Univerzita Karlova v Praze

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Katedra farmakologie a toxikologie

STUDIUM TRANSPORTNÍCH MECHANISMŮ LÉČIV V PLACENTĚ A JÁTRECH

Disertační práce

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Obor: Farmakologie

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Hradec Králové 2006

Poděkování

Na tomto místě bych rád poděkoval všem, kteří mi pomáhali během studia a nemalou měrou tak přispěli i k této práci.

Na prvním místě bych chtěl vyjádřit poděkování Doc. PharmDr. Františku Štaudovi, Ph.D., který mě svým odborným a současně přátelským přístupem vedl během studia a jehož rady a připomínky mi byly vždy přínosem.

Poděkovat bych chtěl PharmDr. Martině Čečkové, Ph.D. za její kamarádský přístup a podnětnou spolupráci při řešení problematiky studia a dále PharmDr. Petru Pávkovi, Ph.D. za cenné rady a zkušenosti, které mi předával. Poděkování náleží také všem ostatním spolupracovníkům a kamarádům na Katedře Farmakologie a Toxikologie za poskytnutí příjemných pracovních podmínek.

Chtěl bych také projevit velký dík Dr. Radovanu Haluzovi Ph.D., Dr. Martinu Bunčekovi Ph.D. a dalším členům týmu GENERI BIOTECH s.r.o. nejen za teoretické a praktické zkušenosti, které jsem získal během naší spolupráce, ale také za jejich přátelský přístup.

Rád bych také poděkoval Dr. Lorella Pascolo Ph.D. a celému kolektivu Centro Studi Fegato v Terstu nejen za odborné zkušenosti získané v oblasti výzkumu jaterních transportérů, ale i za příjemně strávených šest měsíců.

V neposlední řadě patří dík mým rodičům a přítelkyni Zlatce za podporu, kterou mi během studia projevovali.

Seznam zkratek

ABC	„ATP binding cassette“, kazeta vážící ATP (též typ transportérů)
BCRP	„breast cancer resistance protein“, transportér
cDNA	„copy DNA“, DNA vzniklá reverzní transkripcí mRNA
MDR	„multidrug resistance“, mnohačetná léková rezistence
MRP	„multidrug resistance related protein“ (typ ABC-transportérů)
NBD	„nucleotide binding domain“, doména vážící nukleotidy
OAT	„organic anion transporter“, transportér organických aniontů
OATP	„organic anion transporting polypeptide“, polypeptid transportující organické anionty
OCT	„organic cation transporter“, transportér organických kationtů
P-gp	P-glykoprotein
RT-PCR	„reverse transcription – polymerase chain reaction“, polymerázová řetězová reakce, které předchází reverzní transkripce
SLC	„solute carrier“ (typ transportérů)
TM	transmembránový

Obsah

I.

ÚVOD A CÍLE PRÁCE

1. Transport farmak přes biologické membrány

Osud léčiva v organismu ovlivňují čtyři základní farmakokinetické děje – absorbce z místa podání, distribuce mezi kompartmenty, biotransformace a exkrece léčiva. Jedním z procesů, který zasahuje do všech těchto dějů, je transport látek přes biologické membrány. Na buněčné úrovni tyto fosfolipidové membrány mohou oddělovat extracelulární prostředí od intracelulárního nebo cytoplazmu od obsahu buněčných vezikul a tím zasahovat do metabolismu léčiv. V mnohých orgánech jsou buňky navzájem těsně spojeny a tím vytváří fyziologickou bariéru mezi dvěma kompartmenty. Aby molekuly léčiva přestoupily mezi jednotlivými kompartmenty, musí překonat nejméně dvě buněčné membrány (Rang, 2003).

Nejrozšířenějšími způsoby transportu léčiva přes buněčné membrány jsou prostá difúze a transport zprostředkovaný membránovými proteiny – transportéry. Dva další způsoby – difúze aquaporiny nebo pinocytóza - jsou méně časté. Prostá difúze je základní transportní mechanismus závislý na koncentračním gradientu léčiva a fyzikálně-chemických vlastnostech léčiva, především míře ionizace, velikosti jeho molekuly a lipofilitě. Transport zprostředkovaný přenašeči je využíván převážně pro větší molekuly a polární látky (Rang, 2003).

2. Význam membránových transportérů ve farmakologii

Počet genů kódujících proteiny membránových transportérů u člověka je odhadován na 883, což představuje přibližně 2,8% lidského genomu (Venter et al., 2001). Proteiny kódované těmito geny zastávají v buňkách důležité role: transportují živiny, odstraňují nepotřebné látky a udržují elektrochemický gradient na membránách. Četná mendelovsky děděná poškození způsobená mutacemi v genech transportérů a kanálů vyzdvihují jejich fyziologický význam.

Podle počtu současně transportovaných látek a směru transportu se transportní děje zprostředkované přenašeči dělí na uniport (jedna látka), symport (dvě látky stejným směrem) a antiport (dvě látky opačným směrem). Dále lze transportní procesy rozlišovat podle energetické závislosti. Aktivní transport využívá energie ATP (tzv. primárně aktivní transport) nebo elektrochemický gradient spřažené látky (tzv. sekundárně aktivní transport), zatímco facilitovaná difúze probíhá pouze ve směru elektrochemického gradientu transportované látky (Baynes, 2005). Na základě těchto parametrů se transportéry v současnosti člení do dvou skupin: (i) ABC transportéry (ABC – „ATP

binding cassette") využívající energii ATP a (ii) SLC transportéry („solute carrier"), které ke své činnosti vyžadují elektrochemický gradient substrátu nebo spřažené látky.

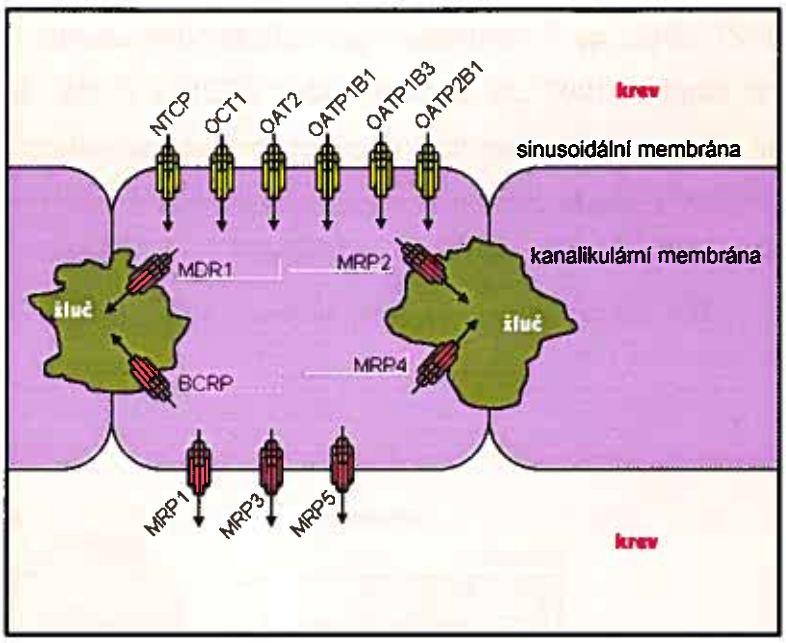
Transportéry zasahují do farmakokinetiky buď tím, že usnadňují nebo naopak brání v přestupu léčiv přes fyziologické bariéry. Vzhledem k tomu, že tyto bariéry jsou obvykle tvořeny vrstvou polarizovaných buněk, má na propustnost bariér vliv také distribuce transportérů mezi apikální a bazolaterální buněčnou membránou. Např. unipolární lokalizace efluxních ABC transportérů je stěžejní pro směr a rozsah prostupu léčiv přes fyziologické bariéry většiny orgánů (Ito et al., 2005).

Vhledem k tomu, že tato práce se zabývá transportními mechanismy v placentě a játrech, budou následující kapitoly věnovány převážně transportním procesům a relevantním transportérům v těchto orgánech.

3. Transportní procesy v játrech

Základní děje odehrávající se v játrech souvisí s bazálním metabolismem, detoxikací a tvorbou žluče. Na hepatobiliární exkreci, která je vedle renální exkrece významnou cestou pro vylučování farmak, se na buněčné úrovni podílí enzymatické a transportní děje v hepatocytu. Eliminace xenobiotik i endogenních látek játry sestává z následujících procesů: (i) vychytávání (uptake) z krve do hepatocytu, (ii) metabolismus a/nebo (iii) sekrece do žluči (Shitara et al., 2005).

Látky z krevního oběhu do hepatocytu vstupují přes sinusoidální (bazolaterální) membránu. V této fázi účinkují především transportéry ze skupiny SLC zprostředkovávající facilitovanou difúzi svých substrátů. ABC transportéry mají naopak stěžejní roli na kanalikulární (apikální) membráně hepatocytu, přes kterou exportují své substráty do žluče proti značnému koncentračnímu gradientu a snižují tak koncentraci svých substrátů v hepatocytu, čímž připravují koncentrační gradient na bazolaterální membráně (Faber et al., 2003) (viz. Obr. 1). ABC transportéry na bazolaterální membráně (MRP1, MRP3) zvyšují svoji expresi při cholestáze a přispívají tak k převedení snížené jaterní clearence na renální clearence (Trauner and Boyer, 2003).



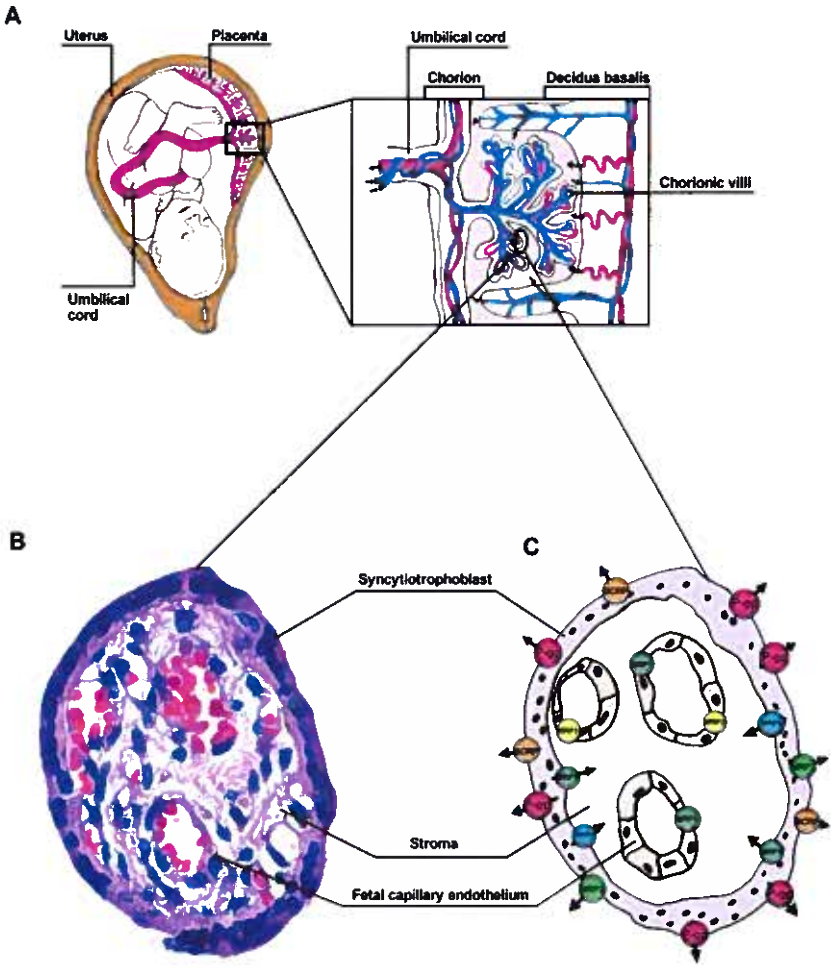
Obr. 1 Schématické znázornění lokalizace transportních proteinů na membránách hepatocytu. ATP-transportéry jsou znázorněny červenou barvou, SLC transportéry žlutou barvou. Upraveno podle (Ito et al., 2005).

4. Transport léčiv přes placentu

Placenta je jednou z tzv. fyziologických bariér oddělujících cirkulaci matky a plodu (Cross, 2006). V současné době se předpokládá, že většina léčiv podaných v průběhu těhotenství může, alespoň do určité míry, přestoupit do cirkulace plodu (Pacifici and Nottoli, 1995; Audus, 1999; Syme et al., 2004). Míra a rychlost přestupu léčiva závisí na (i) fyzikálně-chemických vlastnostech léčiva (molekulová hmotnost, lipofilita, stupeň ionizace, vazebnost na plazmatické bílkoviny), anatomickém uspořádání (např. mezidruhové rozdíly (Carter and Enders, 2004), tloušťka placentární membrány) a fyziologických charakteristikách placenty (průtok krve placentou aj.), podrobněji viz. (van der Aa et al., 1998; Audus, 1999).

V lidské placentě musí léčivo překonat bariéru mnohojaderného syncytiotrofoblastu, resp. jeho apikální a bazální cytoplazmatickou membránu a dále musí projít přes endotel fetálních kapilár (viz Obr. 2). Většina léčiv přechází přes tyto membrány prostou difúzí. Některá léčiva mohou být rozpoznána přenašeči pro endogenní substráty (především ze skupiny SLC transportérů) a placentou mohou procházet facilitovanou difúzí (Ganapathy et al., 2000). Z celkového pohledu je však facilitovaná difúze v transportních dějích zastoupena v menší míře (Syme et al., 2004).

Značný vliv na prostup farmak přes placentu mají ABC transportéry léčiv. Na apikální membráně syncytiotrofoblastu byly lokalizovány transportéry P-gp, MRP2 (St-Pierre et al., 2000; Atkinson et al., 2003) a BCRP (Maliepaard et al., 2001; Litman et al., 2002). V tomto umístění tak mohou zamezovat přestupu svých substrátů z krevního oběhu matky a plnit roli v ochraně plodu. Méně jasná je role transportérů MRP1 a MRP3, které byly lokalizovány v oblasti bazolaterální membrány syncytiotrofoblastu a abluminální straně endotelu (St-Pierre et al., 2000; Atkinson et al., 2003; Nagashige et al., 2003).



Obr. 2 Znázornění struktury lidské placenty. (A) Průřez dělohou na konci těhotenství. V detailu je zobrazena struktura funkční jednotky placenty – kotyledonu. Chorion představuje plodovou část placenty sestávající z choriových klků omývaných mateřskou krví, která vstupuje do intervilózního prostoru spirálovitými artériemi v decidua basalis. Živiny a kyslík procházejí přes vrstvu trofoblastu na choriových klcích, vstupují do krve plodu a do plodu jsou přinášeny umbilikální žílou (červeně). Odkysličená krev je vedena z plodu dvěma umbilikálními artériemi (modře). (B) Řez koncových klků placenty ze třetího trimestru barvený hematoxylin-eosinem (mikrofotografie od Dr. Nachtigala) (C) Schématický nákres řezu koncových klků zobrazující lokalizaci P-gp na apikální mikrovilózní membráně syncitiotrofoblastu a přítomnost dalších efluxních transportérů léčiv. Převzato z (Ceckova-Novotna et al., 2006).

ABC transportéry

Nadrodina ABC transportérů je skupina proteinů s velmi konzervovanou strukturou napříč evolucí od bakterií až po člověka. Tyto proteiny váží ATP a energii uvolněnou při jeho hydrolýze používají k transportu svých substrátů přes membrány a to i proti koncentračnímu gradientu. ABC transportéry přenášejí lipidy, steroly, peptidy, nukleotidy, léčiva, toxické látky a produkty metabolismu přes extra- a intracelulární membrány (Borst and Elferink, 2002). V genomu člověka bylo dosud nalezeno 48 ABC transportérů.

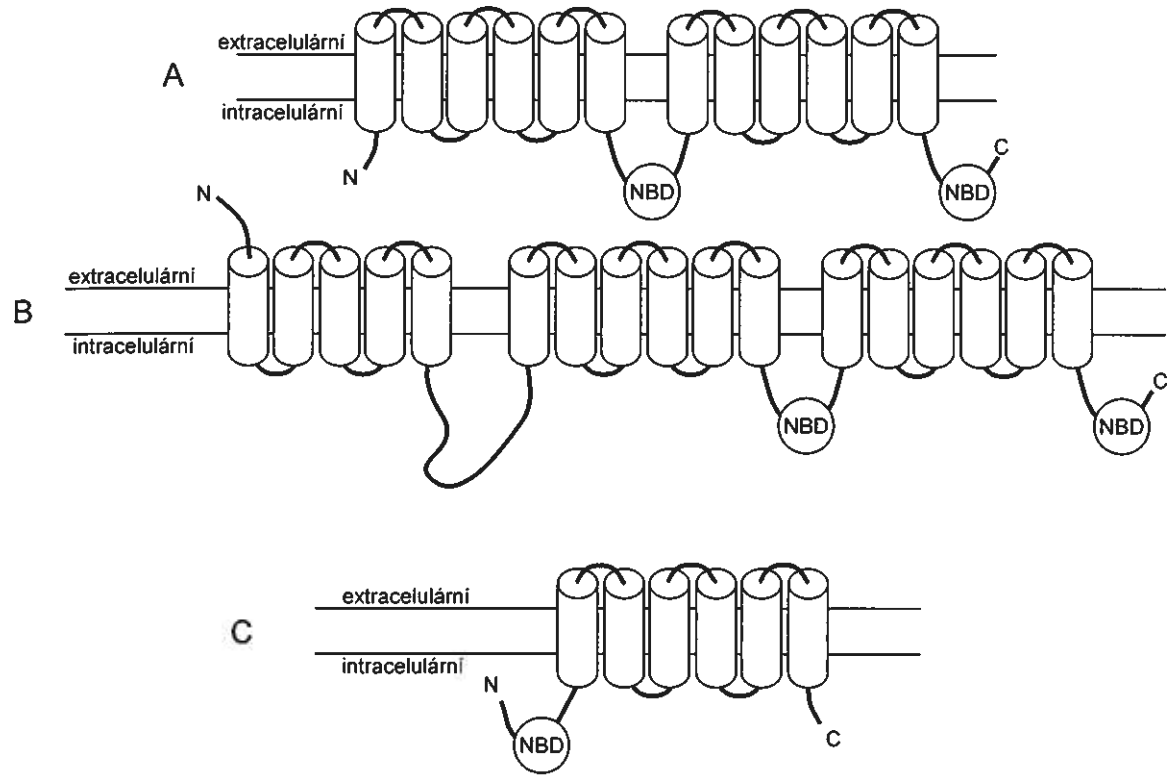
Pohyb substrátů zprostředkovaný ABC transportéry je většinou jednosměrný. V bakteriích jsou tyto přenašeče zapojeny do importu esenciálních živin, které nemohou být přijaty difúzí (cukry, vitamíny, ionty kovů). U eukaryont většina ABC transportérů přesunuje substráty ven z buňky v rámci distribuce látek do jiných orgánů nebo exkrece z organismu. Uvnitř buňky se účastní metabolického procesu tím, že dopravují látky obvykle z cytoplazmy do buněčných organel – endoplazmatického retikula, mitochondrií a peroxizómů.

V eukaryontních organizmech jsou geny kódující ABC transportéry rozptýleny po celém genomu a jejich sekvence jsou mezidruhově velmi konzervované, což dokládá, že většina ABC transportérů existovala již od počátku evoluce. Podle vzájemné podobnosti sekvencí jsou geny pro ABC transportéry členěny do osmi podrodin a savčí ABC transportéry se řadí do sedmi z nich – ABCA – ABCG (Dean et al., 2001).

Pro strukturu ABC transportérů je typická doména schopná vázat ATP (tzv. NBD - „nucleotide binding domaine"). V NBD se nachází vysoce konzervované sekvence aminokyselin - motivy Walker A a B a mezi nimi umístěný C-motiv. Sekvencí těchto motivů se ABC transportéry liší od ostatních ATP-vazných proteinů. Podle počtu NBD se ABC transportéry rozlišují na úplné transportéry se dvěma NBD a poloviční transportéry s jednou NBD (viz Obr. 3). Poloviční transportéry pracují buď ve formě homodimérů nebo ve formě heterodimérů, které vytváří s jiným polovičním transportérem. Kromě NBD lokalizovaných v cytoplazmě obsahují ABC transportéry jednu nebo dvě skupiny transmembránových (TM) domén sestavených z 6-11 α-šroubovic. Z hlediska funkce jsou tyto transmembránové domény zodpovědné za specifitu transportního procesu, zatímco NBD jsou pro ni nepodstatné (Borst and Elferink, 2002).

ABC transportéry začaly být zajímavé z hlediska farmakologie poté, co bylo objeveno, že způsobují mnohačetnou lékovou rezistenci nádorů – MDR („multidrug resistance") (Shen et al., 1986; Ueda et al., 1987). Rezistence nádorů vůči chemoterapii vzniká jako

důsledek schopnosti některých transportérů vylučovat z buňky různá protinádorová léčiva a snižovat tak účinnou koncentraci uvnitř buňky. Později se ukázalo, že stejné transportéry mají význam nejen v protinádorové terapii, ale ovlivňují farmakokinetické procesy jiných léčiv (Szakacs et al., 2006). Tyto farmakologicky relevantní ABC transportéry spadají převážně do tří podrodin – ABCB, ABCC a ABCG.



Obr. 3 Schématické znázornění základních typů struktur ABC transportérů.
A - 12 TM domén a dvě NBD jsou typické P-gp, MDR3, BSEP, MRP4, MRP5, MRP8 a MRP9
B – pro MRP transportéry – MRP1-3 a MRP6-7 je charakteristických dalších 5 TM domén na N konci
C – poloviční transportéry jako BCRP mají pouze šest TM domén a jednu NBD

4.1. ABCB podrodina (MDR/TAP)

Tato podrodina zahrnuje úplné i poloviční transportéry. Úplné mají dvě NBD a dvanáct TM domén a jsou lokalizovány na cytoplazmatické membráně, tyto transportéry jsou kódovány geny *ABCB1*, *ABCB4*, *ABCB5*, *ABCB11*. Poloviční transportéry kódované geny *ABCB2*, *ABCB3*, *ABCB6-10* mají pouze jednu NBD doménu na C-konci a šest TM domén a jsou lokalizovány na membránách intracelulárních vezikul.

Zástupci podrodiny ABCB zastávají v lidském organismu řadu rozličných funkcí. *ABCB1* kóduje gen pro transportér P-gp, studovaný především pro svůj farmakologický význam. Další dva transportéry jsou důležité pro hepatobiliární sekreci - MDR4 kódovaný genem *ABCB4* z hepatocytu do žluče exportuje mastné kyseliny a BSEP („bile salt export pump") kódovaný genem *ABCB11* transportuje do žlučového kanálku žlučové kyseliny, např. taurocholát (Gerloff et al., 1998). Produkty genů *ABCB2* a *ABCB3* – TAP1 a TAP2 – utváří dimer v membráně endoplazmatického retikula do něhož transportují z cytoplazmy peptidy, které pak mohou být spojeny s molekulami HLA I třídy a přítomny na buněčném povrchu (Monaco et al., 1990; Spies et al., 1990). Mutace v genu pro *ABCB7* způsobuje sideroblastickou anémii vázanou na chromozóm X a ataxii (Allikmets et al., 1999; Bekri et al., 2000). (Dean et al., 2001)

4.1.1. *ABCB1* (P-gp, P-gp)

Gen *ABCB1* kóduje transportér P-gp (P-glykoprotein), který je prvním objeveným lékovým transportérem vůbec (Juliano and Ling, 1976). P-gp je sledovaný především pro souvislost s mnohačetnou lékovou rezistencí nádorů vůči chemoterapeutikům - např. paklitaxelu, antracyklinům a *Vinca* alkaloidům. Jeho substrátové spektrum je značně široké a preferuje amfipatické molekuly neutrální nebo mírně bazické povahy, avšak dokáže s nízkou efektivitou transportovat anionty se silným nábojem – např. metotrexát, viz review (Chan et al., 2004). Z chemických struktur interakci s P-gp podporují planární aromatické struktury a přítomnost terciálních aminoskupin. O mechanismu vazby tak širokého spektra substrátů se stále vedou diskuze, ale zřejmě k této substrátové pestrosti přispívá značná konformační přizpůsobivost centrální dutiny v proteinu tvořená transmembránovými doménami s negativně nabitými aminokyselinovými zbytky. Flexibilita dutiny umožňuje mnoho Van der Waalsových interakcí, které nevyžadují přesné umístění substrátu jako v případě vodíkových můstků (Borst and Elferink, 2002).

Kromě nádorové rezistence hraje klíčovou roli v obraně organismu před účinkem xenobiotik a to na třech úrovních: (i) ve sliznici tenkého střeva brání vstřebávání toxinů, (ii) podporuje eliminaci vstřebané látky hepatobiliární a renální sekrecí tím, že na kanalikulární membráně hepatocytů a luminální membráně proximálních tubulů napomáhá efluxu svých substrátů, (iii) omezuje distribuci xenobiotik do vitálně důležitých a citlivých orgánů a tkání (např. mozku, varlat, lymfocytů a plodu) (Fromm, 2004).

V játrech je P-gp exprimován na kanalikulární membráně (Silverman and Schrenk, 1997) a jeho role v hepatobiliární sekreci byla popsána na myších s vyřazenou funkcí obou genů *Abcb1a*/*Abcb1b* (Smit et al., 1998).

V placentě člověka byla exprese *ABCB1* v apikální membráně syncytiotrofoblastu popsána na úrovni proteinu (Sugawara et al., 1988; Cordon-Cardo et al., 1989; Nakamura et al., 1997; Sugawara et al., 1997) i mRNA (Bremer et al., 1992). První transportní studie popisující vliv P-gp v placentě člověka byla provedena v roce 1997 na membránových vezikulech připravených ze syncitiotrofoblastu (Nakamura et al., 1997). Podobně jako jiné efluxní transportéry na apikální membráně syncytiotrofoblastu i P-gp působí jako funkční bariéra vůči účinku xenobiotik, viz. review (Szakacs et al., 2006). Pomocí duální perfúze placenty potkana byl sledován vliv P-gp na transplacentální transport cyklosporinu (Pavek et al., 2001; Pavek et al., 2003). Na význam P-gp v placentě poukazuje příklad použití antivirotika saquinaviru u HIV pozitivních matek před porodem k zabránění vertikálního přenosu infekce z matky na dítě. Tato terapie je neúčinná právě díky funkční bariéře P-gp (Huisman et al., 2001) a nadějí by mohlo být zavedení inhibitorů P-gp (Varma et al., 2003).

4.1.2. *ABCB11* (BSEP)

BSEP kódovaný genem *ABCB11* je hlavním transportérem bilirubinu (Gerloff et al., 1998) a stejně jako P-gp je exprimován na kanalikulární membráně hepatocytu. Jeho exprese je na rozdíl od P-gp soustředěna výhradně v játrech (Nishimura and Naito, 2005). Na rozdíl od MRP2, se kterým sdílí lokalizaci na kanalikulární membráně hepatocytu, nemůže transportovat sulfatované žlučové kyseliny (Akita et al., 2001).

4.2. ABCC podrodina (CFTR/MRP)

Tato podrodina zahrnuje 12 úplných transportérů, jež zastávají spektrum rozličných funkcí a mnohdy nejsou transportéry v pravém slova smyslu. Gen *ABCC7* kóduje protein CFTR, což není transportér, ale chloridový kanál závislý na proteinkináze A. CFTR se podílí na funkci všech exokrinních žláz a mutace v jeho genu způsobují život ohrožující onemocnění cystickou fibrózu. Geny *ABCC8* a *ABCC9*, které kódují receptory SUR1 a SUR2, váží sulfonylureu a ovlivňují draslíkové kanály zúčastněné v sekreci inzulínu. Ostatní geny této podrodiny kódují tzv. MRP transportéry („multidrug resistance related protein"). *ABCC1* (MRP1), *ABCC2* (MRP2), *ABCC3* (MRP3) transportují konjugáty glutathionu a organické anionty. Pro jejich strukturu je typických 5 TM domén na N-konci

(viz. Obr. 3B). Transportéry *ABCC4* (MRP4), *ABCC5* (MRP5), *ABCC11* (MRP8) a *ABCC12* (MRP9) tuto amino-terminální doménu postrádají. *ABCC4* a *ABCC5* způsobují rezistenci vůči nukleosidovým analogům. (Dean et al., 2001)

4.2.1. *ABCC1* (MRP1)

Gen *ABCC1* sousedí s genem *ABCC6*, přičemž jeden z nich vznikl duplikací druhého genu. Produkt genu *ABCC1* je transportér MRP1, který je hlavním efluxním přenašečem konjugátů s glutathionem. V buňkách způsobuje rezistenci k doxorubicinu, daunorubicinu, vinkristinu, kolchicinu a dalším chemoterapeutikům. Svým spektrem substrátů se podobá P-gp, narozdíl od něj však MRP1 vyžaduje, aby látky byly ve formě glutathion-konjugátů, glukuronidů nebo sulfátů (Jedlitschky et al., 1996).

MRP1 transportuje také leukotrien C_4 (LTC_4), který je důležitý pro migraci dendritických buněk. Tato migrace je poškozena u *Abcc1*$^{-/-}$ myší. Zdá se tedy, že MRP1 hraje roli v ochraně buněk před toxicitou a oxidativním stresem a že se podílí ve zprostředkování zánětlivé odpovědi cytokiny (Robbiani et al., 2000).

V buňkách je MRP1 lokalizován na bazolaterální membráně. Takové umístění má v organismu význam v ochraně obsahu uzavřených životně důležitých dutin a tkání před účinem toxinů. Absence MRP1 v Sertoliho buňkách způsobuje zvýšenou léčivy indukovanou destrukci prekurzorů ve spermatogenezi. V plexus choroideus chrání mozkomíšní mok. MRP1 pumpuje konjugované substráty zpět do těla, zatímco efluxní apikální transportéry jako P-gp nebo BCRP by naopak zvyšovaly hladiny toxinů v takových dutinách (Borst and Elferink, 2002).

V játrech je MRP1 za normálních podmínek exprimován jen omezeně a jeho hladina se zvyšuje při porušené biliární sekreci, např. dědičných poruchách apikálně lokalizovaného MRP2 nebo cholestáze (Trauner and Boyer, 2003). V placentě je MRP1 lokalizován v abluminální membráně fetálních kapilár (St-Pierre et al., 2000).

4.2.2. *ABCC2* (MRP2)

ABCC2 kóduje apikální transportér MRP2. MRP2 i MRP1 mají podobné substrátové spektrum avšak tkáňová distribuce MRP2 je značně omezenější než MRP1. MRP2 má významnou úlohu v biliární exkreci xenobiotik i endogenních metabolitů. Podobně jako MRP1 jsou jeho substráty konjugované se sulfátem, glukuronátem nebo glutathionem. Kromě jater je *ABCC2* exprimován také v ledvinách a střevě. MRP2 způsobuje rezistenci

k vinkristinu, vinblastinu, antracyklinům, derivátům kamptotecinu, mitoxantronu, metotrexátu a cis-platině (Borst and Elferink, 2002).

Mutace v genu *ABCC2* způsobují tzv. Dubin-Johnsonův syndrom, při kterém trpí vrozenou konjugovanou hyperbilirubinémií (Paulusma et al., 1997). Za tohoto stavu může bilirubin vstupovat do hepatocytů, kde je konjugován glukuronátem, ale nemůže být exkretován do žluče a vrací se zpět do krevního oběhu. Roli MRP2 pak přebírají bazolaterální transportéry MRP1 a MRP3 (Keppler and Konig, 2000).

4.2.3. *ABCC3* (MRP3)

MRP3 je bazolaterální transportér podobně jako MRP1. Byl nalezen v játrech, trávicím traktu ledvinách (Scheffer et al., 2002). Na sinusoidální membráně hepatocytu je jeho exprese za normálních podmínek nízká a stoupá při cholestáze (Ogawa et al., 2000; Donner and Keppler, 2001; Scheffer et al., 2002).

Na rozdíl od MRP1 a MRP2 nedokáže MRP3 transportovat konjugáty glutathionu (Kool et al., 1999), což by mohlo být vysvětlením, proč buňky produkující rekombinantní MRP3 nejsou rezistentní k chemoterapeutikům, která jsou kotransportována s glutathionem (Konig et al., 1999).

4.2.4. *ABCC4* (MRP4) a *ABCC5* (MRP5)

MRP4 a MRP5, produkty genů *ABCC4* a *ABCC5*, se podobají nejen svojí strukturou, ale také spektrem svých substrátů. Oba jsou transportéry organických aniontů a dokáží transportovat cyklické nukleotidy/nukleosidy a jejich analoga. V buňkách snižují intracelulární koncentraci léčiv odvozených od nukleotidů/nukleosidů - např. antivirotik PMEA (9-(2-fosfonylmethoxyethyl)adenin) a ganciklovir nebo protinádorového terapeutika 6-merkaptopurinu (Borst and Elferink, 2002).

Oba transportéry se podílí na efluxu cyklických nukleotidů a snižují tak, vedle fosfodiesteráz, jejich intracelulární koncentraci. Kromě toho by tímto mechanismem mohly uvolňovat cyklické nukleotidy do extracelulárního prostředí a umožňovat jejich parakrinní působení. V tomto směru koreluje také distribuce a subcelulární lokalizace těchto dvou transportérů v buňkách hladkého svalu (MRP5), denzní granula destiček (MRP5), nervové buňky (oba transportéry) a endotel kapilár (Ritter et al., 2005).

4.3. ABCG podrodina (White)

Struktura těchto polovičních transportérů je jedinečná v obráceném pořadí domén. Na amino-konci těchto proteinů je jediná NBD a TM doména je na C konci (viz. Obr. 3C). Řada proteinů kódovaných geny této podrodiny hraje důležitou roli v transportu sterolů (*ABCG1*, *ABCG4*, *ABCG5*, *ABCG8*); transportéry kódované geny *ABCG5* a *ABCG8* fungují jako homo- i heterodimery a jsou důležité pro export některých živočišných a rostlinných sterolů z enterocytu zpět do lumen tenkého střeva. Jedinci s mutacemi v genech *ABCG5* a *ABCG8* trpí sitosterolémií (Dean et al., 2001). Největšího zájmu ze strany farmakologů doznal transportér BCRP kódovaný genem *ABCG2*.

4.3.1. *ABCG2* (BCRP)

BCRP byl objeven v buněčných liniích rezistentních vůči mitoxantronu, které však neexprimovaly *ABCB1* ani *ABCC1*. BCRP způsobuje rezistenci k antracyklinovým chemoterapeutikům a v genomu buněčných liniích selektovaných topotekanem, mitoxantronem nebo doxorubicinem se objevily multiplikace genu ABCG2. Předpokládá se, že BCRP je funkční jako homodimer, protože buňky získávají rezistenci k výše uvedeným chemoterapeutikům po transfekci samotného *ABCG2* (Staud and Pavek, 2005).

Ve většině rezistentních liniích exprimujících BCRP se vyskytuje pozměněná varianta BCRP, která nemůže transportovat účinně rhodamin 123 a antracykliny. „Wild-type" varianta nese v poloze 482 arginin a mutanty v tomto místě mají threonin nebo glycin. (Dean et al., 2001)

V lidském organismu je BCRP lokalizován v mnoha tkáních. V tenkém a tlustém střevě brání stejně jako P-gp absorpci toxinů a léčiv. Zdá se, že podobnou funkci zastává v placentě v apikální membráně syncytiotrofoblastu a chrání krevní oběh plodu před toxiny v krevním oběhu matky (Jonker et al., 2000; Jonker et al., 2002). V hepatocytech na straně žlučového kanálku se podílí na jaterní exkreci. Potvrzuje se i jeho podstatná úloha v ochranně kmenových buněk před toxickým vlivem porfyrinů, které vznikají při nedostatku kyslíku (Staud and Pavek, 2005).

5. SLC transportéry

Tato velice rozsáhlá skupina transportérů zahrnuje 360 zástupců členěných do 46 nadrodin (http://www.bioparadigms.org), které transportují endo- i exogenní substráty. SLC transportéry zprostředkovávají pohyb molekul přes cytoplazmatické i intracelulární membrány ve směru koncentračního gradientu (uniport, usnadněná difúze) nebo za využití koncentračního gradientu jiné látky (symport nebo antiport). Ke své činnosti nepotřebují energii ATP jako předchozí skupina ABC transportérů.

Skupina SLC transportérů je členěna do 46 nadrodin. Současná klasifikace založená na sekvenční homologii umožňuje řadit do jediné nadrodiny proteiny prokaryontních i eukaryontních organismů. Následující podkapitoly jsou věnovány třem nadrodinám SLCO, SLC22 a SLC10, jejichž mnozí členové zastávají významné role v hepatobiliárním transportu endogenních látek i farmak.

5.1. Nadrodina SLCO

Geny nadrodiny SLCO kódují OATP transportéry ("organic anion transporting polypeptide"). Od první izolace krysího transportéru oatp1 v roce 1994 (Jacquemin et al., 1994) bylo do této rodiny zařazeno 11 lidských transportérů (Hagenbuch and Meier, 2004).

Až na několik zástupců OATP transportérů, které se vyskytují téměř výhradně v játrech (např. OATP1B1 a OATP1B3), jsou OATP transportéry exprimovány ve většině tkání, kde zajišťují Na^+-nezávislý transport aniontů (Hagenbuch and Meier, 2004).

V jejich struktuře je charakteristických 12 TM domén. Mezi TM doménami 9 a 10 se nachází dlouhá extracelulární smyčka, která obsahuje mnoho konzervovaných cysteinových zbytků a tím připomíná zinkové prsty proteinů, které váží DNA. Na hranici mezi extracelulární smyčkou 3 a šestou TM doménou se nachází vysoce konzervovaná sekvence D-X-RW-(I,V)-GAWW-XG-(F,L)-L typická pro OATP nadrodinu. (Hagenbuch and Meier, 2004).

V dalším textu je výklad zaměřen především na lidské transportéry, které jsou významné pro jaterní nebo placentární transport léčiv.

5.1.1. Rodina SLCO1/OATP1

Tato rodina se člení na tři podrodiny SLCO1A-C. U člověka má první podrodina jediného zástupce *SLCO1A2*/OATP1A2 (v původní nomenklatuře OATP-A). Tento transportér byl

transportér byl klonován z jaterní cDNA knihovny (Kullak-Ublick et al., 1995), ačkoli jeho nejvyšší exprese je v mozku a míše (Nishimura and Naito, 2005). OATP1A2 transportuje nejširší spektrum substrátů z OATP transportérů, blíže viz. (Hagenbuch and Meier, 2003). Z jeho značné exprese v mozku se lze domnívat, že napomáhá transportu léčiv a neuroaktivních peptidů do mozku a odstraňování metabolitů z mozku (Hagenbuch and Meier, 2004).

Podrodina genů SLCO1B má dva členy: *SLCO1B1*/OATP1B1 (dříve nazývaný OATP-C) a *SLCO1B3*/OATP1B3 (původně OATP-8). Oba dva proteiny sdílejí 80% aminokyselin v sekvenci a oba jsou exprimovány na bazální membráně hepatocytu (Abe et al., 1999; Konig et al., 2000b; Konig et al., 2000a; Abe et al., 2001). Též substrátová specifita obou přenašečů je velmi podobná a hrají stěžejní roli v jaterní clearence amfipatických látek vázaných na albumin. Jejich substráty zahrnují žlučové aminokyseliny, konjugovaný i nekonjugovaný bilirubin, konjugáty steroidů, hormony štítné žlázy a léčiva – pravastatin, benzylpenicilin, metotrexát, rifampicin (Hagenbuch and Meier, 2003). I přes tuto podobnost existují látky, které jsou selektivně transportovány pouze OATP1B3 jako např. cholecystokinin-8 (CCK-8) (Ismair et al., 2001) nebo kardioglykosidy oubain a digoxin (Kullak-Ublick et al., 2001)

SLCO1C3/OATP1C3 (pův. OATP-F) jako jediný lidský zástupce z podrodiny SLC1C je, podobně jako SLC1A1, exprimován převážně v mozku a míše a v játrech nebyl detekován (Nishimura and Naito, 2005). Vzhledem k tomu, že má vysokou afinitu k tyroxinu a reverznímu trijódthyroninu (Pizzagalli et al., 2002) předpokládá se jeho role ve vývoji mozku.

5.1.2. Rodina SLCO2/OATP2

Tato rodina zahrnuje 5 lidských transportérů členěných do dvou podrodin A a B. Pro jaterní i placentární transport je významným především transportér *SLCO2B1*/OATP2B1 (původně OATP-B), který přestože byl nalezen nejdříve v mozkové tkáni, je nejvíce exprimován v játrech. Narozdíl od transportérů OATP1B1 a OATP1B3 je významná i jeho exprese v jiných orgánech (Kullak-Ublick et al., 2001; St-Pierre et al., 2002; Nishimura and Naito, 2005). S oběma transportéry sdílí v hepatocytu i shodnou lokalizaci na bazolaterální membráně (Kullak-Ublick et al., 2001).

5.1.3. Rodina SLCO3/OATP2

Jediným lidským zástupcem této rodiny je *SLCO3A1*/OATP3A1 (původně OATP-D) izolovaný z lidských ledvin (Tamai et al., 2001). Vyskytuje se ve většině tkání a na úrovni mRNA byla zachycena zvýšená exprese ve varlatech (Nishimura and Naito, 2005).

5.1.4. Rodina SLCO4/OATP4

Zahrnuje 4 lidské transportéry členěné do dvou podrodin (A a C). Transportér *SLC4A1*/OATP4A1 (původně OATP-E) je exprimován ve většině lidských tkání, ačkoli zvýšená exprese byla nalezena v placentě v apikální membráně syncytiotrofoblastu, kde by mohl hrát úlohu v transportu hormonů štítné žlázy (Sato et al., 2003).

5.2. Nadrodina SLC22

Nadrodina SLC22 zahrnuje transportéry bakterií, nižších eukaryont, rostlin a savců, které se účastní uniportu, symportu i antiportu. Transportéry jsou členěny do 18 rodin (Koepsell and Endou, 2004). První člen nadrodiny SLC22 byl krysí *Slc22a1*/Oct1 („organic cation transporter") identifikovaný v roce 1994 pomocí molekulárního klonování (Grundemann et al., 1994).

Pro strukturu SLC22 transportérů je typických 12 transmembránových domén a dvě velké extracelulární smyčky. První smyčka spojuje TM domény 1 a 2 a je lokalizována extracelulárně, druhá smyčka je obrácena směrem dovnitř buňky a spojuje TM domény 6 a 7 (Koepsell and Endou, 2004). Většina zástupců SLC22 nadrodiny je exprimována ve střevě, játrech a ledvinách a hrají proto velice důležitou roli v absorpci a exkreci léčiv (Koepsell and Endou, 2004).

Transportéry SLC22 lze členit do tří podskupin podle substrátové specifity a transportních mechanismů:

První skupina zahrnuje transportéry organických kationtů OCT1-3. Transport zprostředkovaný OCT („organic cation transporter") polarizuje membránu, je nezávislý na Na^+ a je obousměrný. Hnací silou transportu OCT je pouze elektrochemický gradient přenášeného kationtu (Koepsell et al., 2003). U člověka je *SLC22A1*/OCT1 exprimován především v játrech a *SLC22A2* (OCT2) v ledvinách. Naopak *SLC22A3*/(OAT3) je rozšířen i v jiných tkáních (Koepsell et al., 2003).

Druhá skupina zahrnuje transportéry OCTN1-3 a hCT2 a jimi zprostředkovaný transport je dvojího typu. První typ (např. OCTN1) funguje buď jako uniporter pro organické kationty nebo antiporter H^+ a organických kationtů. Druhý typ (např. OCTN2)

pracuje jako uniporter pro organické kationty nebo kotransporter Na^+ a karnitinu (Koepsell and Endou, 2004).

Do třetí skupiny patří transportéry organických aniontů OAT1-5 („organic anion transporter") a transportér močoviny URAT1. Tyto transportéry jsou schopné přemísťovat organické anionty oběma směry (Koepsell and Endou, 2004). U transportérů OAT1 a OAT3 bylo prokázáno, že fungují jako antiportery s divalentními organickými anionty (Sekine et al., 1997; Sweet et al., 2003). U člověka je exprese OAT1 (SLC22A6) a OAT3 (SLC22A8) transportérů lokalizována především v ledvinách (Hosoyamada et al., 1999; Cha et al., 2001; Nishimura and Naito, 2005), kde hrají roli v exkreci celé řady endogenních látek i léčiv, např. nesteroidních protizánětlivých látek, urikosurik, diuretik (Koepsell and Endou, 2004). Tkáňová distribuce OAT2 (*SLC22A7*) je širší, s nejvyšší expresí v játrech (Nishimura and Naito, 2005).

5.3. Nadrodina SLC10

Produkty genů nadrodiny SLC10 jsou stěžejní pro enterohepatální recirkulaci žlučových solí. Transportní systémy účastnící se tohoto procesu hrají roli (i) v transportu žlučových solí z tenkého střeva do krve a (ii) z krve do hepatocytu, přičemž pro oba tyto děje existují dva transportéry:

Transportér ASBT („apical sodium-dependent bile salt transporter"; produkt genu SLC10A2) je exprimován na apikální membráně kartáčového lemu ileocytů. ASBT sekundárně aktivním transportem přenáší molekuly žlučových kyselin z lumen tenkého střeva a umožňuje tím jejich návrat do jater.

Druhý transportér NTCP („Na^+/taurocholate cotransporting polypeptide") je exprimován na bazolaterální membráně hepatocytů, kde extrahuje žlučové kyseliny z portální krve a tím umožňuje jejich resekreci pomocí aktivních transportních systémů na kanalikulární membráně hepatocytu do žluče. (Hagenbuch and Dawson, 2004).

ASBT (*SLC10A2*) je vhodným cílem při terapii hypercholesterolémie, neboť inhibice ASBT vede k přerušení enterohepatální cirkulace žlučových solí, jejich zvýšenému vylučování stolicí a nutnosti zvýšení *de novo* syntézy v játrech. Inhibitory ASBT jsou dvojího typu, (i) dimerní analogy žlučových kyselin nebo (ii) deriváty benzothiazepinů (Geyer et al., 2006).

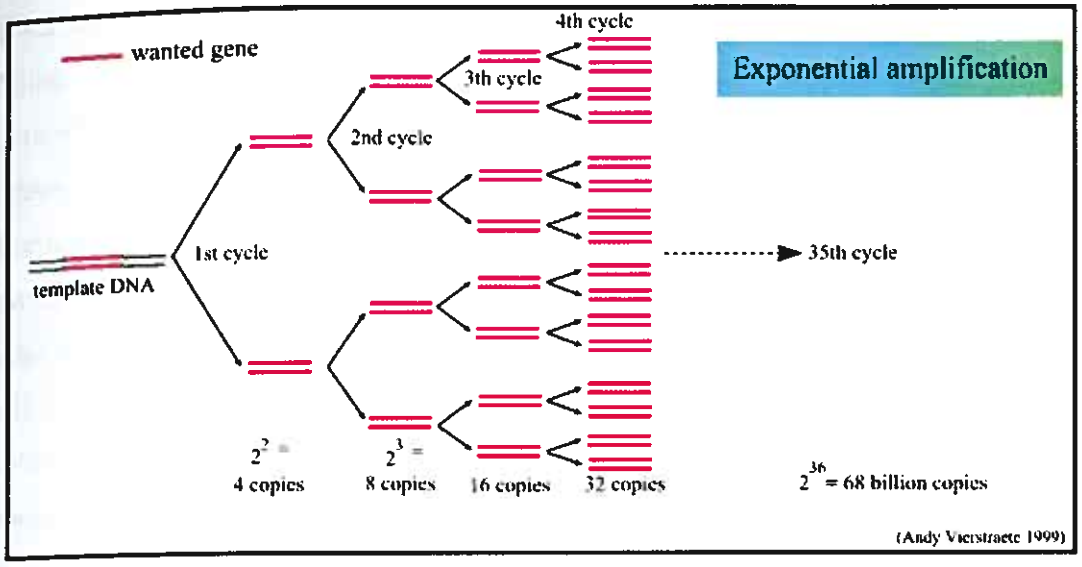
6. Kvantifikace exprese transportérů pomocí real-time RT-PCR

Expresi genu lze sledovat na úrovni transkripční, kdy je objektem zájmu mRNA sledovaného genu, nebo na úrovni translační, která se zaměřuje na finální protein jako výsledek procesu exprese.

V současnosti se pro kvantifikaci na úrovni transkripce nabízí několik metod založených na hybridizaci značené sondy s mRNA: Northern-blotting a *in situ* hybridizace, RPA („RNAse protection assay"), DNA čipy (www.gene-chips.com) a techniky založené na amplifikaci mRNA pomocí RT-PCR. Northern-blotting je jediná metoda poskytující informace o velikosti mRNA, alternativním sestřihu mRNA a integritě RNA (Parker and Barnes, 1999). RPA je vhodná především pro mapování počátku a konce transkripce a rozhraní exonů (Hod, 1992; Saccomanno et al., 1992). *In situ* hybridizace je jedinou metodou pro lokalizaci transkriptů v buňce a tkáních (Parker and Barnes, 1999). Analýza exprese pomocí DNA čipů poskytuje informaci o expresi obrovského množství genů z jediného vzorku (Hartshorn et al., 2005).

RT-PCR, která má oproti předchozím metodám mnohem vyšší citlivost, je založena na enzymatické amplifikaci sekvencí RNA a lze ji použít pro sledování exprese i v jediné buňce (Hartshorn et al., 2005).

PCR („polymerase chain reaction") je cyklický děj, při kterém dochází k exponenciální amplifikaci cílové sekvence DNA (Obr. 4). Aby bylo možné detekovat nebo kvantifikovat sekvenci RNA je třeba převést sekvenci RNA na DNA reverzní transkripcí. DNA vzniklá reverzní transkripcí se nazývá cDNA („complementary DNA"). PCR přístupy pro kvantifikaci je možné rozdělit podle způsobu detekce na end-point metody a real-time metody. End-point metody, ve kterých je kvantita amplikonu hodnocena až po dokončení PCR obvykle gelovou elektroforézou, se dnes používají již jen v omezené míře a to především pro svůj úzký dynamický rozsah a vyšší náročnost na zpracování. V současnosti se většina PCR kvantifikací provádí pomocí metody real-time PCR, při které je kvantita PCR amplikonu sledována během PCR a tím nabízí širší dynamický rozsah než end-point metody (Freeman and Walker, 1999).

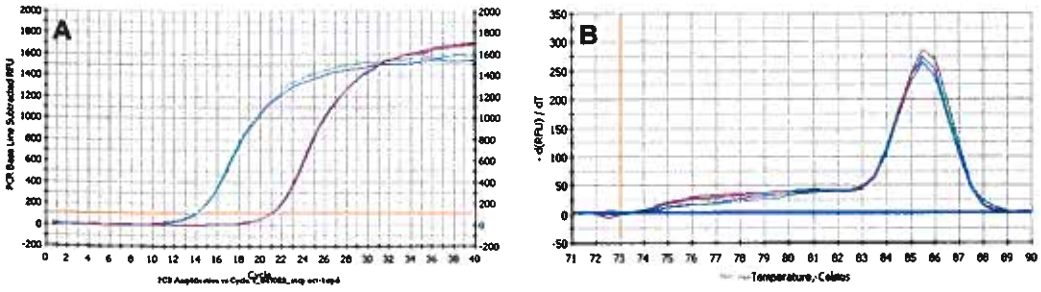


Obr. 4 Schématické znázornění exponenciální amplifikace templátové sekvence v PCR. Během každého cyklu PCR dochází ke zdvojnásobení počtu molekul z předchozího cyklu (http://users.ugent.be/~avierstr/principles/pcr.html).

6.1. Sondy v real-time PCR

Podstatou real-time PCR je použití fluorescenční sondy pro monitorování množství produktů amplifikace v každém cyklu PCR (Obr. 5A). Sondy lze rozdělit na specifické a nespecifické:

Nespecifické sondy jsou barviva, která fluoreskují pouze pokud jsou navázána na dvouřetězcovou DNA a proto mohou detekovat nejen vlastní PCR produkt, ale i primer-diméry a jiné nespecifity. Pokud jsou tyto nespecifické sondy používány, provádí se po dokončení PCR cyklování křivka teploty tání („melting curve"), ze které lze odečíst teplotu tání produktu a vyhodnotit zda byl množen pouze specifický produkt (Obr. 5B). Nejčastěji používanou nespecifickou sondou je Sybr Green I, jehož předností je především nižší cena a univerzální použití (Bustin, 2004).



Obr. 5 (A) Záznam fluorescence z real-time PCR analýzy pomocí Sybr Green I. (B) Záznam z analýzy křivky tání - první derivace závislosti fluorescence na teplotě.

Sekvenčně specifické sondy jsou fluorescenčním barvivem značené oligonukleotidy, které svoji fluorescenční aktivitu zvyšují pouze za přítomnosti amplikonu, k němuž se

mohou hybridizovat. Nezvyšují tedy fluorescenci v přítomnosti primer-dimérů a pod. Většina sekvenčně specifických sond funguje na principu přenosu fluorescenční rezonanční energie (FRET, „fluorescence resonance energy transfer"), při kterém energie vyzářená molekulou fluoroforu (donorem) je absorbována molekulou zhášeče (akceptorem, „quencherem"). Nejvíce používaným typem sond jsou dvojitě značené hydrolyzační sondy známé též pod označením Taq-Man a molekula fluoroforu je umístěna na opačném konci oligonukleotidu než molekula zhášeče. K uvolnění fluorescence dochází po hydrolýze sondy 5'→3' exonukleázovou aktivitou Taq-polymerázy během elongace. Kromě dvojitě značených hydrolyzačních sond se dále používají hybridizační sondy Beacon a Scorpion nebo dvojice jednoduše značených sond (označovaných často jako FRET sondy) (Bustin, 2004).

6.2. Strategie kvantifikace v real-time PCR

Výstupem z real-time PCR je záznam fluorescence v každém cyklu PCR, který má v grafickém zobrazení tvar sigmoidální křivky. Z tohoto záznamu je pro každý analyzovaný vzorek odečtena hodnota C_t („treshold cycle") – počet cyklů při kterém dosáhne fluorescence analyzovaného vzorku prahové hodnoty. Pokud je efektivita amplifikace ideální a v každém cyklu se počet molekul zdvojnásobí:

$$N = N_0 \cdot 2^{C_t}$$

kde N_0 je počet molekul na počátku PCR a N je počet molekul v cyklu C_t. Ne každý PCR systém dosáhne úplné účinnosti a v každém cyklu se nezdvojnásobí počet amplikonů. V tomto případě je třeba upravit vztah na

$$N = N_0 \cdot (E+1)^{C_t}$$

kde E je efektivita reakce.

Výsledek z real-time PCR může být hodnocen podle dvou strategií – relativní nebo absolutní kvantifikace. Absolutní kvantifikace vztahuje výsledky ke kalibrační křivce standardu zatímco relativní kvantifikace pouze vyjadřuje změny v hladině mRNA.

Relativní kvantifikace

V relativní kvantifikaci se navzájem porovnává exprese mezi dvěma vzorky (např. mezi ovlivněným vzorkem a neovlivněnou kontrolou) a tento poměr je vztažen k expresi referenčního genu jako vnitřního standardu. Referenční gen je obvykle tzv. housekeepingový gen, jehož exprese je pokládána za stabilní v kvantifikovaných vzorcích.

Referenční gen lze stanovovat v samostatné reakci nebo současně s cílovým genem v multiplexní reakci (Wittwer et al., 2001).

K výpočtu poměru relativní exprese se používá dvou typů matematických vztahů lišících se podle toho, zda berou zřetel na efektivitu amplifikace v PCR. Základní vztah pro výpočet poměru exprese mezi vzorkem a kontrolou bez zohlednění efektivity se nazývá $\Delta\Delta C_t$ metoda:

$$poměr = \frac{2^{\Delta C_{TG}(kontrola-vzorek)}}{2^{\Delta C_{RG}(kontrola-vzorek)}} = 2^{(\Delta C_{TG}-\Delta C_{RG})} = 2^{\Delta\Delta C_t}$$

kde ΔC_t je rozdíl mezi hodnotami C_t kontroly a sledovaného vzorku pro referenční a cílový gen. Tento přístup lze použít pouze v případě, že efektivita PCR amplifikace cílového i referenčního genu je stejná. Matematický model, který zohledňuje efektivitu, je rozšířenou $\Delta\Delta C_t$ metodou (Pfaffl, 2001):

$$poměr = \frac{(E_{TG})^{\Delta C_{TG}(kontrola-vzorek)}}{(E_{RG})^{\Delta C_{RG}(kontrola-vzorek)}}$$

kde indexem E_{TG} a $C_{t\,TG}$ jsou efektivita amplifikace a C_t hodnoty pro cílový gen a E_{RG} a $C_{t\,RG}$ jsou efektivita amplifikace a C_t hodnoty pro referenční gen.

Velmi často diskutovaným tématem je otázka výběru referenčního genu (Schmittgen and Zakrajsek, 2000; Schmid et al., 2003; Aerts et al., 2004). Dnes se považuje za optimální cestu výběr několika genů, jejichž exprese je nejméně závislá na podmínkách experimentu buď pomocí metody geometrického průměru (Vandesompele et al., 2002; Pfaffl et al., 2004) nebo za využití statistického modelování (Andersen et al., 2004).

Absolutní kvantifikace

V absolutní kvantifikaci je výsledek z PCR porovnáván s kalibrační křivkou standardu a poskytuje informaci o počtu kopií přepočteném na počet buněk, µg RNA nebo množství tkáně. Standardy pro tvorbu kalibračních křivek by měly být stabilní a měly by poskytovat reprodukovatelné výsledky. Standardy jsou používány buď v PCR - rekombinantní DNA, RT-PCR produkt, synteticky připravená DNA a genomová DNA - nebo již v reverzní transkripci - rekombinantní RNA (Bustin, 2000).

Nejčastěji používaným standardem je rekombinantní DNA - zaklonovaný PCR produkt. Tento typ standardu poskytuje velmi dobře reprodukovatelné kalibrační křivky avšak jeho nevýhodou je delší časová náročnost přípravy – amplifikace PCR produktu, izolace a purifikace, klonování, transformace, izolace plazmidu, verifikace a přesné stanovení koncentrace standardu (Bustin, 2000).

7. Cíle předkládané dizertační práce

1. Zavedení metod izolace RNA, reverzní transkripce a kvantitativní real-time PCR pro kvantifikaci exprese transportních genů v lidských a potkaních tkáních a buněčných kulturách.
2. Studium exprese a lokalizace P-gp v placentě potkana v průběhu březosti (v návaznosti na potvrzenou funkci P-gp v terminální placentě potkana in vivo).
3. Porovnání exprese genů *ABCB1* a *ABCG2* v lidské BeWo buněčné linii a terminální lidské placentě.
4. Porovnání exprese 12 genů nadrodin SLCO, SLC22, SLC10 a SLC15 v lidských HepG2 a Chang Liver buněčných liniích jako in vitro modelech pro studium transportních procesů v játrech.
5. Stanovení kinetických charakteristik transportu potenciální diagnostické látky pro magnetickou rezonanci B22956 v buněčných liniích HepG2 a Chang Liver a srovnání s kinetikou endogenní látky taurocholátu.

Podíl doktoranda na předkládaných publikacích:

U kapitoly VI je předkladatel této dizertační práce prvním autorem a u kapitol II, III, IV a V spoluautorem.

Ve studii v kapitolách II a V autor zavedl metody pro end-point RT-PCR detekci exprese *Abcb1a* , *Abcb1b a Abcg2* a s těmito metodami detekoval expresi v potkaní placentě.

Ve studii III autor zavedl metodu pro relativní kvantifikaci exprese *Abcb1a*, *Abcb1b* a *B2m* v tkáních potkana. Dále prováděl odběry potkaních placent v průběhu březosti potkana a se zavedenými metodami provedl real-time RT-PCR analýzu exprese *Abcb1a* a *Abcb1b* v průběhu březosti.

Ve studii IV autor zavedl metody pro absolutní kvantifikaci exprese *ABCG2* na transkripční úrovni a provedl srovnání exprese *ABCG2* a *ABCB1* mezi buněčnou linií BeWo a placentou.

Ve studii uvedené v části VI autor této práce zavedl metodu pro absolutní kvantifikaci exprese na transkripční úrovni 12 SLC transportérů pomocí real-time RT-PCR a provedl analýzu exprese v buněčných liniích HepG2 a Chang Liver. Na těchto liniích dále prováděl „uptake" studie s kontrastní látkou pro magnetickou rezonanci B22956.

Autor sepsal rukopis práce uvedené v kapitole VI. U prací, kde je uveden jako spoluautor, se podílel na sepsání částí týkajících se real-time RT-PCR kvantifikace nebo RT-PCR detekce.

Imunohistochemické lokalizace P-gp a BCRP v placentě prováděli Mgr. Martin Kopecký a PharmDr. Petr Nachtigal, Ph.D. z Katedry lékařských věd Farmaceutické fakulty pod vedením Doc. RNDr. Vladimíra Semeckého, CSc.

Experimenty uvedené v kapitole VI prováděl autor této práce během svého studijního pobytu na Department of BBCM, University of Trieste a Centro Studi Fegato, AREA Science Park Basovizza v Itálii pod vedením Dr. Lorella Pascolo Ph.D. a Prof. Claudio Tiribelli, Ph.D.

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II.

EXAMINATION OF THE FUNCTIONAL ACTIVITY OF P-GLYCOPROTEIN IN THE RAT PLACENTAL BARRIER USING RHODAMINE 123

Pavek P., Staud F., Fendrich Z., Sklenarova H., ***Libra A.****, Novotna M., Kopecky M., Nobilis M., Semecky V. (2003). Examination of the functional activity of P-glycoprotein in the rat placental barrier using rhodamine 123. J Pharmacol Exp Ther 305:1239-1250.*

0022-3565/03/3063-1239–1250$7.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
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JPET 305:1239–1250, 2003

Vol. 305, No. 3
48170/1064316
Printed in U.S.A.

Examination of the Functional Activity of P-glycoprotein in the Rat Placental Barrier Using Rhodamine 123

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Received December 21, 2002; accepted February 25, 2003

ABSTRACT

Rhodamine 123 (Rho123), a model substrate of P-glycoprotein (P-gp), was used to evaluate the functional activity of P-gp efflux transporter in the rat placental barrier. The dually perfused rat-term placenta method was used. In our experiments, the materno-fetal transplacental passage of Rho123 did not meet the criteria of the first-order pharmacokinetics, suggesting an involvement of transporter-mediated process. Inhibitors of P-gp, such as [3′-keto-Bmt^1]-[Val^2]-cyclosporine (PSC833), cyclosporine (CsA), quinidine, and chlorpromazine, increased significantly the materno-fetal transplacental passage of Rho123 in the experiments under steady-state conditions. On the other hand, PSC833, CsA, and quinidine decreased the feto-maternal passage of Rho123. Similarly, in the experiments carried out under nonsteady-state conditions, CsA accelerated the passage of Rho123 in the materno-fetal direction and decreased its passage in the opposite direction. Feto-maternal transplacental clearances of Rho123 were found to be considerably higher than those in the materno-fetal course. Potent P-gp inhibitors, such as PSC833 or CsA, partially canceled the asymmetry. Negligible metabolism of Rho123 into its major demethylated metabolite rhodamine 110 was observed in the rat placenta. Expression of P-gp genes was detected using immunohistochemical, Western blotting, and reverse transcription-polymerase chain reaction methods preferentially in the second rat syncytiotrophoblast layer. In conclusion, these data suggest that P-gp limits the entry of Rho123 into fetuses and at the same time it accelerates the feto-maternal elimination of the model compound. Therefore, it seems plausible that pharmacokinetics of xenobiotics in the rat placental barrier could be controlled by P-gp in both directions.

The function of P-glycoprotein (P-gp) has been proposed to be an ATP-dependent membrane efflux pump, whose primary mode of action is to remove numerous lipophilic compounds from the lipid bilayer and to pump them actively out of cells (Stein, 1997; Ambudkar et al., 1999). In this way, P-gp transfers a multidrug resistance phenomenon (MDR) to tumor cells against a large spectrum of anticancer drugs. Substrates transported by P-gp include a variety of structurally and pharmacologically unrelated, hydrophobic compounds, such as various anticancer drugs, cardiac glycosides, β-blockers, calcium channel blockers, etc. (Ambudkar et al., 1999). P-gp has also been detected on the membranes of a wide range of normal tissues, such as the capillaries forming the blood-brain and blood-testis barriers, apical surface of the gastrointestinal tract, luminal membrane of the renal proximal tubular cells, and hepatocytes. At these sites, P-gp was found to serve as a barrier for the entry of lipophilic xenobiotics into the body and/or to the tissues that are sensitive to their toxic injury.

However, function of P-gp in the placental barrier has been less examined and is still not fully understood. High levels of P-gp have been detected in human and murine syncytiotrophoblast layers, which are the crucial parts of the hemochorial placental barrier (Cordon-Cardo et al., 1990; Sugawara, 1990; Bremer et al., 1992; Trezise et al., 1992; MacFarland et al., 1994; Nakamura et al., 1997; Lankas et al., 1998; Myloma et al., 1999; Smit et al., 1999; Ushigome et al., 2000; Tanabe et al., 2001). P-gp has been demonstrated to be integrated in the microvillous membrane of the human syncytiotropho-

This work was supported by the Grant Agency of the Czech Republic (grants 305/01/0D89 and 305/01/0441), the Grant Agency of the Charles University in Prague (Grant 94/200/C), the Research Center LN00B125, and research Project No. J13/98:11600002 obtained from the Czech Ministry of Education. The results of the article were presented in part at the XIVth World Congress of Pharmacology, San Francisco, 2002 and the 62nd Congress of FIP, Pharmacy and Pharmaceutical Sciences World Congress, Nice, France 2002.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
DOI: 10.1124/jpet.102.048470.

ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistance; Rho123, rhodamine 123; rOCT, rat organic cation transporter; RT-PCR, reverse transcription-polymerase chain reaction; CsA, cyclosporine; QND, quinidine; bp, base pair(s); CPZ, chlorpromazine; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody.

blast that faces directly maternal blood (MacFarland et al., 1994; Ushigome et al., 2000). Transport activity of P-gp in the placental barrier has been examined both in vivo and in vitro (Audus, 1999). Lankas and coworkers revealed that fetuses of CF-1 mice lacking the *mdr1a* gene isoform of P-gp were susceptible to cleft palate malformation induced by avermectin B1a. Conversely, the fetuses of wild-type mice were completely protected against the above-mentioned teratogen (Lankas et al., 1998). Similarly, administration of other P-gp substrates (digoxin, saquinavir, or paclitaxel) to *mdr1a*$^{-/-}$/*1b*$^{-/-}$ knockout mice revealed 2.4-, 7-, and 16-fold higher transplacental transport of these drugs into the fetus compared with wild-type mice (Smit et al., 1999).

The in vitro action of the placental P-gp has been demonstrated in uptake studies using the BeWo cell line (a human choriocarcinoma trophoblastic cell line), primary cultures of the human cytotrophoblasts (Utoguchi et al., 2000), and human trophoblast membrane vesicles (Nakamura et al., 1997). Ushigome et al. (2000) studied the function of P-gp in the placenta using the BeWo cell line cultured in a confluent epithelial monolayer. The study suggests that due to the one-way functional activity of P-gp located in the apical membrane, transport of selected P-gp substrates is higher in the basolateral-to-apical and restricted in the apical-to-basolateral direction. On the basis of all these findings, it is believed that P-gp expressed in trophoblast cells of the placenta limits the entry of its substrates into the fetus by reverse pumping of the compounds from the trophoblast layers back into the maternal bloodstream. Moreover, as the in vitro study using BeWo cells suggests, P-gp could accelerate transplacental passage of P-gp substrates in the feto-maternal direction. Our previous in situ experiments demonstrated that the passage of CsA across the rat placenta is restricted in the materno-fetal direction due to the P-gp activity (Pavek et al., 2001).

The aim of the present article was to examine 1) the functional activity of P-gp in both the materno-fetal and feto-maternal directions using the dually perfused rat placenta method, and 2) to confirm at the level of the intact rat placental barrier the asymmetry of transplacental passage of P-gp substrates described in the in vitro epithelial model of BeWo cells. A fluorescent dye rhodamine 123 (Rho123), which is a well established model substrate for testing the functional transport activity of P-glycoprotein, was used (Masereeuw et al., 1997; van der Sandt et al., 2000). CsA, PSC 833 (a nonimmunosuppressive derivate of CsA), and chlorpromazine were used as selective inhibitors/modulators of P-gp, quinidine was used as a nonselective inhibitor for both P-gp and organic cation transporters (rOCT), and sodium azide was used as an inhibitor of ATP mitochondrial synthesis. Because there was only limited information on P-gp expression in the rat placenta in the current literature, we carried out immunochemical, immunohistochemical, and RT-PCR studies to confirm expression and localization of P-gp gene products in the rat-term placentas.

Materials and Methods

Material and Reagents. Rhodamine 123 and rhodamine 110 (demethylated Rho123) were purchased from Sigma-Aldrich (St. Louis, MO; catalog no. R-8004, batch 127H3707) and Fluka (Buchs, Switzerland; catalog no. 83695, EC.2369447), respectively. Substances of CsA and SDZ PSC833 (PSC 833, [3′-keto-Bmt^1]-[Val^2]-cyclosporine) were obtained thanks to the kindness of Dr. Andrýsek (IVAX Ltd., Opava, Czech Republic). Quinidine sulfate (QND) and diamond fuchsin were purchased from Lachema Ltd. (Brno, Czech Republic). Chlorpromazine chloride (CPZ), bovine serum albumin, RPMI 1640 medium, and other substances were purchased from Sigma-Aldrich. Tris, glycine, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride were obtained from Serva GmbH (Heidelberg, Germany). 4-Nitrophenyl phosphate disodium salt hexahydrate was purchased from Fluka. Acetonitrile and methanol (both HPLC grade) were purchase from Merck (Darmstadt, Germany).

Stock solutions of CsA and PSC833 were used in a concentration of 1 mg/ml containing 19% of ethanol and 1% of cremorphor (v/v). The final concentration of cremorphor in perfusion media was less that 0.01%. Aqueous stock solutions of QND, CPZ, and sodium azide were used in a concentration of 10, 25, and 100 mg/ml, respectively.

Animals. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy (Hradec Králové, Charles University in Prague) and were carried our in accordance with the Guide for the Care and Use of Laboratory Animals, 1996; and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, Strasbourg, 1986.

Pregnant Wistar rats were purchased from Biotest Ltd. (Konárovice, Czech Republic) and were bred in 12/12-h day/night standard conditions with water and pellets ad libitum. Experiments were performed on day 22 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL) in a dose of 40 mg/kg administered intravenously into the tail vein.

Perfusion Method. The method of dually perfused rat placenta was used as described previously (Mohammed et al., 1993; Pavek et al., 2001). Briefly, the placenta was excised and allowed to dive in the heated Ringer saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying the selected placenta and connected with the peristaltic pump bringing Krebs' perfusion liquid containing 1% bovine serum albumin from the maternal reservoir. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and carefully cut so that maternal solution could leave the perfused placenta. The chosen fetus was separated from the neighboring ones by ligatures. The umbilical artery was catheterized using the 24-gauge catheter and connected with the tubing by which the fetal perfusion liquid from the fetal reservoir was supplied. The umbilical vein was catheterized in a similar manner and the selected fetus was removed. After a successful umbilical catheterization, the fetal vein effluent was collected into preweighted glass vials to check a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was discarded. Perfusion experiments did not last longer than 36 min, because the integrity of the placental barrier could be affected in later intervals. This notion is based on our previous experiments with L-[^{3}H]glucose, a marker of paracellular diffusion, where the transplacental passage of L-[^{3}H]glucose started to significantly increase in later intervals of the perfusion (Pavek et al., 2001). Wet weights of the placentas used in experiments were 0.54 ± 0.13 g.

To study the influence of inhibitors on both the materno-fetal and feto-maternal transport of Rho123, experiments were carried out under both steady-state and nonsteady-state conditions as described below.

Evaluation of Rho123 Metabolism in the Rat Placenta. In our pilot experiments, we studied possible placental metabolism of Rho123 during its transplacental passage. In the experiments carried out under both steady-state and nonsteady-state conditions (for experimental designs of steady-state and nonsteady-state experiments see below), Rho123 was present in the maternal perfusion solution in concentrations of 0.65 or 1.3 μM. Fetal effluent samples were analyzed using HPLC.

Effect of CsA on Transplacental Passage of Rho123 under Nonsteady-State Conditions. After successful establishment of the dual perfusion with the Rho123-free and inhibitor-free perfusion

solution, the maternal and/or fetal perfusion inflows were switched to another prewarmed perfusion reservoirs containing examined compounds. The experiments started after 15 s of delay (time 0) to make it possible to fill tubing with the new liquids. The fetal umbilical vein outflow was collected in 5-min intervals into preweighted glass vials for 25 min of the experiment.

To examine possible influence of CsA on the transplacental passage of Rho123 in the materno-fetal direction, CsA was added in a concentration of 40 μM to the maternal reservoir together with Rho123 (1.3 μM). Samples were collected in 5-min intervals from the fetal umbilical vein outflow.

The feto-maternal transplacental passage of Rho123 was followed in experiments where Rho123 was added in a concentration of 1.3 μM into the fetal solution (control experiments). The effect of CsA on the Rho123 feto-maternal passage was examined by simultaneous addition of CsA (40 μM) into the maternal solution and Rho123 (1.3 μM) into the fetal solution at time 0 of the experiment. Samples were collected in 5-min intervals from the fetal umbilical vein outflow.

Effect of CsA, PSC833, QND, CPZ, and Sodium Azide on Transplacental Passage of Rho123 under Steady-State Conditions. In experiments examining the materno-fetal passage of Rho123 under the steady-state conditions, Rho123 (0.65 μM) was brought to the perfused placenta via the catheterized uterine artery immediately after the catheterization. Sample collection started (time 0) after 15-min delay. Within this delay, steady-state conditions for transplacental passage of Rho123 were achieved as suggested by a nearly constant time course of transplacental clearances during the experiment (Fig. 3). Samples were collected in 3-min intervals from the fetal umbilical vein for 36 min of the experiment. In the 12th min of the experiment, examined P-gp inhibitors were added to the maternal reservoir to reach the concentrations of 10 μM (CsA and PSC833), 40 μM (QND and CPZ), and 5 mM (sodium azide), respectively. This experimental design enables observation of the direct effect of the selected inhibitor on the steady-state transplacental passage of Rho123 within one experiment. In addition to this, the steady-state experimental approach eliminates the variability between two groups of experiments carried out under nonsteady-state experiments. On the other hand, the effect of inhibitors on the passage of Rho123 is less evident under steady-state in comparison with the data obtained in the nonsteady-state experiments.

For the examination of the feto-maternal passage of Rho123 under steady-state conditions, fetal solution containing Rho123 (0.65 μM) was used to perfuse the selected placenta via the catheter in the fetal umbilical artery immediately after catheterization. Sample collection started 10 min after the installation of the catheter (time 0). In the 12th min of the experiment, one of the P-glycoprotein inhibitors was added to the maternal reservoir to reach a concentration of 10 μM (CsA and PSC833), 40 μM (QND), and 5 mM (sodium azide), respectively. Samples were collected in 3-min intervals from the fetal umbilical vein for 36 min of experiments.

Our previous results suggested that inhibition of P-gp was more significant when an inhibitor of P-gp was present in the maternal compartment rather than in the fetal one (Pavek et al., 2001). That is why the inhibitors/modulators of P-gp were given into the maternal solution even in the feto-maternal experiments.

In our previous study (Pavek et al., 2001), we also demonstrated that sodium azide (5 mM) increased the transplacental paracellular passage of L-[^{3}H]glucose in later intervals, which could suggest impairment of the placental barrier. Therefore, we conducted experiments with sodium azide only for 27 min after catheterization.

Effect of Maternal Inflow Concentration on the Transplacental Clearance of Rho123. The dependence of the materno-fetal transplacental passage of Rho123 on the concentration of Rho123 in the maternal reservoir was examined in the steady state. Rho123 was brought to the perfused placenta via the uterine artery in various concentrations of 0.42, 0.65, 1.3, 2.0, and 4.0 μM, respectively. Samples were collected in 3-min intervals from the fetal umbilical vein for 30 min of experiments. The mean materno-fetal clearance of Rho123 was calculated for every concentration from all measured intervals.

Calculations. To describe the transfer of Rho123 across the dually perfused rat placenta in the materno-fetal direction in both nonsteady-state and steady-state experiments, its materno-fetal transplacental clearance (CL_{mf}) was calculated according to eq. 1 (Mohammed et al., 1993).

$$CL_{mf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot w_p} \quad (1)$$

where C_{ma} (in micromolar) is the concentration of Rho123 in the maternal reservoir, C_{fv} (in micromolar) is the concentration of Rho123 in the umbilical vein effluent, Q_f (milliliters per minute) is the umbilical flow rate and w_p (in grams) is the wet weight of the placenta. The amount of Rho123 that passed the placenta within any interval in the materno-fetal direction was calculated by multiplication of the volume of perfusion liquid collected from the umbilical vein within this interval by the concentration of Rho123 in the sample. This amount of Rho123 was recalculated per wet weight of the perfused placenta and expressed in nanomoles per gram units. Similarly, the feto-maternal clearance (CL_{fm}) was calculated according to eq. 2.

$$CL_{fm} = \frac{(C_{fa} - C_{fv}) \cdot Q_f}{C_{fa} \cdot w_p} = \left[1 - \frac{C_{fv}}{C_{fa}}\right] \cdot Q_f \cdot \frac{1}{w_p} \quad (2)$$

where C_{fa} (in micromolar) is the concentration of Rho123 in the fetal reservoir entering the perfused placenta via the umbilical artery, C_{fv} (in micromolar) is the concentration of Rho123 in the umbilical vein effluent, Q_f (in milliliters per minute) is the umbilical flow and w_p (in grams) is the wet weight of the placenta. The amount of Rho123 that passed across the perfused placenta in the feto-maternal direction during any interval was calculated as the difference between the concentrations of the fetal artery inflow and the fetal vein outflow (the concentration in the sample) multiplied by the volume of perfusion liquid collected from the umbilical vein within the interval indicated.

In the nonsteady-state experiment, the total cumulative amount of Rho123 that passed across the placenta within 25 min of the experiment was calculated to assess the influence of CsA on the transplacental passage of Rho123 in both directions. To eliminate variations of the cumulative amount caused by the weight of perfused placentas, the amount of Rho123 was expressed as the amount per wet weight of the perfused placenta (nanomoles per gram).

In the steady-state experiments, the effects of P-gp inhibitors given to the maternal solution were evaluated from the following inhibitory ratio (eq. 3).

$$\text{inhibitory ratio} = \frac{X_{12-30}}{X_{0-12}} \quad (3)$$

where X_{12-30} is the amount of Rho123 that passed the placenta from the 12th to 30th minute of experiments (i.e., the period in which an inhibitor was present) and X_{0-12} is the amount of Rho123 that passed the placenta from zero to the 12th minute (i.e., a period with inhibitor-free solution).

If sodium azide was examined as an inhibitor of P-gp, both the materno-fetal and the feto-maternal experiments were performed up to 27 min, and the ratio was calculated as X_{12-27}/X_{0-12}.

In the studies where the effect of maternal inflow concentration on the transplacental clearance of Rho123 was examined, the data were fitted by eq. 4.

$$CL_{mf} = CL_{\text{passive diffusion}} - \frac{CL_{max}}{K_m + C_{ma}} \quad (4)$$

where $CL_{\text{passive diffusion}}$ is a clearance of passive transport of Rho123 across the placenta in the materno-fetal direction, CL_{max} is the maximum reverse feto-maternal clearance of Rho123 mediated by

P-gp function and K_m is the Michaelis-Menten constant of the P-gp-mediated reverse transport.

HPLC Analysis of Rho123 and Its Metabolites in the Perfusion Media. Solid phase extraction of analytes from perfusate samples was performed according to the method of Sweatman et al. (1990) with slight modifications. Visiprep solid phase extraction vacuum manifold (12-port; Supelco, Bellefonte, PA) with SPE columns (Supelclean LC-18, 1-ml tubes; Supelco) were used for the solid phase extraction. The dry extract in the glass tube was reconstituted in 600 μl of the mobile phase, centrifuged, and transferred into a vial of the autosampler. The sample (100 μl) was injected into the chromatographic column.

Chromatographic analyses were performed using chromatograph (Thermo Separation Products, Minneapolis, MN; formerly Spectra Physics). The chromatographic system consisted of a SCM400 solvent degasser, P4000 quaternary gradient pump, AS 3500 autosampler with 100-μl sample loop, SpectraFOCUS high-speed scanning UV-visible detector, SN4000 system controller, and data station (Intel-Pentium 166 MMX, RAM 64 MB, HDD 2GB) with the analytical software ChromQuest 2.1 (ThermoQuest, Inc., San Jose, CA). A LiChroCART 125-4 mm analytical column packed with Purospher RP-18e, 5 μm and precolumn LiChroCART 4-4 mm with the same stationary phase (Merck) were used for analyses. The mobile phase was composed of acetonitrile/0.01 M phosphate buffer, pH 3 (3:7, v/v). Flow rate was 1 ml · min^{-1}.

UV-visible detection was performed either in dual wavelength mode at 500 nm (for rhodamines) and 550 nm (for diamond fuchsin used as an internal standard) or in high-speed scanning mode (range 195–750 nm with 1-nm distance, used for UV-visible spectra collection).

The retention times of Rho110, Rho123, and Diamond fuchsin under the above-mentioned chromatographic conditions were 2.40, 4.09, and 8.44 min, respectively. The whole HPLC analysis lasted 14 min.

Fluorimetric Determination of Rho123 in Perfusion Media. A commercial SIA system with an eight-port selection valve and a fluorometric detector equipped with a flow cell was used for fluorometric determination of Rho123 in samples (Sklenarova et al., 2002).

Cell Line Cultivation and Isolation of the Membrane Fraction from the Cells. The lymphoid macrophage cell line P388 and its resistant P-gp expressing subline P388-MDR were gifts from Dr. St'astny (Institute of Microbiology, Academy of Sciences of the Czech Republic). The membrane fractions of the cell lines were used as positive and negative controls for immunochemical determination of P-gp in the rat placenta. Cells were cultured as was reported previously (St'astny et al., 1999).

Isolation of the Total Membrane Fraction from the Rat Placentas. Rat placentas were collected on the 22nd day of gestation and were homogenized in ice-cold buffer (1:1, v/w) containing 250 mM sucrose, 10 mM Tris, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride, pH 7.4, in a Potter-Elvehjem tissue homogenizer. Homogenized tissues were centrifuged at 15,000g for 15 min at 4°C. The supernatants were further spun at 100,000g for 1 h at 4°C and the pellets containing the membrane fraction were sonicated and resuspended in ice-cold phosphate-buffered saline, pH 7.4.

Isolation of Apical Membrane Fraction of the Rat Syncytiotrophoblast. Apical membrane fraction of the rat placentas was isolated using the method described by Malandro et al. (1996) with slight modifications. Briefly, the total membrane fraction was resuspended in Tris-mannitol buffer (300 mM mannitol, 2 mM Tris base, pH 7.0) and homogenized in a glass homogenizer with Teflon pestle. $MgCl_2$ was added to a final concentration of 10 mM. Then membranes were again homogenized, incubated for 10 min at room temperature, and centrifuged at 2200g for 12 min. The pellet was discarded and supernatant was centrifuged at 100,000g for 60 min to pellet the apical-enriched membrane vesicles. The membrane fractions were resuspended in HEPES-sucrose buffer (300 mM sucrose, 10 mM HEPES-Tris base, pH 7.4) and frozen at −74°C.

Isolation of Basal (basolateral) Membrane Fraction of the Rat Syncytiotrophoblast. The basal membrane fraction of the rat syncytiotrophoblast was isolated according to the method of Malandro et al. (1996). Bicinchoninic acid protein assay reagent kit (Pierce Chemical, Rockford, IL) was used to determine the protein content in the samples. The purity of membrane fractions was analyzed by determination of alkaline phosphatase activity (the marker enzyme of the apical membrane) and Ca^{2+}-ATPase (the marker enzymes of the basolateral membrane, according to the method of Malandro et al., 1996).

Alkaline Phosphate Activity Assay. Apical and total membrane fractions were dissolved in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10) to final protein concentration of 500 μg/ml. 4-Nitrophenyl phosphate disodium salt was added to a final concentration of 0.75 mg/ml. The mixture was incubated for 20 min at 37°C and then absorbance was measured at 410 nm (Helios gamma spectrophotometer; Thermo Spectronic, Rochester, NY).

Western Blotting of P-glycoprotein in the Rat Placenta. The Western blotting analysis was performed to detect P-glycoprotein in the rat placenta membrane fractions. Membrane fractions were suspended in equal volumes of Laemmli sample buffer, and 20 μg of protein per lane was resolved by 7% polyacrylamide SDS-PAGE gel (50 mA) and eletrotransferred (25 V, 300 mA) to nitrocellulose Hybond membranes (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). The nitrocellulose membranes were blocked in 5% blocking solution (Amersham Biosciences UK, Ltd.) and incubated in Tris-buffered saline-Tween 0.01% buffer with murine monoclonal antibody (mAb) C219 (Signet Laboratories, Dedham, MA) diluted 1:500, or with hamster mAb Ab-2/F4 (p170/P-glycoprotein/MDR Ab-2; LabVision Neomarkers, Fremont, CA). The anti-mouse secondary horseradish peroxidase-conjugated antibody (1:1000 dilution) and ECL Western blotting detection kit (both Amersham Biosciences UK, Ltd.) were used for autoradiographic detection of P-gp on FOMA blue medical X-ray films (Foma Bohemia A.S, Hradec Králové, Czech Republic). Densitometric analysis was performed using a high-resolution scanner HP 5400c (Hewlett Packard, Palo Alto, CA) and LabImage gel densitometric software version. 2.62 (Kaplan GmbH, Halle, Germany).

Immunohistochemical Localization of P-gp in the Rat Placenta. Specimens of the placentas were fixed in 4% paraformaldehyde (pH 7.35) or in Bouin's fixative fluid and then were paraffin-embedded. Sections of placentas (thickness, 5 μm) were mounted on an object slide, dewaxed, and rehydrated through a series of ethanol solutions. Endogenous peroxidase activity was blocked with 3% H_2O_2 in 50% methanol solution for 20 min. For heat-induced antigen retrieval, the slides were boiled in 0.1 M Tris-HCl buffer, pH 1.5, for 15 min in a microwave oven at 750 W. Blocking of nonspecific background staining was performed with 10% normal goat serum (Sigma Chemie, Steinheim, Germany) in phosphate-buffered saline (PBS) solution (pH 7.4) for 30 min. Slides were incubated with primary antibody for P-glycoprotein (C219; Signet Laboratories) diluted 1:50 in PBS solution for 15 to 18 h at 4°C. After a PBS rinse, the slides were developed with the secondary antibody goat anti-mouse Ig conjugated to peroxidase-labeled polymer (DAKO EnVision+ ready-to-use; DAKO, Carpinteria, CA). Secondary antibodies were visualized with diaminobenzidine (DAKO DAB substrate-chromogen solution; DAKO) and hematoxylin counterstained. As a control for background staining, control slides were treated in the same manner, except PBS solution was substituted for the primary antibody to P-glycoprotein. Slides were examined using computer image analysis (Hund h500 light microscope, Helmut Hund, Wetzlar, Germany; JVC TK C1380E color video camera, JVC, Tokyo, Japan; LUCIA, version 4.61software, Laboratory Imaging Prague, spol. s r. o., Prague, Czech Republic).

Examination of P-gp Genes Expression by RT-PCR Method. Rat placentas were collected into liquid nitrogen and homogenized in

TRIzol reagent (Invitrogen, Carlsbad, CA) using 1 ml of TRIzol/50 mg of tissue. Total RNA was extracted from the tissue homogenate according to the manufacturer's instructions. RNA integrity was assessed by electrophoresis on a 1% agarose gel. First-strand cDNA was prepared from 1 μg of total RNA with AMV transcriptase (Finnzymes Oy, Espoo, Finland) using oligo(dT) primer under conditions recommended by the manufacturer. cDNA prepared from 3 ng of total RNA was amplified by PCR with HotStar *Taq* polymerase (QIAGEN, Valencia, CA); 3 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM each primer, 0.03 U/μl polymerase; 95°C for 15 min followed by 35 cycles of 95°C for 30 s, 54°C for 1 min, 72°C for 1 min. The sequences of antisense primers were identical for both *mdr1a* and *mdr1b* isoforms: 5'-AGCATTTCTGTATGGTATCTGCAAGC-3'. Sequence of *mdr1a* sense primer was 5'-CTGCTCAAGTGAAAGGGGCTACA-3' (product length 329 bp) and *mdr1b* sense primer 5'-CGCTTCTAATGTTAAAGGGGCTATG-3' (product length 331 bp). β2-Microglobulin was used as a housekeeping gene (B2M antisense: 5'-TACATGTCTCGGTCCCAGGTGA-3'; B2M sense: 5'-TGCCATTCAGAAAACTCCCCA-3', product length 303 bp). Amplified segments were analyzed by electrophoresis on 1.5% agarose gel and visualized using ethidium bromide.

Statistical Analysis. Differences between group mean values were assessed by unpaired Student's t test using STATISTICA software version. 6 (StatSoft, Tulsa, OK). Differences of $p < 0.05$ were considered statistically significant.

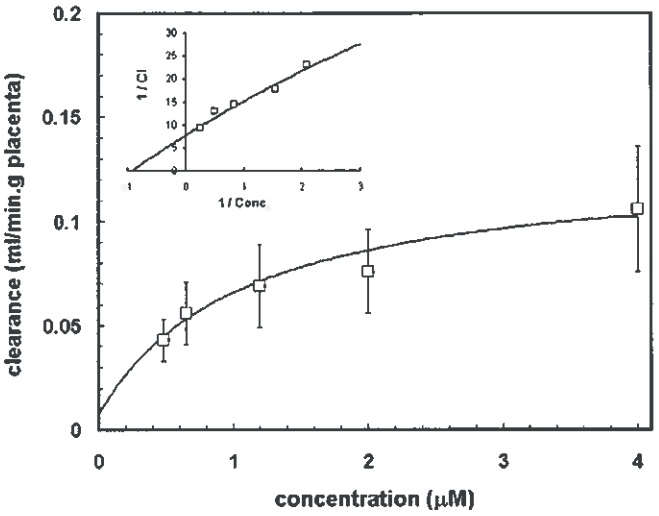

Fig. 1. Saturation kinetics of the materno-fetal passage of Rho123. Dependence of materno-fetal transplacental clearances of Rho123 on the concentration of Rho123 in the maternal inflow medium was examined. Data are expressed as means ± S.D. of at least three experiments. K_m was calculated to be 0.53 ± 0.07 μM, Cl_{max} was 0.07 ± 0.01 ml/min·g, and $Cl_{passive\ diffusion}$ was estimated to be 0.11 ± 0.01 ml/min·g (for details, see *Materials and Methods*). Inset, the figure with the reciprocal values.

Results

Evaluation of Rho123 Metabolism in the Rat Placenta. We detected Rho123 and its major metabolite Rho110 in fetal effluent samples using HPLC (data are not presented). The concentrations of Rho110 in the fetal compartment ranged from 1.9 to 2.5% of Rho123 concentrations entering the placenta from the maternal compartment. None of the used inhibitors had any effect on Rho110 levels in the fetal umbilical effluent samples during experiments. We suppose that the presence of Rho110 in the fetal umbilical effluent is predominantly a consequence of contamination of Rho123 with Rho110 (5.27%) used in our experiments (Rhodamine 123, catalog no. R-8004, batch 127H3707; Sigma-Aldrich). Due to no or negligible biotransformation of Rho123 in the rat placenta, we could employ faster and easier fluorometric method for analysis of Rho123 in our samples.

Effect of Maternal Inflow Concentration on the Transplacental Clearance of Rho123. The materno-fetal transplacental passage of Rho123 was found to be dependent on the maternal inflow concentration in a range of 0.42 to 4.0 μM (Fig. 1). This saturable kinetics of Rho123 indicates that transplacental passage of Rho123 is a transporter-mediated process. After fitting the data to eq. 4, Cl_{max} describing the efficiency of P-gp to pump Rho123 back into the maternal compartment was calculated to be 0.07 ± 0.01 $ml \cdot min^{-1} \cdot g^{-1}$. $Cl_{passive\ diffusion}$ was calculated to be 0.11 ± 0.01 $ml \cdot min^{-1} \cdot g^{-1}$. Therefore, our data indicate that in low concentrations of Rho123 (threshold concentration <0.10 μM), P-gp could completely reverse passive diffusion of Rho123 across the barrier (Fig. 1). In higher concentrations of Rho123 (>4.0 μM), however, P-gp-mediated transport of Rho123 become saturated and is not able to return all passively diffused Rho123 back to the maternal circulation. Unfortunately, fluorometric analysis of Rho123 used in this study did not allow examining lower concentrations of Rho123 than 0.42 μM in the maternal perfusion liquid.

Effect of CsA on Transplacental Passage of Rho123 under Nonsteady-State Conditions. Addition of 30× molar excess CsA to Rho123 solution in the maternal reservoir resulted in an increase of the materno-fetal transplacental clearance (Fig. 2A). Correspondingly, the amount of Rho123 that entered the fetal compartment rose significantly ($p < 0.05$) compared with controls (1.28 ± 0.43 $nmol \cdot g^{-1}$ and 2.17 ± 0.42 $nmol \cdot g^{-1}$, respectively). Doubling the amount of CsA in the maternal solution resulted in a comparable increase in the materno-fetal passage of Rho123 (data not shown). On the other hand, CsA decreased the feto-maternal clearance of Rho123 (Fig. 2B) and significantly lowered the amount of Rho123 passing from the fetal compartment into the maternal one (22.60 ± 2.18 and 19.47 ± 2.54 $nmol \cdot g^{-1}$, respectively).

In control experiments with Rho123 (0.65 μM), its materno-fetal clearances were rising to reach a plateau (steady state) from minute 15 of the experiment (data not shown). In the case of feto-maternal passage of Rho123, steady state was reached within 10 min of the experiments (Fig. 2B). The plateau phase of the transplacental passage of Rho123 enabled us to perform experiments under steady-state conditions (see the following paragraph).

Effect of CsA, PSC833, QND, CPZ, and Sodium Azide on Transplacental Passage of Rho123 in the Steady-State Experiments. Materno-fetal transplacental clearances of Rho123 were nearly constant during the control experiments (Fig. 3). An addition of PSC833, CsA, QND, or CPZ into the maternal reservoir in the 12th min of the experiment to reach a concentration of 10 μM (PSC833 and CsA) or 40 μM (QND and CPZ), resulted in an increase of materno-fetal transplacental passage of Rho123 (Table 1 and Fig. 3; data for CPZ are not shown). The most significant effect on the materno-fetal transplacental passage of Rho123 was observed in the case of PSC833 followed by QND and CsA (Table 1). On the contrary, in experiments where feto-

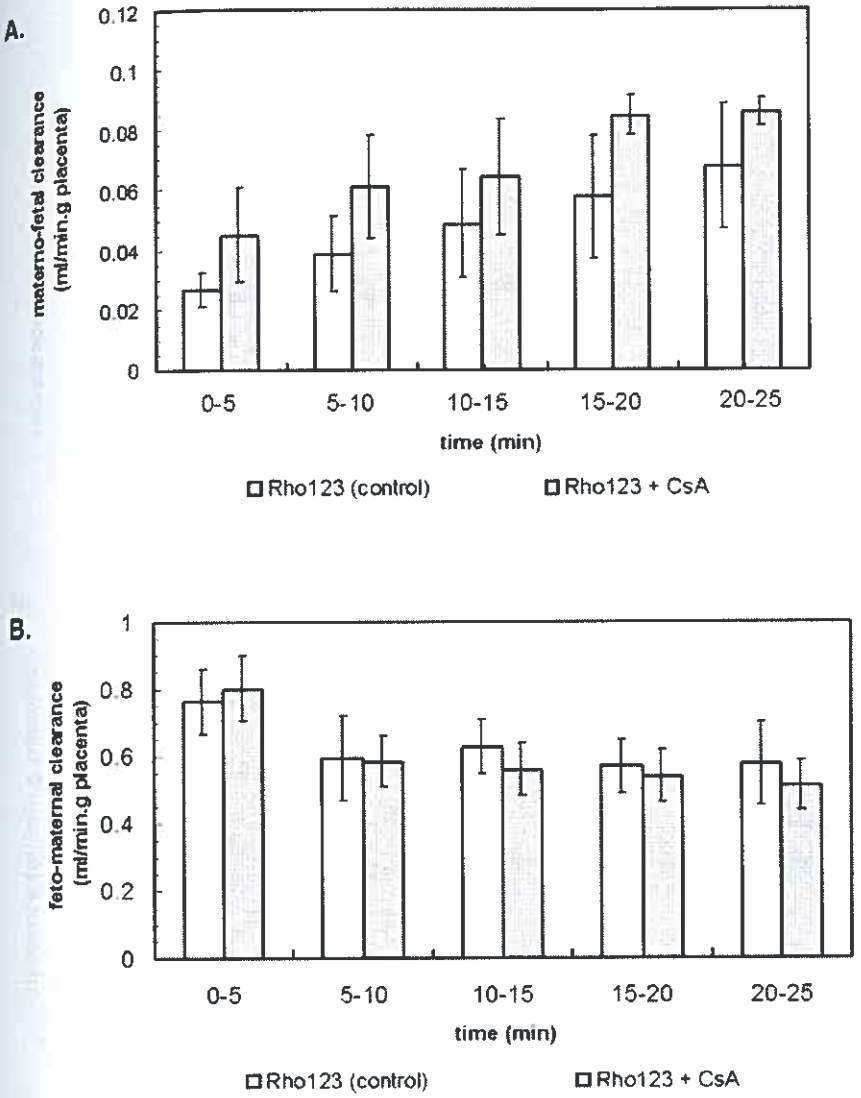

Fig. 2. Influence of CsA on the materno-fetal (A) and feto-maternal (B) transplacental passage of Rho123 in nonsteady-state experiments. Rho123 was given into maternal/fetal reservoir in a concentration of 1.3 μM (control). CsA was present in the maternal solution in a concentration of 40 μM from the beginning of experiments. Data are expressed as means ± S.D. of at least four experiments.

maternal transplacental passage of Rho123 was examined, PSC833, CsA, and QND decreased feto-maternal passage of Rho123 (Table 1 and Fig. 3). QND had the most potent effect on the Rho123 feto-maternal transplacental passage followed by CsA and PSC833 (Table 1). These data indicate that inhibitors of P-gp accelerate materno-fetal passage of Rho123; on the other hand, inhibition of P-gp decreases the feto-maternal passage of Rho123. This corresponds well with the same phenomenon observed in epithelial cell lines cultured in monolayers (placental BeWo, Caco-2, etc.), where the inhibition of a one-way activity of P-gp increases the apical-to-basal and decreases the basal-to-apical transport of P-gp substrates (Yumoto et al., 1999; van der Sandt et al., 2000).

Apart from P-gp specific inhibitors, an ATP-synthesis inhibitor sodium azide was used to study the influence of ATP depletion on the transplacental passage of Rho123. Sodium azide affected the passage of Rho123 across the rat placenta supporting the hypothesis that P-glycoprotein, an ATP-dependent transporter, is involved in the regulation of the passage of Rho123 across the rat placenta (Fig. 4). The impact of sodium azide was comparable with that of PSC833, which is supposed to be one of the most potent specific inhibitors of P-gp (Table 1). On the other hand, the effect of sodium azide on the feto-maternal transplacental passage of Rho123 was not significant (Table 1; Fig. 4).

Comparison of Materno-Fetal and Feto-Maternal Passages of Rho123. Feto-maternal clearances of Rho123 were found to be significantly higher ($p < 0.01$) than clearances in the opposite direction both in steady-state and nonsteady-state experiments (Fig. 5). PSC833 and CsA, however, were able to partly annul this asymmetry between the materno-fetal and feto-maternal passages of Rho123 (Table 2 and Fig. 3). Thus, our results show that the passage of Rho123 across the intact rat placental barrier is asymmetric due to the activity of P-gp similarly as in the case of in vitro cultures of epithelial cell lines (intestinal Caco-2, Yumoto et al., 1999; kidney LLC-PK1:MDR1, van der Sandt et al., 2000). Unlike the placental BeWo epithelial cell line, where inhibition of P-gp lead to the same materno-fetal and feto-maternal passages of P-gp substrates (Ushigome et al., 2000), we did not observe a complete annulment of P-gp function by inhibitors in the intact rat placenta. This discrepancy suggests that transplacental passage across the intact

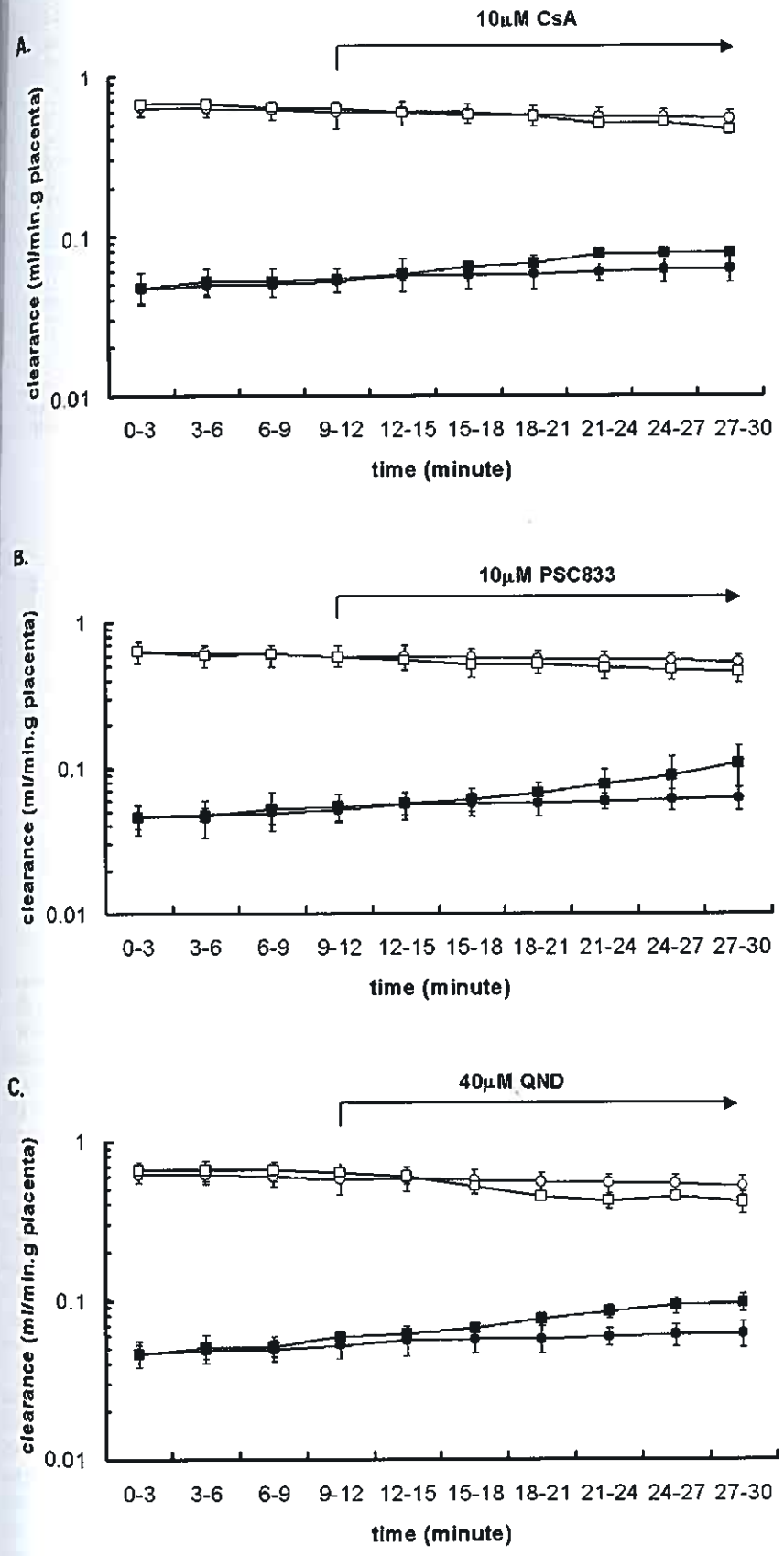

Fig. 3. Influence of CsA, PSC833, and QND on the transplacental passage of Rho123 in the steady-state experiments. In the materno-fetal study, Rho123 was present in the maternal reservoir in a concentration of 0.65 μM (●, control). Inhibitors were added into the maternal solution at the 12th min of experiments to reach 10 μM concentration of CsA (A) and PSC833 (B) or 40 μM concentration of QND (C) (■). In experiments on the feto-maternal passage, Rho123 was present in the fetal reservoir in a concentration of 0.65 μM (○, control). Inhibitors were added into the maternal solution at the 12th min of experiments in the same concentrations as in the materno-fetal study (□). Data are expressed as means ± S.D. of at least four experiments.

TABLE 1
Influence of P-glycoprotein inhibitors on the transplacental passage of Rho123 in both materno-fetal and feto-maternal directions

Inhibitor	Materno-Fetal Passage		Feto-Maternal Passage	
	Ratio[a]	Percentage of control	Ratio[a]	Percentage of control
Control	2.43 ± 0.147	100	1.83 ± 0.06	100.0
Inhibitors of P-glycoprotein				
CsA	2.87 ± 0.39*	118	1.63 ± 0.17*	89.2
PSC833	3.46 ± 0.98*	142	1.69 ± 0.08**	92.5
CPZ	2.85 ± 0.50	117	N.D.	N.D.
Inhibitor of P-gp and rOCT				
QND	3.32 ± 0.74*	136	1.39 ± 0.08***	76.1
ATP synthesis inhibitor				
Sodium azide[b]	1.98 ± 0.18*	134	1.09 ± 0.07	94.0

N.D., not determined.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ to control.
[a] Ratio of the amount of Rho123 that passed the placenta from the 12th to 36th minute of experiments (in the presence of an inhibitor) and the amount of Rho123 that passed the placenta from zero to the 12th minute (without an inhibitor).
[b] Experiments with sodium azide were performed up to the 27th minute; correspondingly, the ratio was calculated as the $amount_{12-27min}/amount_{0-12min}$ and compared with control ratio calculated in the same way.

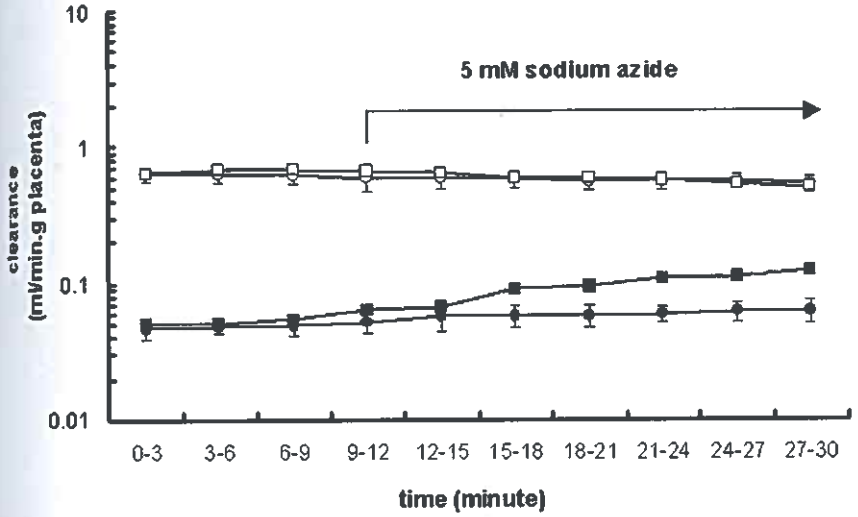

Fig. 4. Influence of sodium azide, an ATP-synthesis inhibitor, on the transplacental passage of Rho123 in the steady-state experiments. In the materno-fetal study, Rho123 was given into maternal reservoir in a concentration of 0.65 μM (●, control). Sodium azide was present in the maternal solution in a concentration of 5 mM from the 12th min of experiments (■). In experiments on feto-maternal passage, Rho123 was present in the fetal reservoir in a concentration of 0.65 μM (○, control). Sodium azide was added into the maternal solution in a concentration of 5 mM at the 12th min of experiments (□). Data are expressed as means ± S.D. of at least four experiments.

placental barrier is a more complex process in comparison with cellular epithelial models.

Western Blotting of P-glycoprotein in Placental Membrane Fractions. P-glycoprotein (molecular mass 150 kDa) was detected in the placental membrane fractions using C219 and Ab-2/F4 monoclonal antibodies (Fig. 6). Monoclonal antibody Ab-2/F4 immunoreacts with P-glycoproteins encoded by human (*MDR1*) and rat (*mdr1a*, *mdr1b*) genes (Huang et al., 2001); mAb C219 shows additional immunoreactivity with *mdr2* and *sister of P-glycoprotein* gene products. The highest levels of P-glycoprotein were found in the apical membrane fraction of the rat syncytiotrophoblast (enrichment for alkaline phosphatase activity, 10.40 ± 0.49). On the other hand, the basal membrane fractions showed weak signal for P-glycoprotein.

Apical and basal membrane isolation methods used in this article (see *Materials and Methods*) have been found to yield the apical membrane fraction of the rat syncytiotrophoblast layer II and the basal membrane fraction of the rat syncytiotrophoblast layer III (Novak et al., 1997). Thus, we suggest that P-glycoprotein is localized predominantly in the apical membrane of the layer II, which forms crucial part of the rat materno-fetal placental barrier.

Immunohistochemical Localization of P-gp in the Rat Placenta. Antigen retrieval immunohistochemistry was performed for localization of P-gp in the rat placenta using mAb C219. The rat chorioallantoic placenta is composed of two distinct zones, junctional and labyrinthine. Strong immunoreactivity of P-glycoprotein was observed only in the inner layers (second or third layer) of the syncytiotrophoblast of the labyrinth zone (Fig. 7). This well corresponds with the fact that the labyrinth zone is thought to be the exchange area of nutriments and drugs between mother and fetuses (the placental "barrier"). Surprisingly, the brush-border membrane of the first syncytiotrophoblast layer, which directly faces maternal blood, was not immunostained as observed in the human placenta (Nakamura et al., 1997; Lankas et al., 1998). Fetal capillaries of the labyrinth zone were without any immunoreactivity. Spongiotrophoblast cells of the rat placental junctional zone, which are important in placental hormone production, were found negative (data not shown). These data further support the hypothesis that P-glycoprotein is present especially in the apical membrane of the second syncytiotrophoblast layer of the rat term placenta.

Expression of Genes Encoding P-gp in the Rat Placenta. Both *mdr1a* and *mdr1b* gene expressions were determined in the rat term placenta by RT-PCR analysis (Fig. 8). We detect mRNAs of both isoforms of mdr1 genes encoding rat P-glycoprotein in the rat placentas on the 22nd day of gestation. Our preliminary results show that expression of

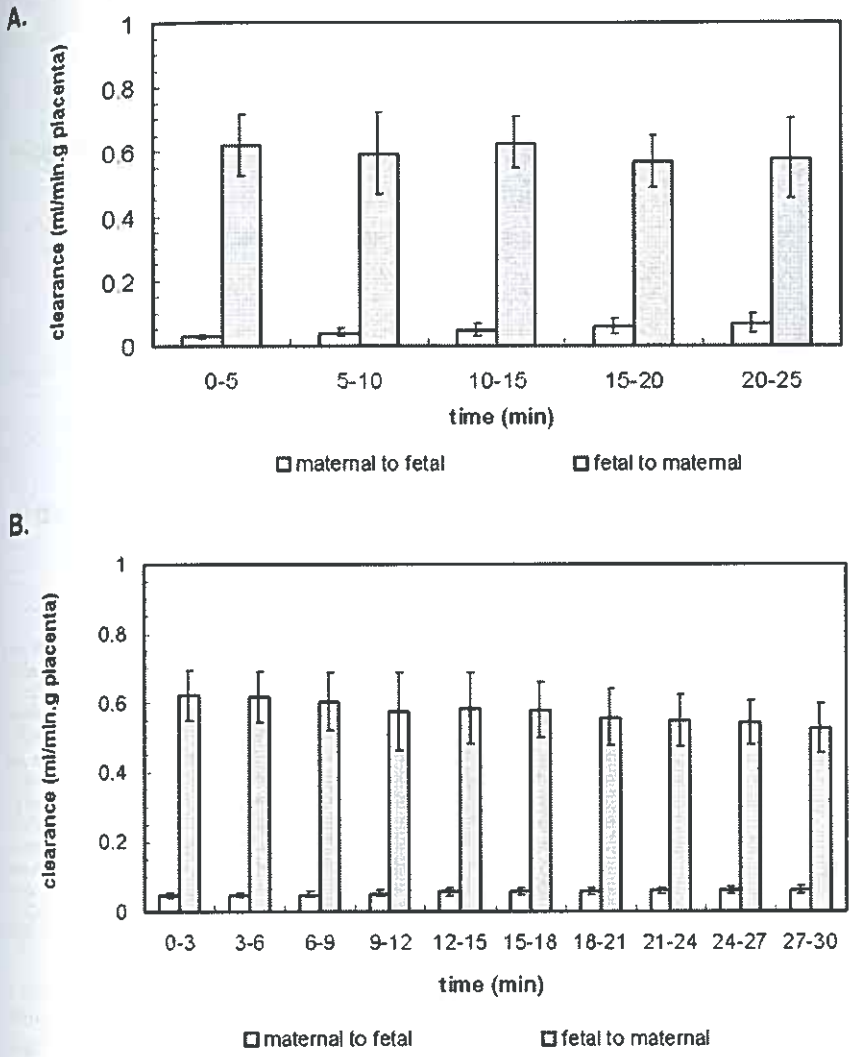

Fig. 5. Comparison of the transplacental passage of Rho123 in the materno-fetal and feto-maternal directions. A, data obtained in the nonsteady-state experiments. Rho123 was given into the maternal/fetal reservoir in a concentration of 1.3 μM. B, data obtained in experiments performed under steady-state conditions. Rho123 was given into the maternal/fetal reservoir in a concentration of 0.65 μM. Materno-fetal clearances were significantly lower ($p < 0.01$) than feto-maternal clearances in all intervals. Data are expressed as means ± S.D. of at least three experiments.

TABLE 2

Influence of P-glycoprotein inhibitors/modulators CsA and PSC833 on both materno-fetal and fetomaternal transplacental clearances (ml · min^{-1} · g^{-1}) of Rho123 across the perfused rat-term placenta under nonsteady-state and steady-state conditions

Values are expressed as the means ± S.D. of at least four experiments.

Direction	Non-Steady-State Experiments[a]		Steady-State Experiments[b]		
	Control (1.3 μM Rho123)	(1.3 μM Rho123) + CsA (40 μM)	Control (0.65 μM Rho123)	(0.65 μM Rho123) + CsA (10 μM)	(0.65 μM Rho123) + PSC833 (10 μM)
Materno-fetal clearance (M → F)	0.05 ± 0.02	0.08 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.05
Feto-maternal clearance (F → M)	0.70 ± 0.07	0.51 ± 0.08	0.54 ± 0.08	0.39 ± 0.03	0.46 ± 0.05
Ratio (F → M/M → F)	12.94	6.32**	8.56	5.22*	4.47*

[a] Mean of transplacental clearances in 20 to 25 min intervals.
[b] Mean of transplacental clearances in 33 to 36 min intervals.
* $p < 0.05$, ** $p < 0.01$; statistically significant difference from control experiments.

mbr1b gene could be dominant in the rat term placenta. Nevertheless, confirmation of these data using real-time RT-PCR method is in progress.

Discussion

P-gp has been demonstrated to be an important determinant of the pharmacokinetics of some lipophilic compounds in various body tissues. However, little is known about the functional expression of P-gp in the placental barrier. Moreover, clinical studies are not feasible in this case because of safety of the developing fetus. Substrates recognized by P-gp include tens of drugs, some of them applied in pregnancy.

The present study examines the functional activity of P-gp in the intact chorioallantoic rat placenta. Both the rat and human placentas are of the hemochorial type, thus the placental barriers of both species are very similar from the morphological and histological points of view. A recent study employing the human trophoblast BeWo cell line brought a new view on the function of P-gp in the placental trophoblast, suggesting that 1) the passage of P-gp substrates is different

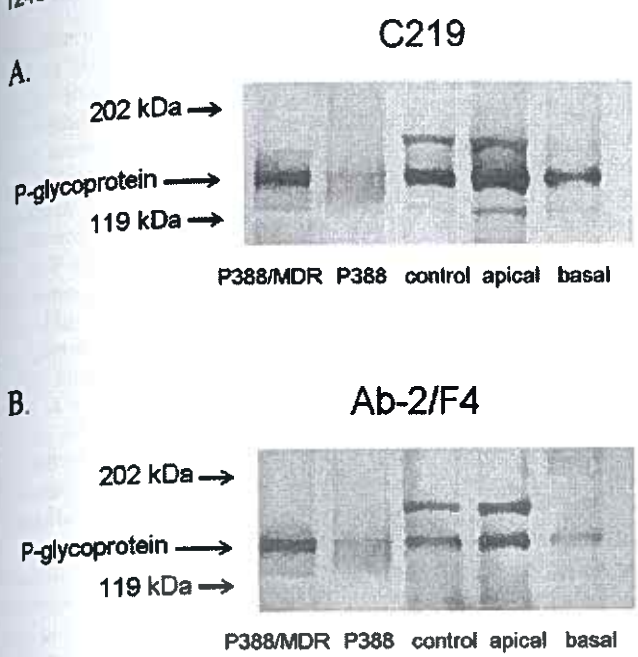

Fig. 6. Western blotting analysis of P-glycoprotein in the rat term placenta. Membrane fractions (20 μg protein/lane) were resolved in a 7.5% gel (SDS-polyacrylamide gel electrophoresis) and transferred onto nylon membranes. Immunoblotting was performed with mAb C219 (1:500) (A) or Ab-2/F4 (1:500) (B) antibodies. Enhanced chemiluminescence reagent was used for chemiluminescence detection. Membranes of multidrug resistant cell line P388/MDR were used as positive control; membranes of cell line P388 without P-glycoprotein were used as negative control. The purified apical membrane fraction (apical) showed more intense bands compared with the total membrane fraction (control) suggesting high level of P-glycoprotein in the apical membrane of the rat syncytiotrophoblast. On the other hand, a weak signal was observed in the case of the purified basal membrane fractions of the rat placental syncytiotrophoblast.

in the apical-to-basolateral and the basolateral-to-apical directions and that 2) P-gp accelerates the passage of some substrates in the basolateral-to-apical direction (Ushigome et al., 2000). On the basis of the in vitro data achieved using BeWo cells, we speculated that, in addition to regulating the materno-fetal passage, P-gp could also stimulate the elimination of its substrates from the fetal circulation in the feto-maternal direction. To confirm this speculation, the pharmacokinetics of Rho123 across the dually perfused rat placenta in situ was investigated. Rhodamine 123, a fluorescent dye, is a well established model compound for the evaluation of the transport activity of P-gp in different sites of the body and for testing of tumor cells for MDR mediated by P-gp (Ludescher et al., 1992; Masereeuw et al., 1997; de Lange et al., 1998; Yumoto et al., 1999; van der Sandt et al., 2000). Nevertheless, some authors suggest that a rOCT could also participate in Rho123 transport (Masereeuw et al., 1997; van der Sandt et al., 2000). To assess the permeability of the intact placental barrier for Rho123, transplacental passage of Rho123 across the placenta was expressed as the transplacental clearance per the wet weight of the placenta (milliliters per minute per gram). Similarly to the permeability coefficient calculated in in vitro epithelial studies, clearance is a constant that characterizes the ability of a drug to pass through the barrier in the in vivo experiments.

The materno-fetal transplacental passage of Rho123 did not show the characteristics of linear pharmacokinetics,

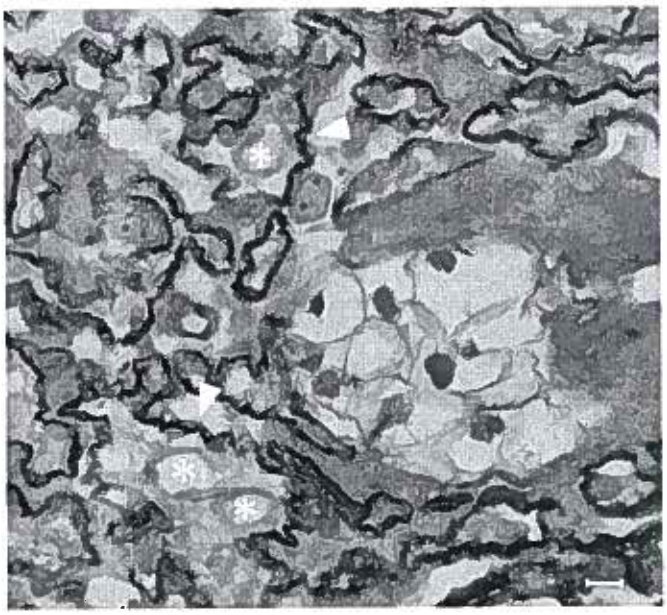

Fig. 7. Immunohistochemical detection of P-glycoprotein in the rat term placenta. The rat placenta was flushed with the perfusion solution via both maternal and fetal vessels and immunohistochemical staining was performed with the monoclonal antibody C219 (1:50 dilution). Strong immunoreactivity is seen in laminas of the syncytiotrophoblast (arrowheads). The fetal capillaries were not positive (asterisks) (hematoxylin stained; scale bar, 10 μm; magnification, 400×).

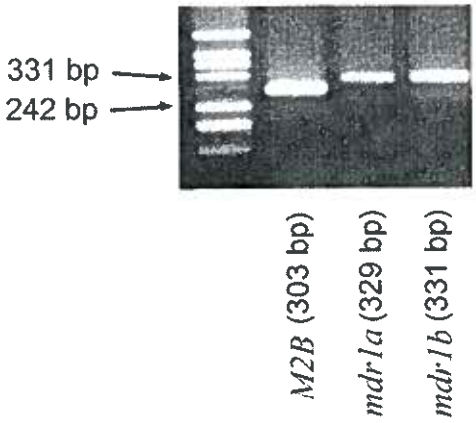

Fig. 8. RT-PCR analysis of rat *mdr1a* and *mdr1b* gene expression in the rat term placenta. PCR products (5 μl) were separated in 1.5% agarose gel and visualized under UV with ethidium bromide. For details, see *Materials and Methods*.

which suggests involvement of a transport process different from passive transport mechanisms (Fig. 1). The same saturation process was found in the case of CsA in the perfused rat placenta, but not in the case of L-[^{3}H]glucose, a marker of passive transport (Pavek et al., 2001). One can speculate that in very low concentrations, P-gp completely returns its substrates back into the maternal compartment. In higher concentrations, however, P-gp becomes saturated and drugs may pass the barrier by passive transport. Under the steady-state conditions, the transplacental clearance of Rho123 was found to be 8.48 times higher in the feto-maternal than in the materno-fetal direction (Table 2; Fig. 3). In BeWo cell monolayers, the transepithelial passages of other substrates of P-gp, such as vinblastine, vincristine, and digoxin, were found to be 6.2-, 3.7-, and 5.0-fold higher, respectively, in the

basolateral-to-apical direction than those in the opposite direction (Ushigome et al., 2000). Thus, the asymmetry is higher in the rat placenta compared with the BeWo model. We found that inhibitors of P-gp, such as PSC833, CsA, and CZP, were able to increase significantly the materno-fetal transplacental passage of Rho123 (Fig. 3 and Table 1). In addition, PSC833 and QND significantly decreased the feto-maternal passage of Rho123 across the placental barrier (Fig. 2 and Table 1). Surprisingly, the inhibitors had less influence on the feto-maternal transport than on the opposite one (Fig. 3 and Table 1). In BeWo cultures, CsA was able to completely cancel the asymmetry of passage, resulting in the same apical-to-basolateral and basolateral-to-apical transport of three examined model compounds (Ushigome et al., 2000). In the intact rat placental barrier, however, P-gp inhibitors did not lead to a complete loss of P-gp function. The feto-maternal clearance of Rho123 was found to be higher than the materno-fetal one even in the last intervals of the experiments. The discrepancies between the data obtained from BeWo cell line and those from the perfused rat placenta demonstrate that the transplacental passage across the intact placental barrier is a more complex process. Other factors, such as presence of additional transport mechanisms, plasma and/or tissue binding, pressures and flows of the perfusion solutions, etc., can influence the transplacental passage of Rho123 across the intact placenta. Despite the fact that syncytiotrophoblast is thought to be the principal barrier component, it seems plausible that others layers, such as endothelia of fetal capillaries, cytotrophoblast cells, and basal laminas, could partly influence transplacental passage of Rho123 in the intact placenta. Because P-gp showed lower effect on the feto-maternal passage of Rho123 in comparison with the materno-fetal passage of Rho123 (Table 1), passive diffusion seems to be important transport mechanism of Rho123 across the membranes in the feto-maternal direction as originally suggested by Stein (1997) in MDR-resistant cell lines. QND influenced the passage of Rho123 to the same extent as specific and highly potent inhibitors of P-gp, such as PSC833 and CsA. Because QND also inhibits rOCT (Pritchard and Miller, 1993), our results suggest that there could be a coinvolvement of an rOCT transporter in the regulation of the transplacental passage of Rho123. P-gp and rOCT1 may provide parallel transport mechanisms in the rat placenta similarly as it was reported in the kidney (Miller, 1995). Sodium azide also increased the materno-fetal passage of Rho123. Thus, ATP-depletion could result in abolition of P-gp protective function and in an increased passage of Rho123 across the placental barrier in the materno-fetal direction. Similar observations have been found in Caco-2 epithelial cell model (Augustijns et al., 1993). Based on these findings, we can speculate that placental hypoxia and placental ATP depletion could result in a decreased function of P-gp, and consequently in a higher exposure of human fetuses to some lipophilic xenobiotics from the maternal circulation. This speculation further emphasizes the importance of studying the functional activity of P-gp in the placental barrier.

To exclude possible interference of Rho123 metabolism with the examined transplacental passage of Rho123, HPLC method was used to determine possible biotransformation of Rho123 in the rat placental barrier. As reported by Sweatman et al. (1990), metabolites of Rho123, such as Rho110 (deacylated metabolite of Rho123) and/or its glucuronide conjugate, are formed in the rat. We found that only negligible amount of Rho123 passing the rat placenta was metabolized into Rho110, and therefore metabolism of Rho123 did not interfere with the study of P-gp-mediated transport processes.

To detect and localize P-gp in the rat placentas, immunochemical and immunohistochemical methods using mAb C219 and Ab-2/F4 have been used. As opposed to the human placental barrier that comprises one syncytiotrophoblast layer, there are three trophoblastic layers in the rat placental labyrinth; two of them are thought to be syncytium (layer II and III). Layer I facing maternal blood is of cellular nature with numerous fenestrations without diaphragmata and thus do not represent a barrier to small compounds (Metz et al., 1976, 1978). Syncytial layers II and III are connected each other by numerous gap junction channels composed preferentially of connexin26 (Metz et al., 1976; Shin et al., 1996). The gap junctions enable exchange of small water-soluble substances, e.g., glucose, between syncytiotrophoblast layers as suggested by Shin et al. (1997) and as was clearly demonstrated by Gabriel et al. (1998) in connexin26-defective mice. We found the highest levels of P-gp predominantly in the apical membranes of the second syncytiotrophoblast layer, which is considered the "barrier" layer of the rat chorioallantoic placenta to maternal blood (Metz et al., 1978; Enders and Blankenship, 1999). Review on both human and rat placental histology and morphology has been published recently by Enders and Blankenship (1999). This localization of P-glycoprotein corresponds well with the function of the membrane. Continuous location of the P-gp in the apical membrane of the syncytiotrophoblast layer II suggests that P-gp is strategically located to face the incoming xenobiotics from the maternal blood before they enter fetal vessels and other fetal tissues. Our results also suggest that both *mdr1a* and *mdr1b* genes encoding rat P-glycoprotein are expressed in the rat-term placenta (Fig. 8). Our preliminary results show that *mbr1b* gene product could prevail over *mdr1a*, however, we are currently using real-time-RT-PCR method to confirm the observation.

We conclude that our results support the hypothesis that P-gp contributes to the barrier function of the placenta. In addition, P-gp was found to accelerate the feto-maternal elimination of its substrates. Both these P-gp functions contribute simultaneously to the protection of the fetus against toxic injury. Consequently, P-gp inhibitors could increase the amount of substrates entering the fetus. Together, the present study emphasizes the importance of assessing the functional activity of P-gp in the human placental barrier since this knowledge could have important toxicological and therapeutic implications in pregnancy.

Acknowledgments

We thank Dana Soucková and Anezka Kunová for excellent assistance with the dually perfused rat placenta method.

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III.

P-GLYCOPROTEIN EXPRESSION AND DISTRIBUTION IN THE RAT PLACENTA DURING PREGNANCY

*Novotna M., **Libra A.**, Kopecky M., Pavek P., Fendrich Z., Semecky V., Staud F. (2004). P-glycoprotein expression and distribution in the rat placenta during pregnancy. Reprod Toxicol 18:785-792.*

Available online at www.sciencedirect.com
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P-glycoprotein expression and distribution in the rat placenta during pregnancy

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Received 25 February 2004; received in revised form 16 April 2004; accepted 27 April 2004

Abstract

P-glycoprotein (P-gp) is a drug efflux transporter that limits the entry of various potentially toxic drugs and xenobiotics into the fetus and is thus considered a placental protective mechanism. In this study, P-gp expression was investigated in the rat chorioallantoic placenta over the course of pregnancy. Three methods have been employed: real-time RT-PCR, western blotting and immunohistochemistry. The expression of *mdr1a* and *mdr1b* genes was demonstrated as early as on the 11th gestation day (gd) and increased with advancing gestation. Western blotting analysis revealed the presence of P-gp in the rat placenta starting from gd 13 onwards. P-gp was localized in the developing labyrinth zone of the placenta on gd 13; from gd 15 up to the term P-gp was seen as a dot like continuous line in the syncytiotrophoblast layers. Our data confirm the presence of P-gp in the rat chorioallantoic placenta starting soon after its development, which may signify the involvement of P-gp in transplacental pharmacokinetics during the whole period of placental maturing.

Keywords: *P*-glycoprotein; Multidrug resistance; Efflux transporter; Placenta; Rat; Trophoblast

1. Introduction

P-glycoprotein (P-gp) is an ATP-dependent membrane efflux pump removing lipophilic drugs and xenobiotics out of cells. It gained worldwide attention two decades ago for its role in the phenomenon of multidrug resistance (MDR) in tumor cells [1]. Since then, P-gp has been detected in a variety of other tissues including epithelial cells of the intestine [2], capillary endothelium of blood-brain [3] and blood-testis barrier [4], and placental trophoblast [4]. Many in vitro and in vivo studies have demonstrated high impact of P-gp on drug pharmacokinetics in these organs (reviewed in [5–8]).

P-gp was found to be present in rodent [9] as well as in human [4,10–13] placenta. In humans, P-gp is a protein product of single *MDR1* gene, whereas two genes (*mdr1a* and *mdr1b*) encode for the transporter in rodents [8]. Rodent *mdr1a* and *mdr1b* appear to be expressed in a tissue specific manner; together they seem to be present in the same tissues as the human P-gp [14]. Both *mdr1* genes were recently found to be expressed in the rat placenta in concentrations exceeding those in liver and kidney, the major organs of excretion [15].

Evidence for the protective role of P-gp to the developing fetus was provided in experiments in *mdr1a* deficient CF-1 mice [16]: fetuses of mdr1a (−/−) mice were extremely sensitive to teratogen isomer of avermectine, a P-gp substrate. Employing *mdr1a*$^{-/-}$/*1b*$^{-/-}$ double knockout mice, Smit and coworkers demonstrated significantly higher transplacental penetration of P-gp substrates digoxin, saquinavir and paclitaxel in *mdr1a*$^{-/-}$/*1b*$^{-/-}$ mice compared to the wild type [17]. Using the method of dually perfused rat placenta in situ, we have recently shown that P-gp limits the entry of drugs from maternal to fetal circulation [18]. In a subsequent study, we described the ability of P-gp to accelerate the feto-maternal elimination of model compounds [19]. Placental P-gp is therefore believed to operate as a protective mechanism by limiting the entry of a variety of xenobiotics into the fetus.

Little is known about the expression and function of placental P-gp over the course of pregnancy. Our previous experiments proved the function of P-gp only in term placentas, however, P-gp was found to be of a great importance in protecting the fetus from potential teratogens also

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0890-6238/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2004.04.014

at earlier stages of pregnancy [16,17]. Several publications have reported on the presence of P-gp in immature as well as in developed human and rodent placenta. In humans, P-gp has been detected in first trimester placenta by immunohistochemistry [11], the expression of *MDR1* gene was demonstrated by in situ hybridization using RNA probes [11] and by RT-PCR [20]. With the application of *mdr1* specific probes, rat *mdr1* genes were found to be expressed in the rat placental labyrinth from the 12th to 20th day of pregnancy [9]. However, none of the methods used in these studies allowed for quantitative evaluation of P-gp expression at different stages of placental development. Furthermore, P-gp was investigated mainly on mRNA but not on the more informative protein level at early stages of pregnancy.

The aim of the present study was to examine placental expression of P-gp on both mRNA and protein levels and to illustrate possible changes in its expression over the course of pregnancy. We took advantage of three techniques to study P-gp expression in the rat chorioallantoic placenta from the 11th to the term 22nd gestation day (gd): (1) we employed real-time RT-PCR to compare the levels of *mdr1a* and *mdr1b* transcripts over the placenta maturing; (2) Western blotting was performed to compare the expression of P-gp on the protein level; (3) immunohistochemical method was used to visualize P-gp localization in the placenta during pregnancy.

2. Materials and methods

2.1. Animals

Female Wistar rats were used for all experimental procedures. Pregnant rats were obtained from Biotest (Konarovice, Czech Republic) and housed in the accredited laboratory at the Faculty of Pharmacy, Charles University. All experiments were approved by the Ethical Committee of the Faculty. Animals were maintained on a 12-h light/dark cycle at 18–20 °C and provided rodent chow and water ad libitum. Gestation day (gd) 0 was established upon detection of coppulatory plug or sperm after overnight mating. Placenta were collected from three dams on gd 11 and from three dams on every day between gd 13 and gd 22. Four to eight placenta were obtained from each dam. Randomly selected placenta were dissected free of endometrium and fetal membranes and either snap-frozen in liquid nitrogen and stored in −80 °C for real-time RT-PCR and Western blotting or fixed in paraformaldehyde for immunohistochemical method.

2.2. Real-time RT-PCR

Total RNA was isolated from pre-weighted placenta using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentration was calculated by A_{260} measurement, RNA integrity was confirmed by electrophoresis on 1% agarose gel.

First strand cDNA was made from 1 μg of total RNA with MMLV transcriptase (Finnzymes, Finland) using oligo(dT)VN primer (Generi-Biotech, Czech Republic) and porcine RNase inhibitor (TaKaRa, BIO, France). Sequences of mRNAs for evaluated genes were obtained from NCBI database. Primers for the target genes *mdr1a* and *mdr1b* and for *β-2-microglobuline* (*b2m*) as a housekeeping gene (Table 1) were designed using the Vector NTI Suite software (Informax, USA).

Primer specificity was determined by 3% agarose gel electrophoresis of RT-PCR products, which resulted in a single product of desired length. Since *mdr1a* and *mdr1b* have common reverse primer, specificity of amplification was verified by restriction analysis. The lengths of restriction fragments obtained from *mdr1a* and *mdr1b* amplicons after incubation with *Xmn*I or *Mbo*I enzymes corresponded with expected sizes. Performing search in BLAST we found no sequence similarities in rat genome. In addition, melting curve analysis on Rotor-gene 2000 real-time cycler (Corbett Research, Australia) was performed, which resulted in single product specific temperatures—*mdr1a* 85.6 °C, *mdr1b* 85.3 °C, *b2m* 86.2 °C.

Real-time PCR analysis was performed on Rotor-Gene cycler. cDNA (10 ng reverse transcribed RNA) was amplified with HotStar Taq polymerase (QIAGEN) under the following conditions: 3 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM each primer, 0.03 U/μl polymerase, SybrGreen I in 1:30,000 dilution; the temperature profile was 95 °C for 15 min; 40 times: 95 °C for 20 s, 55 °C for 30 s, 72 °C for 40 s; 83 °C for 15 s; melting curve program 70–90 °C. Threshold cycle number (C_t) values were acquired at 83 °C, which is the temperature for primer–dimer melting. Each sample of cDNA was amplified in quadruplicates. One of the samples, chosen as the reference calibration sample (calibrator), was analyzed

Table 1
Sequences and specifications of primers, used for real-time RT-PCR quantification

Gene	Accession number	Sequence 5′ → 3′	Product length (bp)	Localization
mdr1a	AF257746	ctg ctc aag tga aag ggg cta ca (f) agc att tct gta tgg tat ctg caa gc (r)	329	2526–2854
mdr1b	AY082609	cgc ttc taa tgt taa agg ggc tat g (f) agc att tct gta tgg tat ctg caa gc (r)	331	2489–2819
β-2-microglobuline	Y00441	tgc cat tca gaa aac tcc cca (f) tac atg tct cgg tcc cag gtg a (r)	303	64–336

f, forward; r, reverse.

in every run. The calibrator was serially (five-fold) diluted (in triplicates) to estimate the efficiency of the reaction. The basic processing of real-time amplification curves was performed on Rotor-Gene 2000 software version 4.6 (Corbett Research, Australia). C_t and slopes of calibration curves were determined. The results were analyzed using Eq. (1) described by Pfaffl [21]:

$$\text{ratio} = \frac{(E_{mdr})^{\Delta C_{t_{mdr}}(\text{calibrator}-\text{sample})}}{(E_{b2m})^{\Delta C_{t_{b2m}}(\text{calibrator}-\text{sample})}}, \quad (1)$$

where E_{mdr} and E_{b2m} are the real-time PCR efficiencies of amplifications of target and housekeeping gene; $\Delta C_{t_{mdr}}$, respectively. $\Delta C_{t_{b2m}}$ are the differencies between C_t of analyzed sample and C_t of calibrator for the target (*mdr*) gene or houskeeping (*b2m*) gene, respectively.

Ratios of samples of the same gd were averaged, the result was expressed as a percentage of the value of gd 22.

2.3. Western blotting

Placenta were homogenized with Potter-Elvehjem homogenizator in ice-cold buffer (1:1, w/v) containing 250 mM sucrose, 10 mM Tris, 5 mM EDTA and protease inhibitors leupeptin (10 μg/ml), pepstatin (10 μg/ml) and PMSF (1 mM), pH 7,4. Crude membrane fractions were obtained through a differential centrifugation of homogenate at 10,000 × *g* for 10 min at 4 °C and subsequent spinning of the supernatant at 36,000 × *g* for 70 min at 4 °C. Protein content was determined in resuspended pelette by bicinchonic acid assay (Pierce Chemicals, Rockford, USA).

Membrane fractions were mixed with 2× SDS sample buffer (1:1) without heating. Proteins (36 μg of protein per line) were separated on 7% polyacrylamide SDS–PAGE gel under constant current (30 mA) and subsequently electrotransferred (25 V) to nitrocellulose Hybond® membrane (Amersham Pharmacia Biotech, Sweden). Unspecific binding sides were blocked by incubating the membrane in 5% blocking solution containing 3% BSA and 2% of blocking agens (Amersham, Pharmacia Biotech, Sweden) and incubated in TBS-Tween 0.01% buffer with murine mAb C219 (Signet Laboratories, Dedham, MA, USA) or mAb MDR Ab-2, clone Ab-2/F4 (NeoMarkers, USA), both diluted 1:500. Anti-mouse secondary horseradish peroxidase-conjugated antibody (1:1000 dilution) and ECL Western blotting detection kit (both Amersham Pharmacia Biotech, Sweden) were used for chemiluminiscence detection of P-gp on FOMA® Blue Medical X-ray films (Foma Bohemia, Czech Republic).

Exposed films were scanned on flatbed scanner HP 5400c (Hewllett-Packard) and densitometrically analyzed with LabImage® gel densitometric software version 2.62 (Kaplan GmbH, Halle, Germany).

The placenta were collected from altogether 33 dams and divided into three groups (11 dams at different stages of pregnancy per group). To average variability in P-gp content in all placenta of one mother, three or four randomly selected placenta of the same dam were pooled and processed together to obtain one sample. Each group formed a set of 11 samples containing placental homogenates of gd 11 and gds 13–22. Each set of samples was processed together at one time (i.e. homogenization, protein content determination and Western blotting analysis) in order to make comparisons across gestational time. Densities of P-gp bands in each set were subsequently related to the density of the 22nd gd band. The results were expressed as three different sets of placental samples of gd 11 and gds 13–22.

2.4. Immunohistochemistry

Specimens of the placenta were fixed in 4% paraformaldehyde (pH 7.35) and then paraffin-embedded. Sections of the placenta (thickness, 5 μm) were dewaxed and rehydrated through a series of ethanol solutions. Endogenous peroxidase activity was blocked with 3% H_2O_2 in 50% methanol solution for 15 min. For heat-induced antigen retrieval, the slides were boiled in 0.1 M Tris–HCl buffer, pH 1.5, for 5 min in a microwave oven at 750 W. Blocking of nonspecific background staining was performed with 10% normal goat serum (Sigma Chemie, Steinheim, Germany) in phosphate-buffered saline (PBS) solution (pH 7.4) for 30 min. Slides were incubated with primary antibody for P-gp (C219; Signet Laboratories) diluted 1:50 in PBS solution for 15–18 h at 4 °C. After PBS rinses, the slides were developed with the secondary goat anti-mouse antibody (DAKO En Vision™, DAKOCytomation, Carpinteria, USA). Secondary antibodies were visualized with diaminobenzidine (DAKOCytomation, Carpinteria, USA) and hematoxylin counterstained. To eliminate background staining, control slides were treated in the same manner, except PBS solution was substituted for the primary antibody to P-gp. Slides were examined using computer image analysis (light microscope Nikon Eclipse E200, Japan; digital camera PixeLINK PL-A642, Vitana Corp., USA, LUCIA software, version 4.71, Laboratory Imaging Prague, Czech Republic).

3. Results

3.1. Expression of mdr1a and mdr1b during pregnancy

The expression of *mdr1* genes in placenta was studied by real-time RT-PCR using relative expression analysis. The levels of *mdr1a/b* transcripts were compared to the expression of *β-2-microglobuline*. Using the mathematical method of Pfaffl [21] the efficiency of PCR was taken into account. Averaged C_t values are plotted in Fig. 1A. The amplification efficiencies of *mdr1a*, *mdr1b* and *β-2-microglobuline* transcripts were 0.962, 0.924 and 0.894, respectively. The equations and correlation coefficients (R^2) of linear regression are shown in Fig. 1.

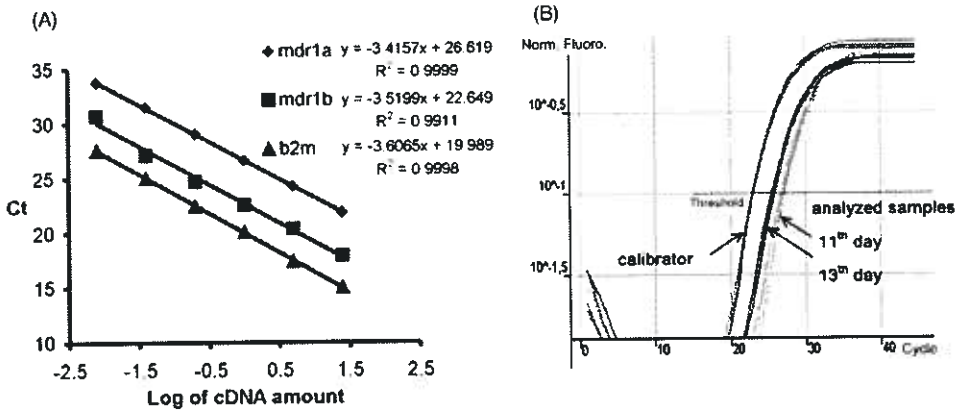

Fig. 1. (A) Standard curves of PCR amplification of mdr1a, mdr1b and *β-2-microglobuline* transcripts are shown. Six-fold serial dilutions were made from calibration sample (one of the cDNA of gd 22). Plotted are C_t values (average of triplicate reactions) vs. amount of reverse transcribed RNA (0.08–25 ng). Data were processed using linear regression analysis. (B) Demonstration of a representative plot of real-time amplification analysis of mdr1a gene in cDNA samples from rat placenta of gd 11 and gd 13. The amount of 10 ng of each cDNA was amplified, the sample of gd 22 was used as a calibrator. Similar analysis was performed for *β-2-microglobuline* as a housekeeping gene. The C_t values of quadruplicates were averaged and processed using Eq. (1).

Significant differences in the expression pattern of both genes (*mdr1a* and *mdr1b*) in the placental samples were observed (Fig. 2). While the concentration of both *mdr1a* and *mdr1b* transcripts increased from gd 11 up to the term, the maximum expression was reached earlier in the case of *mdr1a* than *mdr1b* (gd 19 and gd 22, respectively).

3.2. Western blot analysis of P-gp in placental membrane fractions during pregnancy

In pilot experiments we observed a slight variability in the P-gp expression among placenta of one dam (data not shown). Therefore in subsequent experiments three or four placenta from the same animal were pooled and analyzed as one sample. Furthermore, our data showed no correlation between P-gp expression and weight of the placenta (data not shown).

No P-gp was detected in the placental samples of gd 11 either with C219 or with Ab-2/F4 monoclonal antibody (Fig. 3). P-gp was detectable starting from gd 13 (slight signal observed in two out of three samples employing C219 antibody). P-gp was then found in all placental samples from gd 14 to gd 22 of pregnancy. The monoclonal antibody Ab-2/F4 detected P-gp in one of three samples of gd 14 and in all samples from gd 15 to gd 22. Although the intensity of P-gp expression was rather variable, the levels of P-gp seemed to rise from gd 13 to gd 18 in all three sets of samples. The amount of P-gp on the 22nd day was lower than that of gd 18 in the all three sets.

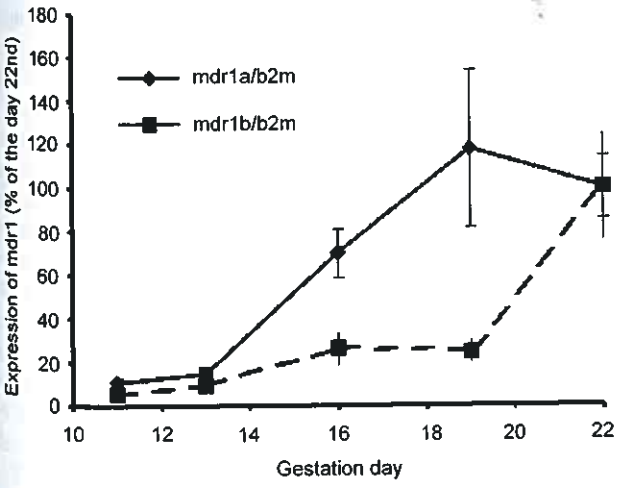

Fig. 2. Relative expressions of *mdr1a* and *mdr1b* genes normalized to the expression of *β-2-microglobuline* (b2m). Plotted are the averages of three measurements. The values are related to gd 22.

3.3. Immunohistochemical localization of P-gp during chorioallantoic placenta maturing

The temporal expression pattern of P-gp in the developing chorioallantoic placenta was investigated by immunohistochemistry at the light microscopy level. No immunopositiv staining for P-gp was found in spongiotrophoblast and trophoblast invading the maternal blood vessels on gd 11 (data not shown). P-gp was detected in the placenta from gd 13 when it occurred in some parts of the developing labyrinth zone as a single continuous line along the interhemal membrane between the maternal blood space and fetal capillaries (Fig. 4A and B). On gd 15, similar pattern of P-gp labelling was found in the labyrinth zone. At higher magnification, P-gp positive labelling formed a dot like continuous line situated in the layers of the syncytiotrophoblast (trophoblast layers II and III). From gd 16 up to the term the expression pattern of P-gp was the same as that on gd 15. The cells of fetal capillaries in the labyrinth zone, spongiotrophoblast and secondary giant trophoblast of the junctional

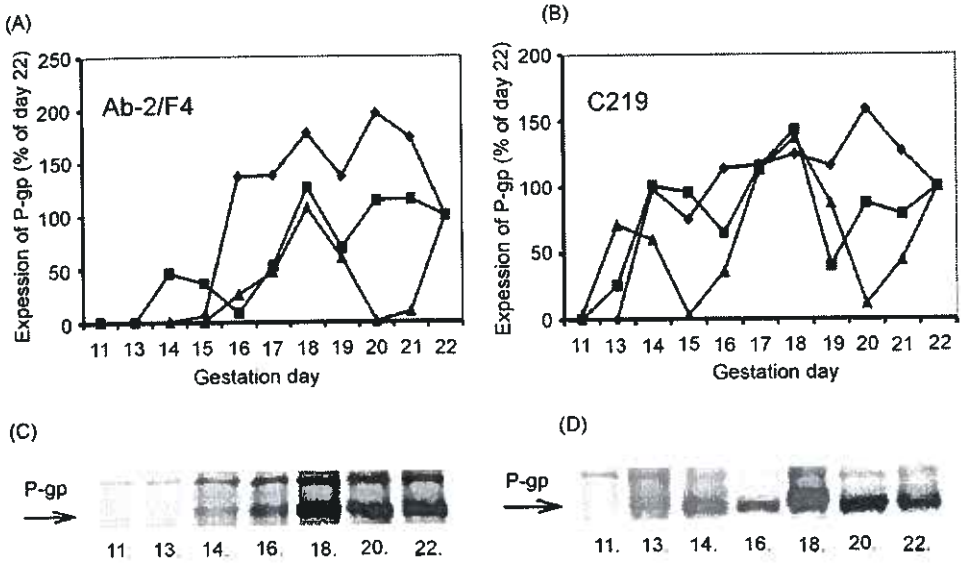

Fig. 3. Expression of P-gp in the rat placenta between gd 11 and gd 22 as detected by Western blotting using monoclonal antibodies Ab-2/F4 (A) and C219 (B). Each row represents a set of samples processed in one turn. No P-gp was detected on gd 11; first signal was found in two out of three samples of gd 13 using C219. (C) and (D) show representative sets of samples with P-gp as a band of about 150 kDa detected by Ab-2/F4 (C) and C219 (D) monoclonal antibodies.

zone were without any immunoreactivity during the course of pregnancy. (Fig. 4C–F).

4. Discussion

P-glycoprotein acts as a membrane efflux pump with broad substrate specifity. By removing xenobiotics out of cells, P-gp plays a significant role in drug absorption and disposition. The importance of P-gp in limiting the transplacental penetration of many potentially toxic xenobiotics was demonstrated by experiments in mice carrying disruption in *mdr1* genes [16,17]. In our recent studies, we have demonstrated the activity of P-gp in situ on dually perfused rat term placenta, using several P-gp substrates and inhibitors [18,19].

In the present study, we analyzed P-gp expression in the rat chorioallantoic placenta during its maturing. The rat placenta is morphologically and histologically similar to the human one, both of them are of hemochorial type. Definitive features of the rat chorioallantoic placenta are well-presented by day 12 of pregnancy (reviewed in [22]); by day 14, choriovitelline placenta regresses and the chorioallantoic placenta is considered to represent the primary unit of maternal-fetal substrate exchange during the last third of pregnancy [23]. We analyzed P-gp expression in the rat placenta from gd 11 up to the term (gd 22), which covers the whole period of chorioallantoic placenta maturing.

The expression of *mdr1a/b* genes in rat placenta during the second half of pregnancy has already been reported [9]. These authors demonstrated the presence of *mdr1* transcripts between gestation days 12 and 20 using common RNA probes for both genes. In the present study, we examined the changes in *mdr1* levels employing the sensitive real-time RT-PCR method using specific primers for both *mdr1a/b* isoforms. We found significant changes in the amount of *mdr1a* and *mdr1b* transcripts over the course of pregnancy and placental development. Both *mdr1* genes were detected as early as on gd 11; however, the levels of *mdr1a* and *mdr1b* transcripts observed on gd 11 were very low. On gd 11, we could not detect P-gp on the protein level by any of the employed immunochemical methods. Therefore we suggest that there is only a negligible amount of P-gp present in the rat chorioallantoic placenta in the early stage of its development and maturing. Although the *mdr1* expression on gd 13 did not significantly differ from that on gd 11, P-gp was already detectable employing the methods of immunohistochemical localization and Western blot analysis. Our real-time PCR data indicate that the expression of both *mdr1a* and *mdr1b* genes tends to increase up to the term reaching a maximum on gd 19 (*mdr1a*) and on the term 22nd day (*mdr1b*). Recently, Leazer and Klaassen [15] observed the expression of *mdr1b* in the rat placenta on gd 21 to be nearly 2.5 times higher than on gd 18, which correlates well with the results presented here. The different patterns in expression of *mdr1a* and *mdr1b* genes show that they are not co-regulated during pregnancy and signify that the expression of both genes probably underlies different regulation pathways. This suggestion is supported by the recent data of Lee [24,25], who provided the evidence that *mdr1a* expression is transcriptionally regulated, whereas the induction of *mdr1a* is regulated post-transcriptionally.

Two different primary antibodies were employed to detect P-gp in plasma membrane by Western blotting. Mouse

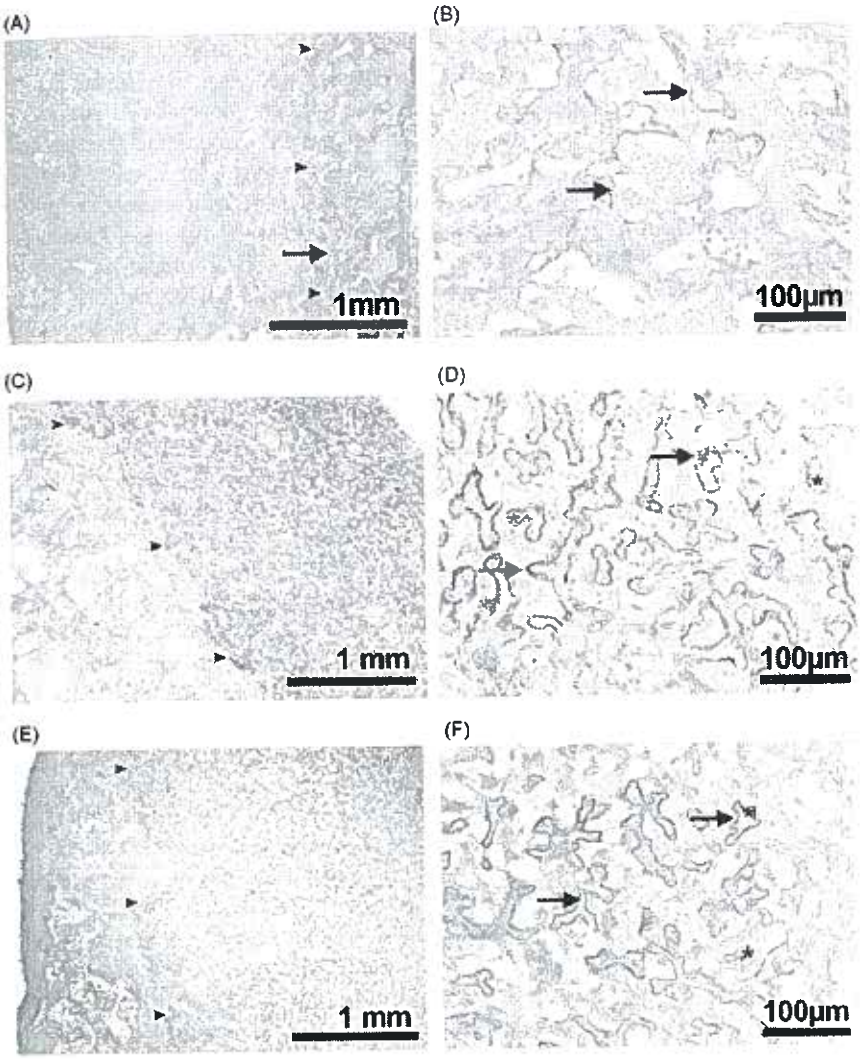

Fig. 4. Light microscopic immunohistochemical staining for P-gp in the rat placenta. (A, B) Placenta at gd 13; (C, D) placenta at gd 17; (E, F) placenta at gd 22. (A) P-gp (arrow) was detected in the developing labyrinth zone of the rat placenta from gd 13 (border between labyrinth and junctional zone: black arrowheads, and junctional zone and maternal decidual tissue: white arrowheads). (B) Detail from the developing labyrinth zone with the positivity for P-gp (arrow) within the syncytiotrophoblast. (C, E) P-gp was localized in the syncytiotrophoblast of the labyrinth zone up to term and this localization did not change during the course of pregnancy. (D, F) At higher magnification, intense staining for P-gp within the layers of syncytiotrophoblast is shown (arrows). Asterisks indicate fetal capillaries.

monoclonal C219 is known to detect one of intracellular epitops of human P-gp [26], whereas Ab-2/F4 recognizes specifically an extracellular epitop [27]. C219 binds to intracellular epitop in all mdr isoforms including mdr2 [28] and detects also sister of P-gp transporter (spgp or bsep) [29,30]. Ab-2/F4 is thought to detect the mdr1 isoforms only [31]. None of the two antibodies is known to be specific for any of P-gp isoforms. Ab-2/F4 thus detects total protein products of *mdr1a* and *mdr1b* genes, C219 antibody binds not only to mdr1a/b but also to a certain extent to the proteins encoded by *mdr2* and *spgp* genes. We found that C219 was able to detect P-gp in some samples of gd 13, whereas no immunoreactivity was observed with Ab-2/F4 monoclonal antibody until gd 15 (apart from one sample of gd 14). No signal was observed even when using higher concentration of Ab-2/F4 antibody (data not presented). The positive signal from C219 could thus reflect mdr2 or spgp expression in the early stages of rat chorioallantoic placenta maturing. However, this seems unlikely since *mdr2* and *spgp* (*bsep*) mRNA expression in term rat placenta is only negligible [15]. Therefore we suggest that these discrepancies are caused mainly by a different affinity of the two monoclonal antibodies to the distinct P-gp epitops. Considering the results obtained with C219 monoclonal antibody we suggest that P-gp is present in the rat placenta from gd 13. P-gp was further detected in all placenta from gd 14 to gd 22, between gd 14 and gd 18 there was a tendency to increase in the intensity of P-gp signal. However, the concentration of P-gp was rather variable

among samples. Although there are no reports on mutations in rat *mdr1* genes that would correlate with P-gp expression, possible polymorphic expression of the genes cannot be excluded as an explanation for the observed variability. In humans, several mutations causing single nucleotide polymorphisms (SNPs) in the MDR1 gene were demonstrated to correlate with placental P-gp expression [32]. Unusual degree of variation in *mdr1* mRNA expression was observed among lung parenchyma from pregnant and also nonpregnant Sprague–Dawley rats [33]. Since the variation was not correlated to biological parameters such as body weight, age, pregnancy or any type of medication, the authors concluded this variability to be caused by polymorphism due to a dominant inherited factor, which stimulates *mdr1* expression. The present study was designed that 3–4 randomly selected placenta of one dam were pooled together to average the variability of P-gp expression among placenta of one dam. However, the variability in P-gp expression observed in western blots could be influenced by a polymorphic expression of *mdr1* in placental samples and by their random selection.

The rat chorioallantoic placenta is composed of two distinct zones: junctional and labyrinth ones, where the labyrinth zone represents the main area of materno-fetal exchange. The rat placenta is hemotrichorial, which indicates that the trophoblast comprises of three layers: a discontinuous layer of cytotrophoblast (layer I) facing maternal blood and two syncytiotrophoblast layers (layers II and III). Because of its fenestrations, layer I is not considered to function as a barrier for penetration of even high molecular weight substances, e.g. horseradish peroxidase (MW 40000) [34]. The apical membrane of layer II is responsible for extraction of nutrients from maternal circulation and forms the main "barrier" for drug penetration. The basal membrane of layer III allows the release of transported substance to the fetal compartment. Both of the membranes thus express transporters, which are necessary for nutrient transfer [23,35,36]. The transport between these two layers is allowed at least partly by gap junctions [36–38].

In human placenta, P-gp was found mainly in the surface of trophoblast [13,16,39]. In rat placenta, the main expression of *mdr1* genes, studied by in situ hybridization, was observed in the trophoblast of labyrinth zone [9]. We confirmed these results performing the immunohistochemical staining of P-gp with C219 antibody. Intensive immunoreactivity for P-gp was found in the developing labyrinth zone of placenta. We report here that P-gp forms a dot like continuous line in syncytiotrophoblast as early as on gd 13 and that the expression patterns do not change until the term. No signal for P-gp was found in the layer I and in the endothelial cells of fetal capillaries in labyrinth or in spongiotrophoblast and secondary giant trophoblast of the junctional zone at any stage of chorioallantoic placenta maturing.

As we previously demonstrated by western blot analysis of the separated membrane fractions [19], P-gp in the term placenta seems to be predominantly localized in the apical membrane of layer II. This supports the hypothesis that the apical membrane of layer II represents the main structure in limiting the xenobiotics transport across the placenta. These data correlate with the localization of P-gp in the syncytiotrophoblast found by immunohistochemistry and support the hypothesis of the protective function of P-gp in the placenta during the whole last third of rat pregnancy.

Apart from the transport and barrier role, mammalian placenta has also endocrine functions including production of steroid hormones and a variety of growth factors [40–42]. We can assume that the expression of placental P-gp on both transcriptional and post-transcriptional levels underlies some control from endogenous factors related to physiological changes in pregnancy. Considering the observed Pgp expression profile and the function of P-gp in rat term placenta [18,19], we assume that P-gp could be involved in transplacental drug transfer starting from gd 13. P-gp may thus protect fetus from potentially harmful xenobiotics during the whole last third of rat pregnancy, when chorioallantoic placenta is the primary unit in feto-maternal exchange. However, P-gp expression does not necessarily have to correlate with the transport activity of the protein. Furthermore, some other mechanisms are involved in the protection of fetus. There are metabolic enzymes present in the placenta and fetus [43–45], which contributes to fetal protection by biotransformation of many xenobiotics. Additionally, P-gp has been found in fetus in early stages of its development [46].

In summary, *mdr1* genes encoding P-gp are expressed in the rat chorioallantoic placenta from the beginning of its establishment as early as on gd 11 and the level of transcripts increases during placenta maturing up to the term. From gd 13, presence of P-gp can already be detected in the interhemal barrier of the developing labyrinth zone. P-gp in the rat placental syncytiotrophoblast shows an increasing expression from the half to the 18th day of pregnancy. Therefore, if the presence of P-gp in the rat placenta is connected with its efflux pump function, placental P-gp could participate in fetal protection soon after the establishment of chorioallantoic placenta.

Acknowledgments

Supported by the Research Center LN00B125 (Czech Republic) and by the Grant Agency of Charles University (Czech Republic), GAUK 84/2003/C/FaF. The authors wish to thank Dr. Martin Buncek (Generi Biotech, Hradec Kralove) for his help with primer design.

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IV.

EXPRESSION AND FUNCTIONAL ACTIVITY OF BREAST CANCER RESISTANCE PROTEIN (BCRP, ABCG2) TRANSPORTER IN THE HUMAN CHORIOCARCINOMA CELL LINE BEWO

*Ceckova M., **Libra A.**, Pavek P., Nachtigal P., Brabec M., Fuchs R., Staud F. (2006). Expression and functional activity of breast cancer resistance protein (bcrp, abcg2) transporter in the human choriocarcinoma cell line bewo. Clin Exp Pharmacol Physiol 33:58-65.*

Clinical and Experimental Pharmacology and Physiology (2006) 33, 58–65

EXPRESSION AND FUNCTIONAL ACTIVITY OF BREAST CANCER RESISTANCE PROTEIN (BCRP, ABCG2) TRANSPORTER IN THE HUMAN CHORIOCARCINOMA CELL LINE BEWO

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SUMMARY

1. Breast cancer resistance protein (BCRP, ABCG2) is a drug efflux transporter that is believed to affect the drug disposition of several drugs and xenobiotics. In the present study, we evaluated the localization and functional expression of BCRP in the human choriocarcinoma cell line BeWo, an *in vitro* model of the human trophoblast, and compared it with the expression of P-glycoprotein (MDR1, ABCB1) as the most widely studied placental transporter. In addition, the expression of *BCRP* at the mRNA level was compared with that of *MDR1* in the human term placenta.

2. Western blotting analysis revealed high endogenous expression of BCRP protein in BeWo cells. Using indirect immunofluorescence microscopy, we found that the BCRP transporter appears to be localized predominantly at the apical plasma membrane. Functional studies showed a significant effect of the BCRP inhibitors GF120918 (5 μmol/L) and Ko143 (1 μmol/L) on mitoxantrone accumulation and, thus, confirmed efflux activity of BCRP in BeWo cells.

3. Using absolute mRNA quantification with real-time reverse transcription–polymerase chain reaction, we found high expression of *BCRP* in BeWo cells, whereas no transcript of *MDR1* (P-glycoprotein), the most extensively studied drug transporter, was detected.

4. In the human placenta, BCRP was localized predominantly in the syncytiotrophoblast layer; however, immunopositivity for the BXP-21 antibody was also observed in fetal vessels of the chorionic villi. The number of *BCRP* transcripts in the human term placenta was found to be more than 10-fold higher compared with the expression of *MDR1*.

5. In conclusion, we suggest that BeWo cells could serve as a suitable *in vitro* model to study trans-trophoblast transport of BCRP substrates and that placental BCRP can play an important role in preventing the accumulation of potentially toxic xenobiotics in the trophoblast cells.

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Received 9 May 2005; revision 15 August 2005; accepted 27 August 2005.

Key words: BeWo cell line, breast cancer resistance protein, drug efflux transporter, pharmacokinetics, placenta, trophoblast.

INTRODUCTION

Breast cancer resistance protein (BCRP, ABCG2) is the most recently discovered drug transporter, belonging to the ATP-binding cassette (ABC) transporter superfamily.[1] Several cytotoxic drugs (e.g. mitoxantrone, methotrexate, topotecan), as well as fluorescent dyes (e.g. Hoechst 33342) and some endogenous substrates, have been found to be transported by BCRP.[2,3] In addition to conferring multidrug resistance to tumour cells, BCRP has been shown to be expressed in various physiological tissues, such as brain capillaries, colon, small intestine, liver or placenta, where it is believed to affect the drug disposition of several drugs and xenobiotics.[2–5]

In the human placenta, high expression of BCRP was originally reported by Allikmets *et al.* using northern blotting.[6] Later, immunohistochemical studies localized BCRP to the trophoblast, the surface layer of placental chorionic villi.[7,8] The role of BCRP in human placenta has not yet been fully elucidated. However, based on considerable overlap in substrate specificity and tissue distribution between BCRP and P-glycoprotein (P-gp; encoded by the multidrug resistance gene *MDR1*), the best-described ABC transporter, BCRP was suggested to contribute to the protective function of the placenta.[9–11] It has been shown in mice that inhibition of Bcrp1 by GF120918, a dual inhibitor of BCRP and P-gp, increased passage of topotecan to the fetus when administered to mdr1a/1b$^{(-/-)}$ mice.[12] Therefore, it was suggested that placental BCRP may play a role in transporting cytotoxic drugs, and possibly toxic xenobiotics, out of the fetus. However, specific localization and the precise role of BCRP in the placenta remain unknown.

Several methods have been introduced to assess the drug transport activity of efflux transporters.[13] To evaluate the transplacental passage of drugs, various techniques have been developed using isolated tissues or cells derived from human placenta.[14] The BeWo choricarcinoma cell line was established as an *in vitro* model to investigate trophoblast cell function.[15] Because BeWo cells display morphological properties and biochemical marker enzymes common to normal trophoblast, they have been used successfully to study the transport and metabolism of endogenous substances such as transferrin,[16,17] IgG[18] or glucose[19] and have also been used in uptake studies involving a variety of membrane transporters.[20–22]

Using the BeWo cell model, the functional activity of P-gp in the trophoblast layer has been examined.[21,23] In addition to confirming the functional expression of P-gp in BeWo cells, these authors emphasised some biochemical properties of the cells being similar to primary cultures of normal human cytotrophoblast and suggested using the BeWo cell line for investigations of the trans-trophoblast transport of nutrients and drugs. High endogenous expression of BCRP has been found previously in several choriocarcinoma cell lines, including BeWo cells.[24] More recently, Ee *et al.* used a new approach of small interfering RNA to knockdown BCRP and observed increased sensitivity of BeWo cells to mitoxantrone and topotecan.[25] However, precise characterization of the expression and function of the BCRP efflux transporter in the BeWo cell line, as one of the *in vitro* models of the placenta, is still lacking.

The aim of the present study was to elucidate, using quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR), immunoblotting and immunofluorescence microscopy, the expression and localization of BCRP in the BeWo cell line and in human placenta. The function of BCRP as a membrane efflux pump in BeWo cells was studied using a fluorescent BCRP substrate, mitoxantrone and inhibitors for BCRP and P-gp transporters. In addition, performing absolute quantification of mRNA using real-time RT-PCR, we were able to compare the expression of *BCRP* with that of *MDR1*, as the most widely studied placental transporter, in both the BeWo cell line and the human term placenta.

METHODS

Tissue samples and cell culture

Human placentas were obtained from five uncomplicated pregnancies after delivery between 36 and 39 weeks gestation. All mothers were non-smokers without any medication during pregnancy. The study was approved by the ethics committee of the Charles University in Prague, Faculty of Pharmacy; informed consent was obtained from all mothers.

Specimens were dissected from the middle villous part of the placenta, snap frozen in liquid nitrogen and stored at −75°C. For immunohistochemistry, samples were immersed in OCT embedding medium (Leica, Wetzlar, Germany), snap frozen in liquid nitrogen-cooled 2-methylbutane and stored at −75°C.

The BeWo cells were purchased from the European Cell Culture Collection (ECACC; Salisbury, Wiltshire, UK). Cells were cultured in Ham's F12 medium adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% fetal calf serum (FCS) and 2 mmol/L L-glutamine. The MDCKII parent cell line and BCRP-transduced MDCKII subline (MDCKII-BCRP), which stably expresses human *BCRP*, have been described and characterized elsewhere.[26] The HCT-8 cells were purchased from the ECACC and were cultured in RPMI medium supplemented with 10% horse serum (HS) and 1 mmol/L sodium pyruvate (NaP). The HepG2 cells were obtained from the American Type Cell Collection (ATCC; LGC Promochem, Teddington, Middlesex, UK) and were grown in minimal essential medium (MEM) supplemented with 1 mmol/L NaP, 0.1 mmol/L non-essential amino acids and 10% FCS. All cell lines were cultured under conditions of 37°C and 5% CO_2/95% humidity.

Quantitative real-time RT-PCR

Total RNA was isolated from cultured cells and from frozen tissue samples using TriReagent (Molecular Research Centre, Cincinnati, OH, USA), according to the manufacturer's instructions. The RNA concentrations were determined from ultraviolet (UV) absorbance at 260 nm, with measurements performed in triplicates. The purity of the isolated RNA was checked by the UV absorbance ratio A_{260}/A_{280} and RNA integrity was confirmed by electrophoresis on a 1% agarose gel. The cDNA was prepared from 1 μg extracted total RNA with MMLV transcriptase using oligo$(dT)_{18}$VN nucleotides and porcine RNase inhibitor. Reference sequences of mRNA for human *BCRP* (GenBank accession no. NM004827) and *MDR1* (GenBank accession no. NM000927) genes were obtained from the NCBI database (http://www.ncbi.nih.gov). Specific primers and probes for the target human *BCRP* and *MDR1* (Table 1) were designed using Vector NTI Suite software (InforMax, Bethesda, MD, USA). Primer specificity was confirmed by agarose gel electrophoresis of RT-PCR products, which resulted in single product of the desired length (179 bp for *BCRP*; 122 bp for *MDR1*). For absolute quantification, pCR plasmids (Invitrogen, Paisley, UK) with subcloned PCR products for *BCRP* and *MDR1* were used as PCR standards. Plasmids were amplified using standard procedures, isolated with a Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany) and sequenced on an ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Real-time PCR analysis was performed on an iCycler (Bio-Rad, Hercules, CA, USA). The cDNA (25 ng transcribed RNA) was amplified using Thermo-Start polymerase (0.04 U/mL; AB-gene, Epsom, UK) under the following conditions: 6 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.1 μmol/L dual-labelled probe and 0.8 μmol/L of each primer for *BCRP* or 0.3 μmol/L of each primer for *MDR1*. The temperature profile was 95°C for 15 min and 40 repeats of a cycle consisting of 95°C for 15 s and 60°C for 45 s. Standard curves were generated from seven decimal dilutions of the corresponding pCR plasmid; the number of copies ranged from 5.10^1 to 5.10^7 copies/25 μL reaction. The analysis of real-time amplification curves and subtraction of threshold (Ct) values was performed using iCycler iQ 3.0 software (Bio-Rad); other calculations were performed in Excel software (Microsoft, Seattle, WA, USA). The absolute number of cDNA copies in each sample was calculated from the calibration curve generated.

Western blotting analysis

The cell membrane fraction of placental tissue was prepared as described previously.[27] The whole cell lysate was prepared from subconfluent cultured cells by scraping the cells and dissolving the phosphate-buffered saline (PBS)-washed cell pellet in ice-cold lysis buffer (50 mmol/L Tris, 1 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT), 1% nonidet P-40 and 0.3% mercaptoethanol) containing protease inhibitors. Lysates were centrifuged at 10 000 *g* for 10 min at 4°C. Samples (100 μg protein) were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride (PVDF) Hybond-P® membrane (Amersham Biosciences, Uppsala, Sweden). After blocking of non-specific binding sites in 5% non-fat dry milk (= blocking buffer), membranes were incubated with BXP-21 antibody (1 : 1000 in 1% blocking buffer; overnight at 4°C). Incubation with corresponding secondary anti-mouse horseradish peroxidase-conjugated antibody was used for recognition of the primary antibody (1 : 2000 in 1% blocking buffer; 60 min at room temperature), followed by enhanced chemiluminiscence detection with an

Table 1 Gene-specific polymerase chain reaction primers and dual-labelled probes

Gene	Forward primer sequence	Probe sequence	Reverse primer sequence	Localization on mRNA
MDR1	TGCTCAGACAGGATGTGAGTTG	ATGACCCTAAAAACACCACTGGAGCATTGA	AATTACAGCAAGCCTGGAACC	2805–2926
BCRP	GCCGTGGAACTCTTTGTGGTAG	ACATCAGCGGATACTACAGAGTGTCATCTT	GCCTTTGGCTTCAATCCTAACA	1534–1712

ECL Advanced Western blotting detection kit (Amersham Biosciences). The membranes were subsequently exposed to FOMA® Blue Medical X-ray films (FOMA Bohemia, Hradec Kralove, Czech Republic). To quantify the bands of interest, exposed films were scanned on a flatbed scanner (HP 5400c; Hewlett-Packard, Houston, TX, USA) and analysed densitometrically with LabImage® gel densitometric software version 2.62 (Kaplan, Halle, Germany).

Cellular uptake experiments

The BeWo, MDCKII and MDCKII-BCRP cells were seeded onto 12-well culture plates and incubated for 2 days to reach at least 75% confluency. Prior to the experiment, the cell culture medium was removed and each well was washed twice with PBS. Cells were then pre-incubated in OPTI-MEM medium (Gibco Invitrogen, Paisley, UK) without inhibitor or in the presence of 5 μmol/L GF120918, 1 μmol/L Ko 143 or 1 μmol/L PSC833 at 37°C in 5% CO_2 for 60 min. After the pre-incubation period, the fluorescent substrate mitoxantrone was added at a final concentration of 20 μmol/L; uptake was performed at 37°C in 5% CO_2 for 60 min. Accumulation was arrested by prompt cooling on ice and washing the cells with ice-cold PBS twice. Cells were trypsinized in ice-cold phenol red-free trypsin/EDTA solution and resuspended in PBS with 2.5% FCS. Mitoxantrone fluorescence in the cells was detected by flow cytometry using a FACSCalibur cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) equipped with a 635 nm red diode laser and 670 nm bandpass filter. Flow cytometry data were processed and analysed using WinMDI ver. 2.8 software (Purdue University, West Lafayette, IN, USA). The mean channel number for each histogram was used as the measure of fluorescence for the calculation of uptake values.

Indirect immunofluorescence microscopy

The BeWo, MDCKII and MDCKII-BCRP cells were seeded onto glass coverslips (13 mm in diameter; $5–10 \times 10^3$ cells/coverslip) and cultured overnight. For placental alkaline phosphatase (PLAP) detection, the polarized BeWo cells were washed with PBS (PBS containing 1 mmol/L $CaCl_2$ and 1 mmol/L $MgCl_2$) and cooled and rabbit antihuman PLAP antibody (1 : 100 in PBS containing 10% goat serum) was bound at 4°C for 60 min to selectively label the apical membrane. Cells were then washed with PBS$^+$, fixed with 4% paraformaldehyd (30 min at room temperature), quenched with 50 mmol/L NH_4Cl and permeabilized with 0.1% Triton X-100 in PBS. All subsequent incubation steps were performed at room temperature. Non-specific binding sites were blocked with 10% goat serum in PBS (blocking buffer). Cells were then washed and incubated with BXP-21 antibody (1 : 100 in blocking buffer) for 1 h. After washing the cells with PBS, primary antibodies were detected using a mixture of the corresponding fluorescent secondary antibodies: goat Alexa 488 anti-rabbit antibody (1 : 1000 in blocking buffer) and goat Alexa 568 anti-mouse antibody (1 : 1000 in blocking buffer; Molecular Probes, Eugene, OR, USA). Nuclei were stained with Hoechst 33342 dye (0.5 μg/mL in PBS for 10 min). Negative controls were obtained by omitting the primary antibody. Cells were mounted in Mowiol and viewed under a Zeiss Axioscope 2MOT (Carl Zeiss, Jena, Germany). Acquisition of images was performed using the Axiocam video camera and Axiovision 3.1 software (Carl Zeiss Vision, Munich, Germany). Images were processed identically using Adobe Photoshop 6.0 software (Adobe, San Jose, CA, USA).

Immunohistochemistry

Sections (5–6 μm) were cut from frozen placenta samples on a cryostat and placed on gelatin-coated slides. Sections were air dried and slides were then fixed for 20 min in cold acetone at –20°C. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in PBS for 15 min. After blocking of non-specific binding sites with 10% normal goat serum in PBS solution (pH 7.4) for 30 min, slides were incubated with primary anti-human BCRP antibody (BXP-21) diluted 1 : 100 in 5% bovine serum albumin (BSA) solution in PBS for 1 h at room temperature. After a PBS rinse, slides were developed with biotinylated goat anti-mouse IgG antibody. Antibody reactivity was detected using Vectastain, horseradish peroxidase-conjugated avidin-biotin complexes (Vector Laboratories, Burlingame, CA, USA) and developed with diaminobenzidine tetrahydrochloride as a substrate and haematoxylin counterstained. To distinguish background staining, control slides were treated in the same manner, except that 5% BSA in PBS solution was substituted for the primary antibody. Slides were examined using computer image analysis with a light microscope Nikon Eclipse E200 (Nikon Instech, Kawasaki, Japan), digital camera PixeLINK PL-A642 (Vitana, Ottawa, Canada) and LUCIA software, version 4.82 (Laboratory Imaging, Prague, Czech Republic).

Materials

Mitoxantrone hydrochloride was purchased from Pliva-Lachema (Brno, Czech Republic). The Ko 143 was kindly provided by Dr Alfred Schinkel's laboratory (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and GF120918 was purchased from GSK (Greenford, England). Cell culture reagents were supplied either by Sigma-Aldrich (St Louis, MO, USA) or by Gibco BRL Life Technologies (Rockvile, MD, USA). The MMLV reverse transcriptase was purchased from Finnzymes (Espoo, Finland) and oligo(dT)$_{18}$VN, primers and dual-labelled probes BHQ1-FAM for real-time RT-PCR were synthesised in Generi-Biotech (Hradec Kralove, Czech Republic). Porcine RNase inhibitor was purchased from TaKaRa BIO (Otsu, Japan). The mouse monoclonal anti-human BCRP antibody BXP-21 and the rabbit polyclonal anti-human PLAP antibody were supplied by Signet Laboratories (Dedham, MA, USA). The secondary HRP-conjugated anti-mouse antibody was purchased from Amersham Pharmacia Biotech. For immunohistochemistry, the secondary goat anti-mouse antibody (DAKO En Vision™) and diaminobenzidine were purchased from DAKOCytomation (Carpinteria, CA, USA). Hoechst 33342 dye (bisbenzimide) was purchased from Sigma-Aldrich and Mowiol 4–88 was supplied by Calbiochem-Novabiochem (La Jolla, CA, USA).

Data analysis

Statistical analysis of differences between two sets of data was performed using Student's unpaired, two-tailed *t*-test. Data are expressed as the mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Quantification of *BCRP* and *MDR1* mRNA in BeWo cells and in the human placenta

To evaluate *BCRP* and *MDR1* expression at the mRNA level in BeWo cells and in human term placentas, we used absolute quantification using real-time RT-PCR. The *BCRP* was found to be expressed in BeWo cells as well as in human term placenta. Surprisingly, the level of *BCRP* transcripts in human placenta was approximately 10-fold higher than that of *MDR1* (Table 2). The amount of *BCRP* transcripts detected in BeWo cells was comparable with that in transfected MDCKII-BCRP cells. However, no *MDR1* could be detected in BeWo cells, even when a higher amount (50 ng) of reverse-transcribed total RNA was used (the detection limit of the method was 1000 transcripts/1 μg transcribed total RNA). Thus, the data indicate that *MDR1* is minimally expressed or even absent in BeWo cells. Conversely, significant amounts of *MDR1* mRNA transcripts were confirmed in cell lines with recognized P-gp activity, hepatocellular carcinoma HepG2 cells and in intestinal HCT-8 cell line[28–31] (Table 2). In addition, *BCRP* expression was also revealed in these two cell lines.

Western blotting analysis

The expression of BCRP at the protein level in BeWo cells and in human term placenta was confirmed by immunodetection of BCRP with the BXP-21 antibody. The BCRP was recognized as an approximate

Table 2 Number of transcripts per μg RNA

Transcripts (10^4)	Human term placenta* Mean ± SD	Minimum–maximum	BeWo	MDCKII-BCRP	HepG2	HCT-8
BCRP	104.4 ± 16.2	77.6–128.1	626.7	704.0	79.7	8.4
MDR1	8.9 ± 2.5	6.3–13.3	ND	ND	72.8	17.0

*Results are based on five term placenta samples from non-medicated, non-smoking mothers. The maximum and minimum values of each transcript are also given.
ND, no transcripts detected.

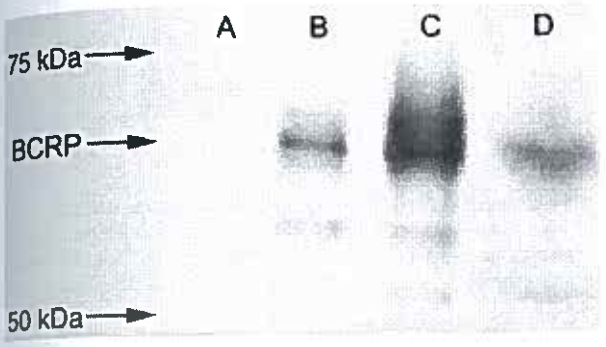

Fig. 1 Immunodetection of breast cancer resistance protein (BCRP). Protein samples, 100 μg of whole cell lysate from MDCKII (negative control; lane A), MDCKII-BCRP (positive control; lane B), BeWo cells (lane C) and 100 μg placental microsomal protein fraction (lane D) were separated on an 8% polyacrylamide gel. Blots were detected with BXP-21 antibody.

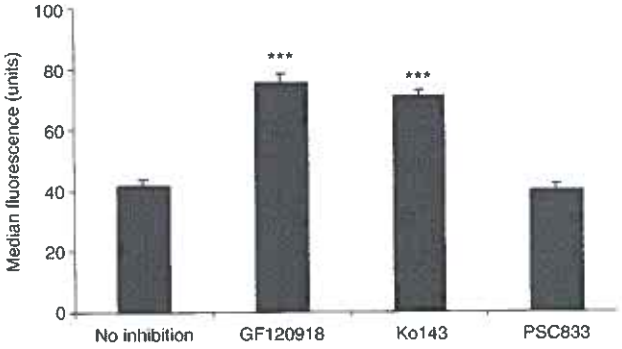

Fig. 2 Cellular uptake of mitoxantrone in the MDCKII-BCRP cell line measured by flow cytometry, showing the accumulation of 20 μmol/L mitoxantrone without any inhibitor or in the presence of 5 μmol/L GF120918, 1 μmol/L Ko143 or 1 μmol/L PSC833, expressed as arbitrary units of mitoxantrone fluorescence. Data are median sample fluorescence, with error bars indicating the standard deviation of at least three experiments. ***$P < 0.001$ compared with control.

70 kDa band in BeWo cells, as well as in human term placenta and in MDCKII-BCRP cells. No signal was observed in non-transfected MDCKII parent cells (Fig. 1). The intensity of the BCRP signal in transfected MDCKII-BCRP cells was approximately sevenfold lower than that of endogenous BCRP expression in BeWo cells, as determined by densitometry analysis from three independent experiments using LabImage® software.

Cellular uptake of mitoxantrone

To investigate the efflux function of BCRP in BeWo cells, we performed uptake experiments with mitoxantrone, a common substrate of BCRP and MDR1.[32] The MDCKII-BCRP and MDCKII parent cells were used in these experiments as a positive and negative control, respectively. As expected, cellular accumulation of mitoxantrone in MDCKII-BCRP cells was significantly increased in the presence of dual BCRP and P-gp inhibitor (5 μmol/L GF120918; $P < 0.001$) and the BCRP-selective inhibitor Ko143 (1 μmol/L; $P < 0.001$; see Fig. 2). Conversely, the P-gp blocker PSC833 (1 μmol/L) had no effect on the amount of mitoxantrone accumulated in the transfected cells. No effect of either BCRP or P-gp inhibitors was observed in parent MDCKII cells.

Similar to the MDCKII-BCRP cell line, mitoxantrone accumulation in BeWo cells was enhanced by 128% in the presence of 5 μmol/L GF120918 ($P < 0.01$) compared with controls. At 1 μmol/L, Ko143 caused an even more pronounced effect, increasing mitoxantrone accumulation by 163% ($P < 0.001$). Significantly increased accumulation of mitoxantrone was also observed in the presence of 1 μmol/L PSC833 ($P < 0.01$), confirming certain efflux activity of P-gp in BeWo cells (Fig. 3).

Localization of BCRP in the plasma membrane

Immunofluorescence microscopy was used to determine the subcellular localization of BCRP in BeWo and MDCKII-BCRP cells. For comparison, detection of a *bona fide* plasma membrane protein, namely PLAP, was included in these experiments. Because MDCKII cells do not express PLAP, this antibody was omitted in both the parent and transfected cell lines. Rabbit anti-PLAP antibody was bound to the polarized cells at 4°C before performing fixation and permeabilization, therefore only the apical membrane of cells was accessible to the antibody. Thus, PLAP was detected exclusively at the apical, microvillous membrane of BeWo cells (Fig. 4f). After permeabilization, both membranes (apical and basolateral) were accessible for detection of BCRP with BXP-21 antibody. According to our observations, the expression pattern for BCRP was found to be similar to that of apical plasma membrane-bound PLAP. Therefore, BCRP appears to be localized at the apical membrane of BeWo cells (Fig. 4e). In agreement with the results of western blotting analysis (see Fig. 1), the fluorescence intensity of the BCRP staining in BeWo cells was considerably higher than that of MDCKII-BCRP cells (Fig. 4a). The specificity of the anti-BCRP antibody was confirmed by the absence of any fluorescence staining in non-transfected MDCKII cells (Fig. 4c).

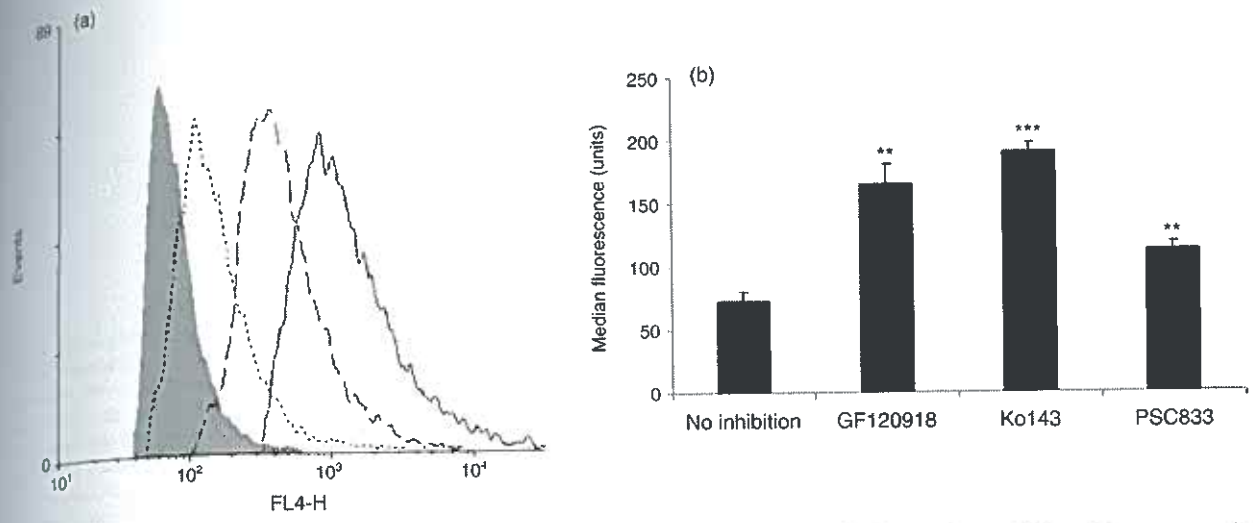

Fig. 3 Cellular uptake of mitoxantrone in BeWo cells measured by flow cytometry. (a) Representative histograms show accumulation of 20 μmol/L mitoxantrone without inhibitors (shaded) and in the presence of 5 μmol/L GF120918 (dashed line), 1 μmol/L Ko143 (solid line) and 1 μmol/L PSC833 (dotted line). (b) Accumulation of mitoxantrone is expressed as arbitrary units of mitoxantrone fluorescence. Data are median sample fluorescence, with error bars indicating the standard deviation of at least three experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

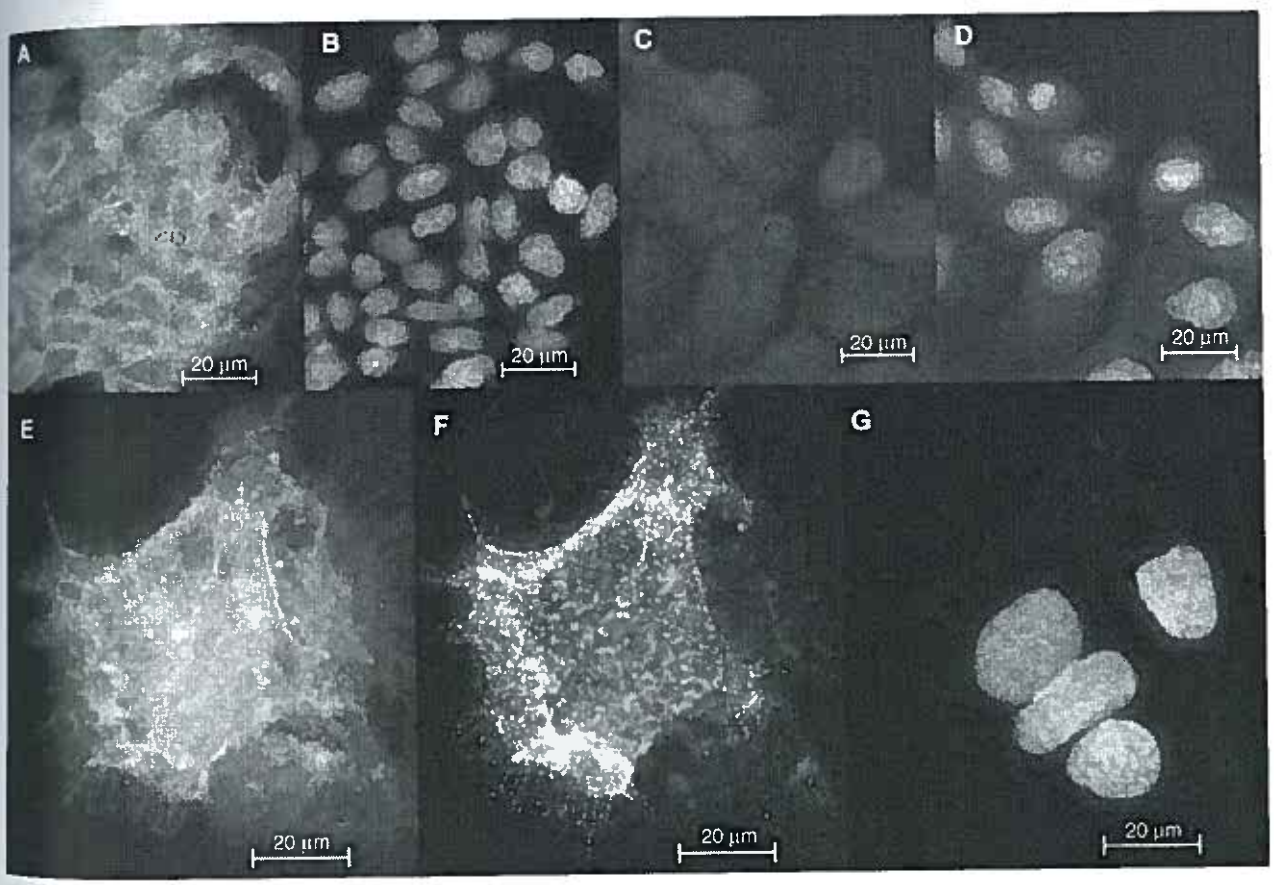

Fig. 4 Indirect immunofluorescence microscopy of BeWo, MDCKII-BCRP and MDCKII cells. Non-permeabilized polarized BeWo cells (e–g) were incubated with rabbit anti-placental alkaline phosphatase (PLAP) antibody. Subsequently, cells were fixed, permeabilized and examined for breast cancer resistance protein (BCRP) expression using anti-BCRP antibody (BXP-21). The MDCKII-BCRP cells (a,b) and MDCKII parent cells (c,d) were used as positive and negative controls for the specificity of BXP-21 antibody, respectively. Nuclei were stained with Hoechst dye (b,d,g). Images were taken at the same exposure time and processed identically in Adobe Photoshop software (Adobe, San Jose, CA, USA) to allow for comparison of staining intensities. Breast cancer resistance protein was found at the cell surface of MDCKII-BCRP cells (a); no staining was present in MDCKII cells (c). In BeWo cells, PLAP was found exclusively at the apical, microvillous membrane (f); BCRP shows a similar expression pattern, indicative of apical localization of the transporter (e).

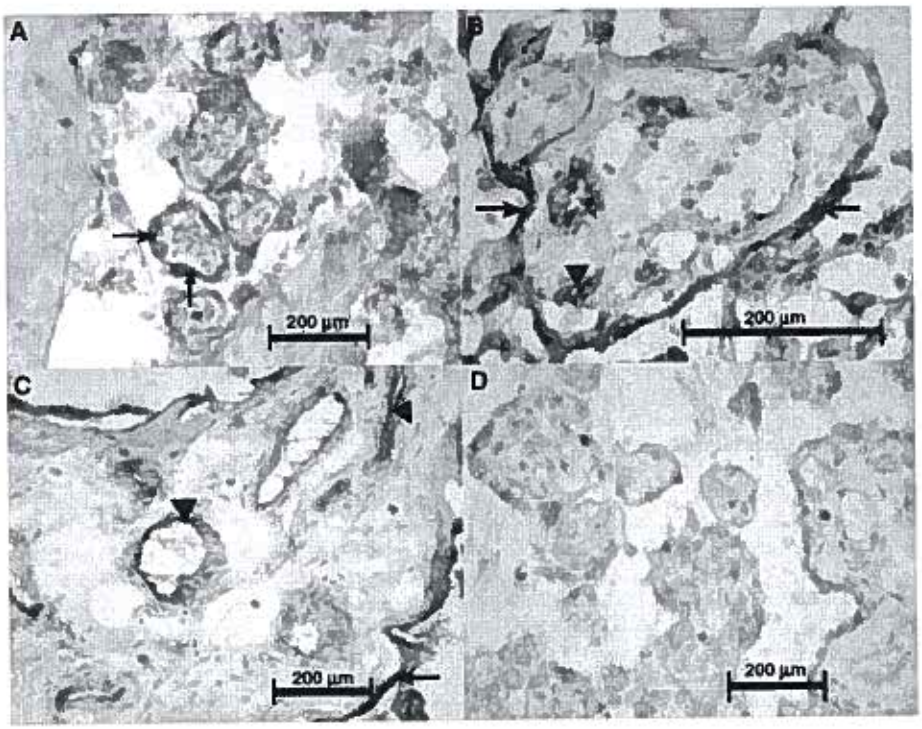

Fig. 5 Immunohistochemistry showing the localization of breast cancer resistance protein (BCRP) in human term placenta. Breast cancer resistance protein expression (arrows) was detected by BXP-21 antibody in the syncytiotrophoblast layer of the free fetal villi (a,b) and in stem fetal villi (c). Moreover, positivity for BCRP is clearly visible in capillaries (arrowheads) in the free fetal villi (b) and in small vessels (arrowheads) in stem villi (c). No BCRP staining is visible in negative controls where primary antibody was omitted (d).

Immunohistochemical detection of BCRP in human placenta

Immunohistochemistry performed on fixed placental sections of four non-medicated term placentas confirmed strong BCRP expression in the syncytiotrophoblast layer in fetal villi. Moreover, intense staining of BXP-21 antibody was also visible in the endothelium of small blood vessels in stem villi. Weaker positivity was observed in some fetal capillaries in the free villi (see Fig. 5).

DISCUSSION

The data from the current study demonstrate that the BCRP transporter is functionally present in the human choriocarcinoma cell line BeWo, an *in vitro* model of human placental trophoblast. As already revealed by Bailey-Dell *et al.*[34] using western blotting, high endogenous expression of BCRP can be found in human choriocarcinoma cell lines, including BeWo cells. In the present study, we have demonstrated high endogenous expression of BCRP in BeWo cells at the mRNA and protein levels. The amount of *BCRP* mRNA transcripts found in BeWo cells was approximately 6.10^6 transcripts/1 µg reverse-transcribed total RNA, which is comparable with that of MDCKII-BCRP cells (Table 2). However, at the protein level, the expression of BCRP in BeWo cells was approximately sevenfold higher compared with MDCKII-BCRP cells. We assume that this discrepancy may be caused by different post-transcriptional modification mechanisms in the canine cells compared with the human cell line.

Using indirect immunofluorescence microscopy, we further investigated the subcellular localization of BCRP on subconfluent BeWo cells cultured on coverslips; under these conditions, cells exhibit polarity and polarized sorting.[33,34] Furthermore, we confirmed the polarity of the cells by staining of the tight junction associated protein ZO-1 (data not shown). The expression of BCRP was compared with that of PLAP. This enzyme is used as an apical membrane marker to follow the isolation and purification of placental brush border membranes[35,36] and is expressed predominantly at the apical membrane of polarized cells.[37] However, basolateral membrane localization of PLAP in BeWo cells and in placental syncytiotrophoblast has also been reported.[38] In the present experiments, we ensured that only apically localized PLAP was labelled. Expression of BCRP was then examined after permeabilization, when both membranes, apical and basolateral, were accessible for BXP-21 antibody. Because the expression pattern of BCRP was similar to that of PLAP, it appears that BCRP is expressed at the apical plasma membrane of BeWo cells (Fig. 4e).

The functional activity of BCRP in the BeWo cell line was demonstrated by uptake experiments using mitoxantrone as a fluorescent substrate for both BCRP and P-gp. Mitoxantrone uptake was 2.3-fold higher in the presence of GF120918 (5 µmol/L), a dual inhibitor of BCRP and P-gp,[39] and even higher accumulation (2.6-fold) was observed in the presence of the BCRP-specific inhibitor Ko143 (1 µmol/L). Because Ko143 is considered to be a specific BCRP inhibitor with only negligible affinity for P-gp,[40] it seems plausible that the increase in mitoxantrone accumulation is caused predominantly by inhibition of the efflux activity of BCRP. Our results correspond with those found in IGROV1/T8 cells[40] and can be explained by a different affinity and potency of GF120918 and Ko143 towards the BCRP transporter. In context with the finding of BCRP being localized predominantly on the apical membrane of BeWo cells, it is obvious that BCRP substrates are pumped out of the cells in the basolateral–apical direction. Because the apical membrane of BeWo cells corresponds with the maternal side of the placental trophoblast,

the efflux activity could predict the enhanced transport of BCRP substrates from the fetal to the maternal compartment.

In the present study, we demonstrated the expression of *BCRP* mRNA in BeWo cells and in human term placenta to be considerably higher than that of *MDR1*, the gene encoding for P-gp. P-Glycoprotein was chosen for comparison because it is the most widely studied placental ABC transporter[9,11] that has also been described in the BeWo cell line.[21–23] Both *BCRP* and *MDR1* were found to be expressed in the human term placenta, with the number of *BCRP* transcripts being one order higher than that of *MDR1*. Surprisingly, we could not identify any *MDR1* transcript in BeWo cells in contrast with the clear detection of *MDR1* in hepatoma HepG2 cells and intestinal HCT-8 cells, which are well known to functionally express P-gp.[28–31] Based on the detection limit of our quantitative real-time RT-PCR system, the expression of *MDR1* in BeWo cells was at least three orders lower than that of *BCRP*. These data correspond well with recently published observations of Atkinson *et al.*,[41] who found no *MDR1* mRNA in BeWo cells obtained from the ECACC and observed only a very faint signal for P-gp using western blotting. In contrast, functional expression of P-gp in the BeWo cell line has recently been demonstrated by two independent groups.[21,23] This discrepancy may be explained by different expression of transporters among particular clones of BeWo cells, as suggested by Atkinson *et al.*[41]

Using immunohistochemical detection with BXP-21 antibody, we observed predominant expression of placental BCRP in the syncytiotrophoblast layer of chorionic villi, confirming the data of Litman *et al.*[7] and Maliepaard *et al.*[8] In addition, immunoreactivity of BXP-21 antibody on placental cryosections was observed in fetal vessels of the placental villi, which has not been reported before. Possible expression of BCRP in fetal vessels would correspond with the general belief that BCRP is expressed in the venous and capillary endothelium.[8] However, the potential role of BCRP in the fetal vessels of the placenta remains to be elucidated.

To conclude, in the present study we demonstrated high expression of BCRP in the choriocarcinoma BeWo cell line at the mRNA and protein levels. The transporter was localized predominantly to the apical plasma membrane and showed intensive efflux activity in the mitoxantrone-uptake study. Surprisingly, expression of *BCRP* in BeWo cells was at least three orders higher than that of *MDR1*. Therefore, caution should be taken when evaluating placental transport of P-gp substrates using the BeWo cell line model. Furthermore, the considerably higher expression of *BCRP* compared with *MDR1* in the human placenta indicates that BCRP could have at least as important a role as P-gp in this tissue, or even be the main ABC transporter of the placenta. Localization of placental BCRP predominantly in the syncytiotrophoblast layer and the apically localized expression of BCRP in the plasma membrane of BeWo cells suggest that placental BCRP can play a significant role in preventing accumulation of potentially toxic xenobiotics in trophoblast cells. Based on our findings, the BeWo cell line seems to be a suitable *in vitro* model to study trans-trophoblast transport of BCRP substrates.

AKNOWLEDGEMENTS

The authors thank Dr Karel Hodik (Faculty Hospital Hradec Kralove, Department of Gynecology and Obstetrics) for providing us with the human placentas and Dr Doris Vokurkova (Faculty Hospital Hradec Kralove, Institute of Clinical Immunology and Allergology) for her assistance in flow cytometry measurements. This work was supported by grants GAUK151/2004/C/FaF 2004 and AKTION 38p17.

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V.

EXPRESSION AND TRANSPORT ACTIVITY OF BREAST CANCER RESISTANCE PROTEIN (BCRP/ABCG2) IN DUALLY PERFUSED RAT PLACENTA AND HRP-1 CELL LINE

Staud F., Vackova Z., Pospechova K., Pavek P., Ceckova M., ***Libra A.****, Cygalova L., Nachtigal P., Fendrich Z. (2006). Expression and transport activity of breast cancer resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line, Journal of Pharmacology and Experimental Therapeutics, provisionally accepted*

EXPRESSION AND TRANSPORT ACTIVITY OF BREAST CANCER RESISTANCE PROTEIN (BCRP/ABCG2) IN DUALLY PERFUSED RAT PLACENTA AND HRP-1 CELL LINE

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Running title: Bcrp in rat placenta

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Non-standard Abbreviations: ABC – ATP-binding cassette, BCRP/Bcrp – human/rodent breast cancer resistance protein; MDR – multidrug resistance; RT-PCR – reverse transcription polymerase chain reaction; P-gp – P-glycoprotein

Recommended section assignment: Absorption, Distribution, Metabolism, and Excretion

ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ABC transporter family that recognizes variety of chemically unrelated compounds. Its expression has been revealed in many mammal tissues including placenta. The purpose of this study was to describe its role in transplacental pharmacokinetics using rat placental HRP-1 cell line and dually perfused rat placenta. In HRP-1 cells, expression of Bcrp, but not of P-glycoprotein, was revealed at mRNA and protein levels. Cell accumulation studies confirmed Bcrp-dependent uptake of BODIPY FL prazosin. In the placental perfusion studies, a pharmacokinetic model was applied to distinguish between passive and Bcrp-mediated transplacental passage of cimetidine as a model substrate. Bcrp was shown to act in a concentration dependent manner and to hinder maternal-to-fetal transport of the drug. Fetal-to-maternal clearance of cimetidine was found to be 25 times higher than that in the opposite direction; this asymmetry was partly eliminated by BCRP inhibitors (2 μM fumitremorgin C or 2 μM GF120918) and completely abolished at high cimetidine concentrations (1 mM). When fetal perfusate was recirculated, Bcrp was found to actively remove cimetidine from the fetal compartment to the maternal one even against concentration gradient and establish a two-fold maternal-to-fetal concentration ratio. Based on our results, we propose a two-level defensive role of Bcrp in placenta: the transporter (i)

reduces passage of its substrates from mother to fetus but also (ii) removes the drug already present in the fetal circulation.

INTRODUCTION

Placenta is an organ that brings maternal and fetal blood circulations to proximity, allowing mutual interchange of nutrients and waste products. On the other hand, placenta forms a barrier to protect fetus against harmful endo- and exogenous compounds from maternal circulation. As a barrier, placenta had for long been supposed to present only a mechanical obstruction formed by fetal endothelia, basal membranes and syncytiotrophoblast. However, over the last two decades, variety of metabolizing enzymes and drug efflux transporters of the ATP-binding cassette (ABC) transporter family have been localized in placental trophoblast (Marin et al., 2004). These proteins are believed to strengthen, in an active and capacity limited manner, placental barrier role and help in protection of the fetus.

Drug efflux transporters of the ABC family are membrane-embedded proteins that limit intracellular concentration of substrates by pumping them out of cell through an active, energy dependent mechanism (Schinkel and Jonker, 2003). The most intensively studied drug efflux transporters to date have been P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug resistance-associated proteins 1 and 2 (ABCC1, ABCC2), all of which were found to be responsible for the phenomenon of multidrug resistance in cancer therapy (Fischer et al., 2005). In addition, due to their extensive distribution in non-tumorous tissues and wide substrate specificity, these proteins significantly affect body disposition of many clinically used drugs. With respect to expression, regulation, function and clinical relevance, the best described of placental ABC transporters to date is P-glycoprotein (Ceckova-Novotna et al., 2006).

Breast cancer resistance protein (BCRP) is the youngest member of this transporter superfamily (Doyle et al., 1998). Its expression has been assessed in many tissues and cells including blood-brain barrier, placenta, intestine, various tumors and side population of stem cells (Staud and Pavek, 2005). Since its tissue distribution and substrate specificity overlaps noticeably with that of P-glycoprotein (P-gp), it is generally believed that these transporters share similar role in protecting pharmacological sanctuaries such as brain and fetus.

Considerable levels of BCRP/Bcrp expression have been detected in placentas of various species. In humans, placental BCRP expression was found to be approximately 10-times higher than that of P-gp (Ceckova et al., 2006). Given the expression pattern and the broad range of substrates including exogenous (drugs, toxins) and endogenous (steroid conjugates, porphyrins) compounds (Staud and Pavek, 2005), it is reasonable to assume that BCRP will be an important component of the placental barrier. Kolwankar et al (Kolwankar et al., 2005) confirmed BCRP function in microvillous membrane vesicles of the human placenta. In addition, we have recently described BCRP expression, localization and function in an *in vitro* placental model, BeWo cell line (Ceckova et al., 2006). The only functional *in vivo* experiments proposing Bcrp activity in the placenta were performed by Jonker et al in transgenic mice (Jonker et al., 2002; Jonker et al., 2000). Nevertheless, transport activity of this efflux protein and its role in transplacental pharmacokinetics has not been fully evaluated to date.

Due to technical constraints and ethical issues, direct investigation of placental drug transfer under *in vivo* conditions in human is not feasible; therefore, several alternative experimental methods have been developed to assess potential risk that drugs in maternal circulation present to fetus (Sastry, 1999). Among these, cell cultures and perfused placentas of various species are widely employed models for mechanistic studies to describe transplacental pharmacokinetics, including transport mechanisms and biotransformation. The dually perfused rat placenta, in particular, is a well established model that has been successfully employed to investigate placental physiology and pharmacology (Kertschanska et al., 2000; Stulc et al., 1995). In our earlier studies, we employed this experimental model to evaluate functional activity of P-gp in the rat placenta (Pavek et al., 2001; Pavek et al., 2003).

In this study, we investigated Bcrp activity both *in vitro*, using HRP-1 rat placental cell line, and *in situ* in dually perfused rat term placenta. A pharmacokinetic model was applied to distinguish between passive and Bcrp-mediated placental transport of cimetidine as a model compound. We provide evidence for striking asymmetry between maternal-to-fetal and fetal-to-maternal transport of cimetidine. This difference is partly lowered by addition of BCRP inhibitors and completely abolished at high substrate concentrations. In addition, using closed perfusion system on the fetal side of the placenta, we are the first to demonstrate that Bcrp not only limits maternal-to-fetal passage of cimetidine but, despite being localized on the maternal-facing side, it also actively removes the drug from the fetal circulation against concentration gradient.

MATERIALS and METHODS

Reagents and chemicals

Cimetidine and radiolabelled [N-*methyl*-^{3}H]Cimetidine were purchased from Sigma-Aldrich (St. Louis, MO, USA) and from Amersham Biosciences (GE Healthcare Life Science, Little Chalfont, UK), respectively. BODIPY FL prazosin, a common BCRP and P-gp substrate, was from Molecular Probes (Karlsruhe, Germany). Specific BCRP inhibitors, Ko143 and fumitremorgin C, were donated by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) or purchased from Alexis (Lausen, Switzerland), respectively. Specific P-gp inhibitors, PSC833 and cyclosporine, were kind gifts from Dr. Andrýsek (Ivax Pharmaceuticals, Opava, Czech Republic). Dual BCRP and P-gp inhibitor, GF120918, was from GSK (Greenford, England). All other compounds were reagent grade.

Cell cultures

For *in vitro* accumulation studies, HRP-1 rat trophoblast cells (Soares et al., 1987), received as a generous gift from Dr. Michael Soares at the University of Kansas Medical Center, were employed. They were maintained in RPMI-1640 culture medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 1 mM sodium pyruvate and 50 μM β-mercaptoethanol. Cells from passages 15-25 were used in experiments described herein.

Animals

All experiments were approved by the Ethical Committee of the Faculty of Pharmacy (Hradec Kralove, Charles University in Prague) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, 1996 and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, Strasbourg, 1986. Pregnant Wistar rats were purchased from Biotest Ltd. (Konárovice, Czech Republic) and were maintained in 12/12-h day/night standard conditions with water and pellets ad libitum. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) in a dose of 40 mg/kg administered into the tail vein.

RNA isolation and RT-PCR analysis

Placentas and kidney as a positive control (Tanaka et al., 2005) were collected on day 21 of gestation from 5 rats. Five randomly selected placentas from each animal were dissected free of maternal tissues and fetal membranes. Immediately after dissection, the organs were frozen in liquid nitrogen and stored at -70°C until analysis. RNA izolation and reverse transcription were performed as described in our previous study (Novotna et al., 2004). Sequences of mRNAs for target genes were obtained from NCBI database; primers for *mdr1a*, *mdr1b* and *bcrp* genes were designed using the Vector NTI Suite software (Informax, Bethesda, MD, USA) and are given in Table 1.

Gene	Accession number	Sequence 5′→3′	Product length (bp)	Localization
mdr1a	AF257746	ctg ctc aag tga aag ggg cta ca (f) agc att tct gta tgg tat ctg caa gc (r)	329	2526-2854
mdr1b	AY082609	cgc ttc taa tgt taa agg ggc tat g (f) agc att tct gta tgg tat ctg caa gc (r)	331	2489-2819
Bcrp	NM181381	cca ctg gaa tgc aaa ata gag (f) cct cat agg tag taa gtc aga cac a (r)	188	1340-1527

Table 1. Sequences and specifications of primers used in RT-PCR

RT-PCR analysis was performed on iCycler iQ (Bio-Rad, Hercules, CA, USA). cDNA was amplified with HotStart Taq polymerase under the following conditions: 3mM $MgCl_2$, 0.2mM dNTP, 0.03 U/μl polymerase, SybrGreen I in 1:100,000 dilution, 0.3 μM each primer; the temperature profile was 95°C for 14 min; 50 times: 95°C for 15s, 60°C for 20s, 72°C for 20s, 72°C for 5 min; melting curve program 72-95°C. Each sample of cDNA was amplified in duplicates. The PCR products were separated by electrophoresis on 2% agarose gel in the presence of ethidium bromide and visualized under ultraviolet light and compared with low molecular weight DNA ladder (25-766bp) (New England BioLabs, Herts, UK).

Western blot analysis

Cell membrane fraction of placental tissue and the whole cell lysate were prepared as described previously (Ceckova et al., 2006; Novotna et al., 2004). Protein contents were determined by BCA™ Protein Assay Detection Kit (Pierce, Rockford, IL, USA). Samples containing 100 μg of cell lysate protein or 40 μg of tissue cell membrane were subjected to electrophoresis on 8% SDS-polyacrylamide gels and subsequently electrotransferred onto

Hybond-ECL membrane (GE Healthcare, Chalfont St. Giles, UK). After blocking in 5 % non-fat dry milk blocking buffer the membranes were probed with mouse monoclonal anti-P-gp antibody C219 (Signet Laboratories, Dedham MA, USA) and rabbit polyclonal anti-ABCG2 antibody M-70 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500 in 1 % blocking buffer; o/n at 4°C). Incubation with corresponding secondary horseradish peroxidase-conjugated antibody (anti-rabbit: 1:2000 in 1 % blocking buffer, anti-mouse 1:1000; 60 min at RT) was used for recognition of the primary antibodies. Immunoreactive proteins were visualized on FOMA® Blue Medical X-ray films (FOMA Bohemia, Hradec Kralove, Czech Republic) by ECL Advance Western blotting detection system (GE Healthcare, Chalfont St. Giles, UK).

Immunohistochemical localization of Bcrp in the rat term placenta

Preparations of the rat placental tissue were performed as described previously (Pavek et al., 2003). The antigen (Bcrp) was unmasked by heating the specimens in sodium citrate buffer (pH 6.0) in a microwave oven at 750W. Slides were incubated with a polyclonal primary antibody for BCRP (M-70; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:10 in BSA (bovine serum albumine) for 15 to 18 h at 4°C. Subsequently, the slides were developed with a secondary antibody – goat anti-rabbit IgG conjugated to peroxidase-labelled polymer (DAKO EnVision™, Carpinteria, USA) for 30 minutes. The reaction was visualized using diaminobenzidine (DAB substrate-chromogen solution, DAKO, Carpinteria, USA) and the sections were counterstained by hematoxylin. Slides were examined using computer image analysis (light microscope Nikon Eclipse E200, Japan; digital firewire camera PixeLINK PL-A642, Vitana Corp. Ottawa, Canada; LUCIA software, version 4.82, Laboratory Imaging, Prague, Czech Republic). Specificity of the immunostaining was assessed by staining with nonimmune isotype-matched immunoglobulins.

Cellular uptake experiments

HRP-1 cells were seeded on 24-well culture plates ($1x10^5$ per well) 2 days prior to experiment. Cell culture medium was removed and cells were washed twice with 500 µl pre-warmed PBS. Cells were then preincubated in OPTI-MEM medium with or without inhibitor (1 µM GF120918, 1 µM Ko143, 25 µM verapamil or 1 µM PSC833) at 37°C in 5 % CO_2 for 60 min before fluorescent substrate, BODIPY FL prazosin (500 nM), was added. Accumulation was allowed for 2 h at 37°C and was arrested by prompt cooling on ice and removal of medium. Cells were washed with ice-cold PBS, lyzed in 1% SDS and fluorescence was measured after 24 hours (Genios Plus, Tecan, Austria). Fluorescence of each well was related to protein content as assessed by BCA™ Protein Assay Detection Kit.

Dual perfusion of the rat placenta

The method of dually perfused rat placenta was used as described previously (Pavek et al., 2003). Briefly, one uterine horn was excised and allowed to dive in the heated Ringer saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying a selected placenta and connected with the peristaltic pump. Krebs' perfusion liquid containing 1 % albumin was brought from the maternal reservoir at a rate of 1

ml/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring ones by ligatures. The umbilical artery was catheterized using 24-gauge catheter and connected with the tubing by which the fetal perfusion liquid from the fetal reservoir was supplied at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighted glass vials to check a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Cimetidine was added to the maternal (in maternal-to-fetal studies) or fetal (in fetal-to-maternal studies) reservoir immediately after successful surgery followed by approximately 10 min stabilization period before sample collection started. Fetal effluent was sampled into pre-weighted vials in 5-minute intervals. Maternal and fetal perfusion pressures were monitored continuously throughout the perfusion experiments. At the end of experiment, placenta was perfused with radioactivity-free buffer, dissolved in tissue solubilizer (Solvable, PerkinElmer, Boston, MA) and its radioactivity measured in order to detect tissue-bound cimetidine.

Effect of cimetidine inflow concentrations and BCRP inhibitors on transplacental clearance

To investigate the effect of cimetidine concentrations on maternal-to-fetal and fetal-to-maternal clearances, cimetidine and [^{3}H]cimetidine as a tracer were added to the corresponding reservoir in the following range of concentrations: 0.005, 0.1, 1, 30, 100 or 1000 μM. The fetal effluent samples were collected in six consecutive 5-min intervals and analyzed for [^{3}H]cimetidine. Transplacental clearance of cimetidine was calculated for every concentration from all measured intervals as described bellow.

To study the effect of BCRP and P-gp inhibitors, fumitremorgin C (2 μM), GF120918 (2 μM), cyclosporine (10 μM) or verapamil (25 μM) was added to the maternal reservoir in the 10th minute of perfusion. Subsequently, transplacental clearance of cimetidine in the period of 0-10 min (without inhibitor) was compared with that in 20-30 min (with inhibitor), leaving the mid-interval of 10-20 min as a stabilization period to achieve a new steady-state after addition of inhibitor. This experimental setup allows for direct observations of inhibitor effect in one animal at different substrate concentrations, reducing possible interindividual variability.

Pharmacokinetic analysis of efflux transport activity in the placenta

Organ clearance concept was applied to mathematically describe maternal-to-fetal and fetal-to-maternal transport of cimetidine. Averaged data from the intervals of 0-10 minute (control) and 20-30 minute (inhibitor) of placental perfusions were used for the following calculations.

Total maternal-to-fetal transplacental clearance (Cl_{Tmf}) of cimetidine normalized to placenta weight was calculated according to equation 1.

$$Cl_{Tmf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot w_p} \qquad (1)$$

where C_{fv} is the concentration of cimetidine in the umbilical vein effluent, Q_f is the umbilical flow rate, C_{ma} is the concentration of cimetidine in the maternal reservoir and w_p is the wet weight of the placenta.

In fetal-to-maternal studies, the ability of the placenta to remove cimetidine from the fetal circulation was expressed as extraction ratio (ER) using equation 2:

$$ER = \frac{(C_{fa} - C_{fv})}{C_{fa}} \tag{2}$$

where C_{fa} is the concentration of cimetidine in the fetal reservoir entering the perfused placenta via the umbilical artery. Total fetal-to-maternal clearance normalized to placenta weight (Cl_{Tfm}) was calculated according to equation 3.

$$Cl_{Tfm} = \frac{ER.Q_f}{w_p} \tag{3}$$

To distinguish between passive and active components of the transplacental movement, the following concept was applied (Fig. 1). Assuming the total transplacental passage of cimetidine being a function of passive diffusion and efflux activity of BCRP, the total transplacental clearance in maternal-to-fetal (Cl_{Tmf}) and fetal-to-maternal (Cl_{Tfm}) direction is described by equations 4 and 5, respectively:

$$Cl_{Tmf} = Cl_{pd} - Cl_{efflux} \tag{4}$$

$$Cl_{Tfm} = Cl_{pd} + Cl_{efflux} \tag{5}$$

where Cl_{pd} is clearance of passive diffusion and Cl_{efflux} expresses the efflux activity of the transporter. Since Cl_{efflux} is a capacity-limited (non-linear) process, it can be expressed in terms of Michaelis-Menten kinetics:

$$Cl_{efflux} = \frac{V_{max}}{K_m + C_{ma(fa)}} \tag{6}$$

where V_{max} is the maximal velocity of the transport, K_m is the concentration at which half the maximal velocity is reached and $C_{ma(fa)}$ is substrate concentration in maternal (C_{ma}) or fetal (C_{fa}) circulation.

In maternal-to-fetal studies, adding Cl_{efflux} into (4) yields the following equation which was used to fit clearance *vs* inflow concentration data:

$$Cl_{Tmf} = Cl_{pd} - \frac{V_{max}}{K_m + C_{ma}} \tag{7}$$

In analogy, when the effect of fetal inflow concentrations on fetal-to-maternal clearance was investigated, data were fitted by the following equation:

$$Cl_{Tfm} = Cl_{pd} + \frac{V_{max}}{K_m + C_{fa}} \tag{8}$$

Radioactivity remaining in the placental tissue after perfusion was less than 0.4 ± 0.06 % of the infused dose in both maternal-to-fetal and fetal-to-maternal studies, regardless of total cimetidine concentration. Therefore, it was ignored in pharmacokinetic modeling. Data were fitted using reciprocal weighting and the numerical module of SAAM II (SAAM Institute, Seattle, WA, USA).

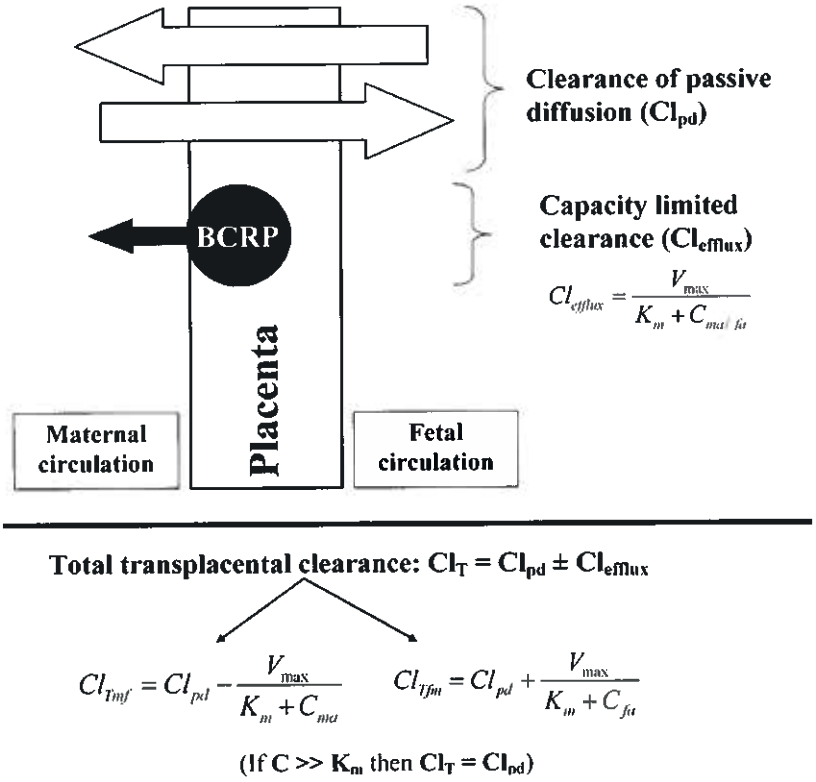

Figure 1. Schematic depiction of pharmacokinetic analysis applied in this study to evaluate efflux transporter activity in the placenta. This model assumes two processes being involved in the transplacental passage: (i) passive diffusion governed by Fick's law (depending mainly on drug's physical-chemical properties, concentration gradient, protein binding and membrane area and thickness) here described as *Clearance of passive diffusion (Cl_{pd})* and (ii) saturable efflux process governed by the rules of Michaelis-Menten non-linear kinetics here described as *Capacity limited clearance (Cl_{efflux})*. Depending on the direction of substrate movement, these two events add up (in fetal-to-maternal direction) or subtract (in maternal-to-fetal direction) to obtain the value of total transplacental clearance (Cl_T). If substrate concentration largely exceeds the Michaelis-Menten constant ($C>>K_m$), then total placental clearance equals to clearance of passive diffusion. Cl_{Tmf} is total transplacental clearance in maternal-to-fetal direction; Cl_{Tfm} is total transplacental clearance in fetal-to-maternal direction.

Statistical analysis

For each group of placental perfusion experiments, the number of animals was $n \geq 4$. Cellular uptake and transport studies are based on $n = 4$. Student's *t*-test or ANOVA analyses were used where appropriate to assess statistical significance. Differences of $p<0.05$ were considered statistically significant.

RESULTS

Expression of Bcrp and P-gp in rat placenta and HRP-1 cells

Using RT-PCR and Western blot analyses, we investigated the expression of Bcrp and P-gp in the rat placenta and HRP-1 cell line and compared it with kidney as a positive control (Tanaka et al., 2005). The PCR products were separated on 2% agarose gel in the presence of ethidium bromide and compared with low molecular weight DNA ladder (25-766bp). The bands corresponding to 329, 331 and 188 bp for *mdr1a*, *mdr1b* and *Bcrp*, respectively, were visualized under the ultraviolet light (Fig. 2A). Expression of *Bcrp* was clearly detected in rat kidney, placenta and HRP-1 cell line samples. In contrast, expression of both *mdr1a* and *mdr1b* (coding for P-gp) was detected only in rat kidney and placenta but not in HRP-1 cell line. Similarly, application of polyclonal anti-Abcg2 antibody M-70 revealed significant levels of Bcrp in the rat placenta, kidneys and HRP-1 cells. Using C219 monoclonal antibody we confirmed the expression of P-gp in the rat placenta and kidneys only while no signal for P-gp was detected in HRP-1 cell lysate (Fig. 2B). This pattern of Bcrp/P-gp expression in rat placental HRP-1 cell line is very similar to what has recently been detected in human choriocarcinoma cell line BeWo (Atkinson et al., 2003; Ceckova et al., 2006; Evseenko et al., 2005).

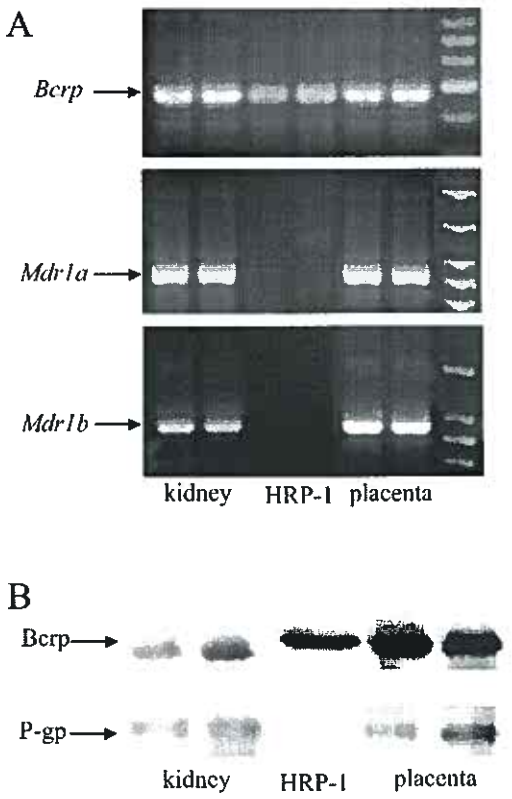

Figure 2. A - mRNA expression of the rat breast cancer resistance protein (Bcrp) and P-glycoprotein (Mdr1a and Mdr1b) in rat kidney, placenta and HRP-1 rat placental cell line. B - Western blot immunoanalysis of protein expression of the rat breast cancer resistance protein (Bcrp) and P-glycoprotein (P-gp) in the rat placenta and kidney and in the HRP-1 rat trophoblast cell line. Kidneys and placentas were randomly sampled from 5 female rats and independently processed as described in Experimental Procedures; two representative samples are shown for each tissue.

Immunohistochemical localization of Bcrp in the rat term placenta

Localization of Bcrp expression in the rat term placenta was investigated by immunohistochemistry at the light microscopy level. Four different rat placentas (gestation day 21) were used for the experiments. The rat placenta is composed of two morphologically different zones, the junctional zone and the labyrinth zone. Bcrp was detected in the inner layers of the syncytiotrophoblast (layer II and III) of the labyrinth zone only (Fig. 3). No Bcrp staining was visible in either layer I or the fetal capillaries. This expression pattern of Bcrp in the rat term placenta closely resembles that of P-gp described in our earlier studies (Novotna et al., 2004; Pavek et al., 2003). Our findings correspond well with the general perception of the labyrinth being the area of materno-fetal exchange.

Fig. 3

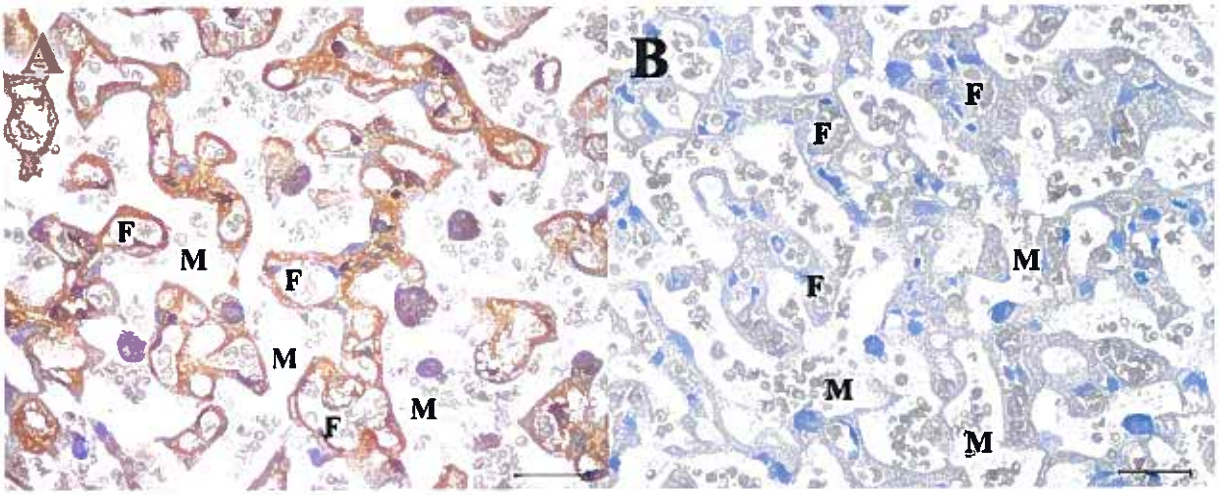

Figure 3. Immunohistochemical localization of Bcrp in the labyrinth of rat term placenta.
Immunohistochemical staining was performed with the monoclonal antibody M-70 (1:10 dilution). (A) Strong reactivity for Bcrp is visible in the inner layers of syncytiotrophoblast (brown color). The fetal capillaries of the syncytiotrophoblast do not reveal any positivity. For negative control (B) the slides were treated in the same manner, except non-immune isotype-matched immunoglobulins were substituted for the primary antibody to Bcrp. F – fetal capillaries, M – maternal blood (hematoxylin counterstained; scale bar, 50 μm; magnification 400x)

Bcrp efflux activity in HRP-1 cell line

To investigate Bcrp and/or P-gp activity in HRP-1 placental cells, BODIPY FL prazosin (500nM) as a common substrate of BCRP and P-gp was added to the cells and the effect of Bcrp and/or P-gp inhibitors was observed. In agreement with gene expression data, only BCRP specific inhibitor, Ko143 (1 μM), and dual BCRP and P-gp inhibitor, GF120918 (1 μM), increased BODIPY FL prazosin accumulation by more than 100% ($p<0.01$). In contrary, P-gp specific inhibitors, PSC833 (1 μM) and verapamil (25 μM) did not affect BODIPY FL prazosin accumulation suggesting undetectable activity of P-gp in the HRP-1 cell line (Fig. 4).

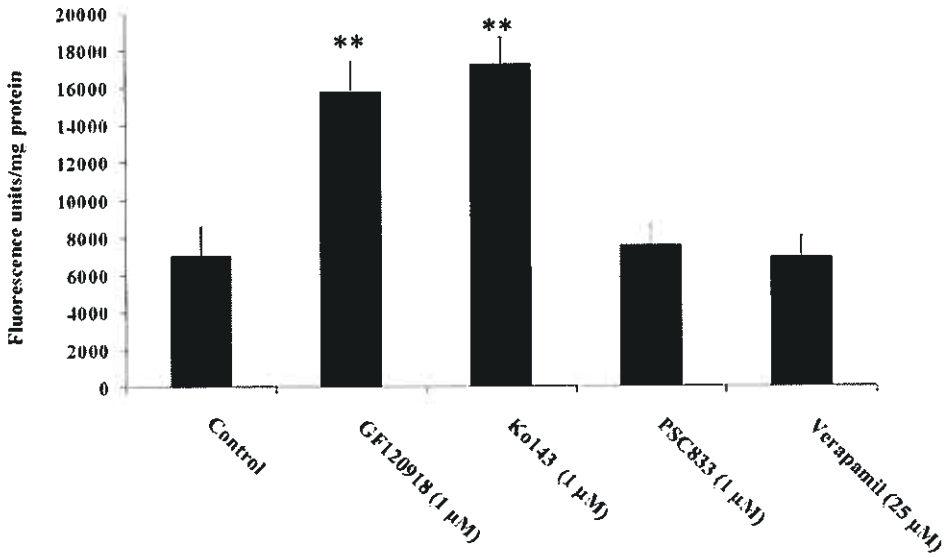

Figure 4. Uptake of common BCRP and P-gp substrate, BODIPY FL prazosin, by rat placental HRP-1 cells. BODIPY FL prazosin was added to cells without inhibitor (control) or in the presence of BCRP or P-gp inhibitors. Both GF120918 and Ko143 (BCRP inhibitors) significantly increased accumulation of BODIPY FL prazosin in the cells. P-gp selective inhibitors (PSC 833 and verapamil) had no effect on BODIPY FL prazosin accumulation. Data are presented as means ± SD of four experiments. ** $p<0.01$ compared with control.

Consistency of perfusion experiments

To determine a steady-state period suitable for inhibitor studies in both maternal-to-fetal and fetal-to-maternal experiments, placenta was first perfused with 0.1 μM cimetidine for 50 minutes. If no inhibitor was added, we observed steady clearances for the whole period. When a BCRP inhibitor was added to the maternal perfusate in the 10^{th} minute of perfusion, it took about 5-10 minutes to achieve a new steady-state (data not shown). Therefore, to evaluate the effect of inhibitor on cimetidine transplacental passage, samples from 0-10 min interval of perfusion were averaged and compared to those collected in 20-30 min interval.

Effect of BCRP and P-gp inhibitors on transplacental passage of cimetidine

To test interactions of cimetidine with placental Bcrp or P-gp, dual and/or specific inhibitors of these transporters were added to maternal reservoir. Addition of BCRP inhibitors, GF120918 (2 μM) or fumitremorgin C (2 μM), caused significant change in transplacental clearance of cimetidine (Fig. 5). Interaction of cimetidine with human P-gp has recently been ruled out using MDR1-transfected MDCKII and LLC-PK1 cell lines (Pavek et al., 2005). To exclude any confounding effects of rat P-gp in transplacental passage of cimetidine, P-gp inhibitors, cyclosporine (10 μM) or verapamil (25 μM), were tested. Although these compounds increased maternal-to-fetal clearance of rhodamine123, a P-gp substrate, in our previous study (Pavek et al., 2003), they did not interfere with transport of cimetidine. Therefore, transplacental passage of cimetidine does not seem to be affected by P-gp and cimetidine can be used as a marker compound to functionally analyze efflux activity of Bcrp in the rat placenta. Inhibitory effect observed after addition of GF120918 may be ascribed to Bcrp blockade only, although we cannot exclude possible contribution of other, yet unidentified GF-sensitive cimetidine transporter.

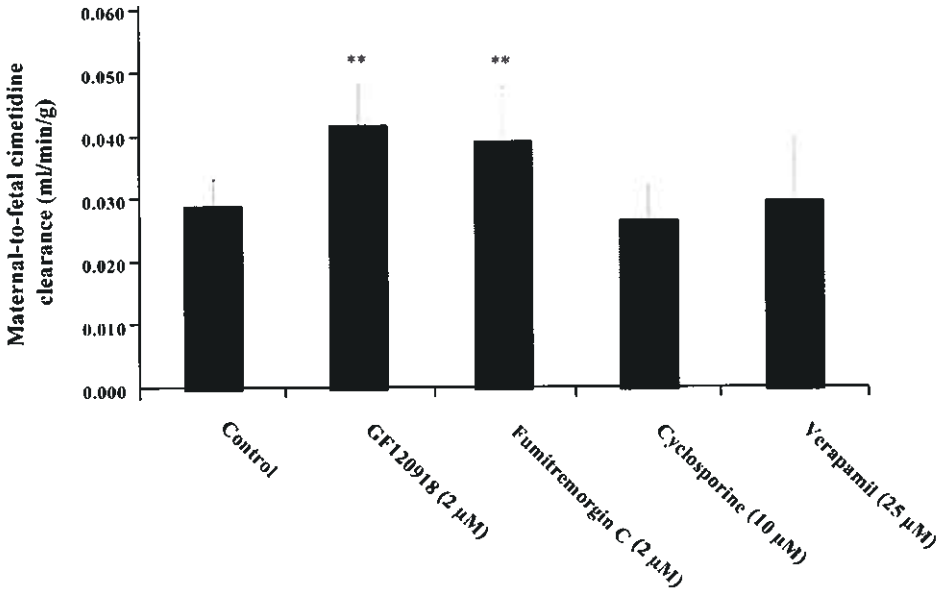

Figure 5. Effect of Bcrp and P-gp inhibitors on maternal-to-fetal clearance of cimetidine. Cimetidine and [^{3}H]cimetidine tracer were added to the maternal compartment in a concentration of 0.1 μM, its radioactivity was measured in fetal venous outflow, and total transplacental clearance was calculated by eq. 1. Inhibitor was added to the maternal perfusate in the 10th min. Only BCRP inhibitors, GF120918 and fumitremorgin C, affected transplacental clearance of cimetidine while P-gp inhibitors, cyclosporine and verapamil, had no significant effect. Data are presented as means ± SD of at least four experiments. ** $p<0.05$ compared with control.

Effect of inflow cimetidine concentrations on transplacental clearance

Cimetidine was infused to maternal or fetal side of the placenta in concentrations of 0.005, 0.1, 1, 30, 100, and 1000 μM. In both maternal-to-fetal and fetal-to-maternal transport studies, increase in cimetidine concentration caused significant change in transplacental clearance, confirming non-linearity of the process and involvement of a capacity-limited mechanism (Figs. 6 and 7). Furthermore, addition of a BCRP inhibitor significantly affected clearances at lower cimetidine concentrations while at concentrations above 30 μM, inhibitor was rather ineffective.

Fitting experimental data with equations 7 and 8 provided pharmacokinetic parameters describing passive and Bcrp-mediated transplacental passage of cimetidine (Table 2). It is evident, that passive movement across the placenta (described by Cl_{pd}) is independent of direction and of inhibitor used. On the other hand, capacity limited clearance (Cl_{efflux}) is a concentration- and inhibitor-dependent parameter. At substrate concentrations largely exceeding the Michaelis-Menten constant ($C>>K_m$) the transporter is saturated, the non-linear fraction of equations 7 and 8 approaches zero and both equations are reduced to linear processes only ($Cl_T = Cl_{pd}$); under these conditions, transplacental pharmacokinetics is beyond any quantifiable effect of efflux transporter and is governed exclusively by passive diffusion. This has been experimentally achieved in both maternal-to-fetal and fetal-to-maternal studies when cimetidine concentration was increased to 1 mM. Furthermore, since addition of inhibitor caused no change in transplacental clearance of 1 mM cimetidine (see Figs. 6 and 7), it is reasonable to assume that at high substrate concentrations, drug-drug interactions will have no effect on penetration of BCRP substrates through placenta.

Comparing maternal-to-fetal and fetal-to-maternal clearances revealed great asymmetry in favor of fetal-to-maternal direction. This was most evident at low cimetidine concentrations (0.005 μM), where fetal-to-maternal clearance was almost 25 times higher than that in the opposite direction (Fig. 8). At a concentration of 1 mM, however, both maternal-to-fetal and fetal-to-maternal clearances of cimetidine reached identical values of 0.042 ml/min/g confirming saturation of Bcrp and limited role of its efflux activity. This experimental value corresponds well with the calculated clearance of passive diffusion (Cl_{pd} ~ 0.041 - 0.043 ml/min/g), see Table 2.

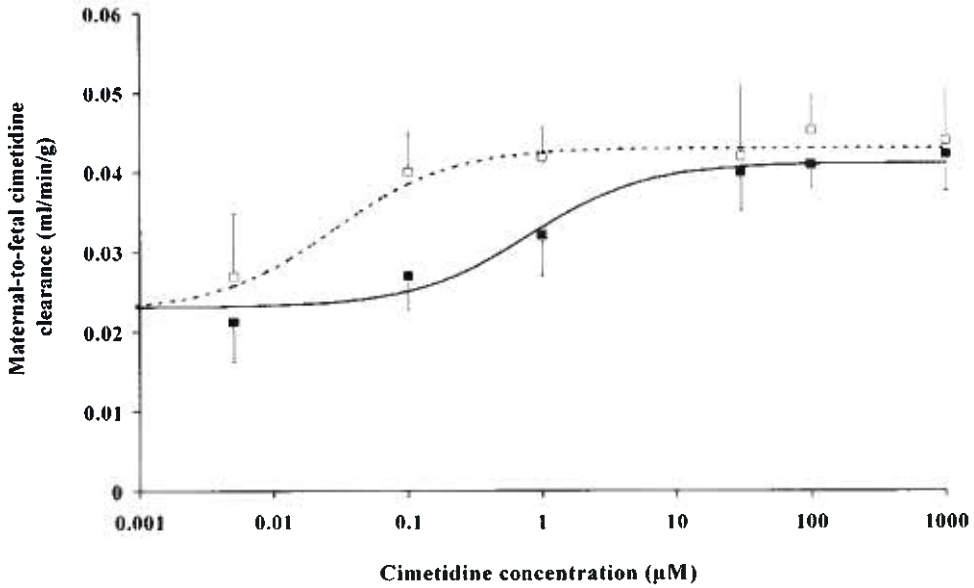

Figure 6. Transport of cimetidine across the dually perfused rat placenta in maternal-to-fetal direction. Cimetidine with [^{3}H]cimetidine tracer were added to the maternal reservoir, its radioactivity was measured in fetal venous outflow, and total transplacental clearance was calculated by eq. 1. Changes of clearance with increasing cimetidine concentration confirm non-linearity of the event and involvement of a saturable mechanism. Inhibitor (2 μM GF120918) was added to block Bcrp activity. At the highest cimetidine concentration tested (1 mM), clearance reached the value of 0.042 ml/min/g and inhibitor activity was negligible. Experimental values are presented as means ± SD of at least four experiments; the lines represent the best fit of these data to eq. 7. Note the sigmoid shape of the lines with the lower plateau delineating combined effect of passive clearance and efflux activity of Bcrp and the upper plateau representing clearance of passive diffusion alone. ■ – cimetidine without inhibitor, □ – cimetidine with GF120918

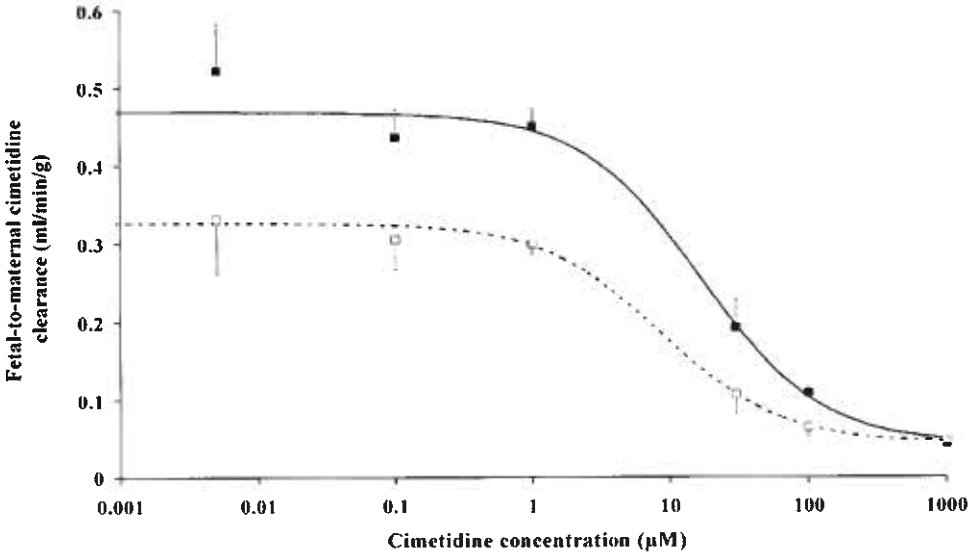

Figure 7. Transport of cimetidine across the dually perfused rat placenta in fetal-to-maternal direction. Cimetidine with [^{3}H]cimetidine tracer were added to the fetal reservoir, its radioactivity was measured in fetal venous outflow, and total transplacental clearance was calculated by eq. 3. Changes of clearance with increasing cimetidine concentration confirm non-linearity of the event and involvement of a saturable mechanism. Inhibitor (2 μM GF120918) was added to block Bcrp activity. As in maternal-to-fetal transport, at the highest cimetidine concentration tested (1 mM), clearances reached the value of 0.042 ml/min/g and inhibitor activity was negligible. Experimental values are presented as means ± SD of at least four experiments; the lines represent the best fit of these data to eq. 8. Note the sigmoid shape of the lines with the upper plateau delineating combined effect of passive clearance and efflux activity of Bcrp and the lower plateau representing clearance of passive diffusion alone. ■ – cimetidine without inhibitor, □ – cimetidine with GF120918

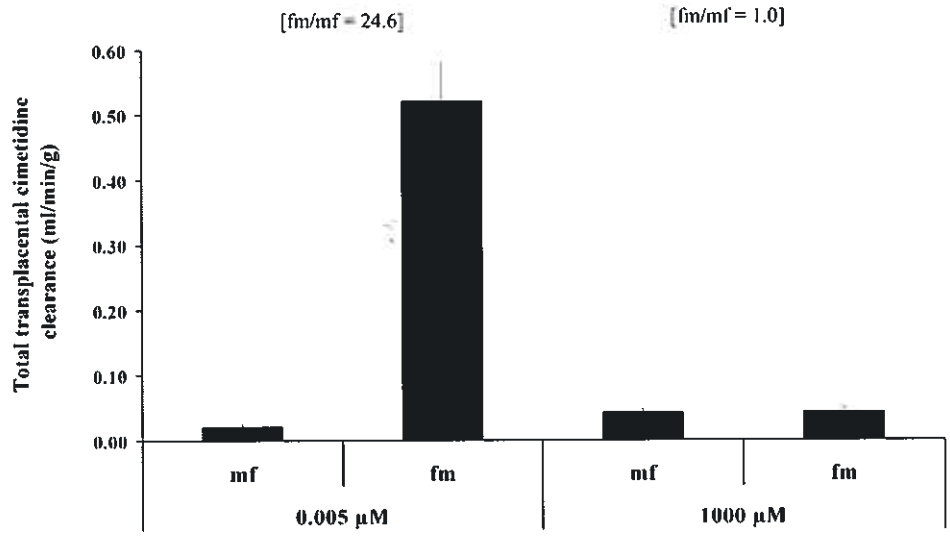

Figure 8. Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions. Cimetidine with [^{3}H]cimetidine tracer were added to the maternal or fetal compartment, its radioactivity was measured in fetal venous outflow, and total transplacental clearances were calculated by eq. 1 or 3, respectively. At low substrate concentrations (0.005 μM), Bcrp efflux activity caused almost 25-times higher clearance in fetal-to-maternal direction. At high substrate concentrations (1000 μM), however, this ratio equalized. Numbers in parenthesis show ratio of fm to mf clearance.

	Maternal-to-fetal transport		Fetal-to-maternal transport	
	control	inhibitor	control	inhibitor
Cl_{pd} (ml/min/g)	0.041	0.043	0.042	0.042
V_{max} (nmol/min/g)	0.014	0.00057	7.14	2.47
K_m (μM)	0.80	0.028	16.7	8.71

Table 2. Pharmacokinetic parameters of transplacental passage of cimetidine. Pharmacokinetic parameters were obtained by fitting experimental data with equations 7 and 8. Cl_{pd} – transplacental clearance occurring by passive diffusion; V_{max} – maximal velocity of Bcrp mediated transport; K_m - concentration of cimetidine at which half the maximal velocity is reached. GF120918 (2 μM) added to the maternal compartment was used as an inhibitor.

Bcrp transports cimetidine from fetus to mother against concentration gradient

To investigate the capability of Bcrp to remove its substrate from fetal circulation, both maternal and fetal sides of the placenta were infused with equal concentrations of cimetidine and after 10 minute stabilization period, the fetal perfusion liquid (10 ml) was recirculated for 60 min. Samples (200 μl) of fetal perfusate were collected every 10 minutes from the fetal reservoir and [^{3}H]cimetidine concentration was detected. This experimental setup assures steady concentration on the maternal side of the placenta and enables investigations of maternal/fetal concentration ratio. At a low drug concentration (5 nM), cimetidine in the fetal circulation steadily decreased and stabilized after about 40 min of perfusion. Fetal to maternal concentration ratio reached a value of 0.49 towards the end of the experiment. Decrease in fetal cimetidine was blocked by co-infused inhibitors (GF120918 or fumitremorgin C), see Fig. 9A. at a high cimetidine concentration (1 mM), maternal and fetal concentrations remained unchanged throughout the perfusion period with fetal/maternal concentration ratio close to 1 (Fig. 9B). These findings demonstrate the potency of placental Bcrp to remove, in a capacity-limited manner, its substrate from fetal compartment and to establish a concentration gradient between maternal and fetal circulations.

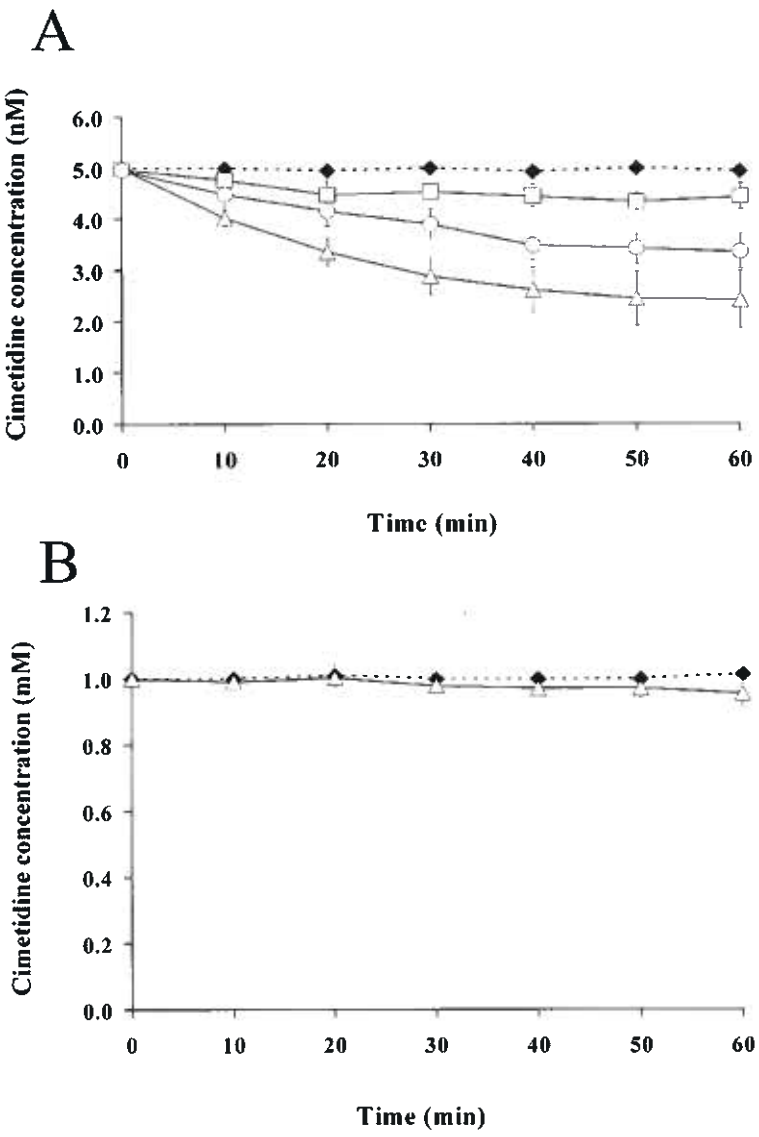

Figure 9. Elimination of cimetidine from the fetal circulation by placental Bcrp. Cimetidine and [^{3}H]cimetidine tracer were added to both maternal (closed symbol) and fetal (open symbols) circulations at equal concentrations. Fetal perfusate (10 ml) was recirculated and sampled for 60 minutes. At low cimetidine concentrations of 5 nM (A), fetal cimetidine decreased from 5 nM down to 2.4 nM and stabilized after 40 min of perfusion. This decrease was inhibited by both BCRP inhibitors, GF120918 (2 μM) and fumitremorgin C (2 μM). At high cimetidine concentrations of 1 mM (B) no decrease in fetal compartment was observed suggesting saturation of the transporter. Data are presented as means ± SD of three experiments.

◆ – maternal cimetidine concentration
□ - fetal cimetidine concentration with GF120918
○ - fetal cimetidine concentration with fumitremorgin C
△ – fetal cimetidine concentration without inhibitor

DISCUSSION

Detailed knowledge of transplacental kinetics of drugs is essential with respect to fetal safety, fetal medication and drug-drug interactions during pregnancy. Apart from physical-chemical properties, placental passage of many drugs is controlled by interactions with biotransformation enzymes and/or efflux transporters. It is widely believed that enzymes and drug efflux transporters form an active component of the placental barrier that helps protect fetus against maternal toxins (Marin et al., 2004). In addition, it seems plausible, that these proteins may, to at least some extent, actively metabolize/transport compounds already present in the fetal circulation. Using rat placental perfusion, we have previously demonstrated that 11β-hydroxysteroid dehydrogenase type 2 metabolizes both maternal and fetal corticosterone with a comparable potency (Staud et al., 2006). Similarly, P-gp has been confirmed to favor fetal-to-maternal transport of its substrates in perfused rat (Pavek et al., 2003) or human placenta (Molsa et al., 2005; Sudhakaran et al., 2005). In the present study, we focused on functional analysis of the latest of ABC drug efflux transporters, Bcrp, using dually perfused rat term placenta and rat placenta-derived cell line, HRP-1.

To date, only few studies have reported on BCRP activity in placenta and these are mainly based on *in vitro* models. Very recently, Kolwankar et al (Kolwankar et al., 2005) employed placental microvillous membrane vesicles to confirm BCRP function in the human placenta. Subsequently, we have described BCRP expression, localization and function in an *in vitro* placental model, BeWo cell line (Ceckova et al., 2006). Here we used rat placental HRP-1 cell line derived from placental labyrinth region at midgestation (Soares et al., 1987). This cell line has previously been employed to study several aspects of placental physiology (Morris Buus and Boockfor, 2004; Shi et al., 1997; Soares et al., 1989), metabolism (Xu et al., 2005) or nutrient transport (Novak et al., 2001; Zhou et al., 2003). To our knowledge, however, no studies have been performed so far to investigate expression and/or activity of ABC drug efflux transporters in this *in vitro* model. Bcrp expression was revealed at both mRNA and protein levels. Surprisingly, we did not detect any expression of *mdr1a* or *mdr1b* genes coding for rat P-gp, neither did we find any signal by Western blotting. Consistent with gene and protein expression results, uptake studies revealed only Bcrp activity, while P-gp did not affect cell accumulation of BODIPY FL prazosin. These data are similar to what has previously been observed in human choriocarcinoma cell line BeWo (Atkinson et al., 2003; Ceckova et al., 2006; Evseenko et al., 2005) where only BCRP was found to be functionally expressed, while P-gp activity was negligible. Lack of expression and function of P-gp in the HRP-1 cell line makes this model inappropriate to investigate the transplacental transport of P-gp substrates. On the other hand, it may well serve as a tool to study Bcrp role in transplacental pharmacokinetics as its efflux activity will not interfere with that of P-gp.

The only functional *in vivo* studies on BCRP activity in the placenta so far have been performed by Jonker et al in transgenic mice (Jonker et al., 2002; Jonker et al., 2000). Using GF120918 as a BCRP inhibitor in P-gp-deficient mice, these authors revealed twofold higher transport of topotecan to fetuses, suggesting Bcrp as a maternal-fetal barrier to passage of xenobiotics across the placenta (Jonker et al., 2000). In a subsequent study in *Bcrp1*$^{-/-}$ mice, two-fold higher ratio of fetal topotecan concentration to maternal plasma concentration was observed compared with *Bcrp1*$^{+/+}$ fetuses (Jonker et al., 2002).

However, comprehensive evaluation of BCRP role in transplacental pharmacokinetics is still lacking.

In the present study, cimetidine was infused to maternal or fetal side of dually perfused rat placenta in various concentrations to evaluate the role of Bcrp in maternal-to-fetal and fetal-to-maternal transport. Cimetidine has been chosen as a model substrate for its convenient properties: it is a BCRP substrate which is not recognized by human P-gp (Pavek et al., 2005), it weakly binds to plasma proteins, and its biotransformation by placental enzymes is negligible (Schenker et al., 1987). In addition, cimetidine passive diffusion through biological membranes is delayed by its physical-chemical properties as shown in transepithelial passage (Pavek et al., 2005) or placental transport (Ching et al., 1987; Schenker et al., 1987); this seems to be an important feature to study substrate interactions with an efflux transporter (Eytan et al., 1996; Lentz et al., 2000).

At low cimetidine concentrations (0.005 μM), fetal-to-maternal clearance was 25-fold higher than clearance in the opposite direction. These findings confirm that Bcrp causes asymmetry in transplacental clearances by returning substrates coming from maternal side and facilitating transport of drugs from fetus to mother. Comparing Michaelis-Menten constants, we found K_m for fetal-to-maternal direction 20 times higher than that for maternal-to-fetal direction. We assume this difference is caused by polarized localization of Bcrp on the maternal side of the placenta (Litman et al., 2002); as a result, a compound administered to the fetal circulation needs to pass through fetal tissues to reach the transporter. This suggests that much higher cimetidine concentrations are needed to saturate Bcrp transporter during fetal-to-maternal passage than in the opposite direction.

The localization of Bcrp on the apical, maternal-facing membrane of the rat placenta observed in this study is identical with that of P-gp (Novotna et al., 2004; Pavek et al., 2003) and suggests that the transporter is important in preventing entry of potential toxins into the fetal compartment. This assumption has been functionally validated in this paper using perfused placenta. Furthermore, we bring evidence that, despite its maternal-facing localization, Bcrp can also actively remove drugs already present in the fetal compartment and establish a pronounced maternal/fetal concentration gradient. To investigate this potential, both maternal and fetal sides of the placenta were perfused with equal concentrations of cimetidine and fetal perfusate was re-circulated. Under this setup, we observed significant difference between maternal and fetal cimetidine concentrations as soon as 10 minutes after initiation of the experiment, confirming that Bcrp can actively remove its substrate from the fetal compartment. Since decrease in cimetidine concentration continued even at later intervals (dropping by more than 50 % within 60 minutes of perfusion), it is evident that Bcrp can transport this compound from fetus even against concentration gradient.

Interestingly, several studies on cimetidine placental transfer were published two decades ago with intriguing results. When investigated in the dually perfused human placenta, two papers concluded that transport of cimetidine occurred by passive diffusion and was rather slow (Ching et al., 1987; Schenker et al., 1987). Moreover, when studied in sheep, large cimetidine gradient between mother and fetus was observed (Mihaly et al., 1983). In a follow-up study, the authors concluded that an active transporter from the fetal to the maternal circulation is responsible for this discrepancy (Ching et al., 1985). Since all the above mentioned studies were performed in the "pre-efflux-transporter era", possible role of a drug efflux transporter in the transplacental pharmacokinetics of cimetidine could not

have been presumed. Nevertheless, our present findings suggest that BCRP is the transporter responsible for limited maternal-to-fetal passage and large maternal/fetal concentration ratio of the compound.

Regarding BCRP expression in human tissues, relatively high mRNA levels were observed in placenta, liver and small intestine with lower expression in the kidney, heart and brain (Doyle et al., 1998). In rodents, on the other hand, different mRNA distribution pattern was indicated by Tanaka et al (Tanaka et al., 2005) who found high expression levels in kidney, small and large intestine while lower levels were found in other tissues including brain and placenta. Based on these observations, the authors suggested limited importance of placental Bcrp in rodents (Tanaka et al., 2005). In contrast, functional role of placental Bcrp has been proposed in mice by Jonker et al (Jonker et al., 2002; Jonker et al., 2000) and thoroughly assessed in the rat placenta in our study. Therefore, mRNA expression levels do not have to necessarily correlate with transport potency of the protein since there is a number of other factors that will determine its functional activity, such as posttranscriptional/ posttranslational modifications in protein expression as well as strategic localization of BCRP along the maternal interface.

In conclusion, we provide evidence for functional expression of Bcrp in the rat placenta. Based on our findings, we propose a two-level defensive role of placental BCRP: the transporter (i) reduces passage of its substrates from mother to fetus but also (ii) removes the drug already present in the fetal circulation even against concentration gradient. Given the broad range of BCRP substrates, including exogenous and endogenous compounds, BCRP seems to be an important component of the placental barrier playing a significant role in protection and detoxication of the fetus.

ACKNOWLEDGMENTS

The authors wish to thank Anezka Kunova and Dana Souckova for their skilful assistance in perfusion experiments.

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FOOTNOTES

This work was supported by grants 151/2004/C/FaF and 104/2005/C from Grant Agency of Charles University, Czech Republic.

VI.

MOLECULAR DETERMINANTS IN THE TRANSPORT OF BILE ACID DERIVED DIAGNOSTIC AGENT IN TUMORAL AND NON-TUMORAL CELL LINES OF HUMAN LIVER

Libra A., *Fernetti C., Lorusso V., Visigalli M., Anelli P.L., Staud F., Tiribelli C., Pascolo L. (2006) Molecular determinants in the transport of bile acid derived diagnostic agent in tumoral and non-tumoral cell lines of human liver, Journal of Pharmacology and Experimental Therapeutics, submitted*

MOLECULAR DETERMINANTS IN THE TRANSPORT OF A BILE ACID DERIVED DIAGNOSTIC AGENT IN TUMORAL AND NON-TUMORAL CELL LINES OF HUMAN LIVER

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Footnotes:
This work was supported by grants from MIUR (FIST 2002/359Ric to C.T.); an Erasmus fellowship (to A.L.); Fondazione Cassa di Risparmio di Trieste (FCRT-01); Fondo Studio Fegato- ONLUS; and career development grants from Bracco Imaging (to C.F. and L.P.).
Part of this work was presented at The FEBS Congress and IUBMB Conference 2005, Budapest, Hungary, and appeared in abstract form (FEBS Journal 272, 2005, Suppl 1: C3-045P).
There is no conflict of interest.

Abbreviations:
CE-MRI: Contrast-enhanced Magnetic Resonance Imaging; OATP: Organic Anion Transporting Polypeptide; OAT: Organic Anion Transporter; OCT: Organic Cation Transporter; PEPT: Peptide Protein Transporter; NTCP: Sodium dependent Taurocholate co-transporting Polipeptide; CCK-8: cholecystokinin octapeptide; BSP: bromosulphophthalein; hLST: human Liver Specific Transporter.

Running title: Hepatic transport of B22956 contrast agent

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ABSTRACT

Contrast-enhanced Magnetic Resonance Imaging (CE-MRI) is a valuable technique for the diagnosis of liver diseases. Gadocoletic acid trisodium salt (B22956/1) is a new contrast agent showing high biliary excretion and thus potentially advantageous in hepatobiliary imaging. The aim of the study was to investigate the molecular mechanisms of hepatic transport of B22956 in a cellular model of hepatic tumor. B22956 ion uptake was measured in tumoral (HepG2) and non-tumoral (Chang liver) hepatic cell lines. Absolute quantitative real-time RT-PCR analyses, using cloned PCR products as standards, were performed on total RNA of HepG2, Chang liver cells and normal liver to evaluate the transcription of twelve transport genes: *OATP-A*, *OATP-B*, *OATP-C*, *OATP-D*, *OATP-E*, *OATP-8*, *OAT2*, *OAT3*, *OCT1*, *NTCP*, *PEPT-1*, *PEPT-2*. B22956 transport was more efficient in Chang liver than in HepG2 cells. and was inhibited by CCK-8, a specific substrate of OATP-8. Real-time RT-PCR analyses revealed different transcription profiles in the tumoral and non-tumoral cell lines. Compared to normal liver, the expression of *OATP-C*, *OATP-D* and *OATP-8* was greatly repressed in HepG2 cells, while *OATP-B* and *OATs* expression was either maintained or increased. On the contrary, in Chang liver cells, *OAT* genes were undetectable, while the expression of *OATP-D*, *OATP-E* and *OATP-8* was similar to normal liver. Transport studies and gene expression analyses indicated that B22956 ion is a good substrate to the liver specific OATP-8, reported to be poorly expressed or absent in human liver tumors. Therefore, B22956 ion may be helpful in detecting hepatic neoplastic lesions by CE-MRI.

INTRODUCTION

Magnetic Resonance Imaging (MRI) produces high-resolution three-dimensional maps delineating morphological features of the tissues analyzed. Differential contrast in soft tissues depends on endogenous differences in water content, relaxation time and/or diffusion characteristics of the specimen of interest.

The specificity of MRI can be further increased by using exogenous contrast agents such as the Gadolinium chelates (Artemov, 2003). The development of molecular targeted MRI contrast agents directed to specific tissue entities could dramatically expand the range of MRI applications (Lorusso *et al.*, 2005). Due to the intrinsically low sensitivity of MRI, high local concentrations of the contrast agents at the target site are required to evoke effective MR contrast, and this means that the contrast agent should recognize targeted cells with high affinity and sensitivity (Weinmann *et al.*, 2003). Several compounds have been studied as potential paramagnetic contrast agents for MRI in liver and only few of them are in the late-phase clinical trials or have received marketing authorization. These agents include water soluble paramagnetic chelates with specific uptake in the hepatocytes, such as Teslascan® (Mangafodipir trisodium, MnDPDP, GE Healthcare, Chalfont St. Giles, UK), MultiHance® (Gadobenate dimeglumine, Gd-BOPTA, Bracco Imaging SpA, Milan, Italy), and Primovist® (Gadoxetic acid disodium, Gd-EOB-DTPA, Schering AG, Germany) (Weinmann *et al.*, 2003). These three agents are characterized by a rather high

hepatic uptake followed by rapid biliary excretion, although the primary route of elimination of these compounds is renal.

Recently, Gadocoletic acid trisodium salt or B22956/1 (Bracco Imaging SpA, Milan, Italy), a new gadolinium based MR contrast agent, has been formulated to be used as a blood pool agent (Cavagna *et al.*, 2002). The compound is a derivative of gadopentetate to which a bile acid-like lateral chain has been linked with the intent to increase the binding to the plasma proteins and thus assuring a long permanence in the circulation. Pharmacological studies in animal models and in healthy human volunteers have shown that, differently from Gd-BOPTA and Gd-EOB-DTPA, the biliary excretion is the primary route of elimination of the contrastographically active moiety B22956 ion (La Noce *et al.*, 2002).

Recent studies also indicated that the ATP dependent transporter MRP2 (ABCC2), localized on the canalicular membrane domain of hepatocytes, is responsible for the presence of B22956 ion in bile, while the ubiquitarious basolateral transporter MRP1 (ABCC1) could be involved in contrasting urinary elimination (Lorusso *et al.*, 2002).

Until now no data have been produced on the mechanism for the basolateral hepatic uptake of this compound. Nevertheless its bile acid-like molecular structure leads to hypothesize that B22956 ion is a potential substrate for the basolateral organic anion transporting polypeptides (OATPs) of the liver (Meier and Stieger, 2002), and that these proteins are responsible for a specific hepatic clearance.

Organic anion transporting polypeptides (OATPs) are a family of transport proteins, which are expressed in multiple tissues and involved in the intake or elimination from the body of a wide range of structurally unrelated compounds. Several members of this family have been largely studied for their role in the hepatic clearance of albumin-bound substrates, ranging from endogenous bile acids and estrogens to many exogenous amphipathic drugs (Hagenbuch and Meier, 2003). It has been demonstrated that Gd-EOB-DTPA is substrate to the rat Oatp1, but not the human OATP-A (van Montfoort *et al.*, 1999), while contrasting evidences have been presented for Gd-BOPTA (Planchamp *et al.*, 2005; Planchamp *et al.*, 2004; Pastor *et al.*, 2003; Pascolo *et al.*, 1999). The latter Gd-complex is not a substrate for human OATP-A and linear transport kinetics have been proposed in rat liver plasma membrane vesicles (Pascolo *et al.*, 1999). More recent studies provided indirect evidence on a role of OATP in the hepatic transport of this contrast agent (Pastor *et al.*, 2003; Planchamp *et al.*, 2004; Planchamp *et al.*, 2005).

The most important OATPs of the human liver are OATP-C (also called human Liver Specific Transporter, hLST) and OATP-8, while OATP-A, the first OATP cloned from human liver, seems to be expressed at much lower extent (Jung *et al.*, 2001; Hagenbuch and Meier, 2003). OATP-C has been reported to be down regulated during neoplastic transformation in the liver (Kinoshita and Miyata, 2002), and it was also shown that OATP-C and OATP-8 are not expressed in HepG2 cell line, which is a widely used model of human hepatic carcinoma (Cui *et al.*, 2003).

In the present study we report on the assessment of transport studies for B22956 ion in two human liver derived cell lines: HepG2 used as a model of hepatocellular carcinoma, and Chang liver, used as model of non-tumor liver cells (Seow *et al.*, 2001b; Seow *et al.*, 2001a; Wirth *et al.*, 1995). Absolute quantitative RT-PCR analyses were also performed to evaluate the effective expression of 12 drug transport genes (*OATP-A, OATP-B, OATP-C, OATP-D, OATP-E, OATP-8, OAT2, OAT3, OCT1, PEPT-1, PEPT-2, NTCP*), mainly

organic anion, cation and peptide transporters (van Montfoort *et al.*, 2003; van Montfoort *et al.*, 2001). The gene expression was assessed in the two cell lines and compared to normal liver to understand the transporters potentially involved in the "*in vivo*" targeting of the contrast agent B22956 ion and to confirm the applicability of the cell models.

MATERIAL AND METHODS

Chemicals

B22956/1 is the trisodium salt of a derivative of gadopentetate and its chemical name according to CAS is trisodium [(3β,5β,12α)-3-[[(4S)-4-[bis[2-[bis[(carboxy-κO)methyl]amino-κN]ethyl]amino-κN]-4-(carboxy-κO)-1-oxybutyl]amino]-12-idroxycholan-24-oato(6-)] gadolinate (3-). In aqueous solution B22956/1 dissociates into sodium ions and the contrastographically active component B22956 ion that will be indicated as B22956 throughout the text. B22956 (or Gd-B22950) is the code name for the Gadolinium complex with the ligand B22950.

[^{147} Pm] Cl_3 in HCl was purchased from ICN (Irvine, CA, USA), and used to prepare stock solutions of [^{147} Pm]-B22950 with a specific activity of 89.6 mCi/mmol as previously described (Lorusso *et al.*, 2002). Labeled solutions of Gd-B22950 were obtained by adding the appropriate amount of the solution of [^{147} Pm] complex to the solution of the corresponding Gd-B22950. [^{147} Pm] and Gd complexes were prepared as sodium salts. The purity of each compound was assessed by HPLC and resulted greater than 99%. The [^{147} Pm] labeled compound used for *in vitro* studies will be referred as labeled B22956 throughout the text.

[^{3}H] taurocholate (specific activity 3.47 Ci/mmol) was obtained from NEN Life Science (Boston, MA, USA). CCK-8 has been prepared by peptide synthesis as previously reported (Accardo *et al.*, 2004) and kindly supplied by Department of Chemistry IFM, University of Torino, Torino, Italy. All other chemicals were of the highest purity commercially available.

Uptake in cell cultures from human liver

HepG2 and Chang liver cell lines were obtained from Istituto Zooprofilattico Sperimentale di Brescia (Italy). The two cell line were cultured in DMEM, with 10% (v/v) FBS and 1% antibiotics (10,000 U/mL penicillin and 10 mg/mL streptomycin), under standard conditions. The cells were routinely maintained in 75 cm^2 Falcon flasks for 3 days, then harvested by exposure to a solution of 0.25% trypsin and 0.02% EDTA, and transferred onto 35 mm diameter Petri dishes at a density of 5 x 10^5 cells/cm^2. The day after, the cells were used for the uptake experiments. Media were from Celbio, Milan, Italy and plastic from Falcon, Becton Dickinson, Franklin Lakes, NJ, USA.

All uptake studies were carried out at 37°C. Cells grown on dishes were washed once with 1.5 mL of fresh culture medium and uptake was initiated by the addition of 1.0 mL uptake medium consisting of DMEM with 10 to 200 µM labeled compounds (labeled B22956 or [^{3}H] taurocholate). After incubation at different time intervals (from 0 to 180 minutes), uptake was stopped by removing the uptake medium and then by immediately washing the cells three times with 1.5 mL of ice-cold HANKS' solution (Sigma-Aldrich, Milan, Italy).

Non specific transport (binding) was measured by incubation at 4°C or by short incubations (20 sec) at room temperature.
The cells were then solubilized into 1 mL of 2% SDS/0.2 N NaOH, and 500 µL of the cell lysate were added to 10 mL of scintillation liquid (Filtercount, Packard, Groningen, The Netherlands), and radioactivity counted. Protein content was measured on an aliquot of the cell lysate, using the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich, Milan, Italy) and the overall transport was expressed as pmol of substrate/mg prot.
Uptake studies were also performed by the addition to the transport media of OATP substrates, such as bromosulphophthalein (BSP), taurocholate (all from Sigma-Aldrich, Milan, Italy) and CCK-8.

RNA extraction and reverse transcription

Total RNA from cell cultures was extracted using TRI-Reagent (Sigma-Aldrich, Milan Italy) following manufacturer instructions. The concentration of RNA and its purity was measured spectrofotometrically and its integrity was checked by assessing the sharpness of ribosomal RNA bands on a 1% agarose gel.
The total RNA from normal human liver tissue was purchased from Ambion (Cambridge, UK) and 1µg of RNA was transcribed using iScript kit (Bio-Rad, Hercules, CA, USA) following the instruction of manufacturer. The cDNA samples were stored at -20°C.

Primers and standards for real-time PCR analysis

Primers for real-time RT-PCR were designed using Vector NTI® Suite software (InforMax, Frederick, MD, USA). One of the primers of each gene spans an exon-exon boundary to avoid amplification of genome DNA. The primer sequences were submitted to BLAST search to avoid amplification of other than desired sequences. The sequences, access codes to reference sequences and other specifications of primers are given in Table 1. The specificity of primers was checked by electrophoresis of PCR products on 3% agarose gel. All PCR products migrated as a single band at the expected size. In each run the melting curves of analyzed samples were compared to the melting curves of plasmid standards.
The pCR plasmids (pCR XL-TOPO or pCR 4Blunt-TOPO, Invitrogen, Carlsbad, CA, USA) with inserted PCR products were prepared by using appropriate cloning kits (Invitrogen, Carlsbad, CA, USA) as previously reported (Ceckova, M., A. Libra, et al. (2006). "Expression and functional activity of breast cancer resistance protein (bcrp, abcg2) transporter in the human choriocarcinoma cell line bewo." Clin Exp Pharmacol Physiol 33(1-2): 58-65.) and used as standards for real-time PCR analysis. The cDNA from HepG2 cell line served as the source for PCR amplification of *OATP-B, OATP-E, OAT2, OAT3, OCT1, PEPT-1* and *NTCP* transcripts. The cDNA from Chang liver was used to generate amplicons of *OATP-8, OATP-C, OATP-D* and *PEPT-2* transcripts and PCR products; the OATP-A amplicon was generated from normal human liver cDNA.
Successful cloning was tested by PCR by using specific primers for each insert and following electrophoresis of PCR products on 3% agarose gel. The plasmids were isolated from colonies carrying PCR positive plasmid for each gene using Qiagen Plasmid Midi Kit (Qiagen, Hilden, Germany). Their concentration was determined by UV absorbance A_{260} measurement and the quality assessed from UV absorbance A_{260} : A_{280} ratio. The plasmids were then sequenced on ABI PRISM 310 DNA sequencer (Applied Biosystems, Foster

City, CA, USA) and the sequences of inserts compared to the reference sequences from NCBI database. All of the plasmids contained the inserts of desired sequence.

Official protein and gene symbol	Trivial name	Accession number	Primer sequence 5' → 3' (f - forward, r - reverse)	Localization	Prod. length (bp)	Prod. Tm (°C)
OATP1A2, (*SLCO1A2*)	**OATP-A**	NM_134431[a]	CCATTGGAACGGGAATAAACA (f) TCTCTTCAGATTTCATACACCTCA (r)	2136 - 2351	216	84.0
OATP2B1, (*SLCO2B3*)	**OATP-B**	NM_007256	GGACAACAGCCAGGTTTTCTACAC (f) AGGAAGGGCACCACCAGATG (r)	1851 - 1958	108	88.5
OATP1B1, (*SLCO1B1*)	**OATP-C**	NM_006446	TAAGGCTAACATCTTATTGGGAGTC (f) CACAGCAGTAAAACATGAGAATTTGG (r)	1250 - 1382	133	79.5
OATP3A1, (*SLCO3A1*)	**OATP-D**	NM_013272	AGCCCTGGACCCCTACTCG (f) CCCGTGAGATTCGTGCTGTTG (r)	1582 - 1716	135	86.5
OATP4A1, (*SCLO4A1*)	**OATP-E**	NM_016354	CACCAGTTGAAGGACAGCAG (f) AGGAGCCAGATGGAGAGAGG (r)	1253 - 1341	89	86.5
OATP1B3, (*SLCO1B3*)	**OATP-8**	NM_019844	CCGTATTTTTTGGAAGGGTCTAC (f) TTCTTTCATTGTCCGATGCC (r)	1897 - 2034	138	79.5
OAT2 (*SLC22A7*)	NLT	NM_153320[a]	TGCTAGTGTCCTCCGATATGA (f) GAAGTGAACAGGTAGGCAGTG (r)	1649 - 1748	100	84.5
OAT3 (*SLC22A8*)		NM_004254	CCCACAGTCATCAGGCAAACA (f) AGGGCGGTGATCCCGTAGA (r)	1448 - 1584	137	86.5
OCT1 (*SLC22A1*)	HOCT1	NM_003057[a]	CGCCGAGAACCTTGGGAGAAA (f) ACGACATCGCCGCAAAACATC (r)	1686 - 1795	110	84.5
NTCP (*SLC10A1*)	NTCP1	NM_003049	GGACATGAACCTCAGCATTG (f) AATGAGAACCAGGACCAGTG (r)	424 - 586	163	85.5
PEPT-1 (*SLC15A1*)		NM_005073	CACCTCCTTGAAGAAGATGGCA (f) GGGAAGACTGGAAGAGTTTTATCG (r)	1121 - 1225	105	86.0
PEPT-2 (*SLC15A2*)		NM_021082	TTTGTCTCTCTACACTGAGCATTC (f) CTGCTTTCTGTATCCTTTACCATC (r)	1453 - 1563	111	81.5

Table 1. Genes and primers. The accessing numbers of RefSeq mRNA sequences and sequences of primers used for real-time PCR analyses to evaluate the expression of transporter genes. [a]) More than one transcript variant is available and the primers for these genes span common area for all transcript variants.

Real-time RT-PCR analysis

Real-time PCR was performed using iCycler (Bio-Rad, Hercules, CA, USA). For each gene analyzed, 50 ng of reverse transcribed RNA was amplified using SybrGreen PCR amplification master-mix (Bio-Rad, Hercules, CA, USA), following manufacturer instructions. The temperature profile was: 95°C for 3 min; 50 times: 95°C for 15 sec, 60°C for 15 sec, 72°C for 20 sec; melting curve program 72°C – 95°C. The calibration curves for each gene amplification were obtained from series of decimally diluted plasmid standards. All the calibration curves had the correlation coefficient $\geq$ 0.99%. The amplification efficiencies varied between 0.98 and 1.02. The triplicates of cDNA samples were amplified along with the decimally diluted plasmids in quantities $5x10^6$ - 50 copies in one run of cycler for each gene. A non-template control for all genes was included in each run. The quantities of transcripts in the samples were extrapolated from calibration curves.

Statistical analysis

Results are expressed as mean ± SD of three assays per condition. Statistical analysis was performed using ANOVA combined with Student's two-tailed t test. Differences among the conditions were considered significant at $p<0.05$.

RESULTS

Characterization of taurocholate uptake

The transport kinetics of [^{3}H] taurocholate in the two cell lines are shown in Figure 1. When measured at 50 μM [^{3}H] taurocholate, both Chang liver and HepG2 showed a rapid initial uptake of the bile acid (Figure 1, panel A and panel C), with similar steady-state levels after 10 minutes of incubation. The two cell lines showed similar [^{3}H] taurocholate transport in terms of time course and kinetic constants. The Michaelis-Menten constants for [^{3}H] taurocholate transport were obtained at 1 minute of incubation and calculated using a mathematical fit according to the equation: $V = ((Vmax * [S]) / (Km + [S])) + Dc * [S]$, in which Dc is the diffusive component of transport. In Chang liver cells (Figure 1, panel B) the apparent Km was 149.4 ± 57.9 μM, the maximal uptake (Vmax) 97.3 ± 32.9 pmol/mg prot/min, and the diffusive component was negligible (Dc was 0.0 ± 0.05). Similarly, in HepG2 cells (Figure 1, panel D) the Km was 174.6 ± 48.1 μM and Vmax 194.5 ± 55.0 pmol/mg prot/min; the diffusive component was also negligible (Dc was 0.0 ± 0.05). These data indicate the presence in both cell lines of one or more carrier mediated mechanisms for [^{3}H] taurocholate with comparable affinity and capacity. It should be noted however that, in spite of a similar affinity, a significantly greater accumulation of the bile acids was found in HepG2 (Vmax HepG2 > Vmax Chang liver cells, $p = 0.02$). The transport of [^{3}H] taurocholate measured in HepG2 is in a good agreement with what previously reported (Lee *et al.*, 2001).

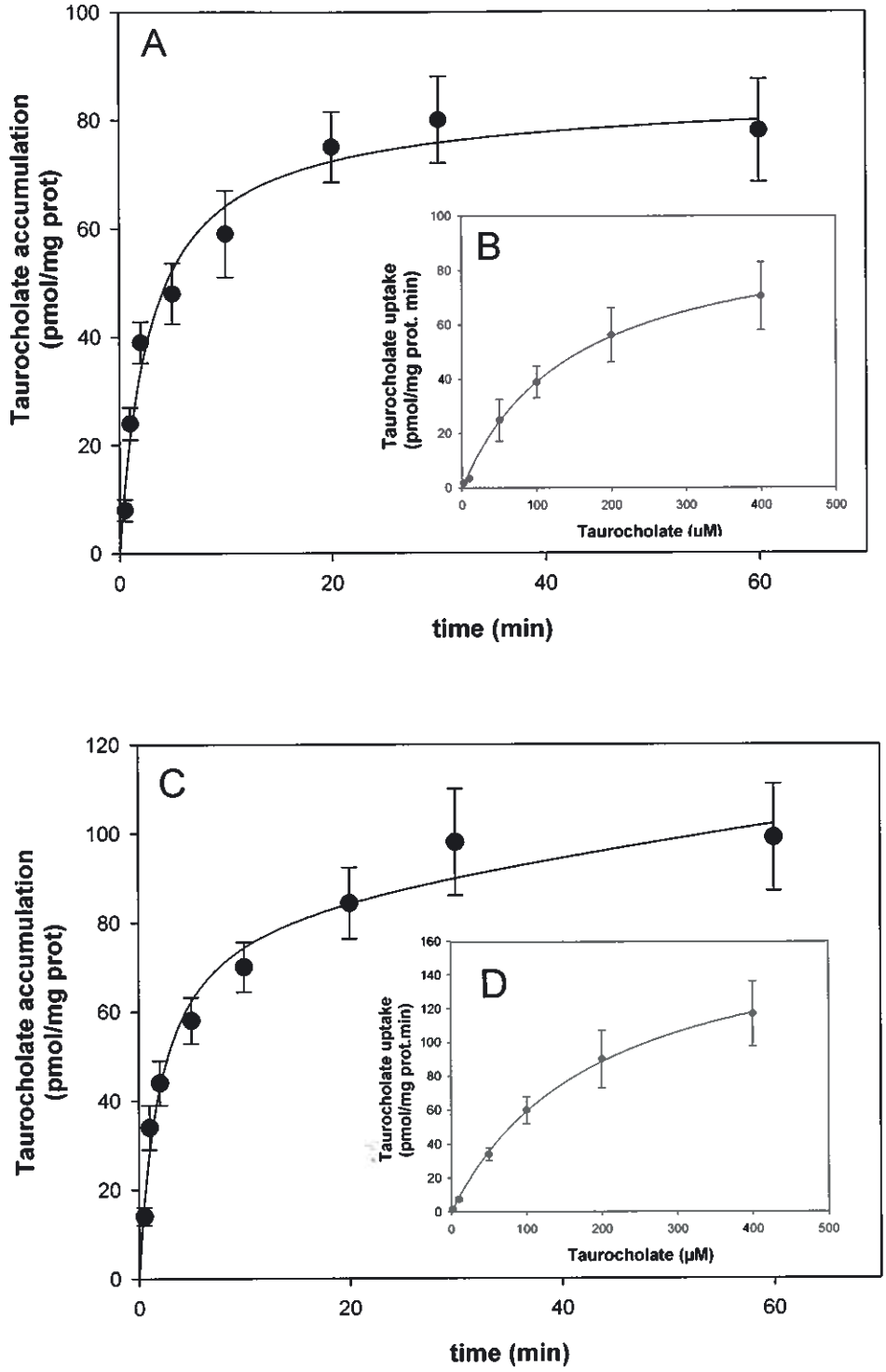

Figure 1. Characterization of [^{3}H] taurocholate uptake in Chang liver cells (A and B) and HepG2 cells (C and D). Panel A shows the time-course of taurocholate measured at a concentration of 50 µM in Chang liver cells. Inset B represent the concentration dependence of transport measured for 1 minute of incubation. Michaelis-Menten constants: Km = 149.4 ± 57.9 µM; Vmax = 97.3 ± 32.9 pmol/mg prot/min. Panel C shows the time-course of taurocholate measured at a concentration of 50 µM in HepG2 cells. Inset D represent the concentration dependence of transport measured for 1 minute of incubation. Michaelis-Menten constants: Km = 174.6 ± 48.1 µM; Vmax = 194.5 ± 55.0 pmol/mg prot/min. Data are expressed as means ± SD for three different experiments, performed in triplicate.

Characterization of B22956 uptake

The transport of B22956 in Chang liver and HepG2 cells was much slower than that of taurocholate. As shown in Figure 2 (panels B and D), the accumulation of the contrast agent increases linearly over the time in the first 60 minutes of incubation in both cell lines. Since the uptake was linear in this time frame, incubation times of 15 and 20 minutes were chosen to measure initial uptake in HepG2 and Chang liver cells respectively, and various concentrations were tested in order to evaluate kinetic constants. In Chang liver cells, B22956 uptake was saturative with a Km of 369 ± 138 μM, while the Vmax was 3270 ± 440 pmol/mg prot/20 min (Figure 2, panel A). The uptake of the contrast agent was also saturative in HepG2 cells (Figure 2, panel C) with an apparent affinity (Km value of 377 ± 47 μM) similar to that found in Chang liver cells but a Vmax (704 ± 41 pmol/mg prot/15 min) significantly lower. The overall efficiency transport, expressed as the ratio Vmax/Km was around 3.5 times higher in Chang than in HepG2 cells.

As shown in Table 2, B22956 transport experiments were performed in the presence of organic anions known to be substrates for OATP transporters (Hagenbuch and Meier, 2003). When transport was evaluated at low B22956 concentration (50 μM), the presence of equimolar concentration of either taurocholate or BSP reduced the uptake by 50% in Chang liver cells, but not in HepG2 cells. Differently, the addition of CCK-8 (20 μM), the OATP-8 substrate (Ismair *et al.*, 2001), reduced by more than 80% B22956 uptake both in Chang liver and HepG2 cells. To better define the inhibitory effect of taurocholate and CCK-8, experiments were repeated at higher B22956 concentration (200 μM). In Chang liver cells, low taurocholate concentrations (10 and 50 μM) reduced B22956 transport by 30-40%, while CCK-8 (20 and 50 μM) confirmed a strong inhibitory effect reducing transport by 60% or more. CCK-8 but not taurocholate, was still effective in reducing the contrast agent transport in HepG2, although the effect was rather weak with a decrease of 20-25% over the control.

The inhibition studies indicate that OATP proteins are involved in the B22956 transport in both cell lines, with a crucial role of OATP-8 suggested by the consistent reduction observed in the presence of low CCK-8 concentration, particularly in Chang liver cells. Of notice was the observation that transport of 50 μM taurocholate was not inhibited by the presence of B22956 (100 μM) and CCK-8 (20 μM), either in Chang liver or in HepG2 (data not shown), suggesting that several transporters contribute to the cell accumulation of taurocholate or B22956 and points to partial sharing of different mechanisms.

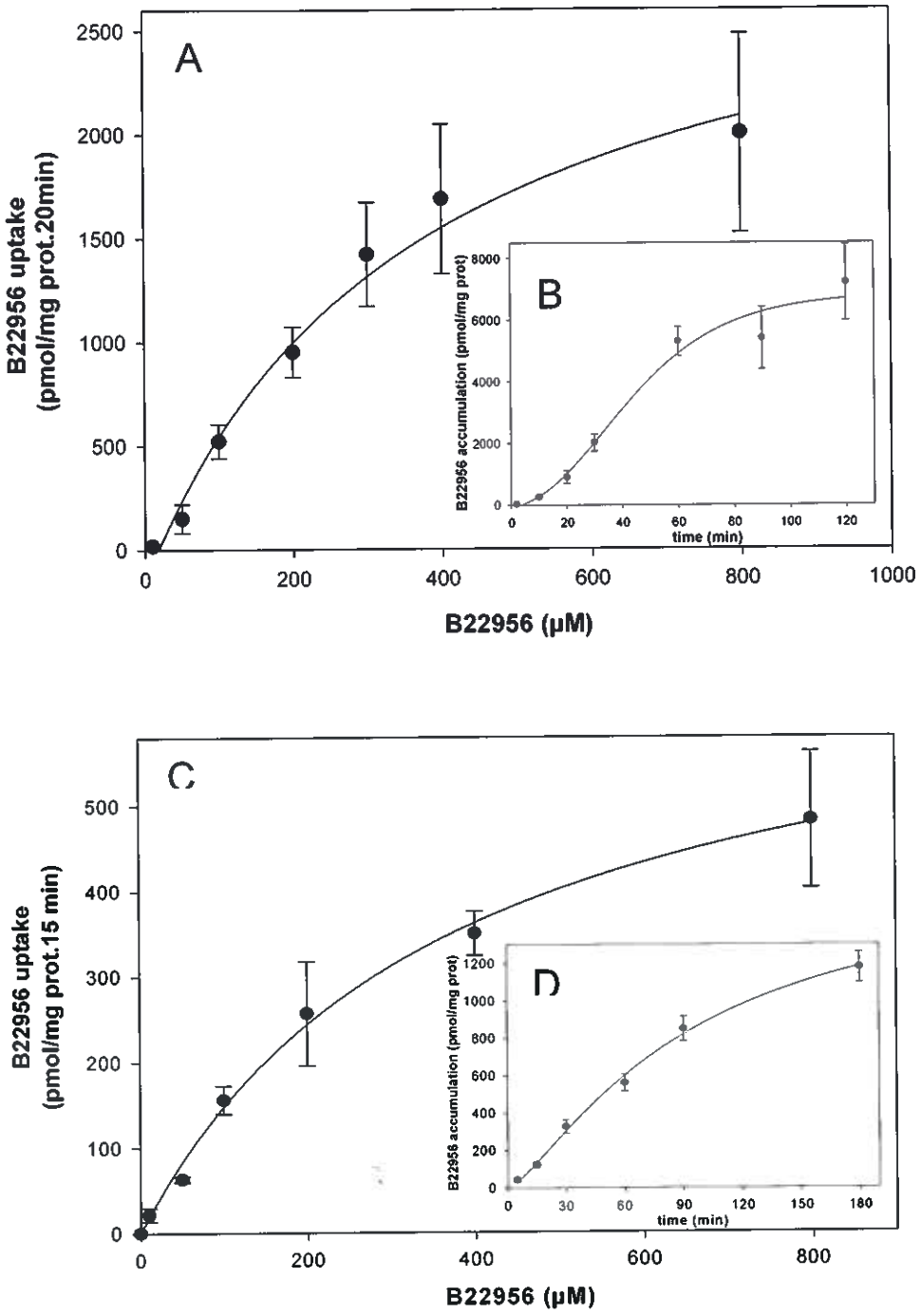

Figure 2. Characterization of B22956 transport in Chang liver cells (A and B) and HepG2 cells (C and D). Panel A shows the saturation kinetic of B22956 uptake in Chang liver cells. Transport was measured at different concentration after incubation of 20 minutes. Data were fitted by non linear regression and Michaelis-Menten constants were calculated: Km = 369 ± 138 µM; Vmax = 3270 ± 0.440 pmol/mg prot/20 min. Inset B shows the time course. Chang liver cells were incubated with 200 µM of B22956 for the indicated times. Panel C shows the saturation kinetic of B22956 uptake in HepG2 cells. Transport was measured at different concentration after incubation of 20 minutes. Data were fitted by non linear regression and Michaelis-Menten constants were calculated: Km = 704 ± 41 µM; Vmax = 377 ± 47 pmol/mg prot/20 min. Inset D represent the time course. HepG2 cells were incubated with 200 µM of B22956 for the indicated times. Data are expressed as means ± SD for three different experiments, performed in triplicate.

B22956 at 50 µM

Inhibitor (µM)	Chang liver Total pmol/mg prot/20 min	Chang liver % remaining	HepG2 Total pmol/mg prot/15 min	HepG2 % remaining
None	202 ± 15	100%	65.3 ± 8.1	100%
Taurocholate (50 µM)	101.7 ± 13.4	50.3%	61.8 ± 7.3	94.6%
BSP (50 µM)	90.1 ± 12.1	44.6%	58.2 ± 6.6	89.1%
CCK-8 (20 µM)	39.9 ± 5.5	19.7%	11.9 ± 17.5	18.2%

B22956 at 200 µM

Inhibitor (µM)	Chang liver Total pmol/mg prot/20 min	Chang liver % remaining	HepG2 Total pmol/mg prot/15 min	HepG2 % remaining
None	980.8 ± 79.1	100%	258.8 ± 35.3	100%
Taurocholate (10 µM)	673.5 ± 22.4	68.7%	251.1 ± 20.4	97.0%
Taurocholate (50 µM)	591.2 ± 18.9	60.2%	240.1 ± 32.2	96.6%
CCK-8 (20 µM)	392.2 ± 21.8	40.0%	208.4 ± 20.3	80.7%
CCK-8 (50 µM)	332.5 ± 17.5	34.0%	193.4 ± 15.3	74.7%

Table 2. Inhibition studies on B22956 uptake. The transport of B22956 was measured at a concentration of 50 or 200 µM, in the presence or in the absence of various OATP substrates.
Results are reported for the experiments conducted in the two hepatic cell lines.

Quantitative RT-PCR analyses

To determine which transporters may mediate B22956 transport in Chang liver and HepG2 cells and which may account for the different accumulation, we tested for the presence of various organic anion transporter transcripts from OATP family (namely *OATP-A*, *OATP-B*, *OATP-C*, *OATP-D*, *OATP-8*, *OATP-E*) and OAT family (*OAT2*, *OAT3*), which are reported to be expressed in liver cells (Hagenbuch and Meier, 2003). To better understand the pharmacological meaning of the data, transporters other than organic anion transporting polypeptides were also analyzed. In particular we assessed the expression of the sodium dependent taurocholate co-transporter *NTCP*, the organic cation transporter *OCT1* and the peptide transporters *PEPT-1* and *PEPT-2* (van Montfoort *et al.*, 2003). The expressions of the selected transporters were analyzed in Chang liver and HepG2 cells, and compared to the levels found in a human liver tissue from a healthy donor.
The results shown in Figure 3 represent a comparison of the gene expression of the different genes in both cell lines, expressed as log units of transcripts per µg of total RNA. The data indicate that HepG2 and Chang liver cells express the almost the same amount of *OATP-E*, *PEPT-2*, and the liver specific *OATP-C* genes. Significantly higher expression of *OATP-B* was observed in HepG2 cells as compared to Chang liver cells (see Table 3). Conversely, the transcripts of *OATP-D* and *OATP-8* were more abundant in Chang liver cell, while the expression of *OATP-A* has not been detected in any cell line. The transcripts for *OAT2*, *PEPT-1*, *NTCP* and *OAT3* were detected only in HepG2 cells (see Table 3).

Real-time PCR analysis confirmed the expression of all the observed genes in normal human liver, with the exception of *OAT3* (Figure 3). Differently from what reported in cell lines (Lee *et al.*, 2001) and similarly to what reported in tissues (Briz *et al.*, 2003), *OATP-A* was significantly expressed in normal liver. This seems to indicate that OATP-A could be not specific of the hepatocyte, but most probably of other less abundant cell types in liver. The other investigated OATPs were expressed at high and quite comparable levels. This is particularly true for the so called liver-specific *OATP-C* that is found at the greatest expression level, second only to that registered for *OCT1*.

As shown in Figure 3, HepG2 cells mimic the expression profile of the normal liver in a larger number of transporters than Chang liver cells. In line with previous reports (Lee *et al.*, 2001), HepG2 cells do not express significant levels of *OATP-C* and *OATP-8*, two hepatospecific transporters that seem to be greatly repressed in liver tumors (Cui *et al.*, 2003).

The expression of *OATP-B*, *OATP-E*, *OAT2*, *OCT1*, *NTCP*, *PEPT-1* and *PEPT-2* is maintained in HepG2 cells with detectable *OAT3*.

The Chang liver cells resemble normal liver in the expression of a lower number of transporters and of particular interest is the maintained expression of *OATP-8*, *OATP-E* and *OATP-D*. This kind of cells misses completely the expression of *OATs* and *NTCP* genes.

Gene	**HepG2**		**Chang liver**		**Human Normal Liver**	
	Log N	**S.D.**	**Log N**	**S.D.**	**Log N**	**S.D.**
OATP-A	N.D.		N.D.		2.65	0.08
OATP-B	5.35	0.05	2.83	0.20	6.18	0.00
OATP-C	2.00	0.36	2.38	0.15	6.43	0.02
OATP-D	1.67	0.45	5.94	0.11	4.63	0.02
OATP-E	6.19	0.02	5.97	0.07	5.28	0.02
OATP-8	2.51	0.22	4.06	0.03	5.08	0.00
OAT2	3.99	0.08	N.D.		6.04	0.08
OAT3	1.99	0.79	N.D.		N.D.	
NTCP	2.99	0.32	N.D.		4.77	0.04
OCT1	4.12	0.02	4.12	0.10	6.69	0.00
PEPT-1	3.16	0.08	N.D.		4.34	0.03
PEPT-2	4.49	0.04	4.08	0.05	3.76	0.09

Table 3. Real time RT-PCR analyses and transporters expression. The log of number of transcripts of selected genes in 1 µg of RNA from HepG2 cells, Chang liver cells and human liver tissue. Measures were made at least in triplicate and repeated in three or more total RNA extracts from the cell lines and one total RNA extract from human liver.

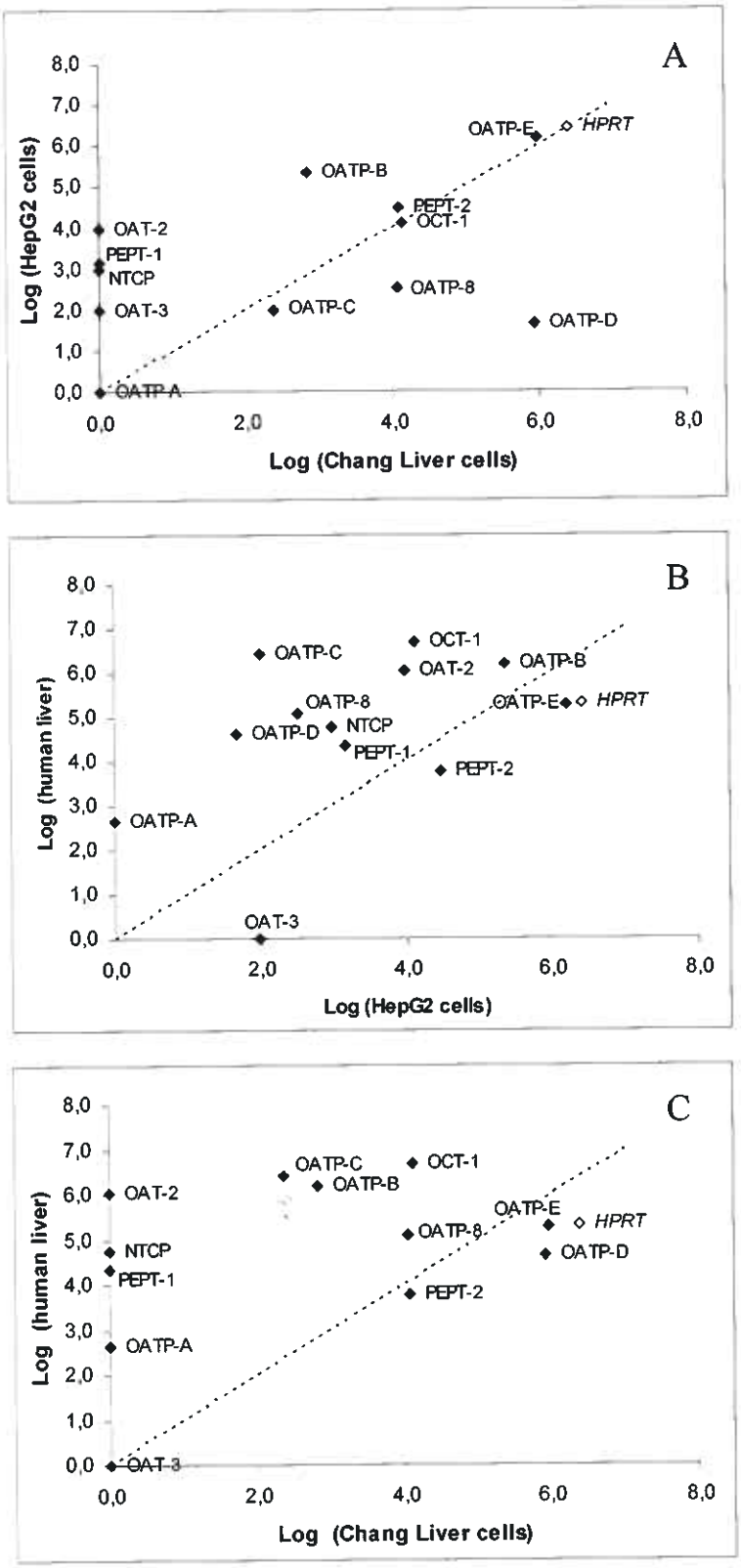

Figure 3. Comparison of transporter genes expression. Panel A: Number of transporter gene transcripts in HepG2 and Chang liver cells. Panel B: Number of transporter gene transcripts in liver tissue and HepG2 cells. Panel C: Number of transporter gene transcripts in liver tissue and Chang liver cells. The numbers of transcripts were normalized to the total RNA amount. The data are plotted as log of transcript number per 1 µg of RNA. The transversal dotted lines indicate equal expression in the cell line.

DISCUSSION

Pharmacological studies of new and old drugs take great advantage of the actual genetic knowledge on the cell membrane transport mechanisms, particularly in liver, kidney and physiological barriers such as blood brain barrier and placenta. This is the case of the contrast media for Magnetic Resonance Imaging (MRI), which is a class of diagnostics formulated to remain in the blood stream for the sufficient time interval to acquire images, to not accumulate in organs and to be rapidly cleared by kidney or, in specific cases, by liver (Lorusso *et al.*, 2005). When studying liver specific MRI contrast agents, biochemical and pharmacological studies were useful to demonstrate that two media (Gd-BOPTA and Gd-EOB.DTPA) that are currently used in clinical practice are substrates to the known canalicular transport system MRP2 (ABCC2) (de Haen *et al.*, 1996). While the biliary excretion of Gd-BOPTA and Gd-EOB.DTPA in rats ranges from 50 to 70% of the administered dose (Lorusso *et al.*, 2005), in humans the biliary excretion of the two contrast agents is much lower (Lorusso *et al.*, 2005). The reasons for such great differences may be explained considering that the hepatic clearance of a drug is conditioned by the uptake mechanisms driving the passage of the compounds from blood into the hepatocytes. This reasoning is supported by the evidence that Gd-EOB.DTPA is substrate to the rat Oatp1, but not the nearest human orthologue OATP-A (Pascolo *et al.*, 1999).

Previous studies indicated that B22956/1 is also a potential hepatospecific contrast agent, since its biliary excretion is much higher than that of Gd-EOB.DTPA and Gd-BOPTA, although mainly mediated by the ABCC2 transporter (Lorusso *et al.*, 2002). No data has been produced until now on the basolateral uptake mechanism in liver for this compound.

In this study we demonstrate that B22956 is substrate to OATP proteins and in particular OATP-8 is involved in the hepatic accumulation of the compound. Since the lateral chain of this new Gd complex derived from the DTPA backbone is a bile acid, it was important to compare transport kinetics of B22956 with those of taurocholate. The present results demonstrate that these two anions share only some transport mechanisms, possibly those mediated by the OATPs.

The transport kinetics of taurocholate were similar in HepG2 and Chang liver cells, and comparable with those previously described (Lee *et al.*, 2001). The taurocholate uptake in HepG2 cells seems to be mainly related to the OATP-E as suggested by the high transcript levels and high affinity to taurocholate (Hagenbuch and Meier, 2003), although the additional role of the very low expressed *OATP-8* and of the relevant *OAT2* (Ugele *et al.*, 2003) must be considered. The OATP-B involvement is excluded because taurocholate is not substrate to this transporter (Meier and Stieger, 2002). The role of NTCP should also be marginal considering the low transcript levels and, more important, previous studies demonstrating the absence of NTCP protein expression in HepG2 cells (Lee *et al.*, 2001; Kullak-Ublick *et al.*, 1996; Glasova *et al.*, 2002). In Chang liver cells the taurocholate uptake is similar to that found in HepG2 cells. From the quantitative analyses of the transcripts of the different transporters in Chang liver cells, it can be concluded that OATs and NTCP proteins do not contribute to taurocholate accumulation as these proteins are absent. On the contrary, OATP-D, OATP-E and OATP-8 are present and may all potentially contribute to the uptake of the bile acid.

The characteristics of the B22956 uptake are different in the two cell lines. While the apparent Km values of the kinetics were comparable, the Vmax found in Chang liver cells

was significantly higher than in HepG2 cells (3270 ± 440 pmol/mg prot/20 min vs. 704 ± 41 pmol/mg prot/15 min, respectively). The inhibition studies (Table 2) clearly indicate that the molecular mechanisms involved are different with only a partial overlap with those accounting for the transport of taurocholate. Taurocholate was effective in inhibiting B22956 uptake in Chang liver cells while it was ineffective in HepG2 cells. In addition, the transport of B22956 in HepG2 was not inhibited by BSP, while the cholephilic anion showed a clear inhibition in Chang liver cells. The uptake at low concentrations of B22956 in HepG2 was affected by CCK-8, a high affinity substrate for OATP-8. In Chang liver cells, OATP-8 expression was 40 times higher than in HepG2. This difference may explain why CCK-8 inhibits B22956 uptake by 60% at a CCK-8:B22956 ratio of 1:10 (Table 3). Differently from HepG2 cells, in Chang liver cells also taurocholate inhibited B22956 uptake, its effect evident both at low and high concentrations of the contrast agent, with the maximum at equimolar concentration. Since taurocholate is transported with different affinities by various OATPs (Kullak-Ublick *et al.*, 2000), it is not surprising that, on the other side, B22956 was unable to inhibit taurocholate uptake (data not shown).

Taken together, these data show that B22956 is transported by OATP-8 with a relatively high affinity, as demonstrated by the inhibition studies. Other transporters such as OATP-D and OATP-E seem to be involved in Chang liver cells, although with lower affinity. In HepG2 cells, the low expression of *OATP-8* seems sufficient to account for B22956 transport; OATP-B and OAT2 are other transporters that could be considered for their additional role.

These results may have a great practical relevance considering that the compound B22956 is an MRI contrast agent already in the process to be used in human diagnostic. Demonstrating that B22956 is taken up by hepatic cells by carrier mediated systems and that differently expressed transporters correspond to a different accumulation, points to a possible use of the contrast agent to discriminate diseased from healthy cells. Particularly, it has been reported that OATP-C and OATP-8 expression is greatly repressed in hepatic tumors. Since OATP-8, which is almost exclusively expressed in the liver (Ismair *et al.*, 2001), is involved in the hepatic accumulation of B22956, it may be expected that hepatic tumors have a lower accumulation of this contrast agent.

HepG2 and Chang liver cells are the most often used models of hepatic cells. While Chang liver cells are considered an *in vitro* model of non malignant liver, HepG2 cells are widely used cellular system for well differentiated hepatocarcinoma. No comparison has been reported so far in terms of drug accumulation and expression of basolateral transporters on different cellular models for liver disease. In the present study we report the comparison of the uptake and the expressions at RNA level of different transport proteins to better characterize the two widely used cell lines.

As shown in Figure 3, Chang liver cells retain a high levels of expression of only some OATPs, but loose or greatly reduce the expression of *OAT2*, *NTCP* and *PEPT-1*, which are reported to be abundant in normal liver (Alcorn *et al.*, 2002). The analysis on HepG2 cells suggests that during tumorogenesis not only *OATP-8* and *OATP-C* are repressed, as already reported (Cui *et al.*, 2003), but also *OATP-D* and *NTCP* expression may be affected. Quite surprisingly OATs expression is maintained and possibly increased as compared to normal liver. All these results are likely to be useful for therapeutic considerations on various families of drugs.

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VII.

SOUHRN / SUMMARY

Membránové transportéry mají vedle enzymů podstatný vliv na pohyb léčiva v organismu. Spektrum a míra exprese transportérů a jejich lokalizace na cytoplazmatických membránách buněk pak ovlivňuje průběh základních farmakokinetických dějů absorpce, distribuce, metabolismu a exkrece léčiva.

Tématicky je možno původní práce, které jsou součástí této dizertace, rozdělit do dvou oblastí podle orgánů, jejichž transportní procesy byly studovány. První čytři původní práce se zabývají expresí, lokalizací a funkční aktivitou efluxních transportérů P-gp a BCRP, v lidské a potkaní placentě a v buněčné linii BeWo odvozené od placentárního choriokarcinomu. V poslední práci byly studovány transportéry, které jsou zapojeny do vychytávání MRI diagnostické látky B22956/1 v buněčných liniích HepG2 a Chang Liver používané jako modely pro studium jaterního transportu.

V první práci byla potvrzena funkční exprese P-gp (*Abcb1*) v placentě potkana na konci březosti. V této studii byla pomocí duální perfúze potkana *in situ* prokázána funkce P-gp v omezení přestupu léčiv do plodu a dále jeho schopnost aktivně pumpovat své substráty z krve plodu zpět do cirkulace matky.

Protože však nebylo zřejmé, zda je P-gp přítomen (a může tak plnit svou ochrannou roli) v placentě po celou dobu březosti experimentálního zvířete a zda se jeho exprese v průběhu dozrávání placenty mění, byla v návaznosti na předchozí studii zkoumána přítomnost P-gp v placentě v průběhu březosti potkana. Na mRNA úrovni byla exprese obou genů kódujících potkaní P-gp, *Abcb1*a a *Abcb1*b, studována pomocí relativní kvantifikace metodou real-time RT-PCR a vztažena k expresi housekeepingového genu beta-2-mikroglobulinu. Pomocí Western-blottingu byl pak P-gp detekován rovněž na proteinové úrovni. Imunohistochemická metoda byla použita pro sledování exprese a především lokalizace P-gp v placentě v průběhu březosti potkana.

Naše studie prokázala přítomnost mRNA transkriptů obou genů kódujících P-gp již v 11. dni gestace. V této době již končí vývoj chorioallantoické placenty potkana a placenta začíná plnit svoji transportní funkci. Během dalšího dozrávání placenty se exprese obou genů dále zvyšovala. Pomocí Western-blottingu byl P-gp detekován od 13. až do konečného 22. dne březosti potkana. Imunohistochemická analýza potvrdila přítomnost placentárního P-gp již ve 13. dni březosti, kdy byl P-gp lokalizován ve vyvíjející se labyrintové zóně placenty. Od 15. dne až do konce březosti byla již imunopozitivita pro P-gp pozorována ve vrstvě trofoblastu. Naše data tak naznačují, že P-gp je přítomen v trofoblastu placenty brzy po jejím vzniku a může tak pravděpodobně plnit svou úlohu v transplacentární farmakokinetice po celou dobu březosti potkana.

V naší další práci jsme se zaměřili na studium teprve nedávno objeveného lékového efluxního transportéru BCRP kódovaného genem *ABCG2*. Jeho lokalizace a funkční exprese byla sledována na placentární linii BeWo, která je používaná jako in vitro model trofoblastu. Na úrovni mRNA transkriptů pro BCRP jsme prokázali výraznou endogenní expresi tohoto transportéru v BeWo buňkách, stejně jako v lidské terminální placentě. Přítomnost BCRP byla dále potvrzena na proteinové úrovni v BeWo buněčné linii i v lidské placentě imunodetekcí pomocí Western blottingu s použitím monoklonální protilátky BXP-21. Funkční akumulační studie prokázala významnou aktivitu BCRP v odčerpávání svých substrátů ven z buněk. Pomocí nepřímé imunofluorescenční mikroskopie jsme dále studovali subcelulární lokalizaci BCRP v BeWo buňkách. Lokalizace tohoto transportéru byla patrná především na mikrovilózní apikální membráně, která odpovídala té straně vrstvy trofoblastu, jež je v přímém kontaktu s mateřskou krví. Protože BeWo buňky představují model trofoblastu, zdá se být pravděpodobné, že BCRP je schopen odčerpávat své substráty ve fetomaternálním směru ven z buněk této vrstvy. Imunohistochemická lokalizace BCRP v lidské placentě potvrdila výraznou expresi BCRP právě ve vrstvě trofoblastu, přestože imunopozitivita pro BCRP byla patrná i v endotelových buňkách fetálních cév. Uvedená data tak naznačují, že BCRP, podobně jako P-gp, pumpuje léčiva a xenobiotika zpět do krevního oběhu matky a pravděpodobně tak poskytuje ochranu plodu před potenciálně toxickým účinkem těchto látek. Uvedené poznatky získané *in vitro* jsme posléze sledovali na *in situ* perfundované placentě potkana, kdy jsme funkční aktivitu placentárního BCRP prokázali pomocí modelového substrátu cimetidinu.

Na transkripční úrovni jsme dále pomocí absolutní kvantifikace porovnávali hladiny exprese genů pro BCRP (*ABCG2*) a P-gp (*ABCB1*) v lidské placentě. Počet transkriptů *ABCG2* více než desetinásobně převyšoval počet transkriptů *ABCB1*. Na základě těchto výsledků se domníváme, že BCRP je přinejmenším stejně důležitým, ne-li důležitějším lékovým transportérem v placentě jako P-gp.

Cílem poslední práce, která byla vypracována na základě mezinárodní spolupráce mezi Centro studi fegato, University of Trieste a Farmaceutickou fakultou, bylo studium molekulárních mechanismů jaterního transportu diagnostika B22956/1 pro magnetickou rezonanci na jaterních buněčných modelech. B22956/1 je nová kontrastní látka pro magnetickou rezonanci, která vykazuje značnou biliární exkreci a proto se zdá být vhodnou látkou pro hepatobiliární zobrazení. Její transportní vlastnosti jsme srovnávali

s taurocholátem, jako typickým zástupcem endogenních látek, které podléhají biliární exkreci.

Kinetika transportu byla sledována pomocí vychytávacích („uptake") experimentů na buněčných liniích Chang Liver a HepG2, které jsou považovány za *in vitro* buněčné modely nenádorových a nádorových jaterních buněk. Tyto pokusy nám ukázaly, že transportní parametry taurocholátu byly velmi podobné v obou buněčných liniích, zatímco B22956/1 je vychytáván 3,5 krát účinněji do Chang Liver buněk než do HepG2 buněk.

V obou buňkách byla pozorována inhibice vychytávání B22956/1 v přítomnosti cholecystokininu-8 (CCK8), specifického inhibitoru transportéru OATP-8 (SLCO1B3), ačkoli tento efekt byl u HepG2 buněk patrný pouze při nízké koncentraci B22956/1. Inhibiční studie dále ukázaly, že transport B22956/1 do Chang Liver buněk je inhibován taurocholátem i bromsulfoftaleinem narozdíl od transportu v HepG2 buňkách, na které tyto inhibitory neměly vliv.

Vzhledem k tomu, že není známo, jaké transportéry se podílí na vychytávání B22956/1, provedli jsme absolutní kvantitativní real-time RT-PCR analýzu exprese genů pro transportéry OATP-A, OATP-B, OATP-C, OATP-D, OATP-E, OATP-8, OAT2, OAT3, OCT1, NTCP, PEPT-1, PEPT-2 v liniích HepG2 a Chang Liver a ve zdravé jaterní tkáni. Výsledky ukázaly, že ve srovnání s jaterní tkání byla v HepG2 buňkách snížena exprese transportérů OATP-C (*SLC1B1*), OATP-D (*SLCO3A1*) a OATP-8 (*SLCO1B3*), zatímco exprese OATP-B (*SLCO2B1*), OAT2 (*SLC22A7*) a OAT3 (*SLC22A8*) byla podobná nebo zvýšená. Naopak v Chang Liver buňkách transkripty OAT2 (*SLC22A7*) a OAT3 (*SLC22A8*) detekovány vůbec a hladina OATP-D (*SLCO3A1*), OATP-E (*SLCO4A1*) a OATP-8 (*SLCO1B3*) byla podobná jako v játrech.

Z výsledků inhibičních experimentů usuzujeme, že OATP-8 (*SLCO1B3*) lze pokládat za transportér, který se podílí z velké většiny na vychytávání B22956/1. V Chang liver buňkách by dále mohly být do vychytávání zapojeny transportéry OATP-D (*SLCO3A1*) a OATP-E (*SLCO4A1*), avšak s mnohem menší afinitou. Z výsledků na HepG2 buňkách je vidět, že OATP-8 se podílí na transportu i přes jeho nízkou expresi a že do transportu by mohly být zapojeny i OATP-B (*SLCO2B1*) a OAT2 (*SLC22A7*). Vzhledem k tomu, že OATP-8 (*SLCO1B3*) je transportér exprimovaný výhradně v játrech a jeho exprese je značně potlačena v jaterních nádorech, dalo by se očekávat, že kontrastní látka B22956/1 bude akumulována v jaterních nádorech méně a proto by mohla být užitečným diagnostikem pro rozlišení nádorové a zdravé jaterní tkáně.

Together with enzymes membrane transporters have substantial influence on movement and fate of drugs in the organism. Their spectrum, expression extent and localization on cytoplasmic membranes have an effect on basic pharmacokinetic processes of absorption, distribution, metabolism and excretion (ADME).

Thematically, this thesis can be split into two fields according to the organs whose transport processes were investigated. The first four original studies deal with the role of efflux transporters P-glycoprotein and BCRP in human and rat placenta and in BeWo cell line derived from human choriocarcinoma. In the last study transporters that could participate in B22956/1 uptake in liver or in cell line derived from liver were investigated.

In the first study the functional expression of P-glycoprotein (*Abcb1*) in the rat placenta was confirmed at the end of pregnancy. By use of in vivo dually perfused rat placenta the ability of P-gp to actively pump its substrates from the fetal blood back to the maternal circulation was demonstrated.

At the mRNA level the expression of both genes coding for P-gp, *Abcb1*a and *Abcb1*b, was investigated using relative quantification by real-time RT-PCR method and compared to expression of a housekeeping gene *beta-2-microglobulin*. Using of Western blotting the placental P-gp was detected at the protein level. Immunohistochemistry was employed to localize P-gp in rat placenta.

Our study revealed the presence of mRNA transcripts of both genes encoding P-gp as soon as on the 11th day of gestation, which corresponds with the day when the definitive features of chorioallantoic rat placenta are well-presented and the organ begins to fulfill its physiological roles. The expression of both genes further increased up to the term. By means of Western-blotting, P-gp was detected from the 13th up to the 22nd day of pregnancy. Immunohistochemical analysis confirmed the presence of P-gp in the developing labyrinth zone of placenta of 13th gestation day; starting from 15th gestation day up to the term immunopositivity for P-gp was observed in the trophoblast layer. Our data thus indicate that P-gp is expressed in the placenta soon after its development and, therefore, can play a role in transplacental pharmacokinetics during the whole period of pregnancy.

In our subsequent study we aimed to examine the recently discovered BCRP drug efflux transporter encoded by *ABCG2* gene. Its localization and functional expression was studied employing human placental cell line BeWo as an in vitro model of placental barrier. At the level of mRNA transcripts we revealed a high endogenous expression of the transporter in

BeWo cells, as well as in human term placenta. The presence of BCRP was further confirmed in BeWo cells and in human term placenta using Western blotting with BXP-21 monoclonal antibody. Functional accumulation studies with mitoxantrone (a substrate of P-gp and BCRP) and inhibitors of BCRP and P-gp demonstrated significant activity of BCRP in pumping its substrates out of the cells. Employing indirect immunofluorescence microscopy, we further studied the subcellular localization of BCRP in BeWo cells. BCRP appears to be localized predominantly at the microvillous apical membrane of the cells, which corresponds to the part of trophoblast layer that is in direct contact with maternal blood. Since BeWo cells represent a model for trophoblast cells it seems plausible that BCRP is able to pump its substrates out of the trophoblast layer the feto-maternal direction. Imunohistochemical localization of BCRP in the human placenta confirmed high expression in the trophoblast layer, however, the immunopositivity for BCRP was also apparent in the endothelial cells of fetal vessels. The above data indicate that BCRP seems to provide (similarly to P-gp) protective role to fetus while pumping potentially toxic drugs and xenobiotics back to the maternal circulation. These findings were further confirmed in experiments on *in situ* perfused rat placenta in which functional activity of placental BCRP was described using cimetidine as a model substrate.

Using absolute quantitative real-time RT-PCR we futher studied the expression levels of *ABCG2* (coding for BCRP) and *ABCB1* (coding for P-gp) in mature human placenta. Surprisingly, the number of *ABCG2* transcripts exceeded ten times the number of *ABCB1* transcripts ten times. On the basis of these results we assume that BCRP is at least as important drug transporter as P-gp.

The aim of the last study, which was carried out on the basis of international cooperation between Centro Studi Fegato, University of Trieste and Department of Pharmacology and Toxicology of our faculty, was to investigate the molecular mechanisms of hepatic transport of MRI contrast agent B22956/1 in hepatic cellular models. Gadocoletic acid trisodium salt (B22956/1) is a new contrast agent showing high biliary excretion and thus is potentially advantageous in hepatobiliary imaging. The transport parameters of this agent were compared with taurocholate, as a typical endogenous compound that is excreted into the bile.

Uptake experiments were performed in Chang Liver and HepG2 cell lines that are considered as in vitro models of tumoral and non-tumoral cells. These experiments showed that transport parameters of taurocholate are very similar in both cell lines, while B22956/1 is taken up 3,5 times quicker by Chang Liver cells than by HepG2 cells.

Inhibition studies further demonstrated that the uptake of B22956/1 into Chang Liver cells is inhibited by taurocholate and bromosulphophthalein in contrast to HepG2 cells, which were not influenced by these inhibitors. In addition the uptake of B22956/1 into Chang Liver cells was greatly inhibited by cholecystokinin-8 (CKK8), which is specific inhibitor of OATP-8 (SLCO1B3) transporter.

Because no information is available about transport proteins involved in uptake mechanisms of B22956/1 in liver, we have performed absolute quantitative real-time RT-PCR analysis PCR in cell lines HepG2 and Chang Liver and healthy human liver tissue to evaluate the expression genes for: OATP-A, OATP-B, OATP-C, OATP-D, OATP-E, OATP-8, OAT2, OAT3, OCT1, NTCP, PEPT-1, PEPT-2. The results have shown that compared to normal liver, the expression of OATP-C (SLC1B1), OATP-D (SLCO3A1) and OATP-8 (SLCO1B3) was greatly repressed in HepG2 cells, while expression of OATP-B (SLCO2B1), OAT2 (SLC22A7) a OAT3 (SLC22A8) was either maintained or increased. On the contrary, in Chang liver cells, transcripts of OAT2 (SLC22A7) and OAT3 (SLC22A8) were undetectable, while the expression of OATP-D (*SLCO3A1*), OATP-E (*SLCO4A1*) and OATP-8 (*SLCO1B3*) was similar to normal liver.

We conclude that B22956 is transported by OATP-8 (*SLCO1B3*) with a relatively high affinity as demonstrated by the inhibition studies. Other transporters such as OATP-D (*SLCO3A1*) and OATP-E (*SLCO4A1*) seem to be involved in Chang liver cells, although with lower affinity. In HepG2 cells, the relatively low expression of OATP-8 (*SLCO1B3*) seems to be sufficient to account for B22956 transport; OATP-B (*SLCO2B1*) and OAT2 (*SLC22A7*) are other transporters that could also be involved in the process. Considering that OATP-8 (*SLCO1B3*) is a transporter expressed exclusively in the liver and its expression is greatly repressed in liver tumors, it may be assumed that B22956/1 will be accumulated with lower extent into hepatic tumors and, therefore, it could serve as a useful diagnostic agent for discrimination between tumoral and intact liver tissue.

VIII.

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1. Původní práce publikované v odborných časopisech

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