12535577. Publisher's official version: http://dx.doi.org/10.1016/S0169-409X(02)00174-6
Open Access version: http://kuscholarworks.ku.edu/dspace/.

Paper citation:
Young, A.M., Allen, C.E., and Audus, K.L.* (2003) Efflux transporters of the placenta. *Adv. Drug Del. Rev. 55*, 125-132. PMID: 12535577

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Keywords:
P-glycoprotein; MDR1; MRP; BCRP; Drug efflux; Placental barrier; Multidrug resistance

Abstract:
The use of pharmaceuticals during pregnancy is often a necessity for the health of the mother. Until recently, the placenta was viewed as a passive organ through which molecules are passed indiscriminately between mother and fetus. In reality, the placenta contains a plethora of transporters, some of which appear to be specifically dedicated to removal of xenobiotics and toxic endogenous compounds. Drug efflux transporters such as P-glycoprotein (P-gp), several multidrug resistant associated proteins (MRPs) and breast cancer resistant protein (BCRP) may provide mechanisms that protect the developing fetus. Bile acid transporters may also play a role in exporting compounds back into the maternal compartment. Steroid hormones directly influence the level of expression and function in some of these transporters. Investigating the link between the hormones of pregnancy and these drug efflux transporters is one possible key in developing strategies to deliver drugs to the mother with minimal fetal risk.

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Acknowledgements

The studies summarized from Dr. Audus' laboratory were supported by NIH grants P01HD-3-9878 and N01DA-4-7405.

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1. Introduction

Historically, the human placenta has been viewed as a protective barrier as well as a site for nutrient and waste exchange between mother and fetus [1]. However, instances of drug-induced birth defects have been in part blamed on the placenta's apparent "leakiness" to maternal blood-borne agents. Accordingly, the medical community generally discourages the use of all drugs during pregnancy. In reality, many women require drug therapies during pregnancy for illnesses such as asthma, diabetes, and HIV [1]. The unfortunate aspect of current research in this area is that due to the unique anatomical properties of the human placenta, no animal model exists that can reliably predict the potential side effects of a drug taken during pregnancy [2]. Therefore, in current practice a woman takes a drug out of necessity in pregnancy against the risk of possible adverse effects on the fetus and possible teratogenic effects which can only be assessed at birth.

Recently, a group of transporters in the ATP binding cassette (ABC) superfamily have been found in the placenta. These include the multidrug resistant gene product 1 (MDR1) also known as P-glycoprotein (P-gp), several of the multidrug resistance associated proteins (MRPs), and breast cancer resistance protein (BCRP). All these proteins were originally implicated in multidrug resistance in tumors [3-5]. However, further research has demonstrated that these transporters are distributed throughout many normal tissues of the body. The transporters seem to be concentrated in organs involved in detoxification processes (liver, kidney, etc.) [6].

ABC transporters exhibit a very broad substrate structure specificity including many xenobiotics as well as endogenous compounds such as steroids. Table 1 summarizes some of the ABC transporters and their substrate specificities. The general consensus about the physiological function of the ABC transporters is that they provide protection from a diverse

group of xenobiotics [6]. In addition, they may protect tissues from harmful effects of endogenous compounds. For example, MDR1 or P-gp is believed to regulate glucocorticoid levels in the adrenal glands [7].

Current research is focusing on the role of efflux proteins in the human placenta and the implications for drug distribution. Early studies suggest that P-gp serves to export xenobiotics into the maternal blood supply, thereby *reducing* chemical exposure in the fetal compartment [8]. The rate-limiting barrier in the placenta to permeation of substances between mother and fetus is a single layer of trophoblasts separates the maternal blood from the fetal capillaries [2]. In early pregnancy, the placenta is primarily composed of cytotrophoblasts, which continually fuse together to form multinucleate syncytiotrophoblasts as pregnancy progresses. Fetal capillaries, supplied by the umbilical artery and drained by the umbilical vein, are found within "fingerlike" structures, villi, within the placenta. Syncytiotrophoblasts form the outer layer of a villus and are bathed in the maternal blood on one side and are immediately adjacent to fetal capillaries on the opposite side. Figure 1 illustrates the unique anatomical arrangement of the placental barrier (syncytiotrophoblast layer) and the fetal capillaries. Understanding the role of efflux transporters in the trophoblast layer is a crucial element in devising new drug delivery strategies for pregnant women. Recently, primary cultures of human cytotrophoblasts and trophoblast-like cell-lines such as BeWo, JAr, and HRP-1 have aided in this process [9-11].

Steroid modulation of efflux transporter expression and activity is one possible key component. Hormonal levels vary widely among women, but the placenta produces roughly 1 millimole of progesterone per day at 40 weeks gestation [12]. Progesterone has been shown to affect P-gp activity in other cell types, so investigation of its influence in the placenta is vital [13]. Modulating placental efflux transporters through hormone-related mechanisms may

provide an opportunity to prepare the placenta for administration of drugs during pregnancy to effectively minimize the risk to the fetus. The following discussion summarizes the current knowledge regarding prominent placental efflux proteins.

2. P-glycoprotein

MDR1 or P-gp is the best characterized drug efflux transporter in the placenta. P-gp's size ranges from roughly 160 to 190 kilodaltons, and it is composed of 12 putative transmembrane domains (TMDs) and 2 nucleotide binding domains (NBDs) with ATPase activity [14].

Cordon-Cardo et al. used immunohistochemistry to show broad P-gp expression throughout body tissues, including placental trophoblasts [6]. Due to the presence of P-gp in a wide variety of tissues, each with a different physiological function, the researchers asserted that P-gp's function in normal tissues might not be limited to export of xenobiotics. In addition, despite the fact that the primary sequence is conserved, tissue-specific function of P-gp is a possibility.

There exists some controversy regarding the level of expression during stages of pregnancy. Most researchers believe that P-gp is expressed and active throughout pregnancy [15,16], particularly cytotrophoblasts [15,17]. Macfarland and colleagues argued that P-gp activity decreased in the later stages of pregnancy and was not limited to trophoblasts [3]. Subsequent research with microvillar membranes of term human trophoblasts refutes that claim [17].

Recent studies in mice have shown detrimental effects to the fetus when P-gp is either knocked out or inhibited. Lankas et al. found that mdr1a knockout mice lack placental P-gp and

have an increased fetal susceptibility to avermectin-induced teratogenicity [18]. Further experiments with bred heterozygotes for mdr1a and mdr1b showed that homozygous negative offspring had the most accumulation or drug exposure, followed by heterozygotes, then homozygous positive offspring. In addition, the administration of P-gp inhibitors completely inhibited placental P-gp activity in heterozygous dams [19]. These two studies suggest that placental P-gp is active and necessary in reducing fetal drug exposure.

In vitro models have also been used to explore the cellular level role of P-gp in placenta. Pavek et al used dually perfused rat placenta to monitor the transplacental passage of cyclosporine. As previously shown, P-gp acted to export the drug back into the maternal compartment [20]. Cell-lines derived from human choriocarcinomas, though not identical to the human syncytiotrophoblast layer forming the placental barrier *in vivo*, have been extremely valuable tools in assessing P-gp expression, activity, and modulation in the placenta. The BeWo cell line, for instance, has been shown to have similar biochemical markers and permeability properties when compared to trophoblasts [9]. Unlike primary cultures, BeWo cells form confluent, polarized monolayers in culture, so they can be used to study transplacental transport of drugs. Utoguchi et al. showed the increased accumulation of the P-gp substrates Calcein AM and vinblastine in the presence of a variety of inhibitors, as well as a Western blot demonstrating protein expression [21]. Another group found that the basolateral (fetal) to apical (maternal) transport of P-gp substrates exceeded the apical to basolateral transport. In the presence of known P-gp inhibitors, the polarized transport was negated. Vesicles prepared from of the apical and basolateral membranes also indicate that P-gp is localized to the maternal surface of trophoblasts [22].

The gene encoding P-gp, MDR1, has polymorphisms that occur with relative frequency among the population. Immunohistochemical staining reveals that several mutations resulted in a decreased level of placental P-gp [23]. The clinical implications in the human placenta, however, are currently unknown and/or are unrecognized.

Another important element to consider when studying placental P-gp is the effect of steroids. While the exact nature of steroid modulation of P-gp is unknown, many other studies support a link between the two. Glucocorticoids are widely regarded as P-gp substrates. Gruol and Bourgeois modified known steroids to produce compounds that were potent P-gp inhibitors as well as glucocorticoid receptor agonists [7]. Through the study of steroid analogues, the substituents required for transport have been determined. The most efficiently transported substrates have 11, 17, and 21 hydroxyl groups [7]. Vo and Gruol found that 2 and 20 carbon keto groups and a 17 hydroxyl group are ideal for P-gp inhibitory activity. In studies in which murine mdr1 mutations were transfected into human cells and steroid accumulation was monitored, the resultant mutations altered P-gp's ability to recognize steroids, especially those with the17α hydroxyl and 20 keto oxygen constituents [24].

Progesterone, the primary hormone of pregnancy, lacks all three hydroxyl groups necessary for P-gp transport, yet it is shown to bind to the protein [25]. Depending on the study, progesterone has shown both inhibitory (above 10 μM) and stimulatory (below 1 μM) effects on P-gp transport [13,25]. In their efforts to identify specific binding sites on P-gp, Shapiro and colleagues found that progesterone binds at a nontransporting, allosteric site and stimulation of Hoechst 33342 (H site) and Rhodamine 123 (R site) transport in the presence of 1 μM progesterone [25]. Although progesterone's effects specifically on placental P-gp have not been demonstrated, Yang et al. have performed experiments monitoring the effect of

progesterone on P-gp in pregnancy tissues. Progesterone was able to inhibit ^{3}H-azidopine photoaffinity labeling of P-gp in the endometrium of the gravid uterus in mouse. In addition, they demonstrated that progesterone was able to reverse vinblastine resistance in multidrug resistant cells [26].

Progesterone and chemically-related compounds have been examined to assess the relationship between structure and P-gp binding. One such study found that 5β progesterone metabolites were more effective P-gp inhibitors than 5α, suggesting a stereochemical interaction with the protein [27]. In another set of experiments, C-7 analogues of progesterone enhanced doxorubicin accumulation in P-gp expressing cells. Some of the analogues inhibited P-gp as effectively as cyclosporin A and verapamil. One of the compounds used also showed decreased affinity for progesterone receptors, decreasing the likelihood of progesterone toxicity [28].

Since progesterone is an endogenous and relatively nontoxic molecule, there has been much interest in co-administering it with antitumor agents to reverse multidrug resistance so common in cancer therapies. A phase I trial of doxorubicin and high dose progesterone showed that the steroid was able to increase doxorubicin activity without altering the pharmacokinetics. Other actions would be required to reduce systemic toxicity, but progesterone shows potential as a clinical P-gp inhibitor [29].

The steroid and xenobiotic receptor (SXR) is a transcription factor that has been shown to induce proteins that are designed for xenobiotic efflux and metabolism, such as P-gp and cytochrome P450 3A4 [30]. Steroid hormones, in particular the pregnanes, increase the levels of P-gp in various cell lines [31,32]. Obviously, this could have a major impact in the placenta where P-gp is present and progesterone (as well as other pregnanes) is in abundance.

While direct clinical evidence is still lacking, placental P-gp likely *reduces* xenobiotic accumulation in the fetal compartment. The effects of steroids are still unclear, however, their overall roles seems to be a modulation of xenobiotic and hormonal transport across the placenta.

3. Multidrug Resistance-associated Proteins (MRP)

Multidrug Resistance-associated Proteins (MRPs) are a separate family of efflux transporters. At this point, there are eight known MRPs, six of which have been fully sequenced [33]. Their size and function vary greatly. MRPs 1,2,3 and 6 have seventeen putative transmembrane domains, as opposed to the twelve found in MRPs 4 and 5. It appears that MRPs 1 through 3 are organic anion transporters. Often, the drugs transported are glutathione or glucuronate conjugates. Neutral drugs can also be cotransported with glutathione [34]. Seelig and colleagues determined that the substrate specificity for MRP1, in terms of electron donating groups, is quite similar to P-gp. The main difference is that P-gp shows a preference in transporting organic cations [35]. MRPs 4 and 5 appear to be nucleotide analogue pumps [36].

MRPs are distributed throughout the body, particularly the liver. MRP2 is believed to be a major transporter for biliary excretion of organic anions. Individuals with certain MRP2 mutations show symptoms of Dubin-Johnson syndrome, a condition characterized by hyperbilirubinemia [37]. MRPs 4 and 5 can readily transport cyclic nucleotides, so they purportedly play a role in cellular signaling pathways. There are few proposed physiological functions for the other MRPs, but they remain largely unknown [36].

The presence of MRPs in the placenta is somewhat controversial. Different laboratories have confirmed and have denied the presence of MRP1 in synctiotrophoblasts [38,39]. Using immunofluorescent staining of placental slices, St. Pierre et al. found MRPs 1 to 3 on the

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maternal surface of trophoblasts surface. In the case of MRP1, most of the signal was detected in the fetal capillaries as opposed to the synctiotrophoblasts [4].

BeWo cell studies have also been used to determine the extent of MRP expression. In situ RT-PCR was used to show MRP1 expression in the cell line [40] and additional studies showed that MRP1 and 5 were present. In the same study, MRP1 and 5 were observed in first trimester and third trimester placentas. In functional studies, the efflux of unconjugated bilirubin by BeWo cells was inhibited by MK571, an MRP inhibitor [41].

The overall effect of the MRPs on xenobiotic removal is unknown. However, a growing body of evidence suggests that, much like P-gp, it can function to reduce xenobiotic accumulation in the fetal compartment.

4. Breast Cancer Resistance Protein (BCRP)

Breast Cancer Resistance Protein (BCRP) is a newly discovered ABC transporter isolated from human breast cancer cells that were selected with doxorubicin in the presence of verapamil. Recently, proteins with minor amino acid differences were cloned from mitoxantrone resistant colon cancer cells and human placenta cells. These new proteins were named mitoxantrone reistance (MXR) and placenta ABC (ABCP) transporters, respectively [42]. One difference between BCRP and the other ABC transporters is the fact that it is composed of only one transmembrane region and one ATP-binding domain while other transporters are composed of two transmembranes regions and two ATP-binding domains [43]. BCRP's homology with the Drosphilia white gene family, a white eye pigment gene, suggests that BCRP requires heterodimerization or homodimerization in order to function in the transport activity of cytotoxic agents [44]. For this reason, BCRP is referred to as a half- transporter.

Consistent with P-gp and the MRPs, BCRP also confers resistance to a variety of drugs. The drug displaying the highest resistance due to BCRP appears to be mitoxantrone. Lesser but still significant resistance is observed with anthracyclines, daunorubicins, doxorubicin, camptothecins [45] and its derivatives, mainly topotecan and SN-38. BCRP also displays cross-resistance to many topoisomerase I inhibitors. Some cross-substrate recognition with P-gp and BCRP is evident. The inhibitor, GF120918, for example, is perceived as a multi-inhibitor because it is found to be highly effective at reversing both P-gp mediated and BCRP mediated multidrug resistance [46]. BCRP's drug resistance is complex due to it's existence as a half-transporter. BCRP is believed to dimerize in order for it to participate in transport activity that results in different substrates corresponding to different dimerization partners. Differing affinities for substrates could also result from polymorphisms or mutations in BCRP [47]. Lysotracker is the only known substrate that is specific for BCRP alone. Other substrates shared with MDR are mitoxantrone, bisantrene, topotecan, prazosin, and rhodamine 123. The substrates shared by all three, MDR, MRP, and BCRP, are daunorubicin, doxorubicin, and epirubicin [48].

Work is in progress to find cell lines that express BCRP. So far the most pronounced resistance is found in the human breast, placenta, colon and gastric cell lines. Lesser but still significant resistance is observed in the human myeloma, small-cell lung, pancreatic, fibrosarcoma, and leukemia cell lines [49]. The highest resistance is observed in sublines of these various cell lines that were selected with different cytotoxic drugs. Therefore, it is believed that cells or cell lines that express low levels of BCRP may be readily induced after continuous exposure to chemotherapeutic agents.

Two newly developed monoclonal antibodies, BXP-34 and BXP-21, have been used to characterize the cellular localization of BCRP in normal human tissue. BXP-34 is suitable for immunoprecipitation and immunohistochemistry experiments, but not Western blots. On the other hand, BXP-21 is used is in Western blots as well as immunocytochemistry and

immunohistochemistry [5]. Consequently, the tools for characterizing BCRP are still emerging and the role of this transporter in moving endogenous substances or xenobiotics in the placenta is currently unknown.

Although BCPR's precise physiological role is not known, many scientists have made speculated on functions depending on the location of the protein *in vivo*. In the placenta, BCRP is believed to function in the maternal-fetal barrier by effluxing drugs away from the fetus, which results in protection of the fetus. BCRP is hypothesized to be important in preventing the intestinal (re-)uptake of drugs. Finally, BCRP has been found in the kidney suggesting that it might play a role in mediating hepatobiliary excretion of transported drugs [50].

5. Bile Acid Transporters

The transport of bile acids between maternal and fetal compartments is essential in maintaining a healthy pregnancy. MRP and BSEP (bile salt export pump, or sister P-gp) expression are interrelated, and can be induced by various steroid hormones and xenobiotics [51]. Xie and colleagues found that stimulation of SXR conferred resistance to lithocholic acid toxicity [52]. Although BSEP has not been found in placenta, potentially, SXR serves as a control center for a whole host of transporters as yet unknown. The hormonal activation of SXR makes the placenta an especially susceptible target, particularly in view of recent studies by Pascual et al. [53]. Pascual et al. used the placental efflux of bile acids to attempt development of a novel drug delivery technique that would reduce fetal cisplatin exposure. Cisplatin is used to treat ovarian cancer during pregnancy, often with detrimental effects to the fetus. By

attaching the drug to glycocholic acid, Pascual et al. were able to significantly reduce the cisplatin levels in both the maternal and fetal organs in a rat model [53]. The transporters involved were not identified specifically, but previous research found an anion exchanger system between bile acid and bicarbonate on the basolateral membrane of the trophoblast [54,55]. Although bile acid transporters are still relatively uncharacterized with respect to multidrug resistance, the Pascual et al. [53] study suggests the feasibility of considering certain efflux mechanisms in therapy schemes to reduce fetal drug exposure.

6. Conclusion

Ideally, a pregnant woman could take the drugs needed for optimum health, while doing no harm to the fetus. A realistic goal is to exploit tissue mechanisms that would reduce fetal drug exposure and recent evidence suggests that efflux transporters might be targeted to modulate drug distribution across the placental barrier. Understanding the complex nature of the functions at the molecular and cellular level, and the hormonal modulation of these efflux transporters will help to further this endeavor.

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Young, A.M., Allen, C.E., and Audus, K.L. (2003) Efflux transporters of the placenta. *Adv. Drug Del. Rev. 55*, 125-132. PMID: 12535577. Publisher's official version: http://dx.doi.org/10.1016/S0169-409X(02)00174-6
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Table 1. Summary of major drug efflux transporters and their association with the placenta

Transporter	Placental location	Substrates	Refs.
P-gp (MDR1)	Trophoblast	Organic cations, steroids, anticancer agents	3,6,13,20,25,35
MRP1	Trophoblast but predominantly in fetal capillary	Organic anions, glutathione and glucuronate conjugates	4,33,35
MRP2	Trophoblast	Similar to MRP 1 and 3	4,33
MRP3	Trophoblast	Similar to MRP 1 and 2	4,33
MRP4-5	Unknown	Nucleotide analogs	33,36
MRP6-8	Unknown	Unknown	33,36
BCRP(MXR, ABCP)	Trophoblast	Anticancer agents	5,44,45

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Figure legends

Figure 1. Illustration of the anatomical arrangement of the syncytiotrophoblast (placental barrier) and the fetal capillaries as would appear in a cross-section of a human placental villus.

Figure 1.