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VÝZNAM EFLUXNÍCH TRANSPORTÉRŮ LÉČIV PRO OCHRANU PLODU

Dizertační práce

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Obor: Farmakologie

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Hradec Králové 2010

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu literatury a v práci řádně citovány.

Lenka Hahnová

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Seznam zkratek

ABC	"ATP-binding cassette" – skupina transportérů vázajících ATP
BCRP/Bcrp	lidský/zvířecí "breast cancer resistance protein"
FDA	"Food and Drug Administration"
NBD	„nucleotide binding domain" – nukleotidy vázající doména
P-gp	P-glykoprotein
PK	farmakokinetika
RT-PCR	"reverse transcription – polymerase chain reaction" – reverzní transkripce - polymerázová řetězová reakce
TMD	„transmembrane domain" – transmembránová doména

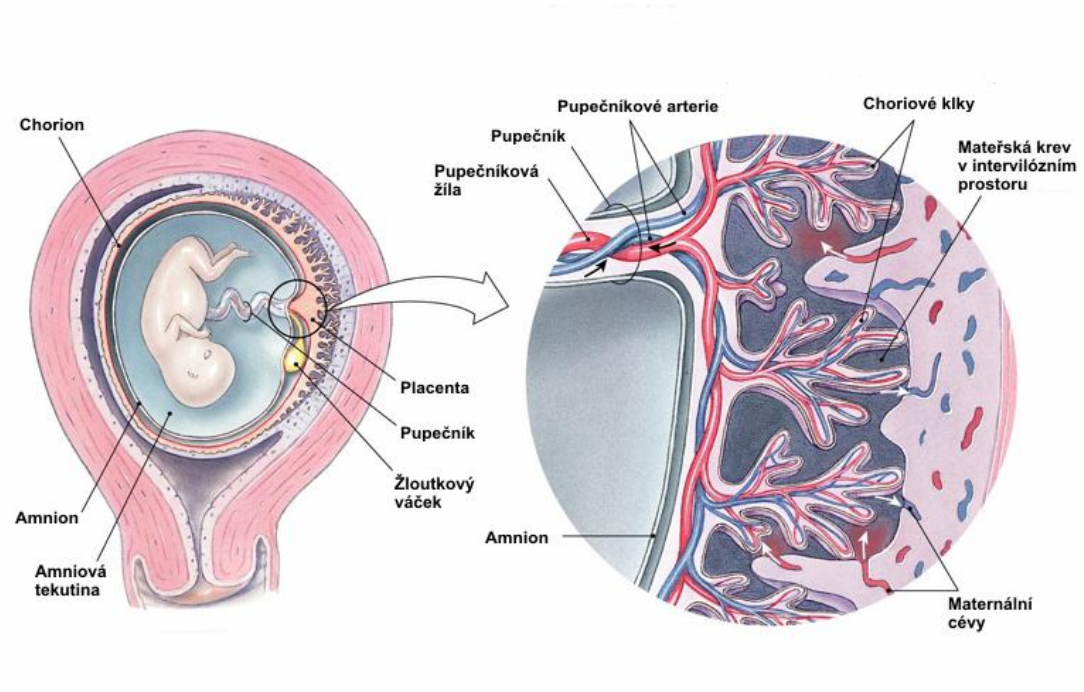
Obsah

I
ÚVOD A CÍLE PRÁCE

1 Struktura a funkce placenty

Placenta je orgán diskovitého tvaru, který přivádí do těsné blízkosti krevní oběhy matky a plodu a zároveň zabraňuje jejich smísení. Tato tkáň plní celou řadu funkcí, které jsou nezbytné pro optimální růst a vývoj plodu. Kromě zajišťování přísunu živin, výměny plynů a exkrece odpadních látek produkuje také škálu hormonů, enzymů a dalších látek ovlivňujících průběh celého těhotenství (Syme et al., 2004).

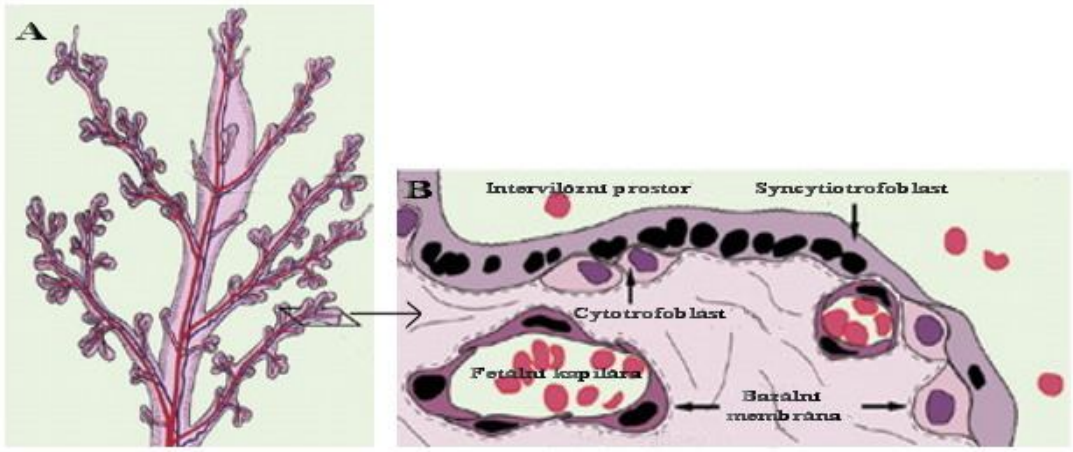
Lidská placenta je tvořena částí plodovou (chorion frondosum) a částí mateřskou (decidua basalis). Decidua basalis vybíhá v septa rozdělující zralou placentu na 20 – 40 funkčních jednotek – kotyledonů. Každý kotyledon je tvořen stromem choriových klků obsahujících pojivovou tkáň a kapilární síť, v níž proudí plodová krev přiváděná a odváděná pupečníkovými cévami (Obr. 1). Choriové klky jsou omývány mateřskou krví, která je spirálními arteriemi přiváděna do intervilózního prostoru. Povrch choriových klků odděluje plodovou a mateřskou krev a vytváří tak vlastní placentární bariéru.



Obr. 1 *Schematické znázornění struktury placenty. Převzato a upraveno z:* http://www.colorado.edu/intphys/Class/IPHY3430-200/image/26-19.jpg

Placentární struktura vykazuje značné mezidruhové rozdíly, na jejichž základě lze savčí placenty rozdělit na tři základní typy: (a) hemochoriální (člověk, potkan, myš, králík), (b) endoteliochoriální (kočka, pes) a (c) epiteliochoriální (ovce, prase, kůň) (van der Aa et al., 1998; Syme et al., 2004). V lidské placentě je bariéra oddělující mateřskou a plodovou krev tvořena dvěma buněčnými vrstvami, trofoblastem a endotelem fetálních kapilár (Obr. 2B). Zpočátku vývoje placenty tvoří trofoblast jednobuněčný mitoticky aktivní cytotrofoblast, který postupně fúzuje v mnohojaderný syncytiotrofoblast představující hlavní složku placentární bariéry. V důsledku úbytku cytotrofoblastu se zmenšuje tloušťka placentární bariéry z počátečních 10 µm až na pouhých 1 - 2 µm v terminálním stádiu těhotenství (van der Aa et al., 1998).

Mnohojaderné soubuní syncytiotrofoblastu má povahu polarizované vrstvy. Apikální membránu (orientovanou na mateřskou stranu) tvoří mikrovilózně zvlněný kartáčový lem, který značně zvětšuje absorpční povrch trofoblastu. Bazální membrána orientovaná na plodovou stranu mikroklky postrádá. K polarizaci mimoto přispívá také rozdílná distribuce enzymů, receptorů a transportních proteinů na obou stranách membrány (Ganapathy et al., 2000; Syme et al., 2004; Ganapathy and Prasad, 2005).



Obr. 2 *Schematické znázornění placentární bariéry.* ***A*** *– choriový klk a síť fetálních kapilár,* ***B*** *– vlastní placentární bariéra. Převzato a upraveno z: http://www.pnas.org/content/suppl/2004/04/03/0401434101.DC1/01434Fig5.jpg*

2 Farmakoterapie v těhotenství

Užívání léků v průběhu těhotenství je často nezbytné zejména z důvodu závažného onemocnění matky - epilepsie, hypertenze, gestační diabetes, nádorové onemocnění, infekce apod. (Garcia-Bournissen et al., 2003; Shiverick et al., 2003), ale stoupá i počet případů, kdy je nutné podávat léčiva matce za účelem terapie plodu - fetální tachykardie, omezení přenosu HIV infekce z matky na plod apod. (Mirochnick, 2000; Ito, 2001; Taylor and Low-Beer, 2001; Oudijk et al., 2002; Capparelli et al., 2005). Výsledky čtyřleté multicentrické studie provedené v USA v letech 1996 - 2000 ukázaly, že 64 % těhotných žen bylo v průběhu 270 dnů před porodem předepsáno léčivo jiné než vitamínový či minerální doplněk stravy. Z toho značná část žen (10 %) obdržela přípravek D nebo X kategorie FDA, které jsou považovány za potenciální teratogeny (Andrade et al., 2004; Andrade et al., 2006).

Jelikož u většiny léčiv neexistují detailní farmakokinetické a farmakodynamické studie, farmakoterapie probíhá často bez dostatečných znalostí efektivity a bezpečnosti terapeutických látek. Podrobné prostudování mechanismů ovlivňujících ochranu plodu je tak pro optimalizaci farmakoterapie v těhotenství nezbytné.

3 Mechanismy ochrany plodu

Po velkou část těhotenství představuje placenta hlavní mechanismus pro ochranu plodu před potenciálně toxickými látkami přítomnými v mateřské krvi. Kromě léčiv či návykových látek se může jednat také o polutanty a toxiny pocházející z ovzduší, jídla nebo pití. Převážná většina těchto látek prostupuje placentou mechanismem pasivní difúze na základě koncentračního gradientu. Faktory ovlivňující pasivní difúzi vycházejí jednak z fyzikálně-chemických vlastností látek (lipofilita, stupeň ionizace, vazba na plazmatické proteiny, molekulová hmotnost), ale také z vlastností placentární bariéry (průtok krve placentou, gradient pH, tloušťka bariéry, velikost povrchu). Při přestupu z krve matky do krve plodu musí každá látka překonat nejprve apikální a bazální membránu syncytiotrofoblastu a následně i endotel fetálních kapilár. Tyto membrány tvoří tzv. **mechanickou složku** placentární bariéry.

Molekuly některých fyziologických i cizorodých látek mohou být rozpoznávány jako substráty membránových přenašečů fungujících buď na základě koncentračního gradientu (usnadněná difuze), nebo spotřeby energie (aktivní transport) (van der Aa et al., 1998). Aktivní transportéry působí buď ve směru materno-fetálním pro přenos živin, nebo ve směru opačném pro exkreci xenobiotik. Druhá skupina je označována jako **efluxní lékové transportéry** a představuje spolu s biotransformačními enzymy **aktivní složku** placentární bariéry.

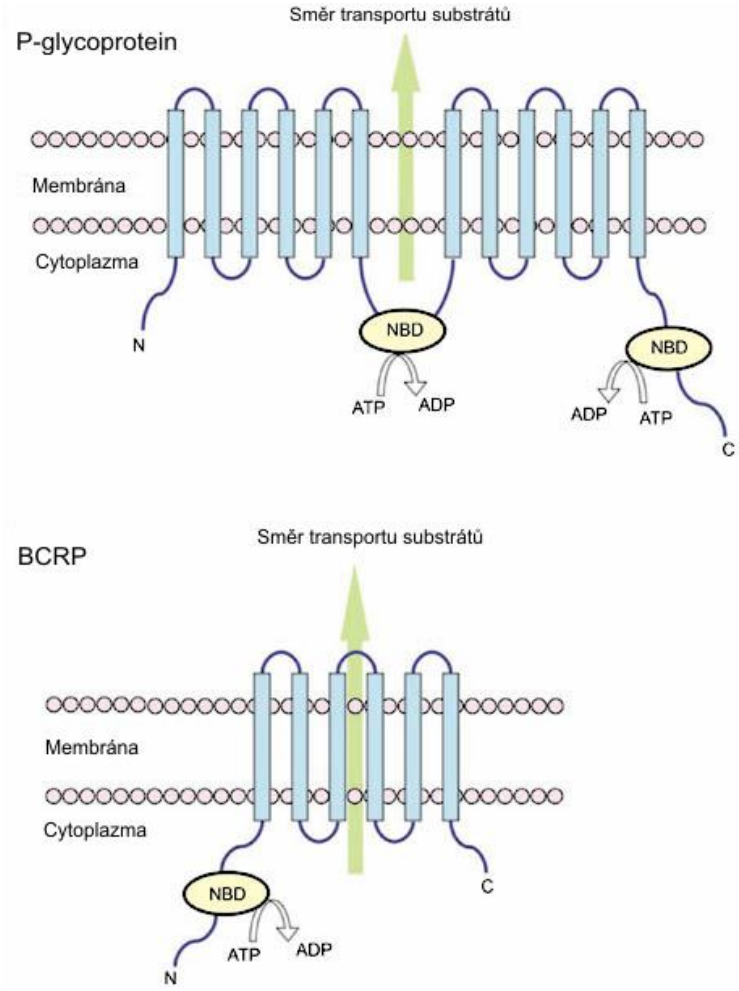
Výsledky studií z posledních let naznačují, že kromě placenty se na aktivní ochraně plodu mohou podílet i další struktury jako fetální membrány a orgány samotného plodu (Aye et al., 2007; Kalabis et al., 2007; Aleksunes et al., 2008). Podrobnější informace k této problematice uvádí přehledový článek autorky této dizertační práce v oddílu II „Fetoprotective activity of BCRP (ABCG2); expression and function throughout pregnancy“.

4 Lékové efluxní transportéry

Skupina lékových efluxních transportérů zahrnuje membránové transportní proteiny, které jsou za spotřeby energie (nejčastěji z hydrolýzy ATP) schopné „pumpovat“ různorodé látky ven z buňky i proti koncentračnímu gradientu. Pro svou závislost na spotřebě ATP jsou řazeny do velké rodiny ATP-binding cassette (ABC) transportérů. Do současné doby bylo u člověka popsáno již 48 ABC transportérů, které jsou klasifikovány do tří podskupin, ABCB, ABCC a ABCG (Marin et al., 2004; Syme et al., 2004). Vyskytují se v řadě tkání (ledviny, střeva, játra, mozek, varlata, placenta atd.), kde svou účastí na absorpci, distribuci a exkreci široké škály látek plní především ochrannou a detoxikační funkci. Některé ABC transportéry byly lokalizovány také v nádorové tkáni, v níž se podílí na fenoménu tzv. mnohočetné lékové rezistence vůči cytotoxickým látkám (Kavallaris, 1997; van der Kolk et al., 2002; Perez-Tomas, 2006).

Základní struktura ABC transportérů je vždy stejná; sestává z různého počtu transmembránových domén (TMD), tvořených šesti transmembránovými segmenty, a nukleotidy vázajících domén (NBD), které se nacházejí na N-konci nebo C-konci molekuly proteinu. Podle počtu domén jsou efluxní transportéry označovány buď jako „plné“ transportéry (např. P-gp), které jsou tvořeny dvěma až třemi TMD a

dvěma NBD, nebo jako „polo-“ transportéry (např. BCRP), tvořené pouze jednou TMD a jednou NBD (Obr. 3). Původně se předpokládalo, že transportéry z druhé zmíněné skupiny musí pro vytvoření funkčního celku homo- nebo heterodimerizovat (Doyle et al., 1998; Miyake et al., 1999; Litman et al., 2001). Dle současných studií však „polo-“ transportéry fungují jako homotetramery (Xu et al., 2004) nobo homooktamery tvořené čtyřmi homodimery (McDevitt et al., 2006).



Obr. 3 *Základní struktura ABC transportérů P-gp a BCRP tvořená různým počtem TMD a NBD. Převzato a upraveno z Lin et al. (2006).*

4.1 P-glykoprotein

P-glykoprotein (P-gp/MDR1/ABCB1) byl objeven v roce 1976 a v současnosti je nejlépe popsaným lékovým efluxním transportérem. Zatímco u člověka je protein o velikosti 170 kDa, který se chová jako efluxní transportér, kódován pouze genem *MDR1*, u hlodavců je zapotřebí dvou genů *mdr1a* a *mdr1b*

(Schinkel, 1997). Zpočátku byl P-gp zkoumán především pro svou schopnost způsobovat mnohočetnou lékovou rezistenci vůči cytostatikům u nádorových buněk (Roninson, 1987; Goldstein et al., 1991; Bosch and Croop, 1996; Goldstein, 1996), pozdější studie však prokázaly jeho expresi také v mnoha fyziologických nenádorových tkáních. P-gp je lokalizován na apikální membráně epitelu tenkého i tlustého střeva, v endoteliálních buňkách mozkových kapilár a varlat, v syncytiotrofoblastu placenty, na kanalikulární membráně jaterních hepatocytů, na apikální membráně renálních proximálních tubulů, atd. (Thiebaut et al., 1987; Cordon-Cardo et al., 1989), kde se podílí na farmakokinetických procesech absorpce, distribuce a eliminace látek (Schinkel, 1998; Smit et al., 1999; Jonker et al., 2000).

Mezi substráty P-gp patří celá škála chemicky a strukturně odlišných látek. Nejčastěji se jedná o neutrální či mírně bazické organické molekuly o velikosti od 200 Da do téměř 1900 Da, které náleží do různých farmakoterapeutických skupin, např. cytotoxické látky, HIV proteázové inhibitory, antibiotika, opioidy, antiemetika či diagnostická barviva rhodamin 123 nebo Hoechst 33342 (viz reviální práce Schinkel and Jonker, 2003; Fromm, 2004).

Dosavadní poznatky týkající se exprese a funkce P-gp v placentě jsou přehledně shrnuty v práci Ceckova-Novotna et al. (2006). V lidské placentě byla exprese P-gp prokázána jak na genové (Bremer et al., 1992), tak na proteinové úrovni (Cordon-Cardo et al., 1989). *MDR1* gen byl detekován ve vrstvě placentárního syncytiotrofoblastu již v první polovině těhotenství (Mylona et al., 1999). Výsledky studií na lidských a zvířecích placentách naznačují, že zatímco u potkana exprese P-gp v průběhu gestace stoupá (Novotna et al., 2004), u člověka a myši je tomu naopak (Gil et al., 2005; Kalabis et al., 2005; Sun et al., 2006). Tato diskrepance je pravděpodobně způsobena odlišnými mechanismy podílejícími se na regulaci exprese P-gp u jednotlivých živočišných druhů.

První studie demonstrující funkční aktivitu P-gp v placentě byla provedena na membránových vezikulách připravených z trofoblastu lidské placenty (Nakamura et al., 1997). Uptake substrátů calceinu-AM a vinblastinu v přítomnosti P-gp inhibitorů prokázal funkční expresi P-gp jak v primárních kulturách cytotrofoblastu, tak v buněčné linii BeWo (Utoguchi et al., 2000). Exprese P-gp v jednotlivých klonech BeWo buněk se však může lišit (Atkinson et al., 2003; Ceckova et al., 2006), proto je potřeba brát tento fakt v potaz při používání tohoto *in vitro* modelu. Veškeré studie provedené na P-gp deficientních myších prokázaly důležitou roli P-gp v ochraně

plodu před průnikem potenciálně toxických látek či terapeutik (Lankas et al., 1998; Smit et al., 1999; Huisman et al., 2001). Studie provedené v naší laboratoři na modelu duálně perfundované potkaní placenty přímo demonstrovaly vliv P-gp na transplacentární farmakokinetiku cyklosporinu (Pavek et al., 2001) a rhodaminu 123 (Pavek et al., 2003). Rozdílná distribuce farmak na maternální a fetální straně placentární bariéry způsobená aktivitou P-gp byla popsána též ve studiích s perfundovanou lidskou placentou *in vitro* (Molsa et al., 2005; Sudhakaran et al., 2005; Nekhayeva et al., 2006; Nanovskaya et al., 2008).

Součástí této dizertační práce je studium vlivu P-gp na transplacentární farmakokinetiku jeho substrátů rhodaminu 123 a BODIPY FL prazosinu s použitím modelu duálně perfundované potkaní placenty (Cygalova et al., 2009) – viz kapitola V.

4.2 Breast cancer resistance protein

Breast cancer resistance protein (BCRP/ABCG2) je více než 10 let známý ABC transportér náležící do podrodiny G, objevený nezávisle třemi výzkumnými laboratořemi (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999). Allikmets pojmenoval tento protein pro jeho vysokou expresi v lidské placentě ABCP (placentární ABC transportér). Brzy poté byl BCRP lokalizován v buněčných membránách střeva, jater, prsní žlázy, mozku a dalších fyziologických tkání (Allen and Schinkel, 2002; Doyle and Ross, 2003; Staud and Pavek, 2005). Podobně jako u P-gp byly také u BCRP odhaleny interakce s různorodými chemicky odlišnými látkami, od endogenních molekul až po fluorescenční barviva, chemoterapeutika a další léčiva (Doyle and Ross, 2003; Mao and Unadkat, 2005; Staud and Pavek, 2005; Krishnamurthy and Schuetz, 2006). Pozoruhodný je částečný překryv v substrátové specifitě s P-gp, avšak na rozdíl od něj je BCRP schopen transportovat také kladně i záporně nabité molekuly, organické anionty a sulfátové konjugáty. Stejným efluxním mechanismem, jakým způsobuje rezistenci buněk vůči cytostatikům, ovlivňuje BCRP farmakokinetické parametry svých substrátů a přispívá k ochraně citlivých struktur těla před potenciálně toxickými látkami.

Vysoká placentární exprese BCRP/Bcrp byla nejprve objevena u člověka (Allikmets et al., 1998) a později i u dalších savců (Jonker et al., 2000; Tanaka et al., 2005; Wang et al., 2006). V lidské placentě je BCRP exprimován převážně v

apikální membráně trofoblastu (Maliepaard et al., 2001), což vede k předpokladu, že se podobně jako P-gp podílí na omezení průchodu cizorodých látek placentární bariérou. Kromě toho se zdá, že BCRP přispívá k regulaci syntézy hormonů v placentě (Grube et al., 2007) a má podíl na ochraně trofoblastu před cytokiny indukovanou apoptózou (Evseenko et al., 2007a; Evseenko et al., 2007b). Pokud jde o změny exprese BCRP/Bcrp v průběhu těhotenství u různých živočišných druhů, nebylo dosud dosaženo jednoznačných výsledků. Zdá se však, že hladina transportéru je nejvyšší ve střední fázi gravidity a ke konci pozvolna klesá (Tanaka et al., 2005; Yasuda et al., 2005; Meyer zu Schwabedissen et al., 2006; Cygalova et al., 2008) – podrobněji viz oddíly II a IV této práce.

Transportní aktivita BCRP byla zkoumána jak s použitím *in vitro*, tak *in vivo* metod. Na modelu membránových vezikul izolovaných z lidské terminální placenty byl prokázán BCRP zprostředkovaný transport mitoxantronu a glyburidu s použitím BCRP inhibitoru novobiocinu (Kolwankar et al., 2005; Gedeon et al., 2008). Také buněčné linie odvozené z lidské či zvířecí placenty (BeWo, JAr, JEG-3, HRP-1) a kultury primárního trofoblastu náleží díky vysoké endogenní expresi BCRP/Bcrp mezi hojně používané modely pro studium efluxní aktivity tohoto transportéru v placentě (Bailey-Dell et al., 2001; Ceckova et al., 2006; Evseenko et al., 2006; Staud et al., 2006). *In vivo* byla poprvé fetoprotektivní role placentárního Bcrp1 naznačena ve studii na *P-gp* „knockoutovaných“ myších, kdy inhibice Bcrp1 pomocí GF120918 vedla k významnému zvýšení přestupu topotekanu z matky do plodu (Jonker et al., 2000). Podobně byl pro sledování farmakokinetiky průchodu BCRP substrátů nitrofurantoinu a glyburidu přes placentu využit model *Bcrp1* deficientních myší (Zhang et al., 2007; Zhou et al., 2008). V naší laboratoři jsme vliv BCRP na farmakokinetiku modelových substrátů prokázali *in situ* metodou duálně perfundované placenty potkana (Staud et al., 2006; Cygalova et al., 2009) – viz kapitoly III a V této práce.

5 Metody studia lékových efluxních transportérů placenty

Z etických, ale i technických důvodů nelze detailně sledovat farmakokinetiku transplacentárního přestupu léčiv u těhotných žen; proto byla vyvinuta řada alternativních technik a modelů, které situaci *in vivo* u člověka do určité míry

nahrazují. Většina informací o účincích exogenních látek na průběh těhotenství a vývoj plodu je tak získávána ze studií prováděných na zvířecích modelech a/nebo s využitím modelů placentární bariéry, které umožňují sledovat transport a metabolismus látek v placentě za fyziologických nebo experimentálních podmínek (viz přehledové články Bourget et al., 1995; Sastry, 1999; Bode et al., 2006; Vahakangas and Myllynen, 2006; Mitra and Audus, 2008). Tyto studie jsou prováděny na buněčných, resp. zvířecích modelech *in vitro, in situ* nebo *in vivo*.

5.1 Metody *in vitro*

Jak primární kultury placentárního trofoblastu, tak buněčné linie získané z lidské či zvířecí nádorové tkáně (např. BeWo, Jeg-3, JAr, HPR atd.) představují široce užívaný *in vitro* model materno-fetální bariéry. Ačkoli data získaná ze studií prováděných v těchto podmínkách neodrážejí plně fyziologické a biochemické změny probíhající v placentě, poskytují cenné informace zejména při provádění pilotních experimentů s novými typy molekul. Navíc mohou částečně nahradit zvířecí modely, čímž významně omezují počty experimentálních zvířat v jednotlivých studiích. Jelikož byla u výše zmíněných buněčných linií prokázána podobná exprese transportních proteinů a metabolických enzymů jako v původním trofoblastu (Ceckova et al., 2006; Evseenko et al., 2006; Staud et al., 2006; Serrano et al., 2007), hodí se pro sledování transportu a biotransformace látek v placentární bariéře.

5.2 Metody *in situ*

Metoda duálně perfundované lidské či zvířecí placenty náleží k technicky velmi náročným, avšak významným přístupům pro detailní studium transplacentární farmakokinetiky. Tato technika umožňuje pozorovat transport látek jak v materno-fetálním, tak ve feto-maternálním směru. S využitím specifických substrátů či inhibitorů lze na tomto modelu studovat význam jednotlivých transportních proteinů v placentární bariéře a ochraně plodu před cizorodými látkami.

Hlavní nevýhodou perfundovaného placentárního systému je omezení jeho využití pouze na třetí trimestr gestace a tudíž nemožnost studovat procesy probíhající

do té doby. Navíc je při perfuzích zvířecích placent nutno brát v úvahu mezidruhové odlišnosti ve struktuře placentární bariéry. Naopak nespornou výhodou zvířecích modelů je možnost sledovat změny v transportní a metabolické aktivitě placentární bariéry způsobené například prenatální terapií vybranými lékovými induktory či inhibitory, což u člověka není z etických důvodů možné.

5.3 Metody *in vivo*

Pro studium placentární bariéry *in vivo* metodami jsou často využívána březí experimentální zvířata. Tento přístup umožňuje analyzovat koncentrace testované látky v jednotlivých orgánech a stanovit její distribuci v těle matky i plodu. Dále lze na těchto modelech sledovat možný teratogenní potenciál testovaných látek, jejich vliv na fertilitu, růst, vznik malformací a celkové přežití plodu. Pro objasnění fyziologických funkcí vybraných transportérů v placentě se využívají také geneticky manipulovaná experimentální zvířata, nejčastěji myši, postrádající gen kódující určitý protein. Na těchto modelech lze pak v porovnání se zvířaty standardního fenotypu sledovat rozdíly v průběhu gravidity a vývoji plodu. Studie na geneticky modifikovaných zvířatech přispěly mimořádně i k objasnění role placentárního P-gp a Bcrp (Smit et al., 1999; Jonker et al., 2000; Huisman et al., 2001; Zhang et al., 2007; Vlaming et al., 2009).

6 Cíle předkládané dizertační práce

Hlavním cílem této práce bylo studium exprese a detoxikační role efluxních transportérů ve fetoplacentární jednotce. Dílčími cíli pak bylo:

1. Studium exprese a transportní aktivity Bcrp v buněčné linii HRP-1 a jeho vlivu na intracelulární kumulaci farmak.

2. Studium exprese a role Bcrp v placentě potkana, zejména z hlediska ovlivnění farmakokinetických parametrů transplacentárního průchodu léčiv.

3. Studium exprese a funkční aktivity Bcrp v placentě a plodu potkana v závislosti na stádiu březosti.

4. Ověření účinnosti transportní aktivity Bcrp a P-gp v placentě potkana s ohledem na fyzikálně-chemické vlastnosti modelových substrátů.

Tato dizertační práce je předložena jako soubor publikací, bezprostředně se týkajících tématu práce. Kapitoly III, IV a V již byly uveřejněny v odborných časopisech, kapitola II byla do odborného časopisu zaslána k posouzení.

7 Podíl doktorandky na předkládaných publikacích

U kapitol II, IV a V je předkladatelka této dizertační práce první autorkou, v případě kapitoly III pak spoluautorkou.

V práci uvedené v části III autorka spolupracovala při provádění akumulačních experimentů na placentární buněčné linii HRP-1 a podílela se na zpracování perfuzních vzorků pro jejich následnou analýzu.

Ve studii IV koordinovala autorka provádění infuzních experimentů, prováděla odběr vzorků plodů a placent, a dále je zpracovávala pro následnou analýzu jak transplacentárního průchodu cimetidinu do plodů, tak exprese Bcrp na úrovni mRNA pomocí zavedené metody real-time RT-PCR.

Ve studii uvedené v části V vedla provádění experimentů s duální perfuzí placenty potkana, zpracovávala získané vzorky a analyzovala je.

Předkladatelka dizertační práce sepsala všechny rukopisy, u nichž je první autorkou.

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II
FETOPROTECTIVE ACTIVITY OF BCRP (ABCG2); EXPRESSION AND FUNCTION THROUGHOUT PREGNANCY

Hahnova Cygalova L, Ceckova M, Staud F: Fetoprotective activity of BCRP (ABCG2); expression and function throughout pregnancy.

Fetoprotective activity of BCRP (ABCG2); expression and function throughout pregnancy

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Abstract

Detailed knowledge of transplacental pharmacokinetics, including the role of drug efflux transporters is crucial for optimizing drug choice, dosage schemes and to avoid/exploit possible drug-drug interactions on placental efflux transporters.

BCRP is the latest member of drug efflux transporters family that is being expressed in the placenta and fetal tissues. Since BCRP transports a wide range of substrates from many pharmacotherapeutic groups, including oral antidiabetics, antivirals, or anticancer compounds, its significant role in fetal protection and detoxication is presumed.

In this review we summarize current knowledge on BCRP expression and function in the fetoplacental unit during gestation as well as regulation and clinical significance of the transporter for drug therapy in pregnant women.

Introduction

The developing fetus is a highly sensitive and fragile tissue whose uncomplicated development primarily depends on optimal *in utero* conditions. Placenta is obviously considered as the crucial organ responsible for successful ontogeny. Its main role consists of the interchange of gasses, nutrients, fetal waste products and other endogenous and exogenous substances by bringing the maternal and fetal circulations into close apposition. Since placenta and fetus originate from the same basis and neither is able to function prosperously without the company of the other, it is necessary to understand the whole arrangement as an entity, described as a fetoplacental unit. The collaborative symbiosis of the fetoplacental unit is often mentioned in connection with steroid hormones synthesis; placenta is dependent on

the supply of appropriate precursors from maternal as well as fetal adrenal glands. The arising product progesterone is then transported back into fetal adrenal gland and transformed into testosterone or dehydroepiandrosterone sulfate, undergoing a conversion to estrogens in placenta again.

Protection of fetal organs against maternal toxins would be another example in which placental and fetal mechanisms work in accord. During the first three months of gestation, drugs and chemicals which are present in the maternal blood are easily delivered to the developing embryo due to the absence of functional placental barrier. Placenta starts fulfilling its protective role in early second trimester and from the middle stages of pregnancy, placenta is almost exclusively in charge of the protection of the developing fetus. Towards the term, however, it is supposed that the fetus itself starts to contribute to the multiple roles of fetoplacental unit, such as metabolism (Vagnerova et al., 2008) or efflux transport (Kalabis et al., 2007; Cygalova et al., 2008) in order to take them over completely after delivery.

Placenta has long been considered a mechanical barrier only, passively protecting the fetus by membranes separating the maternal and fetal compartments i.e. – syncytiotrophoblast, thin layer of connective tissue and vascular fetal endothelium. Over the last two decades, however, functional components have been localized in the syncytiotrophoblast that actively protects the fetus against xenobiotics from mother – these are biotransformation enzymes and ABC drug efflux transporters. While the clinical importance of drug metabolizing enzymes remains to be elucidated (Syme et al., 2004), ABC drug efflux transporters have been experimentally evidenced to play an important role in transplacental pharmacokinetics, fetal protection and detoxication (Young et al., 2003; Huls et al., 2009).

To date, many of the 48 members belonging to the ATP-binding cassette (ABC) family have been revealed in the placenta as well as in fetal tissue at the mRNA and/or protein level. Regarding functional expression, a large amount of information remains to be experimentally clarified; nevertheless, so far the best characterized and clinically relevant efflux transporters seem to be P-glycoprotein (P-gp) (Ceckova-Novotna et al., 2006) and breast cancer resistance protein (BCRP) (Mao, 2008).

Breast cancer resistance protein (BCRP, ABCG2) was first detected in a breast cancer cell line MCF-7, selected for resistance to doxorubicin, mitoxantrone and daunorubicine (Doyle et al., 1998). In parallel, high expression of a transporter with

the same structure was discovered in placenta, giving it the name "placenta-specific ABC transporter" (ABCP) (Allikmets et al., 1998). Since then BCRP has become one of the most intensively studied drug transporters, whose abundant expression was detected in many hematological malignancies and solid tumors indicating the contribution of BCRP to the multidrug resistance (MDR) phenotype in cancer chemotherapy (Bart et al., 2004; Xu et al., 2007). Importantly, BCRP has also been found to be expressed in various normal tissues, affecting pharmacokinetics of several xenobiotics as well as a number of physiologic substances (overviewed in the works of Staud and Pavek, 2005; Mao and Unadkat, 2005).

BCRP in physiological tissues

BCRP has been revealed in both human and animal tissues; however expression profiles differ significantly among species. Whilst in mice the highest expression of Bcrp1, the mouse analogue of BCRP, was found in the kidney and placenta (Wang et al., 2006a), in rat it was the intestine and male kidney (Tanaka et al., 2005). Analysis of a variety of normal human tissues revealed the highest expression of BCRP in the placenta followed by liver, brain and small intestine (Doyle et al., 1998; Langmann et al., 2003). Extensive expression of BCRP in tissue barriers, such as the blood-brain barrier (BBB), placenta or testis suggests that BCRP plays an important role in protection of sensitive tissues against xenobiotics. Studies in Abcg2-deficient mice resulted in increased intestinal uptake of a large scale of BCRP substrates compared to wild-type mice, providing strong evidence for the role of BCRP in limiting oral drug absorption (van Herwaarden et al., 2003; Merino et al., 2005; Sesink et al., 2005; Merino et al., 2006). Studies in animal models also confirmed involvement of BCRP in biliary (Merino et al., 2005; Zamek-Gliszczynski et al., 2006) and urinary (Mizuno et al., 2004) excretion of drugs, xenobiotics or endogenous compounds. The protective impact of BCRP in the BBB still appears somewhat controversial; while several studies provide evidence for functional expression of BCRP in the BBB, (Breedveld et al., 2005; Enokizono et al., 2007; Oostendorp et al., 2009), outcomes of some other studies suggest only limited role of this transporter in the brain (Lee et al., 2005; Zhao et al., 2009). BCRP was also found to be a highly conserved feature in populations of primitive stem cells from a wide variety of sources (Geschwind et al., 2001; Zhou et al., 2001; Scharenberg et al., 2002). The phenomenon that BCRP mRNA falls dramatically with the differentiation of pluripotent stem cells (Zhou et

al., 2001; Scharenberg et al., 2002) argues sharply for the protective function of BCRP in progenitor cells.

It can be assumed that the role of BCRP in fetal organs is similar to that in the adult organism and that cooperation between placental and fetal BCRP exists in the protection of fetus during its development. In this review we aim to summarize the current knowledge on BCRP expression and function in the placental and fetal tissues and to evaluate its role in protecting the developing fetus throughout pregnancy.

Methods to study BCRP in the placenta

Due to ethical restrictions and technical limitations to perform studies in pregnant women, great deal of current knowledge on BCRP expression and function in the fetoplacental unit relies on alternative approaches; these include in vitro cell line models, ex vivo perfused placenta and in vivo transgenic animals. Here we restrict ourselves only to a brief summary of experimental techniques to study BCRP and other ABC efflux transporters in the fetoplacental unit; for detailed descriptions we refer the reader to papers by Sastry (1999), Bode et al. (2006), Vahakangas and Myllynen (2006) and Mitra and Audus (2008).

In vitro methods

Cell cultures derived from the placental trophoblast or cell lines generated from malignant tissues of human or animal origin (e.g. BeWo, Jeg-3, JAr, HRP etc.) present an extensively used model of the materno-fetal barrier.

Before using any cell-based model, verification of transporter expression is imperative as not all placenta-derived cell lines express the same profile of efflux transporters as the native organ. Actually, even different clones of the same cell line, such as human choriocarcinoma BeWo, show various expression and activity of efflux transporters (Atkinson et al., 2003; Ceckova et al., 2006). We assessed that BeWo cell line (Ceckova et al., 2006) as well as rat placental HRP cells (Staud et al., 2006) express prominent levels of functional BCRP and may, therefore, present a useful tool for in vitro studies. Recently, Beghin et al. (2009) have developed and characterized a primoculture of Wistar rat trophoblast from the labyrinth zone of placenta and evaluated stable expression of Bcrp throughout the culture period. Therefore, these and other trophoblast and choriocarcinoma cell lines, in which

BCRP/Bcrp expression has been detected (Bailey-Dell et al., 2001; Evseenko et al., 2006; Serrano et al., 2007), offer suitable in vitro models to study the transport of BCRP substrates through the placental barrier.

Although in vitro models help reduce the need of experimental animals in pharmacological research, data obtained from these studies cannot fully reflect the physiological and biochemical changes in placenta; they do, however, offer a valuable tool especially for carrying out pilot experiments.

In situ methods

The method of dually perfused term human or animal placenta belongs to technically demanding yet invaluable approaches for detailed studies of transplacental pharmacokinetics. This technique enables to observe transport of substances in both maternal-to-fetal and fetal-to-maternal directions; with the use of specific substrates and/or inhibitors and concentration-dependent experiments, this approach allows for investigation of individual transport proteins or metabolic enzymes in the placental barrier. The main drawback of the perfused placental system is that it is restricted to the last days of gestation and cannot be used to study processes occurring earlier in the course of pregnancy. Nevertheless, both human (Myllynen et al., 2008) and animal (Staud et al., 2006; Cygalova et al., 2009) placenta perfusions have significantly contributed to the knowledge of BCRP/Bcrp function in the placenta as described further in the text.

In vivo methods

Genetically modified pregnant experimental animals, especially mice, are frequently used for studies of the placental barrier by in vivo techniques. This experimental approach allows analysis of the tested substance concentration in individual organs and assessing its distribution in the maternal or fetal body. Genetically modified mice from dr. Schinkel's laboratory were the first to show the importance of placental BCRP for the fetal protection (Jonker et al., 2000; Jonker et al., 2002). Many other studies using this method have followed and contributed to the field of placental transporters invaluably (Zhang et al., 2007; Vlaming et al., 2009).

Infusion of pregnant rats with a BCRP substrate and subsequent collection of fetal organs enabled us to study the activity of placental and fetal BCRP and the

fetoprotective role of the transporter at different stages of gestation (Cygalova et al., 2008).

It is important to stress out, that neither of the above models presents a satisfactory alternative to human situation in vivo. As such, several studies using different experimental approaches need to be performed and the results treated in a complex manner.

Maternal-to-fetal passage of BCRP substrates; pharmacokinetic considerations

In the transfer of BCRP substrates across the placenta, both passive and active forms of transport are involved; therefore, the net transplacental clearance in fetal-to-maternal or maternal-to-fetal direction (Cl_{fm} and Cl_{mf}, respectively) is a function of both passive diffusion and efflux transporter activity (Staud et al., 2006; Cygalova et al., 2009).

Passive diffusion of a drug across the placenta is governed by Fick's law and depends on physical-chemical properties of the molecule (such as lipid solubility, degree of ionization), surface area and thickness of the placenta, and transplacental concentration gradient. On the other hand, transporter-mediated transfer is a capacity-limited process that can be expressed in terms of Michaelis-Menten kinetics. Both types of transport can be expressed in terms of drug clearance, i.e. clearance of passive diffusion (Cl_{pd}) and efflux clearance (Cl_{efflux}).

As efflux transport in the placenta runs in fetal-to-maternal direction only, Cl_{efflux} is added to Cl_{pd} in fetal-to-maternal direction and subtracted from Cl_{pd} in maternal-to-fetal direction as follows:

$$Cl_{fm} = Cl_{pd} + Cl_{efflux} \quad (1)$$

and

$$Cl_{mf} = Cl_{pd} - Cl_{efflux} \quad (2)$$

In clearance concept, transport mediated by BCRP can be expressed as:

$$Cl_{efflux} = \frac{V_{\max}}{K_m + C_{ma(fa)}} \tag{3}$$

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. Adding equation 3 to 1 and 2 gives the final formulas describing transplacental passage of BCRP substrates:

$$Cl_{fm} = Cl_{pd} + \frac{V_{\max}}{K_m + C_{fa}} \tag{4}$$

and

$$Cl_{mf} = Cl_{pd} - \frac{V_{\max}}{K_m + C_{ma}} \tag{5}$$

Similarly to other drug efflux transporters, it can be assumed that BCRP will only result in noticeable effects in transplacental pharmacokinetics (and fetal protection) if the rate of active transport for a certain compound is considerable in comparison to passive diffusion rate, i.e. $Cl_{pd} \sim Cl_{efflux}$. If Cl_{pd} is substantially larger than Cl_{efflux}, BCRP activity will be overwhelmed by the passive diffusion of the component and Cl_{efflux} in equations 1 and 2 will have negligible effect on Cl_{mf} or Cl_{fm}, respectively. In other words, drugs with high lipid solubility (and therefore fast passive diffusion) will be less affected in their placental passage by BCRP (or other ABC transporters) than drugs with low lipid solubility. We have recently demonstrated this hypothesis experimentally by studying transplacental pharmacokinetics of various ABC transporter substrates with different lipid solubility. We observed that a rise in lipid solubility increases the passive diffusion and at the same time decreases the efflux transporter effectiveness. Even in the case of BODIPY FL prazosin, a dual substrate of both P-gp and BCRP, combined effect of both transporters was suppressed by high lipid solubility of the molecule and, therefore, rapid clearance by passive diffusion (Cygalova et al., 2009). Therefore, simple statement that a compound is a substrate of one or more placental drug efflux transporter(s) is not sufficient to forecast its lower transport from mother to fetus as the effectiveness of drug efflux transporters is markedly reduced in highly lipophilic drugs.

It is reasonable to assume that the rules outlined above will hold true also for other ABC drug efflux transporters (such as P-gp) and in other body tissues (such as intestinal or blood brain barrier).

BCRP in the embryonic phase of gestation

Embryonic phase is characterized as a period from oocyte fertilization to the formation of organ basics. The phase does not cover proportionally the same period of gestation in various species: it takes eight weeks in human and proportionally much longer time, 16 and 17.5 gd (i.e. almost 84% of gestation), in mouse and rat, respectively. Research within the early stages of gestation is obviously very difficult especially from the ethical and technical point of view. Therefore, only restricted information regarding BCRP in the embryonic fetoplacental unit in humans is available to date (Fig. 1A).

Placenta

In human, circulation of fetal blood in the placental vessels does not begin until the 21st gestation day (gd) (Enders and Blankenship, 1999). The placenta is not fully differentiated until the third month of pregnancy; therefore, there is almost no placental barrier during the embryonic period to prevent drugs and other chemicals from entering the fetal circulation (Mihaly and Morgan, 1983).

Only restricted information concerning human BCRP ontogenesis during the embryonic phase of pregnancy has been published so far. Yeboah et al. (2006) examined BCRP expression in human placentas from the 6th to 41st week of pregnancy and unveiled lower protein levels of BCRP at early stages compared to the end of gestation.

In mouse and rat, almost one quarter of the entire 19 and 21 days gestation period, respectively, occurs prior to any stable physical connection between blastocyst and uterus. The placenta becomes completely functional in the middle of pregnancy (Enders and Blankenship, 1999); shortly afterwards, Bcrp expression in the rodent placentas was found to reach its maximum and decline thereafter (Yasuda et al., 2005; Wang et al., 2006a; Kalabis et al., 2007; Cygalova et al., 2008). In more detail, Yasuda et al. (2005) revealed significantly lower levels of Bcrp mRNA and protein on the 20th gd compared to gd 14. This is in accord with our recent study on Bcrp expression in rat from the 12th gd, which showed 3 times higher expression of

Bcrp mRNA on gd 15 compared to gd 21 (Cygalova et al., 2008). Investigation of Bcrp1 expression in murine placenta during pregnancy revealed a peak of protein level on gd 15 (Wang et al., 2006a). In another study, the highest Bcrp1 mRNA expression within the mouse placenta was detected on gd 9.5 with a significant decrease thereafter (Kalabis et al., 2007). In this work, however, expression of Bcrp1 protein, as assessed by imunohistochemistry and Western immunoblots, did not change significantly during pregnancy. Using immunohistochemical methods, BCRP/Bcrp1 has been shown to be strongly expressed on the apical side of the placental syncytiotrophoblast in human and mouse (Wang et al., 2006a; Yeboah et al., 2006; Grube et al., 2007). Correspondingly, rat Bcrp was localized in the labyrinth zone of the placenta, which separates the maternal blood from the fetal vessels by trophoblast cells (Staud et al., 2006).

The extensive expression of BCRP in the placenta even during earlier phases of gestation indicates protective mechanism for the developing fetus. This hypothesis was firstly tested in P-gp knockout mice on 15.5th gd. Inhibition of Bcrp1 by GF120918, a dual inhibitor of BCRP and P-gp, increased the fetal concentration of topotecan (a BCRP substrate) by 2-fold after administration to the pregnant mice, compared with that in the control animals (Jonker et al., 2000). Another study by the same group confirmed fetoprotective role of placental BCRP in Bcrp1(-/-) mice using pheophorbide-a as a BCRP substrate (Jonker et al., 2002). Similarly, Zhang et al. (2007) observed 5 times greater fetal plasma area under the concentration-time curve of a BCRP substrate nitrofurantoin in the Bcrp knockout mice than in the wild-type pregnant mice on gd 15. We reported that in rat the relative amount of another BCRP substrate cimetidine, which penetrated from maternal circulation to the fetus, was highest on gd 12 and decreased to one tenth thereafter (Cygalova et al., 2008). Due to extensive expression of BCRP mRNA (Allikmets et al., 1998; Doyle et al., 1998), similar effect of BCRP can be expected also in human placenta.

Embryonic membranes

The placenta, however, does not present the only contact between fetal and maternal environments. Amniotic fluid is another way by which drugs and other chemicals from mother circulation can enter the fetus. Aleksunes et al. (2008) have studied the expression of various ABC transporters in mouse amniotic membranes from mid- to late gestation and revealed that the expression of Bcrp1 mRNA and

protein on gd 14 is 2- and 6-fold higher, respectively, compared to placenta. Additionally, Bcrp protein was localized in epithelial cells of the rodent visceral yolk sac from gd 9.5 with maximal values of mRNA from gd 12.5 to 14 (Kalabis et al., 2007; Aleksunes et al., 2008). Supposedly, the transporter functions in fetal membranes include exchange of endobiotics and metabolic by-products, paracrine signaling and fetal protection; however, the exact role remains to be elucidated.

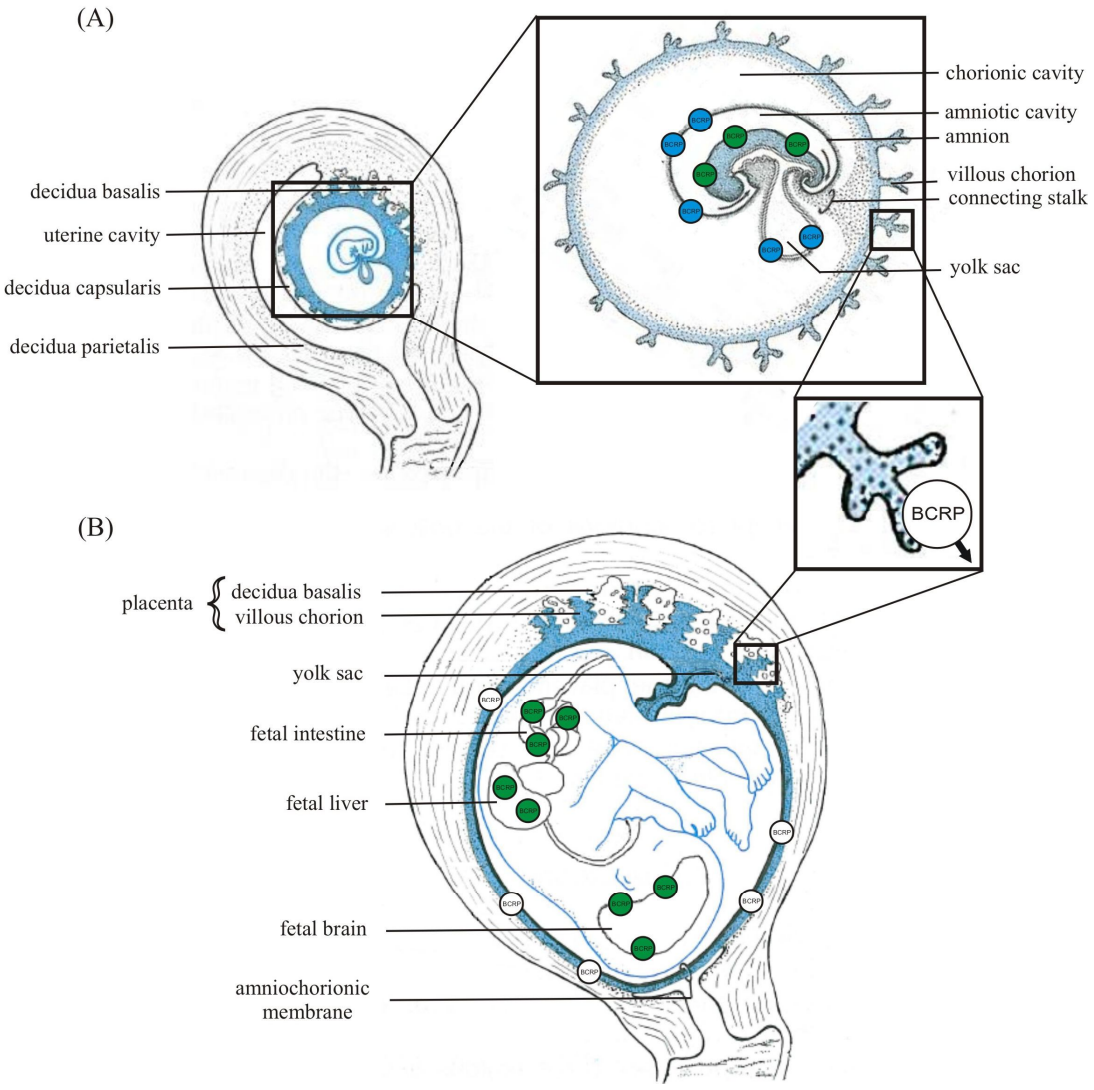

Fig. 1. Drawings of sagittal sections of the gravid uterus in the fourth (A) and twenty-second (B) week showing expression sites of BCRP within placenta, fetal membranes and developing fetus. White colour of the transporter shows locations of BCRP found in human, blue and green coloured transporter represents supposed expression according to findings in mouse or both mouse and rat, respectively. (addapted according to Keith L. Moore, Before We Are Born, 1989)

Embryo

Using in situ hybridisation and immunohistochemical staining, Bcrp1 mRNA and protein have been assessed within the mouse conceptus as early as on gd 9.5 (Kalabis et al., 2007). Our recent studies performed in rat demonstrated expression of Bcrp mRNA in the whole embryo from the 12th gd with a rising tendency towards the end of gestation (Cygalova et al., 2008). These findings indicate that during the later embryonic stage the placenta plays the main role in the protection of embryo, however embryonic own defence mechanisms are developing in order to possibly participate on placenta's protective functions during further gestational phases.

BCRP in the fetal phase of gestation

Besides intense growth, the fetal phase of gestation is characterized by proceeding of tissue and organ differentiation, which has already begun in the embryonic phase. Apart from organs being formed and prepared for the transition from intrauterine to extrauterine environment, placenta undergoes changes connected with its aging such as subchorionic or perivillous fibrin or venous blood accumulation or calcifications. Nevertheless, towards the end of gestation, fetal protection begins to be assured also by fetal mechanisms.

Placenta

Compared to the embryonic stage, substantially more information regarding BCRP expression (Fig. 1B) and function in the fetal phase of pregnancy has been obtained. The first study performed in syncytial microvillous plasma membranes isolated from human placenta didn't find any significant changes in BCRP mRNA and protein expression in the course of pregnancy (Mathias et al., 2005). However, this study did not examine tissues between the 17th week of gestation and term and was conducted with limited collection of tissue samples in which considerable variation in both mRNA and protein levels of BCRP was observed. Further studies examining BCRP expression in placentas from the 6th to 41st week of pregnancy demonstrated no changes in the mRNA levels throughout gestation; on the other hand, Western blot analysis unveiled increasing BCRP protein levels towards the end of gestation (Yeboah et al., 2006). In contrast, another deep analysis performed on a large number of human tissue samples, employing real-time RT-PCR and Western blot, revealed approximately two times lower expression of BCRP in the group of

term placentas (39 ± 2 week) compared to placentas at preterm (28 ± 2 week) (Meyer zu Schwabedissen et al., 2006). Conversely to the variable experimental outcomes on BCRP expression in human placenta, very comparable results have been achieved in all studies conducted in murine and rat placenta suggesting that the expression of Bcrp1/Bcrp is significantly lower at term compared to the previous stages of pregnancy (Yasuda et al., 2005; Wang et al., 2006a; Kalabis et al., 2007; Aleksunes et al., 2008; Cygalova et al., 2008).

Regarding functional expression of BCRP in the placenta, studies in transgenic mice were the first to confirm fetoprotective activity of the transporter (Jonker et al., 2000; Jonker et al., 2002). Using dually perfused rat placenta we have recently suggested that Bcrp not only reduces passage of its substrates from mother to fetus but also removes the drug already present in the fetal circulation against a concentration gradient (Staud et al., 2006; Cygalova et al., 2009). In the human placenta, BCRP activity was first reported using ATP-dependent uptake of mitoxantrone by microvillous membrane vesicles (Kolwankar et al., 2005) and subsequently confirmed at the whole organ level using in situ perfusion of isolated cotyledon; Pollex et al. (2008) showed that the presence of a BCRP inhibitor in the perfusion system caused a significant increase in the fetal-to-maternal concentration ratio of glyburide. Using the same technique, Myllynen et al. (2008) demonstrated that BCRP decreases the materno-fetal transfer of a food-born chemical carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP).

Fetal membranes

Bcrp expression in murine fetal membranes was found to peak in midgestation (gd 12.5) and then dramatically decrease with the progress of gestation (Kalabis et al., 2007). On gd 17 the mRNA expression was about 2-fold lower than on gd 14, however still twice as high as in placenta on the same gd (Aleksunes et al., 2008).

Immunostaing of BCRP protein was observed in human term amnion, chorion and decidua with no alteration in expression by labour (Aye et al., 2007; Yeboah et al., 2008). In contrast to cell accumulation studies, which revealed BCRP to be functionally active in human amnion epithelial monolayer cultures, transwell transport studies using intact amnion membranes failed to demonstrate significant BCRP-mediated vectorial transport (Aye et al., 2007).

These observations support the hypothesis that BCRP/Bcrp expressed in fetal membranes can play an additional role in the exchange of endobiotics and metabolic by-products, paracrine signalling and fetal protection throughout and up to the end of gestation. However, lack of vectorial transport argues more for the protection against intracellular accumulation of xenobiotics and endogenous metabolites present in the amniotic fluid (Aye et al., 2007). Additionally, vicinity of these membranes to the amniotic fluid, where the oxygen tension is low, predestinates BCRP to play a role in cellular survival under this hypoxic environment as suggested by Krishnamurthy and Schuetz (2005).

Fetus

Prominent expression of Bcrp1 mRNA and protein have been detected in fetal liver, kidney, intestines and brain capillaries of mice by in situ hybridisation and immunohistochemical staining on gd 18.5 (Kalabis et al., 2007). Investigation of rat fetuses on gd 18 and 21 resulted in detection of Bcrp mRNA in the liver, intestine and brain tissue (Cygalova et al., 2008). Apart from the liver, in which no difference in Bcrp expression between gd 18 and 21 was revealed, intestine was shown to express higher level of the transporter on gd 21 and brain on gd 18. When the expression of Bcrp in the whole fetal tissue was examined, 6.9 and 7.4-fold increase in Bcrp mRNA expression in fetuses on gds 15 and 18, respectively, was found, compared to gd 12 (Cygalova et al., 2008). Interestingly, the level of Bcrp mRNA expression in the rat liver shortly before birth was 15 times higher than that of the adult organ and gradually decreased to adult levels. Similarly, in the cerebellum Bcrp mRNA levels at birth were already at 1–1.5 times the adult level, and further increased between Days 11 and 26 to about 2–2.5 times the adult level, followed by a decrease to adult levels at Day 42 (de Zwart et al., 2008).

Employing infusion of cimetidine, a BCRP substrate, to the circulation of pregnant rat, we investigated Bcrp activity in the placenta at different stages of pregnancy; we found the ratio of fetal-to-maternal cimetidine concentrations to be almost 10 times higher on gd 12 compared to gds 15, 18 and 21 (Cygalova et al., 2008). In addition, we tested the role of Bcrp in drug disposition in the fetal organism, observing low penetration of cimetidine to fetal brain (Cygalova et al., 2008).

Considering abundant expression and function of BCRP in the fetal tissue, it can be speculated that with advancing gestation not only the BCRP expressed in the placenta, but also BCRP in fetal membranes and tissues can participate on the lowered exposure of fetus to various environmental substances.

Other than fetoprotective role of BCRP in the placenta

Abundant expression of BCRP in the placenta suggests the protein might play, in addition to fetal protection and detoxication, other role(s) in this tissue. Syncytiotrophoblast, the most important component of the placental barrier, is created by cytotrophoblasts differentiation and fusion, a process employing first steps of apoptosis. Recently, Evseenko et al. (2007c) have shown that BCRP, whose expression is up-regulated during trophoblast fusion (Evseenko et al., 2006), may represent one of the mechanisms protecting the cells from apoptosis termination. Suppression of ABCG2 expression by siRNA in BeWo cell model led to a marked increase in phosphatidylserine externalisation followed by accumulation of ceramides and increased apoptosis. These findings suggested a novel role of ABCG2 as a survival factor during the formation of the placental syncytium (Evseenko et al., 2007c). These authors also found decreased BCRP expression in placentas from pregnancies complicated with fetal growth restriction (FGR), a condition frequently associated with placental insufficiency and hypoxia. Although another study demonstrated strong up-regulation of BCRP in JAr cells by low oxygen condition (Krishnamurthy and Schuetz, 2005), no evidence of a relation between FGR, BCRP expression and hypoxia has been obtained (Evseenko et al., 2007a).

The importance of periconceptional intake of multivitamin supplements containing folic acid has been confirmed in randomized trials on birth defects (reviewed in Jenkins et al., 2007). Deficiency of folates, which are essential for a wide spectrum of biochemical reactions, can lead to several key pathologies in fetuses including neural tube defects and congenital heart defects. BCRP, which is currently the only recognized ABC transporter that exports mono- and polyglutamates of folates, has been shown to be down-regulated under conditions of both long- and short-term folate deprivation, suggesting that BCRP plays essential role in the maintenance of cellular folate homeostasis (Ifergan et al., 2004; Ifergan et al., 2005). Therefore not only folate deficiency alone but also increased exposure to

xenobiotics due to consequently lowered BCRP expression can jeopardize correct fetal development.

Regulation of BCRP expression

BCRP expression has been reported to be under the control of both endogenous and exogenous factors; in the placenta, gestation-related hormones are among the most intensively studied factors regulating BCRP expression (Ee et al., 2004b; Imai et al., 2005; Zhang et al., 2006; Wang et al., 2007). Furthermore, certain pathological conditions, such as infection or inflammation, may also affect expression of ABC efflux transporters including BCRP (Petrovic et al., 2007). In addition, over 80 naturally occurring sequence variations in the *ABCG2* have been reported (Robey et al., 2009) that may cause interidividual variations in expression and function of placental BCRP (Kobayashi et al., 2005; Atkinson et al., 2007). As a consequence, the protective role of the placenta may vary not only intraindividually, i.e. at different stages of pregnancy as discussed elsewhere in this review, but also interindividually, i.e. among population.

Various hormonal factors have been investigated for the possibility to regulate the expression of BCRP in the placental tissue. Identification of estrogen response element (ERE) in the promoter of BCRP (Ee et al., 2004b) led to studies on pregnancy-related hormones impact on the BCRP expression. Concerning estradiol (E_2), both up-regulation (Ee et al., 2004a; Ee et al., 2004b; Evseenko et al., 2007b) and down-regulation of BCRP (Imai et al., 2005; Wang et al., 2006b) have been observed in mammalian cells. According to a study in which BCRP was driven by its endogenous promoter, expression of BCRP is more likely up-regulated by 17β-estradiol (Zhang et al., 2006). The effect of progesterone (P_4) on the expression of BCRP in BeWo cell line was examined by two groups also with contradictory results. After treatment with similar concentration of P_4, Yasuda et al. (2005) observed reduction in the expression levels of BCRP mRNA and protein; on the contrary, Wang et al. (2006) detected increase in BCRP expression in the same cell line. In addition, combination of P_4 and E_2 further increased up-regulation of BCRP via E_2 mediated induction of progesterone receptor B (PR_B) mRNA. The authors suggested that PR_B endogenous expression in BeWo cells is low and that E_2 induction through estrogen receptor (ER) is needed to exert PR_B function (Wang et

al., 2006b). On the other hand, in a follow up study from the same laboratory, progesterone effect on the BCRP expression via progesterone receptor A (PB_A) was found to be rather insignificant (Wang et al., 2008). Testosterone alone was not found to significantly affect BCRP expression; however when combined with E_2, testosterone increased BCRP mRNA and protein levels in BeWo cells, possibly through ERα–mediated induction of testosterone receptor (TR) (Wang et al., 2007). Other pregnancy-related hormones, human placental lactogen and human prolactin in combination with E_2, were also reported to induce BCRP expression at mRNA and protein levels (Wang et al., 2007).

Down-regulation of BCRP mRNA and protein expression in trophoblast cells was observed after tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) treatment (Evseenko et al., 2007b). Similarly, in the placentas of endotoxin-treated rats, a significant time- and dose-dependent down-regulation of BCRP at both mRNA and protein levels was demonstrated (Petrovic et al., 2008). As a result of lowered BCRP expression in the placenta, these authors observed a significant increase in the distribution of glyburide, a BCRP substrate, into fetal tissues of these rats. Since elevated levels of several pro-inflammatory cytokines and endotoxins are believed to be associated with some pregnancy disorders, such as placental insufficiency, preeclampsia, gestational diabetes or microbial inflammation, decreased protective function of BCRP against various xenobiotics should be taken into account.

Several environmental toxins as well as medical compounds were shown to regulate expression of many placental proteins, including biotransformation enzymes and transporters. To date, however, only little information is available regarding the effect of these factors on expression of placental BCRP. In the study of Kolwankar et al. (2005), maternal smoking induced CYP1A1 levels in the placenta, but had no effect on expression or activity of placental BCRP. These findings suggest that women who smoke during pregnancy are not at risk of altered xenobiotic transport, at least by BCRP, as compared with nonsmoking pregnant women (Kolwankar et al., 2005). In a study by Myllynen et al. (2008) a 48-hour exposure to 1 μM PhIP, a food-born chemical carcinogen, induced mRNA expression of ABCG2 in BeWo choriocarcinoma cells. However, a clear down-regulation of ABCG2 expression was seen after the exposure to 10 μM PhIP after a 48-hour exposure suggesting posttranscriptional regulation of ABCG2 protein expression.

So far over 80 naturally occurring sequence variations in the *ABCG2* gene with varying frequency among ethnic groups have been reported (Imai et al., 2002; Backstrom et al., 2003; Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005). The nonsynonymous C421A single nucleotide polymorphism (SNP) that results in a glycine to lysine (Q141K) amino acid change has been linked to decreased plasma membrane expression of ABCG2 (Imai et al., 2002; Morisaki et al., 2005) and consequently altered drug pharmacokinetics (Sparreboom et al., 2004; Sparreboom et al., 2005; Zamboni et al., 2006). The SNP G34A results in poor localization of the protein and a resultant decrease in efflux activity (Mizuarai et al., 2004). Both G34A and C421A occur at a relatively high frequency in most ethnic populations (Zamber et al., 2003; Kobayashi et al., 2005). To date, only restricted knowledge about the effect of BCRP polymorphisms on its expression and activity in the placenta is available. In placentas of the Japanese population, Kobayashi et al. (2005) illustrated that the C421A variant of the *BCRP* gene led to decreased BCRP protein level caused by posttranscriptional regulation rather than changes in mRNA expression because polymorphism-dependent changes in mRNA expression were not observed. In addition, a tissue-dependent allele-specific expression profile of *BCRP* is suspected (Kobayashi et al., 2005).

From the information above it is evident that several endogenous as well as exogenous factors along with gene polymorphisms affect expression of placental BCRP at mRNA and protein levels. However, many other studies will be necessary to quantify possible effects of these factors on intra-/interindividual variations in BCRP expression and its protective function in the placenta.

Clinical significance and future perspectives

Recent pharmacoepidemiological studies have indicated that 64% of all pregnant women met the prescription of a drug other than vitamin or mineral supplement during 270 days before delivery (Andrade et al., 2004). In another study, 1.1% pregnant women received a teratogenic drug and 5.8% women received U.S. FDA category D or X drugs (Andrade et al., 2006). It needs to be pointed out that it is not only the mother that is the target of therapy. Some compounds, such as digoxin (Ito, 2001; Oudijk et al., 2002) or antiretrovirals (Mirochnick, 2000; Taylor and Low-

Beer, 2001; Capparelli et al., 2005) are frequently used for fetal, i.e. transplacental therapy. In this case, mother takes the medicine and transplacental passage has to occur to ensure drug availability for treating the fetus. For these reasons, it is obvious, that detailed knowledge of transplacental pharmacokinetics, including the role of efflux transporters, is crucial for optimizing drug choice, dosage schemes and to avoid/exploit possible drug-drug interactions on placental efflux transporters. Since BCRP transports a wide range of substrates from many pharmacotherapeutic groups (see Table 1), including e.g. oral antidiabetics, antivirals, anticancer compounds, its significant role in transplacental pharmacokinetics may be assumed. In the following text we summarize recent information on BCRP role in several clinically relevant situations related to pregnancy.

Gestational diabetes mellitus (GDM) is a typical example of pregnancy complications, affecting about 5% of women, that needs to be medically treated. Parenteral use of insulin is the obvious drug of first choice, however, under certain situations in which insulin is inaccessible or inconvenient, oral antidiabetics might be considered a suitable alternative. Many of these low molecular compounds, in contrast to insulin, cross the placenta by passive diffusion and may present a potential hazard such as severe neonatal hypoglycemia or even malformations for the developing fetus. Glyburide, a second generation sulfonylurea derivative, seems to be an exception, reaching very low fetal concentration. Elliot et al. were the first to observe insignificant transport of glyburide across in situ perfused human placental cotyledon (Elliott et al., 1991; Elliott et al., 1994). The low permeability of the drug through the placenta was originally attributed to its high plasma protein binding and short elimination half-life (Koren, 2001; Nanovskaya et al., 2006). Recently, however, it has been revealed in human as well as animal models that transplacental passage of glyburide is controlled by BCRP (Gedeon et al., 2008; Pollex et al., 2008; Zhou et al., 2008; Cygalova et al., 2009). A randomized, controlled trial failed to detect measurable glyburide levels in umbilical cord blood after delivery and concluded that glyburide and insulin are equally efficient for treatment of GDM at all levels of disease severity (Langer et al., 2000; Langer et al., 2005). A recent meta-analysis comparing benefits and risks of oral diabetes agents with insulin in women with GDM found no substantial maternal or neonatal outcome differences with the use of glyburide or metformin compared with insulin (Nicholson et al., 2009). Based

on recent clinical trials, glyburide seems to be as safe and effective as insulin in the management of GDM and owing to its ease of administration, convenience and low cost, glyburide was forecasted to become the first line of medical treatment in patients with gestational diabetes mellitus within the next few years (Melamed and Yogev, 2009).

Epilepsy is one of the most common neurological diseases, affecting about 0.5 to 1% of pregnant women (Myllynen et al., 2005). If seizures are not sufficiently controlled in pregnancy, they may result in maternal trauma, placental abruption, fetal hypoxia, and increased risk of congenital malformations. Many antiepileptic compounds (AEDs) are used during pregnancy to treat epilepsy despite their known teratogenicities. As suggested by Atkinson et al. (2007), fetal exposure to AEDs may be influenced by drug transporting proteins in the placenta, including P-gp, multidrug resistance protein (MRP) 1, and BCRP. Genetic variations in the expression and activity of these transport proteins may also influence fetal exposure to AEDs and the risk of teratogenicity (Atkinson et al., 2007). However, in vitro and in vivo studies indicated that fetal concentrations of many AEDs were equivalent to or even exceeded those in the maternal circulation questioning the role of ABC transporters in transplacental passage of AEDs (Myllynen et al., 2005). In addition, we performed a series of in vitro studies to test interactions between commonly used AEDs of all generations with BCRP. We found that none of the tested compounds (phenobarbital, phenytoin, ethosuximide, primidone, valproate, carbamazepine, clonazepam, and lamotrigine) was a substrate or inhibitor of BCRP (Cerveny et al., 2006). Based on these studies, the relevance of BCRP to fetal protection against AEDs remains questionable.

Current strategy to treat HIV infected patients consists of highly active anti-retroviral therapy (HAART) that involves administration of multiple antiretroviral drugs acting at different steps of the HIV life cycle. Treatment of HIV-infected pregnant women is an example of clinical situations in which transplacental passage of the drug is desirable to medicate not only the mother but also fetus to reduce the rate of vertical viral transmission (Gulati and Gerk, 2009). However, since many antiviral drugs, especially protease/transcriptase inhibitors, are substrates of P-gp and/or BCRP and show rather limited maternal-to-fetal transport (Forestier et al.,

2001; Wang et al., 2003; Wang et al., 2004; Molsa et al., 2005; Sudhakaran et al., 2005; Gavard et al., 2006; Sudhakaran et al., 2008), inhibition of placental BCRP (or other efflux transporters) would be profitable. Importantly, many of anti-HIV drugs, including ritonavir, saquinavir, or nelfinavir, are recognized BCRP inhibitors (Gupta et al., 2004; Weiss et al., 2007). Ritonavir, a protease inhibitor, is often included in HAART not only for its own anti-HIV activity, but also as an inhibitor of biotransformation enzymes and efflux transporters, thus causing an increase in concentrations of the HIV protease inhibitors (Gulati and Gerk, 2009). We may speculate that ritonavir inhibits also placental and fetal BCRP and, therefore, increases transplacental passage and fetal concentrations of concomitantly administered anti-HIV drugs.

Several case reports in the literature suggested that placental BCRP and/or other efflux transporters may play an important role in protecting the fetus against anticancer agents. For example, successful maternal treatment outcome as well as normal fetal delivery and development were reported in a 21-year-old white woman diagnosed with Burkitt's lymphoma at 26 weeks' gestation and aggressively treated till the end of pregnancy. The systemic intensive polychemotherapy included doxorubicin, a BCRP/P-gp substrate (Lam, 2006). In another report, no congenital anomalies were observed in a baby delivered by 41-year-old woman affected with multiple sclerosis that conceived during therapy and continued treatment with mitoxantrone, a BCRP and P-gp substrate until 29th week of pregnancy (De Santis et al., 2006). In addition, mitoxantrone (potential human teratogen contraindicated during pregnancy) administered during the second trimester has also been reported without adverse fetal outcome (Azuno et al., 1995). These studies are definitely not conclusive, but they do demonstrate potential usefulness of some so far contraindicated drugs during pregnancy and the necessity of collecting and retrospectively analyzing all clinical data from the past.

Urinary tract infections (UTIs) are a common complication of pregnancy, caused by several anatomical and hormonal changes occurring in pregnant women. If left untreated they can lead to pyelonephritis, low-birth-weight infants, premature delivery, and, occasionally, stillbirths (Lee et al., 2008); therefore, prompt treatment is extremely important. Nitrofurantoin, U.S. FDA category B drug, has long been

considered an effective and safe agent in the therapy of UTIs during the whole pregnancy (Ben David et al., 1995; Czeizel et al., 2001). The relative safety of the drug in pregnancy might be, at least partly, explained by its affinity to BCRP transporter; limited levels of nitrofurantoin observed in fetal tissues of wild-type compared to Bcrp1(-/-) pregnant mice suggest that BCRP is very likely the transporter that restricts fetal exposure to nitrofurantoin in humans (Zhang et al., 2007).

Conclusion

The aforementioned observations summarize the current knowledge on expression and function of BCRP efflux transporter in the fetoplacental unit and its role in fetal protection and detoxication. Drug-drug interactions, stage of pregnancy, maternal and fetal genotype, gene regulations and polymorphic variants - all these factors influence, alone or in conjunction, transplacental pharmacokinetics and subsequently drug levels in the fetal circulation.

It is obvious that detailed understanding of the pharmacology of placental and fetal BCRP will have advantageous impact on drug use during pregnancy. However, to date, our knowledge of BCRP transporter in the placenta is still too limited to be fully exploited in clinical trials or settings. Many other retrospective pharmaco-epidemiological analyses, preclinical testings at cellular, animal, and organ levels will be necessary to elucidate the complex issue of materno-fetal transport of medical compounds.

Table 1. Selected substrates and inhibitors of BCRP

Substrates	***Inhibitors***
Anticancer compounds	**Acridone carboxamide derivative**
mitoxantrone, bisantrene, BBR-390	GF120918
methotrexate	***Aspergillus fumigatus* product**
topotecan, irinotecan, diflomotecan, SN-38	fumitremorgin C and anologues Ko132, Ko134
NB-506, J-107088	tryprostatin A
CI1033	**Kinase inhibitors**
imatinib, gefitinib, erlotinib	gefitinib (Iressa, ZD1839)
flavopiridol	imatinib (Gleevec, STI571)
daunorubicin, doxorubicin, epirubicin	EKI-785
Antivirotics	CI1033
lamivudine, zidovudine, abacavir	UCN-01
Antibiotics	**Pipecolinate derivative**
ciprofloxacin, ofloxacin, norfloxacin,	biricodar (VX-710)
erythromycin	**Immunosuppressants**
Calcium channel blockers	cyclosporine A, tacrolimus, sirolimus
azidopine, dipyridamole	**Calcium channel blockers**
H_2-antihistaminics	dipyridamole, nicardipine, nitrendipine,
cimetidin	nimodipine
Antidiabetics	**Antibiotic**
glyburide	novobiocin
Chemotherapeutics	**Estrogens**
nitrofurantoin	estron
HMG-CoA reductase inhibitors	diethylstilbestrol
rosuvastatin, pitavastatin, cerivastatin	**Estrogen antagonits**
Endogenous molecules	tamoxifen and derivates (TAG-11,
estrone-3-sulfate	TAG0139)
17β-estradiolsulfate	**Rauwolfia alkaloid**
17β-estradiol 17-(β-glucuronide)	reserpin
dehydroepiandrosterone	**Flavonoids**
protoporphyrin IX	chrysin, biochanin A, quercetin,

Phytoestrogens	gensitein
genistein, daidzein, coumestrol, quercetin,	**HIV protease inhibitors**
Vitamins	ritonavir, saquinavir, nelfinavir
folic acid	**Proton pump inhibitor**
riboflavin (vitamin B_2)	omeprazole
biotin (vitamin B_7)	
vitamin K_3	
Organic molecules	
BODIPY-prazosin	
Hoechst 33342	

Summarized from: Doyle and Ross (2003), Schinkel and Jonker (2003), Sarkadi et al. (2004), Mao and Unadkat (2005), Staud and Pavek (2005), Ahmed-Belkacem et al. (2006), Robey et al. (2009), Vlaming et al. (2009).

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III

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: Expression and transport activity of breast cancer resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line. J Pharmacol Exp Ther. 2006 Oct;319(1):53-62.

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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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ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is a member of the ATP-binding cassette transporter family that recognizes a 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 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 fumitremorgin C (2 μM) or *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (GF120918; 2 μM) and abolished at high cimetidine concentrations (1000 μM). When fetal perfusate was recirculated, Bcrp was found to actively remove cimetidine from the fetal compartment to the maternal compartment even against a concentration gradient and to establish a 2-fold maternal-to-fetal concentration ratio. Based on our results, we propose a two-level defensive role of Bcrp in the rat placenta in which the transporter 1) reduces passage of its substrates from mother to fetus but also 2) removes the drug already present in the fetal circulation.

Placenta is an organ that brings maternal and fetal blood circulations into proximity, allowing mutual interchange of nutrients and waste products. Conversely, placenta forms a barrier to protect the fetus against harmful endo-and exogenous compounds from maternal circulation. As a barrier, the human and rodent placenta had for long been supposed to present only a mechanical obstruction formed by fetal endothelia, basal membranes, and syncytiotrophoblast. However, over the past two decades, a 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; Syme et al., 2004). These proteins are thought to strengthen, in an active and capacity-limited manner, placental barrier role and help in protecting 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 (P-gp; ABCB1), breast cancer resistance protein (BCRP; ABCG2) and multidrug resistance-associated proteins 1 and 2 (ABCC1 and ABCC2), all of which were found to be responsible for the phenomenon of multidrug resistance in cancer therapy (Fischer et al., 2005). In addition, because of their extensive distribution in nontumorous tissues and wide substrate specificity, these proteins

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ABBREVIATIONS: ABC, ATP-binding cassette; P-gp, P-glycoprotein; BCRP/Bcrp, human/rodent breast cancer resistance protein; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; RT-PCR, reverse transcription-polymerase chain reaction; mdr/MDR, multidrug resistance; bp, base pair(s); ANOVA, analysis of variance; Ko143, 3-(6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12*a*-octahydro-pyrazino[1′,2′:1,6]pyrido[3,4-*b*]indol-3-yl)-propionic acid *tert*-butyl ester; PSC833, [3′-keto-Bmt[1]]-[Val[2]]-cyclosporine.

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).

BCRP is the most recently described member of the ABC 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). Because its tissue distribution and substrate specificity overlap noticeably with that of P-gp, it is generally thought that these transporters share a 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 and toxins) and endogenous (steroid conjugates, and porphyrins) compounds (Staud and Pavek, 2005), it is reasonable to assume that BCRP may be an important component of the placental barrier. 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.

placental physiology and pharmacology (Stulc et al., 1995; Kertschanska et al., 2000). In our previous studies, we used this experimental model to evaluate functional activity of P-gp in the rat placenta (Pavek et al., 2001, 2003).

The aim of the present study was to assess the effect of Bcrp on transplacental passage of its substrates. We investigated Bcrp activity both in vitro, using HRP-1 rat placental cell line, and in situ in dually perfused rat term placenta. Using these techniques, we describe Bcrp as an active component of the rat placental barrier that limits maternal-to-fetal and facilitates fetal-to-maternal transport of its substrates.

Materials and Methods

Reagents and Chemicals. Cimetidine and radiolabeled [*N-methyl*-^{3}H]cimetidine were purchased from Sigma-Aldrich (St. Louis, MO) and from GE Healthcare (Little Chalfont, Buckinghamshire, UK), respectively. BODIPY FL prazosin, a common BCRP and P-gp substrate, was obtained from Invitrogen (Carlsbad, CA). Specific BCRP inhibitors Ko143 and fumitremorgin C were donated by Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) or purchased from Alexis Corporation (Lausanne, Switzerland), respectively. Specific P-gp inhibitors, PSC833 and cyclosporine, were gifts kindly provided by Dr. Andrýsek (Ivax Pharmaceuticals, Opava, Czech Republic). Dual BCRP and P-gp inhibitor GF120918 was from GlaxoSmithKline (Greenford, UK). 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 (University of Kansas Medical Center, Kansas City, KS) were used. They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. Cells from passages 15 to 25 were used in experiments described herein.

Animals. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles Univer-

(Novotna et al., 2004). Sequences of mRNAs for target genes were obtained from National Center for Biotechnology Information data base; primers for *mdr1a*, *mdr1b*, and *bcrp* genes were designed using the Vector NTI Suite software (Informax, Bethesda, MD) and are given in Table 1.

RT-PCR analysis was performed on iCycler iQ (Bio-Rad, Hercules, CA). cDNA was amplified with HotStart *Taq* polymerase under the following conditions: 3 mM $MgCl_2$, 0.2 mM dNTP, 0.03 U/μl poly-

TABLE 1
Sequences and specifications of primers used in RT-PCR

Gene	Accession No.	Sequence 5′→3′	Product Length	Localization
			bp	
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

merase, SYBR Green I in 1:100,000 dilution, and 0.3 μM of each primer; the temperature profile was 95°C for 14 min; 50 times: 95°C for 15 s, 60°C for 20 s, 72°C for 20 s, and 72°C for 5 min; and melting curve program was 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 (25–766 bp) (New England BioLabs, Hertsfordshire, UK).

Western Blot Analysis. Cell membrane fractions of placenta tissues and whole-cell lysates were prepared as described previously (Novotna et al., 2004; Ceckova et al., 2006). Protein contents were determined by BCA Protein Assay detection kit (Pierce Chemical, Rockford, IL). 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). After blocking in 5% nonfat dry milk blocking buffer, the membranes were probed with mouse monoclonal anti-P-gp antibody C219 (Signet Laboratories, Dedham MA) and rabbit polyclonal anti-ABCG2 antibody M-70 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (1:500 in 1% blocking buffer overnight 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 room temperature) 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).

Immunohistochemical Localization of Bcrp in the Rat Term Placenta. Preparation of rat placental tissue was performed as described previously (Pavek et al., 2003). The antigen (Bcrp) was unmasked by heating the specimens in sodium citrate buffer, pH 6.0, two times for 8 min each in a microwave oven at 750 W. Slides were incubated with a polyclonal primary antibody for BCRP (M-70; Santa Cruz Biotechnology, Inc.) diluted 1:10 in bovine serum albumin for 15 to 18 h at 4°C. Subsequently, the slides were developed with the secondary antibody goat anti-rabbit IgG conjugated to peroxidase-labeled polymer (DAKO EnVision, Dako North America Inc., Carpinteria, CA) for 30 min. The reaction was visualized using diaminobenzidine (diaminobenzidine substrate-chromogen solution; Dako North America Inc.), and the sections were counterstained by hematoxylin. Slides were examined using computer image analysis (Nikon Eclipse E200 light microscope; Nikon, Tokyo, Japan; PixeLINK PL-A642 digital firewire camera; Vitana Corp., Ottawa, ON, Canada; and 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 (1×10^5/well) 2 days before the experiments. Cell culture medium was removed, and cells were washed twice with 500 μl of prewarmed 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 and lysed in 1% SDS, and fluorescence was measured after 24 h (Genios Plus; Tecan, Salzburg, 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). In brief, one uterine horn was excised and submerged in heated Ringer's 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 fetuses by ligatures. The umbilical artery was catheterized using 24-gauge catheter connected to the fetal reservoir and perfused 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 preweighed glass vials to check a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments as described previously (Pavek et al., 2001). At the end of experiment, placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue, and dissolved in tissue solubilizer (Solvable; PerkinElmer Life and Analytical Sciences, Boston, MA), and its radioactivity was measured to detect tissue-bound cimetidine.

Two types of perfusion systems were used in this study. For pharmacokinetic analysis of concentration-dependent transplacental passage of cimetidine, both maternal and fetal sides of the placenta were perfused in open-circuit systems, without recirculation of the perfusate. 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 an approximately 10-min stabilization period before sample collection started (time 0). Fetal effluent was sampled into preweighed vials in 5-min intervals and analyzed for [^{3}H]cimetidine.

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-min stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (200 μl) were collected every 10 min from the maternal and fetal reservoirs, and [^{3}H]cimetidine concentration was measured. This experimental setup ensures steady cimetidine concentration on the maternal side of the placenta and enables investigations of maternal/fetal concentration ratio; any net transfer of the drug implies transport against a concentration gradient and is evidence for active transport.

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 maternal or fetal reservoir, respectively, in one of the following concentrations: 0.005, 0.1, 1, 30, 100, or 1000 μM. The inflowing cimetidine concentration was maintained constant for the duration of the experiment; transplacental clearances of cimetidine were calculated for every concentration from all measured intervals as described below.

To study the effect of BCRP and P-gp inhibitors, 2 μM fumitremorgin, 2 μM GF120918, 10 μM cyclosporine, or 25 μM verapamil was added to the maternal or fetal reservoir in the 10th min of perfusion. Subsequently, transplacental clearance of cimetidine in the period of 0 to 10 min (without inhibitor) was compared with that in 20 to 30 min (with inhibitor), leaving the mid-interval of 10 to 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, 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 in open-circuit perfusion system. Averaged data from the intervals of 0 to 10 min (control) and 20 to 30 min (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 eq. 1.

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

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 eq. 2 (Shargel and Yu, 1993):

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

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 eq. 3:

$$Cl_{Tfm} = \frac{ER \cdot Q_f}{w_p} \quad (3)$$

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 Cl_{Tmf} and Cl_{Tfm} are described by eqs. 4 and 5, respectively:

$$Cl_{Tmf} = Cl_{pd} - Cl_{efflux} \quad (4)$$

$$Cl_{Tfm} = Cl_{pd} + Cl_{efflux} \quad (5)$$

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

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

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 eq. 4 yields the following equation, which was used to fit clearance versus inflow concentration data:

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

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

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

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).

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

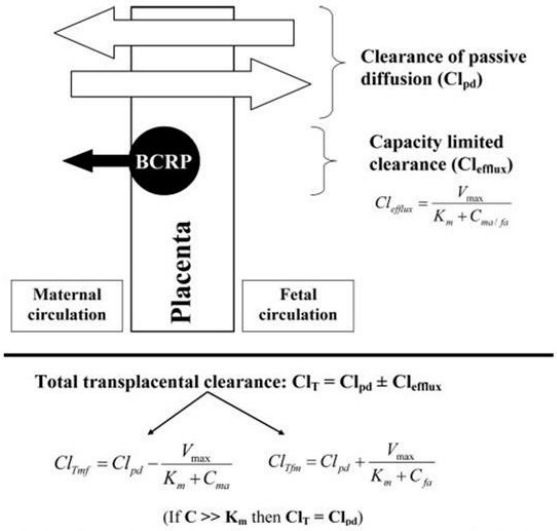

Fig. 1. Schematic depiction of pharmacokinetic analysis applied in this study to evaluate efflux transporter activity in the placenta. This model assumes two processes involved in the transplacental passage: 1) passive diffusion governed by Fick's law (depending mainly on drug's physical-chemical properties, concentration gradient, protein binding, and membrane area and thickness), here described as Cl_{pd} and 2) saturable efflux process governed by the rules of Michaelis-Menten nonlinear kinetics, here described as 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 Cl_T. If substrate concentration largely exceeds the Michaelis-Menten constant ($C \gg K_m$), then total placental clearance equals to clearance of passive diffusion. Cl_{Tmf} and Cl_{Tfm} are described under *Pharmacokinetic Analysis of Efflux Transport Activity in the Placenta*.

Results

Expression of Bcrp and P-gp in Rat Placenta and HRP-1 Cells. RT-PCR and Western blotting were used to investigate the expression of Bcrp and P-gp in the rat placenta and HRP-1 cell line and compared with that in kidney as a positive control (Tanaka et al., 2005). 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 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. Likewise, 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, whereas no signal for P-gp was detected in HRP-1 cell lysate (Fig. 2B).

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 placentas (gestation day 21) were used for the experiments. The rat placenta is composed of two morphologically different zones, the junctional zone (maternal blood spaces separated by trophoblastic trabeculae that do not contain fetal blood vessels) and the labyrinth zone (maternal blood separated from fetal blood vessels by trophoblast cells). Bcrp was detected in the inner

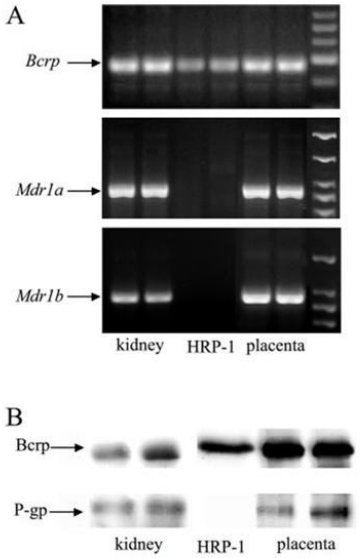

Fig. 2. A, mRNA expression of the rat Bcrp and P-glycoprotein (Mdr1a and Mdr1b) in rat kidney, placenta, and HRP-1 rat placental cell line. B, Western blot immunoanalysis of protein expression of the rat Bcrp and P-gp in the rat placenta and kidney and in the HRP-1 rat trophoblast cell line. Kidneys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

layers of the syncytiotrophoblast (layers II and III) of the labyrinth zone only (Fig. 3). No Bcrp staining was visible in either layer I or in the fetal capillaries.

Bcrp Efflux Activity in HRP-1 Cell Line. To investigate Bcrp and/or P-gp Activity in HRP-1 Placental Cells, 500 nM BODIPY FL prazosin 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% ($p < 0.05$). In contrast, 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).

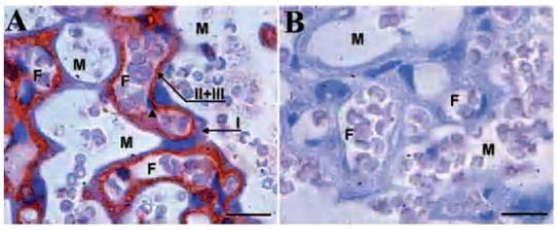

Fig. 3. Immunohistochemical detection of Bcrp in the labyrinth of rat term placenta. Immunohistochemical staining was performed with the monoclonal antibody M-70 (1:10 dilution). A, strong reactivity for Bcrp is visible in the inner layers (II and III) of syncytiotrophoblast. Fetal capillaries or layer I of syncytiotrophoblast do not reveal any positivity. B, for negative control, the slides were treated in the same manner, except nonimmune isotype-matched immunoglobulins were substituted for the primary antibody to Bcrp. F, fetal capillaries; M, maternal blood. Arrowhead shows nucleus of endothelial cell of the fetal capillary, and arrows point to layer I and layers II + III of syncytiotrophoblast (hematoxylin counterstained). Scale bars, 30 μm.

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 min. 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 10th min of perfusion, it took approximately 5 to 10 min to achieve a new steady state (data not shown). Therefore, to evaluate the effect of inhibitor on cimetidine transplacental passage, samples from the 0- to 10-min interval of perfusion were averaged and compared with those collected in 20- to 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 Madin-Darby canine kidney II 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 GF120918-sensitive cimetidine transporter.

Effect of Inflow Cimetidine Concentrations on Transplacental Clearance. Cimetidine was infused to maternal or fetal side of the placenta at one of the following concentrations: 0.005, 0.1, 1, 30, 100, or 1000 μM. In both maternal-to-fetal and fetal-to-maternal transport studies, increase in cimetidine concentration caused significant change in transplacental clearance, confirming nonlinearity 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, whereas at concentrations above 30 μM, inhibitor was rather ineffective.

Fitting experimental data with eqs. 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. Conversely, Cl_{efflux} is a concentration- and inhibitor-dependent parameter. At substrate concentrations largely exceeding the Michaelis-Menten constant ($C \gg K_m$), the transporter is saturated, the nonlinear fraction of eqs. 7 and 8 approaches zero, and both equations are reduced to linear processes only [total transplacental clearance (Cl_T) = Cl_{pd}]; under these conditions, transplacental pharmacokinetics is beyond any quantifiable effect of efflux transporter and is

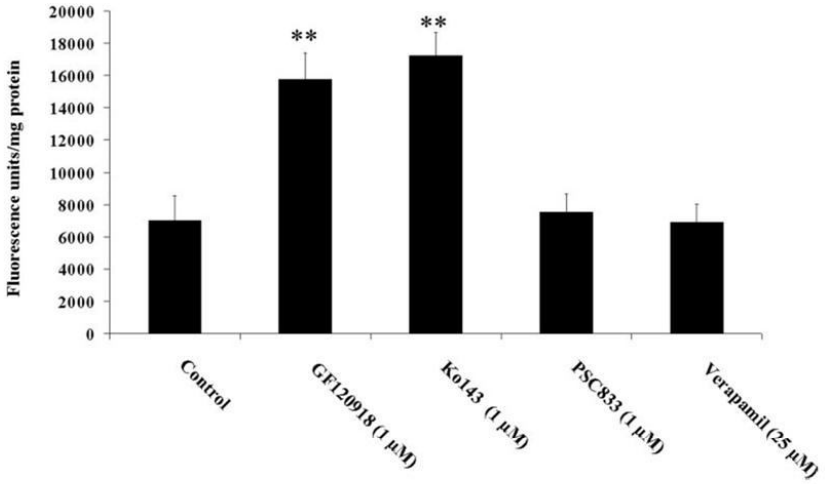

Fig. 4. Uptake of common BCRP and P-gp substrate BODIPY FL prazosin by rat placental HRP-1 cells. BODIPY FL prazosin was added to cells without inhibitor (control) or in the presence of BCRP or P-gp inhibitors. Both GF120918 and Ko143 (BCRP inhibitors) significantly increased accumulation of BODIPY FL 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 ± S.D. of four experiments. One-way ANOVA followed by Bonferroni's test was used; **, $p < 0.05$ compared with control.

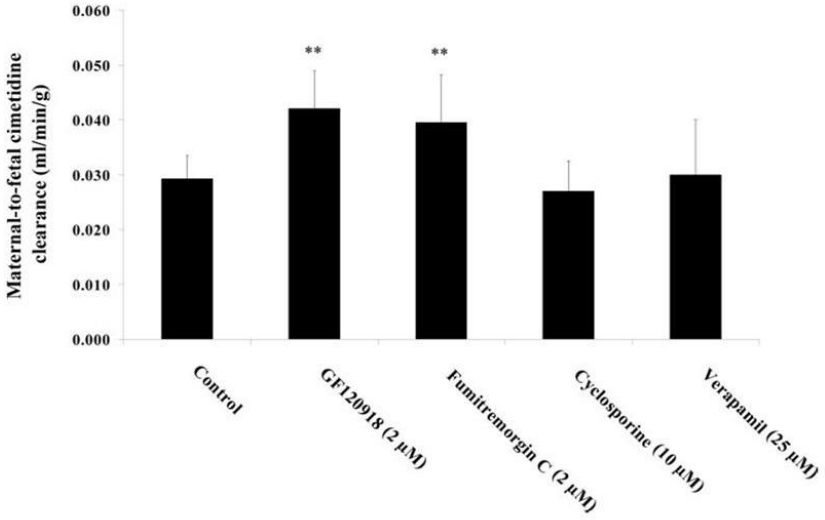

Fig. 5. Effect of Bcrp and P-gp inhibitors on maternal-to-fetal clearance of cimetidine. Cimetidine and [^{3}H]cimetidine tracer were added to the maternal compartment at a concentration of 0.1 μM, and its radioactivity was measured in fetal venous outflow. Total transplacental clearance was calculated by eq. 1 (see *Materials and Methods*). Inhibitor was added to the maternal perfusate in the 10th min. Only BCRP inhibitors GF120918 and fumitremorgin C affected transplacental clearance of cimetidine, whereas P-gp inhibitors cyclosporine and verapamil had no significant effect. Data are presented as means ± S.D. of six experiments. One-way ANOVA followed by Bonferroni's test was used; **, $p < 0.05$ compared with control.

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 1000 μM. Furthermore, because addition of inhibitor caused no change in transplacental clearance of 1000 μM cimetidine (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 ($p < 0.05$) than that in the opposite direction (Fig. 8). At a concentration of 1000 μM, 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; Table 2).

Bcrp Transports Cimetidine from Fetus to Mother against a Concentration Gradient. To investigate the potential of Bcrp to remove its substrate from fetal circulation, cimetidine was added to both maternal and fetal reservoirs at equal concentrations of 0.005 or 1000 μM and fetal perfusate was recirculated. At a low drug concentration (0.005 μM), cimetidine in the fetal circulation steadily decreased and stabilized after approximately 40 min of perfusion. Fetal-to-maternal concentration ratio reached a value of 0.49 toward the end of the experiment. Decrease in fetal cimetidine was blocked by co-infused BCRP inhibitors (GF120918 or fumitremorgin C; Fig. 9A). At a high cimetidine concentration (1000 μM), maternal and fetal concentrations remained unchanged throughout the perfusion period with fetal/maternal concentration ratio staying close to 1

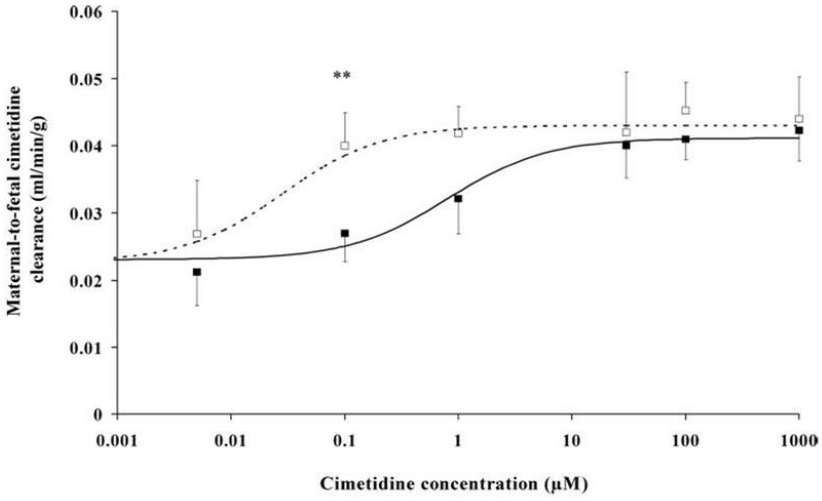

Fig. 6. Transport of cimetidine across the dually perfused rat placenta in maternal-to-fetal direction. Cimetidine with [^{3}H]cimetidine tracer was added to the maternal reservoir, and its radioactivity was measured in fetal venous outflow. Total transplacental clearance was calculated by eq. 1 (see *Materials and Methods*). Changes of clearance with increasing cimetidine concentration confirm nonlinearity 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 (1000 μM), clearance reached the value of 0.042 ml/min/g, and inhibitor activity was negligible. Experimental values are presented as means ± S.D. 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. Asterisks indicate significant ($p < 0.05$) effect of inhibitor. ■, cimetidine without inhibitor; □, cimetidine with GF120918.

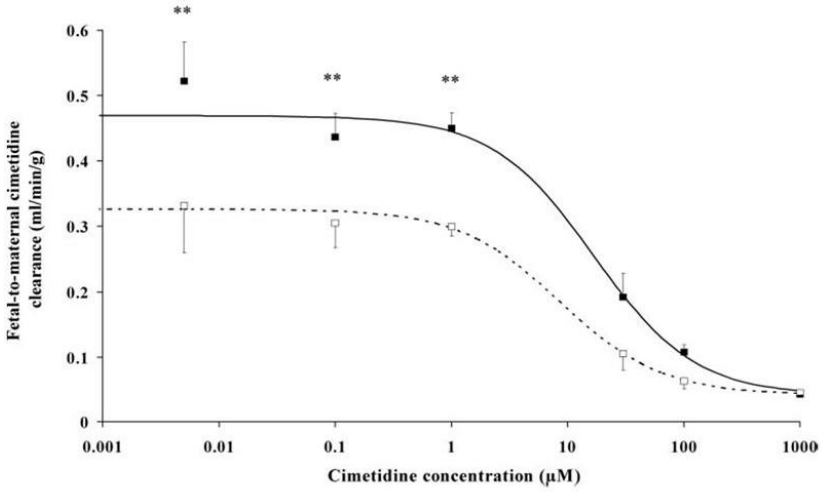

Fig. 7. Transport of cimetidine across the dually perfused rat placenta in fetal-to-maternal direction. Cimetidine with [^{3}H]cimetidine tracer was added to the fetal reservoir, and its radioactivity was measured in fetal venous outflow. Total transplacental clearance was calculated by eq. 3 (see *Materials and Methods*). Changes of clearance with increasing cimetidine concentration confirm nonlinearity 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 (1000 μM), clearances reached the value of 0.042 ml/min/g, and inhibitor activity was negligible. Experimental values are presented as means ± S.D. 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 top plateau delineating combined effect of passive clearance and efflux activity of Bcrp and the bottom plateau representing clearance of passive diffusion alone. Asterisks indicate significant ($p < 0.05$) effect of inhibitor. ■, cimetidine without inhibitor; □, cimetidine with GF120918.

(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.

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 thought 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). Likewise, 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 a few studies have reported on BCRP activity in placenta, and these are mainly based on in vitro models. Very recently, Kolwankar et al. (2005) used 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, the BeWo cell line (Ceckova et al., 2006). In the present study, we used rat placental HRP-1 cell line derived from placental labyrinth region at mid-gestation (Soares et al., 1987). This cell line has previously been used to study several aspects of placental physiology (Soares et al., 1989; Shi et al., 1997; Morris Buus and Boockfor, 2004), 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 placental 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, whereas 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., 2006) where only BCRP was found to be functionally expressed, whereas 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. In contrast, it may well serve as a tool to study Bcrp role in transplacental pharmacokinetics, because 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 in transgenic mice (Jonker et al., 2000, 2002); however, detailed evaluation of BCRP role in transplacental pharmacokinetics is still lacking. In the present study, cimetidine was used to comprehensively describe the role of Bcrp in maternal-to-fetal and fetal-to-maternal transport. Cimetidine was chosen as a model substrate for its convenient properties; it is a BCRP substrate that 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).

The localization of Bcrp on the apical, maternal-facing membrane of the rat placenta closely resembles that of P-gp described in our previous studies (Pavek et al., 2003; Novotna et al., 2004). This finding suggests that Bcrp is, like P-gp, important in preventing entry of potential toxins into the fetal compartment. This assumption has been functionally validated in the present study by means of rat perfused placenta: at low cimetidine concentrations (0.005 μM), maternal-to-fetal clearance was 25-fold lower than clearance in the opposite direction. These data confirm that Bcrp causes asymmetry in transplacental clearances in rats by returning substrates coming from maternal side and facilitating transport of drugs from fetus to mother. Interestingly, pharmacokinetic modeling revealed Michaelis-Menten constant for fetal-to-maternal direction to be 20 times higher than that for

TABLE 2

Pharmacokinetic parameters of transplacental passage of cimetidine

Pharmacokinetic parameters were obtained by fitting experimental data with eqs. 7 and 8. GF120918 (2 μM) added to the maternal compartment was used as an inhibitor.

	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

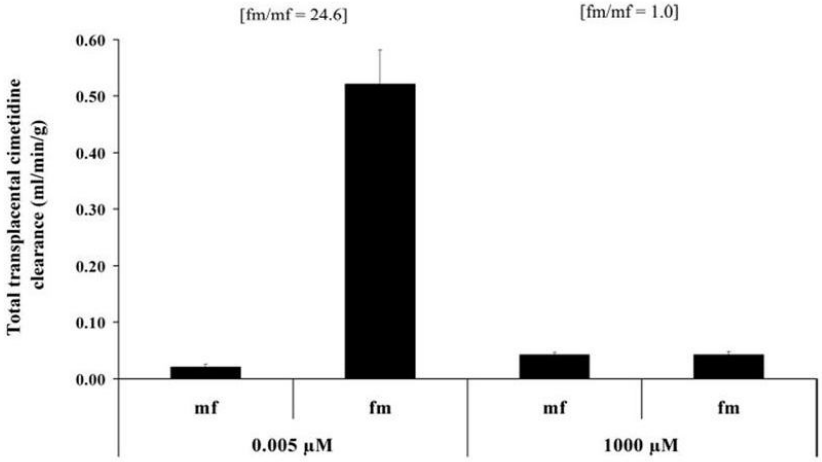

Fig. 8. Ratio of clearances between fetal-to-maternal (fm) and maternal-to-fetal (mf) directions. Cimetidine with [^{3}H]cimetidine tracer was added to the maternal or fetal compartment, and its radioactivity was measured in fetal venous outflow. Total transplacental clearances were calculated by eq. 1 or 3, respectively (see *Materials and Methods*). 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 parentheses show ratio of fm-to-mf clearance.

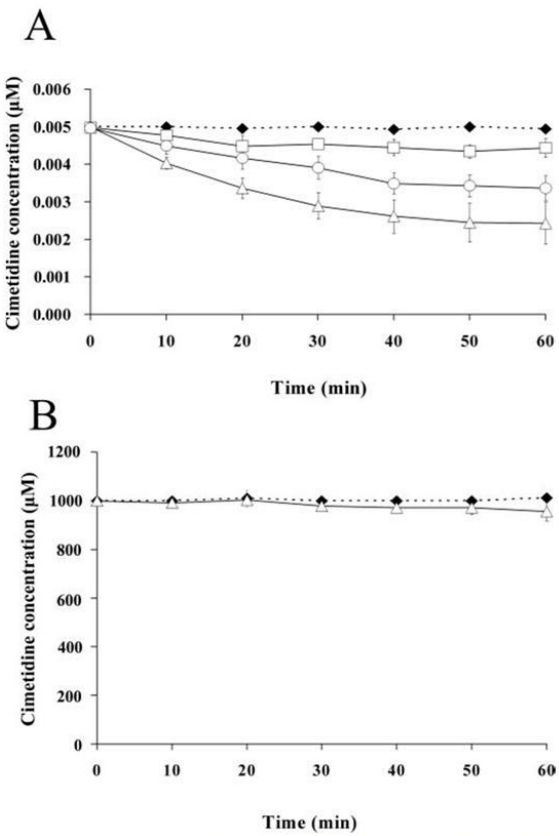

Fig. 9. Elimination of cimetidine from the fetal circulation by placental Bcrp. Cimetidine and [^{3}H]cimetidine tracer were added to both maternal (closed symbol) and fetal (open symbols) circulations at equal concentrations. Fetal perfusate (10 ml) was recirculated and sampled for 60 min. At low cimetidine concentrations of 0.005 μM (A), fetal cimetidine decreased from 0.005 μM down to 0.0024 μM 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 1000 μM (B), no decrease in fetal compartment was observed, suggesting saturation of the transporter. Data are presented as means ± S.D. of three experiments. ◆, maternal cimetidine concentration; □, fetal cimetidine concentration with GF120918; ○, fetal cimetidine concentration with fumitremorgin C; △, fetal cimetidine concentration without inhibitor.

maternal-to-fetal direction. We assume this difference is caused by polarized localization of Bcrp on the maternal side of the placenta; 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.

To investigate the potential of Bcrp to remove drugs already present in the fetal compartment, both maternal and fetal sides of the placenta were perfused with equal concentrations of cimetidine and fetal perfusate was recirculated. After short equilibration period, we observed significant decrease in fetal cimetidine concentrations, confirming that Bcrp can actively remove its substrate from the fetal compartment. Because decrease in cimetidine concentration continued even at later intervals (dropping by more than 50% within 60 min of perfusion), it is evident that Bcrp in rats can pump this compound from fetus to mother even against a concentration gradient.

Interestingly, several studies on cimetidine placental transfer were published two decades ago with intriguing results. When studied in sheep, large cimetidine gradient between mother and fetus was observed (Mihaly et al., 1983). In a follow-up study, the authors suggested that an active transporter from the fetal to the maternal circulation might be responsible for this discrepancy (Ching et al., 1985). In contrast, when investigated in the dually perfused human placenta, two papers concluded that transport of cimetidine was very slow and occurred by passive diffusion with lack of saturation kinetics (Ching et al., 1987; Schenker et al., 1987). These contrasting findings might be explained by interspecies differences; however, one has to realize that these studies were performed before efflux transporters were discovered and described, with limited range of cimetidine concentrations, and without the option to use appropriate inhibitors. Therefore, possible role of a drug efflux transporter in the transplacental pharmacokinetics of cimetidine could not have been taken in account. Our present findings suggest that Bcrp is the transporter responsible for limited maternal-to-fetal passage and large maternal/fetal concentration ratio of cimetidine in rats. However, BCRP activity in perfused placentas of other species must be elucidated before a final conclusion is drawn.

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, however, a different mRNA distribution pattern was indicated by Tanaka et al. (2005). They found high expression levels in kidney and small and large intestine, and 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. (2000, 2002) 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, because there are a number of other factors that determine its functional activity, such as post-transcriptional/post-translational modifications in protein expression as well as strategic localization of BCRP along the maternal interface.

In conclusion, functional expression of Bcrp in the rat placenta and rat placental HRP-1 cell line was confirmed in this study. A pharmacokinetic model was applied to distinguish between passive and Bcrp-mediated placental transport of cimetidine as a model substrate. 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 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, despite being localized on the maternal-facing side, actively removes cimetidine from the fetal circulation against concentration gradient. Based on our findings, we propose a two-level defensive role of placental BCRP in which the transporter 1) reduces passage of its

substrates from mother to fetus but also 2) removes the drug already present in the fetal circulation even against a concentration gradient. Given the broad range of BCRP substrates, this transporter seems to be an important component of the rat placental barrier playing a significant role in protection and detoxication of the fetus.

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IV

ROLE OF BREAST CANCER RESISTANCE PROTEIN (BCRP/ABCG2) IN FETAL PROTECTION DURING GESTATION IN RAT

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Role of breast cancer resistance protein (Bcrp/Abcg2) in fetal protection during gestation in rat

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ABSTRACT

Breast cancer resistance protein (BCRP/ABCG2) is an ABC family drug efflux transporter expressed in a number of physiological tissues including placenta. Here we investigated the expression and function of Bcrp in the rat placenta and fetus during pregnancy. We show that the expression of Bcrp mRNA in placenta peaks on gestation day (gd) 15 and declines significantly to one third up to term. In fetal body tissue, 6.9 and 7.4-fold Bcrp mRNA increase was detected on gds 15 and 18, respectively, compared to the early gd 12. The expression of Bcrp mRNA in fetal organs on gds 18 and 21 is also demonstrated. Additionally, the function of placental and fetal Bcrp during pregnancy was studied by fetal exposure to cimetidine infused to the maternal circulation. The relative amount of drug that penetrated to fetus was highest on gd 12 and decreased to one tenth thereafter. Studies on cimetidine distribution in fetus revealed 2- and 4.4-times lower penetration to the brain on gds 18 and 21, respectively, compared to the whole fetal tissue. Our results indicate that the rat fetus is protected by Bcrp against potentially detrimental substances from gd 15 onwards. Moreover, we propose that the protection of fetus by placental Bcrp is further strengthened by fetal Bcrp.

1. Introduction

Detailed knowledge of all mechanisms influencing drug transfer from mother to fetus is necessary for optimization of pharmacotherapy during pregnancy. Placenta is an organ that brings the maternal and fetal circulations into close proximity, thus allowing the interchange of nutrients, waste products and xenobiotics between the two individuals. Drug transfer across the placenta is, among others, influenced by drug efflux transporters, members of ATP binding cassettes (ABC) family. These membrane-embedded proteins are able to actively pump xenobiotics out of trophoblast cells, strengthening thereby placental barrier role and protecting the fetus (Young et al., 2003; Marin et al., 2004; Unadkat et al., 2004). To date, the best characterized placental drug efflux transporter, regarding its expression, function, regulation and clinical relevance, is P-glycoprotein (P-gp/MDR1/ABCB1) (Ceckova-Novotna et al., 2006).

Breast cancer resistance protein (BCRP/ABCG2) is a relatively novel ABC transporter that was first observed in a breast cancer cell line MCF-7 (Doyle et al., 1998). In parallel, its high expression was disclosed in placenta granting the transporter the name "placenta-specific ABC transporter" (ABCP) (Allikmets et al., 1998). BCRP was subsequently localized in a number of other mammalian physiological tissues, such as epithelia of the intestine and colon, duct and lobules of the breast, liver canalicular membranes and brain capillaries (Allen and Schinkel, 2002; Doyle and Ross, 2003; Staud and Pavek, 2005). BCRP has been found to interact with a broad variety of chemically unrelated compounds ranging from endogenous substances to fluorescent dyes, chemotherapeutic agents and some other drugs (Doyle and Ross, 2003; Mao and Unadkat, 2005; Staud and Pavek, 2005).

Placental expression of BCRP/Bcrp was described in various mammalian species (Allikmets et al., 1998; Doyle et al., 1998; Jonker et al., 2000; Tanaka et al., 2005; Wang et al., 2006). In human placenta, BCRP was predominantly localized to the syncytiotrophoblast layer; however, it was also observed in fetal vessels of the placental chorionic villi (Maliepaard et al., 2001; Ceckova et al., 2006; Yeboah et al., 2006). The levels of BCRP mRNA transcripts were found to be considerably higher than those of MDR1 mRNA encoding P-gp (Ceckova et al., 2006). Functional activity of placental Bcrp was first demonstrated by Jonker et al. (2000, 2002) in studies performed in transgenic mice. Using dually perfused rat term placenta, we have recently demonstrated that not only does Bcrp reduce passage of its potentially toxic substrates from mother to fetus but also removes the drug already present in the fetal circulation (Staud et al., 2006). This suggests that placental BCRP is an important component of the placental barrier playing a significant role in protection and detoxication of the fetus.

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Recently, several studies aimed to evaluate expression of BCRP/Bcrp in placentas of various species over the course of pregnancy. In the rat placenta, reduction in the expression level of Bcrp mRNA and protein from the mid stage to the end of gestation has been observed (Yasuda et al., 2005). Two recent studies on mouse placenta reported peaked Bcrp1 levels on gestation day (gd) 15 (Wang et al., 2006) and a gestation-dependent decrease in the expression of Bcrp1 mRNA from gds 9.5 to 18.5 (Kalabis et al., 2007). In human placenta, the current data on BCRP expression during pregnancy remain rather inconsistent (Mathias et al., 2005; Meyer zu Schwabedissen et al., 2006; Yeboah et al., 2006).

None of the above mentioned studies investigated the function of BCRP or its contribution to the protection of fetus during pregnancy. There are also no data concerning expression of Bcrp at mRNA level in rat fetus and its organs during the intrauterine development. The aim of this work was to evaluate the protective role of placental and fetal Bcrp by studying the exposure of rat fetus to a BCRP substrate administered to the mother during pregnancy. We also examined the expression of Bcrp at mRNA level in placentas and fetal tissues in order to estimate the contribution of these organs to the protection of fetus against potentially detrimental substances.

2. Materials and methods

2.1. Experimental animals

Female Wistar rats were used for all experiments. Pregnant rats were purchased from Biotest (Konarovice, Czech Republic) and maintained at 12/12-h light/dark standard conditions with water and pellets ad libitum. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) 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 (Strassbourg, 1986).

Gd 0 was established upon detection of copulatory plug of sperm after overnight mating. Experiments were performed on gds 12, 15, 18 and 21 using 4 or 5 dams in each group. Fasted rats were anesthetized with pentobarbital (Nembutal; Abbot Laboratories, Abbott Park, IL) in a dose of 40 mg/kg administered into the tail vein.

2.2. Analysis of Bcrp mRNA expression during pregnancy by real-time RT-PCR

One to three randomly selected placentas and fetuses were collected on gds 12, 15, 18 and 21 from each dam. Liver, intestine and brain were dissected free from 1 to 3 randomly selected fetuses of each dam on gds 18 and 21. Immediately after dissection the tissues were frozen in liquid nitrogen and stored at −70 °C until analysis.

Total RNA from collected tissue samples was isolated and reverse transcribed as described previously (Ceckova et al., 2006). Sequences of mRNAs for evaluated genes were obtained from National Center for Biotechnology Information database; primers for *Bcrp* gene and for housekeeping genes *Hypoxanthine guanine phosphoribosyltransferase* (*Hprt*) and *β2-microglobuline* (*b2m*) were designed using the Vector NTI Suite software (Informax, Bethesda, MD) and are given in Table 1.

Real-time RT-PCR analysis was performed on iCycler iQ (Bio-Rad, Hercules, CA). cDNA was amplified with DyNAzyme polymerase (Finnzymes, Espoo, Finland) under the following conditions: 2.5 mM $MgCl_2$, 0.2 mM dNTP, 0.3 μM of each primer; 0.024 U/μl polymerase and SYBR Green I in 1:100,000 dilution; the temperature profile was 94 °C for 10 min; 45 times: 94 °C for 15 s, 60 °C for 20 s and 72 °C for 20 s; 72 °C for 5 min; and melting curve program was 72–95 °C. Each sample of cDNA was amplified in triplicates. One of the samples chosen as the reference calibrator sample (calibrator) was analyzed in every run. Standard curves were generated from multiple threefold dilutions of one sample in order to estimate the efficiency of the reaction. The analysis of real-time amplification curves and subtraction of threshold (Ct) values was performed using iCycler iQ 3.0 software (Bio-Rad) and relative quantification was calculated as described previously (Novotna et al., 2004).

2.3. Fetal exposure to cimetidine during pregnancy

Pregnant rats on gds 12, 15, 18 and 21 were anesthetized and the jugular vein (for the injection of radioisotope) and the carotid artery (for blood sampling) were cannulated. The rats were infused for 60 min with a solution containing 14.0 nmol cimetidine (Sigma–Aldrich, St. Louis, MO) per 1 ml and 100 g of animal, including trace amount of radiolabeled [*N-methyl*-^{3}H]cimetidine (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The steady state, as determined by maternal blood sampling, was reached 40 min after the beginning of the experiment. The concentration of cimetidine in maternal blood at steady state ranged from 150 to 360 nM, which is well below saturating concentration (Staud et al., 2006).

At the end of infusion (60 min) animals were sacrificed and 1–4 randomly selected fetuses of each dam were sampled. On gds 18 and 21 fetal liver, intestine and brain from 1 to 2 fetuses were excised. Fetal tissue and blood samples were treated with tissue solubilizer Solvable (PerkinElmer, Boston, MA), and after mixing with Scintillation liquid (PerkinElmer Life and Analytical Sciences, Boston, MA) their radioactivity was measured. The amount of drug that reached the fetus was expressed as feto-maternal ratio of the amount of [*N-methyl*-^{3}H]cimetidine in 1 g of fetal tissue over the drug concentration in maternal plasma.

2.4. Statistical analysis

The infusion experiments are based on a number of animals $n \geq 4$ for each gd. For subsequent RT-PCR analysis the number of samples in groups of placentas, fetuses and fetal organs was between 5 and 11 for each particular gd. In all cases the standard deviation was calculated from the entire group of samples, since every fetus from one dam is considered to be genetically original. The two-sided, unpaired Student's *t*-test or one-way ANOVA followed by Tukey's test were used, where appropriate, to assess statistical significance.

3. Results

3.1. Expression of Bcrp in placentas and fetuses during pregnancy

The expression of Bcrp at mRNA level in rat placentas, fetuses and fetal organs of different stages of pregnancy was evaluated by real-time RT-PCR analysis. The level of Bcrp mRNA was normalized to the level of both Hprt mRNA and b2m mRNA. However, after performing the analysis of housekeeping stability according to Pfaffl et al. (2004), Hprt was chosen as a normalization gene for its relatively stable expression among placentas, fetuses and examined fetal organs on all studied gds (data not shown).

Bcrp mRNA expression was detected in all placentas of each examined gd (12, 15, 18 and 21). The expression profile peaked on gd 15 ($p < 0.05$, when compared to the other investigated gds), and declined to the levels of gd 12 during gds 18 and 21 (Fig. 1).

When the expression of Bcrp in the whole fetuses was investigated, 6.9 and 7.4-fold increase in Bcrp mRNA expression in fetuses of gds 15 and 18, respectively, was found, compared to gd 12 (Fig. 1).

3.2. Expression of Bcrp in fetal organs during pregnancy

Among fetal organs of the preterm 18th and term 21st gds the highest expression of Bcrp was found in the liver and intestine; conversely, the lowest level of Bcrp mRNA was measured in brain

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

Gene	Accession no.	Sequence 5′ → 3′	Product length (bp)	Localization
Bcrp	NM181381	cca ctg gaa tgc aaa ata gag (f) cct cat agg tag taa gtc aga cac a (r)	188	1340–1527
Hprt	NM012583	gct ata agt tct ttg ctg acc tgc (f) acc agc aag ctt gca acc tta (r)	283	238–520
b2m	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.

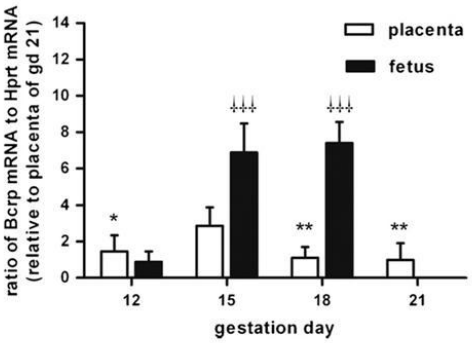

Fig. 1. Relative expression of placental and fetal Bcrp mRNA at different stages of pregnancy, as determined by real-time RT-PCR. Expression of *Bcrp* was normalized to the level of *Hprt* and expressed relatively to placental sample of gd 21; bars are means with S.D.; $n = 5–11$ placentas or fetuses (of at least 4 dams for each gd); $^{*}p < 0.05$, vs. placenta on gd 15; $^{**}p < 0.01$ vs. placenta on gd 15; ↓↓↓, $p < 0.001$ vs. fetus on gd 12; one-way ANOVA followed by Tukey's test.

tissue. We did not observe any difference in the expression of Bcrp mRNA between liver of the 18th and the 21st gd. However, significantly higher Bcrp mRNA expression was found in intestine of gd 21 compared to gd 18 ($p < 0.05$). On the contrary, lower level of Bcrp mRNA ($p < 0.05$) was detected in brain of gd 21 compared to gd 18 (Fig. 2).

3.3. Fetal exposure to cimetidine during pregnancy

The amount of cimetidine that reached the fetus after the infusion to dams on gds 12, 15, 18 and 21 was highest on gd 12 ($p < 0.001$). The ratio of fetal-to-maternal cimetidine concentrations was almost 10 times higher for fetuses of gd 12 in comparison to fetuses of the 15th, 18th and 21st gd; the average values reached 1.180, 0.105, 0.135 and 0.120, respectively (Fig. 3). These data indicate poor protection of fetus against cimetidine at the very early stage of pregnancy; from gd 15 onwards, however, the exposure of fetus to cimetidine from maternal circulation seems to be efficiently limited by placental barrier.

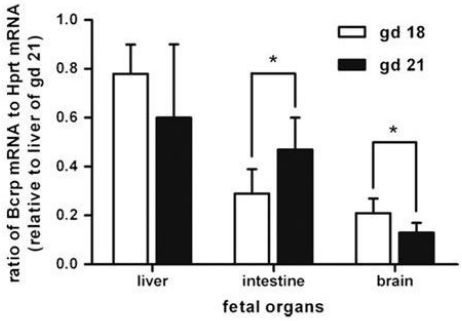

Fig. 2. Relative expression of Bcrp mRNA in fetal organs on preterm gd 18 and term gd 21, as determined by real-time RT-PCR. Expression of *Bcrp* was normalized to the level of *Hprt* and expressed relatively to a sample of liver on gd 21; bars are means with S.D.; $n = 6–8$ fetal organs (of at least 4 dams for each gd); $^{*}p < 0.05$ between intestines on gds 18 and 21 and between brains on gds 18 and 21; Student's *t*-test.

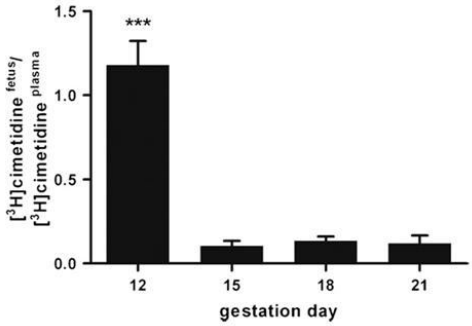

Fig. 3. Exposure of fetus to cimetidine during pregnacy. Cimetidine with [^{3}H]cimetidine tracer were administered i.v. to pregnant rats on gds 12, 15, 18 and 21. One hour after the beginning of the infusion radioactivity in fetal tissue and maternal plasma was measured. Feto-maternal ratio of the amount of [*N-methyl*-^{3}H]cimetidine in 1 g of fetal tissue over the drug concentration in maternal plasma shows pronounced protection of fetal body from the 15th gd; bars are means with S.D.; $n = 4–10$ fetuses (of 4 dams for each gd); $^{***}p < 0.001$ vs. gds 15, 18 and 21; one-way ANOVA followed by Tukey's test.

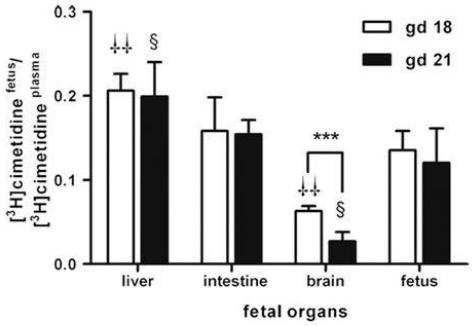

Fig. 4. Distribution of cimetidine to fetal tissues. Cimetidine with [^{3}H]cimetidine tracer were infused to pregnant rats on preterm gd 18 and term gd 21 and its radioactivity was measured in fetal tissues and maternal plasma 60 min after the beginning of the administration. Ratio of the amount of [*N-methyl*-^{3}H]cimetidine in 1 g of fetal tissues over the drug concentration in maternal plasma illustrates differences in cimetidine distribution among fetal organs; bars are means with S.D.; $n = 4–5$ fetal organs (of 4 dams for each gd); $^{***}p < 0.001$ between brains on gds 18 and 21; ↓↓, $p < 0.01$ vs. fetus on gd 18; $^{§}p < 0.05$ vs. fetus on gd 21; Student's *t*-test.

3.4. Distribution of cimetidine into fetal organs

When the post-infusion amount of cimetidine was measured in fetal organs of gds 18 and 21, high amount of the drug was found in liver compared to the whole fetal tissue ($p < 0.01$ and $p < 0.05$, respectively). On the contrary, low amounts of cimetidine were detected in brains of both gds 18 and 21 compared to the whole fetal tissue ($p < 0.01$ and $p < 0.05$, respectively), suggesting presence of a protective mechanism in the fetal blood–brain barrier. In addition, we observed significantly lower amount of cimetidine in brains of gd 21 compared to gd 18 ($p < 0.001$); the average values were 0.027 and 0.063, respectively (Fig. 4). The amounts of cimetidine measured in fetal intestine were comparable to those found in total fetal tissue (Fig. 4).

4. Discussion

BCRP is a relatively novel ABC family transporter physiologically present in many tissues with protective or elimination function

(Doyle and Ross, 2003; Staud and Pavek, 2005). Its expression in adult rat was revealed in many organs, including the placenta, intestine, liver, brain and kidney (Tanaka et al., 2005). The role of BCRP in the mature placenta has been clearly recognized in preventing the fetus against xenobiotics from maternal circulation. In our previous study Bcrp was described as an active component of the rat placental barrier that not only limits materno-fetal transport of cimetidine but also facilitates feto-maternal clearance of the substrate (Staud et al., 2006). Similar results were obtained in mice using nitrofurantoin as a BCRP substrate (Zhang et al., 2007). However, the functional activity of placental BCRP at different stages of gestation has not been elucidated to date.

Several studies evaluating BCRP/Bcrp expression in the placenta during pregnancy have been published so far with contradictory results. In the initial study on human placenta, no changes in the BCRP protein and mRNA levels with gestational age were revealed (Mathias et al., 2005). However, another study examining whole placenta-tissues from 6th to 41st week of pregnancy reported considerable increase in the BCRP protein levels at term, although the levels of BCRP mRNA did not change with progression of gestation (Yeboah et al., 2006). Recently, pronounced BCRP protein has been observed in the group of preterm placentas (28 ± 1 week of pregnancy) compared to term placentas (39 ± 2 week of pregnancy) (Meyer zu Schwabedissen et al., 2006). Studies on mouse placenta showed the peak in Bcrp1 expression on the 15th gd (Wang et al., 2006) and a gestation-dependent decrease in the expression of Bcrp1 mRNA from gds 9.5 to 18.5 (Kalabis et al., 2007). Our study examined the expression pattern of Bcrp at mRNA level in rat placentas and fetuses over the course of pregnancy. No significant differences in the Bcrp expression at mRNA level among placentas of gds 12, 18 and 21 were observed. However, a threefold increase in the Bcrp mRNA expression was found on gd 15 compared to gd 21. This is consistent with other studies showing a reduction of Bcrp at mRNA level from mid-stage to the end of gestation in rat placenta (Yasuda et al., 2005) as well as in mouse placenta (Wang et al., 2006; Kalabis et al., 2007).

A very recent study conducted in mice has shown positivity for Bcrp1 mRNA in mouse conceptus from the mid-stage of pregnancy (Kalabis et al., 2007). In our study we observed very low expression of Bcrp at mRNA level in rat fetus of gd 12, which augmented significantly through gds 15–18 (6.9 and 7.4-fold increase of Bcrp mRNA expression, respectively). We presume that increasing expression of Bcrp in fetal tissues as pregnancy progresses strengthens the protective role of placenta against substances potentially toxic for the developing fetus.

In addition to the profile of Bcrp mRNA expression, we have also evaluated the role of Bcrp in the exposure of fetus to a potentially toxic substrate over the course of pregnancy. Cimetidine was chosen as a model substrate for its convenient properties; it is a well established BCRP substrate that 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). Although cimetidine is also transported by rat organic cation transporters (Oct1 and Oct2) (Tahara et al., 2005) and by multidrug and toxin extrusion protein 1 (MATE1) (Ohta et al., 2006), we do not expect their role in transplacental pharmacokinetics based on their low expression in the placenta (Leazer and Klaassen, 2003; Terada et al., 2006). Here we show that the amount of drug that reached the fetus from the maternal circulation was 10 times higher on gd 12 compared to gds 15, 18 and 21, which indicates lower protection of fetus at the early stage of pregnancy. The feto-maternal ratio of [*N-methyl*-^{3}H]cimetidine did not differ among gds 15, 18 and 21, suggesting involvement of Bcrp protective mechanism from the mid-pregnancy up to term, although involvement of other transporters cannot be excluded.

Confronting the results of Bcrp expression at mRNA level with our functional data we show that although the placental expression of Bcrp mRNA decreases from the 15th gd onwards, the penetration of Bcrp substrate to the fetus remains as low as at the mid-gestation. When trying to clarify this discrepancy by Western blotting and immunohistochemistry, we were able to achieve reproducible results on gd 21 only; data shown in Staud et al. (2006). On earlier days of gestation, cross-reactivity contaminated the results despite several antibodies were used (data not shown). Nevertheless, our previous study (Novotna et al., 2004) as well as studies of others (Evseenko et al., 2006; Yeboah et al., 2006) have demonstrated that the levels of mRNA do not have to correlate tightly with the protein expression. Thus we propose that the inconsistency of our findings can be caused by posttranscriptional and/or posttranslational modifications, delayed gene expression or perhaps by contribution of so far unknown transporters.

In the mouse fetus, the immunopositivity for Bcrp1 has been detected in major excretory, absorptive and barrier tissues such as the liver, intestine and brain capillaries (Kalabis et al., 2007). Kalabis et al. (2007) suggested that the decrease in Bcrp1 mRNA expression in placenta may be counter-balanced by the increase in Bcrp1 expression in fetal organs. Here we demonstrate significant Bcrp expression at mRNA level in the rat fetal liver, intestine and brain of the preterm 18th and term 21st gds. The expression profile of placental and fetal Bcrp mRNA revealed in our study seems to be in accordance with this hypothesis, however, despite the relatively low expression of Bcrp mRNA in placentas on gd 21, pronounced transport activity of Bcrp was demonstrated in our previous study in ex-vivo perfused rat term placenta (Staud et al., 2006). Therefore, we assume that the protective function of placental Bcrp is strengthened, rather than substituted, by fetal Bcrp during the intrauterine development.

The contribution of Bcrp to the biliary excretion of its substrates was showed in mouse as well as in rat liver (Merino et al., 2005; Zamek-Gliszczynski et al., 2006). Fetal liver is able to carry out bile acid synthesis from early stages of gestation and transfers them to the placenta through so far not fully elucidated ways (Marin et al., 2005). It was reported that in adult rat the liver contributes about 30% of total systemic clearance of cimetidine (Henderson et al., 1988). Our study revealed higher concentration of cimetidine in fetal liver compared to the whole fetal tissue, which can be given by strong vascularization of this organ and also by activity of hepatic Bcrp. Additionally, uptake cation transporters Oct1 and Oct2 could influence the amount of cimetidine found in fetal liver. However, expression and function of these transporters in fetal organs has not been confirmed yet.

The presence of Bcrp in fetal intestine, observed at mRNA level on preterm gd 18, may also contribute to the protection of fetus by restricting the absorption of BCRP substrates being excreted by liver to the intestinal lumen. We observed significantly higher expression of intestinal Bcrp mRNA on the term 21st gd compared to preterm gd 18, which seems to reflect the maturation of the transport and enzymatic systems in the organ soon before birth.

The importance of Bcrp in blood–brain barrier (BBB) appears somewhat controversial. Cisternino et al. (2004) showed that Bcrp is able to limit the brain uptake of mitoxantrone and prazosin (both substrates of P-gp and Bcrp) in *mdr1a*$^{(-/-)}$ mice. Functionality of Bcrp in the BBB was also demonstrated in a recent study employing primary cultures of rat brain endothelial cells (Perriere et al., 2007). However, Lee et al. (2005) proposed only a restricted function of Bcrp in BBB. We observed that the post-infusion amount of cimetidine found in fetal brain was much lower compared to the whole fetal tissue, which could indicate presence of a protective mechanism that restricts penetration of potentially harmful BCRP substrates to fetal brain. Despite the fact that the Bcrp mRNA

expression decreased from gds 18 to 21, the amount of the drug that penetrated to brain on the 21st gd was significantly (2.3 times) lower compared to gd 18, proposing an increase in the activity of defensive mechanisms in rat fetus towards the term.

In summary, our study demonstrates Bcrp expression and activity in placenta and fetus during pregnancy. The expression of Bcrp at mRNA level in placenta peaked on gd 15 and decreased thereafter. However, our functional studies showed that the exposure of fetus to a BCRP substrate cimetidine was highest on gd 12 and dropped to one tenth through the rest of gestation. A considerable increase was observed in the expression of fetal Bcrp at mRNA level from gd 12 through gds 15 and 18. Bcrp mRNA expression was found in liver, intestine and brain of fetuses on both preterm gd 18 and term gd 21. We conclude that protection of the fetus is provided by placental Bcrp from the 15th gd until the birth and is further strengthened by fetal Bcrp as the pregnancy progresses.

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V

TRANSPLACENTAL PHARMACOKINETICS OF GLYBURIDE, RHODAMINE 123 AND BODIPY FL PRAZOSIN; EFFECT OF DRUG EFFLUX TRANSPORTERS AND LIPID SOLUBILITY

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Transplacental Pharmacokinetics of Glyburide, Rhodamine 123, and BODIPY FL Prazosin: Effect of Drug Efflux Transporters and Lipid Solubility

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ABSTRACT

Breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp) are the most abundantly expressed ATP-binding cassette (ABC) drug transporters in the placenta. They recognize a large, partly overlapping spectrum of chemically unrelated compounds and affect their transplacental passage. In this study we investigate the effect of Bcrp and P-gp on the transplacental pharmacokinetics of their specific and common substrates employing the technique of dually perfused rat placenta. We show that the clearance of rhodamine 123 (P-gp substrate), glyburide (BCRP substrate) and BODIPY FL prazosin (P-gp and BCRP substrate) in fetal-to-maternal direction is 11, 11.2 and 4 times higher, respectively, than that in the maternal-to-fetal direction. In addition, all of these substances were found to be transported from the fetal compartment even against concentration gradient. We thus demonstrate the ability of placental ABC transporters to hinder maternal-to-fetal and accelerate fetal-to-maternal transport in a concentration-dependent manner. However, by means of pharmacokinetic modeling we describe the inverse correlation between lipid solubility of a molecule and its active transport by placental ABC efflux transporters. Therefore, in the case of highly lipophilic substrates, such as BODIPY FL prazosin in this study, the efficacy of efflux transporters to pump the molecule back to the maternal circulation is markedly limited.

The need to medicate women for various reasons, even during pregnancy, is often inevitable. A recent multicenter study monitoring pregnancies from 1996 to 2000 in the United States revealed that a drug other than a vitamin or mineral supplement was prescribed for 64% of all pregnant women during 270 days before delivery (Andrade et al., 2004). Moreover, 5 to 10% of them received Food and Drug Administration category D or X drugs, classified as potential teratogens (Andrade et al., 2006). These findings emphasize that it is important to understand the pharmacokinetics of the transport of these medications across the placental barrier and to assess their possible risk for the developing fetus.

Drug efflux transporters of the ATP-binding cassette (ABC) transporter family were originally investigated in association with the phenomenon of multidrug resistance in cancer therapy (Kavallaris, 1997; van der Kolk et al., 2002; Pérez-Tomás, 2006) because they are capable of actively pumping their substrates out of cells even against a concentration gradient. Later on, some of these membrane-embedded proteins were also localized in "normal" tissues, such as the liver, kidney, intestine, brain, or placenta, affecting body disposition of many xenobiotic compounds (Schinkel and Jonker, 2003; Leslie et al., 2005).

In the placenta, the best described and most important drug efflux transporters seem to be P-glycoprotein (P-gp) (Ceckova-Novotna et al., 2006) and breast cancer resistance protein (BCRP) (Mao, 2008). Their placental expression in humans and in some experimental animals has been found to be much higher than in most other tissues (Bremer et al., 1992; Doyle et al., 1998; Maliepaard et al., 2001; Leazer and Klaassen, 2003; Wang et al., 2006). Expression, localization, and functional activity of P-gp and BCRP in the human and rat placenta has been described (Bremer et al., 1992; Doyle et al., 1998; Maliepaard et al., 2001; Pávek et al., 2001; Staud et al., 2006). In the human placenta, significantly higher expression was found for BCRP compared with P-gp (Ceckova et al., 2006). Moreover, we have revealed that the placental expres-

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ABBREVIATIONS: ABC, ATP-binding cassette; BCRP, human breast cancer resistance protein; Bcrp, rodent breast cancer resistance protein; BP, BODIPY FL prazosin; P-gp, P-glycoprotein; GF120918, *N*-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; PK, pharmacokinetics; GLB, glyburide; AP, antipyrine; Rho123, rhodamine 123; FTC, fumitremorgin C; mf, maternal-to-fetal; fm, fetal-to-maternal.

sion and transport activity of P-gp and Bcrp changes during pregnancy in the rat (Novotna et al., 2004; Cygalova et al., 2008). Functional studies indicate that P-gp and BCRP transport a large variety of molecules, ranging from endogenous substrates to chemotherapeutic agents and environmental toxins (Schinkel and Jonker, 2003; Mao and Unadkat, 2005; Staud and Pavek, 2005). Considerable overlap in substrate recognition and in tissue distribution between BCRP and P-gp presumes their shared effect in placental detoxication processes.

In the present study we used the technique of dually perfused rat placenta in an open or closed perfusion setup (Staud et al., 2006) to evaluate the effect of P-gp and Bcrp on transplacental pharmacokinetics (PK) of their substrates. Concentration-dependent studies, specific inhibitors and PK modeling have been used to assess the efficacy of these proteins to hinder maternal-to-fetal (mf) and accelerate fetal-to-maternal (fm) transport. BODIPY FL prazosin (BP), a common substrate of both P-gp and BCRP (Kimchi-Sarfaty et al., 2002; Hori et al., 2004), was used to test whether the number of transporters involved in the drug transfer is reflected in its transplacental pharmacokinetics. Finally, correlations between lipid solubility of the molecules and their passive diffusion and/or active transport were investigated.

Materials and Methods

Reagents and Chemicals. Glyburide (GLB; 1-[[*p*-[2-(5-chloro-*o*-anisamido)-ethyl]phenyl]-sulfonyl]-3-cyclohexylurea), a BCRP substrate, and [cyclohexyl-2,3-^{3}H(N)]glyburide ([^{3}H]GLB) (50.2 Ci/mmol) were obtained from Sigma-Aldrich (St. Louis, MO) and from PerkinElmer Life and Analytical Sciences (Boston, MA), respectively. BODIPY FL prazosin [BP; boron, [1-(4-amino-6,7-dimethoxy-2-quinazolinyl)-4-[3-[5-[(3,5-dimethyl-2*H*-pyrrol-2-ylidene-κN)methyl]-1*H*-pyrrol-2-yl-κ*N*]-1-oxopropyl]piperazinato]difluoro-, (T-4)-], a common BCRP and P-gp substrate, was purchased from Invitrogen (Carlsbad, CA). Rhodamine 123 [Rho123; 2-(6-amino-3-imino-3*H*-xanthen-9-yl)benzoic acid methyl ester], a P-gp substrate, was obtained from Sigma-Aldrich. Antipyrine (AP; 2,3-dimethyl-1-phenyl-3-pyrazolin-5-one), a marker of passive diffusion, and [*N*-methyl-^{14}C]antipyrine ([^{14}C]AP) (55 mCi/mmol) were purchased from Sigma-Aldrich and American Radiolabeled Chemicals (St. Louis, MO), respectively. Specific BCRP inhibitor fumitremorgin C [FTC; 9′*R*-(9′α(4*S**(*R**)),9′aβ))-4-(2-(1-(acetyloxy)-2-methylpropyl)-4-oxo-3(4*H*)-quinazolinyl)-1′,3,4,9′a-tetrahydro-1′-hydroxy-2′,2′-dimethylspiro-(furan-2(5*H*),9′-(9*H*)imidazo(1,2-a) indole)-3′,5(2′*H*)-dione] and a dual P-gp and BCRP inhibitor GF120918 were from Alexis Corporation (Lausanne, Switzerland) and GlaxoSmithKline (Greenford, UK), respectively. All other compounds were reagent grade.

Animals. Pregnant Wistar rats were purchased from Biotest Ltd. (Konarovice, Czech Republic) and maintained in 12:12-h day/night standard conditions with pellets and water ad libitum. Experiments were carried out on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (40 mg/kg; Nembutal; Abbott Laboratories, Abbott Park, IL) administered into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Czech Republic) and were performed 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, France, 1986).

Dual Perfusion of the Rat Placenta. The method of dually perfused rat term placenta was used in our study, as described previously (Staud et al., 2006). In brief, one uterine horn was excised and submerged in heated Ringer's 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% dextran 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 fetuses by ligatures. The umbilical artery was catheterized by use of a 24-gauge catheter connected to the fetal reservoir and perfused 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 preweighed glass vial to check for a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Maternal and fetal perfusion pressures were maintained at levels close to physiological values and monitored continuously throughout the perfusion experiments as described previously (Pávek et al., 2001).

Two types of perfusion systems were used in this study (Staud et al., 2006):

1. For pharmacokinetic analysis of concentration-dependent transplacental passage both maternal and fetal sides of the placenta were perfused in open-circuit systems, without recirculation of the perfusate. The tested substance was added to the maternal (in mf studies) or fetal (in fm studies) reservoir immediately after successful surgery followed by an approximately 5-min stabilization period before sample collection started (time 0). Fetal effluent was sampled into preweighed vials at 5-min intervals and analyzed either fluorometrically for Rho123 and BP or radiometrically for [^{3}H]GLB and [^{14}C]AP.

2. To investigate the potential of Bcrp and P-gp in removing their substrates from fetal circulation, both maternal and fetal sides of the placenta were infused with equal concentrations of the tested substance and after 5-min stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (250 μl) were collected every 10 min from the maternal and fetal reservoirs, and concentration of the tested substance was measured. This experimental setup ensures steady substance concentration on the maternal side of the placenta and enables investigations of fetal-to-maternal concentration ratio; any net transfer of the drug implies transport against concentration gradient and provides an evidence of active transport.

To standardize the perfusion experiments, AP with trace amount of [^{14}C]AP was infused to the maternal or fetal side of the placenta in concentrations of 0.25 or 100 μM, and transplacental clearances were calculated.

Effect of Substrate Concentration on Transplacental Clearance in the Presence or Absence of Inhibitors. To investigate the effect of various concentrations of Rho123 and GLB on mf and fm clearances, Rho123 or GLB with a trace amount of [^{3}H]GLB were added to the maternal or fetal reservoir, respectively, in the following concentrations: 0.05, 0.1, 0.5, 1, 10, 30, or 100 μM for Rho123 and 0.01, 0.2, 1, 10, 100, 500, or 1500 μM for GLB. In the case of BP, only the concentration of 0.25 μM was examined because of the low solubility of the compound in water. The inflowing concentration of the substances was maintained constant during the experiment. Transplacental clearances of the aforementioned substances were calculated for every concentration from all measured intervals as described below.

BCRP-specific inhibitor FTC (2 μM), or P-gp and BCRP common inhibitor GF120918 (2 μM) were added to both the maternal and fetal reservoirs at the beginning of the perfusion to study the effect of BCRP and P-gp on the transplacental movement of the substances.

Effect of P-gp and Bcrp on Fetal-to-Maternal Equilibrium of Their Substrates. To examine the effect of Bcrp and P-gp on the fetomaternal concentration ratio at equilibrium, both maternal and fetal sides of the placenta were infused with equal concentrations of the investigated compound. Low and high substrate concentrations

of each substrate were used; these concentrations were taken from sigmoid curves of concentration-dependent studies described above and considered nonsaturating and saturating, respectively. In particular, Rho123 was infused at a concentration of 0.5 or 100 μM; GLB was infused at a concentration of 0.2 or 100 μM. BP, because of its low solubility in water, was infused only at a low concentration of 0.25 μM. FTC (2 μM) or GF120918 (2 μM) were added to both maternal and fetal reservoirs to inhibit the transporters and to demonstrate their effect on fetal-to-maternal equilibrium.

Pharmacokinetic Analysis of Efflux Transport Activity in the Placenta. The clearance concept has been adopted to describe transplacental pharmacokinetics of ABC substrates. Assuming that both passive and active transports are involved in the net transplacental passage of ABC substrates, total transplacental clearance in fm or mf direction (Cl_{fm} and Cl_{mf}, respectively) is a function of passive diffusion (Cl_{pd}, governed by Fick's law) and efflux transporter activity (Cl_{efflux}, governed by saturable kinetics). Because efflux transport in the placenta runs in the fetal-to-maternal direction only, Cl_{efflux} is added to Cl_{pd} in the fm direction and subtracted from Cl_{pd} in the mf direction as follows:

$$Cl_{fm} = Cl_{pd} + Cl_{efflux} \quad (1)$$

and

$$Cl_{mf} = Cl_{pd} - Cl_{efflux} \quad (2)$$

Because Cl_{efflux} is a capacity-limited process, it can be expressed in terms of Michaelis-Menten kinetics:

$$Cl_{efflux} = \frac{V_{max}}{K_m + C_{ma(fa)}} \quad (3)$$

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.

Adding eq. 3 to eqs. 1 and 2 gives the final equations, which were used to fit clearance versus inflow concentration data:

$$Cl_{fm} = Cl_{pd} + \frac{V_{max}}{K_m + C_{fa}} \quad (4)$$

and

$$Cl_{mf} = Cl_{pd} - \frac{V_{max}}{K_m + C_{ma}} \quad (5)$$

Data were fitted by use of reciprocal weighting and the numerical module of SAAM II (SAAM Institute, Seattle, WA). Total maternal-to-fetal transplacental clearance (Cl_{mf}) normalized to placenta weight was calculated according to eq. 6

$$Cl_{mf} = \frac{C_{fv} \cdot Q_f}{C_{ma} \cdot W_p} \quad (6)$$

where C_{fv} is the drug concentration in the umbilical vein effluent, Q_f is the umbilical flow rate, C_{ma} is the concentration in the maternal reservoir, and W_p is the wet weight of the placenta. Total fetal-to-maternal clearance normalized to placenta weight (Cl_{fm}) was calculated according to eq. 7.

$$Cl_{fm} = \frac{(C_{fa} - C_{fv})Q_f}{C_{fa} W_p} \quad (7)$$

where C_{fa} is the drug concentration in the fetal reservoir entering the perfused placenta via the umbilical artery.

At very low substrate concentrations, the role of passive diffusion in net transplacental clearance is minimized. Therefore, the ratio between clearances in fetal-to-maternal and maternal-to-fetal direction (Cl_{fm}/Cl_{mf}) at low substrate concentrations was used in this study as a measure of transporter efficiency.

Statistical Analysis. For each group of placental perfusion experiments, the number of animals was $n \geq 3$. Student's t test or one-way analysis of variance followed by Bonferroni's test were used where appropriate to assess statistical significance. Differences of $p < 0.05$ were considered statistically significant.

Results

Effect of Substrate Inflow Concentrations on Transplacental Clearance in Maternal-to-Fetal and Fetal-to-Maternal Direction. The maternal or fetal side of the placenta was infused with various concentrations of Rho123 (0.05, 0.1, 0.5, 1, 10, 30, or 100 μM) or GLB (0.01, 0.2, 1, 10, 100, 500, or 1500 μM). In both mf and fm transport studies, increase in substrate concentration resulted in significant change in transplacental clearance; plotting transplacental clearances versus inflowing substrate concentrations revealed sigmoid curves in both mf and fm directions (Figs. 1 and 2) confirming involvement of capacity-limited mechanisms. Fitting experimental data with eqs. 4 and 5 provides description of passive and active components of transplacental passage (Table 1). It is evident that the passive movement across the placenta is comparable in both mf and fm directions for both substrates. Cl_{efflux}, however, is a concentration-dependent parameter. At high substrate concentrations, the transporter becomes saturated, Cl_{efflux} ap-

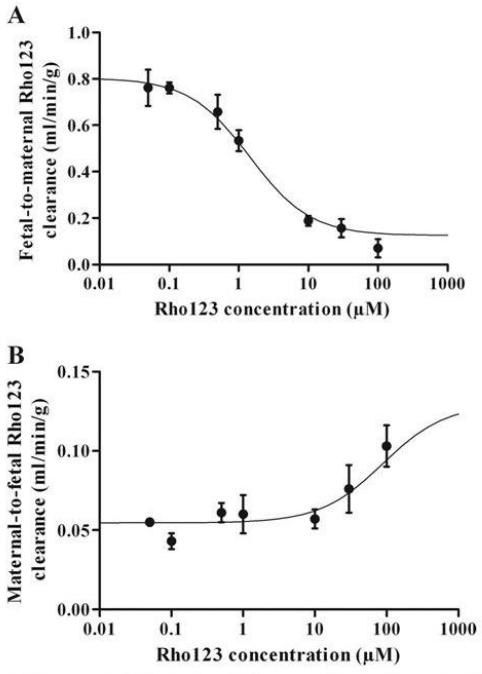

Fig. 1. Transport of rhodamine 123 across the dually perfused rat placenta in the fetal-to-maternal (A) and the maternal-to-fetal (B) direction. Changes of clearance with increasing Rho123 concentration confirm the nonlinearity of the processes and involvement of a saturable mechanism. Experimental values are presented as means ± S.D. of at least three experiments; the line represents the best fit of these data to eqs. 4 (A) and 5 (B).

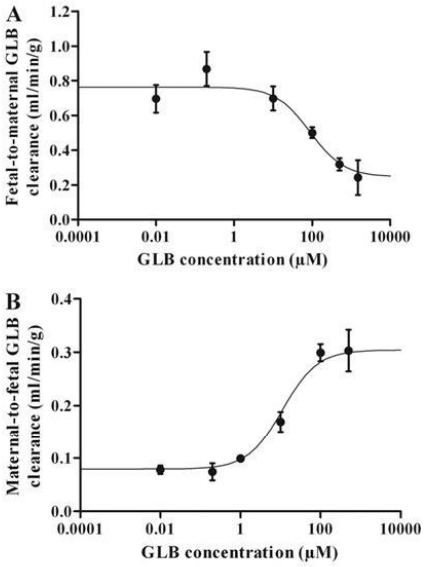

Fig. 2. Transport of glyburide across the dually perfused rat placenta in the fetal-to-maternal (A) and the maternal-to-fetal (B) direction. Changes of clearance with increasing GLB concentration confirm the nonlinearity of the processes and involvement of a saturable mechanism. Experimental values are presented as means ± S.D. of at least three experiments; the line represents the best fit of these data to eqs. 4 (A) and 5 (B).

proaches zero, and eqs. 4 and 5 transform into simple linear processes, i.e., $Cl_{mf} = Cl_{fm} = Cl_{pd}$.

Fetal-to-Maternal Versus Maternal-to-Fetal Clearances: Effect of Concentration and Inhibition. Comparing fm and mf clearances at low substrate concentrations (0.5 μM for Rho123, 0.2 μM for GLB, and 0.25 μM for BP), significantly higher transport of all substances in the fm direction was observed compared with that in the opposite direction (Fig. 3). Addition of GF120918 caused 2.4-, 5.3-, and 1.6-fold decrease in the Cl_{fm}/Cl_{mf} ratio of Rho123, GLB, and BP, respectively (Fig. 3). At high substrate concentrations (100 μM for Rho123 and 500 μM for GLB), mf and fm clearances reached similar values (Figs. 1 and 2), confirming saturation of transporting proteins and limited role of their efflux activity.

In contrast, no statistically significant differences between fm and mf clearances of AP, a model compound of passive diffusion, at either low or high concentrations (0.25 and 100 μM) were found (Fig. 3D). These observations demonstrate solely passive transplacental transfer of AP with no involvement of active transporters and validate the usefulness of our model.

Effect of P-gp/Bcrp on Fetal-to-Maternal Equilibrium of their Substrates. To investigate the potential of P-gp and Bcrp to remove their substrates from fetal circulation, Rho123, GLB, or BP were simultaneously infused to both the maternal and fetal side of the placenta at equal concentrations of 0.5 or 100 μM for Rho123, 0.2 or 100 μM for GLB, and 0.25 μM for BP. In this experimental setup, fetal perfusate was recirculated for 60 min. At low drug concentrations of all tested compounds, a steady decrease in the drug amount in the fetal reservoir with stabilization after approximately 40 min of perfusion was observed; this decline was blocked by coinfusion of P-gp and/or BCRP inhibitors GF120918 or FTC (Fig. 4). At high Rho123 and GLB concentrations (100 μM), maternal and fetal concentrations remained unchanged throughout the perfusion period with the fetal-to-maternal concentration ratio staying close to 1.0 (Fig. 5). Through these findings we demonstrate the capacity of P-gp and Bcrp to remove their substrates from the fetal compartment and to maintain a significant concentration gradient between maternal and fetal circulations.

As expected, in the case of AP no decrease in fetal drug concentration was observed at either low (0.25 μM) or high (100 μM) drug concentration with fetal-to-maternal concentration ratio values close to 1.0 (Figs. 4D and 5C).

Effect of Lipid Solubility on Efflux Transporter Effectiveness in Transplacental Pharmacokinetics. To investigate the effect of lipid solubility on efflux transporter effectiveness, the obtained PK parameters for both specific and common P-gp and BCRP substrates were used. cLogP (logarithm of the partition coefficient between *n*-octanol and water) values were calculated by ChemBioOffice 2008 (CambridgeSoft Corp., Cambridge, MA), which exploits the increment system adding contributions of every atom based on its atom type. Data concerning cimetidine were taken from our previous article (Staud et al., 2006).

Cl_{fm}/Cl_{mf} at low substrate concentrations was considered as a parameter illustrating transporter effectiveness (Fig. 3), whereas Cl_{pd} describes passive movement of drugs across the placenta. When plotting these two parameters against cLogP it is evident that a rise in lipid solubility increases passive diffusion and, at the same time, reduces the effect of the efflux transporter (Fig. 6).

Discussion

The role of placental ABC drug efflux transporters, especially P-gp and BCRP, in transplacental PK has become a widely discussed issue (Ceckova-Novotna et al., 2006; Mao, 2008). They have been localized and functionally described in many in vitro and in situ models, including BeWo cell line (Utoguchi et al., 2000; Ceckova et al., 2006; Evseenko et al., 2006), and perfused human (Kraemer et al., 2006) or rat

TABLE 1
Pharmacokinetic parameters of transplacental passage of Rho123 and GLB
Pharmacokinetic parameters were obtained by fitting experimental data with eqs. 4 and 5 for fm and mf transport, respectively. Data are presented as mean ± S.D.

	Rho123		GLB	
	Fetal-to-Maternal Transport	Maternal-to-Fetal Transport	Fetal-to-Maternal Transport	Maternal-to-Fetal Transport
Cl_{pd} (ml/min/g)	0.125 ± 0.022	0.129 ± 0.015	0.294 ± 0.058	0.304 ± 0.043
V_{max} (mmol/min/g)	0.97 ± 0.12	6.56 ± 0.47	45.8 ± 5.84	2.53 ± 0.24
K_m (μM)	1.42 ± 0.19	88.0 ± 16.8	88.5 ± 11.6	11.2 ± 1.41

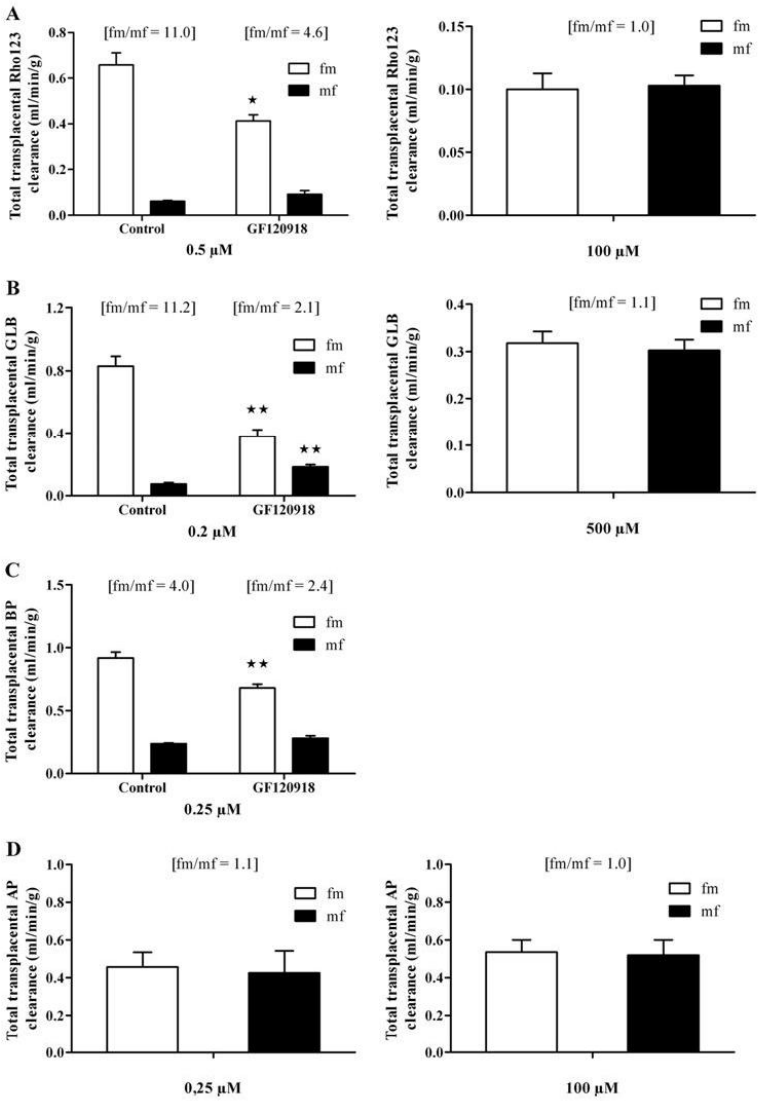

Fig. 3. Ratio of clearances between fm and mf directions at low (left column) and high (right column) substrate concentrations. In the case of Rho123 (A), GLB (B), and BP (C) administered at low concentrations, significantly higher clearances in the fm than in the mf direction were observed. Addition of GF120918 to maternal and fetal circulations significantly decreased this asymmetry. At high concentrations, no differences between fm and mf clearances were detected. In the case of AP (D), a marker of passive diffusion, it is evident that its transplacental pharmacokinetics is not concentration-dependent and, therefore, not affected by either P-gp or Bcrp. Numbers in parentheses show the ratio of the fm-to-mf clearance; bars are means ± S.D. of at least three experiments; Student's t test was used; *, $p < 0.05$; **, $p < 0.01$ versus control.

placenta (Pávek et al., 2001; Staud et al., 2006). It is obvious that these transporters limit mf and possibly also augment fm passage of many xenobiotics. However, these transporters are not omnipotent and the role of other factors, such as physical-chemical properties or plasma protein binding, in the placental transport must not be overlooked. We have previously confirmed the functional activity of placental drug efflux transporters, P-gp (Pavek et al., 2003) and Bcrp (Staud et al., 2006) by use of the model of dually perfused rat placenta. In the latter study, we proposed a pharmacokinetic model describing transplacental transport of ABC substrates that allows for separate quantification of both passive and active events of the process. The aim of the present article was to investigate and compare transplacental passage of several ABC substrates and quantify the effect of drug efflux transporters and/or lipid solubility.

Glyburide is one of the sulfonylureas intensively studied during the past decade within the search for alternative treatment of gestational diabetes. In situ perfusions of human placental cotyledon have revealed that GLB crosses the placenta to fetal compartment in insignificant amounts (Elliott et al., 1991, 1994). Likewise, a randomized, controlled trial failed to detect measurable GLB levels in umbilical cord blood of infants born to mothers that were treated with the drug (Langer et al., 2000). This low permeability of the drug through the placenta was originally attributed to high plasma protein binding (99.8%) and short elimination half-life (Koren, 2001; Nanovskaya et al., 2006). Recently,

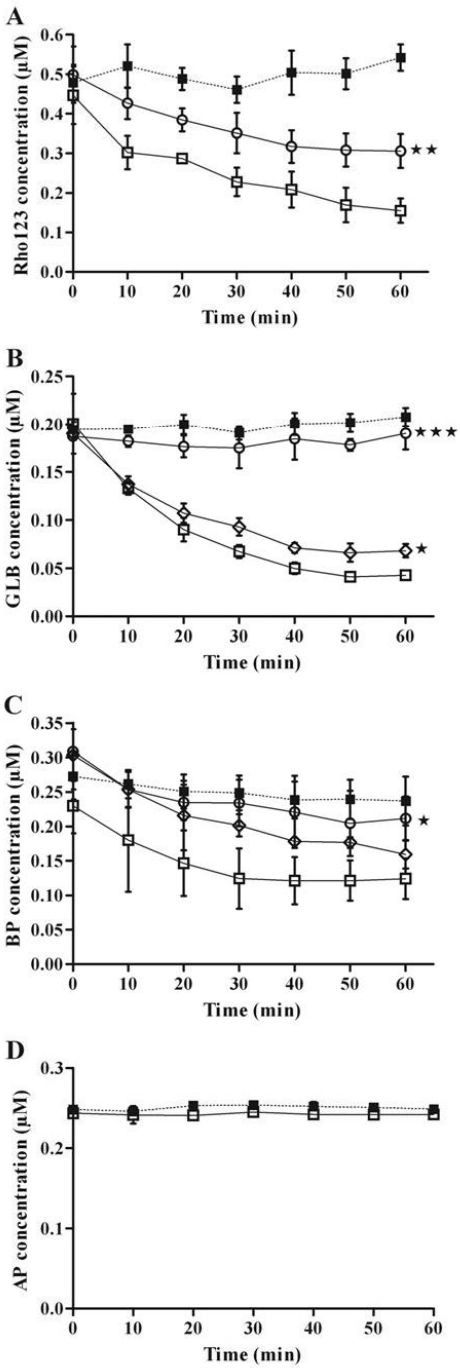

Fig. 4. Elimination of Rho123 (A), GLB (B), and BP (C) from fetal circulation by placental P-gp and/or Bcrp at low Rho123 (0.5 μM), GLB (0.2 μM), and BP (0.25 μM) concentrations. Fetal Rho123, GLB, and BP concentrations decreased to 0.207, 0.049, and 0.121 μM, respectively, and stabilized after 40 min of perfusion. This decline was, in part, inhibited by GF120918 (2 μM) or fumitremorgin C (2 μM). Concentrations of AP (D) remained stable in both circulations throughout the whole experiment, confirming lack of active transport of the molecule. Experimental values are presented as means ± S.D. of at least three experiments; ■, maternal concentration; ○, fetal concentration with GF120918; ◇, fetal concentration with fumitremorgin C; □, fetal concentration without inhibitor; Student's *t* test or one-way analysis of variance followed by Bonferroni's test were used; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with control (fetal concentration without inhibitor).

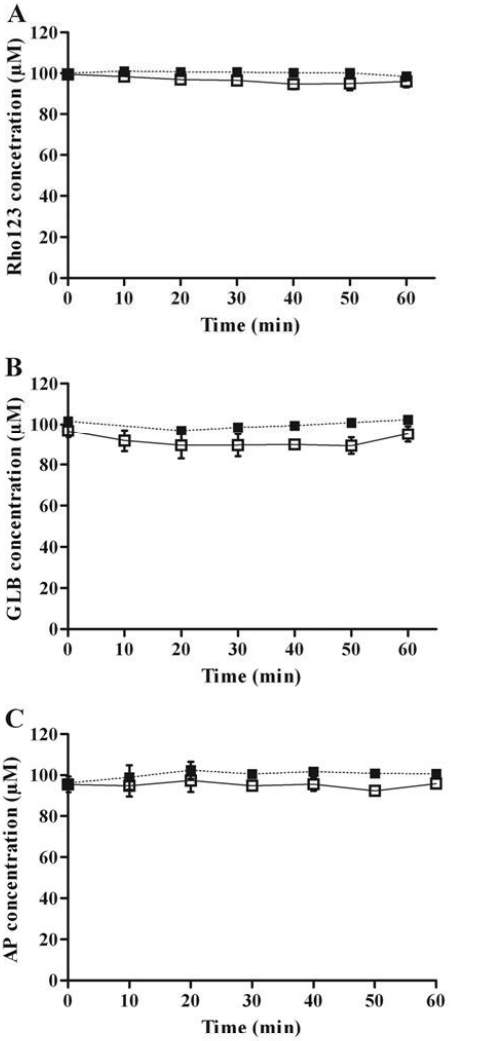

Fig. 5. Elimination of Rho123 (A), GLB (B), and AP (C) from fetal circulation at high concentration (100 μM). At this concentration, no decrease in fetal compartment was observed, suggesting saturation and limited activity of the transporters. Data are presented as means ± S.D. of at least three experiments. ■, maternal concentration; □, fetal concentration.

Kraemer et al. (2006) provided the first direct evidence, using in vitro close-circle perfusions of a human placental cotyledon, that GLB is actively effluxed by a transporter other than P-gp. Shortly afterward, other studies suggested

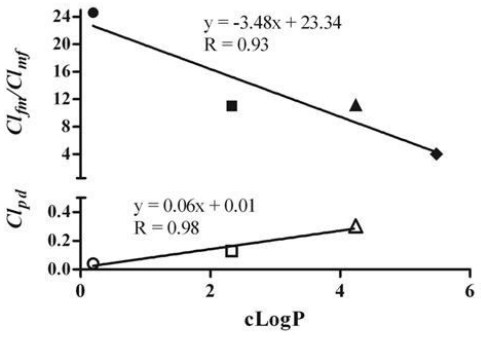

Fig. 6. Effect of the *n*-octanol/water partition coefficient (cLogP) on clearance of passive diffusion (Cl_{pd}) (○, □, △) and on efflux transporter effectiveness (expressed by C_{fm}/C_{mf} clearance ratio) (●, ■, ▲, ♦). Cimetidine (●, ○), Rho123 (■, □), GLB (▲, △), and BP (♦). Data concerning cimetidine were taken from our previous article (Staud et al., 2006). This figure suggests that the higher the lipid solubility of a compound, the higher the clearance of passive diffusion and the lower the effect of transport proteins on transplacental passage.

interaction of GLB with placental BCRP by use of specific inhibitors of a group of various transporters (Gedeon et al., 2006, 2008; Pollex et al., 2008; Zhou et al., 2008). In contrast to human studies, when tritium-labeled GLB was injected into pregnant rat, the fetal-to-maternal radioactivity ratio was 0.535, similar to diazepam (0.641) (Sivan et al., 1995). The authors concluded that GLB crosses the rat placenta and should be considered with caution in the treatment of gestational diabetes. This result was ascribed to interspecies differences (Langer et al., 2000), but was not confirmed in our study. In our experimental setup of dually perfused rat term placenta, albumin was replaced by dextran in the perfusion liquid to avoid contamination of results by plasma protein binding. Subsequently, a broad range of GLB concentrations was tested to unveil nonlinearity of mf and fm transport of the compound. Fitting the placental clearances versus drug concentrations to our PK model resulted in sigmoid curves for both directions, suggesting involvement of an active transport. Comparing fm and mf clearances at low GLB concentrations (0.2 μM), fm clearance was 11.2-fold higher than clearance in the opposite direction. Addition of GF120918 inhibitor reduced this asymmetry to 2.1. Strong effect of BCRP was observed also in the fetal-recirculation experimental setup in which the fetal-to-maternal concentration ratio of GLB toward the end of the experiment was 0.2; addition of inhibitor (2 μM GF120918) reversed this ratio to 0.92. Our data are thus in agreement with those obtained from human (Gedeon et al., 2006; Pollex et al., 2008) and mouse (Zhou et al., 2008) placentas and confirm GLB interaction with rat placental Bcrp. In addition, by use of a fetal recirculation setup, we also evidently demonstrate the ability of Bcrp to transport GLB from fetus to mother even against a concentration gradient, which is in accord with the results obtained by a dual perfusion system on the isolated human placental lobules (Pollex et al., 2008). Our results thus confirm extensive impact of Bcrp on GLB transport across the rat placenta; however, the possible effect of other transporters cannot be excluded.

Rhodamine 123, a fluorescent dye, was established as a model compound for P-gp-mediating transport in various sites of the body (Masereeuw et al., 1997; van der Sandt et al., 2000). In our previous article (Pavek et al., 2003), Rho123 was shown to interact with placental P-gp. Here, we confirm these findings by use of a wide range of inflow Rho123 concentrations in both mf and fm directions and by use of a highly effective inhibitor GF120918 (de Bruin et al., 1999). Unlike Pavek et al. (2003), we have omitted albumin from the perfusion buffer so that the net transfer of Rho123 could be measured without any distorting effect of protein binding. Infusion of Rho123 to the maternal or fetal side of the placenta resulted in a nonlinear relationship between clearance and drug concentration, as observed in studies with GLB in this study, or recently with cimetidine (Staud et al., 2006). The low-concentration plateau of the sigmoid line represents the combined effect of passive clearance and efflux transporter activity; the high-concentration plateau delineates clearance of passive diffusion alone. In addition, we show that the concentration of Rho123 in the fetal compartment decreased by 70% within 60 min of the recirculation experiment, confirming the ability of P-gp to remove its substrate even against a concentration gradient.

BODIPY FL prazosin was included in this study because it is a highly lipophilic compound transported by both P-gp and BCRP (Kimchi-Sarfaty et al., 2002; Hori et al., 2004). With use of this "dual substrate" we aimed to test whether the number of transporters involved in drug transfer is reflected in the transplacental PK. Contrary to our expectations, however, the ratio of BP clearances between fm and mf direction (4.0) was the lowest among all tested substances (Rho123, 11.0; GLB, 11.2) indicating rather limited transporter effectiveness (Fig. 3). Furthermore, addition of a P-gp and BCRP inhibitor, GF120918, caused only 21% increase in mf transport of BP, whereas, in the case of cimetidine, the increase was 45% (Staud et al., 2006). Likewise, when testing the elimination of various substrates from the fetal compartment by fetal reservoir recirculation, BP concentration decreased by 2-fold after the stabilization period, whereas the concentration of GLB decreased by 4-fold. We therefore suggest that the number of efflux transporters involved in placental transport of a substrate does not necessarily correlate with its placental transfer. It seems plausible that other characteristics, such as physical-chemical properties, lipid solubility, in particular, may outweigh the effect of efflux transporters.

In our PK model (Staud et al., 2006) we hypothesized, that drugs with higher lipid solubility and therefore faster passive diffusion will be less affected in their placental passage by ABC transporters than drugs with low lipid solubility. This hypothesis has been demonstrated in this study; when plotting Cl_{pd} and Cl_{fm}/Cl_{mf} ratio against cLogP it is evident that a rise in lipid solubility increases the passive diffusion and, at the same time, decreases the efflux transporter effectiveness (Fig. 6). This relationship provides a reasonable explanation for the transplacental passage of BP. Despite the fact that this substrate is transported by both P-gp and BCRP, the combined effect of these transporters on the transplacental passage of BP seems to be suppressed by high lipid solubility of the molecule and, therefore, rapid clearance by passive diffusion. Therefore, the simple statement that a compound is a substrate of one or more drug efflux transporter(s) is not sufficient to forecast its lower transport from mother to fetus.

In summary, the role of P-gp and Bcrp in the transplacental pharmacokinetics of Rho123, GLB, and BP has been described. These efflux transporters were confirmed to limit the entry of their substrates to fetal circulation and pump them from fetus to mother against concentration gradient. However, the effectiveness of drug efflux transporters is markedly reduced in highly lipophilic drugs.

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VI
SOUHRN

Placenta představuje jedinečné spojení mezi matkou a plodem zajišťující řadu funkcí nezbytných pro správný průběh těhotenství a vývoj jedince. Jednou z hlavních funkcí placenty je výměna plynů, přívod živin do plodu a odvod jeho metabolických zplodin. Mimoto plní placenta také roli endokrinní, metabolickou a především ochrannou, jelikož matka je v průběhu těhotenství vystavována, ať už nevědomě či záměrně, celé škále pro plod toxických látek. Dříve se předpokládalo, že fyziologickou bariéru mezi cirkulacemi matky a plodu tvoří pouze buněčné vrstvy syncytiotrofoblast a endotel fetálních kapilár. Nedávno však bylo zjištěno, že kromě této mechanické složky disponuje placenta i ochrannou složkou aktivní, která je realizována působením efluxních transportérů a biotransformačních enzymů lokalizovaných v polarizované vrstvě syncytiotrofoblastu.

Efluxní transportéry jsou membránové proteiny, které jsou díky své afinitě k ATP schopny aktivně vypumpovávat velké množství různorodých molekul ven z buňky. Dosud nejlépe prostudovanými lékovými efluxními transportéry jsou P-glykoprotein (P-gp) a „breast cancer resistance protein“ (BCRP), které významným způsobem ovlivňují kinetiku transportu látek přes placentu. Hlavním cílem této dizertační práce bylo studium exprese placentárních transportérů Bcrp a P-gp a jejich vlivu na transport farmak přes placentu.

V první práci jsme s využitím metod RT-PCR, Western blottingu a imunohistochemie prokázali přítomnost Bcrp v terminální potkaní placentě i buněčné linii HRP-1. Při paralelním testování P-gp jsme popsali značnou expresi v placentě, avšak v placentárních buňkách HRP-1 nebyl tento transportér detekován. Přítomnost Bcrp v buněčné linii HRP-1 jsme potvrdili i na úrovni funkční s využitím inhibitorů Ko143 a GF120918. Jejich účinkem jsme pozorovali zvýšení akumulace fluorescenčně značeného substrátu BODIPY FL prazosinu do buněk o více než 100%. Stejně tak byla touto akumulační studií prokázána absence efluxní aktivity P-gp, což potvrdilo jeho nedetekovatelnost na úrovni mRNA a proteinu. Dále jsme v této práci studovali vliv Bcrp na transplacentární prostup modelového substrátu cimetidinu s využitím metody duálně perfundované potkaní placenty. Sledování transportu látky ve směru materno-fetálním a feto-maternálním prokázalo značnou asymetrii, která byla částečně snížena přidáním BCRP inhibitorů fumitremorginu C a GF120918, a zcela eliminována při vysokých koncentracích cimetidinu. Tato práce přinesla dva základní poznatky týkající se protektivní role BCRP v placentě, a to že

transportér nejen redukuje prostup substrátů z matky do plodu, ale také aktivně odstraňuje léčiva v cirkulaci plodu již přítomná.

Přestože ochranná aktivita Bcrp v placentě na konci březosti byla první studií potvrzena, stále nebylo zřejmé, zda je transportér Bcrp přítomen v placentě experimentálního zvířete po celou dobu březosti a zda se jeho exprese a funkce v návaznosti na gestační období mění. V naší další práci jsme tudíž sledovali expresi placentárního Bcrp v průběhu březosti potkana na úrovni mRNA a pozorovali signifikantně vyšší množství Bcrp transkriptů v 15. než v 12., 18. a 21. dni. V další části této studie jsme ve fetální tkáni kvantifikovali sedminásobně vyšší expresi Bcrp mRNA ke konci gestace v porovnání s 12. dnem. Ochrannou roli placentárního a fetálního Bcrp jsme zkoumali sledováním množství modelového Bcrp substrátu cimetidinu ve fetální tkáni po infuzní aplikaci do krve matky. Ve 12. dni gestace jsme pozorovali desetinásobně vyšší expozice plodu cimetidinu vzhledem k ostatním sledovaným dnům. Navíc srovnání postinfuzní hladiny substrátu v plodu odhalilo podstatně nižší průnik do mozku než do celkové fetální tkáně. Tyto poznatky podpořily naši hypotézu, že rostoucí exprese Bcrp ve fetální tkáni může s postupujícím těhotenstvím posilovat protektivní roli placenty před látkami pro vyvíjející se plod potenciálně toxickými.

Překrývající se substrátová specifita a podobná tkáňová distribuce transportérů P-gp a BCRP naznačují také jejich společnou úlohu na detoxikačních procesech placenty, proto jsme se rozhodli naši další práci zaměřit na posouzení efektu P-gp a Bcrp na transplacentární farmakokinetiku (PK) jejich substrátů. S využitím specifických inhibitorů, koncentrační škály modelových látek a PK výpočtů na modelu duálně perfundované potkaní placenty jsme potvrdili účinek P-gp a Bcrp ve smyslu omezení materno-fetálního (mf) a urychlení feto-maternálního (fm) transportu. Otázku, zda počet transportérů zapojených do transferu látky může ovlivnit její transplacentární PK, jsme se pokusili zodpovědět aplikací duálního substrátu P-gp a BCRP – BODIPY FL prazosinu (BP) na náš model perfundované potkaní placenty. Navzdory očekávání byl však u BP poměr clearance ve fm a mf směru (4,0) nejnižší mezi sledovanými látkami (cimetidin – 24,6; rhodamin 123 - 11,0; glyburid - 11,2). Obdobných výsledků bylo dosaženo také při testování eliminace týchž substrátů z kompartmentu plodu s využitím recirkulace fetálního rezervoáru, což naznačilo, že vliv fyzikálně-chemických vlastností, zejména liposolubility, může převážit nad efektem proteinů P-gp a Bcrp na transplacentární

transport BP. Tuto hypotézu jsme v naší studii potvrdili PK modelováním. Ze vztahu mezi pasivním/aktivním transportem a liposolubilitou vyplynulo, že vzestup rozpustnosti látky v tucích zvyšuje pasivní difuzi a zároveň snižuje účinnost lékových transportérů.

Závěrem lze konstatovat, že výsledky našich studií potvrzují expresi a funční aktivitu Bcrp a P-gp v placentě potkana a kvantifikují vliv těchto efluxních transportérů na ochranu a detoxikaci plodu. Z poslední studie je však zřejmé, že relativní efektivita efluxních transportérů v transplacentární farmakokinetice může být limitována dalšími faktory, jako je lipofilita sledovaných molekul.

VII
SUMMARY

Placenta, which represents a unique link between the mother and fetus, fulfills many functions essential for normal course of pregnancy and uncomplicated development of the fetus. Nutrient supply, gas exchange and metabolic waste product removal belong to its main roles. In addition, placenta serves as an endocrine, metabolic, immune and protective organ, since during pregnancy mother may be, either unconsciously or deliberately, exposed to a wide range of substances toxic for the fetus. Originally, it was supposed that the physiological barrier between maternal and fetal circulation is created only by cellular layers of syncytiotrophoblast and endothelium of fetal capillaries. However, it has been demonstrated that besides this mechanical component of protection, activity of drug efflux transporters and metabolic enzymes localized in the polarized syncytiotrophoblast layer contribute considerably to the protective function of placental barrier.

Efflux transporters are ATP dependent membrane proteins capable of actively removing different molecules out of cells. So far, the best described drug efflux transporters are P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP), significantly affecting kinetics of transplacental passage of various substances.

The main goal of this work was to study the expression of placental transporters Bcrp and P-gp and their effect on the transport of drugs across the placenta. In the first study the presence of Bcrp in the rat term placenta and placental cell line HRP-1 was demonstrated by RT-PCR, Western blotting and immunohistochemistry. Simultaneously, we detected the expression of P-gp in the rat placenta but not in HRP-1 cell line. We confirmed the presence of Bcrp in the HRP-1 cells also functionally using inhibitors, Ko143 and GF120918, which increased accumulation of a fluorescent BCRP substrate BODIPY FL prazosin to the cells by more than 100%. Consistently with the results of expression studies no activity of P-gp was observed in the HRP-1 cell line. Furthermore, we investigated the impact of Bcrp on the transplacental transfer of a model substrate cimetidine using dually perfused rat placenta. We observed a considerable asymmetry between the materno-fetal and feto-maternal transport of the substrate, which was partly decreased by BCRP inhibitors fumitremorgin C and GF120918 and entirely eliminated at high cimetidine concentrations. This study clearly demonstrated that BCRP not only reduces the passage of drugs from the mother to the fetus but also actively removes the drug already present in fetal blood, even against concentration gradient.

Although we described the protective activity of placental Bcrp at the end of gestation, its expression and function in the earlier stages of gestation remained unclear. In our following study, therefore, we analyzed the expression of placental Bcrp mRNA during the course of pregnancy in rat and observed significantly higher amount of Bcrp transcripts on gestation day (gd) 15 compared to gd 12, 18 and 21. In the next part of this study we quantified a 7-fold higher level of Bcrp mRNA in fetal tissues at the end of gestation compared to the 12^{th} gd. Furthermore, we studied the function of placental and fetal Bcrp by fetal exposure to a model substrate, cimetidine, infused to the maternal circulation. The relative amount of drug that penetrated to the fetus was highest on gd 12 and decreased to one tenth thereafter. Moreover, we have revealed much lower penetration of cimetidine to the brain compared to the whole fetal tissue. Our results indicate that increasing expression of Bcrp in fetal tissues can strengthen the protective role of placental Bcrp as pregnancy proceeds.

Overlapping substrate specificity and similar tissue distribution shared by P-gp and BCRP suggest their common role in detoxication processes of the placenta. Therefore, in the final study we aimed to evaluate the effect of P-gp and Bcrp on the transplacental pharmacokinetics (PK) of their substrates using the model of dually perfused rat placenta. Specific inhibitors, various concentrations of model substrates and PK modeling have been applied to assess the efficacy of these proteins to hinder maternal-to-fetal (mf) and accelerate fetal-to-maternal (fm) transport. Using a dual substrate of P-gp and BCRP, BODIPY FL prazosin (BP), we tried to answer the question whether the number of transporters involved in the transfer of a substance is reflected in its transplacental PK. Contrary to our expectations, however, the ratio of BP clearances between fm and mf direction (4.0) was the lowest among all tested substances (cimetidine – 24.6; rhodamine – 11.0; glyburide – 11.2). When testing the elimination of the same substrates from the fetal compartment by fetal reservoir recirculation, similar results were obtained, suggesting that effect of physical-chemical properties, especially liposolubility, can outweigh the impact of P-gp and Bcrp on the transplacental transport of BP. This hypothesis was confirmed in our study by PK modeling. Based on the relationship between passive/active transport and liposolubility we conclude that a rise in lipid solubility increases the passive diffusion and, at the same time, decreases the efflux transporter effectiveness.

In conclusion, the results of our studies confirm expression and functional activity of Bcrp and P-gp in the rat placenta and quantify the role of these transporters in fetal protection and detoxication. However, the last study indicates that the relative effectiveness of efflux transporters in transplacental pharmacokinetics may be limited by other factors, such as drug lipid solubility.

VIII
SEZNAM PUBLIKOVANÝCH PRACÍ

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