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ROLE OF MEMBRANE TRANSPORTERS, P-GLYCOPROTEIN AND MRP1, IN THE PLACENTAL TRANSFER OF DRUGS

With Special Reference to Saquinavir and Quetiapine

by

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To my dad and to my family

ABSTRACT

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Drug transporting membrane proteins are expressed in various human tissues and blood-tissue barriers, regulating the transfer of drugs, toxins and endogenous compounds into or out of the cells. Various *in vitro* and animal experiments suggest that P-glycoprotein (P-gp) forms a functional barrier between maternal and fetal blood circulation in the placenta thereby protecting the fetus from exposure to xenobiotics during pregnancy. The multidrug resistance-associated protein 1 (MRP1) is a relatively less studied transporter protein in the human placenta.

The aim of this study series was to study the role of placental transporters, apical P-gp and basal MRP1, using saquinavir as a probe drug, and to study transfer of quetiapine and the role of P-gp in its transfer in the dually perfused human placenta/cotyledon. Furthermore, two *ABCB1* (encoding P-gp) polymorphisms (c.3435C>T, p.Ile1145Ile and c.2677G>T/A, p.Ala893Ser/Thr) were studied to determine their impact on P-gp protein expression level and on the transfer of the study drugs. Also, the influence of the P-gp protein expression level on the transfer of the study drugs was addressed. Because P-gp and MRP1 are ATP-dependent drug-efflux pumps, it was studied whether exogenous ATP is needed for the function of ATP-dependent transporter in the present experimental model.

The present results indicated that the addition of exogenous ATP was not necessary for transporter function in the perfused human placental cotyledon. Saquinavir and quetiapine were both found to cross the human placenta; transplacental transfer (TPT_{AUC} %) for saquinavir was <0.5% and for quetiapine 3.7%. Pharmacologic blocking of P-gp led to disruption of the blood-placental barrier (BPB) and increased the placental transfer of P-gp substrate, saquinavir, into the fetal circulation by 6- to 8-fold. In reversed perfusions P-gp, MRP1 and possibly OATP2B1 had a negligible role in the fetal-to-maternal transfer of saquinavir. The TPT_{AUC} % of saquinavir was about 100-fold greater from the fetal side to the maternal side compared with the maternal-to-fetal transfer. P-gp activity is not likely to modify the placental transfer of quetiapine. Higher P-gp protein expression levels were associated with the variant allele 3435T, but no correlation was found between the TPT_{AUC} % of saquinavir and placental P-gp protein expression.

The present results indicate that P-gp activity drastically affects the fetal exposure to saquinavir, and suggest that pharmacological blockade of the P-gp activity during pregnancy may pose an increased risk for adverse fetal outcome. The blockade of P-gp activity could be used in purpose to obtain higher drug concentration to the fetal side, to prevent diseases (for example to decrease virus transfer to fetal side) or to treat sick fetus.

Key words: P-glycoprotein, MRP1, placenta, saquinavir, quetiapine, blood-placental barrier, *ABCB1*, genotype, polymorphism, adenosine triphosphate

TIIVISTELMÄ

Melissa Rahi

SOLUKALVON KULJETUSPROTEIINIEN, P-GLYKOPROTEIININ JA MRP1:N MERKITYS ISTUKAN LÄÄKEAINELÄPÄISEVYYDESSÄ -erityisesti sakinaviirin ja ketiapiinin

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Solukalvon kuljetusproteiineja, on useissa ihmisen elimissä ja veri-kudos esteissä, joissa ne säätelevät lääkkeiden, toksiinien ja endogeenisten aineiden kudosläpäisevyyttä soluun sisälle tai sieltä ulos. Useat *in vitro* ja eläinkokeet osoittavat, että P-glykoproteiini (P-gp) muodostaa toiminnallisen esteen äidin ja sikiön verenkierron välille istukassa ja siten suojelee sikiön altistumista vieraille aineille raskauden aikana. Multidrug resistance-associated protein 1 (MRP1) on vielä suhteellisen vähän tutkittu solukalvoproteiini ihmisen istukassa.

Tämän tutkimussarjan tarkoituksena oli tutkia istukan solukalvon kuljetusproteiinien, sekä apikaalisen P-gp:n, että basaalisen MRP1:n toimintaa, käyttämällä sakinaviiriä koetinlääkeaineena ja tutkia avoimella istukkaperfuusiomenenetelmällä ketiapiinin läpäisevyyttä istukassa sekä P-gp:n roolia sen kudosläpäisevyydessä. Lisäksi tutkimussarjassa selvitettiin kahden *ABCB1* (P-gp:a koodaava geeni) polymorfian (c.3435C>T, p.Ile1145Ile ja c.2677G>T/A, p.Ala893Ser/Thr) vaikutusta P-gp:n ilmentymiseen istukkakudoksessa ja tutkittavien lääkeaineiden kudosläpäisevyyteen sekä määritettiin istukassa ilmentyvän P-gp:n proteiinitason vaikutus tutkittavien lääkeaineiden kudosläpäisevyyteen. P-gp:n ja MRP1:n toiminta on ATP (adenosiinitrifosfaatti)- riippuvaista, minkä vuoksi aluksi selvitettiin onko ATP:n lisääminen tarpeellista tässä tutkimusmallissa ATP-riippuvaisen solukalvon kuljetusproteiinin toiminnan kannalta.

Tutkimustulokset osoittivat, että ATP:n lisääminen ei vaikuta P-gp:n toimintaan istukkaperfuusioissa. Sakinaviiri ja ketiapiini läpäisevät istukan; sakinaviirin istukkaläpäisevyys äidin puolelta sikiön puolelle oli <0.5% ja ketiapiinin istukan läpäisevyys 3.7%. P-gp:n toiminnan farmakologinen estäminen johti istukka-veri esteen häiriöön ja lisäsi P-gp substraatin, sakinaviirin, istukan läpäisykykyä sikiön puolelle 6-8 kertaisesti. Toisen suuntaisessa eli takaisin perfuusiossa (sikiöstä äitiin) P-gp:lla, MRP1:llä ja mahdollisesti OATP2B1:llä oli mitätön rooli sakinaviirin istukan läpäisevyydessä. Sakinaviirin istukanläpäisevyys oli noin 100 – kertaa suurempi perfuusioissa sikiöstä äitiin verrattuna perfuusioihin äidistä sikiöön. P-gp:n toiminta ei näytä vaikuttavan ketiapiinin istukan läpäisevyyteen. *ABCB1* 3435T varianttialleeliin liittyi korkeampi P-gp:n proteiinin ilmentymisenmäärä istukassa, mutta tällä ei ollut merkitystä sakinaviirin istukkaläpäisevyyden määrään.

Nämä tulokset osoittavat, että P-gp:n toiminta merkittävästi vaikuttaa sikiön altistumiseen sakinaviirille ja osoittaa, että P-gp:n toiminnan farmakologinen estäminen raskauden aikana voi johtaa suurentuneeseen altistukseen sikiön kannalta haitallisille aineille tai P-gp:n toiminnan estämistä voidaan käyttää hyväksi, esimerkiksi, estämään virusinfektiota sikiössä tai lääkitsemään sikiötä.

Avainsanat: P-glykoproteiini, MRP1, istukka, sakinaviiri, ketiapiini, veri-istukkaeste, *ABCB1*, genotyyppi, polymorfismi, adenosiinitrifosfaatti

CONTENTS

ABBREVIATIONS

ABC	ATP-binding cassette
ABCB1	Gene symbol of P-glycoprotein
Abcb1a/b	Gene symbol of P-glycoprotein (mouse)
ABCC1	Gene symbol of MRP1
Abcc1	Gene symbol of Mrp1 (mouse)
c.	for a cDNA sequence
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under curve
AUC_{FA}	Area under curve in fetal artery
AUC_{MV}	Area under curve in maternal venous outflow
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein
BPB	Blood-placental barrier
BSEP	Bile salt export pump
CSF	Cerebrospinal fluid
CYP	Cytochrome P450
FIC1	Familiar intrahepatic cholestasis 1
LLOQ	Lower limit of quantification
MDR	Multidrug resistance
MDR1	Synonym of P-glycoprotein
mRNA	messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
Mrp	Multidrug resistance-associated protein in mouse
OAT	Organic anion transporter
OATP	Organic anion transporting protein
OCT	Organic cation transporter
OCTN	Novel organic cation transporter
p.	for a protein sequence
P-gp	P-glycoprotein
PXR	Pregnane X receptor
rpm	rounds per minute
SD	Standard deviation
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin reuptake inhibitor
TI	Transfer index
TPT	Transplacental transfer
TPT%	Transplacental transfer percentage
TPT_{AUC} %	Absolute fraction of the dose crossing the placenta
TPT (%) at 120min	The end-perfusion drug transfer (percentage)

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV.

I Rahi M, Heikkinen T, Hakkola J, Hakala K,Wallerman O, Wadelius M, Wadelius C, Laine K. 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: 65-71.

II Mölsä M#, Heikkinen T, Hakkola J, Hakala K, Wallerman O, Wadelius M, Wadelius C, Laine K. Functional role of P-glycoprotein in the human blood-placental barrier. Clin Pharmacol Ther 2005; 78: 123-31.

III Rahi M, Heikkinen T, Härtter S, Hakkola J, Hakala K, Wallerman O, Wadelius M, Wadelius C, Laine K. Placental transfer of quetiapine in relation to P-glycoprotein activity. J Psychopharmacol. 2007; 21: 751-6.

IV Rahi M, Heikkinen T, Hakala K and Laine K. The effect of probenecid and MK-571 on the feto-maternal transfer of saquinavir in dually perfused human term placenta. Submitted.

#Melissa Rahi (née Mölsä).

1 INTRODUCTION

The role of transporter proteins in tissue permeability of drugs has been shown to be important. An ATP-dependent drug efflux pump, P-glycoprotein (P-gp), has been extensively studied regarding multidrug resistance in tumor cells (Juliano and Ling 1976, Tan et al. 2000, Modok et al. 2006, Zhou 2008). P-gp was first described in hamster´s ovary tumor cells, preventing access of anticancer agents to cells as a result of P-gp overexpression (Juliano and Ling 1976). Expression of P-gp has been identified widely, for example, in the capillary endothelial cells of the brain (blood-brain barrier), intestine, kidney, liver, testes and placenta (blood-placental barrier) (Thiebaut et al. 1987, Cordon-Cardo et al. 1989). Many of these tissues are extremely important for the absorption, distribution and elimination of drugs.

In the placenta, maternal and fetal circulations are isolated from each other with a placental barrier. This functional barrier contains the syncytiotrophoblasts with various transporters, which affect the pharmacokinetics and pharmacodynamics of drugs by effluxing (throwing out of the placenta) or influxing (throwing into the placenta) drugs and compounds (van der Aa 1998, Unadkat et al.2004). P-gp is expressed on the apical (maternal) side of syncytiotrophoblasts, effluxing drugs back to the maternal circulation (Ushigome et al. 2000, Mathias et al. 2005), whereas multidrug resistance-associated protein 1 (MRP1) is considered to be basal efflux transporter and to facilitate transfer of compounds to the fetal circulation as an efflux transporter (Unadkat et al. 2004, Bakos et al. 2007, Myllynen et al. 2007).

MRP1 has been shown to have a crucial role in permeability of drugs, for example, in the brain and in gastrointestinal tissues, but its functional role in human placenta is not clear (Wijnholds et al. 2000, Johnson et al. 2001). Recent studies indicate that many commonly prescribed drugs are substrates of P-gp and MRP1, and many of the drugs also inhibit their function (Haimeur et al. 2004, Marzolini et al. 2004). The immunodeficiency virus protease inhibitor, saquinavir, is a well established substrate of P-gp (Smit et al. 1999) and MRP1 (Williams et al. 2002). Quetiapine (Meats 1997) is a widely used atypical antipsychotic drug and considered to be a substrate of P-gp (Boulton et al. 2002, Ela et al. 2004), although there is also a contradictory study (Grimm et al. 2005). Only little is known about the use of quetiapine during pregnancy.

The main objective of this study was to clarify the role of placental transporters in the transfer of saquinavir and quetiapine by modulating the function of these transporters with well-established inhibitors. Earlier, contradictory findings regarding the relationship between the *ABCB1* polymorphisms and P-gp protein expression level and possible changes in P-gp function have been published. In this thesis, we also studied these associations.

2 REVIEW OF THE LITERATURE

2.1 Human placenta as a model in research of drug permeability

2.1.1 Blood-placental-barrier (BPB)

The human placenta is a complex transport system, a barrier, between the mother and the developing fetus. The umbilical cord (consisting of two umbilical arteries and one umbilical vein) and the placenta form a particular entity (Fig. 1). Structurally, the human placenta is formed of 10-40 compartments, cotyledons, which act as a vascular unit in the placenta (Myren et al. 2007). In cotyledon, two circulations are isolated from each other with a placental barrier. This barrier regulates the exchange of endogenous components, nutrients, gases, wastes and foreign molecules, including drugs, between these two circulations. This barrier consists of a villous tree containing fetal capillary endothelia and a trophoblast cell layer (fetal blood), and this villous tree is surrounded by the intervillous space (maternal blood) (van der Aa 1998, Young et al. 2003, Unadkat et al. 2004) (Fig. 2). The trophoblast layer contains syncytiotrophoblast cells with various transporters (Fig. 3). The apical side of syncytiotrophoblast border is bathed by the maternal blood, and facing fetal side lies the basal membrane which borders the fetal blood. The syncytiotrophoblast layer is indeed in direct contact with maternal blood. (Moe et al. 1995, van der Aa 1998, Ganapathy et al. 2000, Ushigome et al. 2000, Unadkat et al. 2004).

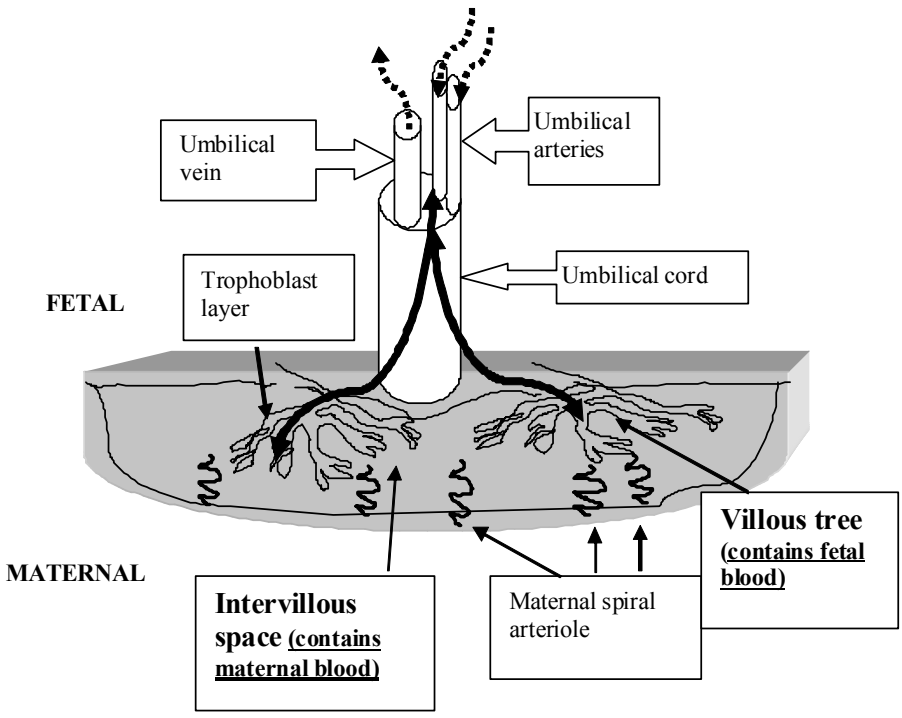

Figure 1. Scheme of placental circulation.

Most compounds and drugs cross the placenta. Principally, compounds with low molecular weight (< 600 Da), high lipophilicity, low degree of ionization or low protein binding can cross the placenta by simple diffusion according to the concentration gradient. Large molecules, like proteins and antiglobulins can pass the placenta by pinocytosis, while very small molecules go through the placenta via small pores. (Bourget et al. 1995, Sastry 1999, Myllynen et al. 2007, Myren et al. 2007). Immunoglobulin G (IgG) is the only one of the major five classes of antibodies which is transferred across the placenta and attends to fetal immunity. IgG pass the placenta in coated vesicles actively with bound receptor molecules. (Kane et al. 2009).

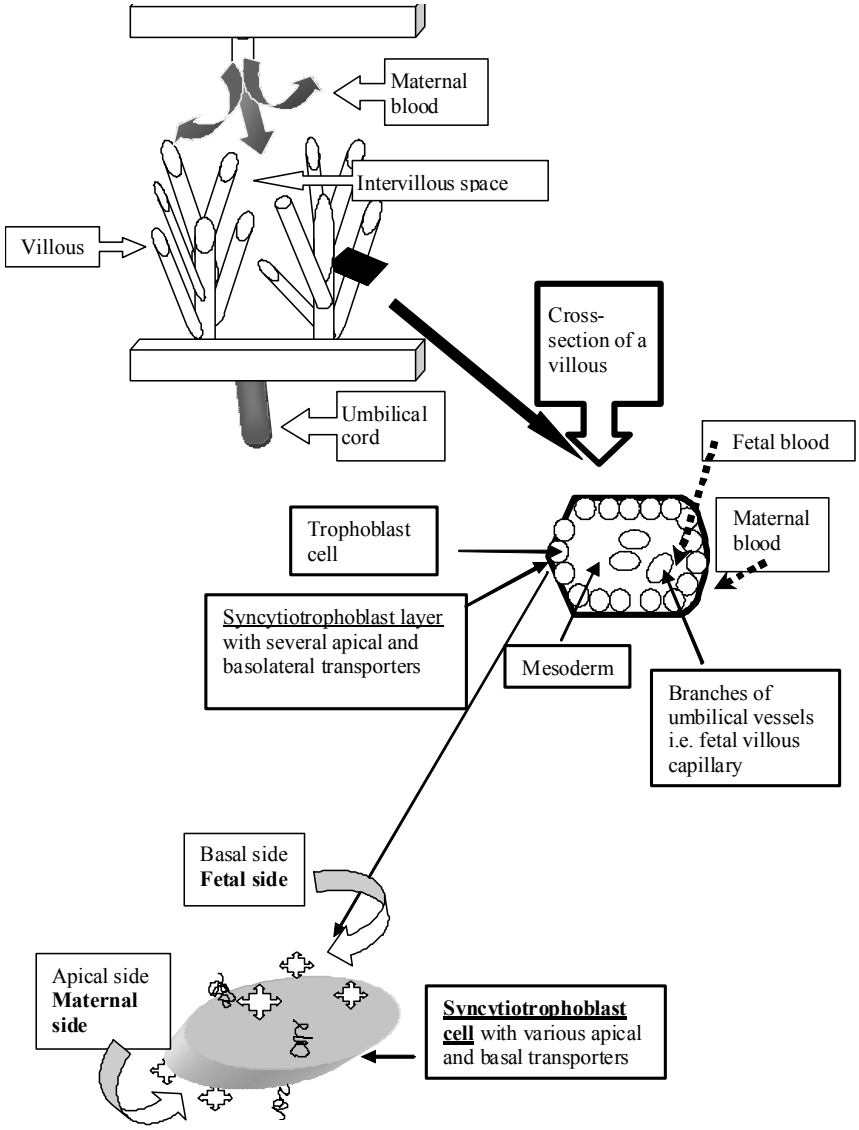

Figure 2. Schematic presentation of blood-placental barrier: villous trees contain fetal capillary endothelia (fetal blood) and trophoblast cells, surrounded by intervillous space (containing maternal blood). The presentation is adopted and modified from Ushigome et al. 2000.

Nevertheless, transfer across the placenta is often mediated by active transporter proteins localized in the syncytiotrophoblast. The passage of the compounds from one blood compartment to another requires their movement across the apical and basal (basolateral) membranes of the syncytiotrophoblast (Unadkat et al. 2004). The active transporter proteins are expressed on both the apical and basal membrane of the syncytiotrophoblast (Moe A 1995, van der Aa 1998, Ganapathy et al. 2000, Ushigome et al. 2000). The transporters can be efflux transporters, which throw compounds back to the circulation (out of the placenta) or influx transporters, facilitating transfer of compounds into the placenta. The transfer is regulated also by influx-efflux (equilibrative transporters) transporters, which transport substrates into or out of the placenta i.e. into or out of the fetal or maternal blood flow depending on the direction of the concentration gradient (Unadkat et al. 2004). It has been suggested that some of the apical and basal transporters can act together allowing special compounds movement directly across placenta (Grube et al. 2007). This has been observed also with transporters in human hepatocytes (Su et al. 2004).

2.1.2 Apical and basal drug transporters in human placenta

In addition to passive transfer, the active placental transporters mediate transfer of drugs and endogenous compounds between maternal and fetal compartments. Several transporters have been shown to be present in placenta. Most transporters are found in extensively studied rodents, but an increasing amount of transporters is found in the human placenta (Ganapathy et al. 2000, Leazer et al. 2003, Unadkat et al. 2004, Evseenko et al. 2006). The functional role of these various transporters has been clarified only partly.

To date a great number of drug transporters have been found in the human placenta; P-glycoprotein (P-gp, MDR1), multidrug resistance protein 3 (MDR3), breast cancer resistance protein (BCRP), multidrug resistance-associated protein 1-5 and 7-8 (MRP1-5, 7-8), organic anion transporting polypeptide 1A2, 2B1, 1B1, 3A1, 4A1, 1B3 (OATP1A2, OATP2B1, OATP1B1, OATP3A1, OATP4A1, OATP1B3), organic cation transporter 3 (OCT3), novel organic cation transporters/carnitine transporters 1 and 2 (OCTN1, OCTN2), novel organic anion transporter 1 and 4 (OAT1 and 4), human equilibrative nucleoside transporters 1 and 2 (hENT1 and 2) familial intrahepatic cholestasis 1 (FIC1), the bile salt export pump (BSEP), sodium-dependent organic anion transporter (SOAT), norepinephrine transporter (NET), serotonin transporter (SERT) and sodium/multivitamin transporter (SMVT). (Wang et al. 1999, Ganapathy et al. 2000, St-Pierre et al. 2002, Langmann et al. 2003, Patel et al. 2003, Sato et al. 2003, Ugele et al. 2003, Lahjouji et al. 2004, Syme et al. 2004, Unadkat et al. 2004, Mathias et al. 2005, Meyer zu Schwabedissen et al. 2005a,b, Sata et al. 2005, Geyer et al. 2007, Serrano et al. 2007, Zhou and You 2007, May et al. 2008). (Table 1). Each transporter protein is encoded by a known gene (genes are also listed in Table 1). The expression of transporter in the human placenta is shown in Figure 3.

Table 1. Examples of transporters known to express in the human placenta in mRNA or protein level.

Transporter		**Gene symbol**
BCRP (protein)	Breast cancer resistance protein	*ABCG2*
BSEP (*) (protein)	Bile salt export pump	*ABCB11*
FIC1 (*) (mRNA)	Familiar intrahepatic cholestasis 1	*ATP8B1*
hENT1 and 2 (*) (protein)	Human equilibrative nucleoside transporters 1 and 2	*SLC29A1* *SLC29A2*
MDR3 (protein)	Multidrug resistance protein 3	*ABCB4*
MRP1 (protein) **MRP2 (protein)** **MRP3 (protein)** **MRP4 (mRNA)** **MRP5 (protein)** **MRP7 (mRNA)** **MRP8 (mRNA)**	Multidrug resistance- associated protein 1-5, 7-8 (i.e. Multidrug related protein)	*ABCC1-5, ABCC7 and ABCC11*
NET (protein)	Norepinephrine transporter	*SLC6A2*
OAT1 (mRNA) **OAT4 (protein)**	Organic anion transporter 1 and 4	*SLC22A6* *SLC22A11*
OATP1A2 (OATP-A) (mRNA)	Organic anion transporting polypeptide 1A2	*SLCO1A2*
OATP1B1 (OATP-C) (mRNA)	Organic anion transporting polypeptide 1B1	*SLCO1B1*
OATP1B3 (*) (OATP-8) (mRNA)	Organic anion transporting polypeptide 1B3	*SLCO1B3*
OATP2B1 (OATP-B) (protein)	Organic anion transporting polypeptide 2B1	*SLCO2B1*
OATP3A1 (OATP-D) (mRNA)	Organic anion transporting polypeptide 3A1	*SLCO3A1*
OATP4A1 (OATP-E) (protein)	Organic anion transporting polypeptide 4A1	*SLCO4A1*
OCT3 (protein)	Organic cation transporter 3	*SLC22A3*
OCTN1 (protein) **OCTN2 (protein)**	Novel organic cation/carnitine transporter 1-2	*SLC22A4* *SLC22A5*
P-gp (protein)	P-glycoprotein	*ABCB1*
SERT (protein)	Serotonin transporter	*SerT*
SMVT (*) (protein)	Sodium/multivitamin transporter	*SLC5A6*
SOAT (*) (mRNA)	Sodium-dependent organic anion transporter	*SLC10A3*

(*) Information limited

OATP nomenclature is shown according to Hagenbuch and Meier 2004 (former name in brackets). References below refer to if transporter is expressed in mRNA or protein level in human placenta:

BCRP (Mathias et al. 2005), BSEP (Patel et al. 2003, Serrano et al. 2007), FIC1 (Patel et al. 2003), hENT1 and 2 (Govindarajan et al. 2007), MDR3 (Evseenko et al. 2006a), MRP1 (Nagashige et al. 2003), MRP2 (St-Pierre et al. 2000), MRP3 (Pascolo et al. 2003), MRP4 and MRP7 (Langmann et al. 2003), MRP5 (Meyer zu Schwabedissen et al. 2005b), MRP8 (Bera et al. 2001), NET (Ramamoorthy et al. 1993), OAT1 (Hosoyamada et al.1999), OAT4 (Ugele et al. 2003), OATB1A2, OATP1B1, OATP1B3 and OATP3A1 (Ugele et al.2003), OATP2B1 (St-Pierre et al. 2002), OATP4A1 (Sato et al. 2003), OCT3 (Sata et al. 2005), OCTN1 (Ganapathy et al. 2005), Evseenko et al. 2006), OCTN2 (Grube et al. 2005), P-gp (Mathias et al. 2005), SERT (Balkovetz et al. 1989), SMVT (Wang et al. 1999, Prasad et al. 1999) and SOAT (Geyer et al. 2007).

Active transporters have been shown to exist on both the apical and the basal side of syncytiotrophoblast (Ganapathy et al. 2000). The basal (or basolateral), fetal-facing membrane of the syncytiotrophoblast is known to express at least MRP1 (Nagashige et al. 2003), MRP5 (Meyer zu Schwabedissen et al. 2005b), MDR3 (Evseenko et al. 2006a), OATP2B1 (St-Pierre et al. 2002), OCT3 (Sata et al. 2005) and OAT4 (Ugele et al. 2003). OATP1B3 is reported to be basolateral uptake transporter (Ugele et al 2003, May et al 2008) and SOAT (SLC10A3) is found to be highly expressed in human placenta and expressed likely in basal side of syncytiotrophoblast as an uptake transporter (Geyer et al. 2007), but the information of those is still limited. There is some contradiction of MRP1 localisation in human placenta. MRP1 is shown to have basal membrane localisation in human placental trophoblast (Nagashige et al. 2003), however, St-Pierre et al. (2000) found some evidence of protein expression in the apical syncytiotrophoblast. (Fig. 3).

The apical side i.e. the maternal-facing membrane of syncytiotrophoblast expresses at least; P-gp (Ushigome et al. 2000), BCRP (Grube et al. 2007), MRP2 (St-Pierre et al. 2000, Meyer zu Schwabedissen et al. 2005a), MRP3 (St-Pierre et al. 2000) OATP4A1 (Sato et al. 2003), hENT1 (Unadkat et al. 2004), OCTN1 (Ganapathy et al. 2005, Evseenko et al. 2006b), OCTN2 (Lahjouji et al. 2004, Grube et al. 2005), NET (Ganapathy et al. 2000), SERT (Ganapathy et al. 2000) and SMVT (Wang et al. 1999). (Fig.3). The information on hENT2 is still limited, but it has been suggested to be expressed on the apical side of syncytiotrophoblast (Govindarajan et al. 2007). Also, the function direction of the transporter proteins (influx and/or efflux transporter) when it is known, is shown by arrows in Figure 3.

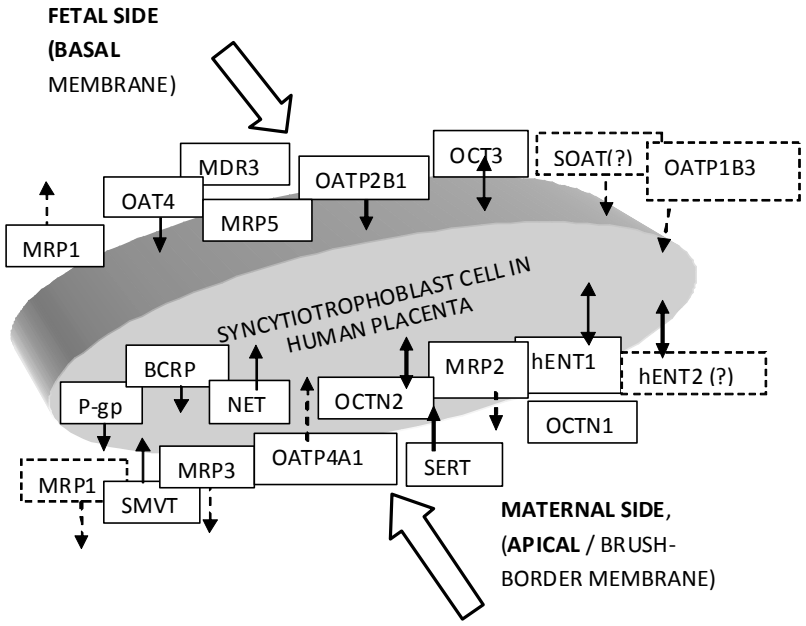

Figure 3. The transporters, which localization is known to be in human placenta (in basal/basolateral or in apical side of the syncytiotrophoblast cell). The function direction of the transporter protein (influx and/or efflux transporter) is shown by arrows. Dotted lines refer to the fact that more information is needed.

Several things affect the placental permeability in addition to transporter activity. Many changes occur in the placenta during pregnancy leading to changes in drug permeability (Myllynen et al. 2007). Such changes include, for example; a remarkable reduction in thickness of basolateral membrane (van der Aa 1998), increase in placental surface area and blood flow (Unadkat et al. 2004) and changes in maternal plasma protein concentrations affecting the permeability of highly protein-bound drugs (Hill et al. 1988, Unadkat et al. 2004).

The expression level of various placental transporters varies during each trimester of pregnancy (Patel et al. 2003, Unadkat et al. 2004, Sun et al. 2006). For example, the P-gp protein expression is highest in early pregnancy, up to about 45-fold higher vs. term placenta, whereafter the amount is reduced towards the end of pregnancy (Mathias et al. 2005). On the other hand, OATP2B1 and BCRP are expressed during the whole pregnancy without any remarkable changes in their expression level (St-Pierre et al. 2002, Mathias et al. 2005).

2.2 Drug transporters in drug permeability

2.2.1 History and significance for clinical pharmacokinetics

Drug transporters modify the pharmacodynamics and pharmacokinetics of drugs by effluxing (throwing out of the cell) or influxing (throwing into the cell) drugs, toxins and biochemical compounds in several tissues and blood-tissue barriers (Unadkat et al. 2004, Liang et al. 2006). The compounds which are substrates for transporters can be transported across biological membrane by facilitated diffusion (does not require energy, depends of electrochemical potential gradient of the substrate) and active transport. The active transporters can be primary active transporters (for example ABC transporters i.e. ATP binding cassette transporters), which use energy directly from ATP or a secondary active transporters (symport or antiport), which use energy from ion-channels. Naturally, some drugs pass membrane simple by the passive diffusion (occurs downhill – down an electrochemical potential gradient, but does not use any transporterprotein) (Goodman and Gilman´s 2006)

Thus, these membrane proteins also form an important part of the cell defence against antineoplastic drugs, which was first observed by the Danish physician Dano. In 1973, Dano demonstrated that multidrug resistant Ehrlich ascites cells were able to decrease intracellular daunorubicin concentration by active transport. Juliano and Ling discovered in 1976 a large glycoprotein in hamster ovary cells and named it P-glycoprotein (P-gp). Later it has been established that overexpression of the *ABCB1* gene (encoding P-gp) in tumor cells is associated with an acquired resistance to anticancer drugs (Ueda et al. 1987). In 1992, Cole et al. identified a new drug pump, the multidrug resistance-associated protein (MRP), which association with multidrug resistance has also been established (Cole et al. 1994, Grant et al. 1994).

Drug transporters are expressed widely in many tissues which are important for the absorption (lung and gut), metabolism and elimination (liver and kidney) of drugs.

These proteins also have a relevant role in maintaining the barrier function in many tissues such as the blood-brain-barrier (BBB), blood-cerebral spinal fluid barrier, blood-testis barrier and the maternal-fetal barrier (placenta) (Thiebaut et al. 1987, Fromm et al. 2004, Chang 2007). Subsequently, several drug transporters have been discovered in several tissues, a number of transporters have been cloned, and our knowledge of the molecular characteristics of individual transporters has increased.

Multidrug resistance (MDR) is a major clinical concern nowadays. The problems it might cause have been seen particularly in cancer treatment. MDR may also cause many drug interactions in everyday therapeutics. Also, overexpression of ATP-binding cassette (ABC) drug transporters, such as P-gp, BCRP and MRP1, may lead to MDR and has been found to be related to worse treatment outcome of cancer and also reduced overall survival (Fardel et al. 1996, Merino et al. 2004, Chang 2007, Zhou et al. 2008). Recent studies have indicated that understanding of MDR and having knowledge to use inhibitors of specific transporters to enable sufficient drug concentration in the target organ, can improve the clinical outcome of drug treatment (Fardel et al. 1996, Chang 2007, Bebawy and Sze 2008).

2.2.2 P-glycoprotein (P-gp)

2.2.2.1 Expression and function of P-gp in extra-placental tissues

Human P-gp, a 170 kDa transmembrane protein, has been widely studied with regard to the multidrug resistance phenomenon in tumor cells (Juliano and Ling 1976, Fardel et al. 1996, Zhou 2008).

P-gp functions as an ATP-depended drug-efflux pump that actively secretes a variety of drugs and toxins out of cells (Juliano and Ling 1976, Cordon-Cardo et al.1989, Nakamura et al. 1997). P-gp is a widely expressed drug transporter in humans (Thiebaut et al. 1987, Fardel et al. 1996, Johnstone et al. 2000) (Fig. 4). In the BBB, among other transporters, P-gp forms a functional barrier and restricts access of various pharmacological agents to the brain (Ramakrishnan 2003, Roberts and Goralski 2008, Zhou 2008). Similarly, P-gp in intestinal tissues can pump xenobiotics back into the gut lumen (Mayer et al. 1996, Sparreboom et al. 1997). A toxicity study with P-gp deficient mice by Lankas et al. in 1998 was among the first investigations to indicate the important role of P-gp in protecting the fetus from toxic compounds.

The functional role of P-gp in various human tissues has been extensively studied because there is evidence that many commonly prescribed drugs are substrates of P-gp, and many of these drugs also inhibit its function (Lin and Yamazaki 2003, Mizuno et al. 2003, Marzolini et al. 2004) (Table 3).

In humans, P-gp (MDR1) is encoded by the *ABCB1* gene (located on chromosome 7), whereas in mice the corresponding encoding genes are; *Abcb1a* and *Abcb1b* (Smit et al. 1999, Marzolini et al. 2004).

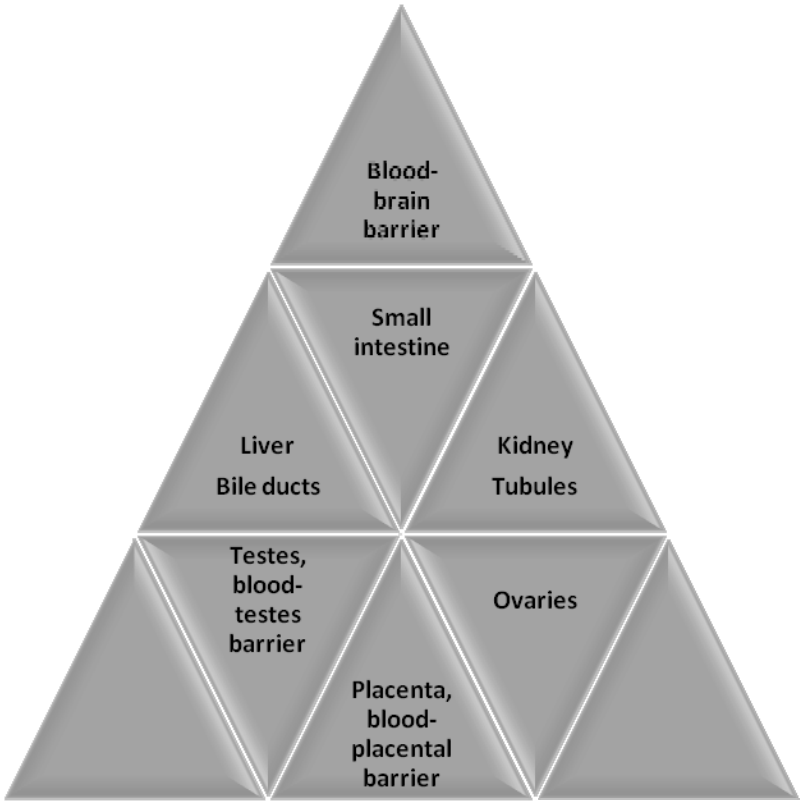

Figure 4. A schematic figure of P-glycoprotein tissue distribution

Several different methods have been developed to study the function of P-gp and the other transporters. There are studies with cultured cells; for example, with primary trophoblast cells, with human placental choriocarcinoma epithelial cells (BeWo cells) or with the Caco 2 cell line (human epithelial colorectal adenocarcinoma cells) (Sastry et al. 1999, Ushigome et al. 2000, Unadkat et al. 2004, Taur and Rodriquez-Proteau 2008). There are also studies with isolated tissues and cells of human placenta; for example, perfused placental cotyledon, villus preparation, trophoblast plasma membrane vesicles and isolated transporters and receptors (Sastry et al. 1999, Ushigome et al. 2000, Forestier et al. 2001). Isolated placental perfusion studies have also been performed in animals (Pávek et al. 2001). P-gp deficient mice have been used to clarify the role of P-gp; Schinkel et al. (1994) generated mice with a homozygous disruption of the *Abcb1a* gene and found that the absence of P-gp leads to a deficiency in the BBB and elevates drug levels in the brain and also in other tissues. Similarly, Smit et al. (1999) found that intravenous administration of P-gp substrates to P-gp deficient mice increased the amount of drug entering the fetal side by several fold compared with wild-type fetuses.

2.2.2.2 Single nucleotide polymorphism (SNP) in the **ABCB1** *gene*

ABCB1 genetic polymorphisms were first identified by Kioka et al. in 1989. More than 100 single nucleotide polymorphisms (SNP) in the *ABCB1* gene have been reported, at

least 19 of which are nonsynonymous, i.e. the SNP can result in amino acid changes (Marzolini et al. 2004, Maeda and Sugiyama 2008). Hoffmeyer et al. (2000) reported the first study of the effects of *ABCB1* genotypes on pharmacotherapy.

The two major, the most studied, polymorphisms in the *ABCB1* gene are the c.3435C>T, p.Ile1145Ile (rs1045642) in exon 26 and c.2677G>T/A, p.Ala893Ser/Thr (rs2032582) in exon 21 (Kim 2002). Synonymous (i.e. can not change encoded amino acid) SNP c.3435C>T, p.Ile1145Ile was the first variant to be associated with altered protein expression, along with increased oral bioavailability of digoxin (Hoffmeyer et al. 2000), apparently due to linkage disequilibrium existing between SNPs c.3435C>T, p.Ile1145Ile in exon 26 and nonsynonymous c.2677G>T, p.Ala893Ser polymorphism in exon 21 (Marzolini et al. 2004). Since c.2677G>T, p.Ala893Ser can result in amino acid change, it is more likely than synonymous SNP c.3435C>T, p.Ile1145Ile to alter P-gp expression (Tanabe et al. 2001). One hypothesis of how the silent mutation can alter the substrate specificity is based on timing of protein formation and thereafter changes in protein folding and affinity (Kimchi-Sarfaty et al. 2007). It is evident that the published studies on the effects of c.3435C>T, p.Ile1145Ile SNP on *ABCB1* activity are conflicting, even when using the same probe drug for the transporter (Kim 2002). For example, Sakaeda et al. showed in 2001 that digoxin plasma levels were, on the contrary, lower in subjects with 3435T allele, thus contradicting the study by Hoffmeyer et al. in 2000. Futhermore, it has been noticed that the prevalence of certain haplotypes varies in one ethnic population versus another (Kim et al. 2001). Although, there are studies of other significant mutations, which correlate with P-gp expression level, such as the SNP c.-129T>C (rs3213619) in exon 1b (Tanabe et al. 2001) or association of c.1199G>A, p.Ser400Asn (rs2229109) in exon 11 with increased resistance of drugs (such as doxorubicin and paclitaxel), the clinical significance of the *ABCB1* polymorphisms with regard to pharmacokinetics seems to be unclear (Zhou 2008).

2.2.2.3 Expression and function of P-gp in placenta

In the human placenta, P-gp is highly expressed in the trophoblast layer (Nakamura et al. 1997), on the apical side of the syncytiotrophoblast cell throughout pregnancy (Mathias et al. 2005). Expression of P-gp is greatest during the first trimester and then the amount decreases towards the end of pregnancy. This finding suggests that the protective role of P-gp is needed most in early pregnancy when the fetus is most vulnerable to the toxicity of foreign compounds during organogenesis. (Mathias et al. 2005, Sun et al. 2006).

P-gp has been found to have an important role in placental drug permeability. Smit and co-workers showed (1999) that the transplacental transfer of the P-gp substrates digoxin, saquinavir and paclitaxel was 2.4 to 16 times higher in P-gp (mdr 1a/1b) knockout mice compared with mice with normal P-gp function. Sudhakaran et al (2005) found in their human placenta perfusion study that the maternal-to-fetal transfer of indinavir (an antiretroviral drug and a substrate of P-gp), was significantly lower than the corresponding fetal-to-maternal transfer. A study employing cultured placental chorioncarcinoma cells suggested that the known P-gp inhibitors cyclosporine A, verapamil and mouse monoclonal antibody anti-P-gp (MRK16)

increased the cellular uptake of the P-gp substrates vinblastine, vincristine and digoxin (Ushigome et al. 2000).

The use of P-gp inhibitors during pregnancy could lead to increased penetration of drugs and environmental toxins through the placenta, which can be harmful for the fetus. It has been shown that P-gp inhibitors Valspodar (PSC833) and GG918 caused a 5- to 7-fold increase in the placental transfer of saquinavir in mice with normal P-gp function, but not in *Abcb1a/1b* knockout mice, lacking functional P-gp (Smit et al. 1999). Also, placental transfer of another P-gp substrate, cyclosporine, was increased by the well-established P-gp inhibitor quinidine in dually perfused rat placenta (Pavek et al. 2001). It has been suggested that the modulation of P-gp could be used to achieve higher drug concentration in the infant of HIV-infected mother and thereby improve prophylaxis in late pregnancy (Smit et al. 1999).

2.2.3 Multidrug resistance associated protein 1 (MRP1)

2.2.3.1 Expression and function of MRP1 in extra-placental tissues

At least 13 members of MRP (MRP1-13), expressed in several tissues, have been identified so far (Borst et al. 2000, Unadkat 2004, Choudhuri and Klaassen 2006, Kruh et al. 2007). Human multidrug resistance-associated protein 1 (MRP1) is a member of the ABC transporter family and its gene (*ABCC1*) is located in chromosome 16 (Haimeur et al. 2004). The *ABCC1* gene encodes this 190 kDa multidrug resistance-associated protein 1 (MRP1), which was originally cloned from a human small cell-lung carcinoma cell line (Cole et al. 1992). MRP1 mediates cellular efflux of glucuronide, glutathione (GSH) and sulphate conjugates and, in addition to this, it also mediates the transfer of drugs, for example, some chemotherapeutic agents (Borst et al. 2000). Sequence variations in this gene might explain some differences in drug response among individuals (Wang et al. 2006). Number of *ABCC1* SNPs has been studied and at least c.1299G>T, p.Arg433Ser (rs60782127) in exon 10 has been shown to results in increased doxorubicin resistance (Choudhuri and Klaassen 2006). The roles of various genotypes on the function of MRP1 are not yet clear (Sharom 2006).

In humans, MRP1 confers resistance to a wide range of chemotherapeutic drugs including anthracyclines and vinca alkaloids by exporting them out of the cells. The list of essential substrates and inhibitors of MRP1 is given in Table 4. (Haimeur et al. 2004). It is obvious that although there are many sequence similarities in MRP1 between different species, there are still eminent differences in their substrate specificity (Haimeur et al. 2004).

The function of MRP1 is significantly enhanced by the presence of glutathione (GSH), and most substrates need to be conjugated with GSH before being transported (Renes et al. 1999, Haimeur et al. 2004). The mechanism of GSH in MRP1-mediated transport is not fully clear, but it has been shown that GSH can improve the transport of certain substrates and also enhance potency of some inhibitors of MRP1 (Haimeur et al. 2004).

MRP1 is widely expressed in the human body, such as the lungs, brain, testis, kidney, colon, peripheral blood mononuclear cells and placenta (Haimeur et al. 2004, Bakos

and Homolya 2007) (Fig. 5). Knowledge of MRP1 has been continuously increasing during the last years because it has been found to be a very important drug transporter in several tissues and to play a role in the development of drug resistance in several cancers (Bakos and Homolya 2007, Munoz et al. 2007). In the brain, the basolateral membrane of the choroid plexus tissue expresses MRP1, which forms a part of the blood-cerebrospinal fluid (CSF) barrier (Haimeur et al. 2004), and Wijnholds et al. (2000) demonstrated with knockout mice that Mrp1 helps to limit accessing of etoposide from blood to CSF.

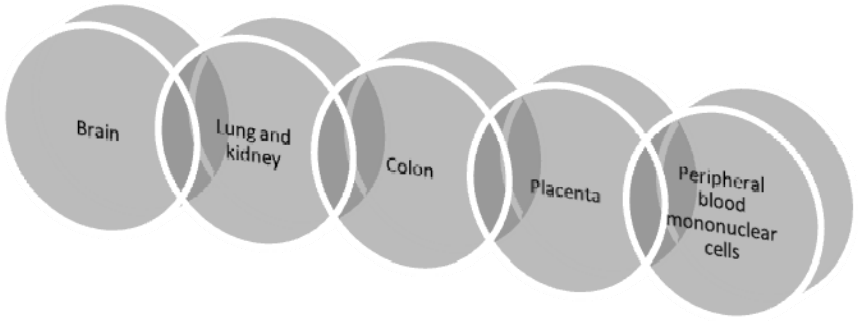

Figure 5. Localization of the relative high amount of MRP in human tissues

2.2.3.2 Expression and function of MRPs in placenta

Human term placenta expresses at least three members of the MRP family: MRP1, MRP2 and MRP3 (St-Pierre et al. 2000). In the syncytiotrophoblast, MRP1 is localized predominantly in the basal, fetal facing plasma membrane (Atkinson et al. 2003, Nagashige et al. 2003), whereas MRP2 (Unadkat et al. 2004, St-Pierre et al. 2000) and MRP3 are apical membrane proteins and MRP3 is expressed also in blood vessel endothelia (St-Pierre et al. 2000). There is however, one study in which some evidence of MRP1 prortein expression was found in the apical syncytiotrophoblast (St-Pierre et al. 2000). MRP transporters are commonly thought to function as an efflux transporters (Unadkat et al. 2004, St-Pierre et al. 2000), thus the basal transporter MRP1 is suggested to facilitate the transfer of drugs into the fetal compartment, i.e. out of the placenta. The true functional role of MRP1 in human placenta has not yet been established.

2.3 Substrates of P-glycoprotein and MRP1

Several drugs and other compounds are known to be substrates of P-gp, for example, anticancer drugs, drugs for hypertension, allergy, infections, immunosuppression and neurological diseases (Marzolini et al. 2004, Marchetti et al. 2007). In Table 3 the distinct substrates, inhibitors and inducers of P-gp are listed according to Marzolini et al. (2004), Marchetti et al. (2007) and Zhou (2008). Some agents, which are substrates of P-gp, are also substrates of the drug-metabolizing enzyme, cytochrome P450 (CYP) 3A4. This can be partly explained by coordinated regulation and partly by colocalization of P-gp and CYP3A4 (Marzolini et al. 2004). Some drugs, which are substrates of P-gp, can also inhibit P-gp-mediated transport of other substrates. For

example, coadministration of paclitaxel (P-gp substrate) and cyclosporine (P-gp substrate and inhibitor) enhances oral bioavailability of paclitaxel i.e. cyclosporine acts as an inhibitor of the intestinal P-gp (Marzolini et al. 2004).

Expression of P-gp is inducible. It has been revealed that the P-gp expression level can be regulated by some nuclear receptors such as the pregnane X receptor (PXR), and these receptors can be activated by some herbs, like St John`s wort (SJW) (Marchetti et al. 2007). Through a similar mechanism, several drugs (for example, paclitaxel and rifampicin) appear to induce *ABCB1* gene expression. According to one study, the intestinal expression P-gp was induced 3.5-fold by rifampicin use as documented by duodenal biopsies in healthy volunteers (Greiner et al. 1999).

Several substrates of MRP1 have been identified, but much less compared with P-gp. Table 4 shows some relevant therapeutic agents, which are substrates or inhibitors of MRP1.

Table 3. Relevant substrates, inhibitors and inducers of P-gp (Marzolini et al. 2004, Marchetti et al. 2007 and Zhou 2008).

Drug	**Substrate**	**Inhibitor**	**Inducer**
Anticancer agents			
Actinomycin D	●		
Daunorubicin	●		●
Docetaxel	●		
Doxorubicin	●		●
Etoposide	●		●
Imatinib	●		
Irinotecan	●		
Mitomycin C	●		
Mitoxantrone	●		●
Paclitaxel	●		●
Teniposide	●		
Topotecan	●		
Vinblastine	●	●	●
Vincristine	●		●
Antihypertensive agents			
Carvedilol		●	
Celiprolol	●		
Diltiazem	●	●	●
Losartan	●		
Nicardipine		●	●
Nifedipine			●
Reserpine		●	●
Talinolol	●	●	
Antiarrhythmics			
Amiodarone		●	
Digoxin	●		
Propafenone		●	
Quinidine	●	●	
Verapamil	●	●	●

Glucocorticoids			
aldosterone	●		
Cortisol	●	●	
Dexamethasone	●		●
Methylprednisolone	●		
Others			
Atorvastatin	●	●	
Bromocriptine		●	●
Colchicine	●		●
Dipyridamole		●	
Elacridar (GF120918)		●	
Emetine		●	
Fexofenadine	●		
Ivermecitin	●		
Loperamide	●		
Mefloquine	●	●	
Methotrexate			●
Midazolam		●	●
Progesterone		●	
Probenecid			●
Retinoic acid			●
Rhodamine123	●		
Spironolactone		●	
Tamoxifen		●	●
Terfenadine	●		
Vecuronium	●		
Antiviral agents			
Amprenavir	●		●
Indinavir	●	●	●
Nelfinavir	●	●	●
Ritonavir	●	●	●
Saquinavir	●	●	●
Antimicrobial agents			
Clarithromycin		●	
Erythromycin	●	●	●
Levofloxacin	●		
Rifampin	●		●
Sparfloxacin	●		
Tetracycline	●		
Doxycycline	●		
Antimycotics			
Itraconazole	●	●	
Ketoconazole		●	
Clotrimazole			●
Immunosuppressants			
Cyclosporine	●	●	●
Sirolimus	●	●	
Tacrolimus	●	●	●
Valspodar (PSC833)	●	●	

Antidepressants			
Amitriptyline	●		
Fluoxetine		●	
Paroxetine		●	
Sertraline		●	
Herbal remedy			
St John`s wort			●
Neuroleptics			
Chloropromazine		●	
Flupenthixol		●	
Phenothiazine			●
Antiepileptics			
Phenobarbital	●		●
Phenytoin	●		●
Antiacids			
Omeprazole		●	
Pentoprazole		●	
Cimetidine	●		
Ranitidine	●		
Opioids			
Methadone		●	
Morphine	●		●
Pentazocine		●	
Antiemetics			
Domperidon	●		
Ondansetron	●		

Table 4. Some relevant substrates and inhibitors of MRP1 (Haimeur et al. 2004)

Substrate	**Inhibitor**
Vincristine	Probenecid
Vinblastine	Sulfinpyrazone
Doxorubicin	Indomethacin
Daunorubicin	VX-710 (Biricodar/Incel)
Epirubicin	Agosterol A
Etoposide (VP-16)	PAK-104P (dihydropyridine)
Irinotecan	Verapamil
SN-38 (metabolite of irinotecan)	Cyclosporine A
Methotrexate	Genistein
Hydroxyflutamide	Quercetin
Flutamide	RU486 (steroid derivative)
Ritonavir	Budesonide
Saquinavir	MK-571

2.3.1 Saquinavir

There is a risk of childhood mortality for children of HIV-infected mothers, caused by maternal virus transfer to the fetus. This risk could be reduced through the treatment with antiretroviral drugs during pregnancy (Brocklehurst and Volmink 2002, Newell

and Thorne 2004). Zidovudine, the first antiretroviral drug, has been shown to reduce the rate of virus transmission from mother to child (Connor et al. 1994, Garcia et al. 1999). Ripamonti et al. concluded in 2007 that atazanavir, a protease inhibitor (PI), crosses the placenta and can potentially provide further protection for the fetus against HIV-infection. Studies have shown that drug transporters, like P-gp, prevent transfer of PIs, saquinavir for example, to the fetal side and can result in minimal fetal exposure and lack of efficacy (Smit et al. 1999).

HIV-infected mothers are treated nowadays with highly active antiretroviral therapy (HAART), i.e. a combination therapy (Newell and Thorne 2004, Keiser et al. 2008). Saquinavir is an antiretroviral drug used in modern HIV therapy (Ananworanich et al. 2008) and a well known substrate of P-gp (Smit et al. 1999). It was the first protease inhibitor (and sixth antiretroviral drug) approved by the Food and Drug Administration (FDA) in 1995 (Tavel 2000). A study employing dually perfused human placental cotyledons indicated that the mean placental transfer of saquinavir was only 1.8% (Forestier et al. 2001). Also, a clinical study in pregnant HIV-positive women confirmed that the transfer of saquinavir from the maternal blood circulation to the fetus is very low (Marzolini et al. 2002, Marzolini and Kim 2005). The previous studies suggest that the placenta protects the fetus from saquinavir exposure.

Saquinavir is also known to be a substrate of MRP1. Williams et al. (2002) showed with cultured cells that saquinavir is transported via MRP1 and also by MRP2. Meaden et al. (2002) found that enhanced expression of MRP1 and P-gp in lymphocytes is associated with lower intracellular accumulation of saquinavir and ritonavir, indicating that both of these protease inhibitors are substrates of P-gp and MRP1. By contrast, HIV protease inhibitors, such as saquinavir, have been shown to be inhibitors of human breast cancer resistance protein (BCRP) (Gupta et al. 2004, Weiss et al. 2007). The information of the association of the other human placental transporters and saquinavir is limited.

The main pharmacokinetic properties of saquinavir are shown in Table 5 (Vanhove et al. 1997, Mirochnick and Capparelli 2004, Pharmaca Fennica 2008, European agency for the Evaluation of Medicinal Products and Roche Pharmaceuticals (U.S.)).

Table 5. Pharmacokinetic properties of saquinavir

Oral absorption	Not good, depends on following food intake
Plasma protein binding	97%
Active metabolite	None/ not known
Passive metabolite	Several
T1/2	1,4h
Gender	Higher concentrations in females
Absolute bioavailability	4% (appr. three fold better with soft gelatine capsules)
Metabolia	Extensive first-pass metabolism. Liver sytocrome P450 CYP3A4 >90%
Excretion	80-90% to feces and 1-3% to urine
Molecular weight	670,86

2.3.2 Quetiapine

The treatment of psychosis during pregnancy is a significant challenge. The adverse effects (weight gain, diabetes, sedation and hypertension) associated with atypical antipsychotics increase fetal risks during pregnancy (Yaeger et al. 2006). The use of atypical antipsychotic medications during pregnancy is preferred, because the older antipsychotic drugs, such as chlorpromazine or fluphenazine, have been associated with prenatal complications in animals (Iqbal et al. 2001). Quetiapine, a dibenzothiazepine derivate (DeVane and Nemeroff 2001), is frequently preferred among women of fertile age because of its low risk of extrapyramidal and sexual side effects, thus it is often used as medication in early pregnancy (Taylor et al. 2003, Green 1999). Moreover, quetiapine is a widely used atypical antipsychotic drug in general population due to its good efficacy in treating symptoms of schizophrenia and because it is well tolerated compared with classical antipsychotics (Meats 1997). Still the data regarding the use of atypical antipsychotic medications, such as quetiapine, in pregnancy are limited (Tenyi et al. 2002, Yaris et al. 2004, Newport et al. 2007).

There is some evidence that supratherapeutic concentrations of quetiapine may cause fetal or embryo toxicity in animals, such as minor soft tissue anomaly, loss of fetal body weight and delays in skeletal ossification, but the overall teratogenic risk is evaluated to be low by the manufacturer (http://www1.astrazeneca-us.com/pi/seroquel.pdf). Newport et al. 2007 quantified the transplacental transfer of four antipsychotic drugs (olanzapine, haloperidol, risperidone and quetiapine) in humans and reported that with olanzapine use there were tendencies toward low birth weight or toward more neonatal intensive care unit admissions after delivery. They also concluded that the human placenta prevents the access of those antipsychotics to the fetal side, and quetiapine demonstrated the lowest placental transfer in that study. There are some (<50) clinical case reports of the use of quetiapine during pregnancy (Tenyi et al. 2002, Yaris et al. 2004, Yaeger et al. 2006, Cabuk et al. 2007) and all without apparent evidence of congenital malformations. A prospective comparative study on the safety of atypical antipsychotics during pregnancy, including quetiapine, did not find any association with an increased risk for major malformations (McKenna et al. 2005). The transplacental transfer of quetiapine in humans was not known before publication of the original publication III.

Two earlier studies indicate that quetiapine is a P-gp substrate (Boulton et al. 2002, Ela et al. 2004), but there is also a contradictory study suggesting that this is not the case (Grimm et al. 2005).

The main pharmacokinetic properties of quetiapine are shown in Table 6 (DeVane and Nemeroff 2001, Pharmaca Fennica 2008 and AstraZeneca (U.S.)).

Table 6. Pharmacokinetic properties of quetiapine

Oral absorption	Good, food has minimal effects
Plasma protein binding	83%
Active metabolite	N-desalkyl quetiapine
T1/2 (quetiapine)	7h
T1/2 (N-desalkyl quetiapine)	12h
Gender	No difference
Absolute bioavailability	Unknown, relative bioavailability nearly complete
Metabolia	Liver sytocrome P450 CYP3A4 mainly
Excretion	73% to urine and 21% to feces
Molecular weight	883,11

2.4 Inhibitors of P-glycoprotein and MRP1

Drug-drug interactions are a major cause of harm complications of drug therapy. Some of the drug interactions can be explained by inhibition or induction of the transporter proteins. An example of a clinically relevant drug-drug interaction is the interaction between digoxin (P-gp substrate) and other cardiac drugs, for example, with verapamil, leading to greater plasma levels of digoxin due to P-gp inhibition (Marzolini et al. 2004). Drug-drug interactions involving the transporters can lead to life-threatening toxicity, especially with the cytotoxic anticancer drugs. Anticancer agents are often dosed close to the maxium-tolerated dose, and thus changes in pharmacokinetics, by other compounds, can even lead to lethal toxicity. (Marchetti et al. 2007).

Several commonly used drugs are inhibitors of P-gp and thus are able to affect the pharmacokinetics of some clinically used drugs, which are substrates of P-gp (Marzolini et al. 2004) (Table 3).

There are two major types of inhibitors, competitive inhibitors (competition for drug-binding sites) or noncompetitive inhibitors (ATP hydrolysis process blocking). Several MDR-reversing compounds such as, for example, P-gp inhibitors, are at various stages of clinical development. The so-called first-generation P-gp inhibitors (e.g. verapamil and cyclosporin A) have low substrate selectivity, an inhibition effect, also on CYP3A4 and require high doses of drugs to reverse MDR. Therefore, new so-called second-generation (e.g. cyclosporin analog i.e. valspodar (PSC833)) and third-generation (e.g. GF120918 i.e. GG918) inhibitors have been developed. These inhibitors have been investigated in different preclinical studies to reverse MDR, thus enhancing drug absorption and increasing drug penetration into target tissues. (Tan et al. 2000, Marchetti et al. 2007). In table 7 is shown some examples of IC50 values (the values of the 50%-inhibitory concentration) of P-gp inhibitors and MRP1 inhibitors with different substrates in different cell lines, showing the wide variety of the values.

Many inhibitors of MRP1 have been identified to date, for example, verapamil and cyclosporine A. Some relevant inhibitors of the MRP1 transporter protein are listed in Table 4. (Haimeur et al. 2004). It has been noticed that the inhibitory effect of some MRP1 inhibitors is enhanced by the presence of GSH, e.g. agosterol A (Haimeur et al. 2004).

2.4.1 Valspodar (PSC833) and GG918

Cyclosporine analog, i.e. valspodar as a second-generation inhibitor and GG918 (also known as GF120918/ elacridar) as a third-generation inhibitor, are known inhibitors of P-gp and employed in different studies in humans and animals (Smit et al. 1999, Mizuno et al. 2003, Marchetti et al. 2007, Ose et al. 2008). GG918 has also been shown to be an inhibitor of BCRP (breast cancer receptor protein) (Kuppens et al. 2007). Valspodar is shown to inhibit MDR3 (Smith et al. 2000).

2.4.2 MK-571 and probenecid

MK-571 and probenecid have been shown to be inhibitors of MRP1 (Renes et al. 1999, Williams et al. 2002, Haimeur et al. 2004). MK-571 has been reported to be an MRP family inhibitor and to have an inhibition effect also at least on MRP2 and MRP4 (Williams et al. 2002, Vellonen et al. 2004, Tian et al. 2006). MK-571 is shown to have inhibitory effect on OATP1B3, OATP2B1 and OATP1B1 (Letschert et al. 2006). Probenecid is also reported to be a non-specific inhibitor of MRP- (Berger et al. 2003), OATP- (organic anion transporter protein) (Janneh et al. 2007) and OAT family (Whitley et al. 2005). Probenecid is reported to inhibit OAT1 and OAT4 from OAT family (Whitley et al. 2005), which are expressed also in placenta.

Table 7. Examples of IC50 values (the values of the 50%-inhibitory concentration) of P-gp inhibitors (valspodar/PSC833 and GG918) and MRP1 inhibitors (probenecid and MK-571) with different substrate in different cell lines, showing the wide variety of the values.

P-gp inhibitors:			
Valspodar (PSC833)	**IC50**	**Substrate**	**Cell line**
Choo et al. 2000	0.11 µM	Digoxin	Caco-2 cells
Drewe et al. 1999	1.13 µM	Saquinavir	Brain endothelial cells
Fisher et al. 1999	2.8 nM	Paclitaxel	MDA/T0.3 (human adenoca cell line)
Kusunoki et al. 1998	0.29 µM	Doxorubicin	LLC-GA5-COL150 cells
Kusunoki et al. 1998	1.06 µM	Vinblastine	(pig renal cell line expressing the human P-gp)
GG918 (GF120918)			
Hyafil et al. 1993	0.16 µM	Azidopine	CHRC5 (chinese hamster ovary cell line)
Luo et al. 2002	0.38 µM	Irinotecan	canine kidney cells
MRP1 inhibitors:			
Probenecid			
Bobrowska-Hägerstrand et al. 2001	100-200 µM	BCPCF (acetoxymethyl ester)	Human erythrocytes
MK-571			
Renes et al. 1999	0.4 µM	Daunorubicin	GLC4/Adr, S1(MRP) (isolated membrane vesicles)
Wu et al. 2005	1.1 µM	DNP-SG (dinitrophenyl S-glutathione conjugate)	Human erythrocytes

2.5 Ex vivo placental perfusion method

Nowadays, it is known, that fetal exposure to drugs and foreign compounds may lead harmful effects on fetus (for example, thalidomide disaster in 1957-1961 or FAS children after alcohol exposure) or have impact on the growth of the fetus (cigarette smoke) (Myren et al. 2007). The pressure for new and more effective drugs is continuous and thus there is a need for methods to study safety of these drugs without harmful effects for mother or fetus.

Several techniques have been developed over the years to study placental transfer of different compounds, nutrients and drugs. Different models have employed placental tissues and cells, for example, perfused placental cotyledon, preparations of trophoblast tissue (including syncytiotrophoblast tissues and plasma membrane vesicles), cultured placental villus tissues, cells and malignant trophoblast cell cultures (Sastry 1999, Myren et al. 2007).

The perfusion studies have been performed with both animal (*in situ* or *in vitro*) and human placentas (Omarini et al. 1992, Ala-Kokko et al. 2000, Pavek et al. 2001, Pollex et al. 2008). *In vivo* models in pregnant animals are helpful, but results of those cannot be extrapolated directly to humans, because human placenta differs clearly between species (Myllynen et al. 2005a, Vähäkangas and Myllynen 2006, Myren et al. 2007). Panigel et al. (1967) first descriped the perfusion of the isolated human placental cotyledon. Afterwards, Panigel´s *in vitro* technique was modified to dual-perfusion technique by Schneider et al. in 1972. The human *ex vivo* placental perfusion model is a unique model to investigate the placental transfer of different therapeutic agents, for example antiepileptic drugs, without exposure of mother or fetus to harmful agents (Myllynen et al. 2005b). Ceccaldi et al. (2007) showed in their human placental perfusion study that enfuvirtide (an antiretroviral fusion inhibitor) does not pass the placenta, suggesting the safe use of this drug during pregnancy.

Placental perfusion method gives also information on placental metabolism, acute toxicity, function of placental transporters and fetal exposure (Myren et al. 2007). Moreover, the ethical issues are of less concern in the ex vivo perfusion method with human placenta (Vähäkangas and Myllynen 2006). The results of placental perfusion method can not be extrapolated directly to *in vivo* condition, because the *in vitro* method is metabolically stabile contrary to *in vivo* condition. Moreover, only term placentas are used in perfusion method and it is known that the amount of some drug transporters and metabolism varies in different gestational age (Vähäkangas and Myllynen 2006, Myren 2007).

There are two different kinds of perfusion methods; an open (not recirculated) and closed (recirculated) perfusion system. The open system is particularly suitable for placental transfer studies, and the recirculation perfusion system, which imitates physiological circumstances, also gives an opportunity to examine the placental metabolism of the compounds (Brandes et al. 1983, Omarini et al. 1992, Myren et al. 2007).

3 AIMS OF THE STUDY

The present study series aimed to investigate the function of BPB regarding the drug transporters, especially the P-gp and MRP1. The specific aims were defined as follows:

1. To study whether addition of exogenous ATP is needed to perfusion medium in the *ex vivo* placental perfusion method, when studying the function of ATP-binding cassette drug transporters.

2. To investigate the placental transfer of quetiapine in isolated perfused human placenta and the role of P-gp in this transfer.

3. To study the effect of inhibition of apically (P-gp) and basally (MRP1) expressed transporters on the maternal-to-fetal and fetal-to-maternal transfer of saquinavir.

4. To study if *ABCB1* polymorphism can affect placental P-gp protein expression levels and to determine if the level of placental P-gp protein expression or the *ABCB1* polymorphism *per se* can affect the function of P-gp, as measured by saquinavir and quetiapine transfer.

4 MATERIALS AND METHODS

4.1 Placentas and the perfusion system

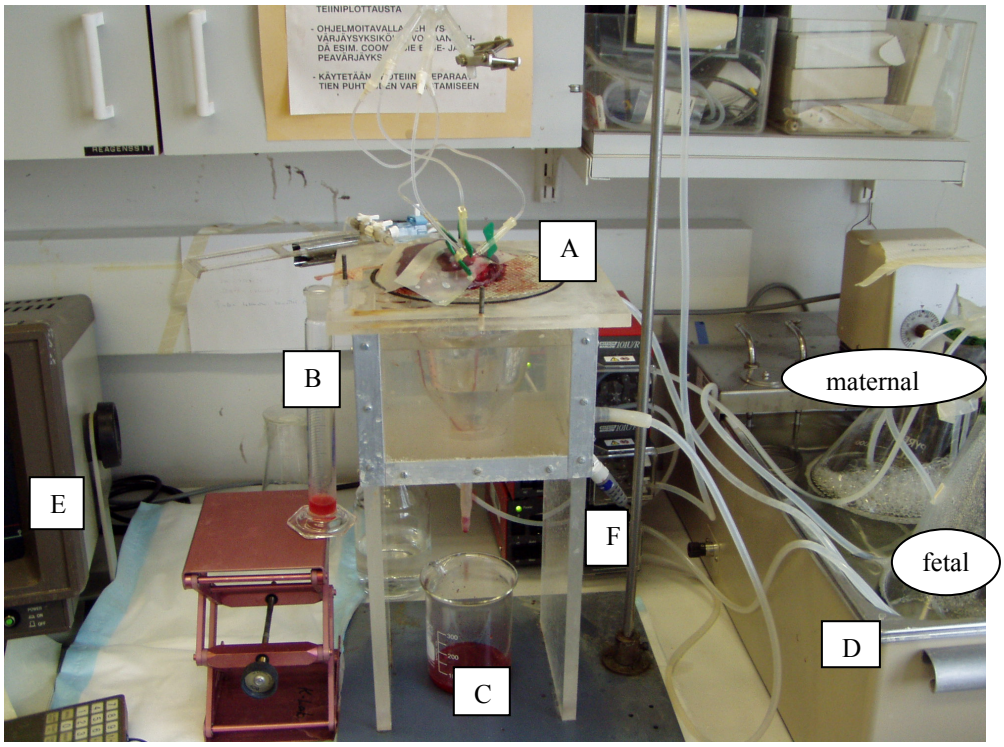

Figure 6. Photo of the perfusion equipment

A) Cotyledon (placenta)
B) Fetal effluent
C) Maternal effluent
D) Warm waterbath with maternal and fetal buffers and study drugs on maternal side (or on fetal side in reversed perfusions).
E) Pressure monitor
F) Pump

The term placentas (at 38 to 42 weeks of pregnancy) were obtained from the Turku University Central Hospital for placental perfusion of saquinavir and quetiapine. The total number of perfused placentas was 83, of which 40 obtained after caesarean section and 43 after normal vaginal delivery. These term placentas were applied for maternal-to-fetal perfusion (n=55) and for fetal-to-maternal perfusion (reversed perfusion) (n=28). Pregnancies were uncomplicated, mothers were healthy and without medication during pregnancy. Mother`s smoking history was not asked, but it has been shown not to affect the expression level or activity of P-gp in human placenta

(Myllynen et al. 2007). The study protocol was approved by the Joint Commission on Ethics of the Turku University and the Turku University Central Hospital. The mothers were fully informed verbally and a written consent was obtained.

The placental perfusion method is well described in the literature (Schneider et al. 1972, Heikkinen et al. 2001). In this study, an open (non-recirculating), dual perfusion method was used to simulate the transplacental transfer of saquinavir and quetiapine during chronic drug therapy. A photo of the perfusion equipment in action and a schematic presentation of the applied perfusion method (Heikkinen et al. 2001) are presented in Figures 6 and 7.

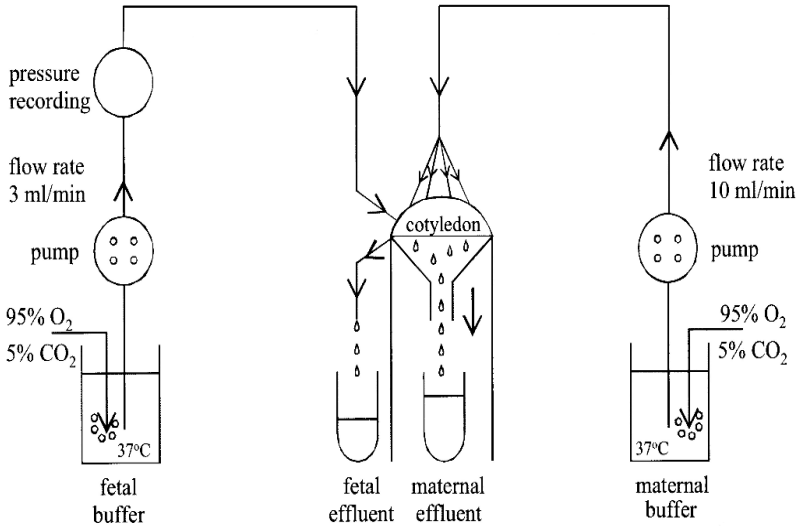

Figure 7. Schematic presentation of the applied perfusion method (Heikkinen et al. 2001)

Immediately after delivery, heparinized 0.9% NaCl solution was injected into the umbilical artery in the delivery room, an intact cotyledon was chosen and the corresponding distal branches from the chorionic artery and vein were cannulated and fixed with garment. The criteria for selecting a good cotyledon have been characterized by Schneider et al. 1972. A cotyledon which was about 4-6 cm in diameter and preferably supplied by a single chorionic artery and vein with the decidual plate intact was chosen (Fig. 8).

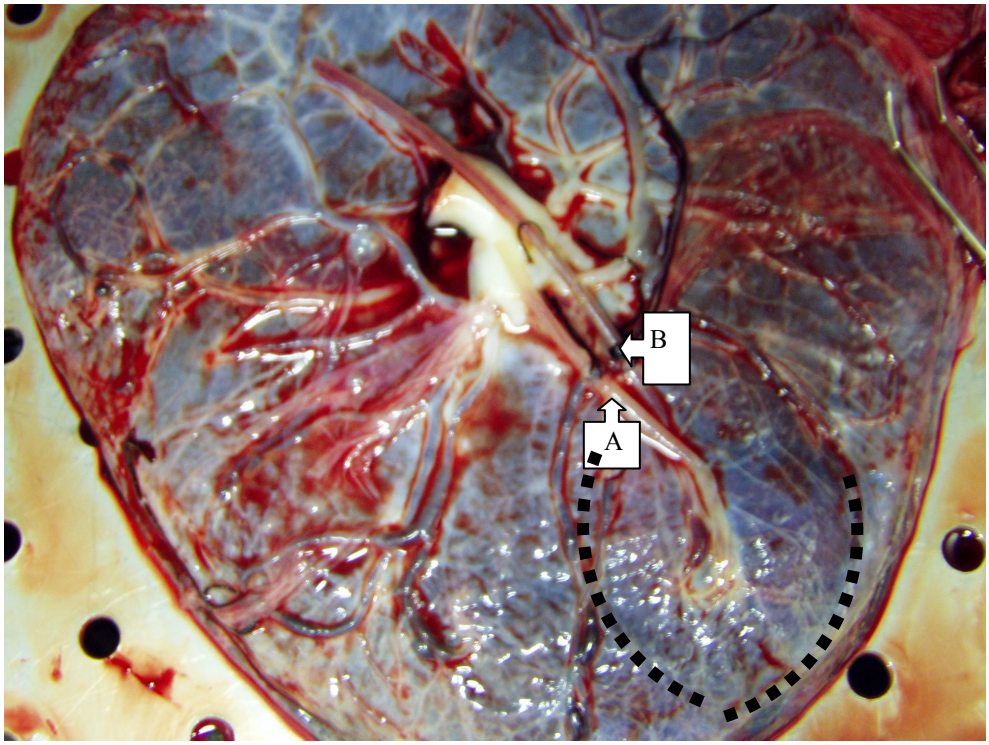

Figure 8. Cannulated placenta before cutting out the selected cotyledon. The cotyledon is outlined with dashed line.

A) Cannulated fetal artery with plastic tube
B) Cannulated fetal vein with plastic tube

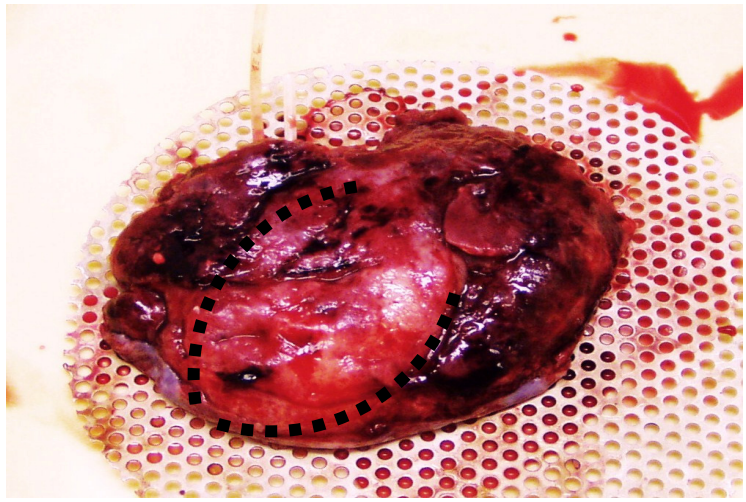

Figure 9. Perfused cotyledon (appears in more pale color than non-perfused part of the placenta) in a piece of cut placenta (maternal surface upwards). Dashed line shows the outlines of the perfused cotyledon.

The cannulated cotyledon was cut out from the placenta and quickly transferred to the perfusion laboratory. The cotyledon was placed on a metal plate with the maternal surface upwards and attached to the perfusion apparatus. For the maternal perfusion, the maternal (decidual) surface of the placenta was pierced with four butterfly needles and the maternal effluent was allowed to drain into a container. On both the maternal and the fetal side, the perfusate was Krebs-Ringer bicarbonate buffer, which contained bovine serum albumin 30 g/l (A-3912, Sigma Chemical Co., USA). The perfusate was maintained at 37˚C and balanced with 95% oxygen and 5% carbon dioxide mixture throughout the perfusion. All perfusions were carried out with a flow rate of 3 ml/min on the fetal side and 10 ml/min on the maternal side. All perfusions were started within 20 minutes after placental delivery.

4.1.1 Maternal-to-fetal perfusions

In all maternal-to-fetal perfusions, the placentas were preperfused for 30 minutes with Krebs-Ringer bicarbonate solution with albumin without the study drugs to stabilize the perfusion system. In the control perfusions, the placentas were perfused for two hours with study drugs saquinavir (Fortovase, Roche, Switzerland, 6.67 µg/ml) or quetiapine (Astra Zeneca, London, United Kingdom, 75 ng/ml) and with a control agent antipyrine (Sigma Chemical Co., USA, 80 µg/ml). Antipyrine was used as a reference drug for passive diffusion dependent placental transfer. The concentration of saquinavir was about 3 times higher than the recommended therapeutic range for clinical use (0.6-2.8µg/ml; Ray et al. 1999), to ensure quantifiable saquinavir concentrations on the fetal side. The concentration of quetiapine corresponded to steady-state serum levels in patients taking this drug (Mandrioli et al. 2002).

In Study I, the maternal perfusate was replaced with a medium containing saquinavir and the reference agent antipyrine, and adenosine 5'-triphosphate (ATP) (A-3377, Sigma Chemical Co., USA) was added to both the maternal and the fetal side. Five different ATP concentrations (0 µmol/l (n=4), 1 µmol/l (n=4), 10 µmol/l (n=4), 100 µmol/l (n=4) and 1000 µmol/l (n=1)) were used. The dose range was based on earlier reports indicating circulating plasma levels of ATP in humans (approximately 1 µmol/l; Gorman 2007). Higher concentrations of ATP were used to ensure the observation of a possible change on saquinavir transfer.

In inhibitor groups (Studies II and III), the placentas were first perfused for 10 minutes with the inhibitors of studied transporters; P-gp inhibitors valspodar (PSC833, Novartis, Switzerland, 2.4 µg/ml) or GG918, (also known as GF120918, GlaxoSmithKline, UK, 0.6 µg/ml). Thereafter, P-gp inhibitors were perfused together with saquinavir or quetiapine and antipyrine throughout the two-hour perfusion. The total perfusion time was two hours and 40 minutes.

Samples for the measurement of the saquinavir, quetiapine and antipyrine concentrations were collected from the fetal venous outflow just before and 10, 20, 30, 40, 50, 60, 80, 100, 120 min after start of drug perfusion, and from the maternal arterial inflow and from the maternal effluent reservoir at 20, 60, 120 min. Samples from maternal arterial inflow were collected to control initial concentrations of the

perfused drugs. After the perfusion, the samples were centrifuged (5 min, 2500 rpm) to remove cellular debris and stored at –20˚C until analysis. In addition, a tissue sample was cut from the placenta for genotyping and for the measurement of P-gp protein expression.

4.1.2 Reversed perfusions

Reversed perfusion studies (fetal-to-maternal perfusion) (Studies II and IV) were performed with 28 placentas. Eight full term placentas were obtained to study the role of P-gp in the transfer of saquinavir from the fetal side to the maternal side (Study II). In Study IV (n=20), we studied the role of basal transmembraine protein, MRP1, in the transfer of saquinavir from the fetal side to the maternal side.

Reversed perfusions were performed using the method described above, but in a reverse manner meaning that the drugs were infused into the fetal artery. Samples were collected from the maternal vein just before infusion, every 10 minutes for 60 min and thereafter every 20 min up to 120 min and from the fetal artery inflow and from the fetal venous outflow at 20, 60 and 120 min after start of perfusion with saquinavir. In the P-gp study (Study II), four placentas were perfused with saquinavir (6.67 μg/ml) and antipyrine (80 μg/ml) only (controls) and four placentas also with the P-gp inhibitor valspodar (PSC833) (2.4 μg/ml). In Study IV, six placentas were perfused with saquinavir (6.67 μg/ml) and antipyrine (80 μg/ml) only (controls) and seven placentas each together with inhibitors MK-571 (Biomol Research Laboratories, Playmouth Meeting, PA, 26.9μg/ml) or probenecid (MP Biomedicals (ICN), 14.3μg/ml).

4.2 Viability of placentas

A blood gas analysis (pH) (Rapidlab 248, Bayer, UK) of the arterial and venous perfusate was performed to test the viability of the placental tissue at the beginning and end of each perfusion. Blood gas analyses (pH) were taken from fetal artery and vein in maternal-to-fetal perfusions and from maternal artery and vein in reversed, fetal-to-maternal perfusions. The flow rates on both the maternal and the fetal side were monitored to assure stabile flow. In addition, the perfusion pressure was monitored throughout the perfusion.

4.3 Drug concentration analyses

Saquinavir and antipyrine

Saquinavir and antipyrine concentrations in the perfusate were determined by high performance liquid chromatography with ultra violet detection. The method for measuring saquinavir and antipyrine in Krebs solution was validated prior to analysis. Five calibration standards were used in the range of 10-1000 ng/ml for saquinavir and 100-25 000 ng/ml for antipyrine.

Three quality control samples of saquinavir and antipyrine were used with the concentrations given in each original publication. The inter-assay variation (percent coefficient of variation) calculated from the quality control samples and the intra-assay variations have been given in the original publications for saquinavir and antipyrine concentrations. The lower limit of quantification (LLOQ) was 10.0 ng/ml for saquinavir and 100 ng/ml for antipyrine. The criterion for acceptable linearity was the calibration standard accuracy of 80-120% for LLOQ and 85-115% for other standards. The criterion for quality control samples was the accuracy of 80-120%.

Quetiapine

Quetiapine was measured by an automated high performance liquid chromatography (HPLC) method with an on-line solid-phase extraction and UV-detection at 254 nm according to a previously published method (Sachse et al. 2006). The detailed method is described in the original publication III. The lower limit of quantification was 5 ng/ml and the day-to-day in-precision ranged between 12.2 %, 3.53 % and 6.22 % for nominal concentrations of 30, 150, and 300 ng/ml quetiapine, respectively.

4.4 Immunoblotting

The detailed methodology information has been reported in the original publication II. In short, for preparation of the membrane fraction, the placental tissue was homogenized and proteins were subjected to SDS-polyacrylamide gel electrophoresis. Thereafter, proteins were transferred onto a Hybond ECL nitrocellulose membrane. Non-specific binding sites were saturated by incubation as reported in the original publication. The membrane was then incubated with JSB-1, a murine monoclonal antibody that binds a cytoplasmic epitope of P-gp and with secondary horseradish peroxidase linked sheep anti-mouse IgG. After washing, the immunoreactive bands were visualized with Chemiluminescent Peroxidase Substrate-1. The immunoreactive bands were quantitated using Quantity One software (Bio-Rad, Hercules, CA, USA).

4.5 Genotyping

The detailed method is described in the original publications I-III. DNA was extracted from frozen placentas using SDS lysis with proteinase K digestion and phenol-chloroform extraction. *ABCB1* was genotyped for the exon 21 (c.2677G>T/A, p.Ala893Ser/Thr) and exon 26 (c.3435C>T, p.Ile1145Ile) SNPs using ABI SNaPshot multiplex SNP detection. The primers used in PCR have been reported in the original publications I-III. The PCR products were purified and pooled. In the SNaPshot reaction, the primers directly adjacent to the variable position were used. Single base extension was performed with fluorescently labelled dideoxynucleotide triphosphates. The products were detected with an ABI 3700 capillary electrophoresis instrument and the results were analysed using ABI Genotyper 3.7. Each sample was run at least twice to avoid genotyping errors.

4.6 Data analysis

The transplacental transfer (TPT) of study drugs (saquinavir, quetiapine and antipyrine) was calculated as the absolute fraction of the dose crossing the placenta (TPT_{AUC} %). The amount of drug on each side was calculated as the area under the drug concentration vs. time curve (AUC (0-2)) multiplied by the corresponding flow:

TPT_{AUC} % = (AUC_{FV} x 3 ml / min) / (AUC_{MA} x 10 ml / min) x 100, where
AUC_{FV} = area under the curve in fetal venous outflow, and
AUC_{MA} = area under the curve in maternal artery.

For reversed perfusions:

TPT_{AUC} % = (AUC_{MV} x 10 ml / min) / (AUC_{FA} x 3 ml / min) x 100, where
AUC_{MV} =area under curve in maternal venous outflow, and
AUC_{FA} = area under curve in fetal artery.

The transplacental transfer index (TI), i.e. the ratio of transplacental transfer between individual drug (saquinavir or quetiapine) and antipyrine, was calculated by dividing the TPT_{AUC} % (individual drug) by the TPT_{AUC} % (antipyrine). All pharmacokinetic results are expressed as mean values and standard deviations (SD).

4.7 Statistical analysis

The statistical analyses of the viability data (pH and perfusion pressure) were carried out with the paired t-test. One-way analysis of variance (ANOVA) with Tukey's test for pairwise comparisons was used to compare the pharmacokinetic variables among the control perfusions and perfusions with the two different inhibitors, to compare the pharmacokinetic variables among the control perfusions and perfusions with the different ATP concentrations and to test influences of the two *ABCB1* polymorphism on the P-gp protein expression level as well as on the transplacental transfer of saquinavir and quetiapine. Unpaired t-test was used for the reversed perfusion pharmacokinetic data in the saquinavir study. Pearson's correlation test was used for all correlations, except that the Spearman rank test was used to test the correlation between P-gp expression and the transfer of quetiapine. The pharmacokinetic data were log-transformed prior to statistical analyses if needed. The two-sided level of statistical significance was set at $p<0.05$.

5 RESULTS

5.1 Viability of placentas

The viability of the placentas was maintained during the two-hour perfusion time. Flow rate, the most important parameter of viability, was unchanged during all perfusions, and no mismatch occurred between fetal and maternal circulation during perfusions. The total number of the obtained placentas was 95 and 83 were accepted for pharmacokinetic analyses, giving a success rate of 87%. Twelve of the obtained cotyledons did not function well during perfusion, or the viability, close to the end stage of the perfusion, was compromised leading to interruption of perfusion. The causes for interruption were, for example; insufficient flow rates, high perfusion pressures in fetal artery, pH out of range or high volume of the maternal venous effluent, which indicated damage in the blood-placental barrier. Four percent of well perfused cotyledons (accepted in the perfusion stage) were discarded later, because of poor performance indicated by negligible transfer of antipyrine. The total number of accepted placentas after pharmacokinetic analyses was 80.

Perfusion pressure, measured from the fetal artery in both maternal-to-fetal and fetal-to-maternal perfusions, remained stabile and stayed within the acceptance range (14-32 mmHg) during all accepted perfusions (Tables 8 and 9). To specify, no statistically significant changes occurred in the perfusion pressures ($p>0.05$) in the first three studies (Studies I-III), but there was a small but statistically significant decrease in perfusion pressure values in the MK-571 group ($p= 0.012$) and in the probenecid group ($p=0.033$) in Study IV. In Study II (maternal-to-fetal perfusions), the pressure measuring device was not available and the other viability indicators like flow rates and pH were observed carefully. It is known that if for some reason the perfusion pressure rises, it immediately affects the flow rates by decreasing the values and thus, unchanged flow rates give indirect evidence for unchanged perfusion pressure.

All pH values were within the acceptance range both in the maternal-to-fetal and in the fetal-to-maternal perfusions (Tables 8 and 9). The more detailed pH data (start and end values) are shown in the viability tables of each original publication. To specify, in the first three studies, all pH values were within the physiologic range ($p>0.05$). The normal physiologic range of newborn cord pH is; artery: 7.05-7.38, vein: 7.17-7.48. In Study IV, all pH values were within the acceptance rate; a modest but statistically significant increase in the perfusate pH was observed in the maternal venous outflow ($p=0.008$) in the probenecid group between the start and the end of the two-hour perfusion.

Antipyrine, which is a freely diffusible drug across the placenta and a good indicator of placental performance, was transferred without differences ($p > 0.05$) in all accepted perfusions ($n=80$). The average flow-corrected transfer (TPT_{AUC} %) of antipyrine was 7.6% in the maternal-to-fetal perfusions and 42.7% in the fetal-to-maternal perfusions (Tables 8 and 9).

Table 8. The viability of the cotyledons in maternal-to-fetal perfusions expressed as pH and perfusion pressure in fetal artery.

pH FA	n=52	7.2 (0.07)
pH FV	n=52	7.2 (0.07)
Perfusion pressure (mmHg)	n=33	20.8 (4.00)
TPT_{AUC} % of antipyrine	n=52	7.6 (5.27)

Data are given as mean values and SD. The beginning and the end values of pH and perfusion pressure are pooled. FA=fetal artery and FV=fetal vena.

Table 9. The viability of all reversely (i.e. fetal-to-maternal direction) perfused cotyledons expressed as pH and perfusion pressure in maternal artery.

pH MA	n=28	7.4 (0.11)
pH MV	n=28	7.3 (0.10)
Perfusion pressure (mmHg)	n=28	19.9 (3.38)
TPT_{AUC} % of antipyrine	n=28	42.7 (16.60)

Data are given as mean (SD). The beginning and the end values of pH and perfusion pressure are pooled. MA= maternal artery, MV= maternal vena.

5.2 Transplacental transfer of study drugs

5.2.1 Maternal-to-fetal perfusions

5.2.1.1 Transfer of saquinavir

The TPT_{AUC} % of saquinavir from the maternal side to the fetal side was low in control placentas (Fig. 11); the mean TPT_{AUC} % of saquinavir was 0.18% (Studies I-II). The transplacental transfer of saquinavir, as measured by TPT_{AUC} % ($p=0.66$) or saquinavir concentration at 120 min ($p=0.67$) was not different between different ATP concentrations or the control perfusions (Study I). The mean placental transfers of saquinavir (TPT%) with and without ATP are shown in Figure 10. The TPT_{AUC} % of saquinavir in maternal-to-fetal perfusions in the control group and after perfusion with P-gp inhibitors valspodar (PSC833) and GG918 are shown in Figure 11. Perfusion with PSC833 increased the TPT_{AUC} % of saquinavir 7.9-fold ($p<0.001$) and perfusion with GG918 6.2-fold ($p<0.001$) (Study II). Also the end-perfusion transfer (%) of saquinavir at 120 min was several-fold higher ($p<0.001$) with both inhibitors (Study II).

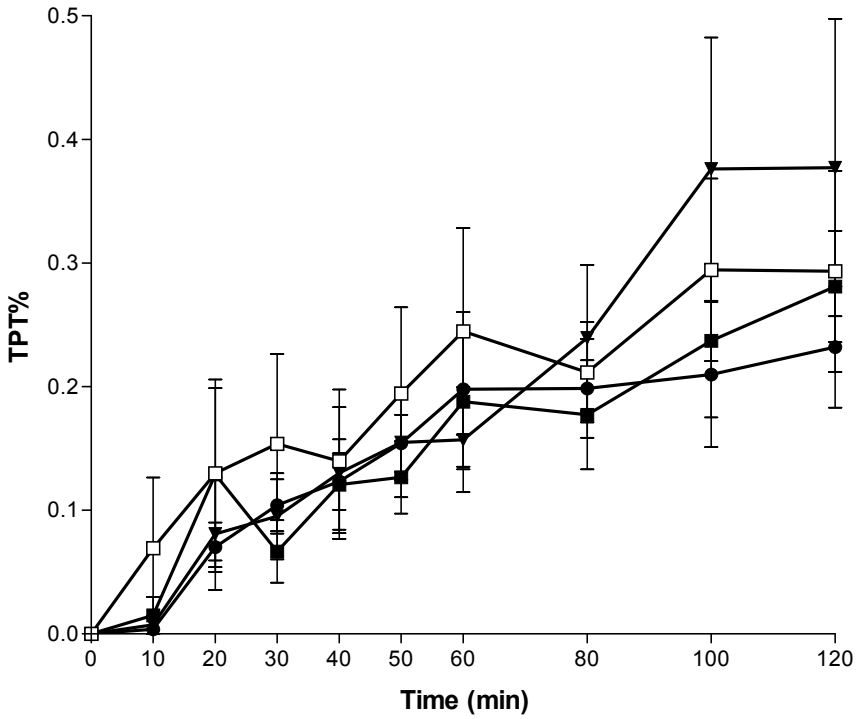

Figure 10. The mean transplacental transfer of saquinavir (TPT%) in the control perfusions: no ATP (n=4; □) and after addition of ATP with 1 μM (n=4; ■), 10μM (n=4;▼) or 100 μM (n=4; ●) in the perfusion medium during the 2-hour perfusion, error bars indicate SEM. (This figure is from original publication I).

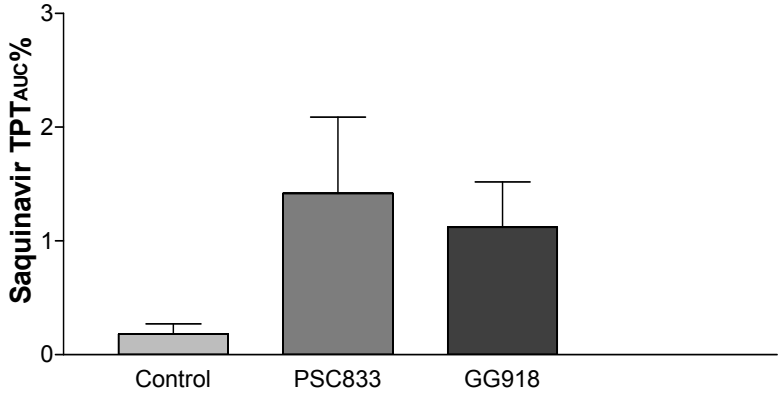

Figure 11. The TPT_{AUC} % of saquinavir in maternal-to-fetal perfusion in the control (n=12; Studies I and II) group and after perfusion with P-glycoprotein inhibitors PSC833 (valspodar) (n=6) and GG918 (n=5). TPT_{AUC}% = transplacental transfer calculated by comparing the absolute amount of drug infused into placenta with the amount of drug found in fetal venous outflow. Error bars indicate SD. P< 0.0001.

5.2.1.2 Transfer of quetiapine

The TPT_{AUC} % of quetiapine in the control perfusions was 3.7% (n=6). The P-gp inhibitors (PSC833 and GG918) had no significant effect on the transfer of quetiapine

as measured by either TPT_{AUC} % (p=0.77) (Fig. 12) or the end-perfusion quetiapine concentration at 120 min (p=0.95) (Study III).

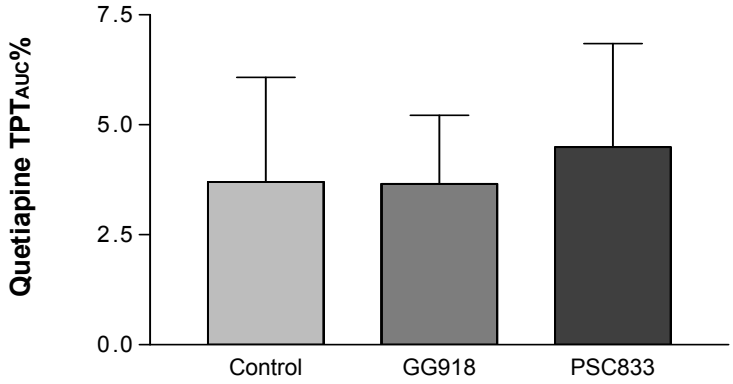

Figure 12. The TPT_{AUC} % of quetiapine in the control group (n=6) and with P-glycoprotein inhibitors GG918 (n=5) and PSC833 (n=6). TPT_{AUC}% = transplacental transfer calculated by comparing the absolute amount of drug infused into placenta with the amount of drug found in fetal venous outflow. Error bars indicate SD. P= 0.77.

5.2.1.3 Transfer of antipyrine

The TPT_{AUC} % of the control agent, freely diffusible antipyrine, was not significantly altered in the ATP perfusion study (p=0.71, Study I) nor after perfusion with the P-gp inhibitors (PSC833 and GG918) (p=0.07, Study II) compared with control perfusions. In addition, no differences were found in the TPT_{AUC} % of antipyrine (p=0.84) between the two inhibitor groups (PSC833 and GG918) and the control group in quetiapine perfusions (Study III).

5.2.2 Fetal-to-maternal perfusions (reversed perfusions)

5.2.2.1 Transfer of saquinavir

The data of all fetal-to-maternal perfusions are shown in Fig. 13. The TPT_{AUC} % of saquinavir to the maternal side in the control group was 16.2 %. The TPT_{AUC} % of saquinavir with P-gp inhibitor (PSC833) and MRP1 inhibitors (probenecid and MK-571) was not significantly different compared with the control perfusions (p=0.28). In Study IV, concomitant perfusion of MK-571 or probenecid showed tendency to reduce the fetal-to-maternal TPT_{AUC} % by 43% (p=0.34) and 24% (p=0.34), but these findings did not reach statistical significance (Fig. 13).

The transfer of saquinavir, in all control placentas, was about 100-fold higher from the fetal side to the maternal side compared with that from the maternal to the fetal side.

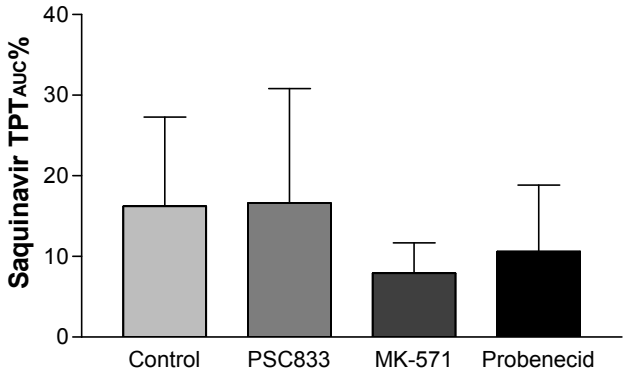

Figure 13. The TPT_{AUC} % of saquinavir (fetal-to-maternal perfusions) in control perfusions (n=10) and with P-gp inhibitor (PSC833, n=4) and with MRP1 inhibitors (MK-571, n=7 and probenecid, n=7). TPT_{AUC}% = transplacental transfer calculated by comparing the absolute amount of drug infused into placenta with the amount of drug found in maternal venous outflow. Error bars indicate SD.

5.2.2.2 Transfer of antipyrine

The employed transporter inhibitors had no statistically significant effect on the TPT_{AUC} % of antipyrine in reversed perfusions (p=0.62, in Study II and p=0.25, in Study IV) compared with controls. The TPT_{AUC} % of antipyrine was about 7-fold higher from fetal-to-maternal perfusions compared with maternal-to-fetal direction within all controls of reversed perfusions (Studies II and IV).

5.3 Effect of *ABCB1* genotype

5.3.1 On saquinavir transfer (maternal-to-fetal)

The *ABCB1* genotype did not seem to affect the TPT_{AUC} % of saquinavir (p= 0.85 in exon 21 group and p= 0.71 in exon 26 group) (Fig. 14). In Figure 14 only placentas from Study I were used to depict the studied association without the potential confounding by P-gp inhibitors.

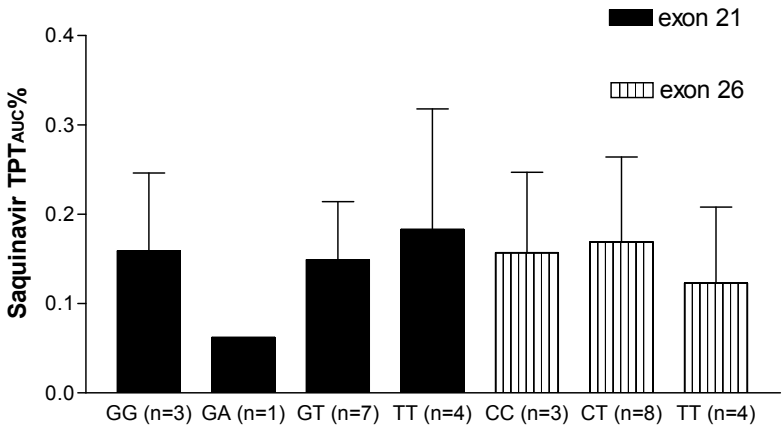

Figure 14. The influence of the c.3435C>T, p.Ile1145Ile (exon 26) and the c.2677G>T/A, p.Ala893Ser/Thr (exon 21) polymorphisms on the placental transfer of saquinavir (n= 15) as expressed by the TPT_{AUC} %. Only one placenta had the GA genotype. Error bars indicate SD.

The *ABCB1* genotype did not affect the PSC833- or GG918-induced change in transplacental transfer of saquinavir either, although it should be noted that the number of placentas was small (for the c.3435C>T, p.Ile1145Ile polymorphism in exon 26 p= 0.46, for the c.2677G>T/A, p.Ala893Ser/Thr polymorphism in exon 21 p= 0.52) (Study II).

5.3.2 On quetiapine transfer

The effect of the *ABCB1* genotype on TPT_{AUC} % of quetiapine is shown in Figure 15. The 3435T allele in exon 26 was associated with somewhat higher placental transfer of quetiapine (p=0.04). The c.2677G>T/A, p.Ala893Ser/Thr polymorphism in exon 21 did not have an overall statistically significant effect on quetiapine transfer (p=0.17), but individuals homozygous for the G-allele (and possibly those with the GA genotype) had a lower quetiapine transfer than others (Fig. 15).

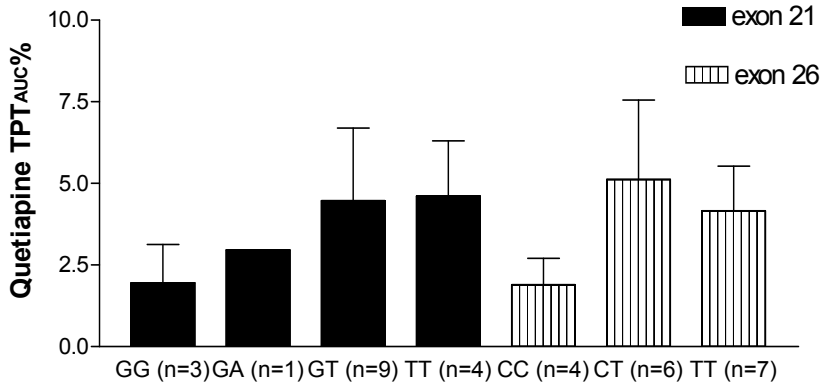

Figure 15. The effect of the *ABCB1* genotype on TPT_{AUC} % of quetiapine. The total number of the studied placentas was 17. Error bars indicate SD.

5.3.3 On P-gp expression

The effect of the *ABCB1* c.2677G>T/A, p.Ala893Ser/Thr polymorphisms in exon 21 and c.3435C>T, p.Ile1145Ile in exon 26 on P-gp protein expression level is shown in Figure 16 (n=44, placentas from Studies I-III). The 3435T allele was associated with a higher protein expression of P-gp (p= 0.009), the placentas carrying the TT genotype had a 29% higher protein expression level compared with the CC genotype (Fig. 16b). There was no significant association with the polymorphism c.2677G>T/A, p.Ala893Ser/Thr on P-gp protein expression level (p=0.44) (Fig. 16a). Figure 17 shows a part of western blot analysis made for this study.

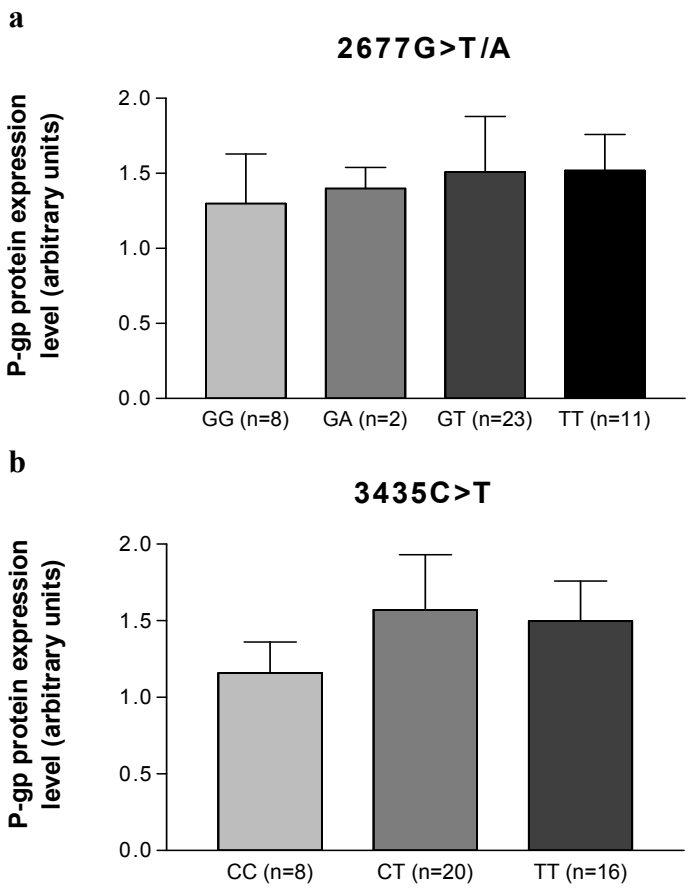

Figure 16. Effect of the *ABCB1* c.2677G>T/A, p.Ala893Ser/Thr polymorphism in exon 21 (a) and c.3435C>T, p.Ile1145Ile polymorphism in exon 26 (b) on P-gp protein expression level (mean). The total number of placentas was 44, from Studies I-III). Error bars indicate SD. (This figure is from original publication I).

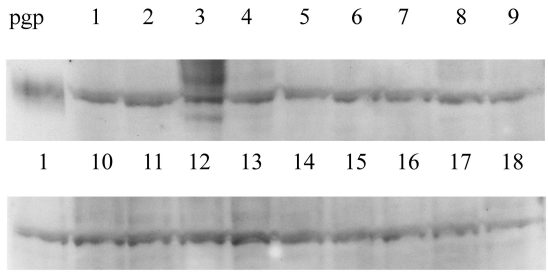

Figure 17. An example of western blot bands from Study III showing expression of P-gp

5.4 P-gp protein expression in human placenta and the transfer of saquinavir and quetiapine

No statistically significant correlation was found between the TPT_{AUC} % of saquinavir (with or without P-gp inhibitors) or TPT_{AUC} % of quetiapine and placental P-gp protein expression in Studies I-III.

6 DISCUSSION

Daily use of pharmaceuticals during pregnancy is needed among some mothers with chronic diseases such as epilepsy, rheumatoid arthritis, HIV or depressive disorders. Temporary need of some drugs, such as antibiotics or antimigraine medication, is common during pregnancy. There is a continuous clinical demand for new and more effective/safer pharmacological treatment options for different disorders during pregnancy, and obtaining information of the use of these options, is a significant challenge.

Several different methods have been used to study the role of membrane transporter proteins and the transfer of drugs. There are studies with cultured cells, perfusion studies in animals and studies in mice lacking P-gp (generated a homozygous disruption of *Abcb1a* gene) (Smit et al. 1999, Ushigome et al. 2000, Staud et al. 2006). However, these cultured cell and animal studies may not be reliable when studying the placental transfer of drugs or factors affecting the function of the human blood-placental barrier (BPB) (Young et al. 2003). Perfusion of the term human placenta offers a good model for the study of drug transfer without exposing the mother or the fetus to the medications. This study series show that the perfusion method with human term placentas is also an elegant way to gain information on the function of placental drug transporters.

6.1 General methodological considerations

6.1.1 Placental perfusion methods

The *ex vivo* human placental perfusion method has been used for over 30 years (Schneider et al. 1972). An open/dual perfusion system, where the perfusates are not recirculated, is especially suitable for placental transfer studies, and the recirculating perfusion model also provides a model to examine the placental metabolism of the substances (Brandes et al. 1983, Omarini et al.1992, Ala-Kokko et al 2000). An *ex vivo*, perfused human placental method, is a safe model, which does not expose either the fetus or the mother to drugs, and, thus, the ethical concerns are very limited. Accordingly, the method offers a possibility to study transplacental transfer of even toxic chemicals, such as cytotoxic drugs or carcinogens (Al-Saleh et al. 2007, Annola et al. 2008, Myllynen et al. 2008). The human placental perfusion method is quite demanding and laborious to build up and use, and thus the number of placentas with this methodology cannot be very high.

The adequate handling of the placenta (or cotyledon) is crucial from the very beginning, already from the delivery room, until the end of each perfusion. Fast and tender preparation, use of heparin for prepared vessels, short distance to the perfusion laboratory and quick connection of the prepared cotyledon to the perfusion apparatus are important factors for the viability of placentas. Vessel preparation of the fetal surface has to take place in the most superficial part of the cotyledon to avoid damaging the BPB during preparation. Damage to the BPB may result in direct

transfer of study drugs and perfusion medium across the placenta. This damage is often diagnosed by high volume of maternal effluent during perfusion or abnormal transfer of antipyrine seen later in pharmacokinetic analyses.

The good viability of the placenta and the stability of the perfusion system are the main criteria for an acceptable perfusion. In this study series, the flow rate, the most important parameter of viability, was unchanged during all perfusions, and no mismatch occurred between fetal and maternal circulation. The perfusion pressures (14-32 mmHg) were within the generally accepted range and measured in fetal artery at the beginning and end of the perfusions (Heikkinen et al. 2002), with the exception of Study IV, where a small but statistically significant decrease in perfusion pressure values was observed in the MK-571 group and the probenecid group. The relatively long perfusion time, as an independent factor, can partly explain the descending trend of the perfusion pressure during the perfusions. In one part of the Study II, in the maternal-to-fetal perfusions, the perfusion pressure device was not available for technical reasons. It is known that if for some reason the perfusion pressure rises, it immediately affects the flow rate by decreasing the values, and therefore other viability indicators (the flow rates and pH) were observed carefully.

All pH values were within acceptance range in this study series (I-IV). A small but statistically significant variation within the pH values in Study IV can probably be explained by the manual oxygenation. Both the fetal and maternal arterial perfusion media are gassed with oxygen-carbon dioxide mixture via two plastic tubes throughout the perfusion. The volume of the gas mixture can be regulated only manually, leading to possible variation in oxygenation. Since antipyrine is freely transferred across the placenta, it is a good surrogate marker of adequate placental function during the perfusion. In this study series the transfer of antipyrine was very stabile and comparable with earlier studies using an identical perfusion methodology (Heikkinen et al. 2001, Heikkinen et al. 2002).

6.1.2 Immunoblotting and genotyping

The P-gp protein expression level of perfused cotyledons was determined to examine the impact of *ABCB1* genotype on P-gp protein expression. Also, the effect of P-gp protein expression on saquinavir and quetiapine transfer was studied. One major limitation in these studies was a relatively small sample size, which may be one reason for statistically nonsignificant results. A larger number of placentas might reveal some variations in results, which went unnoticed in these studies.

A sample of the perfused cotyledon was cut through the whole placental tissue and the maternal side of the obtained sample was marked for the measurement of P-gp protein expression, since P-gp is known to be expressed on the apical side of syncytiotrophoblasts (Mathias et al. 2005). One major chance for bias in Western blot analyses is that it is sometimes difficult to know exactly whether the defined placental tissue is entirely from the fetal or maternal source. These studies were performed with human term placentas, and therefore the results of P-gp protein expression level cannot be generalized to the whole duration of pregnancy. It is known that during the first

trimester of pregnancy, when the functional and protective mechanisms are needed most, the placental expression of the P-gp is at its highest and then it decreases towards the end of pregnancy (Kalabis et al.2005, Mathias et al. 2005, Myllynen et al. 2007). May et al. (2008) suggested, that in late pregnancy other transporters, for example multidrug resistance-associated protein 2 (MRP2), can be more important transporter than P-gp, because MRP2 is increasingly expressed to the end of pregnancy.

In the polymorphic *ABCB1* gene, a number of SNPs have been established in various ethnic populations (Chinn and Kroetz 2007). At least 19 nonsynonymous SNP of *ABCB1* have been reported (Maeda and Sugiyama 2008). In the literature, there are several studies of the effects of *ABCB1* SNPs on altered drug levels and susceptibility to some diseases, but the results are inconsistent and even conflicting, especially with regard to the polymorphism c.3435C>T, p.Ile1145Ile in exon 26 (Marzolini et al. 2004, Kim et al. 2002, Zhou 2008). Therefore, in this study series, we concentrated on the two known *ABCB1* polymorphisms, the c.3435C>T, p.Ile1145Ile polymorphism in exon 26 and the c.2677G>T/A, p.Ala893Ser/Thr polymorphism in exon 21. Accordingly, the possibility remains that some other *ABCB1* polymorphisms may a have higher impact on placental P-gp function. Several studies have shown that SNP c.1199G>A, p.Ser400Asn in exon 11 is associated with increased resistance to anticancer drugs (Zhou 2008). Few studies of the synonymous SNP c.1236C>T, p.Gly412Gly (rs1128503) in exon 12 have been published suggesting that it is a clinically nonrelevant SNP (Marzolini et al. 2004, Zhou 2008). Moreover, these polymorphisms can be linked to some other polymorphisms and thus might affect, for example, protein expression and, possibly, P-gp function in placenta (Marzolini et al. 2004, Zhou 2008). Uncertainty of these associations can lead to incorrect conclusions.

6.2 General discussion

6.2.1 ATP in the perfusion system

The BPB uses ATP-dependent drug transporter proteins to decrease fetal exposure to clinically used drugs (Young et al. 2003). The first aim of this study series was to investigate whether addition of exogenous ATP to the perfusion medium is needed when studying ATP-dependent transporter activities in the employed perfusion setting (Study I). The concentration of ATP was based on earlier reports indicating that circulating plasma levels of ATP in humans are approximately 1 μmol/l (Gorman et al. 2007). We also used higher ATP concentrations to ensure observation of a possible change in substrate transfer. The highest concentration (1000 μmol/l) of added ATP clearly indicated placental toxicity. This present study indirectly suggests that the placenta continues to produce ATP during the placental perfusion, because addition of different amounts of exogenous ATP to the perfusion medium did not change the P-gp-dependent transfer of saquinavir. This result is consistent with previous studies showing that ATP levels remain constant at least for one hour and, possibly, even 11 hours after delivery in perfused human placenta (Young and Schneider 1984, Malek et al. 1996). This finding is relevant for all placental perfusion studies of drugs that are substrates of ATP-dependent transporter proteins, and, especially when studying the function of different ATP-dependent transporters in the perfused human cotyledon.

6.2.2 Placental transfer of study drugs

Saquinavir, an antiretroviral drug used in HIV therapy, was used as a probe drug in these studies to clarify the role of drug transporters, because it is known to be a substrate of P-gp and MRP1 (Smit et al. 1999, Williams et al. 2002). Furthermore, the transplacental transfer of saquinavir from maternal to fetal side was known to be very small (Forestier et al. 2001), suggesting an active role of BPB. The evidence of whether quetiapine is a P-gp substrate is conflicting (Boulton et al. 2002, Ela et al. 2004, Grimm et al. 2005). In this study series, the methods performed first with saquinavir were subsequently used to study transplacental transfer of quetiapine, an uncertain probe drug of P-gp. This widely used atypical antipsychotic drug was an interesting probe drug for this study, because it is used among women of fertile age (Taylor et al. 2003) and sometimes even during pregnancy (off-label) (Newport et al. 2007). Moreover, there is some evidence that quetiapine may cause fetal toxicity in animals at supratherapeutic concentrations (http://www1.astrazeneca-us.com/pi/seroquel.pdf), but the few clinical reports of its use during pregnancy found no evidence of congenital malformations (Tenyi et al. 2002, Yaris et al. 2004, Yaeger et al. 2006).

These present studies indicated that saquinavir and quetiapine transfer across the human placenta, but in different amounts. Transfer of saquinavir was very small; less than 0.5% of the drug added to the maternal side was discovered in the fetal vein during the two-hour perfusion. The TPT_{AUC} % of quetiapine (3.7%) was more than 20-fold compared with the TPT_{AUC} % of saquinavir. The transfer of saquinavir was 97% less and the transfer of quetiapine 29% less than the transfer of the freely diffusible antipyrine, which indicates that the BPB functions as an active obstacle to the transfer of both compounds, but this effect is much more pronounced for saquinavir. The high protein binding of quetiapine (83%) (Green 1999) and saquinavir (98%) (Sudhakaran et al. 2007) may also be one reason for limited placental transfer. However, it is known that the composition of plasma proteins varies between maternal and fetal plasma, resulting in differential protein binding of several drugs (Sudhakaran et al. 2007); thus it needs to be remembered that this study can not give an accurate picture of the *in vivo* condition.

In recent years, the interest of many research groups has been directed to different drug transporters. Especially, the role of the widely expressed P-gp has been understood to be crucial in the pharmacokinetics of different drugs. The function of MRP1 has also been studied diligently, but yet the role of basal transporter proteins, such as MRP1, is generally much less understood, especially in the human placenta. The effect of the modulation of P-gp function and its possible influences on transfer of drugs has been shown in various animal studies; for example, Pavek et al. (2001), showed a significant increase in the placental transfer of the P-gp substrate cyclosporin when perfused with P-gp inhibitors quinidine or chlorpromazine in the rat placenta. Moreover, Lankas et al. showed in 1998 that lack of functional P-gp may predispose to teratogenicity in mice. In that study, all the offsprings of the P-gp-deficient mice which were exposed to a photoisomer of avermectin B1a developed a cleft palate.

Our results in Study II indicated that P-gp has a major functional role in the human BPB. According to our study, the pharmacological blockade of P-gp by valspodar

(PSC833) and GG918 significantly increased (6- to 8-fold) the placental transfer of saquinavir from the maternal circulation to the fetal circulation. Moreover, the mean end-perfusion concentration of saquinavir at 120 minutes was 11- and 6-fold higher in perfusions with valspodar and GG918, respectively, compared with controls. These findings indicate that perfusion of placentas with these inhibitors caused a clear disruption of the BPB in the transfer of saquinavir. The present and earlier results suggest that exposure of the mother to compounds that inhibit P-gp or other transporters during pregnancy could lead to increased penetration of drugs and teratogenic agents to the fetal side (Smit et al. 1999). These inhibitor agents may cause disruption of BPB allowing fetotoxic drugs and environmental toxins to cross the placenta, achieving a detrimental effect on the fetus, although these inhibitors are not teratogenic themselves. On the other hand, modulation of P-gp could be used to treat the fetus during pregnancy, as previously proposed (Smit et al 1999, Ito et al. 2001).

In Study III, we found that the placental transfer of quetiapine was not affected by P-gp inhibition (valspodar and GG918), suggesting that quetiapine is not a substrate of P-gp. However, Holcberg et al. (2003) reported that neither quinidine nor verapamil, which are well known P-gp inhibitors, affected the transplacental transfer of the P-gp substrate digoxin in isolated perfused human placenta. Taking this into account, it is obvious that the placental transfers of different P-gp substrates are not equally affected by P-gp inhibitors. Accordingly, lack of effect of P-gp inhibition on the placental transfer of quetiapine does not exclude the possibility of quetiapine being a P-gp substrate. Again, it should be remembered that our results in the term placenta cannot be directly extrapolated to first and second trimesters of pregnancy, because of different P-gp expression levels (Kalabis et al. 2005, Mathias et al. 2005).

One of the most widely studied SNPs in the *ABCB1* gene, in addition to nonsynonymous c.2677G>T/A, p.Ala893Ser/Thr in exon 21, is the c.3435C>T, p.Ile1145Ile in exon 26 which has been shown to affect the pharmacokinetics of some commonly used P-gp substrates (e.g. loperamide, fexofenadine) in humans (Skarke et al. 2003, Yi et al. 2004). It is a mystery how this silent (synonymous) polymorphism c.3435C>T, p.Ile1145Ile can have influence on the pharmacokinetics of drugs, but the linkage disequilibrium is suggested to be the cause (Marzolini et al. 2004). One other hypothesis for silent SNP´s effect for protein binding affinity is changed conformations in protein binding sites, by altering the timing of protein formation which could lead to changes in protein folding (Kimchi-Sarfaty et al. 2007). In the present studies we wanted to clarify the role of the two known *ABCB1* polymorphisms c.2677G>T/A, p.Ala893Ser/Thr in exon 21 and c.3435C>T, p.Ile1145Ile in exon 26 in the transfer of the study drugs and in the P-gp expression level in perfused cotyledons, because these SNPs are associated with altered P-gp expression in human placenta (Tanabe et al. 2001, Hitzl et al. 2004)). We found in Study III that the variant *ABCB1* 3435T allele was associated with higher placental transfer of quetiapine compared with the 3435C allele, suggesting that this polymorphism may affect the placental transfer of quetiapine. Nevertheless, no association was found between P-gp expression and placental transfer of quetiapine, which (in addition to the negative finding of P-gp inhibition on quetiapine transfer) decreases the likelihood that the *ABCB1* genotype would have a clinically relevant effect on fetal exposure to quetiapine.

Significantly higher placental P-gp protein expression levels were associated with the variant allele 3435T in our studies. This result is not in accordance with previous studies, but it should be noted that the earlier results are contradictory concerning the association of P-gp expression with c.3435C>T, p.Ile1145Ile SNPs in exon 26 (Hizl et al. 2004, Chowbay et al. 2005). Nevertheless, the *ABCB1* genotype did not seem to affect the TPT_{AUC} % of saquinavir and no correlation was found between the TPT_{AUC} % of saquinavir and placental P-gp expression either. The results of this study series may indicate that P-gp and some other ATP-dependent drug transporters, for example, breast cancer resistance protein (BRCP), are the most generously expressed placental drug transporters and, therefore, moderate alterations in the expression level may not lead to function alterations at clinically relevant substrate concentrations.

6.2.3 Reversed perfusions

Placental transporters on the basal surface of the syncytiotrophoblast appear to be less well studied. Studies in mice have suggested that the basal transporter, multidrug resistance-associated protein 1 (Mrp1) plays a significant role in drug pharmacokinetics in various tissues. For example, Wijnholds et al. (2000) showed that in choroid plexus epithelium expressed Mrp1 plays a significant role in the blood-cerebrospinal fluid (CSF) barrier, limiting access of compounds to CSF. In that study, they used a combination of *Abcc1/Abcb1a/Abcb1b* triple-knockout mice (lack of Mrp1 and P-gp), along with *Abcb1a/Abcb1b* double-knockout mice (lack of P-gp) and identified an approximately 10-fold increase in intravenously administered etoposide concentration in CSF in the triple-knockout mice compared with the double-knockout mice, suggesting the crucial role of Mrp1. Furthermore, Johnson et al. (2001) compared mice with combined deficiency of P-gp and Mrp1 with wild-type mice after intraperitoneally administered vincristine and etoposide and observed a crucially increased toxicity in bone marrow and gastrointestinal tissue. However, in P-gp deficient mice, the increase in toxicity was very much lower suggesting the crucial role of Mrp1 in the efflux of these drugs. Human term placenta expresses MRP1 (St-Pierre et al. 2000). MRP1 is localized predominantly in the basal, fetal facing plasma membrane (Atkinson et al. 2003, Nagashige et al. 2003) and has been regarded as an efflux pump i.e facilitating transfer of drugs and other compounds to fetal circulation from the placenta (Unadkat et al. 2004, Myllynen et al. 2007).

Knowing that saquinavir is a substrate of at least some of the transporter proteins present in the basal membrane of the syncytiotrophoblast cells (Williams et al. 2002), we wanted to investigate whether there is active transport of saquinavir from the fetal side to the maternal side by studying the effect of known inhibitors of the basal transporters, MK-571 and probenecid (Renes et al. 2000, Haimeur et al. 2004) and with valspodar (P-gp inhibitor), on the fetal-to-maternal transfer of saquinavir.

The results indicated that the flow-corrected transfer of saquinavir from fetal-to-maternal direction was almost 100-fold greater in fetal-to-maternal direction compared with maternal-to-fetal perfusions. This is consistent with a previous study, indicating that maternal-to-fetal transfer clearance of indinavir, another protease inhibitor, was significantly lower than the corresponding fetal-to-maternal transfer clearance

(Sudhakaran et al. 2005). Besides, the transfer of the reference agent, antipyrine, was also about 7 times greater in the fetal-to-maternal direction compared with the maternal-to-fetal direction of all these perfusion. This result suggests that BPB on the fetal side is more permeable or filled with active transporters, helping the fetus to remove compounds. Earlier evidence supports the existence of active placental influx pumps (throwing compounds into placenta from the fetal compartment), which can even act together with some apical efflux transporters and form a vectorial collaboration in facilitating the compounds from the fetal compartment to maternal blood. Grube et al. (2007), for example, reported of functional interaction between the basal influx transporter OATP2B1 (OATP-B) and the apical efflux transporter BCRP.

In this study series, the perfusions with P-gp inhibitor (valspodar) did not affect the TPT_{AUC} % of saquinavir in reversed perfusions. The TPT_{AUC} % of saquinavir was lower with both MRP1 inhibitors, especially with MK-571 (TPT_{AUC} % 7.9) compared with controls (TPT_{AUC} % 14.0), but this finding was not statistically significant ($p>0.05$), probably due to the high variation of the saquinavir transfer from fetal to maternal side together with our limited sample size.

To guarantee measurable saquinavir concentrations in the maternal venous outflow, the used "fetal" saquinavir concentration was several-fold higher than the supposed clinical fetal concentration would be, as the transplacental transfer of saquinavir to the fetal side is known to be low (Forestier et al. 2001). The results of this study series may be confounded by saturation of the possible basal transport mechanisms because of high saquinavir concentrations. This may also cause masking of the inhibitory effect by the used inhibitors on saquinavir transfer, albeit it is not likely.

The obtained results suggested that the transporter inhibitors valspodar, MK-571 and probenecid did not alter the fetal-to-maternal transfer of saquinavir. Accordingly, it is unlikely that MRP1 plays a significant role in the active removal of saquinavir from the fetus to maternal blood. Probenecid is a strong inhibitor of the OATP transporters, of which at least OATP2B1 is a widely expressed transporter (Mikkaichi et al. 2004, Janneh et al. 2007). Moreover, also MK-571 has been reported to have an inhibitory effect on OATP2B1 (Letschert et al. 2006). Accordingly, the present results may also suggest that there are no major effects by OATP2B1 on the fetal-to-maternal transfer of saquinavir.

6.2.4 Problems with placental perfusion method in studying the transfer of drugs and function of transporters

The methodology of human placental perfusion to study the transfer of drugs has been developed for several decades. Some substrates of the transporter proteins can also inhibit the respective transporter activity. For example, cyclosporine may not be an ideal probe drug for P-gp mediated placental transfer, since it also inhibits the P-gp. Furthermore, specific well-characterized probe drugs as well as model inhibitors for some placental transporters are still lacking, which also partly compromises the interpretation of the present results.

Furthermore, some transporters, for example, Human Equilibrative Nucleoside Transporters 1 and 2 (hENT1 and 2), organic cation transporter 3 (OCT3) and carnitine

transporter 2 (OCTN2) are equilibrative (facilitative), i.e. can act both as influx and efflux transporters depending on the concentration gradient (Unadkat et al. 2004); OATP2B1 alone has also been found to work in both directions (Grube et al. 2007) (Fig. 18). Kovo et al. suggested (2008) in their study, that transplacental transfer of metformin is mediated by a transporter in a dose-dependent manner. Furthermore, the high lipophilicity of drugs and their low molecular weight support transfer across the placenta by passive diffusion without a transporter protein involvement.

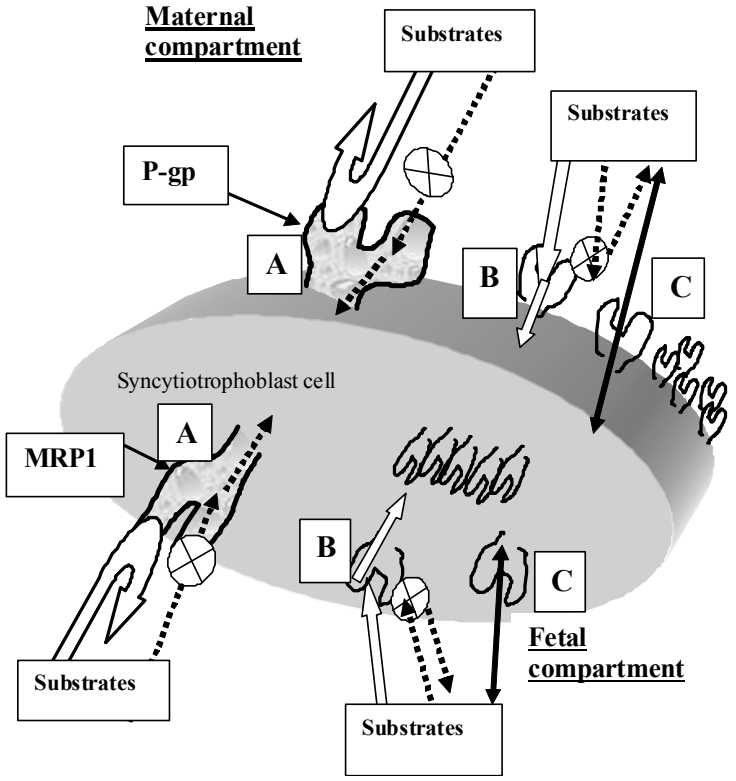

Figure 18. Schematic presentation of syncytiotrophoblast cell with different transporter function in human placenta. A = efflux transporters (throwing substrates out of the cell), B= influx transporters (throwing substrates into the cell) and C= influx-efflux transporters.

6.2.5 Advantages with placental perfusion method in studying the transfer of drugs and function of transporters

The perfusion method using human placenta including obtaining term placentas is quite laborious in studying the mechanisms of placental transfer of compounds. This is problematic since the amount of placentas in perfusion studies is crucial to minimizing the risk of type II statistical errors. Our results suggest, on the other hand, that the human placenta perfusion method could be a valuable tool for characterizing placental pharmacokinetics of compounds relevant for drug therapy during pregnancy. Specifically, it can be used to estimate the effect of maternal drug therapy on the function of P-gp and other transporters.

7 SUMMARY AND CONCLUSIONS

In the present study series, the role of placental transporters (P-gp and MRP1) in the transfer of saquinavir was studied. The placental transfer of quetiapine and the role of P-gp in its transfer were researched. Also, *ABCB1* genetic polymorphism (c.3435C>T, p.Ile1145Ile and c.2677G>T/A, p.Ala893Ser/Thr) was studied to determine their impact on P-gp protein expression level and on transfer of saquinavir and quetiapine. Finally, the influence of P-gp expression level on transfer of the study drugs was characterized.

1. Our results indicate that addition of exogenous ATP did not change the P-gp-dependent transfer of saquinavir, suggesting that the ATP-producing mechanisms are intact in the dually perfused human placenta.

2. Quetiapine (TPT_{AUC} % = 3.7%) and saquinavir (TPT_{AUC} % <0.5%) cross the human placenta.

3. P-gp has a major functional role in the human BPB. Pharmacologic blocking of P-gp can lead to disruption of the BPB and increase the transfer of P-gp substrates, such as saquinavir, to the fetal circulation by several-fold. The increased exposure of the fetus to developmentally harmful drugs or environmental toxins as a result of maternal use of P-gp-inhibiting compounds is a potential mechanism of teratogenicity.
   BPB limits the placental transfer of quetiapine, but P-gp seems not to have a major role in quetiapine transfer. In clinical practice, co-administration of P-gp inhibiting drugs is not likely to increase fetal exposure to quetiapine.

4. P-gp, MRP1 and possibly OATP2B1 have a negligible role in the removal of saquinavir from the fetal circulation. In fetal-to-maternal perfusions, TPT_{AUC} % of saquinavir was about 100-fold greater and TPT_{AUC} % of the reference agent, antipyrine, was 7 times greater compared with maternal-to-fetal direction. These results suggest that BPB on the fetal side is by some mechanisms, unrevealed by the present experiments, more permeable than on the maternal side helping the fetal clearance of compounds.

5. Significantly higher placental P-gp protein expression levels were associated with the *ABCB1* 3435T allele, but no correlation was found between the maternal-to-fetal transfer of saquinavir and the placental P-gp expression level suggesting negligible clinical relevance of this association.

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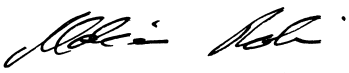

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