1E1, and SULT2A1) in primary cytotrophoblast cells and the trophoblast-like BeWo cell line. These isoforms have been reported to be functional in human placenta. Our results indicated that the phenolic sulfotransferase isoforms, SULT1A1 and SULT1A3, are functional in BeWo, as well as in the primary cytotrophoblast cells. SULT2A1 and SULT1E1 are not functional in either cell type. We also found that chronic exposure to the industrial chemical bisphenol A inhibited SULT1A1 activity. A U-shaped dose-response curve was observed with inhibition (~30-40%) being observed only at intermediate concentrations (10-100 nM). These results suggested that trophoblast cells may be used as a suitable *in vitro* tool to determine the effect of endogenous or exogenous substances on placental sulfotransferase activity. Altered metabolic activity has the chance of altering fetal exposure to drugs and other substances in the maternal circulation. Studies performed in BeWo cells also suggested that one of the roles of the placental efflux transporters is the elimination of sulfate metabolites. The multidrug resistance-associated proteins (MRPs) played a major role in the elimination of 4-nitrophenyl sulfate and acetaminophen sulfate across the basolateral (fetal-facing) and apical (maternal-facing) trophoblast membranes respectively. The

breast cancer resistance protein (BCRP) played a minor role in the elimination of these two sulfate conjugates across the apical membrane.

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Chapter 1: Introduction

1.1 Introduction

During pregnancy the fetus is often exposed to drugs and foreign chemicals from the maternal circulation. For women afflicted with diseases such as diabetes, asthma, epilepsy, and psychiatric disorders, taking drugs during pregnancy is often a necessity. Approximately 50% of pregnant women take prescription drugs and ~4% use drugs that have been found to pose fetal risk in animals and humans [1, 2]. In addition, over-the-counter medicines such as non-steroidal anti inflammatory drugs, antihistamines, and antacids are also commonly consumed during pregnancy. Obvious ethical constraints prevent conducting clinical trials in pregnant women, and hence information is lacking on the fetal exposure and safety of many of the drugs consumed during pregnancy. Unless the objective is to treat the fetus (e.g. to prevent maternal to fetal transmission of HIV), the overall goal of drug delivery during pregnancy is reduction of fetal exposure to drugs.

As the placenta forms the physiological barrier between the mother and the fetus during pregnancy, determining the placental disposition of a drug helps to ascertain the possibility of fetal exposure. Among other placental factors, nutrient transporters, efflux transporters, and metabolic enzymes in the placenta are determinants of trans-placental transfer [3]. The expression and function of placental transporters and enzymes change with gestational age and upon exposure to foreign chemicals. Thus, it is important to determine the types, functions, and regulation of placental enzymes and transporters.

1.2 The placental barrier

Placentation begins 6-8 days post conception when the blastocyst invades the uterine endometrium. The blastocyst consists of an outer epithelial cell layer (the trophoblast) and an inner cell mass (the embryoblast). Mononuclear proliferative, trophoblast cells (cytotrophoblasts) invade the uterine mucosa and fuse together into multinucleated, nonproliferative cells (syncytiotrophoblast). They further develop to form "finger-like projections" known as villi. Villi that contact and spread around the endometrium are known as anchoring villi while villous branches extending into the intervillous space are known as free villi. The combination of the anchoring villi and the decidualized endometrium is known as the basal plate. Maternal blood enters and leaves the intervillous space through the basal plate and in this process the villi are bathed in maternal blood. From the chorionic plate (the placental "boundary" on the fetal side) mesenchymal cells invade the villi and differentiate to ultimately form the fetal capillaries. This arrangement places the trophoblast layer in contact with maternal blood on one side and in close apposition to the fetal capillaries on the other side.

During the first trimester the trophoblast layer consists of an outer syncytiotrophoblast layer (i.e., a multinucleated layer devoid of any lateral cell boundaries) and an underlying cytotrophoblast layer. The latter gradually becomes discontinuous with advancing gestation [4-6]. Therefore, the transfer of solutes from the maternal to the fetal circulation requires passage through three layers of cells – the

syncytiotrophoblast layer, the fetal connective tissue, and the fetal capillary endothelium (Figures 1.2.1 and 1.2.2).

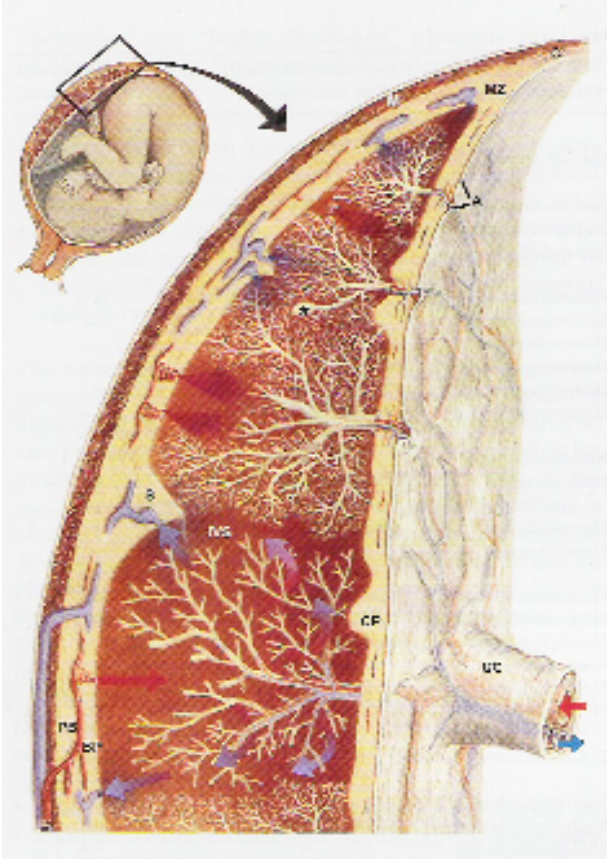


Figure 1.2.1 The mature human placenta showing the villous tree extending from the chorionic plate into the intervillous space where they are surrounded in maternal blood. The figure also shows the villi vascularized by the fetal blood vessels. Abbreviations: BP (basal plate), CP (chorionic plate), IVS (intervillous space), and UC (umbilical cord). The figure has been adapted from Kurt Benirschke, Peter Kaufmann, Rebecca N. Baergen, *Pathology of the human placenta*. 2006: Springer).

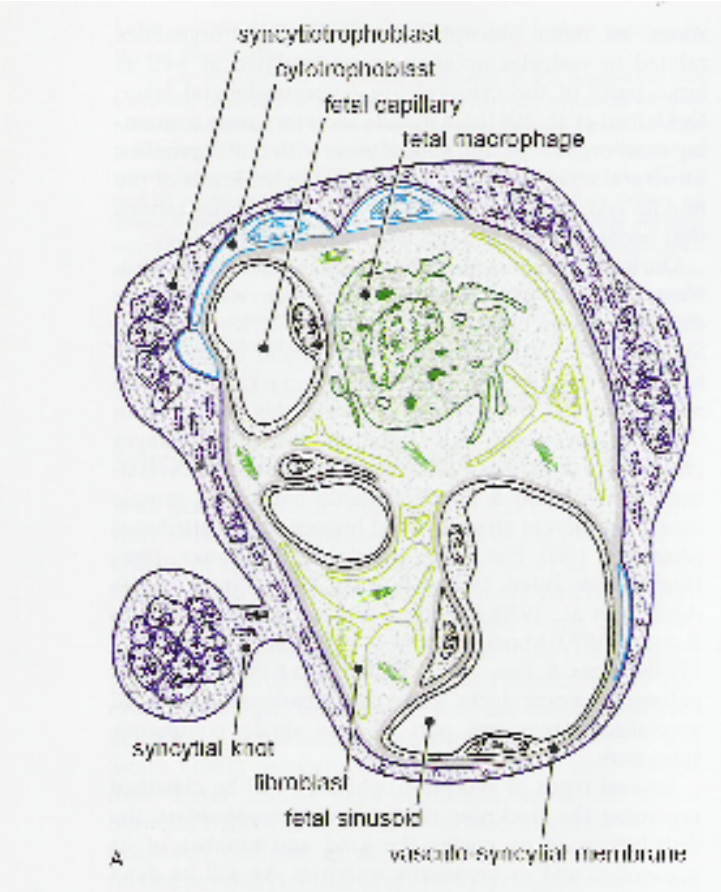


Figure 1.2.2 Cross section of the placental villous tree (The figure has been adapted from Kurt Benirschke, Peter Kaufmann, Rebecca N. Baergen, *Pathology of the human placenta*. 2006: Springer).

Among these three layers, the syncytiotrophoblast layer constitutes the rate-limiting barrier to transfer between the mother and the fetus [4, 6]. The syncytiotrophoblast layer is polarized in nature. The maternal-facing plasma membrane which is in direct contact with maternal blood is a microvillous membrane, while the fetal-facing basement membrane lacks microvilli [4]. In addition to being structurally distinct, the two membranes also differ with respect to the transporters expressed on each of them, which allows directional transport across the placenta [7].

1.3 Models of the placenta

1.3.1 *In vivo* and *ex vivo* models

The human placenta is hemochorial in nature, i.e., the chorionic villi invade and destroy the uterine decidua in such a way that the trophoblast layer is directly bathed in maternal blood [4]. This is in contrast to epitheliochorial placentation (e.g., pig, horse) where the chorionic epithelial cells are in contact with the endometrial epithelium, and endotheliochorial placentation (e.g., dog, cat) where the endometrium is invaded such that the chorion is in contact with the endothelial cells of the maternal capillaries [5]. *In vivo* studies for determining placental transfer or fetal exposure have been conducted in rodents and primates. Both exhibit hemochorial placentation [8, 9]. Animal models take into account maternal as well as fetal pharmacokinetics in determining drug transfer, but suffer from the disadvantages of anatomical and functional placental differences, and different gestational lengths [10].

The *ex vivo* model of the human placenta most representative of *in vivo* settings is the perfused placental cotyledon. The cotyledon is known as the anatomical unit of the placenta and each contains a villous tree. At term, the placenta contains 20-30 cotyledons. These are isolated from term placenta and retain much of the placental structural integrity. The perfused placental cotyledon model has been found to reliably predict not only the mechanism and transfer rates of drugs but in certain cases also the extent of fetal transfer [11]. Another *ex vivo* model is villous tissue fragments. Villous fragments retain structural and functional integrity up to at least 3

hours *in vitro* and can be used to assess drug uptake but not drug transfer. The advantage of this model over cell culture models is that polarization as well as transporter and enzyme expressions are more representative of the *in vivo* situation [12].

1.3.2 *In vitro* models

Cell culture models of the placenta include cytotrophoblast cells isolated from term placenta. In primary culture, the cytotrophoblasts do not proliferate but do spontaneously differentiate into syncytiotrophoblasts. The cells tend to aggregate in the process of differentiation producing large intercellular spaces. This renders primary cultures unsuitable for transport studies but they have been used successfully to assess drug uptake and metabolism [13]. Another advantage is that they allow the study of changes in expression and function of drug transporters and drug metabolizing enzymes, as the cells differentiate from cytotrophoblasts into syncytiotrophoblasts [14].

Other cell culture models include trophoblast-like cell lines (e.g., BeWo, JAr and JEG-3) isolated from malignant choriocarcinomas. The BeWo cell line was derived from a methotrexate-resistant tumor. In culture BeWo cells mainly consist of moderate-sized, mononuclear, proliferative cells that are morphologically cytotrophoblast-like, with a smaller proportion of larger, multinucleated cells that are syncytiotrophoblast-like [15]. Unlike primary cytotrophoblasts, BeWo cells do not

spontaneously differentiate into syncytiotrophoblasts in culture but on treatment with substances that increase levels of cyclic AMP (e.g., forskolin), they differentiate to morphologically represent the syncytiotrophoblasts (i.e., the nuclei cluster, there is no plasma membrane in between the nuclei, a dense microvillar brush border appears on the apical surface). The morphological differentiation is not accompanied by complete biochemical differentiation. Upon forskolin treatment, although human chorionic gonadotropin was produced at higher levels, the expression of placental-alkaline phosphatase and the production of human placental lactogen were not significantly different from what was observed in the control cells [16]. Placental alkaline phosphatase, human chorionic gonadotropin, and human placental lactogen are either expressed or secreted by syncytiotrophoblasts at much higher levels than the cytotrophoblasts.

On the other hand, based on efflux transporter expression profile, it has been suggested that BeWo is more similar to the syncytiotrophoblast than cytotrophoblasts [14]. Grown on transwell inserts the BeWo cells form a polarized, confluent monolayer that exhibits asymmetrical transport of several substances including amino acids, nutrients, cholesterol, folic acid, and neurotransmitters [17-19]. It also exhibits metabolism similar to that of cytotrophoblasts [13]. Other choriocarcinoma cell lines, JAr and JEG-3 have been used to study placental uptake but they are unsuited for trans-trophoblast studies as they do not form confluent monolayers in culture [12]. Most of the work in this thesis has been performed with the BeWo cell line.

1.4 Factors affecting placental drug transfer

As with permeation across any other epithelial or endothelial barrier, drug properties influence the extent of placental transfer. As passive diffusion is the major mode of placental transfer, smaller molecules (molecular weight $\leq$ 600 Da), that are lipophilic, and exhibit a low degree of ionization, easily cross the placenta [3]. Several maternal, placental, as well as fetal factors also affect the fetal/maternal ratio of a drug and this section briefly discusses a few factors.

Maternal factors include metabolism, protein binding, and health conditions which can influence placental blood flow [3]. For example, pregnancy is associated with declining maternal albumin concentrations. This has the potential of increasing the fetal exposure of highly protein-bound drugs as a function of gestation [20].

Fetal factors include protein binding, pH and metabolism. Fetal pH is normally approximately 0.1 units lower than maternal pH but conditions of fetal acidosis, such as is induced during labor, favor the partitioning of basic drugs into the fetal compartment [21, 22]. Fetal serum albumin concentrations are ~40% of maternal serum albumin concentrations in early pregnancy but steadily rise to exceed maternal concentrations near term. Fetal alpha-1 acid glycoprotein concentrations were also shown to increase during pregnancy although they never exceeded maternal concentrations [23]. The change in fetal/maternal protein concentrations can affect the free fraction of the drug in the fetus as a function of gestation. Fetal metabolism is

detected as early as 8-9 weeks of gestation [24]. Although the fetal liver does not express all the cytochrome P450 (CYP) enzymes detected in the adult liver, several CYP isoforms are present. The fetal liver also expresses sulfotransferase and glutathione-S-transferase isoforms, and there is evidence of fetal glucuronidation as well [9, 25, 26].

Placental morphology affects the extent of fetal transfer of drugs whose predominant mode of transport is passive diffusion since diffusional distance decreases (i.e., the syncytiotrophoblast layer decreases in thickness and the newer villi that form are smaller) and surface area increases with advancing gestation.

Placental carrier mechanisms include peptide transporters, amino acid transporters, serotonin transporter, norepinephrine transporter, organic cationic transporters, etc. Structurally similar drugs can compete with the endogenous ligands of these transporters to reach the fetus. For example, the antiepileptic drug gabapentin was reported to have an umbilical cord/maternal plasma ratio of two indicative of fetal accumulation. Placental transfer of gabapentin was proposed to be through the L-type amino acid transporter (LAT-1) [27]. Drugs can also restrict nutrient transport to the fetus to produce profound pharmacological consequences. For instance, amphetamine derivatives inhibit OCTN2-mediated carnitine uptake in placenta [28]. Since the fetus cannot synthesize carnitine on it's own, decreased carnitine transport has the potential of causing cardiomyopathy, muscle weakness, and even sudden infant death [28]. In

addition to the factors mentioned above, placental efflux and metabolism are also determinants of fetal drug exposure. The following section will examine their roles in detail.

1.4.1 Placental metabolism

Placental metabolism is detected as early as the first trimester. CYP1A1 is functional throughout pregnancy [13, 29]. CYP2E1 protein is detected as early as the first trimester but is not functionally active [30]. In term placenta, CYP2E1's protein was detected only in alcoholic patients suggestive of alcohol inducibility, although this exhibited inter-individual variation [31]. Transcripts of several other CYP isoforms have been detected. Some of these are expressed throughout pregnancy but no data is available on their functional activity (e.g., CYP1B1, CYP2F1); others are detected only in the first trimester but not at term (e.g., CYP1A2, CYP3A7) [22, 29, 32]. CYP3A4 which is quantitatively the most abundant CYP in the liver does not display any placental activity [33]. Two steroid-metabolizing isoforms, CYP11A1 (associated with cholesterol side-chain cleavage activity which is the first rate-limiting step in steroid hormone production) and CYP19 (which catalyzes the conversion of androstenedione to estrogens), are constitutively expressed [34]. Selected isoforms of the phase II enzymes, N-acetyltransferase (NAT), glutathione-S-transferase (GST), uridine-diphosphate-glucuronosyl-transferase (UGT), and sulfotransferase (SULT) enzymes are functional in the placenta with UGT activity being greater in the first trimester than at term [30]. The overall pattern of expression and function seems to

indicate that placental metabolism has a more important role during the early stages of pregnancy when fetal metabolism has not developed completely. Further, the lack of functional activity of many placental enzymes has led to the theory that while placental metabolism may influence fetal accumulation of a particular drug, it may be of greater significance from a toxicological view point since placental enzymes have been found to produce mutagenic or toxic metabolites that pose fetal risk.

Exposure to foreign chemicals (e.g., maternal drugs, components of cigarette smoke) can change placental metabolic activity. Smoking induces CYP1A1 and UGT activity, while the anti-HIV drug Azidothymidine induces CYP1A1 and GST, but decreases UGT activity [13, 30, 35]. In a comparative study, it was observed in the placenta of smokers, that CYP1A1 activity and CYP1B1 mRNA were significantly induced, CYP19A1 (aromatase) activity was reduced, while there was no alteration in the genetic expression of phase II enzymes [36]. As CYP1A1, CYP1B1, and CYP19A1 are contributors in the placental estrogen synthesis pathway, it was suggested that smoking may adversely affect this pathway. Cadmium, a component of cigarette smoke, decreases 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) activity in trophoblast, at concentrations similar to its concentrations detected in placenta. It was proposed that *in vivo* this has the chance of producing fetal growth restriction [37].

1.4.1.1 Placental sulfation

Sulfation involves the transfer of a sulfate group from a donor molecule 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to acceptor molecules containing amine, hydroxyl, N-hydroxyl, or sulfhydryl groups. The reaction is mediated by sulfotransferase (SULT) enzymes which may be either cytosolic or membrane-bound. The cytosolic enzymes sulfate small molecules that include endogenous substances (e.g., estradiol, thyroid hormones, bile acids, and neurotransmitters), several drugs and environmental chemicals. Sulfation is mostly a detoxification pathway but in certain cases substrates (e.g., benzylic alcohols derived from polycyclic aromatic hydrocarbons, N-hydroxylated derivatives of homocyclic and heterocyclic amines, nitroalkanes etc.) are activated to produce carcinogenic DNA adducts [38].

The sulfation of several drugs has been detected and several sulfotransferase isoforms have been identified in the placenta [39]. Examination of sections of whole placental tissue revealed that the phenolic sulfotransferase isoforms SULT1A1 and SULT1A3 were functional in all sections of placental tissue of both maternal and fetal origin. Highest activity was reported in sections containing syncytiotrophoblasts [40]. SULT1E1 (estrogen sulfotransferase) protein expression was reported to be low in fractions rich in syncytiotrophoblasts. Other studies have reported abundant estrogen sulfotransferase in the placenta and have localized the protein to the syncytiotrophoblast [41, 42]. Placental dehydroepiandrosterone sulfation was

attributed to SULT2A1 activity [40]. Another isoform, SULT2b1b, which sulfates cholesterol with a high affinity, is localized to the nuclei of syncytiotrophoblast [43].

Among the 13 human cytosolic sulfotransferase genes identified thus far, SULT1A1 is the major sulfotransferase in the human liver. It is also functional in the gastrointestinal tract (GIT) and platelets. SULT1A1 sulfates a wide range of substrates that includes among others estradiol and thyroid hormones[44]. Genetic polymorphisms in SULT1A1 which lead to lower enzyme activity have been associated with upper aerodigestive tract multiple primary neoplasms (UADT-MPN), upper urinary tract urothelial cell carcinoma (UUT-UUC), meningiomal brain tumors, and squamous cell carcinoma of the head and neck (SCCHN) [45-48]. Endogenous substrates of SULT1A3 include neurotransmitters and the enzyme is functional in the gastrointestinal tract and the brain. SULT1E1 sulfates 17β-estradiol with the highest affinity compared to any of the other sulfotransferase isoforms and is functional in the liver, GIT, and the endometrium [49]. SULT2A1 is predominantly functional in the adrenal gland and sulfates steroid hormones (DHEA), cholesterol, and bile acids [44]. SULT2A1, SULT1E1, and SULT1A3 are also well developed in fetal tissues [44]. Although, SULT1A1, SULT1A3, SULT1E1, and SULT2A1 have been extensively characterized in other human tissue, there has not been much work regarding their trophoblast localization. Hence, a part of the objective of this research project was to determine the functional activities of the four sulfotransferase enzymes mentioned above in the BeWo cells and primary cytotrophoblast cells.

1.4.2 Placental efflux transport

The placenta expresses several efflux transporters on both syncytiotrophoblast membranes and the fetal capillary endothelium. Efflux transporters located on the brush border membrane include P-glycoprotein (P-gp/MDR1/ABCB1), the breast cancer resistance protein (BCRP/ABCG2), and multidrug resistance-associated protein (MRP/ABCC) isoforms 1-5 [12]. Efflux transporters located on the basolateral membrane include MRP1 and MRP5 [50, 51]. One of the supposed functions of the efflux transporters located on the apical membrane is the reduction of fetal exposure to drugs and foreign chemicals. Control of fetal exposure to xenobiotics is suggested by several examples. When a fraction of the CF-1 mice naturally lacking the *mdr1a* gene that leads to a loss of placental P-gp were exposed to avermectin during pregnancy, it was observed that fetuses with the genotype *Mdr1a(-/-)* were most susceptible to avermectin-induced cleft palate [52]. Similarly, there was a 3.2 fold greater fetal accumulation of the BCRP substrate topotecan in *Mdr1a/1b(-/-)* knockout mice treated with a dual P-gp and BCRP inhibitor, compared to mice where the inhibitor was not used [8]. In the human perfused placental model, P-gp limited the maternal to fetal transfer of the HIV protease inhibitor indinavir while BCRP limited the maternal-to-fetal clearance of the antidiabetic glyburide [53, 54]. On the other hand efflux transporters on the basolateral membrane are likely to increase fetal exposure to substances. Interestingly, it has been observed that the expression and activity of the efflux transporters change with gestational age. With increasing gestational age, P-gp function decreases and MRP2 function increases [51,

55, 56]. Many of the studies assessing the trans-placental transfer of drugs are performed in *ex vivo* models of the human placenta. The results of these studies often report the extent to which a particular transporter influences fetal accumulation and therefore may not be very reflective of the earlier stages of pregnancy [53, 54].

Genetic polymorphisms in transporters have been postulated to be a reason for the inter-individual differences observed in drug response. Single nucleotide polymorphisms in BCRP and P-glycoprotein have been found to lead to low placental expression but not much work has focused on the effects of polymorphism on trans-placental transfer. Using an *ex vivo* placental model it was observed that one such P-glycoprotein polymorphism that led to low protein expression did not affect the transfer of the P-gp substrate saquinavir [57].

1.4.2.1 Placental sulfate conjugate efflux

While P-gp substrates are neutral or cationic, BCRP and MRP substrates also include anionic compounds. This suggests another role of BCRP and the MRP isoforms i.e., the removal of anionic conjugates. BCRP effluxes sulfate and glucuronide conjugates, while the MRP isoforms eliminate sulfate, glucuronide, as well as glutathione conjugates. This role of the efflux transporters has been seen in the liver, intestine, and kidney [58-64].

Species as well as tissue differences exist with respect to the relative contribution of the efflux transporters in sulfate elimination. For e.g. while in mice BCRP mediated the biliary elimination of acetaminophen sulfate with a minor contribution from MRP2, it was the opposite in rats [60, 61]. In mice, ~30% of the biliary efflux of troglitazone sulfate was via BCRP but BCRP played only a limited role in preventing the oral absorption of troglitazone sulfate [62].

Although it has been proposed that one of the roles of the MRP isoforms and BCRP in the placenta is the elimination of conjugates, this has not yet been investigated. However, altered sulfate transport has been associated with profound toxicological consequences in other tissues. For example, the antidiabetic drug troglitazone was withdrawn from the market due to drug-induced liver toxicity. Troglitazone and troglitazone sulfate inhibit the hepatic bile salt export pump (BSEP) to cause increased hepatic accumulation and hence increased serum levels of bile acids [65]. Troglitazone sulfate is a more potent BSEP inhibitor [65]. MRP2 eliminates troglitazone sulfate. Serum levels of bilirubin were higher in MRP2-deficient rats compared to wild type rats [65]. Thus not only is troglitazone sulfate toxic but its impaired hepatic excretion exacerbates this toxicity. The examples of troglitazone sulfate, as well as the differential involvement of the efflux transporters in sulfate conjugate elimination, underscore the importance of determining the transporters responsible for sulfate elimination in placental tissue.

1.5 Specific objectives

The objectives of this thesis research were to identify the sulfotransferase enzymes functional in trophoblast, determine the induction/inhibition of sulfotransferase enzymes in trophoblast, and elucidate the mechanisms of sulfate elimination from trophoblast cells.

In chapter 2 we assessed the mRNA expression and enzyme activities of selected sulfotransferase isoforms (SULT1A1, SULT1A3, SULT1E1, and SULT2A1) in BeWo cells and primary cytotrophoblast cells. Substrates used for determining SULT1A1, SULT1A3, SULT1E1, and SULT2A1 activities were 4-nitrophenol, dopamine, 17β-estradiol, and dehydroepiandrosterone respectively.

In chapter 2 SULT1A1 was determined to be one of the enzymes functional in BeWo cells. There is much evidence in the literature that indicates that SULT1A1 is inhibited by estrogenic substances on acute exposure. Bisphenol A, exhibits estrogenic properties *in vitro.* It also achieves moderate placental concentrations. In chapter 3 the effects of chronic exposure to bisphenol A on SULT1A1 activity are discussed. It was also examined whether the inhibitory effect was mediated through the nuclear estrogen receptor (ER) β.

In chapter 4 the efflux transporters mediating the elimination of sulfate metabolites from the apical and basolateral trophoblast membranes were determined. BeWo cells

were used as a trophoblast model. Among the efflux transporters that eliminate sulfate metabolites in other tissues, BCRP, MRP1, MRP2, and MRP5 are expressed in BeWo cells. In syncytiotrophoblasts, BCRP and MRP2 are localized to the maternal-facing membrane, while MRP1 and MRP5 are localized to both membranes. Compared to MRP1 or MRP2, BCRP mRNA is upregulated in BeWo cells. This led to the initial hypothesis that BCRP would be responsible for sulfate elimination from the apical (maternal-facing) trophoblast membrane.

Chapter 5 is a summary of the findings and conclusions drawn from each part of the research work. It also lists the future directions.

1.6 References

1. Riley, E.H., et al., *Correlates of prescription drug use during pregnancy.* J Womens Health (Larchmt), 2005. **14**(5): p. 401-9.
2. Andrade, S.E., et al., *Prescription drug use in pregnancy.* Am J Obstet Gynecol, 2004. **191**(2): p. 398-407.
3. Audus, K.L., *Controlling drug delivery across the placenta.* Eur J Pharm Sci, 1999. **8**(3): p. 161-5.
4. Enders, A.C. and T.N. Blankenship, *Comparative placental structure.* Adv Drug Deliv Rev, 1999. **38**(1): p. 3-15.
5. Myren, M., et al., *The human placenta--an alternative for studying foetal exposure.* Toxicol In Vitro, 2007. **21**(7): p. 1332-40.
6. Young, A.M., C.E. Allen, and K.L. Audus, *Efflux transporters of the human placenta.* Adv Drug Deliv Rev, 2003. **55**(1): p. 125-32.
7. Unadkat, J.D., A. Dahlin, and S. Vijay, *Placental drug transporters.* Curr Drug Metab, 2004. **5**(1): p. 125-31.
8. Jonker, J.W., et al., *Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan.* J Natl Cancer Inst, 2000. **92**(20): p. 1651-6.
9. Garland, M., et al., *Placental transfer and fetal elimination of morphine-3-beta-glucuronide in the pregnant baboon.* Drug Metab Dispos, 2008. **36**(9): p. 1859-68.
10. Carter, A.M., *Animal models of human placentation--a review.* Placenta, 2007. **28 Suppl A**: p. S41-7.
11. Tuntland, T., et al., *In vitro models to predict the in vivo mechanism, rate, and extent of placental transfer of dideoxynucleoside drugs against human immunodeficiency virus.* Am J Obstet Gynecol, 1999. **180**(1 Pt 1): p. 198-206.
12. Mitra, P., and K.L. Audus., *In Vitro models and multidrug resistance mechanisms of the placental barrier*, in *Drug Absorption Studies In Situ, In Vitro and In Silico Models*, C. Ehrhardt. and K.J. Kim, Editors, 2008, Springer: New York, NY. p. 368.
13. Avery, M.L., C.E. Meek, and K.L. Audus, *The presence of inducible cytochrome P450 types 1A1 and 1A2 in the BeWo cell line.* Placenta, 2003. **24**(1): p. 45-52.
14. Evseenko, D.A., J.W. Paxton, and J.A. Keelan, *ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast.* Am J Physiol Regul Integr Comp Physiol, 2006. **290**(5): p. R1357-65.
15. Friedman, S.J. and P. Skehan, *Morphological differentiation of human choriocarcinoma cells induced by methotrexate.* Cancer Res, 1979. **39**(6 Pt 1): p. 1960-7.
16. Wice, B., et al., *Modulators of cyclic AMP metabolism induce syncytiotrophoblast formation in vitro.* Exp Cell Res, 1990. **186**(2): p. 306-16.

17. Bode, C.J., et al., *In vitro models for studying trophoblast transcellular transport.* Methods Mol Med, 2006. **122**: p. 225-39.
18. Zhao, H. and H.S. Hundal, *Identification and biochemical localization of a Na-K-Cl cotransporter in the human placental cell line BeWo.* Biochem Biophys Res Commun, 2000. **274**(1): p. 43-8.
19. Liu, F., M.J. Soares, and K.L. Audus, *Permeability properties of monolayers of the human trophoblast cell line BeWo.* Am J Physiol, 1997. **273**(5 Pt 1): p. C1596-604.
20. Loebstein, R., A. Lalkin, and G. Koren, *Pharmacokinetic changes during pregnancy and their clinical relevance.* Clin Pharmacokinet, 1997. **33**(5): p. 328-43.
21. Krishna, B.R., M.I. Zakowski, and G.J. Grant, *Sufentanil transfer in the human placenta during in vitro perfusion.* Can J Anaesth, 1997. **44**(9): p. 996-1001.
22. Myllynen, P., M. Pasanen, and K. Vahakangas, *The fate and effects of xenobiotics in human placenta.* Expert Opin Drug Metab Toxicol, 2007. **3**(3): p. 331-46.
23. Krauer, B., P. Dayer, and R. Anner, *Changes in serum albumin and alpha 1-acid glycoprotein concentrations during pregnancy: an analysis of fetal-maternal pairs.* Br J Obstet Gynaecol, 1984. **91**(9): p. 875-81.
24. Hakkola, J., et al., *Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity.* Crit Rev Toxicol, 1998. **28**(1): p. 35-72.
25. Raijmakers, M.T., E.A. Steegers, and W.H. Peters, *Glutathione S-transferases and thiol concentrations in embryonic and early fetal tissues.* Hum Reprod, 2001. **16**(11): p. 2445-50.
26. Pacifici, G.M., *Sulfation of drugs and hormones in mid-gestation human fetus.* Early Hum Dev, 2005. **81**(7): p. 573-81.
27. Ohman, I., S. Vitols, and T. Tomson, *Pharmacokinetics of gabapentin during delivery, in the neonatal period, and lactation: does a fetal accumulation occur during pregnancy?* Epilepsia, 2005. **46**(10): p. 1621-4.
28. Rytting, E. and K.L. Audus, *Novel organic cation transporter 2-mediated carnitine uptake in placental choriocarcinoma (BeWo) cells.* J Pharmacol Exp Ther, 2005. **312**(1): p. 192-8.
29. Hakkola, J., et al., *Detection of cytochrome P450 gene expression in human placenta in first trimester of pregnancy.* Biochem Pharmacol, 1996. **52**(2): p. 379-83.
30. Collier, A.C., et al., *Metabolizing enzyme localization and activities in the first trimester human placenta: the effect of maternal and gestational age, smoking and alcohol consumption.* Hum Reprod, 2002. **17**(10): p. 2564-72.
31. Rasheed, A., R.N. Hines, and D.G. McCarver-May, *Variation in induction of human placental CYP2E1: possible role in susceptibility to fetal alcohol syndrome?* Toxicol Appl Pharmacol, 1997. **144**(2): p. 396-400.

32. Hakkola, J., et al., *Expression of xenobiotic-metabolizing cytochrome P450 forms in human full-term placenta.* Biochem Pharmacol, 1996. **51**(4): p. 403-11.
33. Glover, D.D., D.J. McRobie, and T.S. Tracy, *Effects of gestational and overt diabetes on placental cytochromes P450 and glutathione S-transferase.* Prim Care Update Ob Gyns, 1998. **5**(4): p. 189.
34. Pasanen, M., *The expression and regulation of drug metabolism in human placenta.* Adv Drug Deliv Rev, 1999. **38**(1): p. 81-97.
35. Collier, A.C., et al., *3'-azido-3'-deoxythymidine (AZT) induces apoptosis and alters metabolic enzyme activity in human placenta.* Toxicol Appl Pharmacol, 2003. **192**(2): p. 164-73.
36. Huuskonen, P., et al., *Microarray analysis of the global alterations in the gene expression in the placentas from cigarette-smoking mothers.* Clin Pharmacol Ther, 2008. **83**(4): p. 542-50.
37. Yang, K., et al., *Cadmium reduces 11 beta-hydroxysteroid dehydrogenase type 2 activity and expression in human placental trophoblast cells.* Am J Physiol Endocrinol Metab, 2006. **290**(1): p. E135-E142.
38. Williams, J.A., et al., *N-Acetyltransferases, sulfotransferases and heterocyclic amine activation in the breast.* Pharmacogenetics, 2001. **11**(5): p. 373-88.
39. Sodha, R.J. and H. Schneider, *Sulphate conjugation of beta 2-adrenoceptor stimulating drugs by platelet and placental phenol sulphotransferase.* Br J Clin Pharmacol, 1984. **17**(1): p. 106-8.
40. Stanley, E.L., et al., *Differential expression of sulfotransferase enzymes involved in thyroid hormone metabolism during human placental development.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5944-55.
41. Miki, Y., et al., *Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues.* J Clin Endocrinol Metab, 2002. **87**(12): p. 5760-8.
42. Suzuki, T., et al., *Steroid sulfatase and estrogen sulfotransferase in normal human tissue and breast carcinoma.* J Steroid Biochem Mol Biol, 2003. **86**(3-5): p. 449-54.
43. Meloche, C.A. and C.N. Falany, *Expression and characterization of the human 3 beta-hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b).* J Steroid Biochem Mol Biol, 2001. **77**(4-5): p. 261-9.
44. Lindsay, J., et al., *Structure, function and polymorphism of human cytosolic sulfotransferases.* Curr Drug Metab, 2008. **9**(2): p. 99-105.
45. Boccia, S., et al., *A case-control study investigating the role of sulfotransferase 1A1 polymorphism in head and neck cancer.* J Cancer Res Clin Oncol, 2006. **132**(7): p. 466-72.
46. Kotnis, A., et al., *Case-control study and meta-analysis of SULT1A1 Arg213His polymorphism for gene, ethnicity and environment interaction for cancer risk.* Br J Cancer, 2008. **99**(8): p. 1340-7.

47. Roupret, M., et al., *Phenol sulfotransferase SULT1A1*2 allele and enhanced risk of upper urinary tract urothelial cell carcinoma.* Cancer Epidemiol Biomarkers Prev, 2007. **16**(11): p. 2500-3.
48. Bardakci, F., et al., *Sulfotransferase 1A1 (SULT1A1) polymorphism and susceptibility to primary brain tumors.* J Cancer Res Clin Oncol, 2008. **134**(1): p. 109-14.
49. Falany, J.L. and C.N. Falany, *Regulation of SULT1E1 expression in Ishikawa adenocarcinoma cells by tibolone.* Steroids, 2006. **71**(10): p. 880-5.
50. Atkinson, D.E., et al., *Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer.* Am J Physiol Cell Physiol, 2003. **285**(3): p. C584-91.
51. Meyer Zu Schwabedissen, H.E., et al., *Expression, localization, and function of MRP5 (ABCC5), a transporter for cyclic nucleotides, in human placenta and cultured human trophoblasts: effects of gestational age and cellular differentiation.* Am J Pathol, 2005. **166**(1): p. 39-48.
52. Lankas, G.R., et al., *Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice.* Reprod Toxicol, 1998. **12**(4): p. 457-63.
53. Sudhakaran, S., et al., *Inhibition of placental P-glycoprotein: impact on indinavir transfer to the foetus.* Br J Clin Pharmacol, 2008. **65**(5): p. 667-73.
54. Pollex, E., A. Lubetsky, and G. Koren, *The role of placental breast cancer resistance protein in the efflux of glyburide across the human placenta.* Placenta, 2008. **29**(8): p. 743-7.
55. Gil, S., et al., *P-glycoprotein expression of the human placenta during pregnancy.* Placenta, 2005. **26**(2-3): p. 268-70.
56. Sun, M., et al., *Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation.* Placenta, 2006. **27**(6-7): p. 602-9.
57. Rahi, M., et al., *Influence of adenosine triphosphate and ABCB1 (MDR1) genotype on the P-glycoprotein-dependent transfer of saquinavir in the dually perfused human placenta.* Hum Exp Toxicol, 2008. **27**(1): p. 65-71.
58. Adachi, Y., et al., *Role of breast cancer resistance protein (Bcrp1/Abcg2) in the extrusion of glucuronide and sulfate conjugates from enterocytes to intestinal lumen.* Mol Pharmacol, 2005. **67**(3): p. 923-8.
59. Mizuno, N., et al., *Impaired renal excretion of 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole (E3040) sulfate in breast cancer resistance protein (BCRP1/ABCG2) knockout mice.* Drug Metab Dispos, 2004. **32**(9): p. 898-901.
60. Zamek-Gliszczynski, M.J., et al., *Multiple mechanisms are involved in the biliary excretion of acetaminophen sulfate in the rat: role of Mrp2 and Bcrp1.* Drug Metab Dispos, 2005. **33**(8): p. 1158-65.
61. Zamek-Gliszczynski, M.J., et al., *The important role of Bcrp (Abcg2) in the biliary excretion of sulfate and glucuronide metabolites of acetaminophen, 4-*

methylumbelliferone, and harmol in mice. Mol Pharmacol, 2006. **70**(6): p. 2127-33.
62. Enokizono, J., H. Kusuhara, and Y. Sugiyama, *Involvement of breast cancer resistance protein (BCRP/ABCG2) in the biliary excretion and intestinal efflux of troglitazone sulfate, the major metabolite of troglitazone with a cholestatic effect.* Drug Metab Dispos, 2007. **35**(2): p. 209-14.
63. Hu, M., J. Chen, and H. Lin, *Metabolism of flavonoids via enteric recycling: mechanistic studies of disposition of apigenin in the Caco-2 cell culture model.* J Pharmacol Exp Ther, 2003. **307**(1): p. 314-21.
64. Brand, W., et al., *Metabolism and transport of the citrus flavonoid hesperetin in Caco-2 cell monolayers.* Drug Metab Dispos, 2008. **36**(9): p. 1794-802.
65. Zamek-Gliszczynski, M.J., et al., *Integration of hepatic drug transporters and phase II metabolizing enzymes: mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites.* Eur J Pharm Sci, 2006. **27**(5): p. 447-86.

Chapter 2: Expression and Functional Activities of Selected Sulfotransferase Isoforms in BeWo Cells and Primary Cytotrophoblast Cells

2.1 Introduction

Metabolism is one of the many potential factors that modify trans-placental drug transfer [1, 2]. Several phase I and phase II metabolic enzymes are active in placenta. This includes the sulfotransferase enzymes. Sulfotransferase (SULT) enzymes catalyze the transfer of a sulfuryl group (SO_3^{-1}) from a physiological donor substrate 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to acceptor substrates containing hydroxyl, amine, N-hydroxyl, or sulfhydryl groups in a process known as sulfation. They can be either cytosolic or membrane-associated. Membrane-bound sulfotransferase enzymes sulfate large biomolecules such as carbohydrates and proteins that are involved in extracellular signaling events. The cytosolic enzymes sulfate small molecules that include steroid hormones, neurotransmitters, and bile acids. Sulfoconjugates are generally inactive but certain sulfated metabolites are pharmacologically active (e.g., minoxidil, morphine). There are also examples of sulfated conjugates being potentially carcinogenic. Many SULT substrates are substrates of the Uridine Diphosphate-Glucuronosyltransferase (UGT) enzymes as well. However, sulfation is a high affinity and low capacity reaction in contrast to glucuronidation which is a high capacity, low affinity reaction.

In human tissues, 13 cytosolic SULT isoforms have been identified so far out of which four isoforms have been very well characterized. These are SULT1A1, SULT1A3, SULT1E1, and SULT2A1. In earlier studies SULT1A1 and SULT1A3 (also known as phenolic sulfotransferases) were referred to as TS-PST/P-PST and

TL-PST/M-PST. The nomenclature was based on their differential thermal stability and substrate specificity, SULT1A1 being referred to as the thermostable phenol-specific phenol sulfotransferase and SULT1A3 as the thermolabile monoamine-specific phenol sulfotransferase. The tissue distributions as well as some of the substrates of these four sulfotransferase isoforms have been listed in table 2.1. Although there is overlapping substrate recognition there is some degree of substrate specificity. For example, 17β-estradiol is sulfated by SULT1A1, SULT1E1, and SULT2A1, but only SULT1E1 mediates sulfation at nanomolar concentrations [3]. High affinity substrates of SULT1A1 include small, planar phenolic compounds while SULT1A3 sulfates monoamines with a high affinity. Endogenous substrates of SULT1A1 include 17β-estradiol and thyroid hormones (T3 and T4). Endogenous substrates of SULT1A3 include catecholamines (dopamine, epinephrine, and norepinephrine). Both SULT1A1 and SULT1A3 also sulfate medicinal drugs. The wide range of substrates accepted by each isoform makes it difficult to assign a particular physiological function but does suggest a major role in xenobiotic elimination.

SULT1E1 (also previously known as estrogen sulfotransferase), exhibits the highest affinity for estrogens among all the sulfotransferase enzymes [4]. As it sulfates 17β-estradiol at physiological concentrations, SULT1E1's physiological functions are most likely concerned with maintaining estrogen balance, and protection of tissues from the untoward effects of estrogen [5, 6].

SULT2A1 sulfates hydroxysteroids such as dehydroepiandrosterone (DHEA). In the liver, SULT2A1 is responsible for majority of the bile acid sulfation [4, 7]. Sulfated DHEA serves as the source of estrogens and androgens in peripheral tissues.

All four isoforms are functional in the liver, with SULT1A1 activity being much higher than that of the others [8]. In contrast to SULT1A1, whose hepatic activity is greater than its intestinal activity, SULT1A3 is predominantly expressed in the intestine with very low hepatic activity [8, 9]. Hepatic SULT1E1 activity has been reported to be similar or greater than small intestinal activity, while SULT2A1 activity is higher in the liver [8, 10]. All four isoforms are expressed in other tissues (table 2.1) but there is lack of information on their relative levels of expression. Thermostable phenol sulfotransferase (SULT1A1) has been reported to be functional in bovine brain microvessel endothelial cells [11]. The presence of sulfotransferase isoforms in the liver, intestine, and blood brain barrier indicates a protective role of the enzymes against xenobiotic exposure.

SULT1A1 and SULT1A3 mRNA levels in the placenta were reported to be much higher than that of SULT1E1, while SULT2A1 was not detected [12]. SULT1A1 and SULT1A3 activities have been detected in term and mid-gestation human placenta [13, 14]. Examination of enzyme activities in placental fractions of maternal as well as fetal origin revealed SULT1A1 and SULT1A3 to be functional in all fractions. Enzyme activities and expression were highest in placental sections that

Table 2.1 Tissue distributions and typical substrates of SULT1A1, SULT1A3, SULT1E1, and SULT2A1

SULT isoform	Substrate	Major sites of expression
SULT1A1	**Endogenous** 17β-estradiol, 3,3'-T_2, dopamine, epinephrine **Exogenous** 4-nitrophenol, 2-naphthol, paracetamol, minoxidil, troglitazone, epicatechin, apomorphine, cis- and trans- 4-hydroxytamoxifen, genistein, daidzein	Liver, platelets, brain, gastrointestinal tract, placenta, lung, kidney.
SULT1A3	**Endogenous** Dopamine, norepinephrine, 2-hydroxy-estradiol **Exogenous** p-nitrocatechol, m-nitrocatechol, 4-nitrophenol, epicatechin, albuterol, apomorphine,ethinyl estradiol, minoxidil, troglitazone	Gastrointestinal tract, platelets, brain, placenta, lung, kidney.
SULT1E1	**Endogenous** 17β-estradiol, estrone, pregnenolone, DHEA **Exogenous** 17α-ethinylestradiol, 3α-hydroxytibolone, 3β-hydroxytibolone, apomorphine, cis-4-hydroxytamoxifen, trans-4-hydroxytamoxifen, minoxidil, troglitazone, naringenin, genistein	Endometrium, mammary epithelium, liver, gastrointestinal tract.
SULT2A1	**Endogenous** DHEA, testosterone, 17β-estradiol, cholesterol, lithocholic acid **Exogenous** 17-ethinyl-estradiol, 3β-hydroxytibolone, budesonide, minoxidil	Adrenal gland, brain, liver, gastrointestinal tract.

Information summarized from Lindsay et al. [15], Chapman et al. [3], Pacifici et al. [16], Baranczyk-Kuzma et al. [11].

predominantly consisted of syncytiotrophoblasts [14]. Similarly SULT2A1 activity was reported to be highest in sections that predominantly consisted of syncytiotrophoblast cells [14]. SULT1E1 has also been localized to the syncytiotrophoblasts [17]. The enzyme activities did not vary as a function of gestational age [14]. There is only limited information available on whether cultured trophoblast cells may serve as suitable *in vitro* models for studying placental sulfation. Tamura et al. have detected mRNA of several sulfotransferase isoforms in BeWo [18]. Since the trophoblast layer consisting of multinucleated syncytiotrophoblast cells and mononucleated cytotrophoblast cells is the rate-limiting barrier in the placental transfer of substances, the BeWo cell-line which is a trophoblast-derived cell line is often used to model the human placenta. In some cases it has been shown to be adequately representative of placental metabolism [19]. The objective of this work was to determine the expression and activities of SULT1A1, SULT1A3, SULT1E1, and SULT2A1 in primary cytotrophoblast cells and in the BeWo cell line.

2.2 Materials and methods

2.2.1 Cell culture

The BeWo cell line (clone b30) was obtained from Dr. Alan Schwartz (Washington University, St. Louis, MO) and cultured as described previously[20]. Passages 29 through 45 of the cells were used in this study. Briefly, cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, St. Louis, MO) containing

10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA) and 1% each of 10,000 U/ml penicillin with 10,000μg/mL streptomycin solution, 200mM L-glutamine solution, and 10mM minimal essential medium nonessential amino acid solution (all three were from Invitrogen, Carlsbad, CA). Cells were maintained at 37°C under 5% CO_2 and 95% relative humidity. The medium was changed every other day. At 70-90% confluence, cells were detached with 0.5% trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO) solution diluted in phosphate buffered saline (PBS) at a 1:10 ratio. Cells were subcultured at a ratio of 1:10. Cytotrophoblast cells were obtained from Dr. Joan S. Hunt (University of Kansas Medical Center, Kansas City, KS). They were isolated from placenta following an uncomplicated caesarean delivery as per the procedure of Petroff et al. [21] and stored under liquid nitrogen until further use.

2.2.2 Homogenate preparation for sulfotransferase assays

BeWo cell homogenate was prepared as described previously with some modifications [22]. Cells at confluence were washed and scraped in PBS containing 0.5 mM EDTA (Sigma, St. Louis, MO) and 1mM phenylmethylsulfonylfluoride (PMSF, Sigma, St. Louis, MO). Cells were then centrifuged, PBS was aspirated off, and the cells were homogenized in 5 mM potassium phosphate buffer (pH 7.4) containing 1.25 mM EDTA, 2.5 mM dithiothreitol (DTT), and Complete Mini Protease Inhibitor cocktail tablets (Roche, Indianapolis, IN) at 4ºC for 20 strokes using a Dounce homogenizer. Homogenates were centrifuged at 13,000 x g for 20

minutes at 4ºC. The supernatant was further centrifuged at 100,000 x g for 1 hour at 4ºC. The resulting high speed supernatant was used for protein determination. It was then diluted 1:1 in a BSA solution (5 mM potassium phosphate buffer, pH 7.4, containing 5 mg/mL BSA) and stored at -70ºC. Cytotrophoblast homogenate was prepared in a similar manner.

2.2.3 Western blot analysis

BeWo cells at confluence were rinsed thrice in PBS and scraped off. The cell suspension was centrifuged at 1,500 rpm for 8 minutes. The pellet was then resuspended in lysis buffer containing 20 mM Tris HCl, 150 mM NaCl, 1 mM PMSF, 1% (v/v) Triton X-100 (all from Sigma, St. Louis, MO), and Complete Mini Protease Inhibitor cocktail tablets (Roche, Indianapolis, IN). This was maintained on ice for 30 minutes. The cell lysate was then centrifuged at 14,000 rpm for 10 minutes at 4ºC. Protein concentration of the supernatant was determined with the BCA assay (Pierce, Rockford, IL) and the supernatant was stored at -80ºC until further analysis. Before electrophoresis, 20-80 μg cytosolic protein was added to loading buffer and loaded onto a 12% Tris-glycine gel (Invitrogen, Carlsbad, CA). Sample pretreatment included testing the effects of boiling and the inclusion of DTT in the samples. Sf-9 insect cell cytosolic extract from insect cells transfected with human SULT2A1 cDNA (Panvera, Carlsbad, CA) served as the positive control. Electrophoresis was performed at 110 V for 2 hours. Protein bands were transferred at 25 V for 4 hours onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA). The membrane was

blocked overnight with 5% (w/v) nonfat milk in TBS buffer (10 mM Tris buffer, pH 8.0, 150 mM NaCl, both from Sigma, St. Louis, MO) at room temperature, washed, and then incubated with a commercially available polyclonal antibody (Panvera, Carlsbad, CA) to SULT2A1 at a 1:500 dilution for 1 hour at room temperature. After incubation the membrane was washed 6 times at 10 minute intervals in 1.5% BSA in TBST buffer (TBS buffer containing 0.1% v/v Tween 20). The membrane was then incubated in secondary antibody (peroxidase-conjugated AffiniPure goat antirabbit IgG, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour. The membrane was washed 6 times at 10 minute intervals in TBST buffer and the protein was visualized by chemiluminescence. The Enhanced Chemiluminescence Western blotting detection reagents were obtained from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK).

2.2.4 Determination of sulfotransferase enzyme activities

Sulfotransferase enzyme assays were performed with the appropriate diagnostic substrates: 4-nitrophenol (Sigma, St. Louis, MO) for SULT1A1, dopamine (Sigma, St. Louis, MO) for SULT1A3, dehydroepiandrosterone (DHEA, (Sigma, St. Louis, MO)) for SULT2A1, and 17β-estradiol (Sigma, St. Louis, MO) for SULT1E1.

Enzyme assays for SULT1A1 and SULT1A3 were a modification of the method of Foldes and Meeks [23]. The complete reaction mixture consisted of 0.4 μM [^{35}S]-3'-phosphoadenosine-5'-phosphosulfate (PAPS, Perkin Elmer, Waltham, MA), varying

concentrations of the acceptor substrates, and BeWo/primary cytotrophoblast protein (20 μg) in 20 mM potassium phosphate buffer (pH 7.4). The final reaction volume was 150 μL. Control reactions did not contain the acceptor substrate. Reactions were started by the addition of PAPS to the other components and incubated at 37^{o}C for 20 minutes. The reaction was terminated by adding 0.1 M barium hydroxide, 0.1 M barium acetate, and 0.1 M zinc sulfate to precipitate the enzyme and the unreacted [^{35}S] PAPS. The sulfated product was determined by liquid scintillation counting. Each assay was performed at least in duplicate.

17β-estradiol (E2) sulfation was measured using a method based on that of Harris et al. [24]. The reaction mixture consisted of 10 μM PAPS, BeWo cytosolic protein (50 μg) in a final volume of 200 μL in 100mM Tris buffer containing 10mM magnesium acetate and 0.1 mM EDTA (pH 7.9). Reactions were started by the addition of [2,4,6,7-^{3}H(N)]-estradiol (0.5 Ci/mmol, Perkin Elmer, Waltham, MA) and incubated at 37^{o}C for 40 min. Reactions were terminated by the sequential addition of ice cold deionized water (200μL) and ice cold dichloromethane (800μL). The tubes were mixed vigorously for 30 sec, the phases were separated by centrifugation at 11,500 x *g* for 5 min, and radioactivity was determined in the aqueous phase that contained sulfated estradiol.

DHEA (Perkin Elmer, Waltham, MA) sulfation was measured using the protocol published by Chen et al. [8]. The reaction mixture consisted of 20 μM PAPS, BeWo

cytosolic protein (50-600 μg), 3 μM or 10 μM DHEA (diluted to 0.4 Ci/mmol) in 50 mM Tris buffer (pH 6.2) in a final volume of 250 μL. After incubation at 37°C for 30 minutes, the reaction was stopped by the addition of 250 μL of 0.25 M Tris buffer (pH 8.7) and 1 mL chloroform. The extraction was repeated twice and an aliquot of the aqueous phase was used for scintillation counting.

2.2.5 Reverse transcription polymerase chain reaction (RT-PCR)

BeWo cells at confluence were washed, scraped in PBS, and then centrifuged to remove the PBS. Cytotrophoblast cells were retrieved from liquid nitrogen, thawed, and centrifuged to remove storage medium. Total RNA was isolated from either BeWo cells or cytotrophoblast using a commercially available phenol-guanidine isothiocyanate solution (TRIZOL reagent, Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. mRNA was purified from total RNA using an Oligotex mRNA Mini Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol and stored at -80ºC until further use. The mRNA was concentrated prior to the experiment by mixing volume enough to give 200 ng with 1/10th the volume of 3M DEPC treated sodium acetate (pH 5.2), 2 μL glycogen (Roche Diagnostics, Indianapolis, IN), and ethanol (at a volume 2.5 fold more than the volume of mRNA). This was stored overnight at -20ºC to precipitate the mRNA. After overnight precipitation, the sample was spun down at 14,000 rpm for 15 minutes at 4ºC. The supernatant was aspirated off, the pellet was washed in 75% ethanol, and the tubes spun down at 14,000 rpm for 15 minutes at 4ºC. Most of the supernatant was aspirated off. The remainder was

dried in room air and reconstituted in RNAse free water prior to reverse transcription. 200 ng mRNA was reverse transcribed and amplified using the Access RT-PCR Introductory System (Promega, Madison, WI). The primer sequences for amplifying SULT1A1, SULT1A3, SULT2A1 and SULT1E1 have previously been published [18, 25, 26] and have been listed in table 2.2. Specific primers were not designed for SULT1A2. Instead, primers were used that can simultaneously amplify SULT1A1, SULT1A2, and SULT1A3 cDNAs [25]. This will henceforth be referred to as SULT1A cDNA. All primers were synthesized by the Biotechnology Support Facility at the University of Kansas Medical Center (Kansas City, KS). The primers utilized for amplifying β-actin transcripts were obtained from Promega (Madison, WI). PCR cycle parameters were according to manufacturer's protocol and were as follows: 1 cycle each of reverse transcription at 48^{o}C for 45 minutes and denaturation for 2 min at 94°C, followed by 40 cycles each of denaturation at 94^{o}C for 30 seconds, annealing at 60^{o}C for 1 minute, and extension at 68^{o}C for 2 minutes. This preceded a final elongation at 68°C for 7 minutes.

SULT1A and SULT1A1 cDNA were further subjected to restriction digest analysis (Sty I and *Hin*d III, Promega, Madison, WI) [25]. The RT-PCR product was purified with the Wizard® DNA Clean-up System (Promega, Madison, WI) and each restriction digest was set up with 200 ng mRNA for 1 hour as per the manufacturer's protocol.

Electrophoresis of all RT-PCR products was performed at 80 V on a 2% agarose gel. The gel was post-stained with 0.5 μg/mL ethidium bromide for 45 minutes, washed four times in distilled water at 10 minute intervals, and viewed with a UV transilluminator.

2.2.6 RT-PCR product sequencing

For sequencing SULT1A1 cDNA, a small portion of the gel below the desired band was cut off and replaced with 1% low-melt agarose. Electrophoresis was resumed until the band migrated into this area. This portion of the gel was then removed and purified with the Wizard® DNA Clean-up System (Promega, Madison, WI). The purified PCR product was then inserted into a pCR®II-TOPO® plasmid vector using a TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). The vector was transformed into One Shot® TOP10F' chemically competent *E. Coli* cells. Colonies were selected from LB plates and broth (Becton Dickinson, Sparks, MD) containing 50 μg/mL kanamycin. Plasmid DNA was isolated and purified with the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI). Plasmid DNA was then subjected to restriction digest (*Hin*d III and Sty I) to verify the orientation of the cloned PCR product. The products were sequenced at the University of Kansas Medical Center Biotechnology Support Facility (Kansas City, KS). cDNA purification, cloning, transformation, and plasmid DNA purification were all performed according to manufacturers' protocols.

Table 2.2 Primer sequences used for reverse transcribing sulfotransferase mRNA and expected base pair sizes of the reaction products

Target sequence	Genbank accession ID	Primers (Forward, Reverse)	Expected size (Expected size after digest)
SULT1A1 (39-1025)	L10819	5'-ATGGAGCTGATCCAGGACAC-3' 5'-TGACCTACCGTCCCAGGCCC-3'	987 (Sty I : 437,550)
SULT1A1 (42-808) SULT1A2 (74-840) SULT1A3 (22-788)	L19955 U28169 X84653	5'-ATGGAGCTGATCCAGGACAC-3' 5'-ATGAAGGGGGAGATGCTGT-3'	767 (Sty I : 437,330) 767 (HindIII: 468,299) 767 (Sty I : 656,111)
SULT1A3 (139-1125)	L19956	5'-ATGGAGCTGATCCAGGACAC-3' 5'-TGAGCCACTGTGCCTGACTC-3'	987
SULT1E1 (389-852)	Y11195	5'-ACCTGAACTTCTTCCTGCC-3' 5'-TCCAGTCTCCTGTAATTCCC-3'	464
SULT2A1 (21-875)	U08024	5'-ATGTCGGACGATTTCTTATG-3' 5'-AATCCCATGGGAACAGCTC-3'	856

2.2.7 Data analysis

SULT1A1 and SULT1A3 enzyme activity data before inhibition was observed was fitted to the Michaelis-Menten equation:

$$V = \frac{V_{max} C}{K_m + C}$$

where V, V_{max}, K_m, and C represent the sulfation rate, maximal sulfation rate, the apparent Michaelis-Menten constant, and the utilized substrate concentration respectively. The IC_{50} values (inhibitor concentration at which sulfation rate is 50% of the controls) for 2,6,dichloro-4-nitrophenol were calculated from the Four-Parameter Logistic Function as follows:

$$y = D + \frac{A - D}{1 + 10^{(x - \log C)B}}$$

where x is the log concentration of the inhibitor and logC is the log of IC_{50}. The IC_{50} values for sodium chloride and the temperature at which enzyme activity was inhibited by 50% were calculated from the Four-Parameter Logistic Function (Linear) as follows:

$$y = D + \frac{A - D}{1 + 10^{(\log(x) - \log C)B}}$$

where x is the concentration of the inhibitor and logC is the log of IC_{50}. Regression was performed using the Sigma Plot software (SPSS, Chicago, IL).

2.3 Results

2.3.1 Sulfotransferase mRNA expression in trophoblasts

RT-PCR was performed to compare mRNA expression between BeWo cells and primary cytotrophoblasts. As can be seen in Figures 2.1 and 2.2, SULT1A3 and SULT1A1 were detected in both BeWo cells and cytotrophoblasts. The results indicated that SULT1A1 was upregulated in BeWo. SULT2A1 was expressed only in BeWo and not in the primary cytotrophoblast cells; SULT1E1 expression in BeWo was weak whereas it was undetectable in the cytotrophoblasts.

The mRNA of another isoform, SULT1A2, has been detected in several human tissues but the protein is expressed at very low levels [27]. This isoform exhibits 96%

homology to SULT1A1 and 90% homology to SULT1A3 at the amino acid level. The recombinant protein exhibits biochemical properties very similar to SULT1A1. The primers used for SULT1A2 were not unique to it but could amplify any of SULT1A mRNAs (SULT1A1, SULT1A2, and SULT1A3) producing a 767 bp product. However, SULT1A2 has a recognition sequence for the restriction enzyme *Hind* III (cleaving the 767 bp product into 468 and 299 bp products) which is absent in both SULT1A1 and SULT1A3 [25]. On the other hand, both SULT1A1 and SULT1A3 have recognition sequences for Sty I which would cleave them into 437 bp and 330 bp, and 656 bp and 111 bp, products respectively. When the 767 bp product was subjected to Sty I restriction digest, bands were observed at 767 bp and 656 bp (Figure 2.4). No bands were observed at the expected positions for SULT1A1. *Hind* III digestion produced only the 767 bp band. This indicates that SULT1A2 mRNA is either not expressed in BeWo, or that SULT1A3 is expressed more abundantly and has been amplified at much higher levels than SULT1A2. However, the results raised doubts about the product obtained with the SULT1A1 specific primers (Figure 2.2). For confirmation this RT-PCR product was subjected to restriction digest by Sty I and fragments were observed at the expected positions (Figure 2.5). Further when this product was sequenced, it gave a good match to human liver SULT1A1 mRNA.

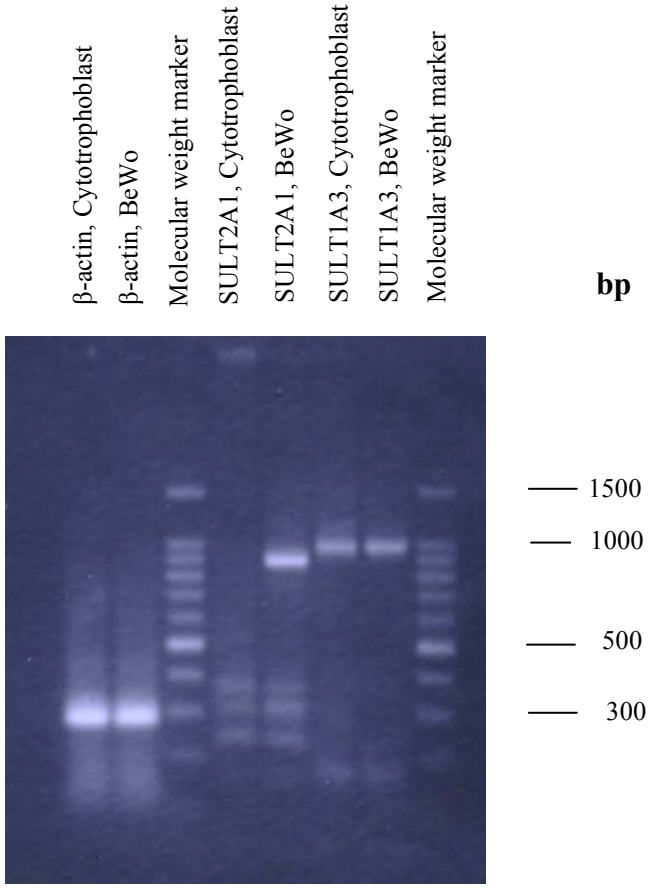


Figure 2.1 Reverse transcription polymerase chain reaction (RT-PCR) analyses of SULT1A3 and SULT2A1 in BeWo cells and in primary cytotrophoblast cells. 200 ng mRNA was used for reverse transcription and polymerase chain reaction amplification of SULT2A1 and SULT1A3. Expected sizes: SULT1A1 (987 bp), SULT2A1 (872 bp).

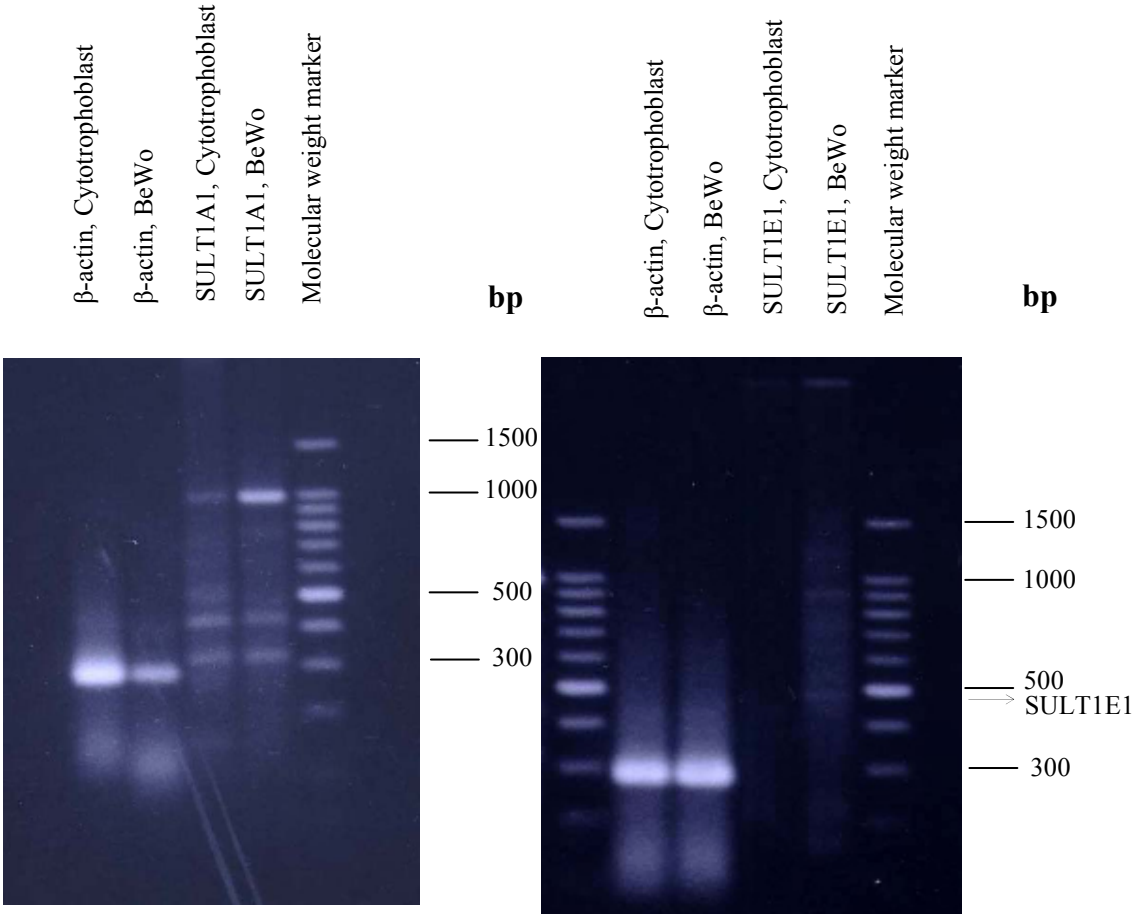


Figure 2.2 Reverse transcription polymerase chain reaction (RT-PCR) analysis of SULT1A1 in BeWo cells and in primary cytotrophoblast cells. 200 ng mRNA was used for reverse transcription and polymerase chain reaction amplification of SULT1A1. Expected size: SULT1A1 (987 bp).

Figure 2.3 Reverse transcription polymerase chain reaction (RT-PCR) analysis of SULT1E1 in BeWo cells and in primary cytotrophoblast cells. 200 ng mRNA was used for reverse transcription and polymerase chain reaction amplification of SULT1E1. Expected size: SULT1E1 (464 bp).

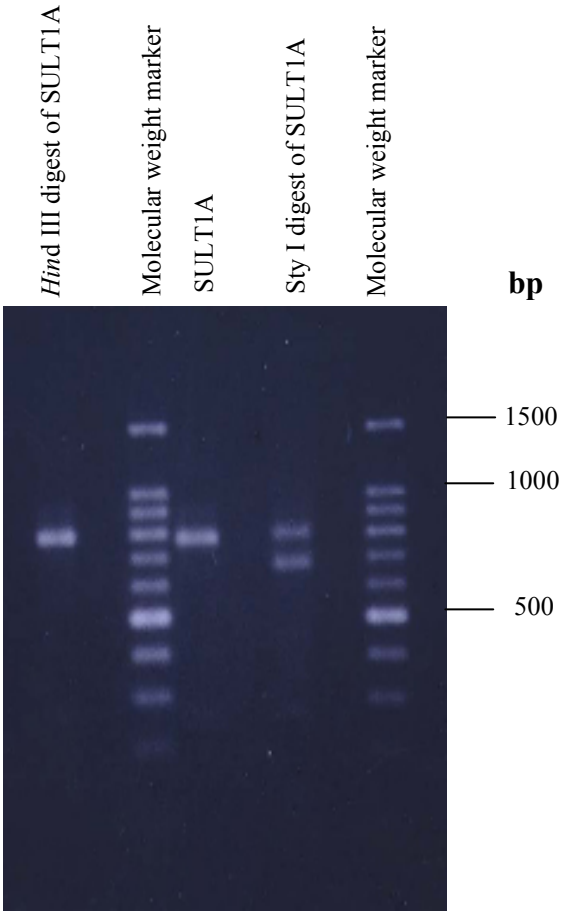


Figure 2.4 Reverse transcription polymerase chain reaction (RT-PCR) analysis of SULT1A cDNA in BeWo after restriction digest with either Sty I or *Hin*d III. 200 ng mRNA was used for reverse transcription and polymerase chain reaction amplification using primers that can code for SULT1A1, SULT1A2, or SULT1A3 (referred to as SULT1A). RT-PCR products were subjected to restriction enzyme digestion with either *Hin*d III or Sty I. Expected sizes: Undigested SULT1A cDNA (767 bp), Sty I digest of SULT1A1 (437 bp and 330 bp), *Hin*d III digest of SULT1A2 (468 bp and 299 bp), and Sty I digest of SULT1A3 (656 bp and 111 bp).

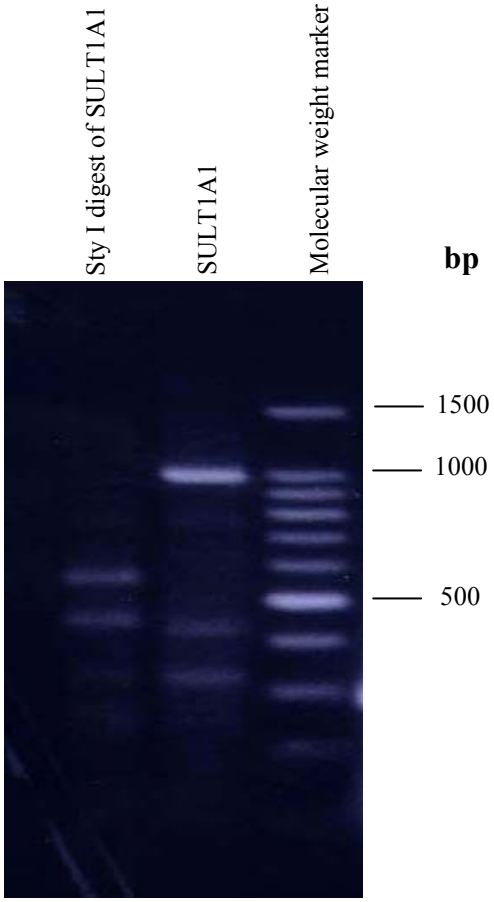


Figure 2.5 Reverse transcription polymerase chain reaction (RT-PCR) analysis of SULT1A1 cDNA in BeWo after restriction digest with Sty I. 200 ng mRNA was used for reverse transcription and polymerase chain reaction amplification of SULT1A1 cDNA. The RT-PCR product was subjected to Sty I digestion. Expected sizes: Undigested SULT1A1 (987 bp), Sty I digest of SULT1A1 (437 bp and 550 bp).

2.3.2 4-nitrophenol and dopamine sulfation activities in BeWo

The enzyme activities of SULT1A1 and SULT1A3 were tested using the diagnostic substrates of these enzymes – 4-nitrophenol for SULT1A1 and dopamine for SULT1A3. Sulfation of both 4-nitrophenol (0.1-160 μM) and dopamine (0.1-80 μM) exhibited substrate inhibition at higher concentrations (Figures 2.6 and 2.7). Sulfation of 4-nitrophenol and dopamine by recombinant SULT1A1 and SULT1A3 respectively have been reported to exhibit substrate inhibition at higher concentrations [28]. Our results indicated SULT1A1 and SULT1A3 mediated sulfation of 4-nitrophenol and dopamine in BeWo cells.

The sulfation profiles before substrate inhibition were fitted to the Michaelis-Menten equation to obtain kinetic parameters. The K_m and V_{max} values have been listed in table 2.3. The apparent K_m value obtained with 4-nitrophenol compared well with SULT1A1-mediated sulfation either in other tissues or with the recombinant protein [3, 22, 29, 30]. These studies have reported K_m values of SULT1A1-mediated sulfation of 4-nitrophenol in the range 0.5-1 μM. In BeWo, the K_m obtained with dopamine sulfation was 0.3-0.7 μM. Though typically SULT1A3-mediated sulfation of dopamine exhibits higher apparent K_m values (3-10 μM) [22, 31], much lower K_m values have also been observed (0.65 μM) [32]. These indicated the possibility of SULT1A1 and SULT1A3 being functional in BeWo. However, there can be overlapping substrate specificities between the SULT isoforms. Thus the sulfation activities were further characterized in the presence of inhibitors.

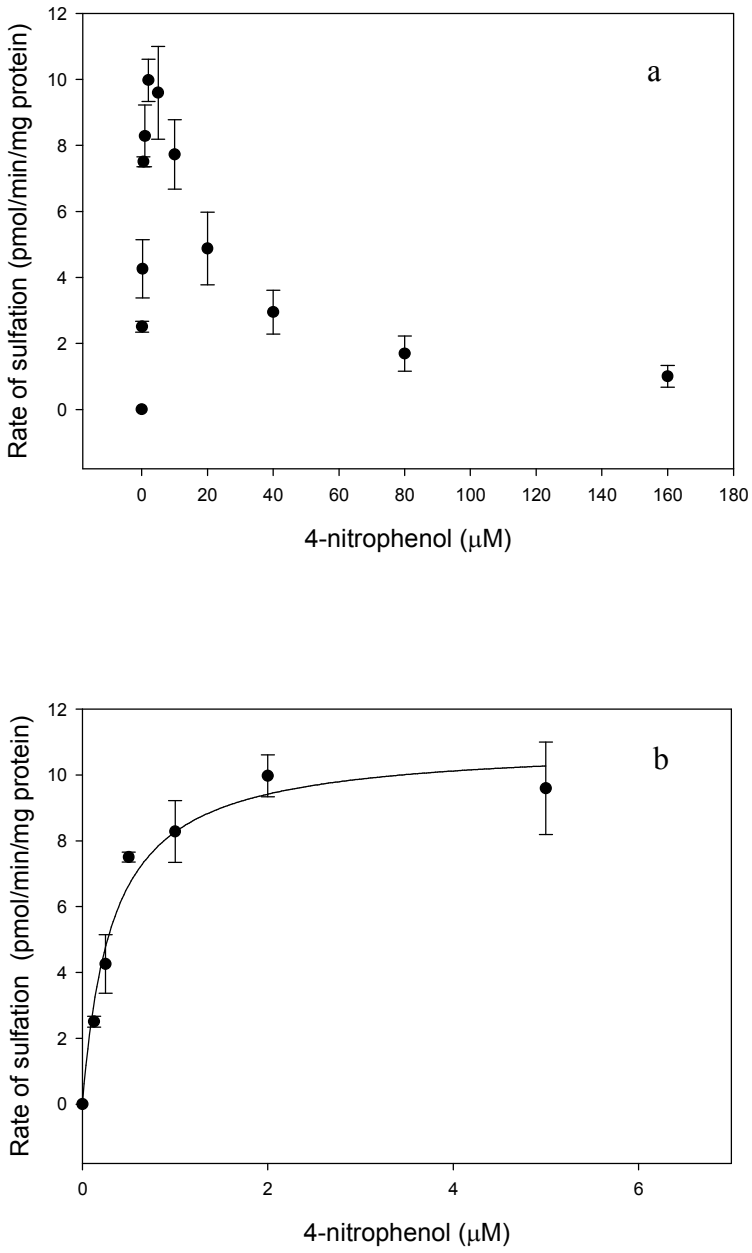


Figure 2.6 4-nitrophenol sulfation (pmol/min/mg protein) in BeWo cells as a function of 4-nitrophenol concentration (Figure 2.6a). Figure 2.6b shows the data up to 5 μM and represents a fit to the Michaelis-Menten equation. Each point represents the mean ± S.D. of three determinations. Each assay was repeated at least twice and K_m was 0.32 – 0.65 μM.

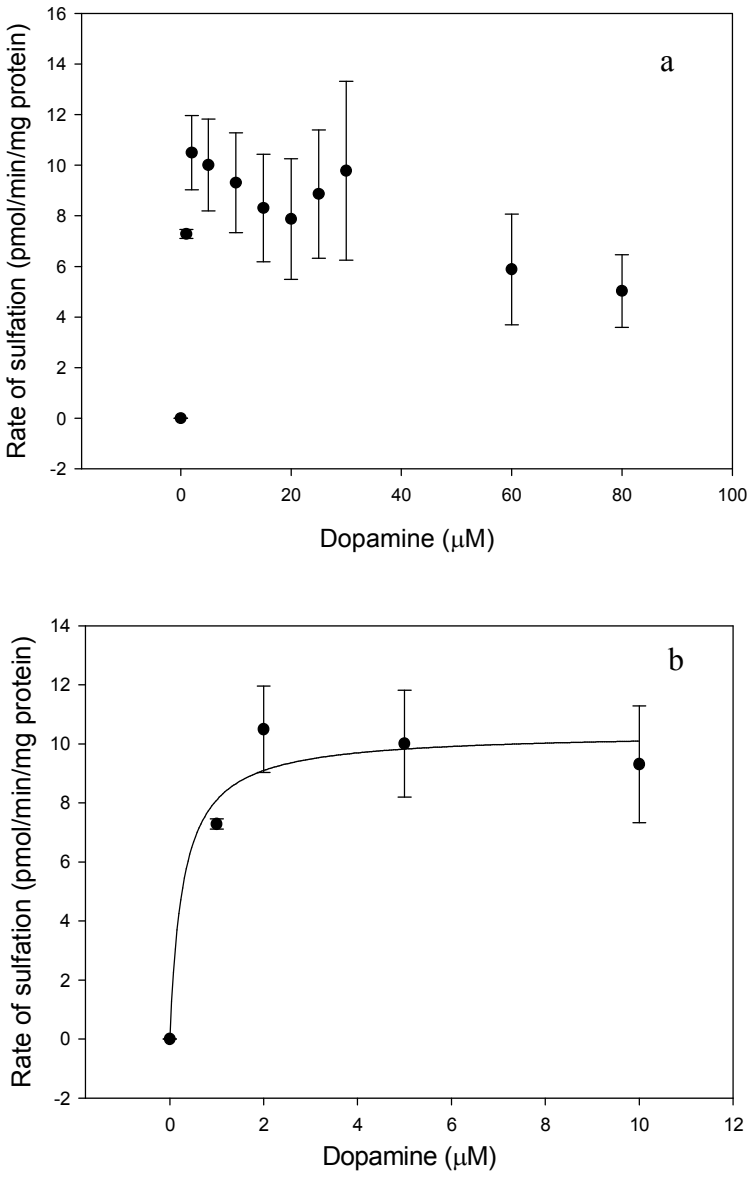


Figure 2.7 Dopamine sulfation (pmol/min/mg protein) in BeWo cells as a function of dopamine concentration (Figure 2.7a). Figure 2.7b shows the data up to 10 μM and represents a fit to the Michaelis-Menten equation. Each point represents the mean ± S.D. of three determinations. Each assay was repeated at least twice and K_m was 0.28 – 0.71 μM.

2.3.3 Effect of inhibitors 2,6-dichloro-4-nitrophenol (DCNP) and NaCl

To verify whether 4-nitrophenol and dopamine sulfation were mediated by SULT1A1 and SULT1A3 respectively, the inhibitory effects of 2,6-dichloro-4-nitrophenol (DCNP) and sodium chloride were tested. Typically SULT1A1 activity is more sensitive to inhibition by DCNP and sodium chloride, than SULT1A3. In BeWo cells, 4-nitrophenol sulfation was more sensitive to inhibition by DCNP than dopamine sulfation, whereas both exhibited similar sensitivities to sodium chloride inhibition (Figure 2.8 and table 2.3). The IC_{50} value for 4-nitrophenol sulfation obtained with DCNP is similar to previously cited values [22, 30, 33, 34] but the IC_{50} obtained with sodium chloride is somewhat higher than that proposed in the literature that puts it between 80-100 mM [22, 30]. The IC_{50} values with DCNP and sodium chloride for dopamine sulfation agreed very well with references of SULT1A3-mediated sulfation of dopamine, which put the values between 0.4-0.6 mM and 180-340 mM respectively [22, 31].

2.3.4 Thermal stability

SULT1A1 and SULT1A3 exhibit differential thermal stability, and this has led to their nomenclature as thermostable and thermolabile phenolic sulfotransferase isoforms respectively. SULT1A1 has been reported to be 50% inactivated between 43-44ºC while SULT1A3 is 50% inactivated between 38.5-40.5ºC [22, 31, 34]. Though there are discrepancies between studies with regards to the absolute values reported, SULT1A1 is relatively more thermostable than SULT1A3 [33]. In BeWo

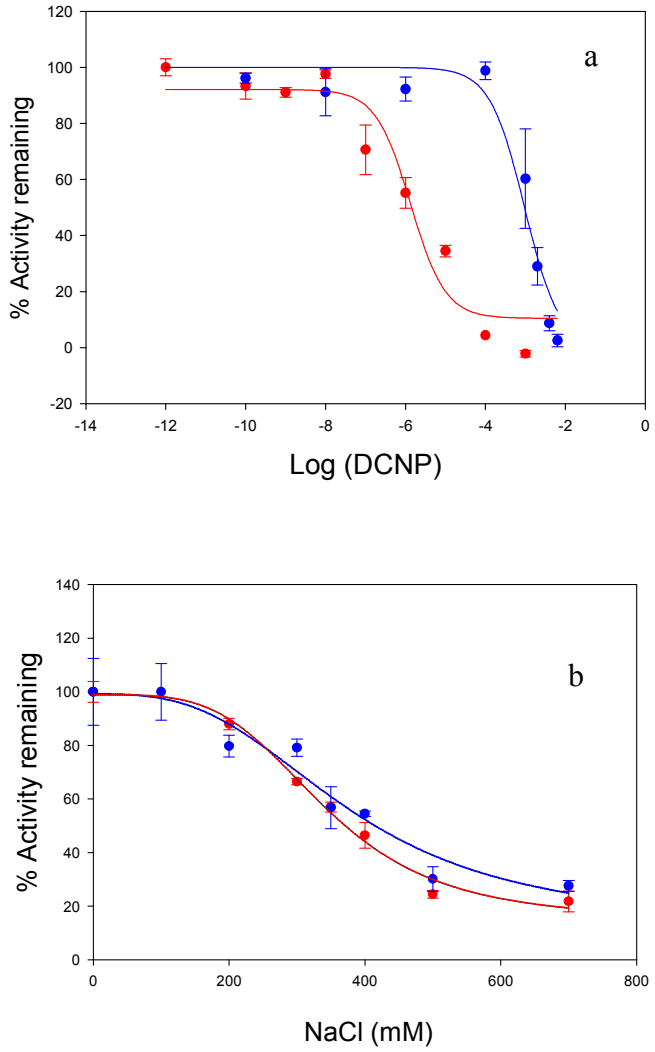


Figure 2.8 Effects of inhibitors on sulfotransferase activities. (a) Effect of 2,6-dichloro-4-nitrophenol (DCNP) on 4-nitrophenol (-o-) and dopamine (-o-) sulfation activities in BeWo. The SULT activity in the presence of an inhibitor was expressed as a percentage of the activity in the absence of an inhibitor. The IC_{50} values for 4-nitrophenol and dopamine were between (0.04-0.1 μM) and (0.95-1.14 mM) respectively. (b) Effect of NaCl on 4-nitrophenol (-o-) and dopamine (-o-) sulfation activities in BeWo. The SULT activity in the presence of an inhibitor was expressed as a percentage of the activity in the absence of an inhibitor. The IC_{50} values for 4-nitrophenol and dopamine were between (300-360 mM) and (200-340 mM) respectively. Each point represents mean ± S.D. of three determinations.

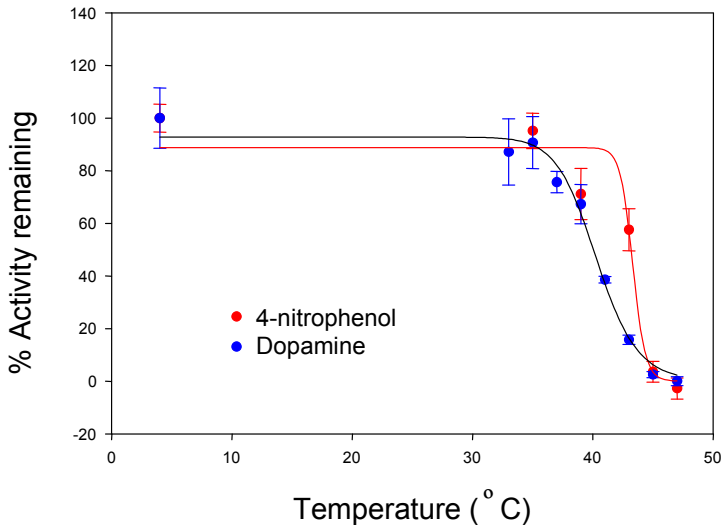


Figure 2.9 Thermal stability of 4-nitrophenol and dopamine sulfation activities in BeWo. The net sulfotransferase activity was expressed as a percentage of the basal (unheated) activities. The 50% inactivation temperatures (T_{50}) were 41.2-43.6ºC and 39ºC for 4-nitrophenol and dopamine respectively. Each point represents mean ± S.D. of three determinations.

Table 2.3 Kinetic parameters obtained from 4-nitrophenol and dopamine sulfation in BeWo cells in the absence and presence of inhibitors.

	Substrate	
	4-nitrophenol	**Dopamine**
K_m (µM)	0.32 – 0.65	0.28 – 0.71
V_{max} (pmol/min/mg)	6.9-10.94	5.9-10.4
IC_{50} (DCNP, µM)	0.04-0.1	943-1135
IC_{50} (NaCl, mM)	305-360	197-338
T_{50} (ºC)	41.2-43.6	39

cells, 4-nitrophenol and dopamine sulfation were 50% inactivated between 41-43ºC and at 39ºC, respectively (Figure 2.9 and table 2.3). These observations matched well

with previously reported values and indicated that in BeWo the enzymes retain distinctive thermal stability properties.

Overall, the results of 4-nitrophenol and dopamine sulfation in the presence of inhibitors and heat, agreed well with previous reports [22, 30, 31, 33, 34]. As these results confirmed that 4-nitrophenol and dopamine sulfation in BeWo were mediated by SULT1A1 and SULT1A3 respectively, protein expression of these two enzymes was not examined in BeWo.

2.3.5 17β-estradiol and dehydroepiandrosterone sulfation activities in BeWo

SULT1E1 activity was assessed with 10-30 nM 17β-estradiol in BeWo cells. The sulfation rate (0.02-0.05 pmol/min/mg) observed was much lower than SULT1A1 or SULT1A3 activities. Genistein is a potent inhibitor of SULT1E1-mediated sulfation of 17β-estradiol [35]. In BeWo cells, 17β-estradiol sulfation was not inhibited by genistein indicating that 17β-estradiol sulfation is probably not mediated by SULT1E1 (Figure 2.10). This combined with the low mRNA levels of SULT1E1 indicates that SULT1E1 is likely not functional in BeWo cells.

It has been reported that SULT2A1 exhibits maximal activity towards DHEA at 3-10 μM [4]. Even though the RT-PCR results indicated abundant expression of SULT2A1 mRNA, there was negligible sulfation of DHEA (3 μM and 10 μM). Increasing protein concentrations did not increase DHEA sulfation. Under the same conditions

sulfation was observed with the recombinant protein. In Western blot (Figure 2.11) no band was observed at 35 kDa (molecular mass of SULT2A1). The native form of the enzyme is present as a homodimer and there are reports of both forms of the enzyme being observed in immunoblots [36]. Our experiments consistently revealed a band at 75 kDa raising the possibility of this being a SULT2A1 dimer. Including reducing agents or eliminating boiling (the latter has been said to cause SULT2A1 protein aggregation) [36] did not produce any change in western blot results, indicating that the 75 kDa band likely results from nonspecific antibody binding.

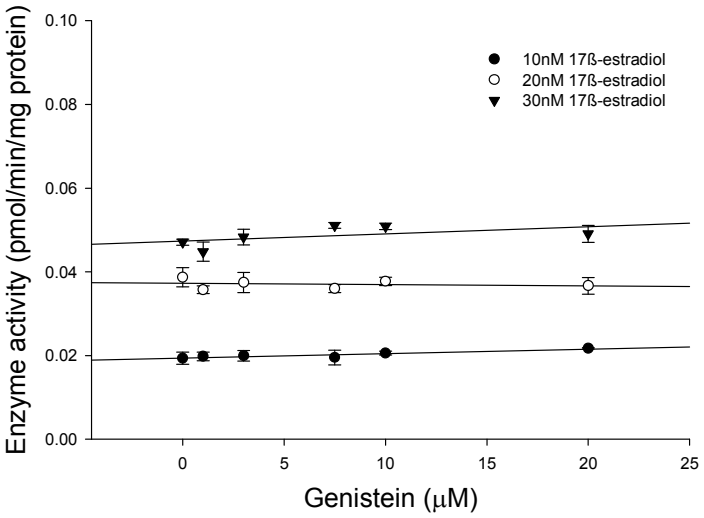


Figure 2.10 Sulfation of 17β-estradiol (10, 20, and 30 nM) in BeWo cells in the presence of genistein. Increase concentrations of genistein did not produce any change in 17β-estradiol sulfation.

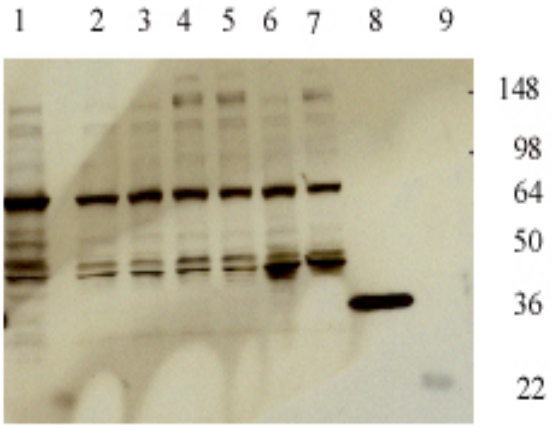


Figure 2.11 Western blot analysis of SULT2A1 in BeWo cells. 40/80 µg of total protein from BeWo cell lysate and 2.5 µg of recombinant SULT2A1 protein were subjected to electrophoresis on a 12% Tris-glycine gel. The membrane was incubated with a SULT2A1 polyclonal antibody. The secondary antibody was peroxidase-conjugated AffiniPure goat anti-rabbit IgG. The membrane was detected by chemiluminescence. Lanes 1, 2: 40 µg BeWo lysate with and without boiling; lanes 3 and 4: 40 µg BeWo lysate + dithiothreitol (DTT) without boiling; lanes 5 and 6: 40 µg BeWo lysate + DTT with boiling; lane 7: 80 µg BeWo cell lysate; lane 8: 2.5 µg of recombinant SULT2A1; lane 9: molecular weight marker.

2.3.6 SULT1A1 and SULT1A3 activities in primary cytotrophoblast cells

SULT1A1 and SULT1A3 activities were then examined in cytotrophoblast cells in the presence of 2,6,dichloro-4-nitrophenol (DCNP) concentrations that previously completely inhibited enzyme activities in BeWo cells (Figure 2.12). At these concentrations of DCNP (1 mM and 4 mM), sulfation of 4-nitrophenol and dopamine were also completely inhibited in cytotrophoblast cells. This indicated that 4-nitrophenol sulfation and dopamine sulfation were being mediated by SULT1A1 and SULT1A3 respectively in the primary cytotrophoblast cells. SULT1A3 activity was similar in the two types of trophoblast (6.9±0.6 pmol/min/mg in BeWo cells vs.

3.8±0.8 pmol/min/mg in primary cytotrophoblast cells) whereas SULT1A1 was upregulated in BeWo cells (21.6±2 pmol/min/mg in BeWo cells vs. 2.8±2.4 pmol/min/mg in the primary cytotrophoblast). The relative pattern of enzyme activities agreed with the mRNA expression of SULT1A1 and SULT1A3.

2.4 Discussions and conclusions

Placental metabolic enzymes mediate the synthesis and metabolism of hormones, as well as the metabolism of foreign chemicals that include medicinal drugs and environmental chemicals [37]. They can also produce carcinogenic adducts. For example, benzo(a)pyrene-7,8-diol-9,10-epoxide DNA adducts were found in the placenta of smokers [38]. The activation of benzo(a)pyrene to these carcinogenic adducts is mediated by CYP1A1 and CYP1A1 is active in the placenta throughout pregnancy. This underscores the importance of studying placental metabolism to not only reduce the extent to which a particular drug reaches the fetus but also to prevent toxic metabolites from reaching the fetus. Placental sulfation includes endogenous substrates (e.g., 17β-estradiol and 3,3'-triiodothyronine) and drugs (e.g., salbutamol, ritodrine, fenoterol, and betanaphthol) [39, 40]. Studies have indicated sulfotransferase activities in several sections of whole placental tissue but not much work has dealt with activities at the cellular level, which constitutes the rate-limiting barrier to trans-placental transfer [14]. Tamura et al. reported SULT1A1 and SULT1A3 enzyme activities in BeWo cells using single concentrations of the diagnostic substrates 4-nitrophenol and dopamine [18]. However, several

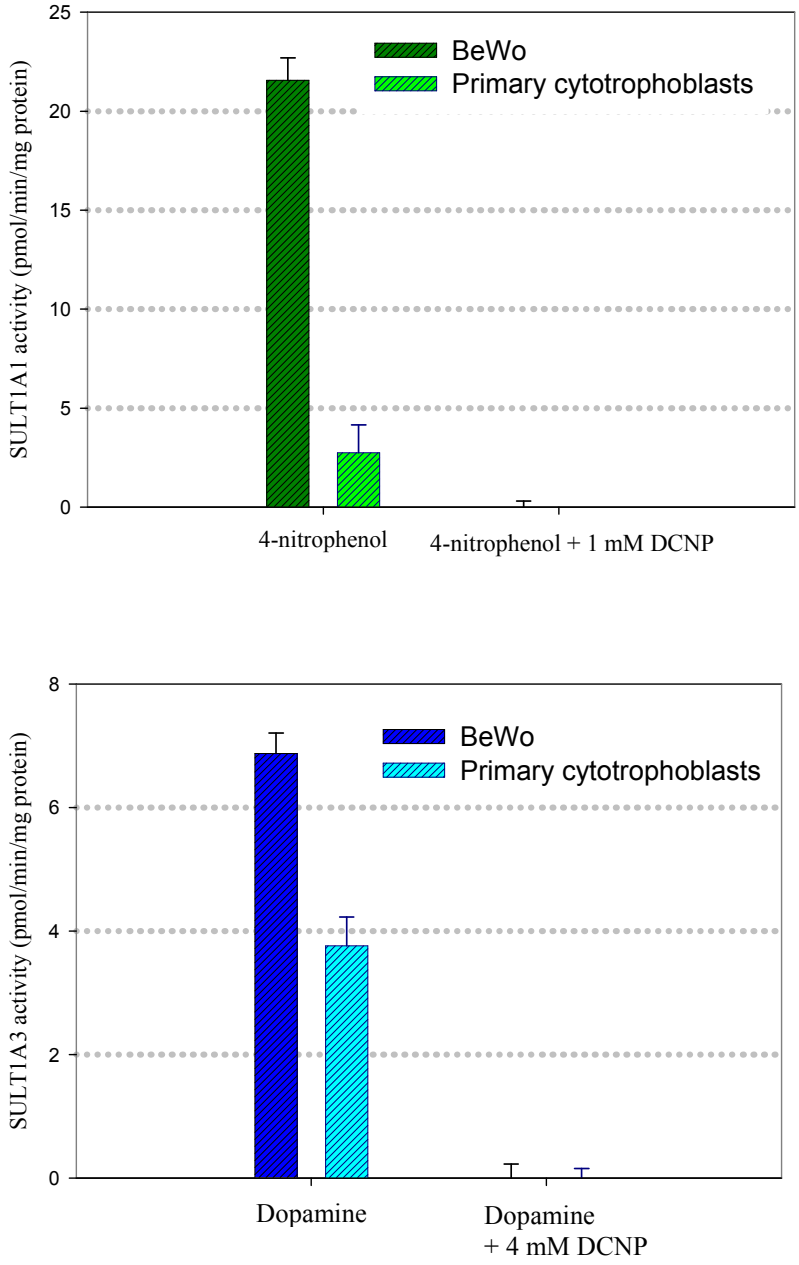


Figure 2.12 4-nitrophenol (4 µM) and dopamine (1 µM) sulfation activities in BeWo cells and primary cytotrophoblasts. Sulfotransferase activities were measured in the presence and in the absence of 1 mM and 4 mM 2,6,-dichloro-4-nitrophenol (DCNP). At these concentrations, DCNP previously completely abolished 4-nitrophenol and dopamine sulfation in BeWo cells.

sulfotransferase isoforms exhibit overlapping substrate specificity. For instance, SULT1A1, SULT1A2, and SULT1B1, all sulfate 4-nitrophenol at low micromolar concentrations and using single substrate concentrations can lead to erroneous identification of sulfotransferase isoforms [3, 41]. Our work examines 4-nitrophenol and dopamine sulfation activities in the presence of prototypical inhibitors of these enzymes and adds on to the work of Tamura et al [18]. Biochemical characteristics, including apparent K_m values, response to inhibitors and thermal stability profiles agreed well with previously reported values. This led to the conclusion that 4-nitrophenol and dopamine sulfations in BeWo cells are being mediated by SULT1A1 and SULT1A3, respectively. When compared to the primary cultures of cytotrophoblast cells, enzyme activities indicated similar SULT1A3 activity in BeWo and the cytotrophoblasts, while SULT1A1 appeared to be upregulated in BeWo cells. This is consistent with the RT-PCR results that revealed similar SULT1A3 mRNA levels but elevated SULT1A1 mRNA in BeWo cells. The enzyme activity assays and western blots indicated that SULT2A1 protein is not expressed in BeWo cells. Although SULT2A1 mRNA was abundantly expressed in BeWo cells it was not expressed in cytotrophoblasts. This indicates that this enzyme is probably not active in trophoblast cells. There appears to be some contradiction about the expression of SULT2A1 in placenta. Stanley et al reported high sulfation rates of DHEA in fractions rich in syncytiotrophoblasts and attributed this to SULT2A1. However, Nishimura et al failed to detect any SULT2A1 mRNA in the placenta [12, 14]. In the work of Stanley et al. DHEA sulfation was detected with 10 μM DHEA. However,

SULT2A1 is not the only isoform that sulfates DHEA at such concentrations. Another sulfotransferase isoform SULT2b1b, which also sulfates hydroxysteroids, is localized primarily to the nuclei of syncytiotrophoblasts [42]. One of the physiological substrates of SULT2b1b is cholesterol [7] which is a precursor for progesterone synthesis. The placenta synthesizes progesterone and it has been speculated that the physiological role of SULT2b1b in the placenta may be prevention of nuclear receptor activation [43]. SULT2b1b and SULT2A1 exhibit similar affinities for DHEA [42]. It is possible that the DHEA sulfation activity reported by Stanley et al. [14] was mediated by SULT2b1b. Another possible reason for the discrepancy observed in our work and the work of Stanley et al. [14] with respect to SULT2A1 function in trophoblast, could be differential enzyme expression between the cytotrophoblasts and syncytiotrophoblasts which has been observed with several transport proteins [44]. BeWo cells consist primarily of cytotrophoblasts while Stanley et al. used placental sections predominantly consisting of syncytiotrophoblasts [14].

Our results indicated that SULT1E1 is not expressed in BeWo cells. Like SULT2A1 there are conflicting reports on placental SULT1E1 expression. In placental fractions rich in syncytiotrophoblasts, SULT1E1 activity was much lower than SULT1A1 and SULT1A3 activities [14]. It has also been reported that SULT1E1 mRNA in placenta is lower by 40 fold or more than SULT1A1 or SULT1A3 mRNA [12]. On the other hand some studies have reported SULT1E1 to be localized to syncytiotrophoblasts

[17]. One possible reason for observing low SULT1E1 activity in BeWo cells could be the presence of steroid sulfatase enzymes. These enzymes exhibit particularly high activity in placental tissue and have been localized to the syncytiotrophoblasts [17, 45].

Overall this study indicates that the phenolic sulfotransferase isoforms SULT1A1 and SULT1A3 are active in trophoblast cells. SULT1A1 sulfates a wide range of substrates that includes steroid hormones, medicinal drugs, as well as procarcinogens. Not much has been reported on the physiological functions of this enzyme in the placenta. Previously Stanley et al. failed to find any correlation between placental SULT1A1 and SULT1A3 activities and thyroid hormone sulfation which led them to conclude that placental sulfation is most likely not responsible for the high levels of T_3 and T_4 sulfate detected in the fetal circulation [14]. One of the endogenous substrates of SULT1A1 is 17β-estradiol, although SULT1A1 sulfates the hormone at much higher concentrations than SULT1E1 [46]. As the syncytiotrophoblast secretes increasing amounts of the 17β-estradiol with advancing gestation [47], it is tempting to speculate that a possible physiological function of the enzyme is maintaining tissue estrogen balance, at least under conditions of high local concentrations.

Endogenous substrates of SULT1A3 include catecholamines (e.g., epinephrine, norepinephrine and dopamine) and catecholestrogens. The majority of plasma catecholamines exist in the sulfated form. While circulating levels of epinephrine and

norepinephrine sulfate levels are around 70-80%, ~95% of circulating dopamine exists in the sulfated form [48]. In patients suffering from hypertension, plasma levels of dopamine sulfate were elevated without any change in the free levels of dopamine, and it has been speculated that genetic defects in the aryl sulfatase gene may cause essential hypertension [49, 50]. In pheochromocytoma, there is an elevation in the plasma levels of catecholamines as well as the catecholamine sulfates [51]. The physiological significance of catecholamine sulfation is not very well understood. Since sulfated catecholamines are inactive at the adrenergic and dopaminergic receptors, it has been suggested that conjugation serves as a protective mechanism against the adverse responses that may be produced from the high circulating levels of catecholamines [7, 52-55]. While studies have not dealt with the role of SULT1A3 in the placenta, similar to its function in other tissues, physiological functions of SULT1A3 in the placenta may be catecholamine inactivation.

Sulfation is well developed in the fetus. Several studies report fetal hepatic SULT1A1 activity (~100-150 pmol/min/mg protein) to be higher than placental activity (~ 20-40 pmol/min/mg protein) [16, 56]. Activities in fetal kidney and adrenal were similar or higher than that in the placenta. Similarly, SULT1A3 activities in fetal gut and liver were respectively 10- and 5- fold higher than that in the placenta [16, 56]. No correlations were found between gestational age (14-35 weeks) and fetal sulfotransferase activity suggesting that fetal sulfation had developed by the 14^{th} week of gestation [16]. Nevertheless, the SULT1A family also appears to be well

developed in the placenta and likely acts as a support of fetal metabolism for fetal protection.

In conclusion our work establishes the phenolic sulfotransferase isoforms SULT1A1 and SULT1A3 to be functional in BeWo cells and in primary cultures of cytotrophoblast cells. A lot of the work on placental metabolism has focused on the effects of cigarette smoke, medicinal drugs, and environmental chemicals on placental enzyme activity, as a way of determining if this may be related to fetal toxicity. There has not been a lot of focus on the effects of foreign chemicals on placental sulfotransferase activity. SULT1A1 catalyses the sulfation of several procarcinogens such as N-hydroxylated aromatic amines, nitroalkanes, and benzylic alcohols to produce nucleophilic DNA adducts [57, 58]. The functional activities of SULT1A1 and SULT1A3 in BeWo cells suggests that this cell line can be used as a suitable model for studying placental sulfotransferase induction/inhibition as a basis for developing an understanding of the role of these enzymes in physiological and disease processes.

2.5 References

1. Nanovskaya, T.N., et al., *Methadone metabolism by human placenta.* Biochem Pharmacol, 2004. **68**(3): p. 583-91.
2. Deshmukh, S.V., T.N. Nanovskaya, and M.S. Ahmed, *Aromatase is the major enzyme metabolizing buprenorphine in human placenta.* J Pharmacol Exp Ther, 2003. **306**(3): p. 1099-105.
3. Chapman, E., et al., *Sulfotransferases: structure, mechanism, biological activity, inhibition, and synthetic utility.* Angew Chem Int Ed Engl, 2004. **43**(27): p. 3526-48.
4. Falany, C.N., *Enzymology of human cytosolic sulfotransferases.* FASEB J, 1997. **11**(4): p. 206-16.
5. Falany, J.L., N. Macrina, and C.N. Falany, *Regulation of MCF-7 breast cancer cell growth by beta-estradiol sulfation.* Breast Cancer Res Treat, 2002. **74**(2): p. 167-76.
6. Kotov, A., et al., *Regulation of estrogen activity by sulfation in human Ishikawa endometrial adenocarcinoma cells.* J Steroid Biochem Mol Biol, 1999. **68**(3-4): p. 137-44.
7. Strott, C.A., *Sulfonation and molecular action.* Endocr Rev, 2002. **23**(5): p. 703-32.
8. Chen, G., et al., *Human gastrointestinal sulfotransferases: identification and distribution.* Toxicol Appl Pharmacol, 2003. **187**(3): p. 186-97.
9. Cappiello, M., L. Giuliani, and G.M. Pacifici, *Differential distribution of phenol and catechol sulphotransferases in human liver and intestinal mucosa.* Pharmacology, 1990. **40**(2): p. 69-76.
10. Furimsky, A.M., et al., *Effect of resveratrol on 17beta-estradiol sulfation by human hepatic and jejunal S9 and recombinant sulfotransferase 1E1.* Drug Metab Dispos, 2008. **36**(1): p. 129-36.
11. Baranczyk-Kuzma, A., K.L. Audus, and R.T. Borchardt, *Catecholamine-metabolizing enzymes of bovine brain microvessel endothelial cell monolayers.* J Neurochem, 1986. **46**(6): p. 1956-60.
12. Nishimura, M. and S. Naito, *Tissue-specific mRNA expression profiles of human phase I metabolizing enzymes except for cytochrome P450 and phase II metabolizing enzymes.* Drug Metab Pharmacokinet, 2006. **21**(5): p. 357-74.
13. Sodha, R.J., V. Glover, and M. Sandler, *Phenolsulphotransferase in human placenta.* Biochem Pharmacol, 1983. **32**(10): p. 1655-7.
14. Stanley, E.L., et al., *Differential expression of sulfotransferase enzymes involved in thyroid hormone metabolism during human placental development.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5944-55.
15. Lindsay, J., et al., *Structure, function and polymorphism of human cytosolic sulfotransferases.* Curr Drug Metab, 2008. **9**(2): p. 99-105.
16. Pacifici, G.M., *Sulfation of drugs and hormones in mid-gestation human fetus.* Early Hum Dev, 2005. **81**(7): p. 573-81.

17. Miki, Y., et al., *Systemic distribution of steroid sulfatase and estrogen sulfotransferase in human adult and fetal tissues.* J Clin Endocrinol Metab, 2002. **87**(12): p. 5760-8.
18. Tamura, H.O., et al., *Expression profiling of sulfotransferases in human cell lines derived from extra-hepatic tissues.* Biol Pharm Bull, 2001. **24**(11): p. 1258-62.
19. Avery, M.L., C.E. Meek, and K.L. Audus, *The presence of inducible cytochrome P450 types 1A1 and 1A2 in the BeWo cell line.* Placenta, 2003. **24**(1): p. 45-52.
20. Bode, C.J., et al., *In vitro models for studying trophoblast transcellular transport.* Methods Mol Med, 2006. **122**: p. 225-39.
21. Petroff, M.G., et al., *Isolation and culture of term human trophoblast cells.* Methods Mol Med, 2006. **121**: p. 203-17.
22. Dubin, R.L., et al., *Thermostable (SULT1A1) and thermolabile (SULT1A3) phenol sulfotransferases in human osteosarcoma and osteoblast cells.* Bone, 2001. **28**(6): p. 617-24.
23. Foldes, A. and J.L. Meek, *Rat brain phenolsulfotransferase: partial purification and some properties.* Biochim Biophys Acta, 1973. **327**(2): p. 365-74.
24. Harris, R.M., et al., *Phytoestrogens are potent inhibitors of estrogen sulfation: implications for breast cancer risk and treatment.* J Clin Endocrinol Metab, 2004. **89**(4): p. 1779-87.
25. Spink, B.C., et al., *SULT1A1 catalyzes 2-methoxyestradiol sulfonation in MCF-7 breast cancer cells.* Carcinogenesis, 2000. **21**(11): p. 1947-57.
26. Taskinen, J., et al., *Conjugation of catechols by recombinant human sulfotransferases, UDP-glucuronosyltransferases, and soluble catechol O-methyltransferase: structure-conjugation relationships and predictive models.* Drug Metab Dispos, 2003. **31**(9): p. 1187-97.
27. Teubner, W., et al., *Identification and localization of soluble sulfotransferases in the human gastrointestinal tract.* Biochem J, 2007. **404**(2): p. 207-15.
28. Barnett, A.C., et al., *Active site mutations and substrate inhibition in human sulfotransferase 1A1 and 1A3.* J Biol Chem, 2004. **279**(18): p. 18799-805.
29. Gamage, N.U., et al., *Structure of a human carcinogen-converting enzyme, SULT1A1. Structural and kinetic implications of substrate inhibition.* J Biol Chem, 2003. **278**(9): p. 7655-62.
30. Anderson, R.J., B.L. Jackson, and D.K. Liebentritt, *Human platelet thermostable phenol sulfotransferase from blacks and whites: biochemical properties and variations in thermal stability.* J Lab Clin Med, 1988. **112**(6): p. 773-83.
31. Kudlacek, P.E., D.L. Clemens, and R.J. Anderson, *Characterization of recombinant human liver thermolabile phenol sulfotransferase with minoxidil as the substrate.* Biochem Biophys Res Commun, 1995. **210**(2): p. 363-9.
32. Dajani, R., et al., *Kinetic properties of human dopamine sulfotransferase (SULT1A3) expressed in prokaryotic and eukaryotic systems: comparison*

with the recombinant enzyme purified from Escherichia coli. Protein Expr Purif, 1999. **16**(1): p. 11-8.
33. Raftogianis, R.B., T.C. Wood, and R.M. Weinshilboum, *Human phenol sulfotransferases SULT1A2 and SULT1A1: genetic polymorphisms, allozyme properties, and human liver genotype-phenotype correlations.* Biochem Pharmacol, 1999. **58**(4): p. 605-16.
34. Anderson, R.J. and D.K. Liebentritt, *Human platelet thermostable phenol sulfotransferase: assay of frozen samples and correlation between frozen and fresh activities.* Clin Chim Acta, 1990. **189**(2): p. 221-9.
35. Nishiyama, T., et al., *Sulfation of environmental estrogens by cytosolic human sulfotransferases.* Drug Metab Pharmacokinet, 2002. **17**(3): p. 221-8.
36. Aldred, S. and R.H. Waring, *Localisation of dehydroepiandrosterone sulphotransferase in adult rat brain.* Brain Res Bull, 1999. **48**(3): p. 291-6.
37. Pasanen, M., *The expression and regulation of drug metabolism in human placenta.* Adv Drug Deliv Rev, 1999. **38**(1): p. 81-97.
38. Manchester, D.K., et al., *Synchronous fluorescence spectroscopic, immunoaffinity chromatographic and 32P-postlabeling analysis of human placental DNA known to contain benzo[a]pyrene diol epoxide adducts.* Carcinogenesis, 1990. **11**(4): p. 553-9.
39. Cappiello, M., et al., *Sulphotransferase and its substrate: adenosine-3'-phosphate-5'-phosphosulphate in human fetal liver and placenta.* Dev Pharmacol Ther, 1990. **14**(1): p. 62-5.
40. Sodha, R.J. and H. Schneider, *Sulphate conjugation of beta 2-adrenoceptor stimulating drugs by platelet and placental phenol sulphotransferase.* Br J Clin Pharmacol, 1984. **17**(1): p. 106-8.
41. Tabrett, C.A. and M.W. Coughtrie, *Phenol sulfotransferase 1A1 activity in human liver: kinetic properties, interindividual variation and re-evaluation of the suitability of 4-nitrophenol as a probe substrate.* Biochem Pharmacol, 2003. **66**(11): p. 2089-97.
42. Meloche, C.A. and C.N. Falany, *Expression and characterization of the human 3 beta-hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b).* J Steroid Biochem Mol Biol, 2001. **77**(4-5): p. 261-9.
43. He, D., et al., *Different subcellular localization of sulphotransferase 2B1b in human placenta and prostate.* Biochem J, 2004. **379**(Pt 3): p. 533-40.
44. Evseenko, D.A., J.W. Paxton, and J.A. Keelan, *ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast.* Am J Physiol Regul Integr Comp Physiol, 2006. **290**(5): p. R1357-65.
45. Suzuki, T., et al., *Steroid sulfatase and estrogen sulfotransferase in normal human tissue and breast carcinoma.* J Steroid Biochem Mol Biol, 2003. **86**(3-5): p. 449-54.
46. Yasuda, S., M. Suiko, and M.C. Liu, *Oral contraceptives as substrates and inhibitors for human cytosolic SULTs.* J Biochem, 2005. **137**(3): p. 401-6.

47. Kallen, C.B., *Steroid hormone synthesis in pregnancy.* Obstet Gynecol Clin North Am, 2004. **31**(4): p. 795-816, x.
48. Kuchel, O. and N.T. Buu, *Conjugated catecholamines and their measurement: some pharmacokinetic aspects.* Curr Med Res Opin, 1983. **8 Suppl 3**: p. 3-8.
49. Yoshizumi, M., et al., *Physiological significance of plasma sulfoconjugated dopamine in patients with hypertension--clinical and experimental studies.* Life Sci, 1996. **59**(4): p. 324-30.
50. Kuchel, O., *Clinical implications of genetic and acquired defects in catecholamine synthesis and metabolism.* Clin Invest Med, 1994. **17**(4): p. 354-73.
51. Kuchel, O., et al., *Free and conjugated plasma catecholamines in hypertensive patients with and without pheochromocytoma.* Hypertension, 1980. **2**(2): p. 177-86.
52. Michel, G.L., et al., *Sulfoconjugated catecholamines: lack of beta-adrenoceptor binding and adenylate cyclase stimulation in human mononuclear leukocytes.* Eur J Pharmacol, 1987. **143**(2): p. 179-88.
53. Kyncl, J.J., et al., *Adrenergic and dopaminergic properties of dopamine sulfoconjugates.* J Cardiovasc Pharmacol, 1985. **7**(6): p. 1198-204.
54. Lenz, T., et al., *O-Methylated and sulfoconjugated catecholamines: differential activities at human platelet alpha 2-adrenoceptors.* Can J Physiol Pharmacol, 1991. **69**(7): p. 929-37.
55. Stokes, A.H., T.G. Hastings, and K.E. Vrana, *Cytotoxic and genotoxic potential of dopamine.* J Neurosci Res, 1999. **55**(6): p. 659-65.
56. Cappiello, M., et al., *Dopamine sulphotransferase is better developed than p-nitrophenol sulphotransferase in the human fetus.* Dev Pharmacol Ther, 1991. **16**(2): p. 83-8.
57. Williams, J.A., et al., *N-Acetyltransferases, sulfotransferases and heterocyclic amine activation in the breast.* Pharmacogenetics, 2001. **11**(5): p. 373-88.
58. Kreis, P., et al., *Human phenol sulfotransferases hP-PST and hM-PST activate propane 2-nitronate to a genotoxicant.* Carcinogenesis, 2000. **21**(2): p. 295-9.

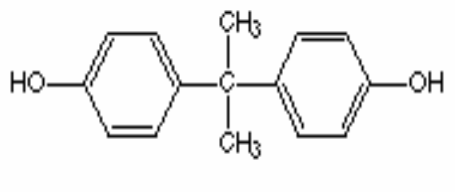
Chapter 3: Effect of Chronic Bisphenol A Exposure on SULT1A1

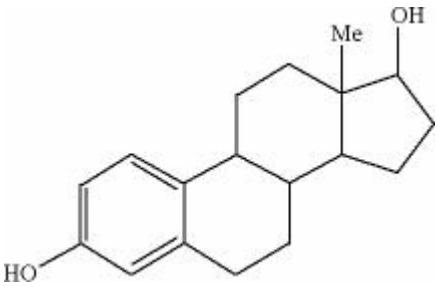
3.1 Introduction

Bisphenol A is widely used in the production of polycarbonate plastics that are used in the manufacture of food and beverage containers, and epoxy resins that are used in linings of metal food and beverage cans, and dental sealants. Bisphenol A is manufactured in large quantities with its annual production capacity exceeding 6 billion pounds in 2003 [1]. The ester bonds linking the monomers of bisphenol A in polycarbonate plastics and resins are hydrolyzed to release bisphenol A. The reaction is accelerated under acidic conditions, basic conditions, and increasing temperature[1, 2]. Leaching increases with repeated use. Human exposure to bisphenol A is believed to be mainly through food [3]. Mean bisphenol A concentrations in random serum samples of the human population following environmental exposure are estimated to be in the range 0.6-2.5 ng/mL [4-7]. Pregnancy does not seem to affect bisphenol A disposition as serum concentrations of bisphenol A in women from early pregnancy did not significantly differ from that of non-pregnant women [8]. Similar levels of bisphenol A in fetal and maternal serum indicate that bisphenol A readily crosses the placenta [8, 9]. Median placental concentrations of bisphenol A are approximately 12.7 μg BPA/kg of placental tissue (range 1-105 μg/kg) [9].

Although nonsteroidal in structure (Figure 3.1.1) bisphenol A mimics the actions of 17β-estradiol and is considered to be a xenoestrogen [10-12]. Bisphenol A binds to the nuclear estrogen receptors (ER) α and β, and plasma membrane-bound ERs [11, 13]. It is also considered to be an endocrine disrupter as neonatal or prenatal

bisphenol A exposure produces severe reproductive tract abnormalities in rodents. Some effects observed in female mice include morphological and functional changes





Bisphenol A 17β-estradiol

Figure 3.1.1 Structures of bisphenol A and 17β-estradiol.

in the ovaries, mammary glands, brain, and reproductive tract [14-16]. In male rodents prostate enlargement, alterations in prostate histoarchitecture, and increased expression of the androgen receptor constitute some of the consequences of perinatal BPA exposure [16]. Maffini et al have documented the effects observed in rodents after perinatal exposure to bisphenol A as shown in Figure 3.1.2 [16]. Some of the above-mentioned *in vivo* effects of bisphenol A were from doses that resulted in plasma concentrations similar to that reported in humans following environmental exposure [15]. Thus there is growing concern over bisphenol A exposures posing a potential threat to human health, especially during organogenesis. Similarly in *in vitro* studies, effects of bisphenol A included cell proliferation in prostatic adenocarcinoma cells, breast cancer cells, and increased prolactin release (which can be linked to

reproductive dysfunctions) in rat pituitary cells [10, 13, 17]. Bisphenol A produced some of these effects at concentrations relevant in humans.

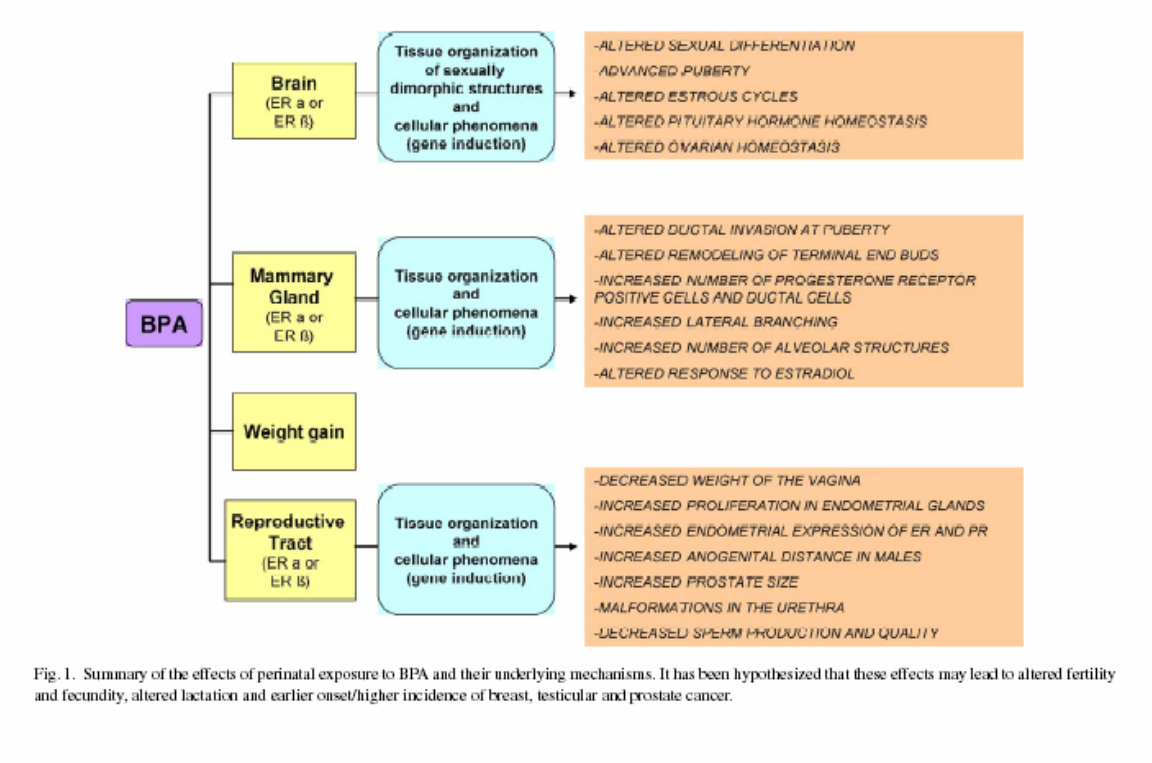


Fig. 1. Summary of the effects of perinatal exposure to BPA and their underlying mechanisms. It has been hypothesized that these effects may lead to altered fertility and fecundity, altered lactation and earlier onset/higher incidence of breast, testicular and prostate cancer.

Figure 3.1.2 Effects of perinatal exposure to bisphenol A in rodents (The figure has been adapted from Maffini et al., Mol Cell Endocrinol, 2006. 254-255: p. 179-86).

In chapter 1 we established the functional activity of the sulfotransferase isoforms SULT1A1 and SULT1A3 in the BeWo cell line. Estrogenic compounds acutely inhibit both SULT1A1 and SULT1A3, but they inhibit SULT1A1 with a much higher potency [18, 19]. Although there is much less information available on the effect of estrogenic substances on sulfotransferase enzymes upon chronic exposure, it has been demonstrated that when breast cancer cells were treated with 4-hydroxy-tamoxifen

for 16 hours, of the 8000 transcripts analyzed only members of the SULT1A family were upregulated [20]. Estradiol also increased SULT1A mRNA levels, although at longer periods of incubation (72 hours). So, taking into account firstly that SULT1A1 is potently inhibited by estrogenic substances; secondly that bisphenol A achieves appreciable placental concentrations and is estrogenic, it would be interesting to observe if bisphenol A produces any effects on SULT1A1 activity in the trophoblast. The objective of this study was to determine the effect of bisphenol A on SULT1A1 activity in trophoblasts upon acute as well as chronic exposure. We have used the BeWo cell line as a trophoblast model.

3.2 Materials and methods

3.2.1 BeWo cell culture

The BeWo cell line (clone b30) was obtained from Dr. Alan Schwartz (Washington University, St. Louis, MO) and cultured as described previously [21]. Passages 29 through 45 of the cells were used in this study. Briefly, cells were grown at 37ºC in an atmosphere of 5% CO_2 and 95% relative humidity. They were maintained in Dulbecco's Modified Eagles Medium (DMEM, Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 1% each of 10,000 U/ml penicillin with 10,000 µg/mL streptomycin, 200 mM L-glutamine, and 10 mM minimal essential medium nonessential amino acid solution (all three from Invitrogen, Carlsbad, CA). At 70-90% confluence, cells were detached with 0.5% trypsin and 0.2% ethylenediaminetetraacetic acid solution (EDTA, Sigma,

St. Louis, MO) diluted in phosphate buffered saline (PBS) at a 1:10 ratio. Cells were then subcultured at a ratio of 1:10.

3.2.2 Cell lysate preparation

BeWo cell lysate was prepared as per the protocol of Ebmeier et al with a few modifications [22]. Cells at 90-100% confluence were washed and scraped up in prewarmed (37ºC) PBS containing 0.5 mM ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO) and 1 mM phenylmethanesulfonylfluoride (PMSF, Sigma, St. Louis, MO). They were then centrifuged, PBS was aspirated off, and the cells were homogenized in 5 mM potassium phosphate (pH 7.4), containing 0.25 M sucrose, 1.25 mM tetrasodium EDTA, 1.5 mM dithiothreitol (all from Sigma, St. Louis, MO), and Complete Mini Protease inhibitor cocktail (Roche, Indianapolis, IN) at 4ºC for 20 strokes using a Dounce homogenizer. Homogenates were centrifuged at 13,000 x g for 20 minutes at 4ºC. The resultant supernatant was further centrifuged at 100,000 x g for 1 hour at 4ºC. Aliquots of the resulting supernatant were used for protein determination using the Bradford protein assay method (Biorad, Hercules, CA). The remaining supernatant was mixed with an equal volume of 5mM potassium phosphate (pH 7.4), containing 5 mg/ml BSA (Sigma, St. Louis, MO) and was stored at -80ºC until further use.

3.2.3 Chronic treatment of BeWo cells with bisphenol A

Bisphenol A (Sigma, St. Louis, MO) was prepared as 1000x stock solutions in ethanol. Cells were plated at a density of 10,000 cells/cm^2. For the 48 hour treatments, cells at ~ 50% confluence were exposed to selected concentrations of bisphenol A. The medium was changed 24 hours later and BPA was included in the change medium at the same concentration. Control cells were cultured in medium containing 0.1% ethanol under the same conditions. Bisphenol A treatments were performed either in serum-containing or serum-free medium. For the 24 hour treatments, cells were exposed to the selected concentrations of bisphenol A in serum-free medium at ~70% confluence. At the end of the dosing period, homogenate was prepared and stored as outlined in section 3.2.2. Incubations with the estrogen receptor inhibitor ICI 182,780 (Tocris Bioscience, Ellisville, MO) were all done in serum-free medium for a period of 24 hours.

3.2.4 Determination of SULT1A1 activity

Sulfotransferase enzyme assays were performed using 4-nitrophenol as a marker substrate of SULT1A1. The enzyme assay was a modification of the method of Foldes and Meeks [23]. The final reaction mixture consisted of substrate 4 μM 4-nitrophenol (Sigma, St. Louis, MO), 1 μM [^{35}S] PAPS (Perkin Elmer, Waltham, MA), and 5 μg BeWo cytosolic protein in 50 mM potassium phosphate buffer (pH 7.4). Control reactions contained DMSO (Sigma, St. Louis, MO) in place of the substrate. The reaction mixture was incubated at 37^{o}C for 20 minutes. The reaction

was terminated by adding 0.1 M barium hydroxide (Sigma, St. Louis, MO), 0.1 M barium acetate (Sigma, St. Louis, MO), and 0.1 M zinc sulfate (Sigma, St. Louis, MO) to precipitate the enzyme and the unreacted [^{35}S]PAPS. The sulfated product was determined by liquid scintillation counting. Each assay was performed at least in duplicate. Enzyme assays measuring the acute inhibitory effect of bisphenol A on SULT1A1 were performed in a similar manner. The reaction mixtures contained both 4-nitrophenol and bisphenol A, whereas the controls contained bisphenol A alone.

3.2.5 Reverse transcription polymerase chain reaction (RT-PCR)

BeWo cells at confluence were washed, scraped in PBS, and then centrifuged to remove the PBS. Total RNA was isolated using a commercially available phenol-guanidine isothiocyanate solution (TRIZOL Reagent, Invitrogen) according to the manufacturer's protocol. mRNA was purified from total RNA using an Oligotex mRNA Mini Kit (Qiagen, Valencia, CA) as per the manufacturer's protocol and stored at -80ºC until further use. The mRNA was concentrated prior to the experiment by mixing the required volume (containing 200 ng RNA) with 1/10th the volume of 3 M DEPC treated sodium acetate (pH 5.2), 2 µL glycogen (Roche Diagnostics, Indianapolis, IN), and ethanol (at a volume 2.5 fold more than the volume of mRNA). This was stored overnight at -20ºC to precipitate the mRNA. After overnight precipitation, the sample was spun down at 14,000 rpm for 15 minutes at 4ºC. The supernatant was aspirated off, the pellet was washed in 75% ethanol, and the tubes spun down at 14,000 rpm for 15 minutes at 4ºC. Most of the supernatant was

aspirated off. The remainder was dried in room air and reconstituted in RNAse free water prior to reverse transcription. 200 ng mRNA was reverse transcribed and amplified using the Access RT-PCR Introductory System (Promega, Madison, WI). The primer sequences for amplifying SULT1A1 were obtained from the literature [24]. The sense primer for SULT1A1 was 5'-ATGGAGCTGATCCAGGACAC-3' and the antisense primer was 5'-TGACCTACCGTCCCAGGCCC-3'. Primers were synthesized by the Biotechnology Support Facility at the University of Kansas Medical Center (Kansas City, KS). The primers utilized for amplifying β-actin transcripts were obtained from Promega (Madison, WI). Cycle parameters were as follows: 1 cycle each of reverse transcription at 48^{o}C for 45 minutes and denaturation for 2 min at 94 °C, followed by 40 cycles each of denaturation at 94^{o}C for 30 seconds, annealing at 60^{o}C for 1 minute, and extension at 68^{o}C for 2 minutes. This preceded a final elongation at 68°C for 7 minutes. Electrophoresis of all RT-PCR products was performed at 80 V on a 2% agarose gel. The gel was post-stained with 0.5 μg/mL ethidium bromide for 45 minutes, washed four times in distilled water at 10 minute intervals and viewed with a UV transilluminator.

3.2.6 Cytotoxicity assays

Cytotoxicity of bisphenol A was assessed using the Quick Cell Proliferation Assay Kit (Biovision, Mountain View, CA) according to manufacturer's protocol. The assay measures cellular mitochondrial dehydrogenase mediated conversion of the tetrazolium salt WST-1 to formazan (which absorbs at 440 nm). Briefly, cells were

cultured on 96-well plates at a density of 10,000 cells/cm^2, in a total volume of 100 µL. They were treated as mentioned in section 3.2.3. Following this 10 µL of the reagent was added to each well, the cells were incubated for 2 hours under standard culture conditions, and absorbance read at 450 nm.

3.3 Results

3.3.1 Bisphenol A as an acute inhibitor of SULT1A1

Bisphenol A has been reported to be a substrate of SULT1A1 (K_m=4.7 µM; specific activity = 965±206 pmol/min/mg protein at a concentration of 50 µM) [25, 26] . In BeWo cell homogenates, however, no sulfation could be detected using bisphenol A (0.6-100 µM) as a substrate (data not shown). Hence it can be assumed that under the utilized assay conditions if BeWo cytosolic protein was incubated with both 4-nitrophenol and bisphenol A, the measured radioactivity would be due to 4-nitrophenylsulfate and not bisphenol A sulfate. Bisphenol A (0.5-50 µM) was incubated along with 4 µM 4-nitrophenol. Bisphenol A significantly inhibited SULT1A1 only at 50 µM (Figure 3.3.1). Higher concentrations of bisphenol A were not tested because at 50 µM bisphenol A is at a much higher concentration than its concentrations detected in random human serum samples.

3.3.2 Effect of chronic exposure to bisphenol A on SULT1A1 activity

Upon chronic (48 hours) exposure, bisphenol A inhibited SULT1A1 at intermediate concentrations but not at lower or higher concentrations to produce a U-shaped non-

monotonic dose-response curve (Figure 3.3.2). Inhibition was observed at 100pM – 1 μM and was statistically significant at 10 nM and 100 nM. The maximal inhibition observed was around 40%. At none of the tested concentrations, bisphenol A affected cell viability (Figure 3.3.3).

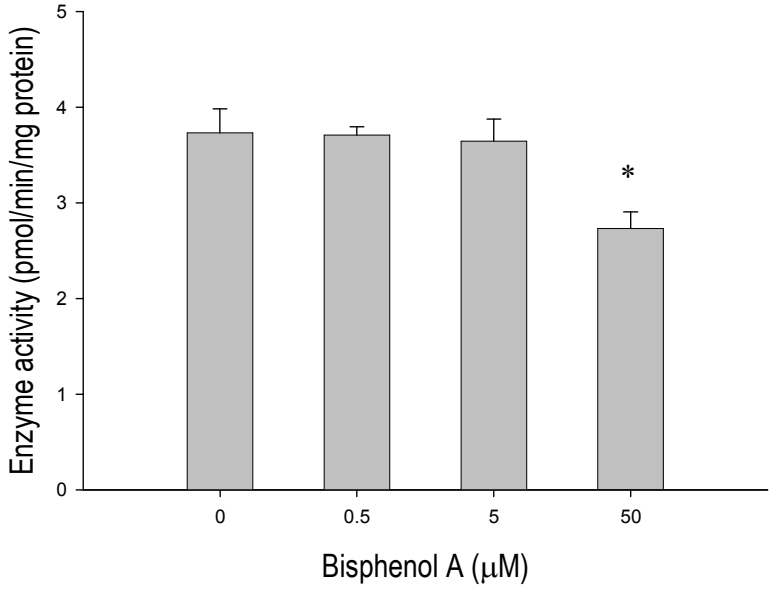


Figure 3.3.1 Effect of acute bisphenol A exposure upon SULT1A1 activity. SULT1A1 activity in BeWo cytosolic homogenate was determined with 4 μM 4-nitrophenol, in the absence and presence of bisphenol A at 37ºC. Control experiments contained bisphenol A at the same concentrations in the absence of 4-nitrophenol. The data has been plotted as mean ± S.E.M (n=3). * denotes a significant difference with respect to the vehicle-treated controls ($p<0.05$).

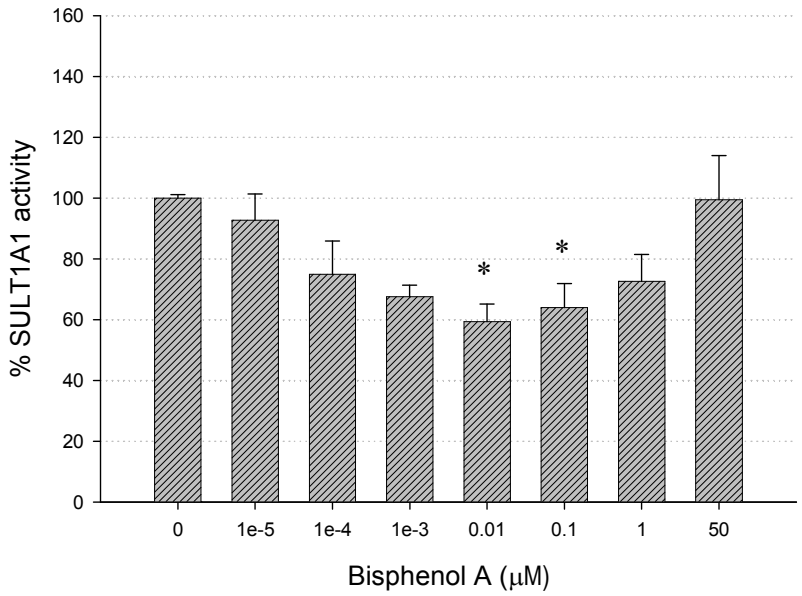


Figure 3.3.2 Effect of chronic bisphenol A exposure upon SULT1A1 activity. BeWo cells were exposed to bisphenol A (10 pM-50 μM) in DMEM containing 10% fetal bovine serum for 48 hours. The cytosolic fraction of BeWo homogenate was then extracted and SULT1A1 activity was determined with 4 μM 4-nitrophenol at 37ºC. The net SULT1A1 activity has been expressed as a percentage of the vehicle-treated controls. Each data point in a particular experiment represents triplicate measurements and each experiment was repeated twice. The data has been plotted as mean ± S.E.M. * denotes a significant difference with respect to the vehicle-treated controls ($p<0.05$).

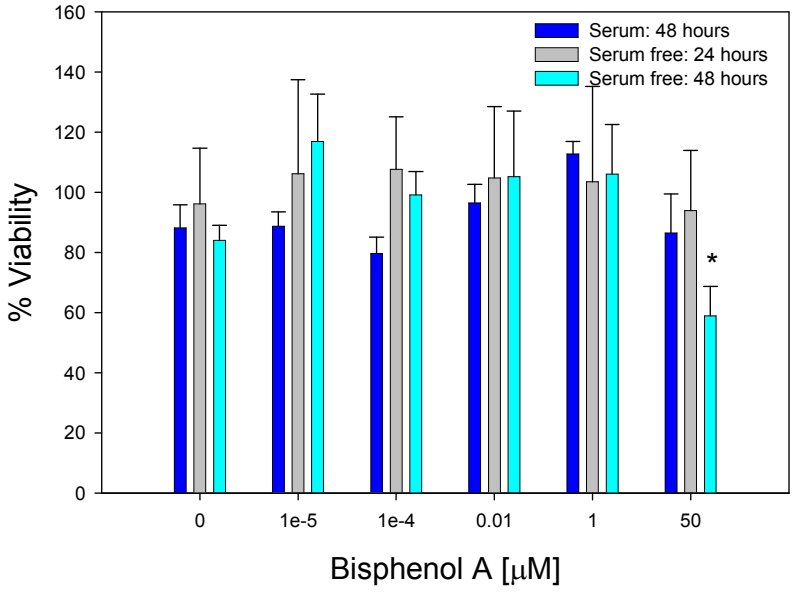


Figure 3.3.3 BeWo cell viability upon bisphenol A exposure. BeWo cells were seeded in 96-well plates. The cells were treated with bisphenol A (10 pM-50 μM) after 48 hours for the 48-hour treatments and after 72 hours for the 24-hour treatments. Treatments were either in serum-free or serum-containing medium. Upon completion of treatment, cytotoxicity was assessed by the WST assay (a modification of the MTT assay) using a commercially available kit. * denotes a significant difference with respect to the vehicle-treated controls ($p<0.05$).

3.3.3 Effect of chronic exposure to bisphenol A on SULT1A1 mRNA expression.

mRNA was extracted from BeWo cells after chronic exposure to bisphenol A for 48 hours. Concentrations tested were 100 nM, 1 μM, and 50 μM. In accordance to what was observed with the enzyme activity assays, at concentrations of 1 μM and 50 μM, there was no change in SULT1A1 mRNA expression with respect to the vehicle-treated controls (Figure 3.3.4). Contrary to what was observed with the enzyme activity data, there was no decrease in SULT1A1 mRNA at 0.1 μM.

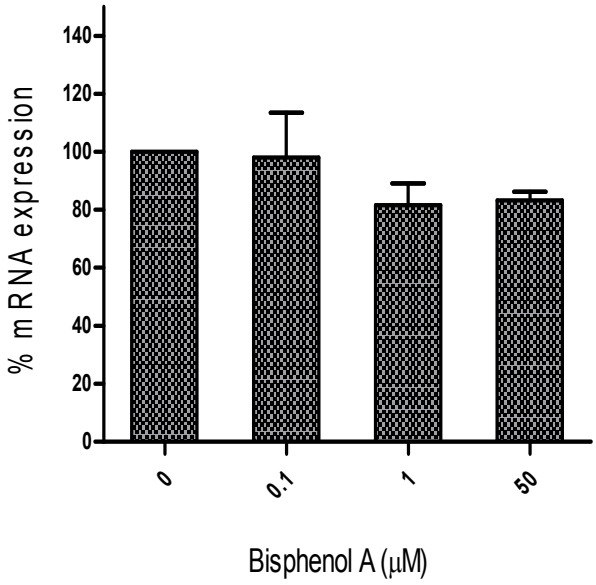


Figure 3.3.4 Effect of chronic bisphenol A exposure on SULT1A1 mRNA expression. BeWo cells were exposed to bisphenol A (100 nM, 1 μM, and 50 μM) in DMEM containing 10% fetal bovine serum for 48 hours. mRNA was then isolated and RT-PCR was performed to detect SULT1A1 mRNA. Each data point in a particular experiment represents triplicate measurements. The data has been plotted as mean ± S.E.M.

3.3.4 Effect of chronic exposure to bisphenol A on SULT1A1 activity (serum-free medium).

The above-mentioned chronic bisphenol A incubations (section 3.3.2) were done in medium containing 10% serum. To eliminate any extra-estrogenic effects, BeWo cells were exposed to bisphenol A in medium without any serum for either 24 or 48 hours. As with treatments in serum-containing medium, chronic bisphenol A exposure resulted in SULT1A1 activity inhibition producing a U-shaped non-monotonic dose-response curve. Statistically significant inhibition was observed only at intermediate concentrations of 10 nM (Figure 3.3.5). SULT1A1 activity was

inhibited at lower and higher concentrations but this was not statistically significant. At these concentrations bisphenol A was not toxic to the cells (Figure 3.3.3).

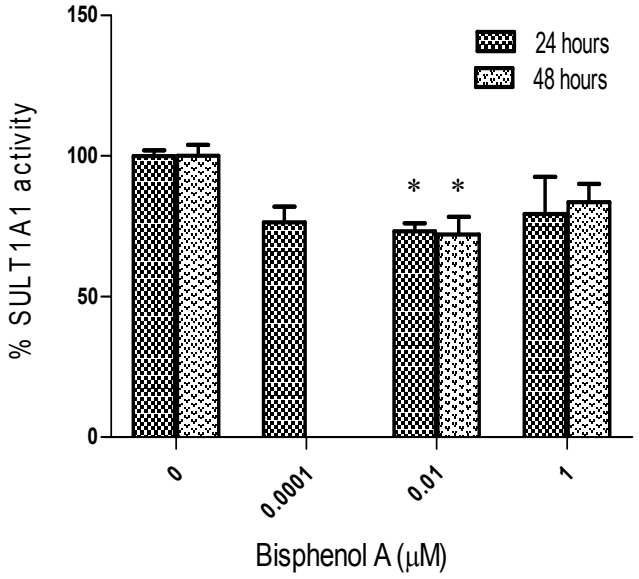


Figure 3.3.5 Effect of bisphenol A on SULT1A1 activity upon chronic exposure to bisphenol A. BeWo cells were exposed to bisphenol A in serum-free medium for 24 or 48 hours. The cytosolic fraction of homogenate was then extracted and SULT1A1 activity was determined with 4 μM 4-nitrophenol at 37ºC. The net SULT1A1 activity has been expressed as a percentage of the vehicle-treated controls. Each data point in a particular experiment represents triplicate measurements and each experiment was repeated twice. The data has been plotted as mean ± S.E.M. * denotes a significant difference with respect to the vehicle-treated controls ($p<0.05$).

3.3.5 Effect of ICI182,780 on bisphenol A mediated inhibition of SULT1A1

To determine if the inhibitory effect of bisphenol A was mediated through ERβ, BeWo cells were exposed to 10 nM bisphenol A in the absence or presence of IC182,780 (10 nM, 100 nM, 1 μM) in serum-free medium for 24 hours. At these concentrations ICI182,780 has previously been shown to inhibit ERβ. If bisphenol A

mediated inhibition of SULT1A1 is via ERβ, then in the presence of ICI182,780 SULT1A1 activity should come back to the levels of the untreated controls. Even in the presence of ICI182,780 SULT1A1 activity was inhibited to the same extent indicating that at least the inhibitory effect of bisphenol A on SULT1A1 was not mediated through ERβ (Figure 3.3.6).

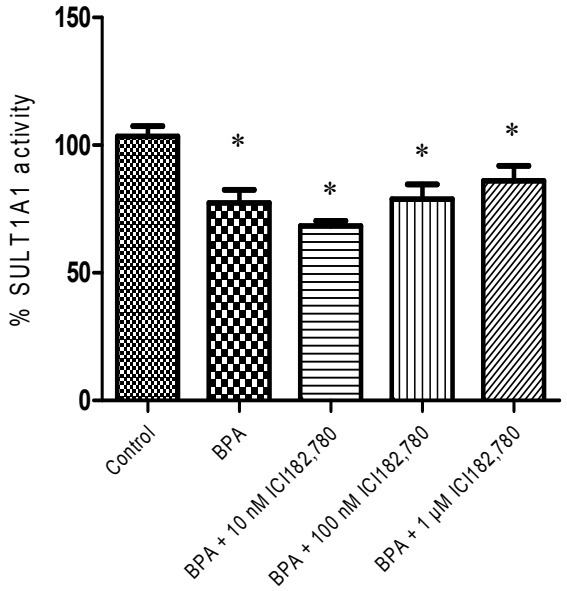


Figure 3.3.6 Effect of bisphenol A on SULT1A1 activity in the presence of ICI182,780. BeWo cells were exposed to 10 nM bisphenol A (BPA) in the presence/absence of the estrogen receptor inhibitor ICI182,780 in serum-free medium for 24 hours. The cytosolic fraction of homogenate was then extracted and SULT1A1 activity was determined with 4 μM 4-nitrophenol at 37ºC. The net SULT1A1 activity has been expressed as a percentage of the vehicle-treated controls. The data has been plotted as mean ± S.E.M (n=3). * denotes a significant difference with respect to the vehicle-treated controls ($p<0.05$).

3.4 Discussions and conclusions

In many cases 4 μM 4-nitrophenol is used as a marker substrate for SULT1A1. We established in chapter 2 that sulfation of 4-nitrophenol in the low micromolar range in BeWo cells is mediated by SULT1A1. Therefore, 4 μM 4-nitrophenol was used to test for SULT1A1 activity. Bisphenol A inhibits SULT1E1 [27] but there are no reports of it being a SULT1A1 inhibitor. Upon acute exposure, bisphenol A inhibited SULT1A1 at 50 μM but not at lower concentrations. As mean serum concentrations of bisphenol A are approximately reported to be in the range 0.6-2.5 ng/mL (~1-12.5 nM), bisphenol A is not an acute inhibitor of SULT1A1 at concentrations at which it was detected in random samples of the human population following environmental exposure. This data agrees with previous reports regarding bisphenol A as an inhibitor of other enzymes (i.e. inhibition is observed only at concentrations much higher than relevant concentrations). For example, bisphenol A inhibited SULT1E1 at 1 μM or higher concentrations. It also inhibits several cytochrome P450 enzymes. However in most of these cases, the reported IC_{50} values were all higher than the serum concentrations of bisphenol A [28, 29].

In contrast to the acute exposure studies, bisphenol A inhibited SULT1A1 activity upon chronic exposure at relevant concentrations. Dose-dependent inhibition was not observed, rather a U-shaped non-monotonic dose-response curve was seen. Statistically significant inhibition was observed at 10 nM and 100 nM, and the maximal inhibition was approximately 40%. In contrast to SULT1A1 activity,

bisphenol A did not affect SULT1A1 mRNA expression, which suggested that the effects of bisphenol A on SULT1A1 were likely post-translational.

Initial experiments were conducted in serum-containing medium. The effective concentration of bisphenol A as well as any other exogenous compound is dependent on protein binding to serum proteins as this modifies the amount available for cellular uptake. Bisphenol A is reported to have a free fraction of ~8% in serum [30]. Therefore we hypothesized that it should inhibit SULT1A1 with a higher potency in serum-free media. In serum-free medium, bisphenol A inhibited SULT1A1 to approximately the same extent (the maximum inhibition observed was around 30%) and in a similar manner, to produce a U-shaped dose-response curve. Statistically significant inhibition was observed only at intermediate concentrations (~10 nM). The same extent of inhibition was observed for either 24 or 48 hour incubations. The U-shaped nature of the curve indicates that bisphenol A is acting through at least two mechanisms. One pathway inhibits SULT1A1 activity while the other induces SULT1A1 activity. The combined effect produces a U-shaped curve. Such non-monotonic dose-response curves have been observed for other estrogenic substances. For example, to test the estrogenicity of several phytoestrogens and environmental estrogens, estrogenic induction of pS2 mRNA was determined in MCF-7 breast cancer cells following incubation of the cells with testosterone. Several of the compounds tested produced U-shaped dose-response curves. The effect was proposed to be due to the phytoestrogens acting through two pathways – at lower

concentrations, the phytoestrogens inhibited aromatase activity (which led to a lesser amount of 17β-estradiol being produced from testosterone) whereas at higher concentrations pS2 mRNA was induced through the estrogenicity of the phytoestrogens [31].

Bisphenol A exerts some of its effects by binding to the nuclear estrogen receptors (ER) α and β. The resulting complex binds to estrogen responsive elements (EREs) on genes to affect transcription. The nuclear estrogen receptor mediated effects of BPA are cell specific as it depends on the type and the levels of the receptor type expressed in each cell. Bisphenol A binds to ERβ with a higher affinity than it does to ERα. Also, the ERβ-bisphenol A complex recruits some co-activators with a greater affinity than the ERα-bisphenol A complex. Further, each has several isoforms and the different isoforms of ERβ differentially affect ERE-dependent gene expression [32]. In addition to these nuclear receptor mediated effects, some of the rapid signaling effects produced by bisphenol A are mediated through the non-classical membrane estrogen receptor (ncmER) [32].

Not much is known about the transcriptional regulation of SULT1A1 in humans. Glucocorticoids induce SULT1A1 mRNA and protein in bovine adrenocortical cells, as well as in rat (both *in vitro* and *in vivo*). In rat hepatocytes, transcriptional induction of SULT1A1 is mediated through both the glucocorticoid receptor (GR) as well as the androgen receptor (AR), with GR mediation predominating over AR

mediation [33]. However, human hepatic SULT1A1 expression is not affected by glucocorticoid treatment [33]. Activators of other nuclear receptors such as the constitutive androgen receptor (CAR), pregnane-X receptor (PXR), and the aryl hydrocarbon receptor (AhR), also did not alter SULT1A1 mRNA levels in primary human hepatocytes [34].

The BeWo b30 subclone used in this study expresses the estrogen receptor β (ERβ) but not ERα [35, 36]. The U-shaped curve indicates that bisphenol A is possibly acting through at least two mechanisms. One of these inhibits SULT1A1 activity while the other induces SULT1A1 activity, and the combined effect results in a U-shaped curve. The presence of the ERα and ERβ inhibitor ICI182,780 did not produce any change in inhibitory effect observed with 10 nM bisphenol A. The inhibitory effect reaches a maximum around 10-100 nM. If this effect is mediated by ERβ, then in the presence of ICI182,780 SULT1A1 activities should be higher than that in the presence of bisphenol A alone. The lack of any effect of ICI182,780 indicates that at least the inhibitory effect of bisphenol A on SULT1A1 is not mediated through ERβ. Future studies should examine whether ERβ is associated with the up-regulation of SULT1A1 activity. If the increase in SULT1A1 activity that is observed at bisphenol A concentrations greater than 10-100 nM, is not observed in the presence of ICI182,780, then this would indicate that the inductive effect of bisphenol A on SULT1A1 is being mediated through ERβ.

In conclusion, we can say that chronic exposure to bisphenol A inhibited SULT1A1 at concentrations detected in random serum samples of the human population. The nature of inhibition suggested that bisphenol A is acting via at least two mechanisms, one of which inhibits SULT1A1 and the other induces SULT1A1. The inhibitory effect of bisphenol A on SULT1A1 was not mediated via ERβ. Altered SULT1A1 activity can potentially modify fetal exposure to sulfotransferase substrates. It can also have other consequences such as changes in estradiol sulfation which is a SULT1A1 substrate. It has been proposed that, reduced estrogen sulfation by polymorphic variants of SULT1A1 that exhibit reduced enzyme activity, may be a possible cause of cancer in estrogen-responsive tissues [37]. On the other hand, the extent of SULT1A1 inhibition (~30-40%) was modest and may not translate into actually altering the extents of fetal exposure as sulfotransferase substrates can be subject to other conjugation pathways such as glucuronidation.

3.5 References

1. vom Saal, F.S. and W.V. Welshons, *Large effects from small exposures. II. The importance of positive controls in low-dose research on bisphenol A.* Environ Res, 2006. **100**(1): p. 50-76.
2. Carrey, F.A., *Organic Chemistry*. 2003, Boston: McGraw-Hill.
3. Dekant, W. and W. Volkel, *Human exposure to bisphenol A by biomonitoring: methods, results and assessment of environmental exposures.* Toxicol Appl Pharmacol, 2008. **228**(1): p. 114-34.
4. Hiroi, H., et al., *Differences in serum bisphenol a concentrations in premenopausal normal women and women with endometrial hyperplasia.* Endocr J, 2004. **51**(6): p. 595-600.
5. Takeuchi, T. and O. Tsutsumi, *Serum bisphenol a concentrations showed gender differences, possibly linked to androgen levels.* Biochem Biophys Res Commun, 2002. **291**(1): p. 76-8.
6. Sugiura-Ogasawara, M., et al., *Exposure to bisphenol A is associated with recurrent miscarriage.* Hum Reprod, 2005. **20**(8): p. 2325-9.
7. Sajiki, J., K. Takahashi, and J. Yonekubo, *Sensitive method for the determination of bisphenol-A in serum using two systems of high-performance liquid chromatography.* J Chromatogr B Biomed Sci Appl, 1999. **736**(1-2): p. 255-61.
8. Ikezuki, Y., et al., *Determination of bisphenol A concentrations in human biological fluids reveals significant early prenatal exposure.* Hum Reprod, 2002. **17**(11): p. 2839-41.
9. Schonfelder, G., et al., *Parent bisphenol A accumulation in the human maternal-fetal-placental unit.* Environ Health Perspect, 2002. **110**(11): p. A703-7.
10. Han, D., H. Tachibana, and K. Yamada, *Inhibition of environmental estrogen-induced proliferation of human breast carcinoma MCF-7 cells by flavonoids.* In Vitro Cell Dev Biol Anim, 2001. **37**(5): p. 275-82.
11. Gutendorf, B. and J. Westendorf, *Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens.* Toxicology, 2001. **166**(1-2): p. 79-89.
12. Hiroi, H., et al., *Differential interactions of bisphenol A and 17beta-estradiol with estrogen receptor alpha (ERalpha) and ERbeta.* Endocr J, 1999. **46**(6): p. 773-8.
13. Wozniak, A.L., N.N. Bulayeva, and C.S. Watson, *Xenoestrogens at picomolar to nanomolar concentrations trigger membrane estrogen receptor-alpha-mediated Ca2+ fluxes and prolactin release in GH3/B6 pituitary tumor cells.* Environ Health Perspect, 2005. **113**(4): p. 431-9.
14. Newbold, R.R., W.N. Jefferson, and E. Padilla-Banks, *Long-term adverse effects of neonatal exposure to bisphenol A on the murine female reproductive tract.* Reprod Toxicol, 2007. **24**(2): p. 253-8.

15. Markey, C.M., et al., *Long-term effects of fetal exposure to low doses of the xenoestrogen bisphenol-A in the female mouse genital tract.* Biol Reprod, 2005. **72**(6): p. 1344-51.
16. Maffini, M.V., et al., *Endocrine disruptors and reproductive health: the case of bisphenol-A.* Mol Cell Endocrinol, 2006. **254-255**: p. 179-86.
17. Wetherill, Y.B., et al., *The xenoestrogen bisphenol A induces inappropriate androgen receptor activation and mitogenesis in prostatic adenocarcinoma cells.* Mol Cancer Ther, 2002. **1**(7): p. 515-24.
18. Pacifici, G.M., *Inhibition of human liver and duodenum sulfotransferases by drugs and dietary chemicals: a review of the literature.* Int J Clin Pharmacol Ther, 2004. **42**(9): p. 488-95.
19. Yasuda, S., M. Suiko, and M.C. Liu, *Oral contraceptives as substrates and inhibitors for human cytosolic SULTs.* J Biochem, 2005. **137**(3): p. 401-6.
20. Seth, P., et al., *Phenol sulfotransferases: hormonal regulation, polymorphism, and age of onset of breast cancer.* Cancer Res, 2000. **60**(24): p. 6859-63.
21. Bode, C.J., et al., *In vitro models for studying trophoblast transcellular transport.* Methods Mol Med, 2006. **122**: p. 225-39.
22. Ebmeier, C.C. and R.J. Anderson, *Human thyroid phenol sulfotransferase enzymes 1A1 and 1A3: activities in normal and diseased thyroid glands, and inhibition by thyroid hormones and phytoestrogens.* J Clin Endocrinol Metab, 2004. **89**(11): p. 5597-605.
23. Foldes, A. and J.L. Meek, *Rat brain phenolsulfotransferase: partial purification and some properties.* Biochim Biophys Acta, 1973. **327**(2): p. 365-74.
24. Tamura, H.O., et al., *Expression profiling of sulfotransferases in human cell lines derived from extra-hepatic tissues.* Biol Pharm Bull, 2001. **24**(11): p. 1258-62.
25. Nishiyama, T., et al., *Sulfation of environmental estrogens by cytosolic human sulfotransferases.* Drug Metab Pharmacokinet, 2002. **17**(3): p. 221-8.
26. Suiko, M., Y. Sakakibara, and M.C. Liu, *Sulfation of environmental estrogen-like chemicals by human cytosolic sulfotransferases.* Biochem Biophys Res Commun, 2000. **267**(1): p. 80-4.
27. Kester, M.H., et al., *Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters.* J Clin Endocrinol Metab, 2002. **87**(3): p. 1142-50.
28. Niwa, T., et al., *Inhibition of drug-metabolizing enzyme activity in human hepatic cytochrome P450s by bisphenol A.* Biol Pharm Bull, 2000. **23**(4): p. 498-501.
29. Niwa, T., et al., *Metabolism and interaction of bisphenol A in human hepatic cytochrome P450 and steroidogenic CYP17.* Biol Pharm Bull, 2001. **24**(9): p. 1064-7.

30. Welshons, W.V., S.C. Nagel, and F.S. vom Saal, *Large effects from small exposures. III. Endocrine mechanisms mediating effects of bisphenol A at levels of human exposure.* Endocrinology, 2006. **147**(6 Suppl): p. S56-69.
31. Almstrup, K., et al., *Dual effects of phytoestrogens result in u-shaped dose-response curves.* Environ Health Perspect, 2002. **110**(8): p. 743-8.
32. Wetherill, Y.B., et al., *In vitro molecular mechanisms of bisphenol A action.* Reprod Toxicol, 2007. **24**(2): p. 178-98.
33. Runge-Morris, M. and T.A. Kocarek, *Regulation of sulfotransferases by xenobiotic receptors.* Curr Drug Metab, 2005. **6**(4): p. 299-307.
34. Hempel, N., et al., *The human sulfotransferase SULT1A1 gene is regulated in a synergistic manner by Sp1 and GA binding protein.* Mol Pharmacol, 2004. **66**(6): p. 1690-701.
35. Jin, H., and Audus, K.L., *Influence of β-Estradiol on Calcein Efflux in Placental BeWo Cells*
36. Jin, H. and K.L. Audus, *Effect of bisphenol A on drug efflux in BeWo, a human trophoblast-like cell line.* Placenta, 2005. **26 Suppl A**: p. S96-S103.
37. Hempel, N., et al., *Human cytosolic sulfotransferase SULT1A1.* Int J Biochem Cell Biol, 2007. **39**(4): p. 685-9.

Chapter 4: Mechanisms of Elimination of Sulfate Metabolites out of BeWo Cells

4.1 Introduction

The placenta has the capacity of promoting elimination of drugs and natural substances by sulfation [1, 2]. Since sulfate metabolites bear a negative charge at physiological pH it is likely that passive diffusion would not be the predominant mechanism of their elimination out of the placenta. This indicates transporter-mediated elimination which can be mediated by either uptake transporters or efflux transporters.

Among the efflux transporters, the breast cancer resistance protein (BCRP) mediates biliary elimination of sulfoconjugates in rat and mice [3, 4]. Similarly human BCRP mediated the elimination of steroid sulfate conjugates and the sulfate conjugates of smaller exogenous molecules in cell-based systems overexpressing this transporter [5-7]. A major part of the work regarding the involvement of multidrug resistance-associated protein isoforms in the excretion of sulfate conjugates has been performed in rats where MRP2 excreted bile acid conjugates as well as xenobiotic sulfate conjugates [6]. MRP3 and MRP4 have been reported to interact with the sulfate conjugates of xenobiotics, steroids and bile acids, while MRP1 has been reported to transport steroid conjugates [6, 8]. Thus literature would suggest that MRP isoforms and BCRP are prime candidates to mediate the transport of sulfated metabolites.

BCRP is abundantly expressed in the placenta with immunohistochemistry revealing predominantly syncytiotrophoblast staining with some staining of the fetal blood

vessels [9, 10]. As far as its syncytiotrophoblast localization is concerned, several studies have reported mainly brush border (maternal-facing) membrane localization with faint signals detectable in the basal (fetal-facing) membrane fractions [5, 11] . MRP2, MRP3 and MRP4 are localized predominantly at the syncytiotrophoblast brush border membrane [12, 13]. MRP1 is expressed in the syncytiotrophoblast and fetal capillary endothelium, with predominant staining in the fetal capillary endothelium [12, 14]. There is conflicting data on the syncytiotrophoblast localization of MRP1. While one study reports predominantly brush border membrane localization [12], the other reported only basal membrane localization [14]. Both of these studies utilized immunoblotting. MRP5 is located predominantly to the basal syncytiotrophoblast membrane, although some expression was also observed in the brush border membrane [15].

Species as well as tissue differences exist with regards to the relative contribution of the efflux transporters responsible for the elimination of sulfates [3, 16, 17]. It is important to identify the transporters responsible for sulfate excretion in order to understand the role of the placenta in the elimination of sulfated metabolites in general, as well as to understand the potential for placental accumulation/fetal exposure of toxic sulfate metabolites.

The objective of the present work was to determine the efflux transporters responsible for the elimination of sulfate conjugates out of trophoblast cells using the BeWo cell

line as a human trophoblast model. The mRNA of BCRP and several of the MRP isoforms are expressed in BeWo cells, and protein expression has been determined for BCRP, MRP1, MRP2 and MRP5 [13, 18-20]. Although there are differences between primary trophoblast cells and BeWo cells with respect to the expression and function of several efflux transporters [13, 18], the BeWo subclone used in this study forms a polarized monolayer of cells when grown on transwells, with the apical and basolateral membranes representing the maternal-facing and fetal-facing trophoblast membranes respectively [21]. This is of particular importance to our study as our objective is to begin describing the transporters responsible for the elimination of sulfate metabolites out of the placenta. A bidirectional transport study is a good way of determining if preferential excretion occurs across any one of the trophoblast membranes. We examined the fate of two common substances for sulfation pathways, 4-nitrophenol and acetaminophen.

4.2 Materials and methods

4.2.1 BeWo cell culture

The BeWo cell line (clone b30) was obtained from Dr. Alan Schwartz (Washington University, St. Louis, MO) and cultured as described previously [22]. Passages 29 through 45 of the cells were used in this study. Briefly, cells were grown at 37ºC in an atmosphere of 5% CO_2 and saturated relative humidity. They were maintained in Dulbecco's Modified Eagles Medium (DMEM, Sigma, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA), and 1%

each of 10,000 U/ml penicillin with 10,000 μg/mL streptomycin solution, 200 mM L-glutamine solution, and 10 mM minimal essential medium nonessential amino acid solution (all three from Invitrogen, Carlsbad, CA). At 70-90% confluence, cells were detached with 0.5% trypsin and 0.2% ethylenediaminetetraacetic acid (EDTA, Sigma, St. Louis, MO) diluted in phosphate buffered saline (PBS) at a 1:10 ratio. Cells were then subcultured at a ratio of 1:10.

4.2.2 Accumulation studies

For accumulation studies cells were seeded onto poly-d-lysine (Sigma, St. Louis, MO) and fibronectin (Sigma, St. Louis, MO) coated plates at a cell density of 12,500 cells/cm^2 as per the protocol of Bode et al [22]. BeWo monolayers were used for the uptake studies once they reached 90-100% confluence (4-6 days). The cells were washed twice in warm (37ºC) HBSS (Sigma, St. Louis, MO) containing 25 mM glucose (HBSS-Glc). They were then allowed to equilibrate in HBSS-Glc at 37ºC on a shaker (~ 50 rpm) for 30 minutes. Cells were then incubated with pre-warmed (37ºC) HBSS-Glc solutions containing mitoxantrone in the presence of either known BCRP inhibitors, fumitremorgin C or chrysin, or 4-nitrophenyl sulfate (0.5-200 μM) or acetaminophen sulfate (0.5-200 μM, all were from Sigma, St. Louis, MO). For the time-dependent studies 20 nM [^{3}H] mitoxantrone (4 Ci/mmol, Moravek Biochemicals Inc., Brea, CA) was used. For the others 10 μM unlabeled mitoxantrone (Sigma, St. Louis, MO) containing 20 nM [^{3}H] mitoxantrone (final specific activity 0.02 Ci/mmol) was used. Fumitremorgin C was used at 5 μM and chrysin at 100 μM. Cells

were incubated for 150 minutes with shaking at 50 rpm At the end of the accumulation period, the dosing solutions were aspirated off and the cells were washed thrice with ice-cold HBSS-Glc. Following this, the cells were lysed for 2 hours at 37ºC with a lysing solution containing 0.5% Triton X-100 (Sigma, St. Louis, MO) in 0.2N NaOH. The cell lysates were analyzed by liquid scintillation counting and their protein content determined by the BCA assay kit (Pierce Chemical, Rockford, IL). Accumulation studies of 500nM BODIPY® FL prazosin (Invitrogen, Carlsbad, CA) was performed in a similar manner with the following exceptions: the dosing solutions were prepared in HBSS-Glc containing 2% BSA (Sigma, St. Louis, MO) and the lysing solution contained 2% Triton X-100. Fluorescence was detected using a Bio-Tek FL600 Microplate Fluorescence Reader (Bio-Tek Instruments, Inc., Winooski, VT), with excitation at 485 nm and emission at 535 nm.

4.2.3 Bidirectional transport studies

For the bidirectional transport studies BeWo cells were seeded onto 0.4 μM pore size Transwell® plates (Costar Corporation, Acton, MA) at a density of 45,000 cells/cm^2. Transwells were coated with human placental collagen (Sigma, St. Louis, MO) prior to seeding as per the method of Bode et al [22]. All experiments were conducted between days 5-8. Stock solutions of 4-nitrophenol, 4-nitrophenyl sulfate, acetaminophen, acetaminophen sulfate, 3-methyl-4-nitrophenol and cimetidine (all from Sigma, St. Louis, MO) were prepared in HBSS, allowed to shake overnight, and filtered prior to use. 0.5 μM fluorescein (Sigma, St. Louis, MO) was prepared in

HBSS. Prior to the experiments HBSS and drug solutions were pre-warmed to 37^{o}C. The volume of solutions added to the apical chamber was 0.5 mL while that added to the basolateral chamber was 1.5 mL. Cell monolayers were incubated in HBSS for 20 minutes following which compound along with 0.5 μM fluorescein was added to either the apical or the basolateral chambers. The cells were then incubated at 37ºC with shaking (50 rpm). At 5, 10, 20, 30, and 45 minutes, 200 μL of the receiver chamber was withdrawn and replaced with fresh HBSS. The samples were then spun down at 2,500 RCF for 15 minutes and the supernatant analyzed by LC-MS/MS. The integrity of the monolayer was assessed by determining the permeability of fluorescein alone in separate monolayers.

The studies examining the effect of inhibitors on the basolateral elimination of 4-nitrophenyl sulfate were performed in a similar fashion. Monolayers were pre-incubated with inhibitor for 30 minutes in both apical and basolateral chambers. Inhibitors used were 25 μM MK-571 (Biomol, Plymouth Meeting, PA), 0.2 μM leukotriene C4 (Biomol, Plymouth Meeting, PA), or 5 μM fumitremorgin C. The preincubation solution was then aspirated off and the cells were incubated with 50 μM 4-nitrophenol solution containing inhibitors. Control cells contained vehicle instead of the inhibitors. The rest of the procedure was as outlined above except that during sampling of the receiver chamber, the volume withdrawn was replaced with HBSS containing inhibitor.

Samples from transport studies were analyzed by LC-MS/MS (LC coupled to Quattro Triple Quadrupole mass spectrometer). All separations were done on a Phenomenex Luna C18 column (2.0 x 50 mm, 5 μM pore size). The injection volume was 20 μL. 4-nitrophenol (PNP), 4-nitrophenyl sulfate (PNPS) and the internal standard 3-methyl-4-nitrophenol (MPNP) were separated using a mobile phase gradient at a flow rate of 0.3 mL/min. Solvent A was water containing 0.1% formic acid, while solvent B was acetonitrile containing 0.1% formic acid. The solvents were held at 95:5 A:B for 2 minutes, ramped to 30:70 A:B from 2 to 4.5 minutes, maintained at 30:70 A:B from 4.5 to 9.5 minutes, changed to 95:5 A:B from 9.5 to 10 minutes and then held at this composition until 15 minutes. They were detected in negative ion mode using multiple reaction monitoring: PNP (138 → 108), PNPS (217.95 → 138.05), and MPNP (152 → 122) (figure 4.2.1). To reduce exposure of the mass spectrometer to possible salts and cellular contaminants in the sample, the HPLC eluent was diverted to waste for the first 4 minutes.

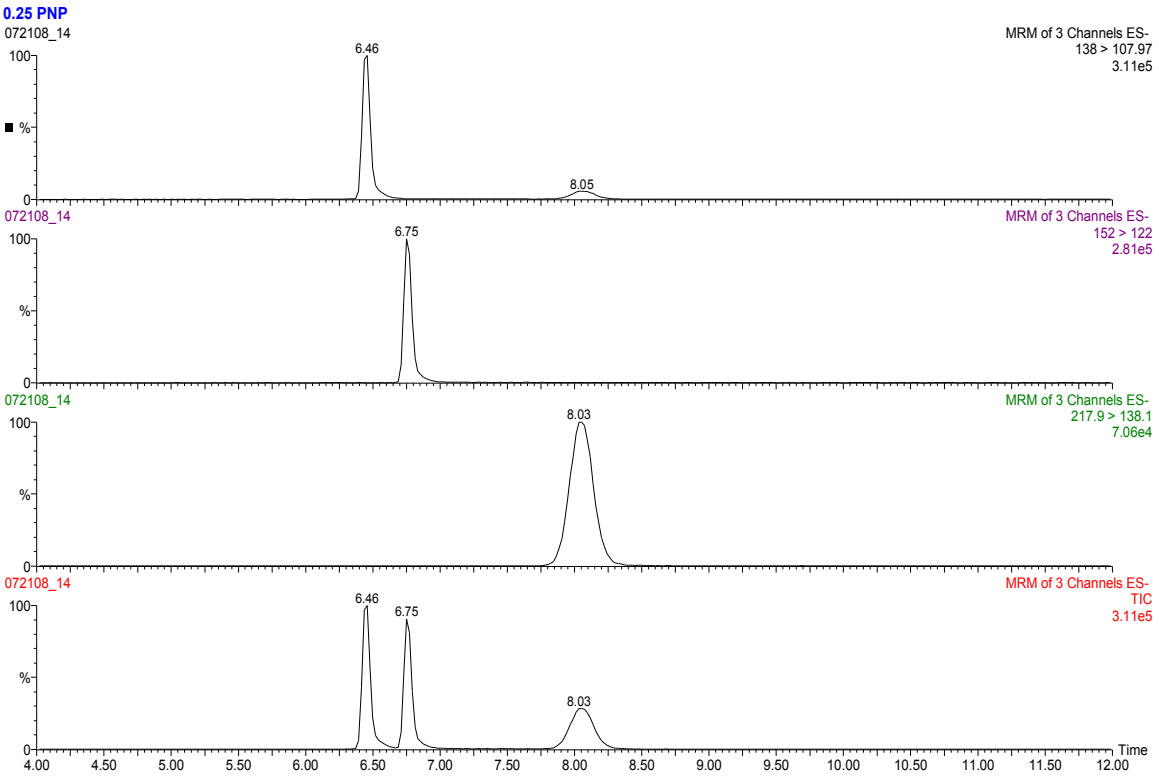


Figure 4.2.1 Representative MS chromatograms of 4-nitrophenol (PNP), 4-nitrophenyl sulfate (PNPS), and 3-methyl-4-nitrophenol (MPNP). The analytes were detected in negative ion mode using multiple reaction monitoring: PNP (138 → 108), PNPS (217.95 → 138.05), and MPNP (152 → 122).

Acetaminophen, acetaminophen sulfate (AS), and the internal standard cimetidine were separated using a mobile phase gradient at a flow rate of 0.3 mL/min. Solvent A was water containing 0.1% formic acid, while solvent B was acetonitrile containing 0.1% formic acid. The solvents were held at 100:0 A:B for 1 minute, ramped to 50:50 A:B from 1 to 2.5 minutes, maintained at 50:50 A:B from 2.5 to 4.5 minutes, changed to 100:0 A:B from 4.5 to 5.5 minutes, and then held at this composition for another 3.5 minutes. AS and cimetidine were detected in negative ion mode: AS (230.1 →

149.8), cimetidine (251.2 → 156.9) as seen in figure 4.2.2. Acetaminophen and cimetidine were detected in positive ion mode: acetaminophen (152 → 110), cimetidine (253 → 117). The HPLC eluent was diverted to waste for the first 3 minutes.

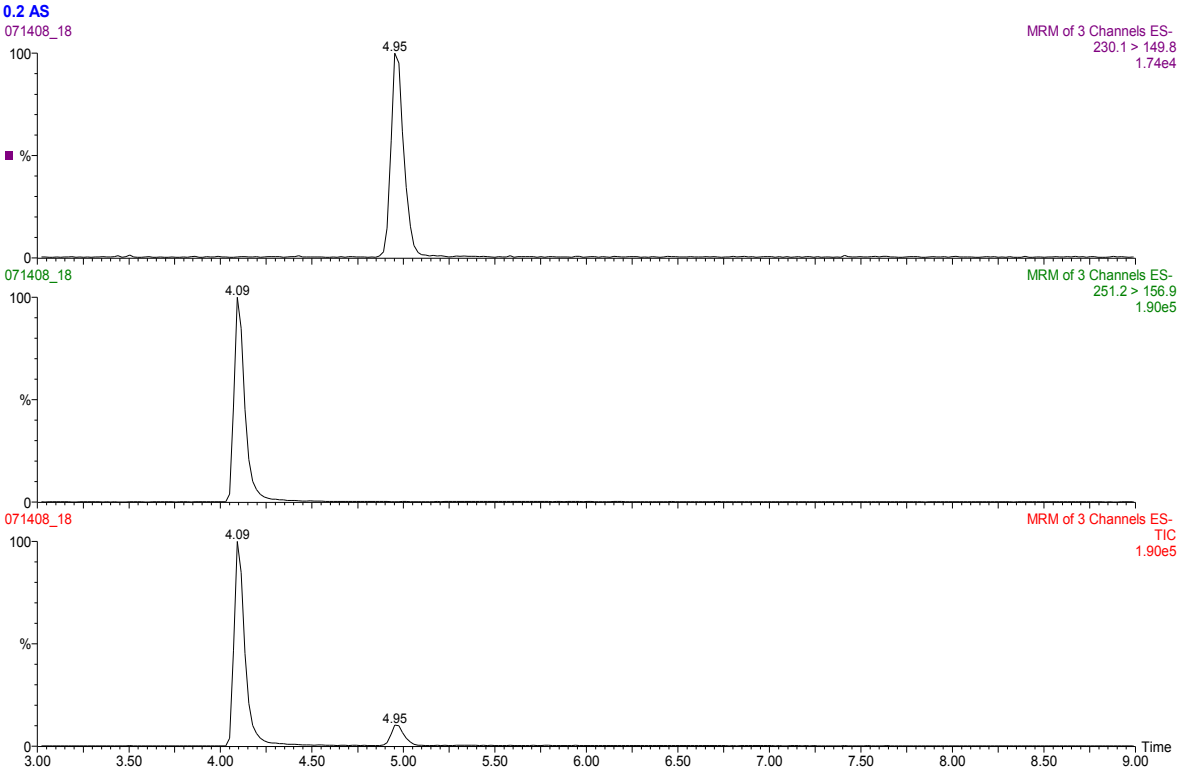


Figure 4.2.2 Representative MS chromatograms of acetaminophen sulfate (AS) and cimetidine. The analytes were detected in negative ion mode using multiple reaction monitoring: AS (230.1 → 149.8), and cimetidine (251.2 → 156.9).

Analytes were quantified from standard curves with the standards prepared in HBSS. Figures 4.2.3, 4.2.4, and 4.2.5 are representative calibration curves of 4-nitrophenol, 4-nitrophenyl sulfate, and acetaminophen sulfate. Standards exhibited good linearity over a calibration range of 5-250 ng/mL (exact concentrations have been listed in the

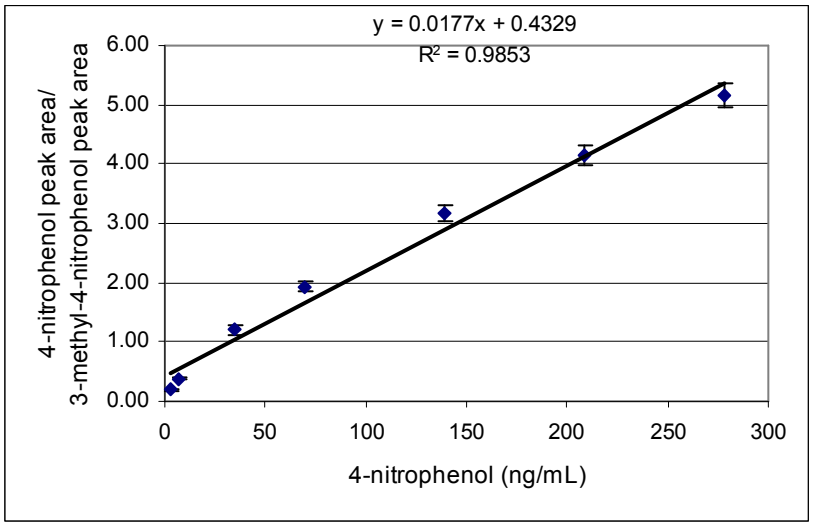


Figure 4.2.3 Calibration curve of 4-nitrophenol. Linearity was observed in the range 3-280 ng/mL.

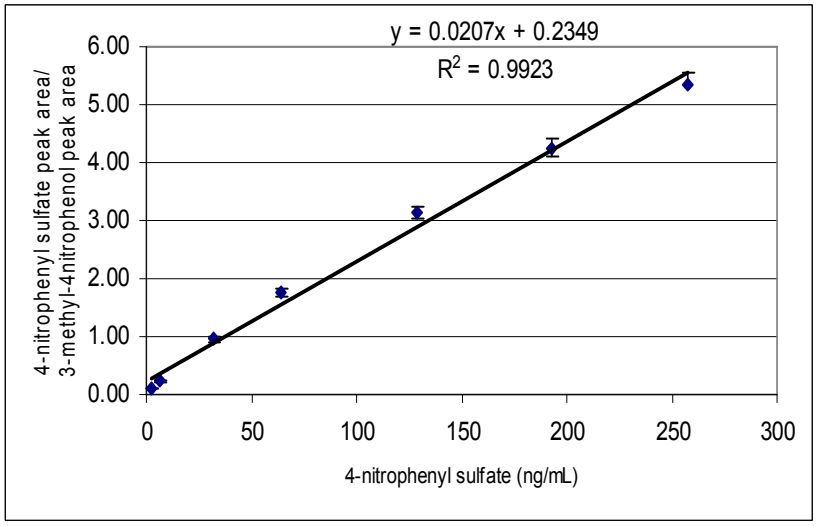


Figure 4.2.4 Calibration curve of 4-nitrophenyl sulfate. Linearity was observed in the range 3-260 ng/mL.

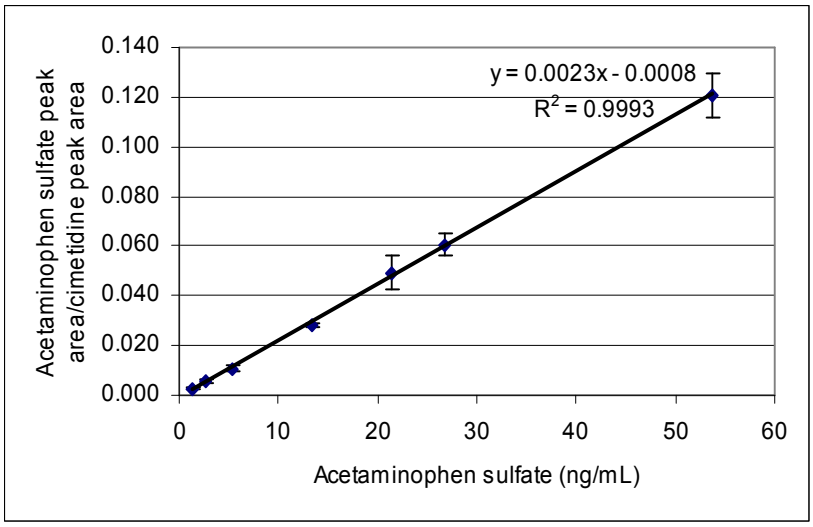


Figure 4.2.5 Calibration curve of acetaminophen sulfate. Linearity was observed in the range 1.3-270 ng/mL.

figure legends). The lower limit of detection for acetaminophen sulfate was 1.4 ng/mL. At the lowest concentrations, 4-nitrophenol and 4-nitrophenyl sulfate exhibited S/N ratios of 440 and 100 respectively.

4.2.4 Efflux studies

For efflux studies, cells were seeded onto poly-d-lysine and fibronectin coated plates at a cell density of 12,500 cells/cm^2 as per the protocol of Bode et al [22]. Plates were used 6-9 days post-seeding. Cells were washed thrice in warm HBSS (37ºC) and pre-incubated with HBSS containing inhibitor for 30 minutes. Inhibitors used were 5 µM and 25 µM MK-571; 10 and 100 µM indomethacin (Sigma, St. Louis, MO); 5 µM fumitremorgin C; 200 µM chrysin; and 0.1 µM and 0.2 µM leukotriene C4. The incubation medium was then aspirated away. The cells were freshly incubated with 1

μM 4-nitrophenol or 250 μM acetaminophen in the presence/absence of inhibitors for 2 hours and 4 hours, respectively. The incubation medium was then collected, spun down at 2,500 RCF for 15 minutes, and analyzed for the sulfated metabolites by LC-MS/MS. The analytical methods were the same as used in section 4.2.3.

4.2.5 Determination of sulfotransferase enzyme activities

BeWo cell lysate was prepared and sulfotransferase activities measured according to the procedure of Hu et al. [23] with slight modifications. BeWo cells at confluence were scraped up in PBS. The cells were centrifuged, PBS was aspirated away, and the cells were then suspended in 50 mM potassium phosphate buffer (pH 7.4) and homogenized on ice for 30 seconds with a Polytron homogenizer. The homogenate was spun down at 14,000 rpm for 15 minutes, the supernatant withdrawn and spun down at 14,000 rpm for 15 minutes again. The resultant supernatant was stored at -80ºC for further use. Protein concentration was determined using a BCA assay kit (Pierce, Rockford, IL). Sulfotransferase activities were measured as follows: Cell lysate at a final concentration of 0.9 mg protein/mL was added to 100μM 3'-phosphoadenosine-5'-phosphosulfate (PAPS, Sigma, St. Louis, MO) and 1 μM 4-nitrophenol/250 μM acetaminophen in a total reaction volume of 100 μL. The mixture was incubated at 37ºC for 2 hours in the case of 4-nitrophenol and 4 hours in the case of acetaminophen. The reaction was stopped by the addition of 25 μL of 94% acetonitrile/6% glacial acetic acid containing the internal standards 3-methyl-4-nitrophenol or cimetidine. The samples were then centrifuged at 14,000 rpm for 15

minutes and the supernatant analyzed by LC-MS/MS. The analytical methods were the same as used in section 4.2.3.

4.2.6 Permeability calculations

The following equation was used to calculate permeability in either the apical to basolateral direction ($P_{app,A\ to\ B}$) or basolateral to apical direction ($P_{app,B\ to\ A}$) [24] :

$$P_{app} = \frac{dC}{dt} \times \frac{V_r}{A} \times C_d$$

Where dC/dt is the change in concentration in the receiver compartment with respect to time, V_r is the volume of the receiver compartment, A is the growth surface area, and C_d is the initial concentration of the compound added to the donor compartment. The permeability directional ratio (PDR) was calculated as follows:

$$PDR = P_{app,\ B\ to\ A} / P_{app,\ A\ to\ B}$$

Metabolite excretion rates (V_{mt}) from the cell was calculated as reported before [25, 26] . Metabolite excretion rates were obtained by monitoring the change of metabolite concentration (C_m) in the receiver chamber as a function of time.

$$V_{mt} = \frac{dC_m}{dt} \times V_r$$

4.2.7 Statistical analysis

Statistical significance was determined using One-way analysis of variance (ANOVA) followed by Dunnett's post-comparison test (GraphPad Prism software) or the student's t-test as deemed appropriate. A p-value of less than 0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Effect of acetaminophen sulfate and 4-nitrophenyl sulfate on the accumulation of mitoxantrone and BODIPY® FL prazosin

BCRP, MRP1 and MRP5 proteins are expressed in BeWo cells [18-20]. Some studies failed to detect MRP2 protein expression in BeWo cells while others have reported weak expression [18-20]. In BeWo cells, BCRP mRNA levels were reported to be much higher than those of MRP1 or MRP2 [27]. These studies did not examine the membrane localization of the transporters in BeWo cells. However, in syncytiotrophoblast BCRP and MRP2 are localized to the apical membrane, while MRP1 and MRP5 are localized to both membranes. BeWo cells consist primarily of cytotrophoblast-like cells [28]. Assuming similar transporter localization in syncytiotrophoblast and BeWo cells, the initial hypothesis was that BCRP would predominantly mediate sulfate metabolite elimination across the apical trophoblast membrane. To examine this hypothesis, the effect of acetaminophen sulfate and 4-nitrophenyl sulfate on the accumulation of the BCRP substrates BODIPY® FL prazosin and [^{3}H]-mitoxantrone was determined. If the sulfates altered accumulation

of the BCRP substrates, it would indicate an interaction of BCRP with the sulfates, and would imply a potential role of BCRP in sulfate elimination.

BODIPY® FL prazosin and [^{3}H]-mitoxantrone were used at concentrations that are typically used for BCRP [29, 30]. Accumulation of both mitoxantrone and BODIPY® FL prazosin reached steady state after the first hour of incubation (Figures 4.3.1 and 4.3.2). The presence of the BCRP-specific inhibitor fumitremorgin C (FTC), on an average, increased the accumulation of BODIPY® FL prazosin by 1.5-2 fold and that of mitoxantrone by 1.3-1.5 fold. The accumulation of both substrates in the presence/absence of FTC was on an average most significantly different at 150 minutes. Hence an accumulation period of 150 minutes was utilized for all subsequent experiments.

4-nitrophenyl sulfate (0.5-200 μM) did not alter the accumulation of mitoxantrone (Figure 4.3.3). In the same concentration range, 4-nitrophenyl sulfate did not affect the accumulation of BODIPY® FL prazosin either except at 200 μM where it produced a statistically significant increase in accumulation suggesting it to be a potential BCRP inhibitor at this concentration (Figure 4.3.4). The increase in accumulation (1.5-1.8 fold) was comparable to that produced by fumitremorgin C (1.5-2 fold). Acetaminophen sulfate (0.5-200 μM) did not affect the accumulation of either mitoxantrone or BODIPY® FL prazosin (Figures 4.3.5 and 4.3.6) suggesting no interaction with BCRP.

The structure of the membrane-bound ATP-binding cassette (ABC) family of transporters reveals that they consist of trans-membrane domains and cytosolic domains. The substrate-binding sites of these transporters are usually located on the trans-membrane domains and substrates gain access to the binding site from the membrane-cytosolic interface [31]. It can be contended that in these experiments the charged sulfates would not be able to permeate the cells and thus would not gain access to the transporter-binding site. BeWo cells, however, have been reported to take up organic anions via temperature-sensitive mechanisms indicating carrier-mediated transport [27]. Although 4-nitrophenyl sulfate or acetaminophen sulfate uptake has not been demonstrated in BeWo cells, both sulfates are taken up by carrier-mediated processes in isolated hepatocytes [32, 33]. BeWo cells produce 4-nitrophenyl sulfate upon incubation with 4-nitrophenol (Figure 4.3.10). To assess if intracellularly-generated 4-nitrophenyl sulfate affected the accumulation of mitoxantrone in a different manner, BeWo cell monolayers were incubated with 4-nitrophenol (0.5-200 μM). We found that under these conditions the accumulation of mitoxantrone decreased by 10-30%. As this was not dose dependent, it indicated that intracellularly generated 4-nitrophenyl sulfate did not interact with BCRP and implied a minor role of BCRP in sulfate elimination.

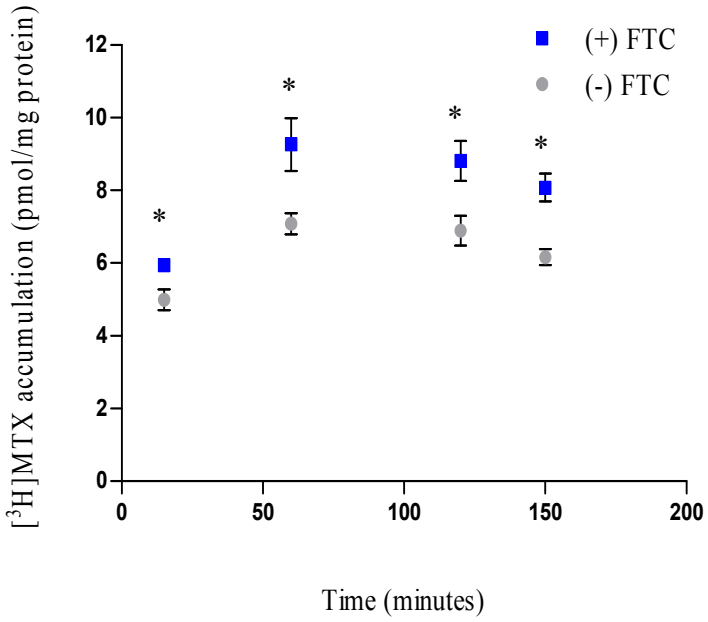


Figure 4.3.1 Time-dependent accumulation of mitoxantrone (MTX) in BeWo cells. Accumulation of 20 nM [^{3}H]-mitoxantrone (4 Ci/mmol) was measured at 37ºC as a function of time, in the absence or presence of the BCRP-specific inhibitor fumitremorgin C (FTC). Each experiment was repeated in triplicate with each point and bar in an individual experiment representing the mean ± S.E.M. of triplicate measurements. * indicates $p < 0.05$.

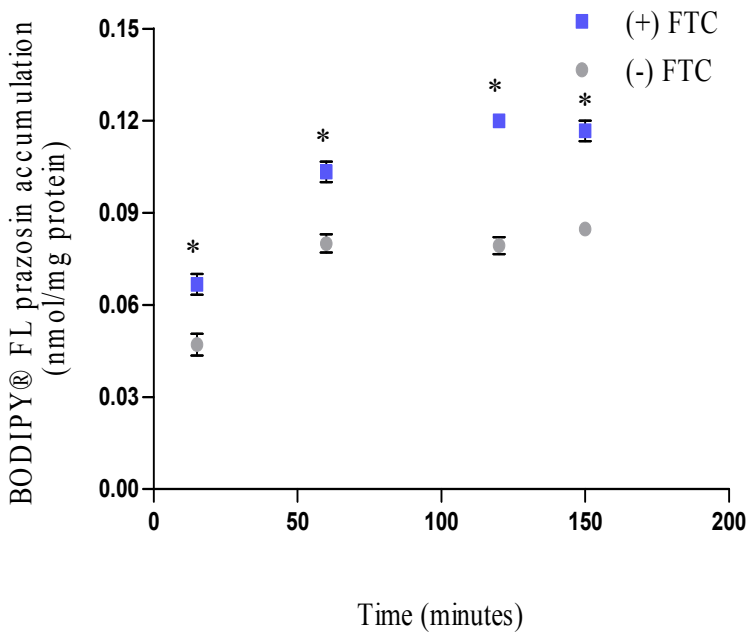


Figure 4.3.2 Time-dependent accumulation of BODIPY® FL prazosin in BeWo cells. Accumulation of 500 nM BODIPY® FL prazosin was measured at 37ºC as a function of time, in the absence or presence of the BCRP-specific inhibitor fumitremorgin C (FTC). Each experiment was repeated in triplicate with each point and bar in an individual experiment representing the mean ± S.E.M. of triplicate measurements. * indicates $p < 0.05$.

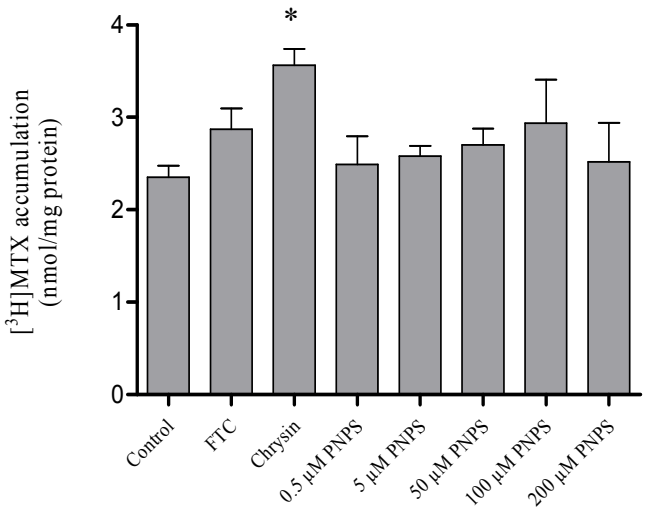


Figure 4.3.3 Effect of 4-nitrophenyl sulfate (PNPS) on the accumulation of mitoxantrone (MTX). Accumulation of 10 μM mitoxantrone (specific activity 0.02 Ci/mmol) was measured in the presence of 4-nitrophenyl sulfate (0.5-200 μM) at 37ºC for 150 minutes. Known BCRP inhibitors fumitremorgin C (FTC) and chrysin, at 5 μM and 100 μM respectively, served as the positive controls. Control cells contained mitoxantrone alone. Each data point in an individual experiment represents quadruplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

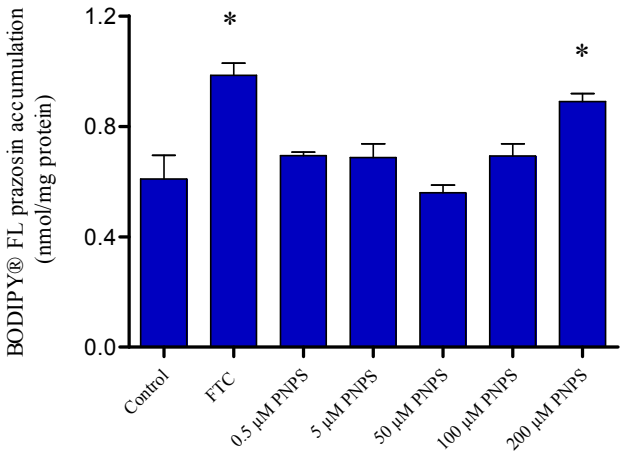


Figure 4.3.4 Effect of 4-nitrophenyl sulfate (PNPS) on the accumulation of BODIPY® FL prazosin. Accumulation of 500 nM BODIPY® FL prazosin was measured in the presence of 4-nitrophenyl sulfate (0.5-200 µM) at 37ºC for 150 minutes. The BCRP inhibitor fumitremorgin C (FTC) at 5 µM served as the positive control. Control cells contained BODIPY® FL prazosin alone. Each data point in an individual experiment represents quadruplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

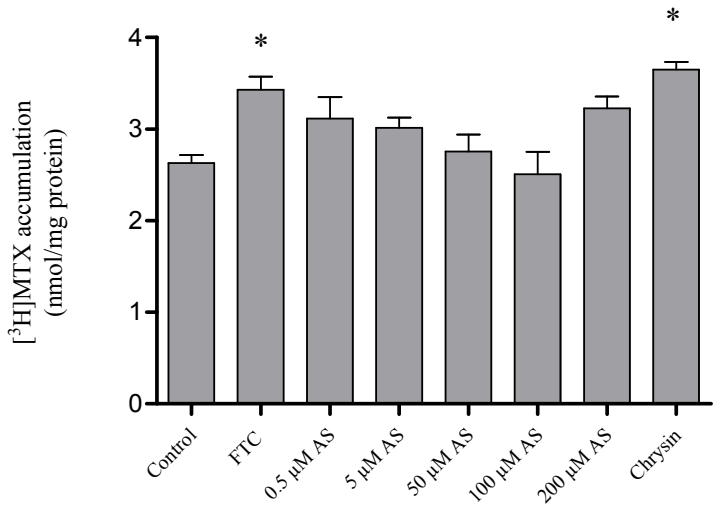


Figure 4.3.5 Effect of acetaminophen sulfate (AS) on the accumulation of mitoxantrone (MTX) in BeWo cell monolayers. Accumulation of 10 µM mitoxantrone (specific activity 0.02 Ci/mmol) was measured in the presence of acetaminophen sulfate (0.5-200 µM) at 37ºC for 150 minutes. Known BCRP inhibitors fumitremorgin C (FTC) and chrysin, at 5 µM and 100 µM respectively, served as the positive controls. Control cells contained mitoxantrone alone. Each data point in an individual experiment represents quadruplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

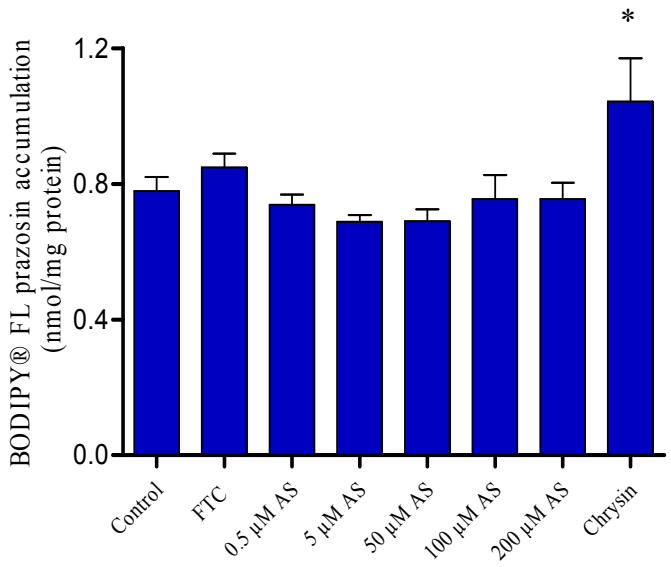


Fig. 4.6 Effect of acetaminophen sulfate (AS) on the accumulation of BODIPY® FL prazosin in BeWo cells. Accumulation of 500 nM BODIPY® FL prazosin was measured in the presence of acetaminophen sulfate (0.5-200 µM) at 37ºC for 150 minutes. Known BCRP inhibitors, fumitremorgin C (FTC) and chrysin, at 5 µM and 100 µM respectively, served as the positive controls. Control cells contained BODIPY® FL prazosin alone. Each data point in an individual experiment represents quadruplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

4.3.2 Transport of 4-nitrophenyl sulfate

Substrates of cellular efflux pumps exhibit asymmetrical apparent permeability coefficients in the apical to basolateral, and basolateral to apical directions. For e.g. a substance exhibiting a PDR of 2 or greater can be considered to be a substrate of an apically-located efflux transporter. The permeability of 0.5, 1 or 5 µM 4-nitrophenyl sulfate was determined. There was no change in the apparent permeability coefficients of 4-nitrophenyl sulfate with change in concentration and the PDR in all

cases was approximately 0.75-1 (Figure 4.3.7). This was not due to 4-nitrophenyl sulfate affecting BeWo monolayer integrity because $P_{app,\ A\text{-}B}$ and $P_{app,\ B\text{-}A}$ of the paracellular marker fluorescein in the presence of 4-nitrophenyl sulfate were similar

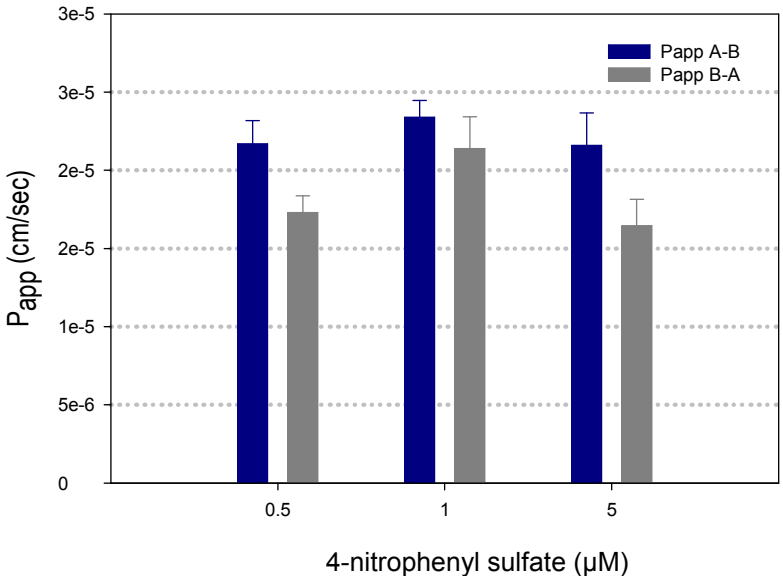


Figure 4.3.7 Bidirectional transport of 4-nitrophenyl sulfate in BeWo cells. The apparent permeability coefficient (P_{app}) of 4-nitrophenyl sulfate was determined by incubating BeWo monolayers with 4-nitrophenyl sulfate (0.5, 1, and 5 µM) at 37ºC for a total period of 45 minutes. P_{app} was determined in the apical to basolateral (A-B), as well as, basolateral to apical (B-A) directions. Each data point in an individual experiment represents triplicate measurements. Data has been plotted as mean ± S.D.

to the values obtained when fluorescein was run alone (data not shown). The PDR ~ 1 indicated passive diffusion to be the main process involved in the transport of 4-nitrophenyl sulfate. Under the conditions utilized, BeWo cells were somewhat leakier paracellularly compared to other epithelial cell monolayers. $P_{app,\ A\text{-}B}$ of the paracellular permeability marker Lucifer yellow in the apical to basolateral direction was determined to be ~ 10 x 10^{-6} cm/sec. This is more than 10-fold higher than what

is considered acceptable for Caco-2 monolayers. It is possible that under such conditions the contributions of passive transport were much higher than that of active transport, and this generated an efflux ratio ~ 1. Bidirectional transport of acetaminophen sulfate was not determined for the same reason.

4.3.3 Excretion of 4-nitrophenyl sulfate upon 4-nitrophenol incubation

BeWo monolayers were incubated with 4-nitrophenol in either the apical or basolateral chambers. The appearance of 4-nitrophenol and 4-nitrophenyl sulfate, were monitored in the receiver chambers. At all concentrations (0.5-50 μM), the PDR of 4-nitrophenol was between 0.7-1 (table 4.1). Neither $P_{app, A-B}$, nor $P_{app, B-A,}$ of 4-nitrophenol underwent saturation with increasing concentrations indicating passive diffusion to be the main route of elimination across both apical as well as basolateral membranes (Table 4.1).

4-nitrophenyl sulfate appeared in both donor and receiver chambers. The amount of sulfate appearing in the receiver chamber increased linearly with time for both basolateral as well as apical elimination (data not shown). The excretion rates of 4-nitrophenyl sulfate (Figure 4.3.8 and table 4.1) across the apical and basolateral membranes did not change as a function of 4-nitrophenol concentration indicating that in the utilized concentration range either metabolism or transport had already been saturated. At all concentrations the excretion rates across both membranes were almost equal. This suggested that the transporter(s) on both membranes eliminate 4-

nitrophenyl sulfate with equal efficiency. Analytical issues prevented examination of apical or basolateral elimination of acetaminophen sulfate under similar conditions.

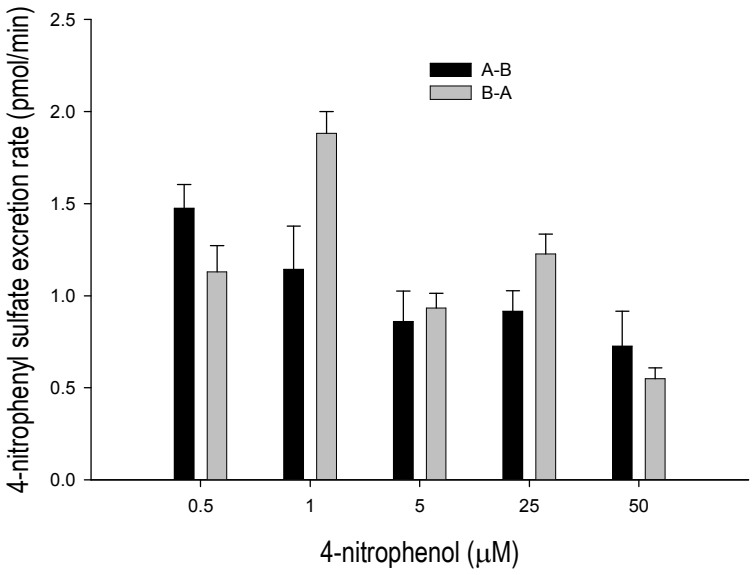


Figure 4.3.8 Excretion of 4-nitrophenyl sulfate as a function of 4-nitrophenol dosing concentration. BeWo cell monolayers were grown on transwells. The apical or basolateral chambers were treated with 4-nitrophenol at 37ºC for 45 minutes. The excretion rate of 4-nitrophenyl sulfate across the basolateral (black bars) and apical (gray bars) membranes was estimated after apical or basolateral exposures, respectively, of 4-nitrophenol. Each data point in an individual experiment represents triplicate measurements. Data has been plotted as mean ± S.D. A-B denotes apical to basolateral transport while B-A denotes basolateral to apical transport.

Table 4.1 Apparent permeability of 4-nitrophenol and excretion rates of 4-nitrophenyl sulfate. BeWo monolayers grown on transwells were treated with 4-nitrophenol in either the apical or basolateral chambers, at 37ºC for 45 minutes. The receiver chamber was periodically sampled for 4-nitrophenol and 4-itrophenylsulfate to determine the apparent permeability coefficient (P_{app}) of 4-nitrophenol, and the excretion rates of 4-nitrophenyl sulfate. Data has been plotted as mean ± S.D (n=3). A-B denotes apical to basolateral transport while B-A denotes basolateral to apical transport.

4-nitrophenol (µM)	4-nitrophenol			4-nitrophenyl sulfate	
	P_{app} x 10^6 (cm/sec)		PDR	Excretion rate (pmol/min)	
	A-B	B-A		A-B	B-A
0.5	34.5±5.07	24.1±2.10	0.70±0.12	1.48±0.13	1.13±0.14
1	33.4±3.19	34.0±2.44	1.02±0.12	1.14±0.24	1.88±0.12
5	46.0±5.74	37.1±4.99	0.81±0.15	0.86±0.16	0.93±0.08
25	33.4±3.19	34.0±2.44	1.02±0.12	0.92±0.11	1.23±0.11
50	21.9±5.25	20.5±1.84	0.94±0.24	0.73±0.19	0.55±0.06

4.3.4 Efflux of the sulfate metabolites across the apical membrane

To determine efflux across the apical membrane, cells were plated onto 12-well uptake plates and at confluence were incubated with 4-nitrophenol (1 µM, 10 µM, or 25 µM) or acetaminophen (10 µM, 50 µM, or 250 µM). The transport medium was analyzed for 4-nitrophenyl sulfate or acetaminophen sulfate. At all concentrations of 4-nitrophenol, the concentrations of 4-nitrophenyl sulfate determined in the transport medium increased linearly with time up to an incubation period of 4 hours (data not shown). Similarly, at all concentrations of acetaminophen, acetaminophen sulfate in the transport medium increased linearly as a function of time (Figure 4.3.9). An

incubation time of 2 hours was selected for future experiments with 4-nitrophenol (1 μM). An incubation period of 4 hours was selected for acetaminophen (250 μM) for improved detection (this has been discussed in section 4.4).

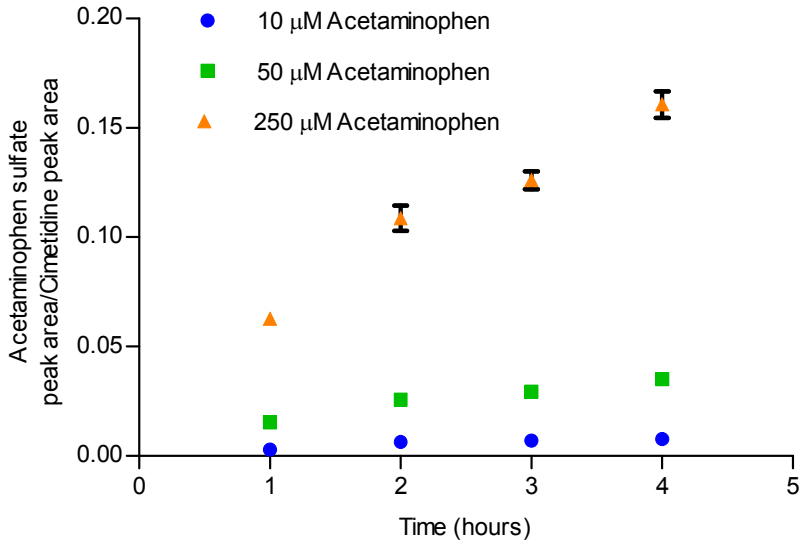


Figure 4.3.9 Appearance of intracellularly generated acetaminophen sulfate in the incubation medium as a function of time. BeWo cell monolayers grown on 12-well plates were incubated with 10, 50, or 250 μM acetaminophen at 37ºC for 1, 2, 3, or 4 hours. Following this, the incubation medium was withdrawn and analyzed for acetaminophen sulfate. Each data point in an individual experiment represents triplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

Efflux of acetaminophen sulfate across the apical membrane underwent a dose-dependent decrease in the presence of the MRP inhibitors MK-571 and indomethacin (Figure 4.3.10). Another MRP inhibitor leukotriene C4 (LTC4) did not produce any effect. 5 μM of the BCRP-specific inhibitor FTC also produced a significant decrease

in efflux; the amount of acetaminophen sulfate effluxed being ~77% of vehicle-treated controls. Much lesser amounts were eliminated at the highest concentrations of MK-571 (~26%) and indomethacin (~58%). Maximum inhibition was produced by 200 μM chrysin (~8%). Similar results were obtained with the apical efflux of 4-nitrophenyl sulfate (Figure 4.3.11). The percentage of 4-nitrophenyl sulfate detected in the transport medium was ~20% in the presence of 25 μM MK-571, ~70% in the presence of 100 μM indomethacin, ~85% in the presence of 5 μM FTC and undetectable in the presence of chrysin. Leukotriene C4 did not produce any effect. All the above values denote the percentage of the sulfate metabolite detected in the transport medium in the presence of inhibitors with respect to the vehicle-treated controls.

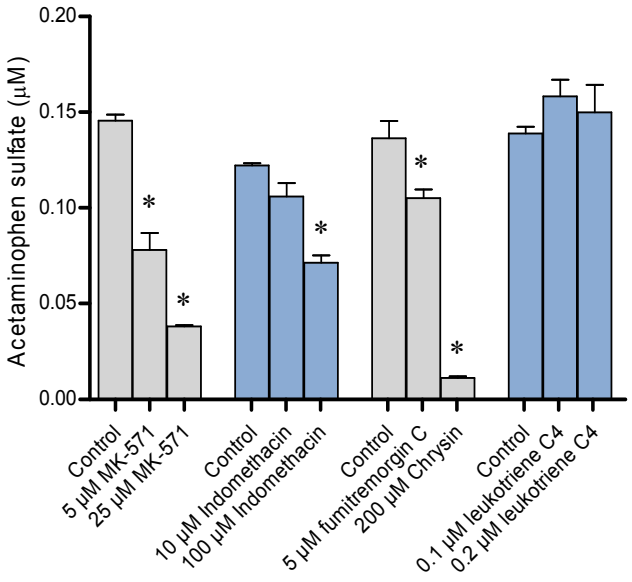


Figure 4.3.10 Effect of transporter inhibitors on the efflux of acetaminophen sulfate from the apical membrane of BeWo cells. BeWo monolayers grown on 12-well plates were incubated with 250 μM acetaminophen at 37ºC for 4 hours, in the presence of MRP inhibitors (MK-571, indomethacin, and leukotriene C4) or BCRP inhibitors (fumitremorgin C and chrysin). Control cells contained the vehicle alone. At the end of 4 hours, the incubation medium was analyzed for acetaminophen sulfate. Each data point in an individual experiment represents triplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$

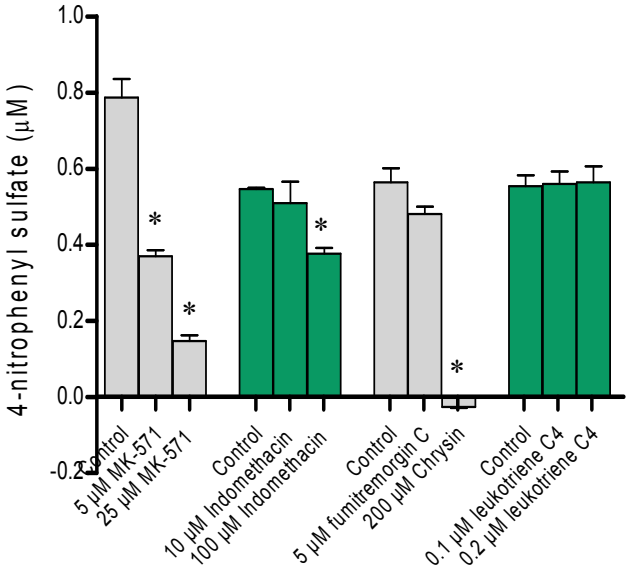


Figure 4.3.11 Effect of transporter inhibitors on the efflux of 4-nitrophenyl sulfate from the apical membrane of BeWo cells. BeWo monolayers grown on 12-well plates were incubated with 1 µM 4-nitrophenol at 37ºC for 2 hours, in the presence of MRP inhibitors (MK-571, indomethacin, and leukotriene C4) or BCRP inhibitors (fumitremorgin C and chrysin). At the end of 2 hours, the incubation medium was analyzed for 4-nitrophenyl sulfate. Each data point in an individual experiment represents triplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

4.3.5 Inhibition of 4-nitrophenyl sulfate elimination across the basolateral membrane

To investigate the transporters involved in the elimination of 4-nitrophenyl sulfate across the basolateral membrane, the apical to basolateral transport of 50 µM 4-nitrophenol was determined in the presence of various inhibitors. Both MRP inhibitors MK-571 (25 µM) and LTC4 (0.2 µM) inhibited the excretion rate of 4-nitrophenyl sulfate across the basolateral membrane by approximately 50% (Figure

4.3.12). 5 μM FTC decreased the excretion rate of 4-nitrophenyl sulfate to the same extent as MK-571 and leukotriene C4, but this was not statistically significant.

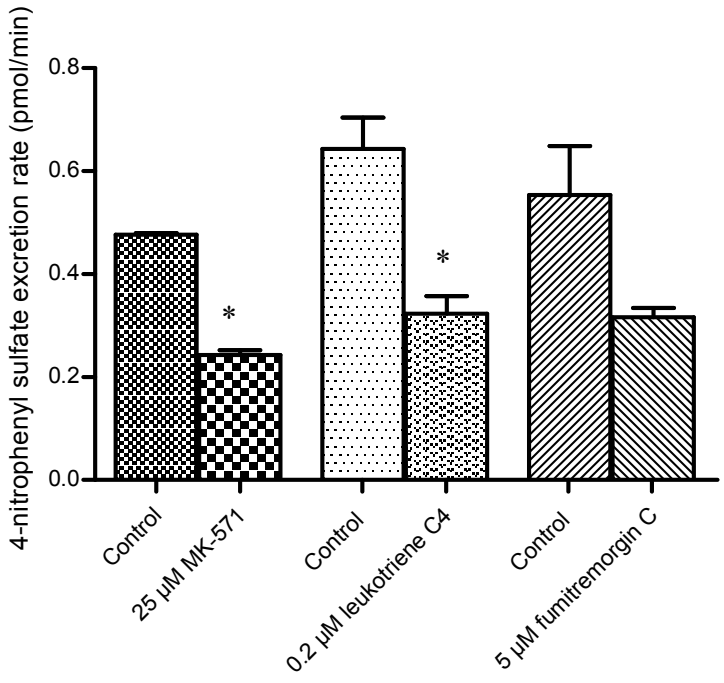


Figure 4.3.12 Effect of transporter inhibitors on the elimination of 4-nitrophenyl sulfate across the basolateral membrane of BeWo cells. The apical chambers of BeWo monolayers grown on transwells were incubated with 50 μM 4-nitrophenol at 37ºC for 45 minutes, in the absence and presence of the different inhibitors. MK-571 and leukotriene C4 are MRP inhibitors whereas fumitremorgin C is a BCRP inhibitor. The excretion rate of 4-nitrophenyl sulfate across the basolateral membrane was obtained by determining the concentrations of 4-nitrophenyl sulfate in the basolateral chamber as a function of time. Each data point in an individual experiment represents triplicate measurements. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

4.3.6 Effect of inhibitors on sulfate metabolite formation in BeWo cell lysate

To determine if the inhibitors used in the preceding section selectively affected transport, their effect on sulfate conjugation was determined. Only inhibitor

concentrations that had produced a statistically significant change in the efflux or transport studies (sections 4.3.4 and 4.3.5) were used. For 1 μM 4-nitrophenol, in the presence of 5 μM MK-571, 25 μM MK-571, 100 μM indomethacin, 200 μM chrysin,

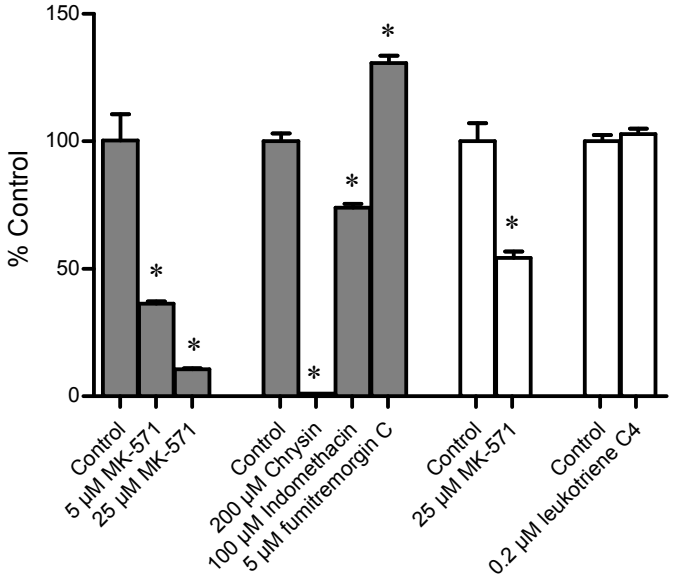


Figure 4.3.13 Effect of inhibitors on the formation of 4-nitrophenyl sulfate in BeWo cells. BeWo cytosolic homogenate was incubated with 1μM or 50μM 4-nitrophenol and 100μM 3'-phosphoadenosine-5'-phosphosulfate (PAPS) for 2 hours at 37ºC in the absence and presence of different efflux transporter inhibitors. The reaction mixture was then analyzed for 4-nitrophenyl sulfate. 4-nitrophenyl sulfate formed in the presence of inhibitors was normalized to the amount produced in the vehicle-treated controls. Solid and open bars indicate that lysate was incubated with 1μM and 50μM 4-nitrophenol respectively. Each data point in an individual experiment represents triplicate measurements and data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

and 5μM FTC, the amount of 4-nitrophenyl sulfate produced was 36%, 11%, 1%, 74%, and 131% of the vehicle-treated controls respectively (Figure 4.3.13). The changes in all these cases were statistically significant. For 50 μM 4-nitrophenol, in

the presence of 25 μM MK-571, 54% of 4-nitrophenyl sulfate was produced. Leukotriene C4 did not produce any change in the amount of 4-nitrophenyl sulfate produced. In contrast, none of the inhibitors changed the formation of acetaminophen sulfate (Figure 4.3.14).

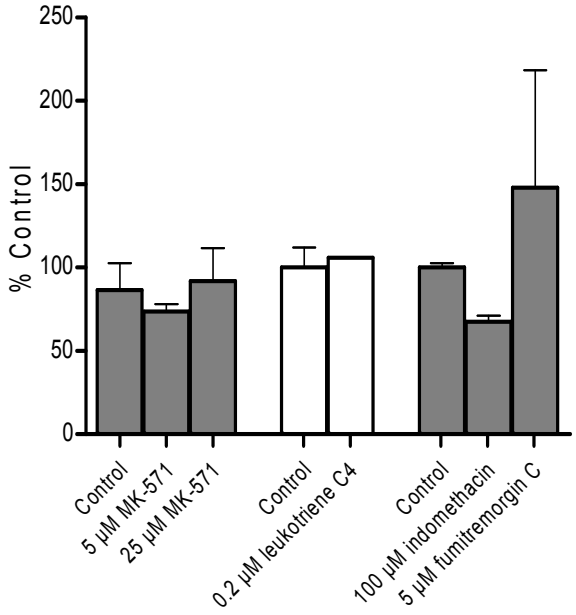


Figure 4.3.14 Effect of inhibitors on the formation of acetaminophen sulfate in BeWo cells. BeWo cytosolic homogenate was incubated with 250 μM acetaminophen and 100 μM 3'-phosphoadenosine-5'-phosphosulfate (PAPS) for 4 hours at 37ºC in the absence and presence of different efflux transporter inhibitors. The reaction mixture was then analyzed for acetaminophen sulfate. Acetaminophen sulfate formed in the presence of inhibitors was normalized to the amount produced in the vehicle-treated controls. Data has been plotted as mean ± S.E.M. * indicates $p < 0.05$.

4.4 Discussions and conclusions

Low oral bioavailability of several drugs has been attributed to extensive intestinal metabolism and excretion of the metabolites back into the intestinal lumen. The

multidrug resistance-associated proteins (MRPs) and the organic anion transporters (OATs) have been reported to be the major transporters responsible for the elimination of sulfate metabolites out of Caco-2 cells. Excretion of the sulfate and glucuronide conjugates of several isoflavones as well as some pharmaceutical drugs were all inhibited to some extent at least by MRP inhibitors MK-571, leukotriene C4, and the OAT inhibitor estrone sulfate in Caco-2 [23, 25, 26]. Interestingly none of these earlier studies examined the involvement of BCRP in the efflux of the sulfate and glucuronide conjugates. In a more recent study, BCRP, but not MRP, significantly decreased apical sulfate metabolite efflux in Caco-2 [34]. This suggests that one of the roles of BCRP in the intestine may be the elimination of sulfate conjugates. This agrees well with reports of BCRP predominantly mediating the biliary excretion of xenobiotic sulfate conjugates in mice [3]. There are tissue-specific and species-specific differences with respect to the relative contributions of the transporters. In contrast to mice, in rats MRP2 primarily mediated the biliary efflux of xenobiotic sulfate conjugates [16]. While BCRP was a major contributor in the biliary efflux of troglitazone sulfate in mice, it only played a minor role in the intestinal mucosal efflux of this sulfate [17]. This differential effect makes it more important to determine which transporters are responsible for conjugate efflux in placenta.

In the accumulation studies acetaminophen sulfate did not affect the accumulation of either BODIPY® FL prazosin or mitoxantrone suggesting that acetaminophen sulfate does not interact with BCRP. 4-nitrophenyl sulfate did not alter the accumulation of

mitoxantrone or BODIPY® FL prazosin, except at 200 μM where it increased BODIPY® FL prazosin accumulation. Hence, 4-nitrophenyl sulfate can be concluded to be an inhibitor of BCRP only at high concentrations. Although not as effective, this is in agreement with the study of Suzuki et al. [35] who showed that 4-nitrophenyl sulfate inhibited BCRP-mediated transport of estrone sulfate in membrane vesicles obtained from mouse lymphoma P388 cells (IC_{50} = 53 μM). The extent to which 4-nitrophenyl sulfate or acetaminophen sulfate increased accumulation was compared with the effects produced by FTC and chrysin. While FTC is a BCRP-specific inhibitor [36, 37], chrysin inhibits BCRP, MRP1, and P-gp [38]. In all experiments the increase in accumulation produced in the presence of chrysin was much higher than that produced by FTC. A possible reason for this could be that BODIPY® FL prazosin and mitoxantrone are being effluxed out by transporters in addition to BCRP. Though BODIPY® FL prazosin and mitoxantrone were used at concentrations typically used to test for BCRP, they are not BCRP-specific substrates. BODIPY® FL prazosin is a substrate of P-gp also [39]; while mitoxantrone is known to be a substrate of MRP1 and P-gp as well [40]. Therefore, we can conclude that in the tested concentration range, acetaminophen sulfate and 4-nitrophenyl sulfate do not interact with BCRP, P-gp, or MRP1. This suggested a minor role of BCRP and MRP1 in apical sulfate elimination.

Bidirectional transport studies of 4-nitrophenyl sulfate did not reveal if it was preferentially eliminated across the apical or basolateral membranes. To avoid any

contributions of paracellular permeability the cells were incubated with 4-nitrophenol and the appearance of intracellularly generated 4-nitrophenyl sulfate was monitored. Further, the excretion rates of 4-nitrophenyl sulfate across either membrane were about the same at all concentrations. These findings indicated that the transporter(s) on both membranes efflux out 4-nitrophenyl sulfate at similar rates. In our hands BeWo monolayer integrity was compromised (i.e. the monolayers lifted off the transwell membranes) at incubation times longer than 60 minutes. Acetaminophen sulfate was not detectable in the transport medium at 60 minutes or less, and hence we were unable to determine the excretion pattern of acetaminophen sulfate across the apical and basolateral membranes.

Efflux assays conducted with BeWo monolayers grown on multiwell plates, allowed for longer incubation times. Upon incubation with 4-nitrophenol or acetaminophen, the amounts of 4-nitrophenyl sulfate and acetaminophen sulfate detected in the transport medium increased linearly as a function of time. Hence, longer incubation times markedly improved the detection of acetaminophen sulfate. Since we monitored the transport medium for the sulfate metabolite, this gave us information on sulfate elimination from the apical trophoblast membrane. Results from the efflux assay indicated that one or more of the MRP proteins are primarily responsible for apical elimination of 4-nitrophenyl sulfate and acetaminophen sulfate with a lesser contribution from BCRP. The MRP inhibitors utilized were not specific to a particular isoform and all the MRP isoforms expressed in BeWo cells (i.e., MRP 1, 2,

and 5), are expressed on the apical syncytiotrophoblast membrane to some extent at least [12, 13, 15]. Further, as studies have reported that MRP2 is either weakly expressed or not expressed in BeWo cells [18-20], it would be reasonable to hypothesize that MRP1 or MRP5 primarily mediate the apical elimination of 4-nitrophenyl sulfate and acetaminophen sulfate. However, the involvement of any MRP isoform in the excretion of 4-nitrophenyl sulfate is complicated by the fact that both MK-571 and indomethacin decreased the formation of 4-nitrophenyl sulfate as well. 100 μM indomethacin inhibited metabolism and apical efflux to approximately the same extent. In the presence of 5 μM and 25 μM MK-571 respectively, only 36% and 11% of PNPS was formed with respect to the vehicle-treated controls, while the amount effluxed were 47% and 19% of the controls respectively. Thus with the inhibitors used, no conclusion could be reached about the role of MRP isoforms in the apical efflux of 4-nitrophenyl sulfate.

The excretion of 4-nitrophenyl sulfate across the basolateral membrane was also significantly decreased in the presence of both MRP inhibitors MK-571 and leukotriene C4. In contrast to MK-571, leukotriene C4 affected only transport and not metabolism. Since out of the MRP isoforms detected in the basolateral syncytiotrophoblast membrane, MRP1 and MRP5 protein are expressed in BeWo cells, it would be reasonable to propose that either MRP1 or MRP5 mediate 4-nitrophenyl sulfate excretion across the basolateral trophoblast membrane. Although FTC did not produce a statistically significant change in 4-nitrophenyl sulfate

elimination across the basolateral membrane, it reduced the excretion rate to almost the same extent as the MRP inhibitors. BCRP is localized to syncytiotrophoblast apical membrane with faint signals observed in basal membrane fractions [5, 11]. It is likely that BCRP inhibition drives 4-nitrophenyl sulfate excretion across a basolateral membrane transporter as has been reported with hesperitin sulfate elimination in Caco-2 [34].

In several cases MK-571 at similar concentrations (as used in this study) or higher has been shown to inhibit sulfate efflux [23, 25, 41, 42]. In many of these cases the effect of the inhibitor on metabolism was not determined. While it has been shown that MK-571 did not affect apigenin sulfation up to a concentration of 100 μM, in this study the metabolism experiment was performed for 30 minutes whereas the intact cell efflux experiments were performed for 2 hours [23]. Our work shows for the first time that MK-571 affected formation and efflux of 4-nitrophenyl sulfate to almost the same extents. This underscores the importance of performing metabolic studies along with transport studies when determining the effect of inhibitors on metabolite efflux.

In mice BCRP plays a major role in the biliary efflux of acetaminophen sulfate with a minor contribution from MRP2 [3]. Sinusoidal elimination of acetaminophen sulfate in mice is mediated by MRP3 and MRP4 [43]. In rat biliary excretion is mediated primarily by MRP2 with a small contribution from BCRP [3, 16]. Our study indicates a major contribution of MRP1/MRP5 in the apical efflux of acetaminophen sulfate

with a minor contribution from BCRP. Compared to acetaminophen sulfate, much less is known about which transporters are responsible for 4-nitrophenyl sulfate efflux in other tissues or even species. In rat liver 4-nitrophenyl sulfate undergoes biliary as well as sinusoidal excretion but the transporters responsible for it's elimination have not been determined [44]. MRP3 and MRP4 on the hepatic basolateral membrane, and MRP2 and BCRP on the hepatic canalicular membrane, play major roles in the elimination of sulfate metabolites [6]. Our data indicates that like acetaminophen sulfate, a portion of the apical efflux of 4-nitrophenyl sulfate is mediated by BCRP while its basolateral excretion is mediated by either MRP1 or MRP5.

In conclusion, we can say that BCRP is responsible for a small portion of the efflux of 4-nitrophenyl sulfate from the apical membrane of BeWo cells, while either MRP1 or MRP5 mediate its basolateral elimination. Apical elimination of acetaminophen sulfate is mediated most likely, primarily by MRP1/ MRP5 with a minor contribution by BCRP. Our study suggests that another role of placental efflux transporters is elimination of xenobiotic sulfate conjugates. None of the inhibitors used in this study completely inhibited the efflux of either acetaminophen sulfate or 4-nitrophenyl sulfate. This suggests the involvement of other transporters such as the organic anion transporters (OATs) or the organic anion transporting polypeptides (OATPs). OAT isoforms partly mediate the intestinal efflux of sulfates of xenobiotics such as raloxifene and apigenin [23, 25]. Similarly several OATP isoforms transport estrone sulfate and dehydroepiandrosterone sulfate [45]. In placenta OATP-E/4A1 and

OATP-B/2B1 are localized on apical and basolateral trophoblast membranes respectively, while OAT4 is localized on the basolateral trophoblast membrane [19, 46]. A physiological function of OAT4 in the placenta is the uptake of sulfoconjugated steroids from the fetus for estrogen synthesis in the syncytiotrophoblast [46], while both OAT4 and OATP2B1 mediate the uptake of sulfated estrogens from the fetal circulation [46]. Future studies should examine if additional roles of placental organic anion transporters and organic anion transporting polypeptides include sulfate elimination out of the placenta. Further, BeWo cells do not express MRP3 or MRP4, which may also contribute to sulfate elimination in the placenta.

4.5 References

1. Sodha, R.J. and H. Schneider, *Sulphate conjugation of beta 2-adrenoceptor stimulating drugs by platelet and placental phenol sulphotransferase.* Br J Clin Pharmacol, 1984. **17**(1): p. 106-8.
2. Stanley, E.L., et al., *Differential expression of sulfotransferase enzymes involved in thyroid hormone metabolism during human placental development.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5944-55.
3. Zamek-Gliszczynski, M.J., et al., *The important role of Bcrp (Abcg2) in the biliary excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in mice.* Mol Pharmacol, 2006. **70**(6): p. 2127-33.
4. Adachi, Y., et al., *Role of breast cancer resistance protein (Bcrp1/Abcg2) in the extrusion of glucuronide and sulfate conjugates from enterocytes to intestinal lumen.* Mol Pharmacol, 2005. **67**(3): p. 923-8.
5. Grube, M., et al., *Organic anion transporting polypeptide 2B1 and breast cancer resistance protein interact in the transepithelial transport of steroid sulfates in human placenta.* Drug Metab Dispos, 2007. **35**(1): p. 30-5.
6. Zamek-Gliszczynski, M.J., et al., *Integration of hepatic drug transporters and phase II metabolizing enzymes: mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites.* Eur J Pharm Sci, 2006. **27**(5): p. 447-86.
7. Imai, Y., et al., *Breast cancer resistance protein exports sulfated estrogens but not free estrogens.* Mol Pharmacol, 2003. **64**(3): p. 610-8.
8. Deeley, R.G. and S.P. Cole, *Substrate recognition and transport by multidrug resistance protein 1 (ABCC1).* FEBS Lett, 2006. **580**(4): p. 1103-11.
9. Maliepaard, M., et al., *Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues.* Cancer Res, 2001. **61**(8): p. 3458-64.
10. Ceckova, M., et al., *Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo.* Clin Exp Pharmacol Physiol, 2006. **33**(1-2): p. 58-65.
11. Evseenko, D.A., et al., *The ABC transporter BCRP/ABCG2 is a placental survival factor, and its expression is reduced in idiopathic human fetal growth restriction.* FASEB J, 2007. **21**(13): p. 3592-605.
12. St-Pierre, M.V., et al., *Expression of members of the multidrug resistance protein family in human term placenta.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(4): p. R1495-503.
13. Azzaroli, F., et al., *Clinical trial: modulation of human placental multidrug resistance proteins in cholestasis of pregnancy by ursodeoxycholic acid.* Aliment Pharmacol Ther, 2007. **26**(8): p. 1139-46.

14. Atkinson, D.E., et al., *Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer.* Am J Physiol Cell Physiol, 2003. **285**(3): p. C584-91.
15. Meyer Zu Schwabedissen, H.E., et al., *Expression, localization, and function of MRP5 (ABCC5), a transporter for cyclic nucleotides, in human placenta and cultured human trophoblasts: effects of gestational age and cellular differentiation.* Am J Pathol, 2005. **166**(1): p. 39-48.
16. Zamek-Gliszczynski, M.J., et al., *Multiple mechanisms are involved in the biliary excretion of acetaminophen sulfate in the rat: role of Mrp2 and Bcrp1.* Drug Metab Dispos, 2005. **33**(8): p. 1158-65.
17. Enokizono, J., H. Kusuhara, and Y. Sugiyama, *Involvement of breast cancer resistance protein (BCRP/ABCG2) in the biliary excretion and intestinal efflux of troglitazone sulfate, the major metabolite of troglitazone with a cholestatic effect.* Drug Metab Dispos, 2007. **35**(2): p. 209-14.
18. Evseenko, D.A., J.W. Paxton, and J.A. Keelan, *ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast.* Am J Physiol Regul Integr Comp Physiol, 2006. **290**(5): p. R1357-65.
19. Young, A.M., *Characterization of efflux transporters of the human trophoblast using BeWo as a model*, in *Pharmaceutical Chemistry*. 2005, University of Kansas: Lawrence.
20. Pascolo, L., et al., *Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells.* Biochem Biophys Res Commun, 2003. **303**(1): p. 259-65.
21. Liu, F., M.J. Soares, and K.L. Audus, *Permeability properties of monolayers of the human trophoblast cell line BeWo.* Am J Physiol, 1997. **273**(5 Pt 1): p. C1596-604.
22. Bode, C.J., et al., *In vitro models for studying trophoblast transcellular transport.* Methods Mol Med, 2006. **122**: p. 225-39.
23. Hu, M., J. Chen, and H. Lin, *Metabolism of flavonoids via enteric recycling: mechanistic studies of disposition of apigenin in the Caco-2 cell culture model.* J Pharmacol Exp Ther, 2003. **307**(1): p. 314-21.
24. Young, A.M., et al., *Tetrazole compounds: the effect of structure and pH on Caco-2 cell permeability.* J Pharm Sci, 2006. **95**(4): p. 717-25.
25. Jeong, E.J., H. Lin, and M. Hu, *Disposition mechanisms of raloxifene in the human intestinal Caco-2 model.* J Pharmacol Exp Ther, 2004. **310**(1): p. 376-85.
26. Chen, J., H. Lin, and M. Hu, *Absorption and metabolism of genistein and its five isoflavone analogs in the human intestinal Caco-2 model.* Cancer Chemother Pharmacol, 2005. **55**(2): p. 159-69.
27. Serrano, M.A., et al., *Expression in human trophoblast and choriocarcinoma cell lines, BeWo, Jeg-3 and JAr of genes involved in the hepatobiliary-like excretory function of the placenta.* Placenta, 2007. **28**(2-3): p. 107-17.

28. Friedman, S.J. and P. Skehan, *Morphological differentiation of human choriocarcinoma cells induced by methotrexate.* Cancer Res, 1979. **39**(6 Pt 1): p. 1960-7.
29. Cerveny, L., et al., *Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents.* Epilepsia, 2006. **47**(3): p. 461-8.
30. Lee, G., et al., *Expression of the ATP-binding cassette membrane transporter, ABCG2, in human and rodent brain microvessel endothelial and glial cell culture systems.* Pharm Res, 2007. **24**(7): p. 1262-74.
31. Sharom, F.J., *Shedding light on drug transport: structure and function of the P-glycoprotein multidrug transporter (ABCB1).* Biochem Cell Biol, 2006. **84**(6): p. 979-92.
32. Sakuma-Sawada, N., et al., *Inhibition of the hepatic uptake of paracetamol sulphate by anionic compounds.* J Pharm Pharmacol, 1997. **49**(8): p. 743-6.
33. Sakuma-Sawada, N., et al., *Hepatic uptake of p-nitrophenyl sulfate by transporter that acetaminophen sulfate shares for uptake: sulfate moiety as a vector for metabolite transport.* Res Commun Mol Pathol Pharmacol, 1997. **97**(2): p. 131-8.
34. Brand, W., et al., *Metabolism and transport of the citrus flavonoid hesperetin in Caco-2 cell monolayers.* Drug Metab Dispos, 2008. **36**(9): p. 1794-802.
35. Suzuki, M., et al., *ABCG2 transports sulfated conjugates of steroids and xenobiotics.* J Biol Chem, 2003. **278**(25): p. 22644-9.
36. Rabindran, S.K., et al., *Reversal of a novel multidrug resistance mechanism in human colon carcinoma cells by fumitremorgin C.* Cancer Res, 1998. **58**(24): p. 5850-8.
37. Rabindran, S.K., et al., *Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein.* Cancer Res, 2000. **60**(1): p. 47-50.
38. Gyemant, N., et al., *In vitro search for synergy between flavonoids and epirubicin on multidrug-resistant cancer cells.* In Vivo, 2005. **19**(2): p. 367-74.
39. Kimchi-Sarfaty, C., J.J. Gribar, and M.M. Gottesman, *Functional characterization of coding polymorphisms in the human MDR1 gene using a vaccinia virus expression system.* Mol Pharmacol, 2002. **62**(1): p. 1-6.
40. Pawarode, A., et al., *Differential effects of the immunosuppressive agents cyclosporin A, tacrolimus and sirolimus on drug transport by multidrug resistance proteins.* Cancer Chemother Pharmacol, 2007. **60**(2): p. 179-88.
41. Zhang, L., et al., *Mechanistic study on the intestinal absorption and disposition of baicalein.* Eur J Pharm Sci, 2007. **31**(3-4): p. 221-31.
42. Walle, U.K., A. Galijatovic, and T. Walle, *Transport of the flavonoid chrysin and its conjugated metabolites by the human intestinal cell line Caco-2.* Biochem Pharmacol, 1999. **58**(3): p. 431-8.
43. Zamek-Gliszczynski, M.J., et al., *Evaluation of the role of multidrug resistance-associated protein (Mrp) 3 and Mrp4 in hepatic basolateral*

excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3-/- and Abcc4-/- mice. J Pharmacol Exp Ther, 2006. **319**(3): p. 1485-91.

44. Higaki, K., et al., *Pharmacokinetic analysis of factors determining elimination pathways for sulfate and glucuronide metabolites of xenobiotics II: Studies with isolated perfused rat liver.* Xenobiotica, 2003. **33**(11): p. 1097-108.
45. Konig, J., et al., *Pharmacogenomics of human OATP transporters.* Naunyn Schmiedebergs Arch Pharmacol, 2006. **372**(6): p. 432-43.
46. Ugele, B., A. Bahn, and M. Rex-Haffner, *Functional differences in steroid sulfate uptake of organic anion transporter 4 (OAT4) and organic anion transporting polypeptide 2B1 (OATP2B1) in human placenta.* J Steroid Biochem Mol Biol, 2008. **111**(1-2): p. 1-6.

Chapter 5: Summary and Future Directions

5.1 Research summary

Placental metabolism and transport have the potential of altering fetal exposure to drugs or natural substances in the maternal circulation. Sulfation of drugs has been detected in the placenta and several sulfotransferase enzymes have been identified in the syncytiotrophoblast layer. Among the efflux transporters known to transport sulfate conjugates, the breast cancer resistance protein (BCRP) and the multidrug-resistance associated proteins 1, 2, 3 and 4 are located on the brush border (maternal-facing) syncytiotrophoblast membrane, whereas MRP1 and MRP5 are located on the basal (fetal-facing) syncytiotrophoblast membrane. Not much is known though about the induction/inhibition of placental sulfotransferase enzymes, or if one of the functions of placental efflux transporters could be the elimination of sulfate metabolites. This work has addressed some of these questions.

In chapter 2 we established that the phenolic sulfotransferase isoforms, SULT1A1 and SULT1A3, are functional in BeWo cells and cytotrophoblast cells, while SULT1E1 and SULT2A1 are not functional in BeWo cells. The overall expression profile is in agreement with what has been reported before by Stanley et al., i.e., in placental fractions rich in syncytiotrophoblasts SULT1A1 and SULT1A3 activities were much higher than that of SULT1E1 [1]. Stanley et al. also reported high SULT2A1 activity in these fractions which is in contrast to our conclusion of SULT2A1 protein not being expressed in trophoblast [1]. A possible reason for the observed difference could be overlapping substrate specificity of different isoforms.

Enzyme activity assays as well as mRNA expression indicated that SULT1A1 was upregulated in BeWo cells whereas SULT1A3 was equivalently expressed in BeWo cells and the primary cytotrophoblast cells.

Chapter 3 examined the effect of bisphenol A on SULT1A1 activity upon acute and chronic exposure. Bisphenol A was an acute inhibitor of SULT1A1 at concentrations much higher than that detected in random human serum samples. Chronic bisphenol A exposure resulted in inhibition of SULT1A1 activity. A U-shaped non-monotonic dose response curve was observed. Maximum inhibition (i.e., activity was reduced to 60-70% of the activity observed in untreated cells) was observed at intermediate concentrations and the inhibition was at relevant concentrations. The nature of SULT1A1 inhibition indicated at least two mechanisms of regulation – one causing inhibition and the other induction of SULT1A1. The estrogen receptor (ER) β inhibitor ICI182780 did not restore SULT1A1 activities to control levels suggesting that the inhibitory effect of bisphenol A on SULT1A1 activity was not mediated via ERβ.

Chapter 4 examined the mechanisms of elimination of the sulfate metabolites of two common SULT1A1 substrates, 4-nitrophenol and acetaminophen. BCRP mediated a minor portion of the elimination of 4-nitrophenylsulfate across the apical membrane, while either MRP1 or MRP5 mediated almost half of its excretion across the basolateral membrane. No conclusion was reached about the involvement of

MRP1/MRP2/ MRP5 in the apical elimination of 4-nitrophenylsulfate as the utilized MRP inhibitors affected the formation of 4-nitrophenylsulfate to almost the same extent that they affected its efflux. The inhibitors, however, did not affect the formation of acetaminophen sulfate leading to the conclusion that MRP1/MRP2/ MRP5 are responsible for the major portion of the apical elimination of acetaminophen sulfate with a minor contribution from BCRP.

5.2 Overall conclusions

This work shows that the BeWo cell line can be used successfully to examine the roles of SULT1A1 and SULT1A3 in placental metabolism. Bisphenol A inhibited SULT1A1 activity upon chronic exposure. The maximal inhibition was ~ 30%. As the effect is moderate and sulfation is a co-factor limited but not an enzyme-limited reaction, it would be interesting to observe if this produces any changes in *in vivo* situations with respect to fetal exposure. This work also shows that another placental function of placental efflux transporters is elimination of sulfate metabolites.

Compared to other tissues, the placenta expresses high quantities of the steroid sulfatase enzyme (STS) whose physiological function in the placenta is the deconjugation of dehydroepiandrosterone sulfate (DHEAS) and 16-hydroxyl DHEAS taken up from the fetus for the *de novo* synthesis of estrogen in the syncytiotrophoblast [2]. It was proposed that OATP2B1 on the basolateral membrane and BCRP on the apical membrane function together to eliminate

dehydroepiandrosterone sulfate taken up from the fetal circulation [3]. In a subsequent study, it was mentioned that the high sulfatase concentrations in the syncytiotrophoblast makes this dual transport system an unlikely *in vivo* process [2]. The same reasoning can be extended against the role of trophoblast efflux transporters in the elimination of sulfate metabolites. However, compared to physiological substrates such as estrone sulfate and dehydroepiandrosterone sulfate, the steroid sulfatase enzyme exhibits a lower affinity for small molecules such as 4-nitrophenylsulfate (K_m = 400 μM for arylsulfatase C) [4], whereas SULT1A1 sulfates small molecules such as 4-nitrophenol at much lower concentrations (i.e., reported K_m values of SULT1A1 for 4-nitrophenol ~ 1 μM) [5, 6]. The differential affinity indicates that in spite of the high expression of steroid sulfatase in the placenta, it is quite possible that the sulfotransferase enzymes and the efflux transporters might act in concert to reduce fetal exposure to sulfate metabolites.

5.3 Future directions

The BeWo cell line expresses several enzymes and transporters that are functional in the whole placenta and this makes it a useful model to predict or estimate the extent of fetal exposure to substances in the maternal circulation. However, one of the disadvantages of trying to determine the degree to which a particular transporter or enzyme influences any process, is the lack of availability of specific substrates. P-gp, MRP1/2, and BCRP, all of which are expressed on the apical trophoblast membrane, recognize a broad range of substrates, and there can also be overlapping substrate

specificity. This could be a reason why in the presence of even a BCRP-specific inhibitor such as fumitremorgin C (FTC), only modest increases were observed in the accumulation of BODIPY® FL prazosin or mitoxantrone, both of which are BCRP substrates but are substrates of other transporters as well. A more conclusive approach towards determining the role of a particular transporter may be achieved in cells over-expressing these transporters. However, determining whether a particular transporter is involved in metabolite elimination or not, is further complicated due to the inhibitors affecting metabolism as well (i.e., as we have shown with the formation of 4-nitrophenylsulfate). Further, cells over-expressing a particular transporter would not help reach a conclusion on the relative contributions of the transporters in sulfate efflux in a particular tissue. One approach maybe to isolate the brush border and basolateral membranes, and then perform the study with sulfated standards in the presence of specific inhibitors of the efflux transporters.

Figure 5.1 depicts all the transporters that may potentially eliminate sulfate metabolites out of syncytiotrophoblasts. In addition to the efflux transporters examined in chapter 4 for sulfate metabolite elimination, mRNA of several organic anion transporters (OATs) as well as organic anion transporting polypeptides (OATPs) are expressed in trophoblast [7, 8]. Protein expression or localization studies have been performed with only a few of these. The organic anion transporter 4 (OAT4) and the organic anion transporting polypeptide 2B1 (OATP2B1) have been localized to the syncytiotrophoblast basolateral membrane, while OATP-E

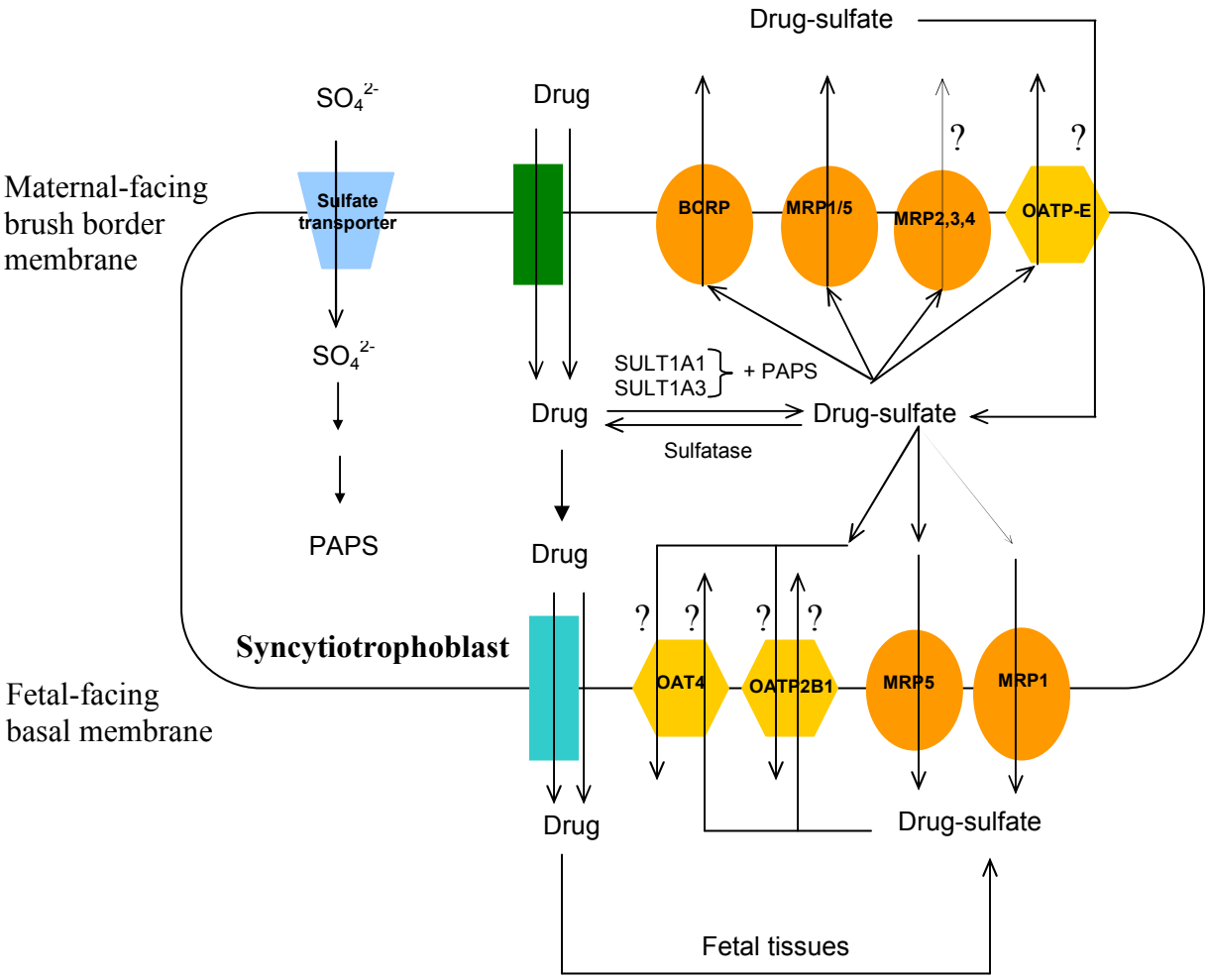


Figure 5.1 General scheme of sulfate metabolite formation and elimination from the syncytiotrophoblast layer. The figure illustrates the transporters on the maternal-facing brush border and fetal-facing basal syncytiotrophoblast membranes, which have been found to, or could potentially play a role in sulfate metabolite elimination.

(OATP4A1) is expressed on the apical syncytiotrophoblast membrane as shown in Figure 5.1 [2, 9]. The physiological function of placental OAT4 has been postulated to be the uptake of sulfated C19 steroids for *de novo* estrogen synthesis in the

placenta while both OAT4 and OATP-2B1 mediate the uptake of sulfoconjugated estrogens from the fetal circulation [2]. It has been suggested that placental OATP-E may mediate the uptake of thyroid hormones from the maternal circulation for transfer to the fetus [10]. OAT isoforms in the kidney eliminate several anionic drugs and in the intestine they have also been shown to expel sulfate metabolites [11, 12]. Whether the placental OAT and OATP isoforms contribute to the elimination of sulfate conjugates has not been examined so far, but this seems more than likely since none of the efflux inhibitors used in our study completely inhibited the efflux of either acetaminophen sulfate or 4-nitrophenylsulfate. Further, MRP3 and MRP4 are expressed on apical syncytiotrophoblast membrane. Both transporters eliminate sulfate metabolites across the hepatic sinusoidal membrane but similar roles in placenta have not been looked into.

In early pregnancy, the trophoblast layer consists predominantly of cytotrophoblasts. With advancing gestation, the cytotrophoblast cells fuse together to form the multinucleated syncytiotrophoblast cells. There are functional differences between the cytotrophoblast cells and the syncytiotrophoblast. For example, BCRP and MRP2 protein expression increased upon differentiation of cytotrophoblasts into syncytiotrophoblasts [13, 14]. The BeWo cell line, which primarily consists of cytotrophoblast-like cells, can be induced *in vitro* to syncytialize into multinucleated syncytiotrophoblast-like cells. The latter would be more reflective of the trophoblast layer of late pregnancy. It may be worthwhile to examine sulfotransferase and efflux

transporter function as the cytotrophoblasts differentiate into syncytiotrophoblasts. Any positive correlation would indicate a combined role of sulfotransferase enzymes and the efflux transporters in fetal protection.

Finally, maternal disease states such as diabetes and intra-hepatic cholestasis of pregnancy (ICP) affect placental metabolism [15]. High altitude pregnancies and other maternal conditions such as hypertension, anemia, pulmonary disease, and preeclampsia can lead to fetal hypoxia which causes intrauterine growth restriction (IUGR) and low birth weight. Under hypoxic conditions several placental uptake or transport systems are modified [16]. Under such circumstances, it may be worthwhile to examine if hypoxia alters sulfotransferase enzyme expression in the placenta, as in rats hypoxia decreased acetaminophen sulfation [17].

5.4 References

1. Stanley, E.L., et al., *Differential expression of sulfotransferase enzymes involved in thyroid hormone metabolism during human placental development.* J Clin Endocrinol Metab, 2001. **86**(12): p. 5944-55.
2. Ugele, B., A. Bahn, and M. Rex-Haffner, *Functional differences in steroid sulfate uptake of organic anion transporter 4 (OAT4) and organic anion transporting polypeptide 2B1 (OATP2B1) in human placenta.* J Steroid Biochem Mol Biol, 2008. **111**(1-2): p. 1-6.
3. Grube, M., et al., *Organic anion transporting polypeptide 2B1 and breast cancer resistance protein interact in the transepithelial transport of steroid sulfates in human placenta.* Drug Metab Dispos, 2007. **35**(1): p. 30-5.
4. Hanson, S.R., M.D. Best, and C.H. Wong, *Sulfatases: structure, mechanism, biological activity, inhibition, and synthetic utility.* Angew Chem Int Ed Engl, 2004. **43**(43): p. 5736-63.
5. Gamage, N.U., et al., *Structure of a human carcinogen-converting enzyme, SULT1A1. Structural and kinetic implications of substrate inhibition.* J Biol Chem, 2003. **278**(9): p. 7655-62.
6. Chapman, E., et al., *Sulfotransferases: structure, mechanism, biological activity, inhibition, and synthetic utility.* Angew Chem Int Ed Engl, 2004. **43**(27): p. 3526-48.
7. Serrano, M.A., et al., *Expression in human trophoblast and choriocarcinoma cell lines, BeWo, Jeg-3 and JAr of genes involved in the hepatobiliary-like excretory function of the placenta.* Placenta, 2007. **28**(2-3): p. 107-17.
8. Hosoyamada, M., et al., *Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney.* Am J Physiol, 1999. **276**(1 Pt 2): p. F122-8.
9. Young, A.M., *Characterization of efflux transporters of the human trophoblast using BeWo as a model*, in *Department of Pharmaceutical Chemistry*. 2005, University of Kansas: Lawrence.
10. Hagenbuch, B., *Cellular entry of thyroid hormones by organic anion transporting polypeptides.* Best Pract Res Clin Endocrinol Metab, 2007. **21**(2): p. 209-21.
11. Hu, M., J. Chen, and H. Lin, *Metabolism of flavonoids via enteric recycling: mechanistic studies of disposition of apigenin in the Caco-2 cell culture model.* J Pharmacol Exp Ther, 2003. **307**(1): p. 314-21.
12. Jeong, E.J., H. Lin, and M. Hu, *Disposition mechanisms of raloxifene in the human intestinal Caco-2 model.* J Pharmacol Exp Ther, 2004. **310**(1): p. 376-85.
13. Evseenko, D.A., J.W. Paxton, and J.A. Keelan, *ABC drug transporter expression and functional activity in trophoblast-like cell lines and differentiating primary trophoblast.* Am J Physiol Regul Integr Comp Physiol, 2006. **290**(5): p. R1357-65.

14. Meyer zu Schwabedissen, H.E., et al., *Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation.* Drug Metab Dispos, 2005. **33**(7): p. 896-904.
15. Myllynen, P., M. Pasanen, and K. Vahakangas, *The fate and effects of xenobiotics in human placenta.* Expert Opin Drug Metab Toxicol, 2007. **3**(3): p. 331-46.
16. Rytting, E. and K.L. Audus, *Effects of low oxygen levels on the expression and function of transporter OCTN2 in BeWo cells.* J Pharm Pharmacol, 2007. **59**(8): p. 1095-102.
17. Aw, T.Y., et al., *Effect of chronic hypoxia on acetaminophen metabolism in the rat.* Biochem Pharmacol, 1991. **42**(5): p. 1029-38.

Appendix: P-Glycoprotein Function is Affected by pH: A Definitive Study with Colchicine.

P-Glycoprotein Function is Affected by pH: A Definitive Study with Colchicine

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Abstract

Purpose. To investigate whether changes in extracellular pH affect the active efflux of colchicine, a non-ionizable P-glycoprotein substrate.

Methods. The effect of pH on P-glycoprotein (Pgp) functionality was studied by testing colchicine in bidirectional Caco-2 and mdr1-MDCK cell permeability assays in which the pH of the apical and basolateral chambers was varied. In addition, drug uptake experiments were conducted under various pH buffer conditions.

Results. The apparent permeability of colchicine in the apical to basolateral direction ($P_{app,A\ to\ B}$) remained unchanged over the investigated pH range except at pH 4.5 where it significantly differed. More distinct changes were observed in the basolateral to apical permeability resulting in the permeability directional ratio (PDR) values being approximately 34 and 9 fold lower at pH values 4.5 and 5.0 compared to that at pH 7.4. The permeability of digoxin was examined at pH values 4.5, 5.0 and 7.4. There is a significant difference in $P_{app,\ A\ to\ B}$ between pH values 4.5 and 7.4. The PDR change is in the same order as that measured for colchicine; 47 compared to 34 fold. The uptake profile of colchicine changed dramatically due to the varying buffer pH. There was no increase in drug accumulation with time at pH values 7.4, 6.0 and

5.5. However, with buffers at pH 4.5 and 5.0 there was an increase in colchicine uptake with time similar to that observed for colchicine with a Pgp inhibitor. For the buffer conditions at pH values 4.5 and 5.0, an approximate 8 and 7 fold increase respectively in colchicine accumulation over 1 hour was observed compared to the accumulation at pH 7.4.

Conclusion. P-glycoprotein function, as assessed by the bidirectional transport of colchicine, is reduced as a result of changes in the extracellular pH. The efflux ratios of digoxin and colchicine were affected by pH to a different extent. The reduction of extracellular pH at or below 5.0 can inactivate Pgp function resulting in higher amount of drug accumulation inside the cells.

A.1 Introduction

Drug transporters play a major role in drug distribution, disposition, as well as chemotherapy resistance.[1-3] One subset of transporters, in particular, has recently been noted as clinically important; the family of ATP-binding cassette (ABC) efflux transporters.[4] An important member of this family, P-glycoprotein (Pgp, MDR1 or ABCB1), was the first mammalian MDR linked transport protein to be discovered[5] and has been studied extensively for over 25 years.[6-9] Despite the many years of study dedicated to the mechanism of action of Pgp, a single model describing how substances are transported does not yet exist.[7,9] The absence of a definitive model has negative implications for the development of effective anti-cancer drugs as the induction of Pgp in tumor cells leads to multi drug resistance which is a major cause of cancer treatment failure. Pgp can also be found in normal human tissues. It is present in the intestine, kidney, liver and brain vascular endothelium and therefore can participate in drug absorption and brain distribution.

It has been shown that Pgp can reduce the oral bioavailability of a drug by reducing its intestinal absorption and by potentially enhancing its first pass metabolism.[10] The pH of the gastrointestinal lumen is one among the many factors that affect the absorption of an orally administered drug. It varies along the different segments of the intestine, going from pH 4.5 beyond the pyloric sphincter which separates the stomach from the duodenum, ranging from 5.0 to 7.5 in the small intestine, and dropping to around 5.0 in the colon.[11,12] Thus acidic and basic drugs are ionized to

different extents along the intestine. The passive absorption of charged drug molecules would be expected to vary as a function of intestinal pH because the lipophilic environment of the intestinal wall favors the permeation of the uncharged species as opposed to the ionized fraction.[13] The pH of the tumor microenvironment plays a similar role in the disposition of an anti-cancer drug. The extracellular pH of tumor tissue is significantly lower than the extracellular pH of normal tissue.[14] Many chemotherapeutic agents are weakly basic and become charged in the tumor microenvironment preventing their intracellular uptake and hence their efficacy.

It is easy to understand how pH can affect the passive diffusion of a drug into a cell (pH-partition hypothesis)[13] but how is Pgp efflux affected by pH conditions? Although there are several published papers in which the effect of pH on the function of the P-glycoprotein efflux transporter has been probed, the available data is somewhat conflicting as some studies have concluded that pH does not affect Pgp functionality while others have deduced that the function is altered by pH.[15-19] One potential reason for this conflicting data was the use of test substrates and inhibitors that do not remain unionized in the pH ranges studied and therefore the effect of ionization on the passive permeability may have confounded the results. In addition some compounds chosen in these studies were substrates for other drug transporters (e.g. daunorubicin which is also a BCRP substrate[20]).

The objective of the present study was to elucidate the effects, if any, of variable apical pH (4.5-8.0) on Pgp activity using Caco-2 cells as a model. In order to isolate and systematically study the effect of pH on Pgp functionality, colchicine was chosen as a neutral Pgp test substrate. The change in extracellular pH, theoretically, should not alter the passive diffusion of colchicine. To specifically test if the observed effects are Pgp-mediated or not, the results obtained in Caco-2 cells were compared with those obtained in mdr1-MDCK cells. If pH-dependent changes in passive permeability and membrane properties can be ruled out, observed differences in the efflux ratio of colchicine can be contributed to Pgp function being affected by changes in the extracellular pH.

A.2 Materials and methods

A.2.1 Materials

Dulbecco's Modified Eagle's Medium (DMEM) buffered with HEPES was obtained from Gibco Invitrogen (Carlsbad, CA). All other cell culture reagents were from Mediatech (Herndon, VA). Transwell plates were obtained from Costar Corporation (Acton, MA). Digoxin and colchicine were purchased from Acros Organics. Alprenolol was purchased from MP Biomedicals, INC. (Solon, OH). Zosuquidar trihydrochloride (LY3359793) was purchased from API Services INC. (Westford, MA), Lucifer yellow, testosterone, sodium acetate trihydrate, 2-(N-morpholino) ethanesulfonic acid (MES) and HEPES were obtained from Sigma-Aldrich (St. Louis, MO). Caco-2 cells originating from a human colorectal carcinoma were acquired

from American Tissue Culture Collection (Manassas, VA). The MDCK cells transfected with the human mdr1 gene (mdr1-MDCK)[21,22] were from Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

A.2.2 Cell culture

Caco-2 cells and mdr1-MDCK cells were grown at 37°C in an atmosphere of 5% CO_2 and 90% relative humidity. Caco-2 cells were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 I.U./ml penicillin and 100 μg/mL streptomycin, and 10 mM nonessential amino acids. The mdr1-MDCK cells were maintained using the same culture conditions with the exception of the media being supplemented with 20 nM colchicine to preserve selective pressure.[23] Confluent cell monolayers were subcultured every 7 days by treatment with 0.25% trypsin containing 1 mM EDTA.

A.2.3 Bidirectional transport studies

Caco-2 cells were seeded at a density of 80,000 cells/cm^2 in 12 well plates on polycarbonate filters (Costar Transwell® cell culture inserts, 12.0 mm diameter, 3.0 micron pore size). Caco-2 cells were grown on filters for 21-25 days. The mdr1-MDCK cells were seeded at a density of 50,000 cells/cm^2 in 12 well plates on polycarbonate filters (Costar Transwell® cell culture inserts, 12.0 mm diameter, 0.4 micron pore size). The mdr1-MDCK cells were grown for 6-8 days. Caco-2 cells of passage numbers 41 to 60 and mdr1-MDCK cells of passage numbers 6 to 14 were

used for all experiments in this paper. Prior to the transport studies, the culture medium was replaced with HBSS at pH 7.4 and equilibrated for 30 minutes at 37^{o}C. For transport studies that involved a pH gradient across the monolayer, the apical chambers contained a drug/buffer solution at pH values varying from 4.5-8.0 while the basolateral chamber contained drug/buffer solution at pH 7.4. HBSS was buffered with 10 mM sodium acetate for pH 4.5, 5.0; 10 mM MES for pH 5.5, 6.0; and 25 mM HEPES for pH 8.0. For pH 7.4, initially HBSS was used which was later changed to HBSS buffered with 25 mM HEPES (an explanation for the change is provided in the Results and Discussion section). For the other studies, both chambers contained drug/buffer solutions at the same pH. Drug solutions were prepared in HBSS (at the adjusted pH value for the experiment) at a concentration of 20 μM for digoxin, colchicine, and testosterone and added to either the apical (AP) or basolateral (BL) sides of the cell monolayers. The volume of solution added to the apical chamber was 0.5 mL while that added to the basolateral chamber was 1.0 mL. The transport experiments were carried out in an oven maintained at 37^{o}C. The plates were shaken at 100 rpm using an orbital shaker (Lab-Line Instruments, Melrose Park, IL) for the duration of the experiment (1 hour). At discrete time intervals, 200 μL of the receiver chamber solution was withdrawn and replaced with fresh buffer solution (sink condition). The integrity of the cell monolayer was assessed by measuring the permeability of the paracellular marker Lucifer yellow (100 μM). If the apparent permeability coefficient of Lucifer yellow was found to be less than 0.3 x 10^{-6} cm/sec, the cell monolayers were considered acceptable to use in experiments. Colchicine,

digoxin, and testosterone samples were analyzed by LCMS (an integrated Cohesive Technologies LX-2 series liquid chromatography system coupled with an Applied Biosystems MDS-SCIEX 4000 Q Trap triple quadrupole mass spectrometer (Foster City, CA)). The Lucifer yellow samples were measured using a Wallac 1420 Multilabel Reader; using a 485 nm excitation and 535 nm emission (Perkin Elmer Life Sciences, Waltham, MA).

A.2.4 Permeability calculations

The following equation was used to calculate apparent permeability[24] in either the apical to basolateral direction ($P_{app,A\ to\ B}$) or basolateral to apical direction ($P_{app,B\ to\ A}$) :

$$P_{app} = dC/dt \times V_r/A \times C_d$$

Where dC/dt is the change in concentration in the receiver compartment with respect to time, V_r is the volume of the receiver compartment, A is the growth surface area, and C_d is the initial concentration of the drug in the donor compartment. Mass balance was calculated for each experiment. In all transport experiments the mass balance was greater than 70%.

A permeability directional ratio (PDR) was calculated as follows:

$$PDR = P_{app,\ B\ to\ A} / P_{app,\ A\ to\ B}$$

A.2.5 Drug uptake studies

All drug uptake experiments were performed using mdr1-MDCK cells seeded in 6 well tissue culture plates at a density of 60,000 cells/mL and grown for 5 days. Prior

to the uptake studies, the cells were washed twice with pre-warmed (37°C) HBSS buffer solution. The buffer solutions at various pH values were prepared as previously mentioned. The cell monolayers were then incubated with 4 mL of test compound solution (1 μM) at 37°C on an orbital shaker (rotation speed 100 rpm) for up to one hour. At discrete time intervals, the drug solution was removed from the wells and cells were washed twice with ice-cold HBSS and lysed in 1 mL of freshly prepared lysis buffer which consisted of 20 mM ammonium acetate/methanol (4:1) and alprenolol (20 ng/mL) as an internal LCMS standard. Contents of each well were centrifuged at 14,000 rpm for 15 minutes and 200 μL of the supernatant was then transferred for quantitation by LCMS. The concentration of the test drug was then measured using a standard curve prepared by spiking known concentrations of the drug into the lysis buffer. The percent uptake was calculated by dividing the amount of drug recovered from the lysed cells by the amount of drug applied to the cells. For the uptake studies with a Pgp inhibitor, the cells were preincubated with a 1 μM solution of zosuquidar[25] for 30 minutes. The cells were then washed with fresh HBSS and the drug/inhibitor solution (1 μM for both) was applied and the experiment was completed as outlined above. Protein content of the mdr1-MDCK cells grown in individual wells of the 6-well plates was determined separately by Quant-iT™ Protein Assay Kit with a Qubit™ fluorometer (Invitrogen™, Carlsbad, CA). The extraction solution containing ammonium acetate and methanol was not used in order to prevent damage to the extracted proteins. Instead, lysis buffer containing TX-100 was used as reported in the literature.[26] Cells were dissolved in 1 mL of freshly prepared lysis

buffer consisting of 1% TX-100 (v/v), 20 mM Tris HCl and 150 mM NaCl. The average protein content per well for the mdr1-MDCK cells (n = 6) was found to be 2.55 ± 0.06 mg/mL and 2.57 ± 0.14 mg/mL for two different cell passages. Based on this data it was concluded that normalization of the uptake data for protein content was not necessary and all drug percent uptake values were reported based on LCMS data (as described above).

A.2.6 Statistical analysis

All experiments were performed at a minimum in triplicate. Statistical significance was calculated with one-way Analysis of Variance (ANOVA) followed by Tukey's post comparison test, or the Student's t-test as appropriate. A p-value of less than 0.05 was considered to be statistically significant. The fold differences mentioned in the paper were derived by dividing the permeability directional ratios.

A.3 Results

A.3.1 Caco-2 cell monolayer integrity

To establish the integrity of the Caco-2 cell monolayers in the pH range of the transport studies (4.5 to 8.0), the permeability of the paracellular permeability marker Lucifer yellow (LY) in several buffers at different pH values was investigated (Table I). In agreement with earlier published reports the cell monolayer did not become leaky upon exposure to various pH conditions.[24,27] Note that the Caco-2 monolayer integrity for each set of passaged cells was routinely tested at pH 7.4. Monolayers

with P_{app} values greater than 0.3 x 10^{-6} cm/sec were considered to be unsuitable for the transport studies.

In another experiment designed to monitor potential changes in the condition of the Caco-2 cell monolayer in the pH range of the planned transport studies, the bidirectional permeability of testosterone was measured at pH values 4.5 and 7.4 (Table II). Testosterone is a neutral compound known to be a substrate of CYP3A4 but not a substrate of Pgp.[28,29] No difference in the permeability directional ratio (PDR) value of testosterone was observed by changing the pH from 7.4 to 4.5 (Table II) providing additional evidence the Caco-2 cell monolayer is remaining intact during the transport experiment.

Table I. Permeability of Lucifer yellow across Caco-2 monolayers as a function of pH (n = 3). AP and BL represent apical and basolateral chambers.

Compound	**pH (AP/BL)**	**$P_{app,A\ to\ B}$ x 10^6 (cm/s)**
LY (100 μM)	4.5 /7.4	0.09 ± 0.01
LY (100 μM)	5.0/7.4	0.12 ± 0.06
LY (100 μM)	7.4/7.4	0.07 ± 0.01
LY (100 μM)	8.0/7.4	0.15 ± 0.03
LY (100 μM)	4.5/4.5	0.08 ± 0.01
LY (100 μM) and 20 μM colchicine	4.5/7.4	0.18 ± 0.04

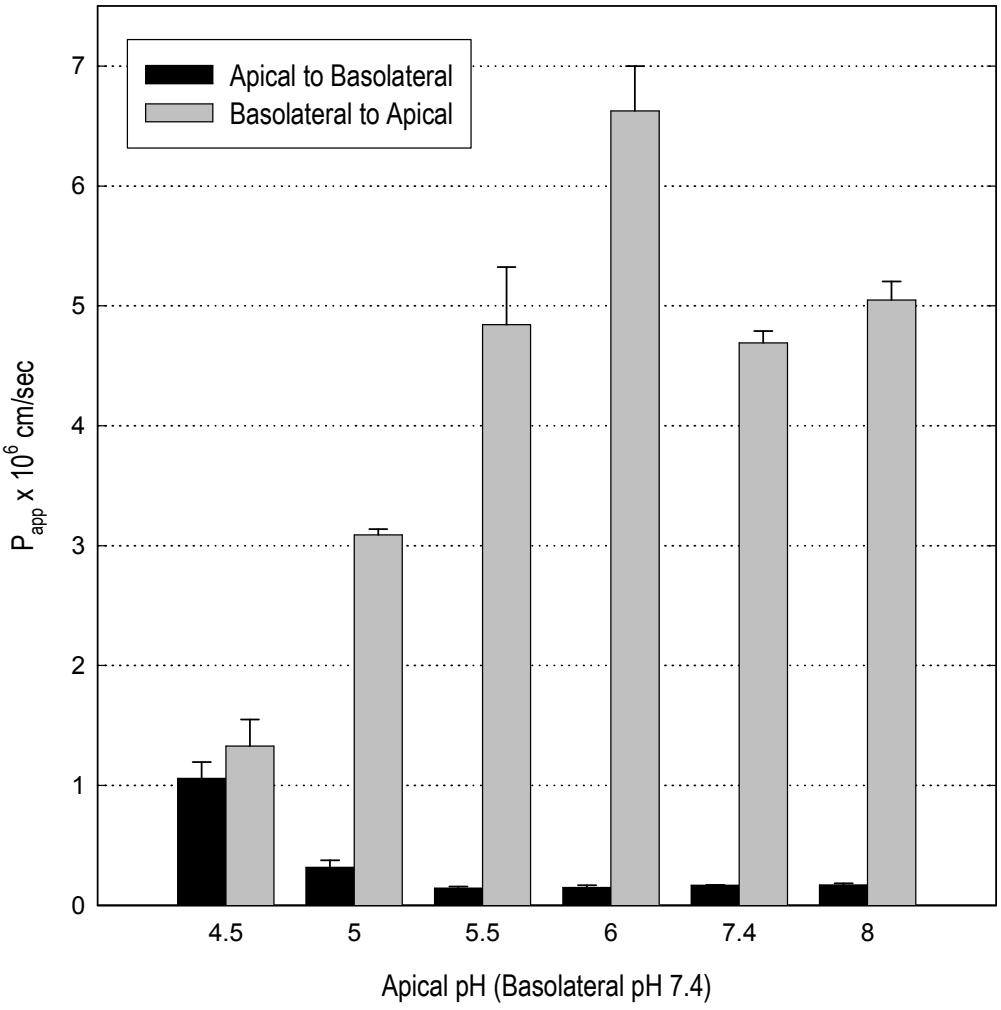
Table II. Permeability of testosterone across Caco-2 monolayers as a function of pH (n = 3). AP and BL represent apical and basolateral chambers. The number in brackets shows the fold change compared with the value at pH 7.4.

pH (AP/BL)	$P_{app, A \text{ to } B} \times 10^6$ (cm/s)	$P_{app, B \text{ to } A} \times 10^6$ (cm/s)	PDR
4.5/4.5	24.0 ± 0.9 (2x)	20.4 ± 0.8 (2x)	0.9 ± 0.1
7.4/7.4	11.0 ± 1.9	10.1 ± 2.8	0.9 ± 0.3

A.3.2 pH-dependent bidirectional transport of colchicine and digoxin across Caco-2 cell monolayers

Since, colchicine has a pK_a of 12.35,[30] within the pH range 4.5-8.0 the concentration of the unionized species is not expected to change. Hence, the passive permeability of colchicine should not be affected by pH. It has been demonstrated previously that the polarized transport of colchicine across Caco-2 cell monolayers is mediated by P-glycoprotein.[31] Generally, compounds exhibiting asymmetrical transport and exhibiting permeability directional ratio (PDR) values of 2 or greater are considered to be substrates of efflux transporters.[24] Observed variances in the PDR values due to pH change would indicate that P-glycoprotein functionality is being affected by pH, thus the permeability of colchicine was examined in the presence of a pH gradient. The apical side pH values were varied between 4.5 and 8.0 while the basolateral side pH was kept at 7.4 (Figure I).

Figure I. Apparent permeability of 20 μM colchicine across Caco-2 cell monolayers as a function of apical pH (basolateral pH 7.4). Each bar represents mean ± SD, (n=3).



Apical pH	$P_{app, A\ to\ B}$ x 10^6 (cm/s)	$P_{app, B\ to\ A}$ x 10^6 (cm/s)	PDR
4.5	0.57 ± 0.08	0.66 ± 0.09	1.2 ± 0.2 (23x)
5	0.24 ± 0.03	2.53 ± 0.11	10.5 ± 1.2 (3x)
5.5	0.14 ± 0.02	4.31 ± 0.36	30.8 ± 5.3
6	0.15 ± 0.03	5.78 ± 0.37	39.7 ± 7.8
7.4	0.17 ± 0.01	4.69 ± 0.10	27.4 ± 1.7
8	0.12 ± 0.02	4.52 ± 0.18	37.4 ± 6.1

The number in brackets shows a significant fold change compared with the value at pH 7.4.

As can be seen in Figure I, the $P_{app,A\ to\ B}$ remained unchanged over the investigated pH range except at pH 4.5 where it significantly differed. More distinct differences were observed in the basolateral to apical permeability resulting in the PDR values being 23 and 3 fold lower at apical pH values of 4.5 and 5.0 from that at 7.4. It has been shown that Pgp mediated efflux is asymmetric with respect to absorptive and secretory transport,[29] thus the larger change in basolateral to apical permeability with respect to the change in apical to basolateral permeability is not surprising.

The permeability of Lucifer yellow was tested in the presence of 20 μM colchicine at an apical pH of 4.5 (the lowest pH value used in the study) to see if the altered PDR was a result of the colchicine affecting the monolayer integrity at lower pH values. As

Table III. pH values at the start and end of the bidirectional Caco-2 permeability studies.

pH (A/B) t = 0	**Apical pH t = 120 min.**		**Basolateral pH t = 120 min.**	
	HBSS	**HEPES**	**HBSS**	**HEPES**
4.5/7.4	5.5	6.0	7.2	7.4
5.0/7.4	5.9	6.1	7.0	7.3
5.5/7.4	5.7	5.7	6.9	7.5
6.0/7.4	6.1	6.1	7.5	7.4
8.0/7.4	8.0	8.1	7.4	7.5

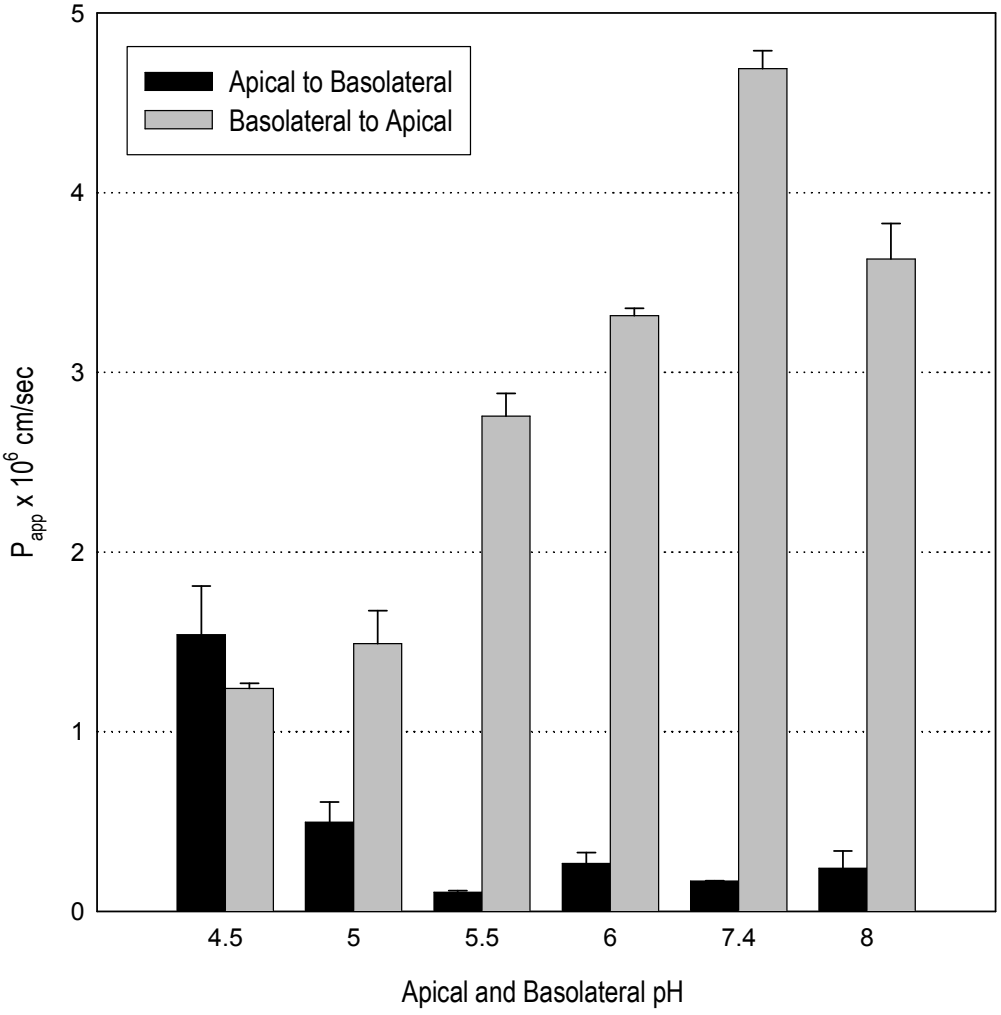
can be seen in Table I, the monolayer was not compromised. However, pH measurement at the end of the experiments indicated that the apical and basolateral chambers were equilibrating (Table III). Changing to a 25 mM HEPES buffered

HBSS solution instead of HBSS alone for maintaining a pH of 7.4 as has been previously reported[15] did not stabilize the initial pH values.

The colchicine permeability experiments were repeated with the same pH on the apical and basolateral sides. In the absence of a pH gradient, the results were qualitatively similar to those obtained with a pH gradient (Figure II). The PDR was 34 fold and 9 fold lower at pH 4.5 and 5.0 compared to the PDR value measured at pH 7.4. The low PDR values obtained at pH 4.5 and 5.0, indicate the possibility of Pgp mediated transport being impaired at the low pH values.

It has been shown previously by Neuhoff et al. that the efflux ratio of digoxin across Caco-2 cell monolayers is independent of apical pH (pH range tested: 5.0 to 8.0).[15] To test if the permeability and PDR differences are specific to colchicine, the permeability of digoxin was examined at pH values 4.5, 5.0 and 7.4. There is a significant difference in $P_{app, A-B}$ between the pH values 4.5 and 7.4 (Table IV). The PDR change is on the same order as measured for colchicine; 47 compared to 34 fold. Compared to the average efflux ratio for digoxin over the pH range 5.0 to 8.0 reported by Neuhoff (8.9 ± 0.8)[15] this finding is quite different. There is a possibility that the inconsistencies are due to the different experimental methodologies, such as a higher stirring rate (450 rpm) and removal of the entire contents of the receiver chamber at experimental time points in the Neuhoff study.

Figure II. Apparent permeability of 20 μM colchicine across Caco-2 cell monolayers as a function of pH (apical and basolateral pH values the same). Each bar represents mean ± SD, (n=3).



pH	$P_{app, A\ to\ B}$ x 10^6 (cm/s)	$P_{app, B\ to\ A}$ x 10^6 (cm/s)	PDR
4.5	1.54 ± 0.27	1.24 ± 0.04	0.8 ± 0.1 (34x)
5.0	0.50 ± 0.11	1.49 ± 0.18	3.0 ± 0.8 (9x)
5.5	0.11 ± 0.01	2.76 ± 0.13	26.2 ± 2.7
6.0	0.27 ± 0.06	3.31 ± 0.04	12.5 ± 2.9 (2x)
7.4	0.17 ± 0.01	4.69 ± 0.10	27.4 ± 1.7
8.0	0.24 ± 0.10	3.63 ± 0.20	15.2 ± 6.1 (2x)

The number in brackets shows a significant fold change compared with the value at pH 7.4.

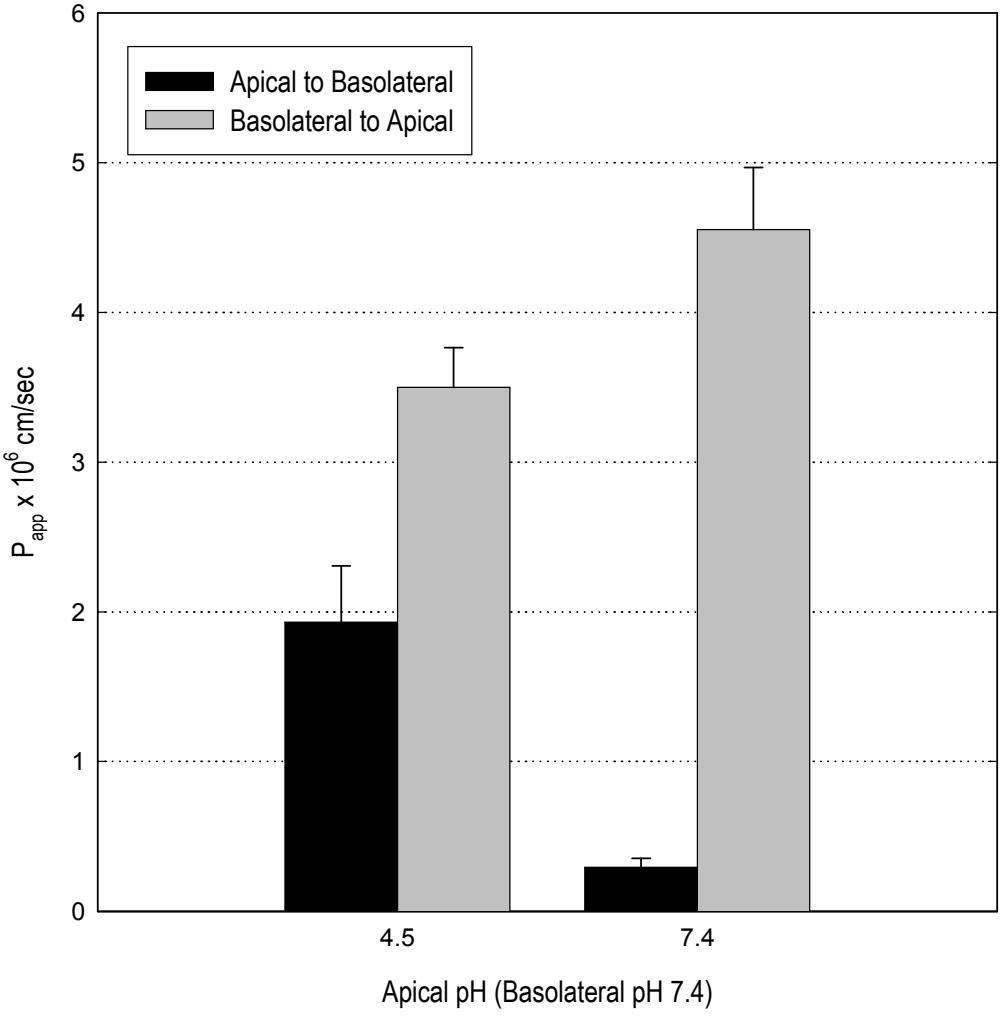
Table IV. Comparison of the bidirectional transport of 20 μM colchicine and digoxin across Caco-2 cell monolayers (same apical and basolateral pH values). The number in brackets shows a significant fold change compared with the value at pH 7.4.

pH	**Colchicine**			**Digoxin**		
	$P_{app, A to B}$ x 10^6 (cm/s)	$P_{app, B to A}$ x 10^6 (cm/s)	PDR	$P_{app, A to B}$ x 10^6 (cm/s)	$P_{app, B to A}$ x 10^6 (cm/s)	PDR
4.5	1.54 ± 0.27	1.24 ± 0.04	0.8 ± 0.1 (34x)	23.5 ± 0.1	24.0 ± 0.3	1.0 ± 1.1 (47x)
5.0	0.50 ± 0.11	1.49 ± 0.18	3.0 ± 0.8 (9x)	0.75 ± 0.07	11.3 ± 0.8	15.2 + 1.8 (3x)
7.4	0.17 ± 0.01	4.69 ± 0.10	27.4 ± 1.7	2.43 ± 0.13	114.0 ± 10.3	47.0 ± 4.9

A.3.3 pH-dependent bidirectional transport of colchicine across mdr1-MDCK cell monolayers

As confirmation that the pH-dependent efflux of colchicine observed in Caco-2 cells is indeed due to Pgp, transport studies in the presence of a pH gradient were conducted using Madine Darby Canine Kidney (MDCK) cells transfected with the human *mdr1* gene.[21,22] As observed with the Caco-2 cell transport experiments, the PDR changes significantly upon lowering the apical side pH from 7.4 to 4.5 (Figure III) in the mdr1-MDCK cell experiments. This data provides further verification that P-glycoprotein functionality is affected by pH.

Figure III. Apparent permeability of 20 μM colchicine across mdr1-MDCK cell monolayers as a function of pH. Each bar represents mean ± SD, (n=3).



pH	$P_{app\ A\ to\ B}$ x 10^6 (cm/s)	$P_{app\ B\ to\ A}$ x 10^6 (cm/s)	PDR
4.5	1.93 ± 0.38	1.24 ± 0.04	1.8 ± 0.4 (9x)
7.4	0.29 ± 0.06	4.55 ± 0.41	15.5 ± 3.5

The number in brackets shows a significant fold change compared with the value at pH 7.4.

A.3.4 pH-dependent uptake of testosterone and colchicine in mdr1-MDCK cells

The uptake of colchicine in mdr1-MDCK cells was studied as a function of the buffer pH value to further investigate the effect of pH on Pgp functionality. The level of colchicine uptake in mdr1-MDCK cells was found to be constant at pH 7.4 for one hour. However, treatment of the mdr1-MDCK cells with the Pgp inhibitor zosuquidar[25] allowed the colchicine cell concentration to increase with time (Figure IV) demonstrating that reduced Pgp activity caused increased drug accumulation. Comparison of the uptake profile for colchicine with and without Pgp-inhibitor shows more than a 6 fold increase in drug uptake in the presence of the inhibitor (Figure IV). Before investigating the effect of pH on colchicine uptake, testosterone was again used as a test control to monitor potential changes in the cell membrane integrity. The transport assay for testosterone showed no pH effect on the permeability directional ratio. Performing an uptake assay with testosterone at pH values 4.5 and 7.4 showed no difference in testosterone uptake with time (Figure V). Instead, a decrease in testosterone recovered from the cell can be observed over time, the decrease being more pronounced at pH 7.4. This is possibly due to the metabolism of testosterone to 6β-OH testosterone by CYP3A4.[32,33] It is reasonable to postulate that CYP3A4 is being effected by pH as well and the metabolism of testosterone was more extensive at pH 7.4 compared to at pH 4.5.

Figure IV. Colchicine (1μM) uptake by mdr1-MDCK cells with and without a Pgp inhibitor

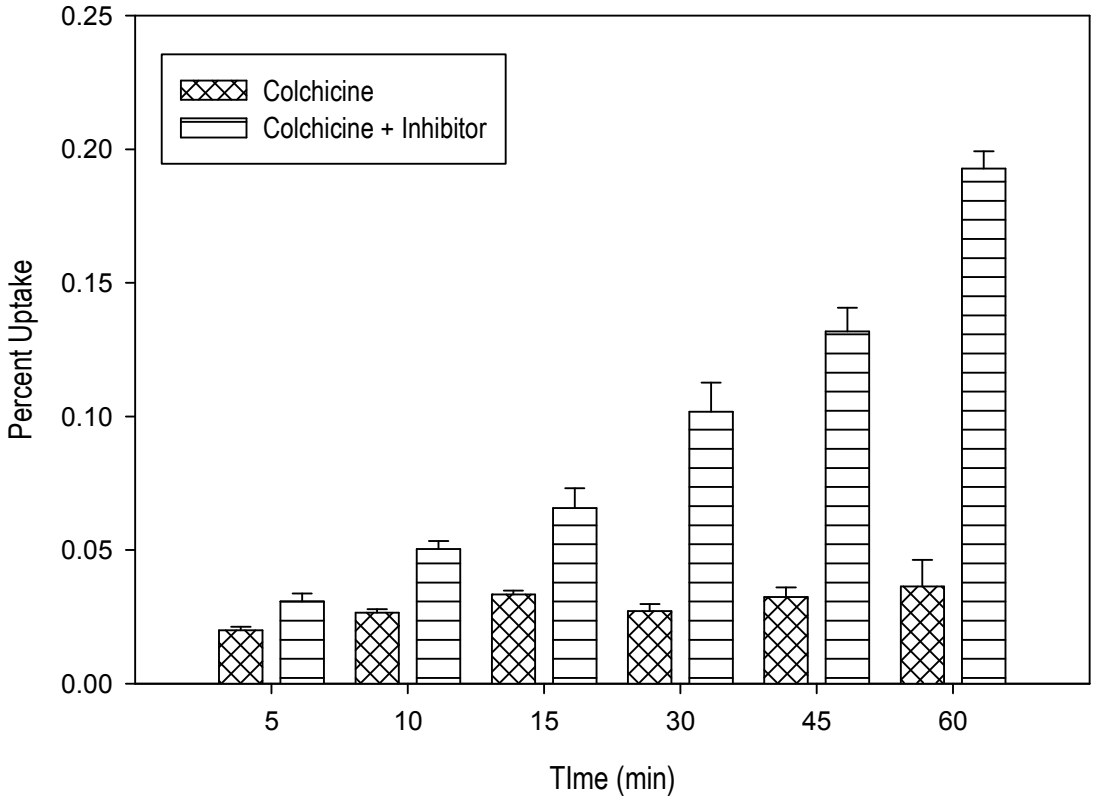
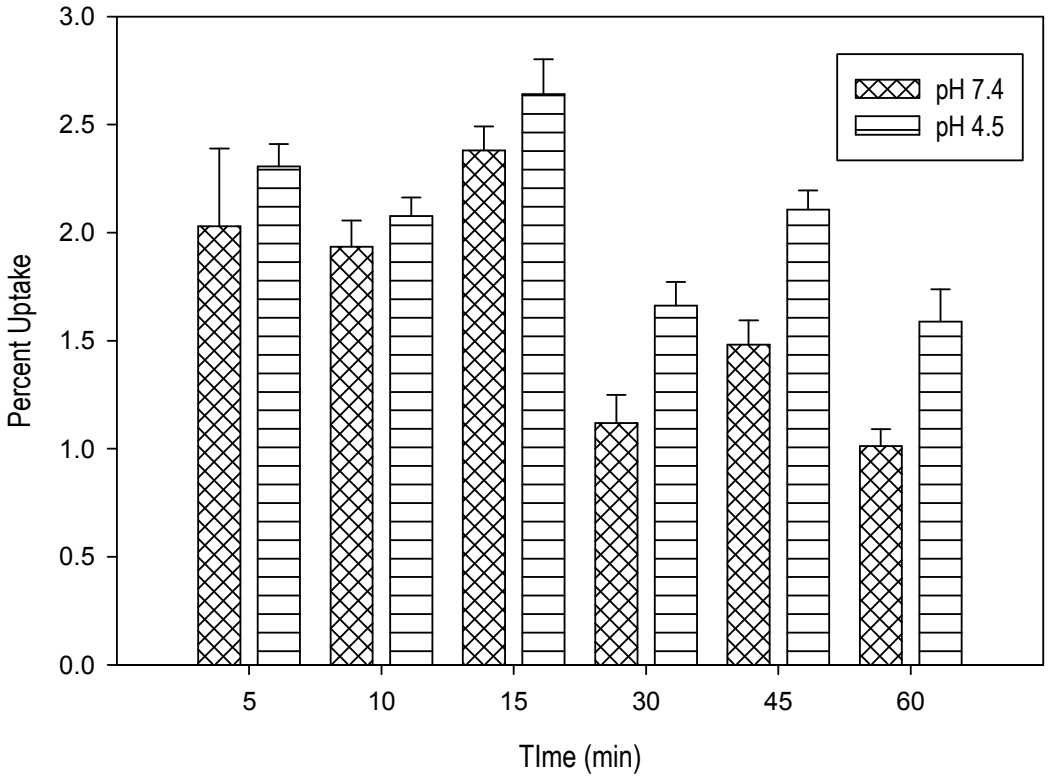
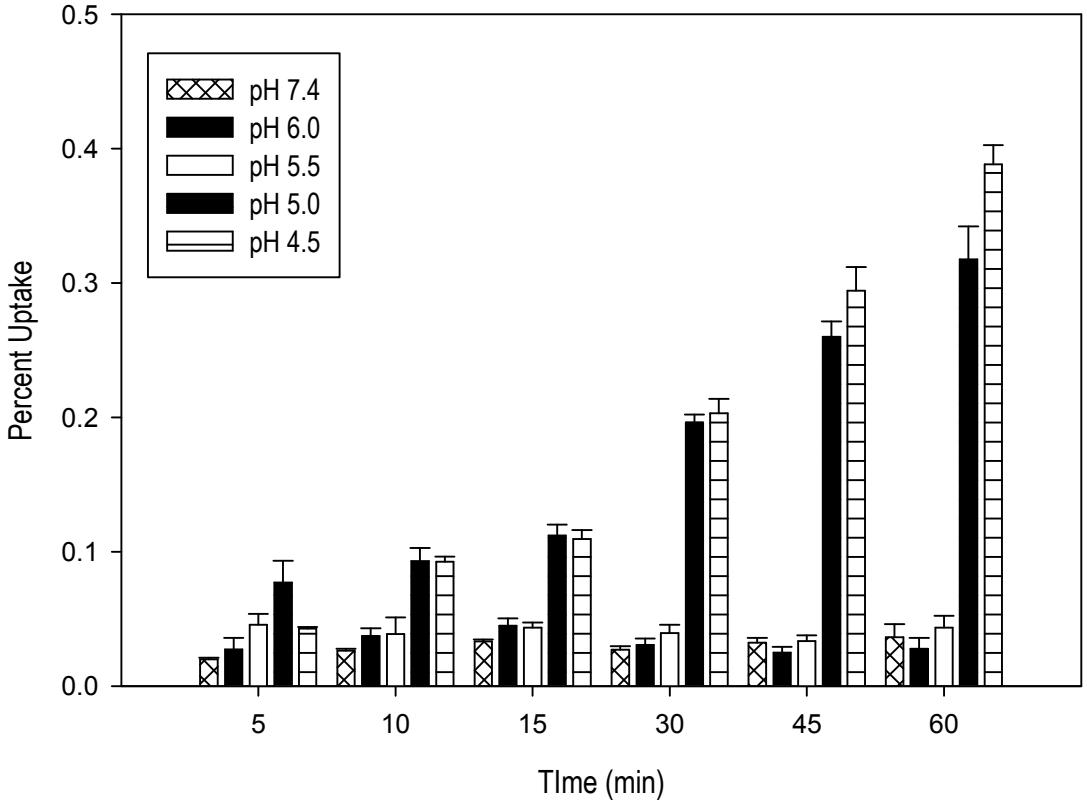


Figure V. Testosterone (1μM) uptake by mdr1-MDCK cells at pH 7.4 and 4.5



In order to monitor the effect of pH on the colchicine uptake profile, mdr1-MDCK cells were incubated with colchicine in different pH buffers for one hour. Figure VI shows a remarkable change of the uptake profile for the drug due to the varying

Figure VI. Colchicine (1 μM) uptake by mdr1-MDCK cells as a function of pH value



buffer pH. There was no increase in drug accumulation with time at pH values 7.4, 6.0 and 5.5. However, with buffers at pH 4.5 and 5.0 there was an increase in colchicine uptake with time similar to that observed for colchicine with the Pgp inhibitor. For the buffer conditions at pH values 4.5 and 5.0, an approximate 8 and 7

fold increase in colchicine accumulation over 1 hour was observed compared to the accumulation at pH 7.4 (Figure VI).

A.4 Discussion

Initially, to simulate *in vivo* conditions, the permeability of 20 μM colchicine was examined in the presence of a pH gradient in Caco-2 cells.[34] Except at pH 4.5, colchicine exhibited apically-directed asymmetrical bidirectional transport (Figure I). The PDR was between 31-37 in the pH interval 5.5-8.0 with no increasing or decreasing trends and the value at pH 7.4 was in agreement with the efflux ratio of colchicine that has been previously reported (PDR = 27.4).[29] In contrast, at pH 5.0 the PDR was approximately 3 fold lower than at pH 5.5, resulting from an almost 60% decrease in $P_{app, B\ to\ A}$ and a 1.7 fold increase in $P_{app, A\ to\ B}$. At pH 4.5, the asymmetry in transport was abolished as witnessed by a PDR of 1.2. Compared with the apparent permeability rates at pH 5.0, this resulted from an approximately 25% decrease in the $P_{app, B\ to\ A}$, and a 2.4 fold increase in the $P_{app, A\ to\ B}$. The PDR at pH values 4.5 and 5.0 were 23 and 3 fold lower than that at pH 7.4 respectively.

The change in the efflux ratio indicates that pH could be affecting Pgp function. It has been demonstrated before that in a pH gradient system, a different extent of ionization in the apical and basolateral chambers can generate a passive efflux ratio leading to an over estimation of the contribution of efflux transporters.[15] Colchicine is a neutral molecule, therefore pH-dependent changes in passive permeability would not be

expected in the investigated pH range and no contribution from a passive efflux ratio would not be generated. Another possibility could be that the altered apparent permeability and efflux ratio are resulting from pH-mediated changes in the properties of cellular membranes. The monolayer integrity was verified by performing permeability experiments with the paracellular permeability marker Lucifer yellow (Table I). If Pgp function was selectively influenced by the change of pH, the permeability of a neutral non Pgp substrate should not have been affected under this conditions. Testosterone is a substrate for CYP3A4 but not for Pgp.[28,32] Results from the testosterone transport experiments show that for testosterone there was a slight difference in permeability rate coefficients with the P_{app} at pH 7.4 two fold less than the P_{app} at pH 4.5 (Table II). Unlike colchicine, there is no change in the permeability directional ratio. Furthermore, the testosterone apparent permeability rate coefficient (A to B) measured at pH 7.4 is in agreement with the published value ($P_{app, A\ to\ B} = 24.9 \pm 3.3 \times 10^{-6}$ cm/s).[35]

This first set of transport experiments was conducted using a pH gradient (apical pH varied, basolateral pH kept at pH 7.4). The pH of the chambers changed during the course of the experiment (Table III) so in order to directly study the effects of pH on Pgp functionality the experiments were repeated in the absence of a pH gradient (same apical and basolateral pH). There were changes in absolute values but no increasing or decreasing trends in the PDR in the extracellular pH range of 5.5-8.0. In accordance with the previous studies there was an increase in efflux ratio going

from pH 4.5 to pH 5.5 (Figure II). At pH 4.5, the PDR was ~3.5 fold lower than that at pH 5.0, while at pH 5.0 it was ~8.5 fold lower than that at pH 5.5. Thus, in studies conducted both in the absence as well as the presence of a pH gradient, a decrease in extracellular pH was accompanied by a decrease in $P_{app, B\ to\ A}$, an increase in $P_{app, A\ to\ B}$, and a decrease in PDR. These results confirm the possibility of reduced function of the transporter at acidic pH conditions.

The apically directed asymmetrical transport of colchicine indicates that it is being transported by an efflux transporter located on the apical membrane of Caco-2 cells. In addition to Pgp, several other efflux transporters are expressed in Caco-2 cells including the breast cancer resistance protein (BCRP) and the multidrug resistance associated proteins MRP2 and MRP3.[36,37] Both BCRP and MRP2 are located on the apical membrane of Caco-2 cells,[38,39] but colchicine has been found to be a substrate of Pgp only.[20] To further confirm that the altered efflux ratio is due to modified Pgp function, the permeability of 20 μM colchicine was measured in mdr1-MDCK cells in the absence of a pH gradient. MDCK cells that have been transfected with the human mdr1 gene are frequently used to screen for Pgp substrates as they lack any significant levels of other transporters.[40] As seen with Caco-2 cells, the asymmetric transport of colchicine observed at pH 7.4 was attenuated at pH 4.5 (Figure III). According to the conclusions drawn from the Caco-2 permeability data, if P-glycoprotein is dysfunctional at acidic pH, then with decreasing pH the $P_{app, A\ to\ B}$ should increase while the $P_{app, B\ to\ A}$ should decrease. In agreement with this

hypothesis, $P_{app, A \text{ to } B}$ increases with decreasing pH but contrary to it, the $P_{app, B \text{ to } A}$ also increased with decreasing pH (Figure III). It has been noted that the different apical membrane compositions of MDCK and Caco-2 cells could result in different orientations of Pgp in the membranes and this could be a possible reason for the different affinities of Pgp substrates/inhibitors observed in the two cell types.[40] Both the reduction of asymmetric transport as well as the increased apical to basolateral permeability observed at pH 4.5, indicate that Pgp mediated transport of colchicine is being reduced under acidic pH conditions.

The observation that Pgp functionality is reduced at low pH values is contrary to other reports in the literature. Altenberg et al. performed efflux studies with the Pgp substrate rhodamine 123 in various drug resistant cell lines and showed that changes in the intra- or extracellular pH environment did not mediate Pgp activity (pH range 5.5-7.5).[20] Thews et al. reported that the reduction of extracellular pH to 6.6 led to an increase of Pgp mediated efflux activity in rat prostrate cells when compared to control cells with an extracellular pH value of 7.4.[18] They found that the Pgp activity was doubled after 3 to 6 hours of incubation in acidic medium (pH 6.6) without an increase in Pgp expression level. They also reported that the cytotoxicity of a Pgp-substrate drug daunorubicin was reduced in the acidic environment. The reduced cytotoxicity was attributed to the higher activity of the efflux transporter under this condition. Since daunorubicin is a basic drug (pK_a of 8.4),[41] the lowered activity could be explained by the pH-partition hypothesis in which the lipid bilayer of the

cell wall favors the permeation of the uncharged species as opposed to the ionized fraction.[13] The transport of basic drugs across cells is governed by the concentration of the uncharged species which in turn is determined by pH.[15,17,24] Thus the uptake of the weakly basic daunorubicin (and related cytotoxicity) is impaired at a low pH. In fact the acidic microenvironment of tumors is one of the confounding factors in developing an effective anti-tumor drug.[42-44]

Since several of the preceding literature reports have utilized uptake experiments to investigate pH and Pgp functionality; a comparable experiment was conducted in which the accumulation of colchicine in mdr1-MDCK cells under various pH conditions was examined. Hafny et al. looked at the colchicine cellular kinetics in immortalized rat brain microvessel endothelial cells.[45] Colchicine uptake was reported to show two phases with an initial rapid entry followed by constant rate of accumulation for 10 nM [^{3}H] colchicine. Equilibrium colchicine concentration was achieved after 45 hours in the concentration range of 0.5 nM to 20 nM. However, Hafny found that increasing the concentration of colchicine to 200 nM reduces the time required for attaining the maximum level of drug uptake to 6 hours. In our studies we used 1 μM colchicine to ensure adequate sensitivity for LCMS detection technique. An initial increasing uptake phase for the drug leading to equilibrium level inside the cell at pH 7.4 was not observed. Instead the amount of colchicine recovered from the cell appeared constant after 5 minutes at pH values 7.4 (Figure IV). It is possible that under this condition the intracellular colchicine level has reached a

maximum in less than 5 minutes. Incubation of the mdr1-MDCK with a Pgp inhibitor (zosuquidar) for thirty minutes prior to the uptake experiment led to increasing colchicine accumulation with time (Figure IV). A similar effect is observed when the uptake experiment is performed with colchicine alone under pH conditions at pH 4.5 and 5.0 (Figure VI) which indicates that at these low pH values Pgp function is diminished.

To see if the same phenomenon could be observed with other Pgp substrates, the bidirectional permeability study with Caco-2 cells was repeated using digoxin (20μM), another neutral Pgp substrate. The digoxin PDR decreased with decreasing pH although a dramatic change was not detected until the pH was lowered to 4.5 (Table IV). Neuhoff et al. have previously demonstrated that the efflux ratio of digoxin (8.9 ± 0.8) across Caco-2 is independent of the apical pH in the range 5.0 to 8.0. Based on this data, Neuhoff concluded that Pgp function is not impacted by pH which is contrary to our findings. There a several notable differences in the experimental design of the two studies such as the length of time, orbital stirrer setting, and test sampling. It is difficult to attribute the differences in data to these variables without further evaluation.

In conclusion, our data indicates for the first time that P-glycoprotein function, as assessed by the bidirectional transport of colchicine, is being reduced as a result of changes in the extracellular pH. The decrease in $P_{app, A \text{ to } B}$ and increase in $P_{app, B \text{ to } A}$

with decreasing pH indicate that at least in the pH range 4.5-5.5 the function of the pump is being impaired with decreasing pH. It appears that the efflux ratios of digoxin and colchicine were affected by pH to a different extent.

The uptake study clearly shows that reduction of extracellular pH at or below 5.0 can inactivate the Pgp function resulting in higher amount of drug accumulation inside the cells. In the future, it might be possible to develop formulations which can reduce the pH of the tumor microenvironment to pH values which leads to transient inactivation of P-glycoprotein efflux. Additional studies of this nature are warranted as these data may provide important insight to the elucidation of the P-glycoprotein efflux mechanism and ultimately the development of a successful chemotherapy for multi drug resistant tumors.

A.5 References

1. R. B. Kim. *Transporters and drug discovery: why, when, and how*. Mol. Pharm. **3**:26-32 (2006).
2. Y. Shitara, T. Horie, and Y. Sugiyama. *Transporters as determinants of drug clearance and tissue distribution*. Eur. J. Pharm. Sci. **27**:425-446 (2006).
3. G. Szakacs, J. K. Paterson, J. A. Ludwig, C. Booth-Genthe, and M. M. Gottesman. *Targeting multidrug resistance in cancer*. Nature Reviews Drug Discovery **5**:219-234 (2006).
4. V. Fischer, H. J. Einolf, and D. Cohen. *Efflux transporters and their clinical relevance*. Mini-Rev. Med. Chem.. **5**:183-195 (2005).
5. C. Chen, J. E. Chin, K. Ueda, D. P. Clark, I. Pastan, M. M. Gottesman, and I. B. Roninson. *Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells*. Cell **47**:381-389 (1986).
6. F. J. Sharom. *Shedding light on drug transport:structure and function of the P-glycoprotein multidrug transporter (ABCB1)*. Biochem. Cell Biol. **84**:979-992 (2006).
7. S. V. Ambudkar, I. Kim, and Z. E. Sauna. *The power of the pump:mechanisms of action of P-glycoprotein (ABCB1)*. Eur. J. Pharm. Sci. **27**:392-400 (2006).
8. R. Callaghan, R. C. Ford, and I. D. Kerr. *The translocation mechanism of P-glycoprotein*. FEBS Lett. **580**:1056-1063 (2006).
9. M. Hennessy, and J. P. Spiers. *A primer on the mechanics of P-glycoprotein the multidrug transporter*. Pharmacol. Res. **55**:1-15 (2007).
10. C. G. Dietrich, A. Geier, and R. P. J. Oude Elferink. *ABC of oral bioavailability: transporters as gatekeepers in the gut*. Gut **52**:1788-1795 (2003).
11. M. V. Varma, and R. Panchagnula. *pH-dependent functional activity of P-glycoprotein in limiting intestinal absorption of protic drugs: kinetic analysis of quinidine efflux in situ*. J Pharm Sci. **94**:2632-43 (2005).
12. A. M. L. Hillery, A. W. Lloyd, and J. Swarbrick. *Drug Delivery and Targeting for Pharmacists and Pharmaceutical Scientists*, Taylor and Francis, New York and London, 2001, p. 152.
13. C. A. Hogben, D. J. Tocco, B. B. Brodie, and L. S. Schanker. *On the mechanism of intestinal absorption of drugs*. J Pharmacol Exp Ther. **125**:275-82 (1959).
14. P. Vaupel, F. Kallinowski, and P. Okunieff. *Blood flow, oxygen, and nutrient supply, and metabolic microenvironment of human tumors: a review*. Cancer Res. **49**:6449-6465 (1989).
15. S. Neuhoff, AL. Ungell, I. Zamora, and P. Artursson. *pH-dependent bidirectional transport of weakly basic drugs across Caco-2 monolayers: implications for drug-drug interactions*. Pharm Res. **20**:1141-8 (2003).

16. K. Goda, L. Balkay, T. Marian, L. Tron, A. Aszalos, and G. Szabo. *Intracellular pH does not affect drug extrusion by P-glycoprotein*. J Photochem Photobiol *B*. **34**:177-82 (1996).
17. G. A. Altenberg, G. Young, J. K. Horton, D. Glass, J. A. Belli, and L. Reuss. *Changes in intra- or extracellular pH do not mediate P-glycoprotein-dependent multidrug resistance*. Proc Natl Acad Sci .*U. S. A*. **90**:9735-8 (1993).
18. O. Thews, B. Gassner, D. K. Kelleher, G. Schwerdt, and M. Gekle *Impact of extracellular acidity on the activity of P-glycoprotein and the cytotoxicity of chemotherapeutic drugs*. Neoplasia **8**:143-52 (2006).
19. C. Lotz, D. K. Kelleher, B. Gassner, M. Gekle, P. Vaupel, and O. Thews. *Role of the tumor microenvironment in the activity and expression of the P-glycoprotein in human colon carcinoma cells*. Oncology Reports. **17**:239-244 (2007).
20. L. Zhang, J. M. Strong, W. Qiu, L. J. Lesko, and S. Huang. *Scientific perspectives on drug transporters and their role in drug interactions*. Mol. Pharm. **3**:62-69 (2006).
21. E. Bakos, R. Evers, G. Szakacs, G. E. Tusnady, E. Welker, K. Szabo, M. De Hass, L. Van Deemter, P. Borst, A. Varadi, and B. Sarkadi. *Functional multidrug resistant protein (MRP1) lacking the N-terminal transmembrane domain*. J. Biol. Chem. **273**:32167-32175 (1998).
22. R. Evers, M. Kool, A. J,. Smith, L. Van Deemter, M. De Haas, and P. Borst. *Inhibitory effect of the reversal agents V-104, GF120918 and pluronic L61 on MDR1 PGP, MRP1 And MRP2 mediated transport*. Brit. J. Cancer **83**:366-374 (2000).
23. Q. Wang, J. D. Rager, K. Weinstein, P. S. Kardos, G. L. Dobson, J. Li, and I. J. Hidalgo. *Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier*. Int. J. Pharm. **288**:349-359 (2005).
24. A. M. Young, K. L. Audus, J. Proudfoot, and M. Yazdanian. *Tetrazole compounds: the effect of structure and pH on Caco-2 cell permeability*. J Pharm Sci. **95**:717-25 (2006).
25. R. L. Shepard, J. Cao, J. J. Starling, and A. H. Dantzig. *Modulation of P-glycoprotein but not MRP1- or BCRP- mediated drug resistance by LY335979*. Int. J. Cancer **103**:121-125 (2003).
26. F. Tang, H. Ouyang, J.Z. Yang, and R. T. Borchardt. *Bidirectional transport of rhodamine 123 and Hoechst 33342, fluorescence probes of the binding sites on P-glycoprotein, across MDCK-MDR1 cell monolayers*. J. Pharm. Sci. **93**:1185-1194 (2004).
27. S. Neuhoff, AL. Ungell, I. Zamora, and P. Artursson. *pH-Dependent passive and active transport of acidic drugs across Caco-2 cell monolayers*. Eur J Pharm Sci. **25**:211-20 (2005).
28. D. J. Waxman, C. Attisano, F. P. Guengerich, and D. P. Lapenson. *Human liver microsomal steroid metabolism: identification of the major microsomal*

steroid hormone 6 beta-hydroxylase cytochrome P-450 enzyme. Archiv Biochem. Biophys. **263**:424-436 (1988).

29. M. D. Troutman, and D.R. Thakker. *Efflux ratio cannot assess P-glycoprotein-mediated attenuation of absorptive transport: asymmetric effect of P-glycoprotein on absorptive and secretory transport across Caco-2 cell monolayers*. Pharm Res. **20**:1200-9 (2003).
30. M. Windholz, S. Budavari, R. F. Blumetti, and E. S. Otterbein. The Merck Index 10th Edition, Merck and Co., INC, Rahway, New Jersey, 1983.
31. Y. H. Li, K. Ito, Y. Tsuda, R. Kohda, H. Yamada, and T. Itoh. *Mechanism of intestinal absorption of an orally active beta-lactam prodrug: uptake and transport of carindacillin in Caco-2 cells*. J. Pharmacol. Exp. Ther. **290**:958-964 (1999).
32. L. M. Chan, A. E. Cooper, A. L. Dudley, D. Ford, and B. H. Hirst. *P-glycoprotein potentiates CYP3A4-mediated drug disappearance during Caco-2 intestinal secretory detoxification*. Journal of Drug Targeting **12**:405-413 (2004).
33. H. A. Engman, H. Lennernas, J. Taipalensuu, C. Otter, B. Leidvik, and P. Artursson. *CYP3A4, CYP3A5, and MDR1 in human small and large intestinal cell lines suitable for drug transport studies*. J. Pharm. Sci. **90**:1736-1751 (2001).
34. S. Yamashita, T. Furubayashi, M. Kataoka, T. Sakane, H. Sezaki, and H. Tokuda. *Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells*. Eur. J. Pharm. Sci. **10**:195-204 (2000).
35. M. Yazdanian, S. L. Glynn, J. L. Wright, and A. Hawi. *Correlating partitioning and Caco-2 cell permeability of structurally diverse small molecular weight compounds*. Pharm. Res. **15**:1490-94 (1998).
36. G. Englund, F. Rorsman, A. Roennblom, U. Karlbom, L. Lazorova, J. Grasjoe, A. Kindmark, and P. Artursson. *Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells*. Eur J Pharm Sci. **29**:269-77 (2006).
37. A. Seithel, J. Karlsson, C. Hilgendorf, A. Bjoerquist, and A. Ungell. *Variability in mRNA expression of ABC- and SLC-transporters in human intestinal cells: comparison between human segments and Caco-2 cells*. Eur J Pharm Sci. **28**:291-9 (2006).
38. C. Q. Xia, N. Liu, D. Yang, G. Miwa, and L. Gan. *Expression, localization, and functional characteristics of breast cancer resistance protein in Caco-2 cells.* Drug Metab Dispos. **33**:637-43 (2005).
39. H. M. Prime-Chapman, R. A. Feam, A. E. Cooper, V. Moore, and B. H. Hirst. *Differential multidrug resistance-associated protein 1 through 6 isoform expression and function in human intestinal epithelial Caco-2 cells*. J Pharmacol Exp Ther. **311**:476-84 (2004).
40. F. Tang, K. Horie, and R. T. Borchardt. *Are MDCK cells transfected with the human mdr1 gene a good model of the human intestinal mucosa?* Pharm. Res. **19**:765-772 (2002).

41. S. Mankhetkorn, and A. Garnier-Suillerot. *The ability of verapamil to restore intracellular accumulation of anthracyclines in multidrug resistant cells depends on the kinetics of their uptake.* Eur. J. Pharmacol. **343**:313-321 (1998).
42. A. De Milito, and S. Fais. *Tumor acidity, chemoresistance and proton pump inhibitors.* Future Oncology **1**:779-786 (2005).
43. DeMilito and S. Fais. *Proton pump inhibitors may reduce tumour resistance*. Expert. Opin. Pharmocother. **6**: 1049-1054 (2005).
44. L. E. Gerweck, S. Vijayappa, and S. Kozin. *Tumor pH controls the in vivo efficacy of weak acid and base chemotherapeutics*. Mol. Cancer Ther. **5**:1275-1279 (2006).
45. B. El Hafny, N. Cano, M. Piciotti, A. Regina, J. M. Scherrmann, and F. Roux. *Role of P-glycoprotein in colchicine and vinblastine cellular kinetics in an immortalized rat brain microvessel endothelial cell line*. Biochem. Pharmacol. **53**:1735-1742 (1997).