Univerzita Karlova v Praze
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Dizertační práce

Interakce vybraných antiepileptik s efluxními lékovými ABC transportéry a nukleárními receptory

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Seznam zkratek

ABC	ATP-binding cassette
ATP	Adenosintrifosfát
BBB	Blood brain barrier
BCRP	Breast cancer resistance protein
cAMP	Cyklický adenosinmonofosfát
CAR	Konstitutivní androstanový receptor (Constitutive Androstane Receptor)
CCRP	Cytoplazmatický CAR vázající protein (CAR Cytoplasmic Retention Protein)
CFTR	Cystic fibrosis conductance regulator
CITCO	6-(4-chlorofenyl)imidazo[2,1-b][1,3]-thiazol-5-karbaldehyd-O-(3,4-dichlorobenzyl)oxim
cGMP	Cyklický guanosinmonofosfát
CNS	Centrální nervová soustava
Coact	Koaktivátor (z anglického coactivator)
CYP	Cytochrom P450
DR	Direct repeat
EMSA	Electrophoretic mobility shift assay
ER	Everted repeat
HEB	Hematoencefalická bariéra
HDAC	Histonové deacetylázy
HSP90	Heat shock protein 90
LS174T	Buněčná linie odvozená od lidského střevního adenokarcinomu
LTC4	Leukotrien C4
MDCKII	Madine-Darby canine kidney II (buněčná linie)
MEF3.8	Buněčná linie odvozená od myších fibroblastů
MRP	Proteiny spojené s vícečetnou lékovou rezistencí (Multidrug Resistance-Associated Proteins)
NBD	Nukleotid vázající doména (Nucleotid Binding Protein)
PCR	Polymerázová řetězová reakce (Polymerase Chain Reaction)
P-gp	P-glykoprotein
PP-2A	Proteinová fosfatáza 2A (Protein Phosphatase 2A)
PXRE	Proximální responzivní element
RT-PCR	Reverzní transkriptázová polymerázová řetězová reakce
PXR	Pregnanový X receptor (synonymum: steroidní X receptor; SXR)
RXRα	Retinoidní X receptor α
SXR	Steroidní X receptor (synonymum: pregnanový X receptor; PXR)
Syn.	Synonymum
UGT	UDP-glukuronosyltransferáza
XREM	Distální responzivní element (Xenobiotics Responsive Element)

Obsah

1. Úvod

1.1. Farmakologie epilepsie

Epilepsie je chronické onemocnění CNS různé etiologie, které postihuje 1 – 2 % populace. Pro toto onemocnění jsou charakteristické opakující se záchvaty nekontrolovaného dráždění neuronů mozku, které se od ostatních liší nestabilitou klidového potenciálu. Elektrické výboje ze zachvácené oblasti jsou na elektroencefalogramu vyjádřeny vysokofrekvenčními impulsy. Lokalizace primárního výboje a jeho další šíření určují příznaky epileptického záchvatu. Záchvaty se tak mohou projevit motorickými, senzitivními a vegetativními (orgánovými) reakcemi. Z farmakoterapeutického hlediska lze epileptické záchvaty rozdělit takto:

- Parciální záchvaty
  - ⇨ Simplexní parciální
  - ⇨ Komplexní parciální záchvat s poruchou vědomí (syn. psychomotorická epilepsie; epilepsie spánkového laloku)
  - ⇨ Sekundárně generalizovaný parciální záchvat

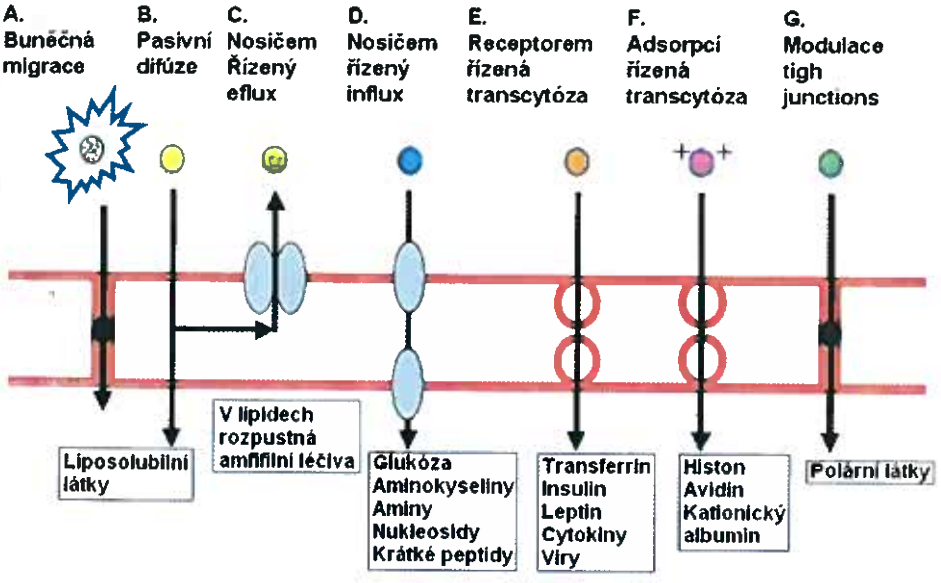
- Generalizované záchvaty
  - ⇨ Absence (syn. *petit mal*)
  - ⇨ Myoklonický záchvat
  - ⇨ Atonický záchvat
  - ⇨ Tonicko-klonický záchvat (syn. *grand mal*)

Antiepileptika jsou skupinou léčiv snižující výskyt motorických a psychických projevů epilepsie tím, že redukují křečovou pohotovost organismu. Základní mechanizmus účinku antiepileptik spočívá ve stabilizaci klidového membránového potenciálu a snížení dráždivosti nervových buněk tvořících paroxysmální výboje. Protože jednotlivé epileptické záchvaty trvají relativně krátce, jejich akutní terapie není možná. Terapie epilepsie antiepileptiky je tedy dlouhodobý proces, který je mnohdy ukončen až úmrtím pacienta. Dnes používaná antiepileptika jsou rozdělena do tří generací. Níže uvádím některé zástupce jednotlivých generací včetně indikací (v závorce).

1. generace: **ethosuximid** (absence u dětí), **fenytoin** (generalizované tonicko-klonické záchvaty, parciální záchvaty, nitrožilně podaný při *status epilepticus*), **primidon**

Transport menších polárních molekul jako jsou glukóza a esenciální aminokyseliny je zajištěn přítomností více či méně specifických transportních mechanismů. Makromolekuly (peptidy a proteiny) jsou transportovány převážně transcytózou, která může být buď receptorově zprostředkovaná nebo na receptorech nezávislá (Begley and Brightman, 2003). Na druhé straně, lipofilní látky mají potenciál procházet přes HEB pasivní difúzí. Rychlost přestupu látek přes HEB pasivní difúzí je dána především mírou jejich rozpustnosti v tucích a dále koncentračním gradientem na obou stranách HEB. Existuje však velký počet výjimek, u nichž přechod přes HEB lipofilitě neodpovídá. U těchto látek bylo prokázáno, že jejich přechod přes HEB je často významně redukován některými efluxními lékovými **ABC transportéry** (ATP-binding cassette) (Begley and Brightman, 2003). Veškeré dosud popsané možnosti transportu látek přes HEB jsou shrnuty na Obrázku 2.

Některé efluxní lékové ABC transportéry byly identifikovány jako klíčové determinanty průchodu mnoha dnes používaných lipofilních léčiv, popřípadě jejich konjugátů či metabolitů přes fyziologické bariéry organismu, včetně již zmíněné HEB (Schinkel and Jonker, 2003).



Obr. 2. Známé možnosti transportu látek přes hematoencefakickou bariéru (HEB). (A) Leukocyty mohou procházet přes HEB skrze buněčná spojení. (B) Lipofilní látky procházejí pasivní difúzí. (C) Některé liposolubilní látky jsou z HEB aktivně pumpovány zpět do krevního oběhu. (D) Nosičem řízený transport, který může být pasivní nebo sekundárně aktivní (glukóza, aminokyseliny, nukleosidy). (E) Receptorově řízená transcytóza, která umožňuje transport makromolekul typu peptidů a proteinů. (F) Adsorpcí řízená transcytóza, jež může být nespecificky spuštěna nabitými makromolekulami. (G) Modulace funkce „tight junctions" řídí částečný nebo úplný průchod polárních látek přes HEB paracelulární cestou. Zpracováno dle publikace Begley and Brightman, 2003.

Pokud jde o strategii léčby epilepsie antiepileptiky, z počátku je cílem monoterapií dosáhnout stavu bez záchvatů. Dávka antiepileptika se zvyšuje do té doby, dokud záchvaty

neustanou nebo dokud se nežádoucí účinky nestanou nepřijatelnými. Pokud se prokáže neúčinnost monoterapie přechází se na antiepileptikum druhé volby, popřípadě na tzv. add-on terapii. Úspěšnost kontroly epileptických záchvatů antiepileptiky se pohybuje okolo 70 %.

Přibližně 30 % pacientů však tvoří skupinu s **farmakorezistentní** (refrakterní) **epilepsií,** která představuje vážný klinický problém spojený s vysilujícími psychosociálními potížemi a se zvýšeným rizikem úmrtí pacienta. Farmakorezistence v epilepsii je definována jako selhání kontroly epileptických záchvatů navzdory užívání kombinace tří nebo více antiepileptik v maximálních tolerovaných dávkách (Sisodiya, 2003). Většina pacientů trpících farmakorezistentní epilepsií je rezistentní k téměř všem klinicky používaným antiepileptikům a to i přesto, že tato léčiva mají rozdílné mechanismy účinku. Tento fakt popírá teorii patologických změn specifických vazebných míst pro antiepileptika a podporuje teorii, že se jedná o nespecifický, pravděpodobně adaptabilní mechanismus jako je snížený up–take antiepileptik do mozku (Sisodiya, 2003; Sisodiya and Thom, 2003). Za možnou příčinu tohoto fenoménu je považována mezi jinými i **up-regulace** a **ektopická** exprese některých ABC transportérů v HEB (Sisodiya, 2003; Sisodiya and Thom, 2003).

Užívání antiepileptik pacienty s sebou často nese riziko více či méně se projevujících nežádoucích účinků. Příkladem takových nežádoucích účinků mohou být sedace, zmatenost, ataxie, hepatotoxicita či teratogenní efekt. Dalšími nežádoucími účinky jsou **farmakokinetické interakce** antiepileptik např. s léčivy užívanými k terapii souběžně probíhajícího onemocnění. Konečným důsledkem takové farmakokinetické interakce může být zvýšení nebo snížení plazmatické koncentrace antiepileptika, stejně tak jako souběžně podávaného léčiva. Zvýšení plazmatické koncentrace antiepileptik velmi často předznamenává vystupňované projevy nežádoucích účinků léčiva, naopak snížení se může projevit epileptickými záchvaty (Patsalos and Perucca, 2003).

Příčinou farmakokinetických interakcí často bývá léčbou podmíněná modulace aktivity a exprese některých ABC transportérů a metabolických enzymů z rodiny cytochromu P450 (Patsalos et al., 2002; Patsalos and Perucca, 2003; Perucca, 2006), které společně určují biodostupnost a rychlost eliminace řady léčiv a tím i výsledný farmakologický efekt a míru projevů nežádoucích účinků (Strolin Benedetti et al., 1990; Strolin Benedetti and Dostert, 1994; Chen and Raymond, 2006). Molekulární mechanismy modulace aktivity a exprese cytochromu P450 a ABC transportérů nebyly doposud plně popsány, avšak předpokládá se, že důležitou úlohu bude mít regulace exprese těchto genů na úrovni transkripce. Pomocí různých experimentálních přístupů bylo zjištěno, že v transkripční regulaci *CYP3A4* a *MDR1* (gen kódující P-glykoprotein) existuje určitá spojitost; např. fenobarbital, rifampicin a klotrimazol

mají schopnost indukovat v buněčných liniích LS 180/WT a LS 180/AD50 jak *CYP3A4*, tak i *MDR1*. Zýšená exprese *MDR1* a *CYP3A4* byla podobně prokázána i v kultuře primárních hepatocytů kultivovaných v přítomnosti dexametazonu (Pichard et al., 1992; Zhao et al., 1993).

Tato pozorování vedla k hypotéze, že regulace *CYP3A4* a *MDR1* je na genové úrovni koordinována pomocí podobného mechanismu. Současné studie ukázaly, že transkripční aktivace těchto genů je kromě jiného řízena dvěma důležitými nukleárními receptory, konkrétně konstitutivním androstanovým receptorem (CAR, NR1I3) a pregnanovým X receptorem (PXR; SXR, NR1I2) (Geick et al., 2001; Goodwin et al., 2002; Burk et al., 2005a; Burk et al., 2005b).

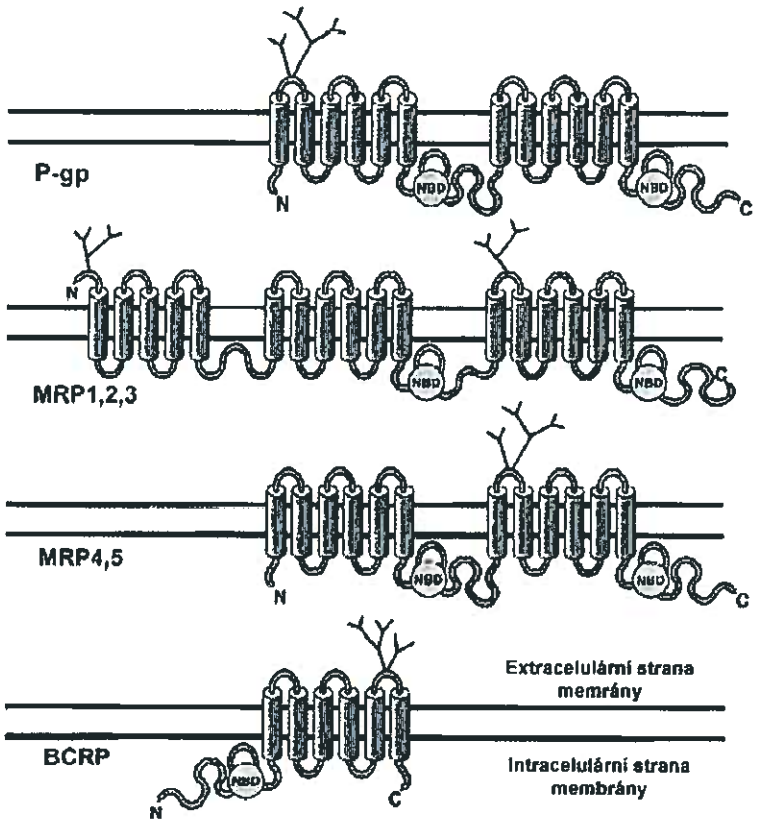
Předkládaná dizertační práce se zabývá:

- Interakcí vybraných antiepileptik s efluxními lékovými ABC transportéry a rolí studovaných ABC transportérů ve vzniku rezistence vůči antiepileptické léčbě.
- Interakcí kyseliny valproové s konstitutivním androstanovým receptorem (CAR) a pregnanovým X receptorem (PXR) a vlivem této interakce na expresi *CYP3A4* a *MDR1*.

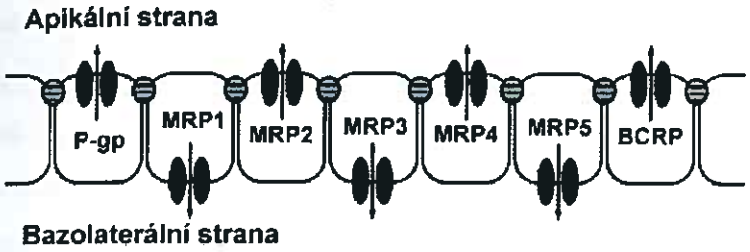
1.2. Interakce antiepileptik s efluxními lékovými ABC transportéry; role studovaných ABC transportérů ve vzniku rezistence vůči antiepileptické léčbě

1.2.1. Charakterizace efluxních lékových ABC transportérů

Efluxní lékové ABC transportéry jsou transmebránové proteiny schopné aktivně, za spotřeby ATP, transportovat strukturálně značně odlišná léčiva a další xenobiotika přes buněčnou membránu i proti výraznému koncentračnímu gradientu (Schinkel and Jonker, 2003). Rodina efluxních lékových ABC transportérů zahrnuje P-glykoprotein (P-gp, MDR1; ABCB1), devět transportních proteinů nazývaných „multidrug resistance-associated proteins" (MRP 1-9; ABC1-6, ABC10-12) a nedávno objevený „breast cancer resistance protein" (BCRP; ABCG2). Všechny efluxní lékové ABC transportéry vykazují do určité míry obdobnou strukturu sestávající se z daného počtu transmembránových domén, intracelulárního ATP-vazebného místa a cukerného zbytku na extracelulární kličce (Obr. 3), přičemž v buněčné membráně mohou být tyto lékové transportéry lokalizovány apikálně nebo bazolaterálně (Obr. 4).



Obr. 3. Sekundární struktura nejznámějších ABC (ATP binding cassette) lékových efluxních transportérů lokalizovaných v plazmatické membráně. NBD ="nucleotide binding domain" = vazebné místo pro ATP. Převzato z přehledového článku Schinkel and Jonker, 2003.



Obr. 4. Lokalizace nejznámějších ABC transportérů v buněčné membráně. Převzato z přehledového článku Schinkel and Jonker, 2003.

V rámci lidského organismu efluxní lékové ABC transportéry do značné míry ovlivňují farmakokinetiku mnoha léčiv a to na úrovni absorpce, distribuce i eliminace. Navíc bylo prokázáno, že některé efluxní lékové ABC transportéry tvoří aktivní složku řady tělesných bariér a chrání tak citlivé tkáně jako např. **mozek**, varle nebo plod před toxickým vlivem xenobiotik (Schinkel and Jonker, 2003). Tato fakta vedla k tomu, že efluxní lékové ABC transportéry a jejich interakce s léčivy se staly v posledních dvou desetiletích intenzivně sledovaným fenoménem, jehož studium nemá jen teoretický význam, ale stále častěji se promítá i do klinické farmakologie a farmakoterapie. Již dnes je známo velké množství lékových interakcí, které lze vysvětlit na základě afinity léčiv k efluxním transportérům (Kakumoto et al., 2002; Takara et al., 2002). Vedle toho, polymorfismus transportérů byl prokázán jako významná příčina variability farmakokinetiky léčiv v populaci (Siddiqui et al., 2003; Sparreboom et al., 2003).

Významná úloha ve farmakokinetice léčiv je připisována především **P-gp**, který je schopný transportovat širokou škálu strukturně odlišných léčiv nejrůznějších farmakoterapeutických skupin. P-gp byl lokalizován ve tkáních důležitých pro dispozici léčiv jako jsou střevo, játra, ledviny, HEB, hematotestikulární bariéra či placenta. P-gp ovlivňuje osud řady léčiv v organismu tím, že omezuje jejich střevní absorpci, brání distribuci do některých tkání a urychluje exkreci těchto léčiv z organismu; viz. přehledové články (Lin, 2003; Lin and Yamazaki, 2003; Fromm, 2004).

MRP transportéry plní vedle pravděpodobné funkce farmakologické řadu rolí fyziologických. Mezi substráty těchto transportérů patří látky povahy organických aniontů, především ve formě konjugátů s glutathionem, glukuronovou kyselinou či sulfáty a dále endogenní látky jako leukotrien C4 (LTC4) a nukleosidové analogy. Funkce jednotlivých MRP transportérů tak spočívá především v jaterní exkreci organických iontů (včetně bilirubinu). Některé MRP transportéry dále pravděpodobně hrají roli v buněčné signalizaci pomocí cAMP a cGMP a v LTC4 regulovaných imunitních reakcích, ABCC7/CFTR má úlohu na protein-kináze A závislého chloridového kanálu v tkáních zapojených v exokrinní činnosti

jako pankreas, střevo a ledviny; přehledně zpracováno v publikacích (Borst et al., 1999; Borst et al., 2000; Dean and Allikmets, 2001; Dean et al., 2001; Borst and Elferink, 2002; Leonard et al., 2003; Schinkel and Jonker, 2003).

BCRP byl popsán jako transportér schopný způsobovat rezistenci nádorových buněk vůči mitoxantronu, doxorubicinu a daunorubicinu (Doyle et al., 1998). Kromě cytotoxických chemoterapeutik zahrnuje spektrum substrátů BCRP transportéru i léčiva z dalších farmakoterapeutických skupin jako jsou například fluorochinolony (Schinkel and Jonker, 2003; Merino et al., 2006; Ando et al., 2007). BCRP, podobně jako P-gp, je přítomen ve tkáních důležitých pro dispozici léčiv, především v játrech, ve střevě, v placentě a HEB. Navíc je BCRP zodpovědný za specifický fenotyp subpopulace kmenových buněk a ochranu buněk před hypoxií (Allen and Schinkel, 2002; Sarkadi et al., 2004; Štaud and Pávek, 2005).

P-gp, BCRP a někteří zástupci skupiny MRP byli intenzivně zkoumáni především pro svou schopnost aktivně vypumpovávat cytotoxické látky ven z nádorových buněk a způsobovat tak jejich rezistenci vůči cytostatikům (Roninson, 1987; Goldstein et al., 1991; Bosch and Croop, 1996; Naito et al., 1995; Kruh et al., 2001; Litman et al., 2000; Ross, 2000).

Z pohledu funkce ABC transportérů lokalizovaných v HEB je v dnešní době pozornost zaměřena především na P-gp, MRP2 a BCRP, u nichž se předpokládá význam v ochraně mozku před potenciálně toxickými xenobiotiky (Cordon-Cardo et al., 1989; Dombrowski et al., 2001; Cooray et al., 2002; Aronica et al., 2005) a také na MRP1, který byl lokalizován v chorioideálním plexu (Rao et al., 1999; Wijnholds, 2002).

1.2.2. Role efluxních lékových ABC transportérů ve vzniku farmakorezistentní epilepsie

Data získaná imunohistochemickými a molekulárně genetickými metodami ukazují, že v mozcích pacientů postižených farmakorezistentní epilepsií způsobenou např. fokální kortikální dysplazií, neuroepiteliálním nádorem nebo hippokampální sklerózou dochází v HEB ke zvýšené expresi P-gp (Sisodiya and Thom, 2003) a MRP2 (Potschka et al., 2003). Tyto proteiny byly za podobných okolností detekovány a lokalizovány i v populaci buněk mozkového parenchymu, kde se za fyziologických podmínek nevyskytují (Aronica et al., 2004).

Provedené studie naznačují, že jmenované proteiny, analogicky k rezistenci nádorů vůči léčbě cytostatiky, jsou schopny snižovat „up-take" antiepileptik do epileptogenních center mozku (Sisodiya, 2003; Sisodiya and Thom, 2003).

Aby bylo možné potvrdit nebo vyvrátit hypotézu, že se zvýšená exprese efluxních lékových ABC transportérů podílí na rozvoji farmakorezistentní epilepsie, je potřeba experimentálně ověřit, zda antiepileptika patří mezi jejich substráty. Jelikož není možné provést studii přechodu antiepileptik přes lidskou HEB, byla tato problematika doposud studována pouze na zvířecích modelech (např. „knockoutované" myši, popřípadě potkani deficientní pro daný transportér). V poslední době jsou experimenty tohoto typu na zvířatech nahrazovány transportními experimenty na monovrstvách buněk, které trvale exprimují daný ABC transportér.

Ze skupiny antiepileptik byl pozorován snížený průchod fenytoinu, fenobarbitalu a karbamazepinu přes **potkaní** HEB způsobený efluxní aktivitou P-gp (Potschka et al., 2001; Potschka and Loscher, 2001; Potschka et al., 2002). Dalšími předpokládanými substráty **potkaního** P-gp jsou gabapentin, felbamat a lamotrigin (Luer et al., 1999; Potschka et al., 2002). Pomocí experimentů na myším modelu a *in vitro* studií za použití transfekovaných buněčných linií bylo zjištěno, že topiramat, fenytoin a levetiracetam jsou substráty **myšího** P-gp (Sills et al., 2002; Baltes et al., 2007). Autoři Baltes a kolektiv navíc na základě výsledků *in vitro* studie provedené na transfekovaných buněčných liniích navrhli, že fenytoin, levetiracetam a karbamazepin by mohly být i slabými substráty **lidského** P-gp (Baltes et al., 2007). Fenytoin a karbamazepin jsou prokázanými substráty **potkaního** Mrp2 (Potschka et al., 2003) a pravděpodobně i **lidského** MRP2 (Baltes et al., 2007).

BCRP je dosud posledním objeveným efluxním lékovým ABC transportérem (Doyle et al., 1998). Na základě publikovaných dat existuje hypotéza, že zvýšená exprese BCRP pozorovaná v HEB některých epileptogenních nádorů je potenciální příčinou snížení průchodu antiepileptik do cílových epileptogenních struktur mozku (Cooray et al., 2002; Aronica et al., 2004; Aronica et al., 2005). Avšak antiepileptikum, které by bylo substrátem BCRP, není doposud známo (Červený et al., 2006).

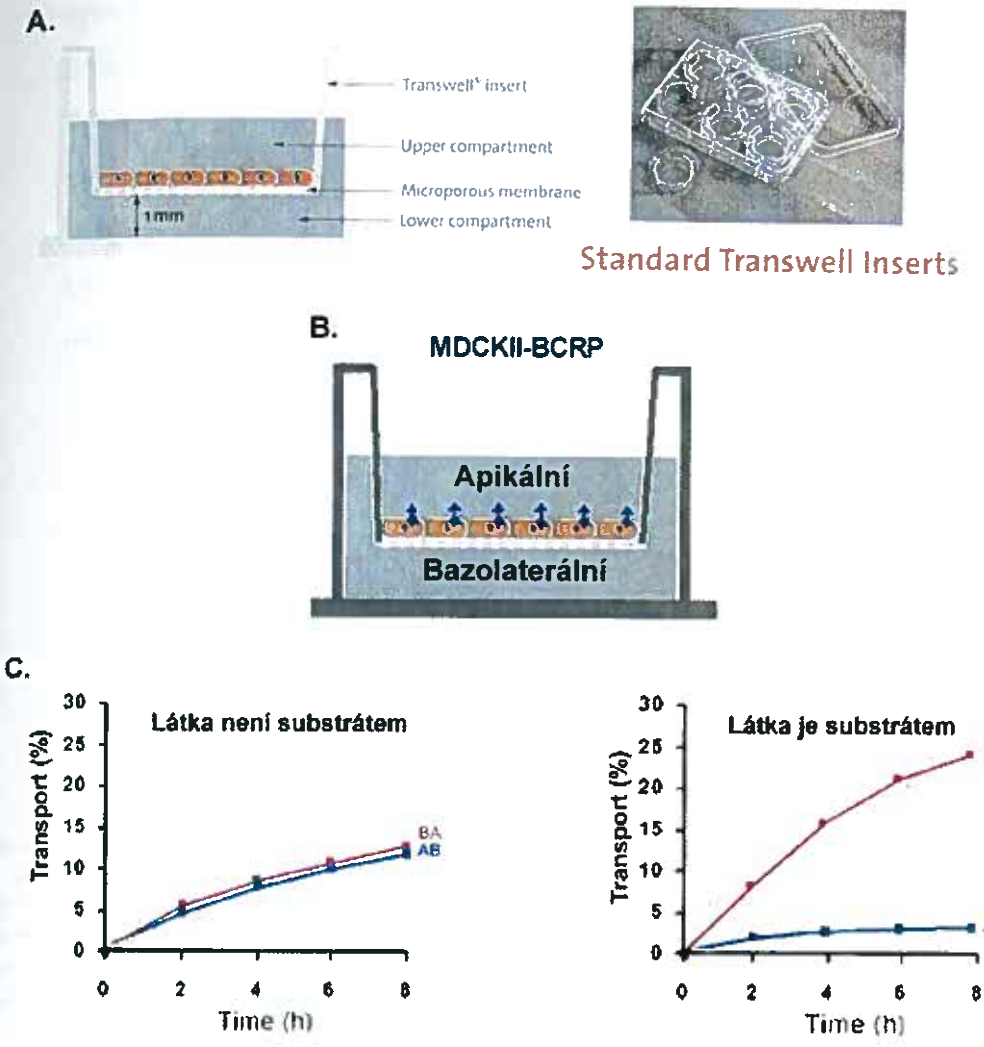
Oblast interakce antiepileptik s ABC transportéry na úrovni exprese a jejich funkční inhibice, stimulace, popřípadě indukce je další relativně neprozkoumaným tématem. Seegers a kol. publikovali studii ukazující, že fenobarbital a fenytoin nemá vliv na expresi P-gp v HEB zdravých potkanů kmene Wistar (Seegers et al., 2002). V jiné publikované studii byl zjištěno, že lamotrigin, karbamazepin, fenytoin a kyselina valproová v koncentracích výrazně vyšších než jsou jejich koncentrace terapeutické mají schopnost slabě inhibovat P-gp (Weiss et al., 2003). Studie zabývající se modulací (inhibice/stimulace) aktivity efluxních lékových transportérů antiepileptiky identifikovaly kyselinu valproovou jako možný mírně působící inhibitor BCRP transportérem zprostředkovaného transportu (Červený et al., 2006).

Z hlediska lékových interakcí jde o poměrně zásadní fenomén. Např. inhibice těchto transportérů by znamenala možnost zvýšeného průniku jiných xenobiotik (substrátů efluxních transportérů) přes HEB a naopak indukce by způsobovala nižší průnik současně podávaných léčiv přes tuto bariéru.

1.2.3. *In vitro* metody použité pro studium interakce antiepileptik s efluxními lékovými ABC transportéry.

V rámci této dizertační práce byly pro studium interakce antiepileptik s efluxními lékovými ABC transportéry použity dvě *in vitro* metody: i) transportní studie a ii) akumulační studie

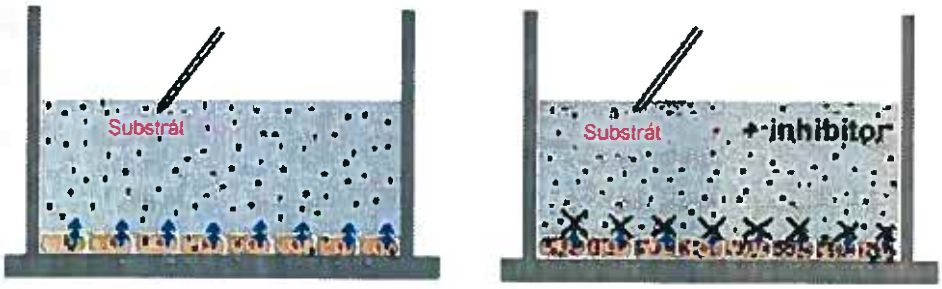
i) **Transportní studie** jsou velice vhodným nástrojem poskytujícím kvalitativní informaci, zda je testovaná látka substrátem daného transportéru či nikoliv. Tyto studie jsou prováděny na nosičích s mikroporózní polykarbonátovou membránou, která rozděluje prostor jamky v kultivačních destičkách na dva kompartmenty, horní (apikální) a dolní (bazolaterální) (Obr. 5A-B). Mikroporózní membrána slouží jako podklad pro vytvoření monovrstvy polarizovaných buněk exprimujících studovaný transportér. Tyto buňky musí být vhodně zvolené, základním kriteriem jejich výběru je adherentní růst s přirozenou tendencí vytvářet monovrstvu na podkladu. Mezi takové patří i námi použité Madin-Darby canine kidney II buňky (MDCKII), které jsou zároveň dostupné i ve formě buněk trvale exprimující v apikální membráně BCRP (Obr. 5B-C), popřípadě MRP2 nebo P-gp transportéry.



Obr. 5. (A) Názorné zobrazení použitých nosičů se semipermeabilní polykarbonátovou membránou. (B) Typické transportní zkoušky jsou založeny na aplikaci testované látky na apikální nebo bazolaterální stranu souvislé monovrstvy polarizovaných epiteliálních buněk a analýze koncentrace aplikované látky v druhém kompartmentu. Takto lze kvatifikovat výsledný transport látky přes monovrstvu, v tomto případě tvořenou MDCKII-BCRP buňkami. Pokud je látka substrátem studovaného transportéru, je její transport přes monovrstvu buněk vlivem transportéru v jednom směru urychlen a ve směru opačném téměř blokován. Naopak pokud látka není substrátem studovaného transportéru, nemůže být pozorována žádná asymetrie v transportu přes monovrstvu a nejsou tedy žádné rozdíly mezi transportem látek v parentní a transportérem transfekované buněčné linii. (C) Příklad časové závislosti transportu testované látky, která je substrátem: transport ve směru apikálně-bazolaterálním (AP) a bazolaterálně-apikálním je porovnatelný (graf vlevo) a která je substrátem studovaného transportéru: ve směru bazolaterálně-apikálním (BA) je transport testované látky urychlen a ve směru opačném (AB) je blokován (graf vpravo).

ii) **Akumulační studie** umožňují studium interakce transportérů s testovanými látkami ve smyslu jejich funkční modulace, konkrétně inhibice nebo stimulace. Tyto studie jsou založeny na akumulaci známého fluorescenčního nebo radioaktivně značeného substrátu v buňkách. Za normální situace je takovýto substrát efluxním transportérem pumpován ven z buněk zpět do kultivačního média. Pokud je testovaná látka modulátorem aktivity transportu

zprostředkovaného studovaným efluxním transportérem, je tímto mechanizmem ovlivněna akumulace substrátu v buňkách. Výsledná akumulace může být detekována v buněčném lyzátu, médiu, či intaktních buňkách (Obr. 6). Látky stimulující transport akumulaci snižují, inhibitory akumulaci substrátu zvyšují. Příkladem inhibitorů BCRP transportéru jsou specifický Ko143 a nespecifický GF121809 (inhibuje taktéž P-gp) (de Bruin et al., 1999; Allen et al., 2002), inhibitorem MRP1 a MRP2 transportérů je MK-571.



Obr. 6. Schéma akumulační studie. Na obrázku vlevo je znázorněna situace, kdy fluorescenční nebo radioaktivně značený substrát, který je přítomen v médiu, pasivní difúzí přechází přes buněčnou membránu. ABC transportér přítomný v apikální membráně pumpuje substrát zpět do média. Na obrázku vpravo je kromě substrátu v médiu přítomen také inhibitor, který znemožňuje eflux substrátu zpět do média. Tímto dochází v porovnání s příkladem na levém obrázku ke zvýšení akumulace substrátu v buňkách. Fluorescence nebo radioaktivita akumulovaného substrátu je posléze měřena v lyzátu nebo intaktních buňkách.

1.3. Interakce kyseliny valproové s konstitutivním androstanovým receptorem (CAR) a pregnanovým X receptorem (PXR); vliv této interakce na expresi *CYP3A4* a *MDR1*

1.3.1. Charakteristika CAR a PXR

CAR a PXR náleží do první rodiny „ligandem aktivovatelných" nukleárních receptorů (Germain et al., 2006). Kromě CAR a PXR patří do této rodiny také receptor pro vitamin D, peroxizomové proliferaci-aktivující receptory α,β,γ a řada dalších (Germain et al., 2006). Rozvoj molekulárně-biologických metod umožnil identifikaci exogenních ligandů těchto nukleárních receptorů (viz. dále), které v naprosté většině způsobují aktivaci transkripce cílených genů. V mnoha případech se jedná o geny kódující biotransformační enzymy a efluxní lékové ABC transportéry. Zvýšení transkripční aktivity biotransformačních enzymů a efluxních lékových ABC transportérů může vést až k jejich indukci na proteinové úrovni, což umožňuje urychlení eliminace potenciálně škodlivých xenobiotik a omezení jejich kumulace v organismu. To je důvodem, proč jsou PXR a CAR někdy označovány jako „xenosenzory" zodpovědné za primární defenzivní mechanismus organismu. Je zajímavé, že tyto nukleární receptory regulují geny podílející se spíše dispozici xenobiotik, než na biotransformaci a syntéze endogenních látek, např. steroidní a thyroidní hormony, bilirubin (Goodwin and Moore, 2004). Důkazem pro toto tvrzení je, že geneticky manipulované myši postrádající geny pro Pxr a Car jsou životaschopné bez vážnějších abnormalit (Zhang et al., 2004).

1.3.2. Mechanismus CAR a PXR zprostředkované transkripční aktivace genů

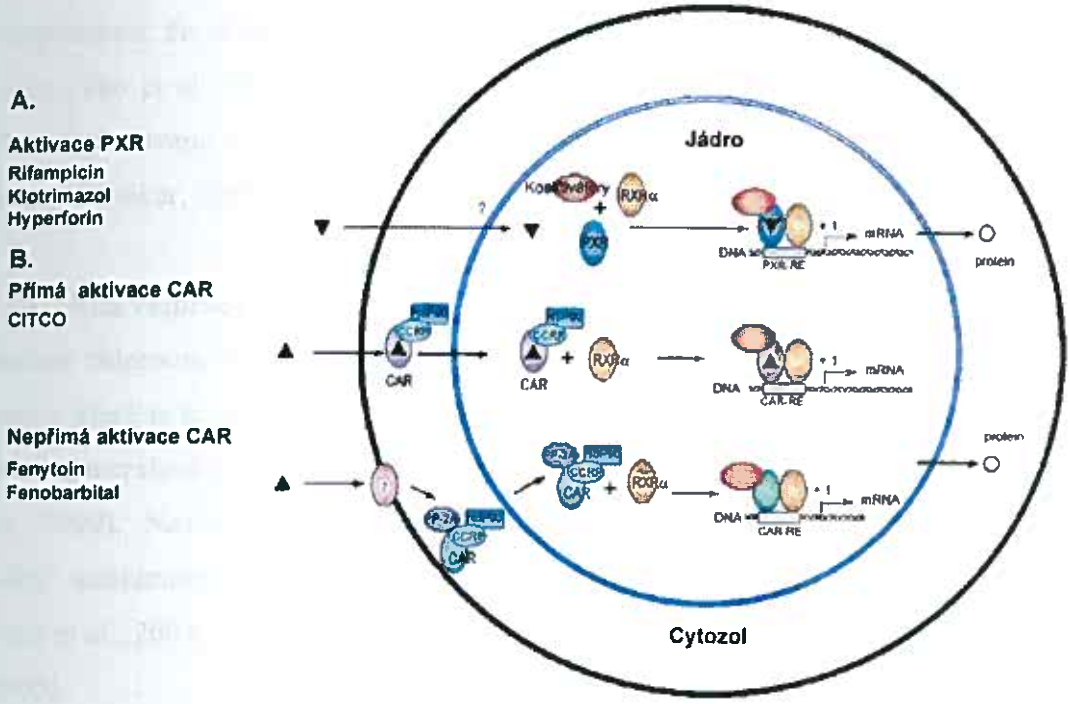
Transkripční aktivace genů zprostředkovaná PXR je podmíněna přímou interakcí aktivátoru s PXR. Naproti tomu interakce aktivátoru s CAR může proběhnout nepřímou nebo přímou cestou (Goodwin and Moore, 2004). Nepřímá aktivace je založena na zvýšené translokaci CAR přítomného v cytoplazmě do buněčného jádra. Tento způsob aktivace nukleárního receptoru je umožněn tím, že CAR jeví určitou míru konstitutivní aktivity, což znamená, že pro vlastní transkripční aktivaci nutně nepotřebuje ligand (Forman et al., 1998; Honkakoski et al., 1998; Kawamoto et al., 1999). Mezi nepřímé aktivátory CAR patří např. antiepileptikum fenobarbital a bilirubin (Honkakoski et al., 1998; Kawamoto et al., 1999; Huang et al., 2003). Přímá aktivace CAR je zprostředkována vazbou ligandu k vazebnému místu CAR. Doposud bylo popsáno jen omezené množství přímých aktivátorů CAR, např. CITCO a antimalarika odvozená od artemisininu (Maglich et al., 2003; Burk et al., 2005b).

Aktivovaná forma nukleárního receptoru CAR nebo PXR vytváří ve většině případů heterodimer s receptorem pro 9-*cis* retinovou kyselinu (RXRα), který se následně váže k responzivnímu elementu v promotorové oblasti cíleného genu a spouští jeho transkripci (Honkakoski et al., 1998). V případě *CYP3A4* jsou nejvíce prozkoumány responzivní elementy CAR a PXR označované **Direct Repeat 3** (DR3) lokalizovaný v oblasti *CYP3A4* promotoru zvané distální responsivní element (-7863/-7208) nebo také XREM (xenobiotic responsive element) a dále **Everted Repeat 6** (ER6) lokalizovaný v promotorové oblasti *CYP3A4* genu (-362/+53) zvané proximální responzivní element (PXRE) (Goodwin et al., 1999; Bombail et al., 2004). V promotoru *MDR1* se heterodimery CAR/RXRα a PXR/RXRα váží na responzivní element zvaný Direct Repeat 4 (DR4), který byl autory Geick a kol. identifikován přibližně 8 tisíc bází před transkripčním startem *MDR1* (Geick et al., 2001). Mechanismus CAR a PXR řízené transkripční aktivace je blíže popsán na Obrázku 7. CAR i PXR regulují kromě *CYP3A4* a *MDR1* transkripční aktivitu mnoha dalších lidských i zvířecích genů. CAR je zapojen v regulaci *Cyp2b1*, *Cyp2b2*, *CYP2B6*, *CYP2C9*, *CYP2C19*, CYP3A5, *UGT1A1*, *Mrp3* a *Mrp4* (Goodwin et al., 2001; Kim et al., 2001; Sugatani et al., 2001; Ferguson et al., 2002; Xiong et al., 2002; Chen et al., 2003; Savkur et al., 2003). PXR je zahrnut v transkripční regulaci značně podobného spektra genů. Mezi cílové geny PXR patří *CYP1A2*, *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5*, *CYP3A7*, *UGT1A1*, *UGT1A2*, *UGT1A3* a *UGT1A4* (Gerbal-Chaloin et al., 2001; Goodwin et al., 2001; Maglich et al., 2002; Gardner-Stephen et al., 2004; Chen et al., 2004).

1.3.3. Ligandy CAR a PXR

V případě CAR je jediným dosud známým výrazně specifickým aktivátorem experimentální sloučenina CITCO, což je vyjádřeno chemicky 6-(4-chlorofenyl)imidazo[2,1-*b*][1,3]-thiazol-5-karbaldehyd-O-(3,4-dichlorobenzyl)oxim. Tato látka preferenčně aktivuje CAR (EC_{50} = 5 nM) a ve výrazně nižší míře také PXR (EC_{50} = 3 μM) (Maglich et al., 2003). Dalšími jsou např. antimalarika odvozená od artemisininu, avšak tyto sloučeniny jeví srovnatelnou afinitu i k PXR (Burk et al., 2005b). Barbiturát fenobarbital a bilirubin, jak již bylo zmíněno, aktivují CAR nepřímo tím, že způsobují zvýšenou translokaci tohoto nukleárního receptoru z cytoplazmy do jádra (Honkakoski et al., 1998; Kawamoto et al., 1999; Goodwin and Moore, 2004). Bylo také rozpoznáno několik kompetitivních inverzních agonistů CAR. Mezi takové látky paří androstanol, androstenol a klotrimazol (Forman et al., 1998; Moore et al., 2000b). Pro PXR již bylo objeveno mnoho strukturně a chemicky odlišných ligandů. Mezi ligandy PXR patří rifampicin, považovaný za modelový aktivátor

PXR (Bertilsson et al., 1998; Pascussi et al., 2000), endogenní kyseliny žlučové (Staudinger et al., 2001; Xie et al., 2001), metabolit progesteronu 5-pregnan-3,20-dion (Jones et al., 2000), rostlinné atidepresivum hyperforin (Moore et al., 2000a), karbamazepin, topotekan, glukokortikoidy a mnoho dalších (Schuetz et al., 2002).



Obr. 7. Modelové znázornění aktivace PXR a CAR. (A) Pregnanový X receptor (PXR) může být aktivován pouze přímou vazbou ligandu. Ligandem PXR jsou např. rifampicin, klotrimazol či rostlinné antidepresivum hyperforin (Bertilsson et al., 1998; Pascussi et al., 2000), (Moore et al., 2000a). (B) CAR je přítomen v cytoplazmě v komplexu s tzv. cytoplazmatickým CAR vázajícím proteinem (CCRP) a molekulárním chaperonem zvaným „heat shock protein 90" (HSP90). CAR je přímo aktivován např. experimentální látkou CITCO. Navíc CAR jeví konstitutivní aktivitu i v nepřítomnosti ligandu. Díky této vlastnosti může být CAR aktivován i nepřímo. Mezi nepřímé aktivátory patří např. antiepileptikum fenobarbital. Signální cesta nepřímé aktivace nebyla doposud obstojně popsána. Oproti přímé aktivaci se zde předpokládá také podíl proteinové fosfatázy 2A (PP-2A), která je vázána na komplex CAR-CCRP-HSP90. Aktivovaná forma PXR a CAR posléze tvoří heterodimer s RXRα, který se váže k responzivní oblasti cíleného genu a způsobuje aktivaci transkripce. V procesu transkripční aktivace se předpokládá také začlenění koaktivátoru (Coacts). Obrázek byl zpracován dle publikace Goodwin and Moore, 2004.

1.3.4. Charakteristika CYP3A4

Cytochrom P450 je rodinou hemoproteinů, které mají významnou úlohu v oxidativním metabolismu mnoha endogenních i exogenních sloučenin (Nelson et al., 1996). Podrodina lidského cytochromu P450 3A má tři významné zástupce: CYP3A4, CYP3A5 a CYP3A7. CYP3A4 je převládající izoformou, která je lokalizována u dospělého člověka převážně v játrech. CYP3A4 je klíčovým enzymem v biotransformaci testosteronu (Waxman et al., 1991), což je důvodem jeho významné úlohy v udržování fyziologických hladin steroidních

hormonů (Goodwin et al., 1999). Naproti tomu se ale CYP3A4 taktéž podílí na bioaktivaci prokarcinogenů pocházejících z vnějšího prostředí, např. benzo(a)pyrenů a jiných derivátů polycyklických aromatických uhlovodíků a karcinogenních mykotoxinů (Li et al., 1995). Je dokumentováno, že CYP3A4 by se mohl podílet na biotransformaci až 60 % terapeutik (Li et al., 1995). Zvýšená exprese některých zástupců rodiny cytochromu P450 včetně CYP3A4 byla detekována v nádorových buňkách (Murray et al., 1993a; Murray et al., 1993b), přičemž je pravděpodobné, že se tento fenomén může podílet na prohloubení rezistence vůči léčbě cytostatiky (Yao et al., 2000). Je zajímavé, že většina rozpoznaných substrátů CYP3A4 je zároveň transportována i P-gp, např. vinka alkaloidy, kolchicin, cyklosporin A a verapamil (Schinkel and Jonker, 2003).

1.3.5. Kyselina valproová

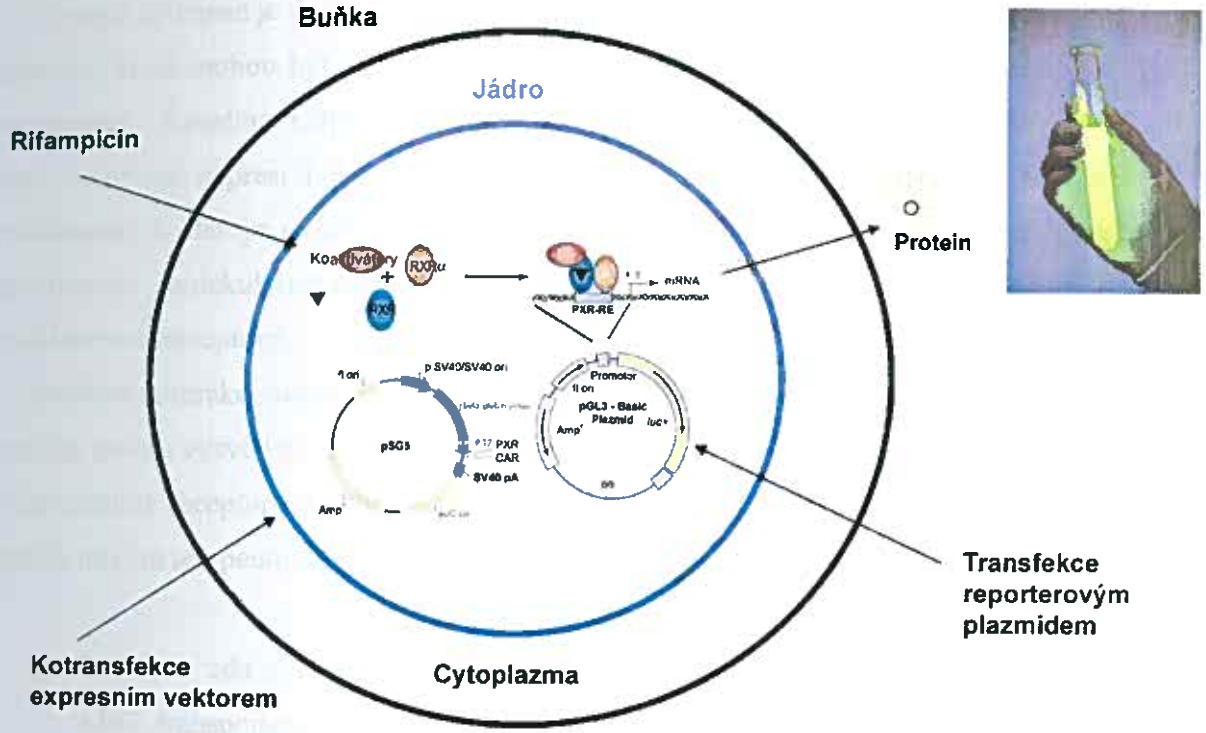
Kyselina valproová je vysoce účinným a dobře snášeným léčivem s antikonvulzivními vlastnostmi, které je řazeno mezi antiepileptika druhé generace. Je používáno hlavně k léčbě primárních generalizovaných tonicko-klonických záchvatů, absencí a záchvatů parciálních (Tanaka, 1999). Navíc byla nedávno kyselina valproová identifikována jako léčivo s potenciální antitumorózní aktivitou schopné inhibovat histonové deacetylázy (HDAC) (Gottlicher et al., 2001; Blaheta et al., 2002; Kramer et al., 2003; Blaheta et al., 2005; Raffoux et al., 2005).

Bylo prokázáno, že kyselina valproová ovlivňuje expresi širokého spektra genů (Bosetti et al., 2004). Některé z těchto změn genové exprese lze vysvětlit např. inhibicí aktivity HDAC (Chen et al., 1999; Phiel et al., 2001; Werling et al., 2001; Bosetti et al., 2004; Eyal et al., 2006) a některé interakcí s nukleárními receptory (Červený et al., 2007).

1.3.6. Molekulárně genetické metody využité pro studium transkripční aktivity a interakce ligandu s recipientní strukturou

Při studiu CAR a PXR zprostředkované transkripční aktivace genů *CYP3A4* a *MDR1* byly využity moderní metody molekulární farmakologie. Jednalo se především o genové reporterové studie, elektroforetickou detekci vazby proteinu k responzivnímu elementu testovaných genů (EMSA – „electrophoretic mobility shift assay") a reverzní trankriptázovou polymerázovou řetězovou reakci v reálném čase (real time RT-PCR). Na Obrázku 8 je popsán model genového reporterového experimentu pro testování PXR zprostředkované transkripční aktivace studovaných genů. EMSA je metodou vhodnou pro studium veškerých interakcí proteinů s DNA. Tímto způsobem je možné vizualizovat interakci proteinů se specifickými

sekvencemi DNA a detekovat tak účast daného proteinu např. v transkripční regulaci testovaného genu. Metoda EMSA je založena na principu, že komplex protein/DNA migruje v nedenaturujícím polyakrylamidovém nebo agarózovém gelu pomaleji než samotná DNA. Ve výsledku můžeme danou interakci hodnotit jak kvalitativně (k interakci proteinu a DNA došlo nebo nedošlo) tak kvantitativně (pomocí různých vyhodnocovacích programů je možné analyzovat intenzity a velikosti signálů).



Obr. 8. Schéma reporterového genového experimentu, který byl použit pro studium interakce kyseliny valproové s CAR a PXR. V obrázku je uveden rifampicin jakožto modelový aktivátor PXR. Celý systém byl založen na současné inkorporaci dvou plazmidů do buňky. Jednalo se vždy o reporterový plazmid nesoucí v promotorové oblasti důležité responzivní elementy specifické pro daný cílený gen a dále strukturní gen pro luciferázu. Druhým plazmidem byl expresní vektor, který nesl virový promotor (např. SV-40) a strukturní gen studovaného nukleárního receptoru (CAR nebo PXR). Takto připravené buňky byly posléze vystaveny působení testované látky (zde je znázorněn rifampicin – modelový aktivátor PXR). Po 24 hodinách byly buňky lyzovány a k lyzátu byl přidán luciferin - substrát luciferázy. Luciferin je luciferázou biotransformován a výsledný produkt jeví luminescenci, která je přístrojově kvantifikovatelná (např. multifunkční přístroj Genios Plus)

1.4. Cíle předkládané dizertační práce

Zvýšená exprese efluxních lékových ABC transportérů v HEB je považována za jednu z příčin selhání antiepileptické léčby. Existuje několik antiepileptik, u nichž se předpokládá, že jejich průchod přes HEB je limitován dvěma nejznámějšími zástupci této skupiny aktivních transportérů, P-gp a MRP2. Antiepileptikum, jehož přechod přes HEB je redukován relativně méně prozkoumaným BCRP transportérem, není však doposud známo.

Terapie epilepsie je dlouhodobý proces, který je často spojen s výskytem mnoha lékových interakcí, které mohou být podmíněné změnou exprese a aktivity cytochromu P450 a ABC transportérů. Kyselina valproová je relativně dobře snášené širokospektré antiepiletikum, které ovlivňuje expresi řady genů inhibicí histonových deacetyláz (HDACi). Na základě publikovaných dat je ovšem zjevné, že v některých případech se navíc musí uplatňovat selektivnější molekulární mechanismus než HDACi, např. interakce kyseliny valproové s nukleárními receptory.

Studium interakcí antiepileptik s efluxními lékovými ABC transportéry je jednou z cest, která by mohla vysvětlit vznik rezistence k antiepileptické léčbě a popis inteakcí antiepileptik s nuklearními receptory a vliv této interakce na expresi genů by v budoucnu mohl otevřít cestu k novým terapeutickým postupům. Konkrétními cíly předkládané dizertační práce bylo:

1. Studovat, zda vybraná antiepileptika jsou substráty sledovaných efluxních lékových ABC transportérů, konkrétně BCRP a objasnit jeho potenciální úlohu ve vzniku rezistence vůči vybraným antiepileptikům v průběhu antiepileptické léčby.

2. Testovat potenciál vybraných antiepileptik inhibovat, popřípadě stimulovat transport zprostředkovaný BCRP transportérem.

3. Studovat interakci kyseliny valproové s nukleárním i receptory CAR a PXR na molekulární úrovni a na tomto základě vysvětlit některé diskrepance v efektu kyseliny valproové na expresi genů, konkrétně *CYP3A4* a *MDR1*.

Tato dizertační práce je předložena ve formě souboru publikací, které již byly otištěny v odborných časopisech.

1.5. Podíl na jednotlivých publikacích

U kapitol 2 a 3 je předkladatel této dizertační práce prvním autorem, v případě 4. a 5. kapitoly pak druhým autorem. Autor disertace sepsal všechny rukopisy, u nichž je prvním autorem. V ostatních případech se podílel jen na úpravách textu.

Autor předkládané dizertační práce prováděl veškeré transportní a akumulační experimenty uvedené v části 2 a dále genové reporterové experimenty, real time RT-PCR analýzu vzorků a testy enzymatické aktivity uvedené v kapitole 3. Ve studii uvedené v části 4 předkládané dizertační práce autor prováděl některé procedury spojené s real time RT-PCR (izolace RNA, reverzní transkripce).

Chemické analýzy z 2. části dizertační práce byly provedeny PharmDr. Janou Malákovou z Ústavu klinické biochemie a diagnostiky Fakultní nemocnice Hradec Králové. Analytická vyhodnocení koncentrace 6β-hydroxytestosteronu z části 3 byly udělány Doc. Evou Anzenbacherovou, CSc. z Ústavu lékařské chemie a biochemie Lékařské fakulty Univerzity Palackého v Olomouci. Mgr. Radim Vrzal a Doc. RNDr. Zdeněk Dvořák, Ph.D. připravili kulturu primárních hepatocytů (část 3). „Electrophoretic mobility shift asssays" (EMSAs) byly provedeny Mgr. Lucií Švecovou z Katedry farmakologie a toxikologie Farmaceutické fakulty v Hradci Králové, Univerzity Karlovy v Praze.

Témata projektů uvedených v předkládané dizertační práci byla vymyšlena PharmDr. Petrem Pávkem, Ph.D., který mi byl zároveň ve všech směrech nápomocný i při vlastní realizaci studií uvedených v kapitole 2 a 3.

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2. Lack of interactions between breast cancer resistance protein (BCRP/ABCG2) and selected antiepileptic agents

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Laboratory Research

Lack of Interactions between Breast Cancer Resistance Protein (BCRP/ABCG2) and Selected Antiepileptic Agents

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Summary: *Purpose:* Recent studies have indicated constitutive expression of efflux transporter, breast cancer resistance protein (BCRP, ABCG2), in endothelial cells of the blood–brain barrier (BBB). In epileptogenic brain tumors such as ganglioma, astrocytoma, anaplastic astrocytomas, or glioma multiforme, strong expression of BCRP in the microvasculature of the BBB was observed. Therefore it was hypothesized that this phenomenon could critically influence the bioavailability of drugs in these tumors and potentially contribute to the failure of antiepileptic treatment. The aim of this study was to test whether some commonly used antiepileptic drugs (AEDs) are substrates transported by human BCRP. In particular, we focused on phenobarbital, phenytoin, ethosuximide, primidone, valproate, carbamazepine, clonazepam, and lamotrigine. Furthermore, the inhibitory potency of these AEDs to BCRP was examined.

Methods: To study substrate affinity of tested AEDs to BCRP, transport experiments were performed in epithelial BCRP-expressing MDCKII-BCRP and MDCKII-parent cell lines cultured on microporous membrane. For detection of inhibitory potency of AEDs to BCRP, accumulation assays were carried out in MEF3.8-BCRP cells with known BCRP substrates, BODIPY FL prazosin and mitoxantrone.

Results: No obvious interactions of tested AEDs with BCRP transporter were observed. Therefore these drugs in relevant therapeutic concentrations are neither substrates nor inhibitors of BCRP.

Conclusions: Based on our in vitro data we can conclude that resistance to treatment with the tested AEDs probably is not caused by the overexpression of BCRP in the BBB of epileptogenic brain tumors. **Key Words:** Breast cancer resistance protein—BCRP—ABCG2—Antiepileptic drugs—Transport—Refractory epilepsy—Transporter.

Epilepsy is a common neurologic disorder affecting ~1–2% of the population. Refractory epilepsy occurs in about one third of cases overall (1,2). Resistance in epilepsy is a multifactorial and drug-nonspecific clinical problem (3). Most patients with refractory epilepsy are resistant to several antiepileptic drugs (AEDs), even though these drugs act by different mechanisms (2). This argues against epilepsy-induced alterations in specific drug targets as a major cause of refractory epilepsy and supports the hypothesis that a nonspecific mechanism such as decreased drug uptake into the brain is involved.

In recent years, attention has been focused on several transmembrane transporters localized in the blood–brain barrier (BBB), especially those belonging to the ABC transporter protein superfamily [e.g. P-glycoprotein (P-gp, ABCB1), multidrug resistance–associated protein 1 (MRP1, ABCC1), multidrug resistance–associated protein 2 (MRP2, ABCC2), and breast cancer resistance protein (BCRP, ABCG2)]. These proteins reduce cytoplasmic accumulation of drugs and xenobiotics by their extrusion out of cells. Some of transporters are suggested to play a role in the limiting of drug penetration across the BBB and thus they could modulate efficacy and central nervous system toxicity of numerous compounds (4–9). Basal expression of P-gp, MRP2, and BCRP was found in endothelial cells of normal human brain (10–14), whereas MRP1 was found only in the choroid plexus epithelium (15,16). Brain expression of these transporters is markedly increased in many cases of refractory epilepsy (3,12,13,17), suggesting that at least in some resistant patients, this phenomenon might contribute to resistance to AEDs. This hypothesis also is supported by the fact that AEDs such as phenytoin (PHT), carbamazepine (CBZ), phenobarbital

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(PB), and lamotrigine (LTG) are recognized P-gp substrates (5,7,9,18), and PHT, CBZ, and valproate (VPA) are suggested substrates of MRP2 (4,6,19,20). Furthermore, recent evidence also indicates a possible role of the C3435T polymorphism of P-gp in refractory epilepsy (21).

Several studies have focused on interactions of AEDs with ABC transporters, considering modulation (induction/inhibition) of transporter activity by these compounds (22,23). With in vitro methods, it was found that LTG, CBZ, PHT, and VPA have inhibitory potency to P-gp, albeit in concentrations that are higher than the therapeutic levels (22). Potential inhibition of other ABC transporters by AEDs has not been studied, although this is a very important clinical aspect in the case of coadministration of two drugs interacting with the same transporter.

Strong expression of BCRP was recently observed by Aronica and colleagues (13) in several epileptogenic brain tumors such as ganglioma, astrocytoma, anaplastic astrocytomas, or glioma multiforme. Consequently, the authors suggested that overexpression of BCRP in these tumors could critically influence the bioavailability of drugs and contribute to failure in epilepsy treatment (13).

The aim of the present study was to test potential interactions of several AEDs with the BCRP transport system. In particular, substrate affinity and inhibitory potency to BCRP were examined for the following AEDs: PB, PHT, ethosuximide (ESM), primidone (PRM), VPA, CBZ, clonazepam (CZP), and LTG. Two cell-based methods were used: (a) in vitro transepithelial transport studies to examine potential affinity of AEDs to BCRP, and (b) accumulation experiments with BCRP substrates to detect inhibitory potency of tested AEDs to BCRP. Transport assays were carried out by using BCRP-expressing MDCKII-BCRP and MDCKII-parent cell lines. The study of inhibitory potencies of selected AEDs was based on accumulation of recognized BCRP substrates, BODIPY FL prazosin (24) and mitoxantrone (MIT) (25), in BCRP-expressing MEF3.8-BCRP cells.

MATERIALS AND METHODS

Chemicals and materials

LTG was purchased from GlaxoSmithKline (Research Triangle Park, NC, U.S.A.), PB, PRM, CBZ, VPA, and ESM were purchased from Sigma, and PHT was from Parke-Davis GmbH (Berlin, Freiburg, Germany), originally manufactured as Epanutin parenteral. CZP was from F. Hoffmann-La Roche Ltd Roche (Basel, Switzerland), manufactured as Rivotril inj. A known BCRP substrate, dietary carcinogen PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) and [^{14}C]PhIP (10 Ci/mmol), were from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). GF120918, a nonselective inhibitor of BCRP, was provided by GlaxoSmithKline (Greenford, England), and Ko143, a selective inhibitor of BCRP, was kindly donated by Dr. A. H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Fluorescent substrates of the BCRP transporter, BODIPY FL prazosin and mitoxantrone (MIT), were from Molecular Probes (Karlsruhe, Germany) and Pliva-Lachema a.s. (Brno, CR) manufactured as Refador inj., 2 mg/ml, respectively. Complete high-glucose DMEM medium, fetal calf serum (FCS), [^{3}H]inulin 40 kDa (0.5–3 Ci/mmol), DMSO, and Triton X-100 were from Sigma. Serum-free Opti-MEM medium was manufactured by Gibco (U.K.). The 24-well plates and flasks for cultivations were from TPP (Trasadingen, Switzerland). Microporous polycarbonate filters (3.0-μm pore size, 24-mm diameter; Transwell 3414) were from Corning (Corning, NY, U.S.A.).

Stock solutions of VPA and ESM were prepared in saline, and stock solutions of PB, LTG, PRM, and CBZ were prepared in DMSO. The amount of DMSO in cultivation media was <0.1%.

Cells

MDCKII (Madine-Darby Canine Kidney) parent cell line, MDCKII cells transfected with cDNA of human *BCRP* (MDCKII-BCRP) and MEF3.8 cell line, spontaneously immortalized embryo fibroblasts derived from triple knockout *mdr1a/b*$^{-/-}$, *mrp1*$^{-/-}$ mice, transfected with cDNA of human *BCRP* (MEF3.8-BCRP) (26). Cell lines were cultured in DMEM complete high-glucose medium with L-glutamine, supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Transport experiments

Transport assays were performed on microporous polycarbonate membrane filters by using MDCKII-parent and MDCKII-BCRP cell lines. Cells were seeded at a density of 1.0×10^6 and cultured to confluence. Transport assays were carried out as reported, with slight modifications (26). At time zero, Opti-MEM medium was added to both compartments (2 ml) and enriched with AED in the appropriate chamber. Transepithelial transport was investigated at three different concentrations for each AED. In particular, PRM, PHT, CBZ, and PB were studied at concentrations of 10, 25 and 100 μM, LTG was investigated at concentrations of 5, 25, and 100 μM, CZP was studied at concentrations of 50 nM, 500 nM, and 25 μM, and VPA and ESM were tested at concentrations of 25, 250, and 500 μM. After 6 h of incubation at 37°C, 400 μl of the solution was taken from the opposite compartment. Concentrations of AEDs were analyzed by high-performance liquid chromatography (HPLC) or fluorescence polarization immunoassay (FPIA). PhIP, a known substrate of BCRP, traced with [^{14}C]PhIP (0.02 μCi/ml), was used in the starting compartment at the concentrations of 2 and 25 μM as a method validator. For detection of PhIP, 50-μl aliquots were taken after 3 and 6 h. The integrity of cell monolayers was tested with radiolabeled

[^{3}H]inulin. Inulin leakage was accepted up to 1% per hour per well. Translocations were observed in both apical-basolateral (AB) and basolateral-apical directions (BA). Results are plotted as the percentage of starting concentrations of AEDs measured in opposite compartments. Data were calculated as the means of transported fractions (%) ±SD from at least three experiments. Ratios of BA to AB translocation after 6-h incubation (r) were calculated for all tested AEDs.

Accumulation assays

Accumulation assays with BODIPY FL prazosin

MEF3.8-BCRP cells were seeded in 24-well plates (1×10^5 cells per well). After they reached subconfluence, medium was aspirated, cells were washed with PBS and preincubated for 60 min at 37°C in Opti-MEM medium with or without inhibitors GF120918 (2 μM) and Ko143 (500 nM) or tested AEDs in different concentrations (see later). Accumulation of BODIPY FL prazosin (500 nM) lasted 2 h at 37°C and was stopped by removing the medium and washing the cells subsequently with ice-cold PBS (1 ml, 1 min), ice-cold PBS with FCS (10%) for 30 min and ice-cold PBS (1 ml, 1 min). Lysis buffer (100 μl; NaCl, 150 mM; Tris-HCl, 50 mM; SDS, 0.1%; Triton, 1%; and sodium deoxycholate, 1% wt/vol) was added and cells were lysed for ≥1 h. PRM, PHT, PB, LTG, and CBZ were studied at concentrations of 25 and 100 μM. CZP was tested at concentrations of 500 nM and 25 μM. VPA was investigated at concentrations 25, 250 and 500 μM, and ESM was tested at concentrations of 25 and 500 μM. Relative cellular accumulation of BODIPY FL prazosin was determined in lysate with the multiplate fluorimeter Genios Plus (Tecan, Grödig, Austria). Excitation and emission wavelengths for BODIPY FL prazosin were 485 and 535 nm, respectively. Background fluorescence of all tested AEDs was checked in appropriate wavelengths and subtracted. Accumulation of BODIPY FL prazosin in control experiments (without AEDs) was set to 100%.

Accumulation assays with MIT

MEF3.8-BCRP cells were seeded in 24-well plates (1×10^5 cells per well). After they reached subconfluence, medium was aspirated, cells were washed with PBS, and then preincubated in Opti-MEM medium with or without inhibitor GF120918 (2 μM) or tested AEDs in appropriate concentrations for 60 min at 37°C. PRM, PHT, PB, LTG, and CBZ were studied at concentrations of 25 and 100 μM; CZP was investigated at concentrations of 500 nM and 25 μM. VPA and ESM were tested at concentrations of 25 and 500 μM. Accumulation of MIT (20 μM) lasted 1 h at 37°C and was stopped by removing the medium and quick washing twice with ice-cold PBS (1 ml). Subsequently cells were trypsinized in ice-cold phenol red dye-free trypsin. Collected cells were resuspended in ice-cold PBS with 2.5% of FCS. Relative cellular accumulation of MIT was determined by flow cytometry by using the FACSCalibur cytometer (Becton-Dickinson, San Jose, CA, U.S.A.). Samples were gated on forward versus side scatter to exclude cell debris and clumps. Excitation and emission wavelengths for MIT were 633 nm and 661 nm, respectively. Fluorescence of accumulated substrate in tested populations of ≥8,000 cells was quantified from histogram plots by using the median of fluorescence. Possible background fluorescence of all tested AEDs and GF120918 was checked in appropriate channels, but the fluorescence was negligible in all cases. Accumulation of MIT in control experiments (without AEDs) was set to be 100%. Flow-cytometry data were processed and analyzed by using WinMDI ver.2.8.

Analytic methods

A reversed-phase liquid-chromatographic method was used for simultaneous determination of PRM, PB, PHT, CBZ, and their internal standard 5-ethyl-5-p-tolylbarbituric acid (27). The compounds were eluted after precipitation of proteins on column Symmetry C18 (250 × 4.6 mm; 5-μm particle size). Separation was performed with a mobile phase of acetonitrile-methanol 50 mM phosphate buffer, pH 6 (13-30-57) at a flow rate of 0.9 ml/min and at 40°C. Detection was at a wavelength of 200 nm.

CZP and internal standard flunitrazepam were extracted after alkalization with borate buffer (pH, 9) into diethyl ether (28). After evaporation of the extract, the residue was reconstituted, and the analytes were separated on symmetry C18 250 × 4.6 mm, 5-μm particle-size column (Waters). HPLC analysis was performed by isocratic flow rate of 1.2 ml/min at 43°C. The peaks were monitored at a wavelength of 220 nm. The mobile phase was prepared by mixing 37% acetonitrile and 63% sodium phosphate buffer, 6 mM, pH 5.8.

For the quantification of LTG, high-performance liquid chromatography was used. Samples were alkalized with 2 M NaOH, and LTG and internal standard BW 725C78 were extracted with ethylacetate (29). Separation was performed on column symmetry C18 150 × 4.6 mm, 5-μm particle size (Waters) with a flow rate of 1 ml/min at 40°C. The isocratic mobile system consisted of acetonitrile and phosphate buffer, 6 mM, pH 6.8 (28:72). The elution of peaks was monitored at a wavelength of 306 nm.

Measurement of ESM and VPA was performed on a TDxFlx instrument (Abbott Laboratories, Abbott Park, IL, U.S.A.) by using fluorescence polarization immunoassay (27).

Detection limits and precisions of analytic methods are summarized in Table 1.

Statistical analysis

Student's unpaired, two-tailed t test was used when appropriate to perform statistical analysis of differences between two sets of data. A value of $p < 0.05$ was considered

TABLE 1. *Specific characteristics of analytic methods used for analysis of tested antiepileptic drugs*

Compounds	Method	Detection limits (μM)	Precision coefficient of variation (%) in three different concentrations (μM)		
PRM	HPLC	1.37	13.1 (6.35%)	35.1 (6.03%)	53 (4.25%)
PB	HPLC	4.74	36.5 (3.92%)	129 (3.49%)	221 (4%)
PHT	HPLC	1.98	17.9 (3.41%)	64.3 (3.08%)	86.8 (3.26%)
CBZ	HPLC	2.12	13.9 (4.01%)	38.9 (3.62%)	71 (3.29%)
LTG	HPLC	1.23	18.35 (8.37%)	50.77 (7.59%)	
CZP	HPLC	0.0099	0.198 (6.5%)	0.792 (2.4%)	
ESM	FPIA	3.89	247.8 (5.26%)	495.6 (4.69%)	849.6 (4.43%)
VPA	FPIA	4.85	259.88 (5.06%)	519.8 (4.35%)	866.25 (4.8%)

Coefficient of variation is calculated as a relative standard deviation from three independent analyses of one sample and represents reproducibility and precision of the method.

PRM, primidone; PB, phenobarbital; PHT, phenytoin; CBZ, carbamazepine; LTG, lamotrigine; CZP, clonazepam; ESM, ethosuximide; VPA, valproic acid; HPLC, high-performance liquid chromatography; FPIA, fluorescence polarization immunoassay.

statistically significant. Errors are represented as standard deviations (SDs) of at least three experiments.

RESULTS

Transport experiments

To test whether selected AEDs are substrates of BCRP, we used an established in vitro method with epithelial cells, parent and transfected with cDNA of the studied transporter, cultured on microporous membranes. This method is based on the fact that if a compound is a substrate of the examined transporter, translocation of the compound across the transporter-expressing monolayer is accelerated in one direction and decreased in the opposite direction. Conversely, when the examined transporter is not involved, no asymmetry can be observed in transport across the transporter-expressing monolayer, and transport does not differ between parent and transporter-expressing cell lines.

In our experiments, we used endothelial MDCKII-BCRP cells, which express human BCRP transporter on the apical membrane. Therefore we expected an increase of transport in the basolateral–apical direction (BA) and a decrease in the apical–basolateral direction (AB); the ratio of BA to AB was >1. PhiP, a recognized BCRP substrate (26), was used as the validation of the method at concentrations of 2 and 25 μM. As expected, we detected a significant asymmetry in its transport across MDCKII-BCRP cells in both concentrations ($r = 25$ and 10, respectively; Fig. 1A), but no asymmetry across MDCKII-parent line (data not shown). This pattern of transport clearly demonstrates PhiP as a BCRP substrate and the validity of the method used.

In our study, we did not observe any significant asymmetry in transport of selected AEDs across MDCKII-BCRP cells in any concentration tested. Transport ratios were within the interval of $r = 0.85$–1.2 (see Fig. 1B–I). Moreover, transport of AEDs across MDCKII-BCRP cells was equivalent to passage across MDCKII-parent cells, with r values being within the interval of 0.9 to 1.2 (data for MDCKII-parent are not shown). These findings clearly show that the selected AEDs are not substrates of the BCRP transporter. Slight asymmetries observed in transepithelial transport of some tested AEDs across both MDCKII-BCRP (see Fig. 1) and MDCKII-parent cells (data not shown) are probably caused by nonspecified endogenous canine transporters.

Accumulation assays with AEDs

Accumulation assays were carried out to investigate inhibitory potencies of tested AEDs to BCRP. This method is based on accumulation of known BCRP substrates by BCRP-expressing cells. BCRP limits the entry of its substrates into the cell line. Inhibitors are able to abrogate its function and increase intracellular accumulation of substrates (see data for GF120918 and Ko143 in Figs. 2 and 3). Two approaches were used to analyze fluorescent BCRP substrates, BODIPY FL prazosin and MIT. Fluorescence of BODIPY FL prazosin was measured in cellular lysate by using a multiplate fluorimeter, and the fluorescence of MIT was measured in intact cells by using a flow cytometer. Experiments were carried out in MEF3.8-BCRP cells (derived from triple knockout *mdr1a/b*$^{-/-}$, *mrp1*$^{-/-}$ mice transduced with cDNA of human BCRP) (26). This cell line is suitable for this type of experiment because of no interference with additional transporters, murine *mdr* and *mrp1*. GF120918 and Ko143 were used as known inhibitors of BCRP.

Accumulation assays with BODIPY FL prazosin

With the exception of VAP at a concentration of 500 μM, tested AEDs did not affect cell accumulation of BODIPY FL prazosin at either of tested concentrations (Fig. 2). VAP (500 μM) caused slight but significant ($p < 0.05$) increase in BODIPY FL prazosin cell accumulation (Fig. 2).

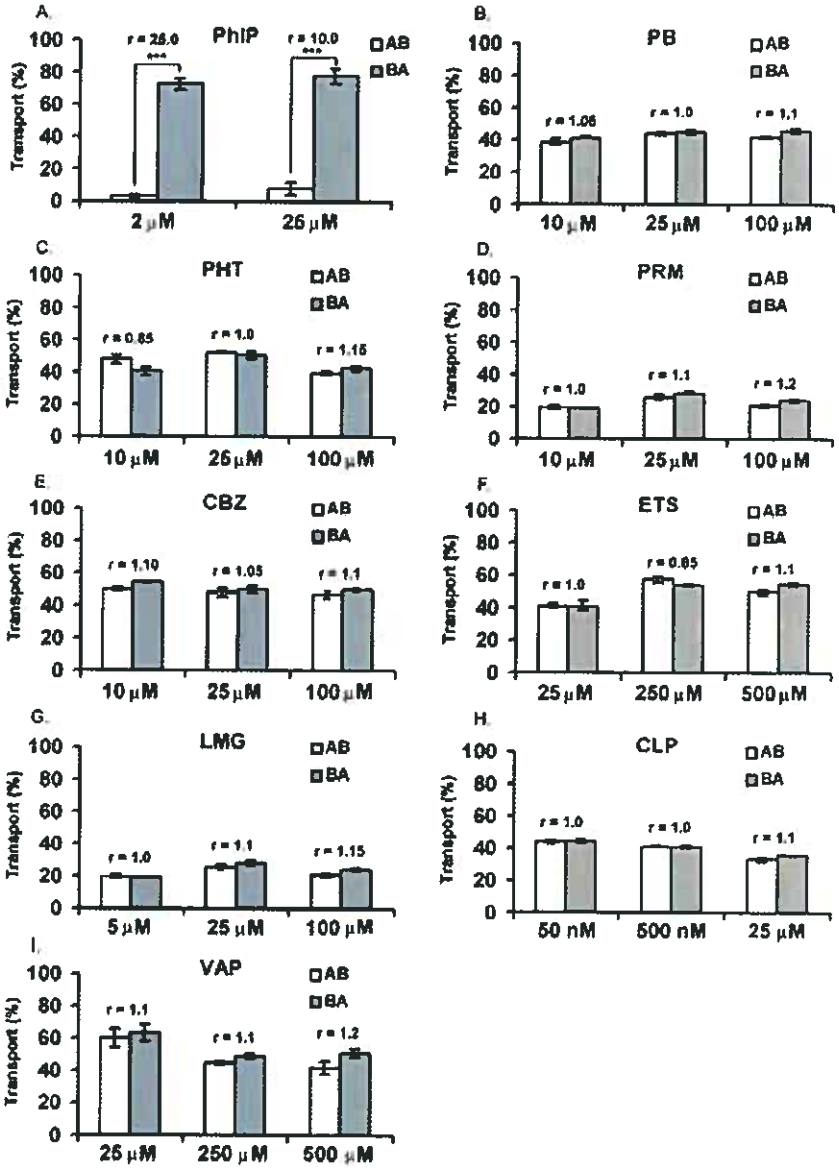

FIG. 1. Transport of antiepileptic drugs (AEDs) across MDCKII–breast cancer resistance protein (BCRP) monolayers. The experiment was started with application of AED (or PhIP) to one compartment (basal or apical). Concentrations of AEDs were measured after 6 h in the opposite compartment. AEDs were tested at the following concentrations: 10, 25, and 100 μM for PB (B), PHT (C), PRM (D), CBZ (E); 25, 250, and 500 μM for ESM (F) and VPA (I); 5, 25, and 100 μM for LTG, and 50 and 500 nM and 25 μM for CZP, respectively. Results are plotted as the percentage of starting concentrations of AEDs in the opposite compartments. Transport of PhIP, a known substrate of BCRP, at the concentrations of 2 and 25 μM demonstrates BCRP-mediated passage of the substance across MDCKII-BCRP cells (A). Translocations of tested substances across MDCKII-BCRP monolayer were studied in apical–basolateral direction (AB) or basolateral–apical directions (BA). Values r (ratios of BA to AB) are shown. Data are presented as mean ± SD; $n \geq 3$. $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

Accumulation assays with MTX

With this method, no significant effect of selected AEDs at all tested concentrations on accumulation of MIT was detected (Fig. 3).

DISCUSSION

Resistance to AEDs is a common problem in epilepsy treatment (2). However, this phenomenon is not well understood and cannot be attributed only to low serum concentrations of AEDs. One of the current hypotheses assumes overexpression of some ABC transporters in the BBB of drug-resistant patients. Transporters such as P-gp and MRP2 have the ability to pump their substrates; including several AEDs, from the BBB back into blood circulation (4–9). Overexpression of these transporters can therefore affect penetration of AEDs across the BBB and results in ineffective concentrations of AEDs in the human brain. Other mechanisms of pharmacoresistance in epilepsy are less well defined and include alterations in drug targets (including genetic factors) as well as inherent risk factors associated with certain seizure etiologies (2,30).

Recently, Aronica et al. (13) detected strong expression of BCRP in endothelial cells of epileptogenic brain tumors such as gliomas (astrocytoma, anaplastic astrocytoma, glioblastoma multiforme, anaplastic oligodendroglioma) and glioneuronal tumors (gangliogliomas). Consequently, the authors suggested that BCRP might

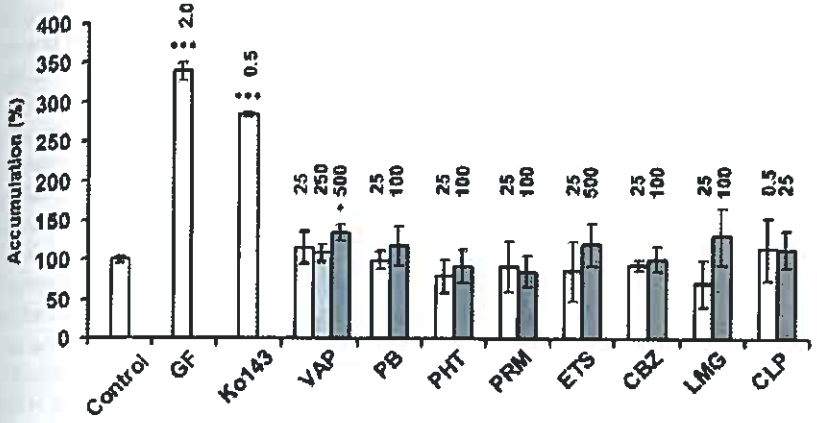

FIG. 2. Effect of antiepileptic drugs (AEDs) on breast cancer resistance protein (BCRP)-mediated transport of BODIPY FL prazosin (500 n*M*) in the MEF3.8-BCRP cell line. Tested concentrations of AEDs or inhibitors are indicated (μM). Results are presented as relative cellular accumulation of BODIPY FL prazosin related to control (100%). GF120918 (2 μM) and Ko143 (500 n*M*) were used as known inhibitors of BCRP. Data are presented as the mean ± SD; n ≥ 3. *p < 0.05, ***p < 0.001.

represent an important barrier against drug access to the brain and could critically influence the bioavailibility of AEDs into these epileptogenic brain tumors. However, interactions of AEDs with BCRP transporter have not been studied. Therefore the aim of our study was to test several AEDs for their interactions with BCRP transporter.

To test substrate affinity of selected AEDs to BCRP, we used a sensitive in vitro transport assay performed in epithelial monolayers cultured on microporous membrane filters. For this purpose, MDCKII-parent cell line and its subline expressing human BCRP were used (26).

Transport assays with tested AEDs were performed in both cell lines to distinguish specific involvement of BCRP in transepithelial passage of tested compounds. In this model, unless a compound is a substrate of the tested transporter, its passage across the transfected cell line is very similar in both directions (AB and BA), and ratio *r* is close to 1. Moreover, the transport pattern of the tested compound is identical in both the MDCKII-parent and the BCRP-expressing subline. Conversely, when a compound is a substrate of the BCRP transporter, r >> 1 for the MDCKII-BCRP cell line but close to 1 in the case of the MDCKII-parent line (see Fig. 1A for PhiP, a known substrate of BCRP). In our study, we tested all selected AEDs in three concentrations that cover their potential therapeutic range of blood concentrations in patients with refractory epilepsy. In our experiments, we did not observe any significant asymmetry in the transport of AEDs across either MDCKII-BCRP or MDCKII-parent cells, regardless of the concentration used. Transport ratios were within the interval of $r = 0.85–1.2$ for both MDCKII-BCRP and MDCKII-parent cell lines (Fig. 1; data for MDCKII-parent line are not presented). These findings provide evidence that tested AEDs are not substrates of the BCRP transporter. We can therefore hypothesize that passive diffusion or some nonspecified transport mechanisms or both might be mechanisms involved in transepithelial transport across MDCKII cell lines.

AEDs also are frequently associated with clinically important drug–drug interactions that often affect safety and effectiveness of drug treatment in epilepsy patients (31,32). However, only limited data exist on the modulation of ABC transporter activity by AEDs. Weiss et al. (22) observed inhibition of P-gp in L-MDR1 cells treated with high supramicromolar concentrations of LTG, CBZ, PHT, and VPA (22). Therefore these authors suggested that the mentioned AEDs might possess the ability to enhance transmission of P-gp substrates to the brain (22). No data exist on possible modulation of BCRP-mediated transport with AEDs.

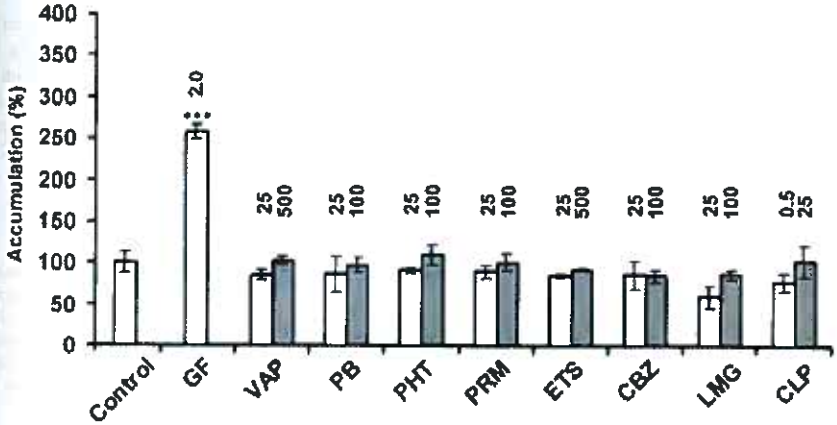

FIG. 3. Effect of antiepileptic drugs (AEDs) on breast cancer resistance protein (BCRP)-mediated transport of MIT (20 μM) in the MEF3.8-BCRP cell line. Tested concentrations of AEDs or inhibitors are indicated (μM). Results are presented as a relative cellular accumulation of MIT related to control (100%). Fluorescence of the accumulated substrate was quantified by using a flow cytometer in populations of ≥8,000 cells from histogram plots by using the median of fluorescence. GF120918 (2 μM) was used as a known inhibitor of BCRP. Data are presented as the mean ± SD; n ≥ 3. ***p < 0.001.

The goal of our accumulation experiments was to detect a potential inhibitory effect of AEDs on BCRP-mediated transport. This study was performed by using two technically independent methods. The first one detects a common fluorescent substrate of BCRP and P-gp, BODIPY FL prazosin (24), in cellular lysate. The second method, using flow cytometry, enables analysis of the fluorescent substrate MIT (25) in intact cells.

We aimed to test the inhibitory potency of AEDs at concentrations that cover their free drug fraction in blood. In the case of CZP, the chosen concentration was higher than its normal therapeutic blood concentrations. Regardless of concentration used, we did not detect any effect of AEDs on transport of BCRP substrates in MEF3.8-BCRP cells, except VPA at a concentration of 500 μM. At this concentration, VPA slightly but significantly increased the cell accumulation of BODIPY FL prazosin ($p < 0.05$), but this finding was not confirmed by the second, principally identical method, with another BCRP substrate, MIT (Figs. 2 and 3.). Therefore we can only speculate whether VPA at a concentration of 500 μM can inhibit BCRP-mediated transport of BODIPY FL prazosin. GF120918 and Ko143 were used in our experiments as known inhibitors of BCRP (33,34). The MEF3.8-BCRP cell line expresses no P-gp, and this fact enables us to use GF120918, the nonselective inhibitor of P-gp and BCRP, as a "specific" BCRP inhibitor. The specific and most potent inhibitor of BCRP, Ko143 (34), was also applied. After treatment of the MEF3.8-BCRP cells with GF120918, we observed a higher cell accumulation of BODIPY FL prazosin compared with that in cells treated with Ko143. This was probably caused by using different concentrations of inhibitors Ko143 (500 nM) and GF120918 (2 μM).

In summary, we suggest that overexpression of BCRP in the BBB is not involved in development of drug resistance to tested AEDs. On the basis of results from accumulation assays, we suppose that tested AEDs in normal therapeutic plasma concentrations do not influence, by means of BCRP inhibition, the pharmacokinetics of BCRP substrates in the BBB or in other tissues expressing BCRP, such as the intestine and placenta.

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3. Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways

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Valproic Acid Induces CYP3A4 and MDR1 Gene Expression by Activation of Constitutive Androstane Receptor and Pregnane X Receptor Pathways

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ABSTRACT:

In our study, we tested the hypothesis whether valproic acid (VPA) in therapeutic concentrations has potential to affect expression of CYP3A4 and MDR1 via constitutive androstane receptor (CAR) and pregnane X receptor (PXR) pathways. Interaction of VPA with CAR and PXR nuclear receptors was studied using luciferase reporter assays, real-time reverse transcriptase polymerase chain reaction (RT-PCR), electrophoretic mobility shift assay (EMSA), and analysis of CYP3A4 catalytic activity. Using transient transfection reporter assays in HepG2 cells, VPA was recognized to activate CYP3A4 promoter via CAR and PXR pathways. By contrast, a significant effect of VPA on MDR1 promoter activation was observed only in CAR-cotransfected HepG2 cells. These data well correlated with up-regulation of CYP3A4 and MDR1 mRNAs analyzed by real-time RT-PCR in cells transfected with expression vectors encoding CAR or PXR and treated with VPA. In addition, VPA significantly up-regulated CYP3A4 mRNA in primary hepatocytes and augmented the effect of rifampicin. EMSA experiments showed VPA-mediated augmentation of CAR/retinoid X receptor α heterodimer binding to direct repeat 3 (DR3) and DR4 responsive elements of *CYP3A4* and *MDR1* genes, respectively. Finally, analysis of specific CYP3A4 catalytic activity revealed its significant increase in VPA-treated LS174T cells transfected with PXR. In conclusion, we provide novel insight into the mechanism by which VPA affects gene expression of *CYP3A4* and *MDR1* genes. Our results demonstrate that VPA has potential to up-regulate CYP3A4 and MDR1 through direct activation of CAR and/or PXR pathways. Furthermore, we suggest that VPA synergistically augments the effect of rifampicin in transactivation of CYP3A4 in primary human hepatocytes.

Valproic acid (VPA) is an effective broad-spectrum anticonvulsant used in the treatment of primary generalized tonic-clonic, absence, and partial seizures (Tanaka, 1999). VPA has recently been identified as an inhibitor of histone deacetylase (HDAC) with potential antitumorous activity that has been studied in several clinical trials (Göttlicher et al., 2001; Blaheta et al., 2002, 2005; Kramer et al., 2003; Raffoux et al., 2005).

VPA is well known to affect mechanisms that control drug disposition such as activity of hepatic biotransformation enzymes or drug binding to plasma proteins (Rogiers et al., 1995; Wen et al., 2001; Perucca, 2006). Generally, VPA is thought to be an inhibitor rather than an inducer of drug-metabolizing enzymes (Perucca, 2006). However, this point of view is currently not supported satisfactorily by comprehensive data published in the literature. Regarding metabolizing enzymes of the cytochrome P450 family, Wen et al. (2001) have demonstrated, using in vitro methods, that VPA in therapeutically relevant concentrations competitively inhibits only human CYP2C9 catalytic activity. On the other hand, there is clear evidence that VPA has potential to up-regulate expression and activity of several rodent and human genes encoding proteins involved in drug disposition. It was found that prolonged exposure of rats to VPA results in the self-inducing metabolism of the agent (Fisher et al., 1991). Moreover, Rogiers et al. (1992, 1995) have found VPA to be a potent inducer of genes of the rat Cyp2b subfamily, in particular, *Cyp2b1* and *Cyp2b2*. Recently, Eyel et al. (2006) have found out that VPA does not affect expression of rat *Cyp3a2*, an ortholog of human *CYP3A4*, whereas valpromide, the primary amide of VPA that reveals no HDAC-

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ABBREVIATIONS: VPA, valproic acid; HDAC, histone deacetylase; CITCO, (6-(4-chlorophenyl) imidazo [2,1-b][1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime); NR, nuclear receptor; RT-PCR, reverse transcriptase polymerase chain reaction; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXRα, retinoid X receptor α (9-*cis* retinoic acid receptor-α); FCS, fetal calf serum; DMSO, dimethyl sulfoxide; DR, direct repeat; ER, everted repeat; HPRT, hypoxanthine-guanine phosphoribosyl transferase; B2M, β_2-microglobulin; PBREM, phenobarbital responsive enhancer module; EMSA, electrophoretic mobility shift assay; ANOVA, analysis of variance.

inhibitory activity, was shown to induce this gene by a nonspecific mechanism. In addition, these authors have suggested that VPA induces expression and activity of human P-glycoprotein (MDR1), a member of the ATP-binding cassette family of drug transporters, in tumor cell lines by the mechanism of HDAC inhibition (Eyal et al., 2006). These summarized data indicate that VPA is capable of altering expression and activity of various cytochromes P450 differently. Moreover, VPA has potential to induce the *MDR1* gene.

We assumed that alteration of expression of these genes could be caused by interaction of VPA with constitutive androstane receptor (CAR; NR1I3) and human pregnane X receptor (PXR; SXR; NR1I2). PXR and CAR are ligand-activated nuclear receptors that act as heterodimers with retinoid X receptor α (RXRα) and up-regulate the transcription of their target genes, such as *CYP3A4* and *MDR1*, by interaction with specific promoter-binding motifs (Goodwin et al., 1999). PXR has been shown to be activated by many structurally and chemically diverse ligands. Examples of human PXR activators include xenobiotics such as rifampicin (Bertilsson et al., 1998; Pascussi et al., 2000), the endobiotic lithocholic acid (Staudinger et al., 2001; Xie et al., 2001), the progesterone metabolite 5-pregnane-3,20-dione (Jones et al., 2000), and the herbal compound hyperforin (Moore et al., 2000a). In the case of CAR, the experimental substance CITCO ((6-(4-chlorophenyl) imidazo [2,1-b][1,3] thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl) oxime), the antimalarial artemisinin drugs, and 5-pregnane-3,20-dione have been recognized as its ligands (Jones et al., 2000; Maglich et al., 2003; Burk et al., 2005b). The barbiturate drug phenobarbital activates CAR indirectly through the increase of CAR translocation from the cytoplasm to the nucleus (Honkakoski et al., 1998; Kawamoto et al., 1999; Goodwin and Moore, 2004). On the other hand, several competitive inverse agonists of CAR have also been discovered such as androstanol, androstenol, and clotrimazole (Forman et al., 1998; Moore et al., 2000b).

In this study, we examined whether VPA controls expression of *CYP3A4* and *MDR1* (ABCB1) genes at the transcriptional level via activation of PXR and CAR pathways. This hypothesis was investigated in the human Caucasian hepatocyte carcinoma (HepG2) and human colon carcinoma (LS174T) cells using several reporter luciferase constructs with major promoter-regulatory sequences of *CYP3A4* and *MDR1*. In addition, using real-time RT-PCR, we investigated CYP3A4 mRNA expression in primary human hepatocytes treated with VPA. Moreover, we studied whether VPA augments binding of CAR/RXRα to several response elements of CYP3A4 (DR3, ER6) and MDR1 (DR4) using the electrophoretic mobility shift assay (EMSA).

Our data indicate that VPA is capable of transactivating both CYP3A4 and MDR1 via interaction with the CAR pathway; however, VPA-mediated activation of PXR pathway controls only *CYP3A4* gene expression. Moreover, we demonstrate that VPA synergizes with rifampicin in transactivation of CYP3A4 in primary human hepatocytes.

Materials and Methods

Cell Lines and Chemicals. The human Caucasian hepatocyte carcinoma HepG2 and human colon adenocarcinoma LS174T cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK) and were used within 25 passages after delivery and maintained in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 1% sodium pyruvate, and 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO). The latter cell line has been previously shown to have highly inducible expression of *CYP3A4* and *MDR1* (Geick et al., 2001).

Androstenol (5α-androst-16-en-3α-ol), rifampicin, VPA (2-propylpentanoic acid), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. CITCO was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). DMSO or water was used as a solvent of VPA when indicated.

Plasmids. A chimeric *p3A4-luc* reporter construct containing the basal promoter (−362/+53) with proximal PXR response element and the distal xenobiotic responsive enhancer module (−7836/−7208) of the *CYP3A4* gene 5′-flanking region inserted to pGL3-Basic reporter vector was described by Goodwin et al. (1999). Plasmid *p*-7975(Δ7012–1804) harboring the enhancer of *MDR1* gene 5′-flanking region (MDR1E) and the basal promoter were constructed with *pGL3-Basic* reporter vector as described by Geick et al. (2001), herein referred to as *pMDR1-luc*. *pMDR1E-SV40-luc* reporter plasmid was constructed by insertion of the enhancer region of *MDR1* gene from −7881 to −7809 into *pGL3-Promoter* plasmid (Promega, Madison, WI) in NheI/BglII cloning sites upstream of a heterologous promoter SV40. CYP2B6 phenobarbital responsive enhancer module (PBREM) reporter gene construct (*pPBREM-SV40-luc*) containing two DR4-type motifs (NR1 and NR2) was prepared from *pGL3-Promoter* plasmid by insertion of the region from −1733 to −1683 of CYP2B6 promoter into NheI/BglII cloning sites upstream of a heterologous promoter SV40. Constructed plasmids were sequenced using a Big Dye Terminator Cycle Sequencing Method (Applied Biosystems, Foster City, CA). The expression plasmid for human PXR receptor, *pSG5-PXR*, was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX). The human CAR expression plasmid *pCR3-CAR* was kindly provided by Dr. M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The expression plasmid *pSG5-hRXRα* encoding hRXRα cDNA was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland).

Transient Transfection and Luciferase Gene Reporter Assays. All transfection assays were carried out using Lipofectamine2000 transfection reagent (Invitrogen, Carlsbad, CA) in cells cultivated in the phenol red-free medium containing 10% charcoal-stripped FCS, 1% sodium pyruvate, and 1% nonessential amino acids according to the manufacturer's instruction.

HepG2 cells (2×10^5 per well) were seeded into 48-well plates and cotransfected with a luciferase reporter construct (0.4 μg/well) and expression plasmid encoding either CAR or PXR (50 ng/well) 24 h later. Subsequently, cells were maintained in medium supplemented with 10% charcoal/dextran-stripped fetal bovine serum containing VPA at appropriate concentrations for 24 h. Luminescence activity was determined with a Genios Plus luminometer (Tecan, Grödig, Austria) in cell lysate using a commercially available luciferase detection system (Promega). In addition, all experiments were performed in parallel using empty *pGL3-Basic* and *pGL3-Promoter* luciferase reporter constructs containing no responsive elements to distinguish nonspecific CAR- and PXR- independent effect of VPA on reporter plasmids used. Luminescence of these samples was taken as background that was subtracted in the final calculation from luminescence of cell samples transiently transfected with reporter plasmids containing response elements of tested genes. Resulting data are presented as ratio of luminescence of treated cell samples to control. Luminescence of each sample has been normalized to its protein concentration determined with the BCA system (Pierce, Rockford, IL).

Real-Time RT-PCR Analysis of CYP3A4 mRNA. LS174T cells (1.2×10^5 per well) were seeded into 24-well plates and cultivated for 24 h. Then cells were transfected with CAR or PXR expression plasmids (400 ng/well) and appropriate cell samples were exposed to VPA at a concentration of 500 μM for 48 h. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The following primers were used for *CYP3A4*: forward primer 5′-TTCAGCAAGAAGAACAAGGACAA-3′, reverse primer 5′-GGTTGAAGAAGTCCTCCTAAGC-3′); for *MDR1*, forward primer 5′-TGCTCAGACAGGATGTGAGTTG-3′, reverse primer 5′-AATTACAGCAAGCCTGGAACC-3′; and for housekeeping genes *HPRT* (hypoxanthine-guanine phosphoribosyl transferase), forward primer 5′-CTGGAAAGAATGTCTTGATTGTGG-3′, reverse primer 5′-TTTGGATTATACTGCCTGACCAAG-3′ and *B2M* (β_2-microglobulin), forward primer 5′-CGTGTGAACCATGTGACTTTGTC-3′, reverse primer 5′-CATCTTCAAACCTCCATGATGC-3′). cDNA was prepared from 1 μg of total RNA with MMLV transcriptase (Finnzymes, Espoo, Finland) using oligo$(dT)_{15}$VN primer (Generi-Biotech, Hradec Králové, Czech Republic) and porcine RNase inhibitor (TaKaRa BIO, Shiga, Japan). Real-Time PCR analysis was performed on an iCycler (Bio-Rad, Hercules, CA). cDNA (40 ng of reverse-transcribed RNA) was amplified with HotStar *Taq* polymerase (QIAGEN,

Valencia, CA) under the following conditions: 3 mM $MgCl_2$, 0.2 mM deoxynucleoside-5′-triphosphate, 0.3 μM each primer, 0.025 U/μl polymerase, SYBR Green I in 1:100.000 dilution, and fluorescein (10 nM). The temperature profile was 95°C for 14 min; 50 times 95°C for 15 s, 60°C for 20 s, 72°C for 30 s, 72°C for 15 s; melting curve program 70–95°C. All samples were run in triplicates simultaneously with negative controls. The processing of real-time amplification curves was performed on iCycler software version 4.6 (Bio-Rad). Pfaffl's method was applied for relative quantification of gene expression normalized to endogenous control (housekeeping) gene (Pfaffl, 2001). Results are presented as the means of at least three experiments.

Isolation and CYP3A4 mRNA Analysis in Primary Cell Culture of Human Hepatocytes. Human tissue samples were obtained according to protocols approved by the local ethics committee of the Palacky University in Olomouc (Czech Republic) complying with the current Czech legislation. Hepatocytes were prepared from lobectomy segments resected from adult patients for medical reasons unrelated to our research program. The tissue encompassing the tumor was dissected by a surgeon and sent for anatomopathological studies, whereas the remaining tissue was used for hepatocyte preparation. No information on the patients was available to us, apart from age, sex, and the reason for surgery. Human liver samples used in this study were obtained from two donors: donor 1, a woman, 69 years old, tumor metastasis; and donor 2, a woman, 46 years old, Caroli syndrome. Hepatocytes were isolated as described previously (Pichard-Garcia et al., 2002). After isolation, the cells were plated on collagen-coated culture dishes at density 1.4×10^5 cells/cm^2. Culture medium was enriched for plating with 2% FCS (v/v) as described previously (Isom et al., 1985). The medium was replaced with serum-free medium the day after, and the cells were allowed to be stabilized for an additional 48 to 72 h before the treatment with VPA (500 μM) or rifampicin (10 μM) or their combination. Cells were maintained at 37°C and 5% CO_2 in a humidified incubator. The effect of VPA on CYP3A4 mRNA expression was tested in periods of 24 and 48 h. The effect of rifampicin and the combination of VPA and rifampicin were studied in a period of 24 h. In the latter experiments, DMSO (0.1%) was used as solvent of both VPA and rifampicin. The level of CYP3A4 mRNA expression was analyzed using real-time RT-PCR according to the protocol mentioned above.

Functional Analysis of CYP3A4 Activity. LS174T cells (6×10^5 per well) were seeded into 12-well plates and cultivated for 24 h. Subsequently, cells were transfected with CAR or PXR expression plasmids (1.2 μg/well) and then exposed to VPA at a concentration of 600 μM for 48 h. After treatment, cells were washed with phosphate-buffered saline and cultivated in serum-free Opti-MEM medium (Invitrogen-Gibco) containing testosterone at a final concentration of 150 μM for 2 h at 37°C. Then, cells were washed with phosphate-buffered saline and lysed with 200 μl of SDS (1%). CYP3A4 activity was detected in total cellular lysate (150 μl) using an established method based on the measuring of a prototypic CYP3A4-mediated testosterone 6β-hydroxylation activity (Guengerich et al., 1986) and modified for the analysis of the metabolite in cell lysate. For the determination of CYP3A4 activity, the metabolite was extracted to dichloromethane. Subsequently, the solvent was evaporated and the sample dissolved in the mobile phase. The concentration of the testosterone metabolite was determined using a Shimadzu Class VP HPLC system (Shimadzu, Tokyo, Japan). Final activity was normalized to sample protein concentration in cell lysate that was measured using the BCA assay according to the manufacturer's instructions (Pierce).

EMSA. Human CAR and RXRα were translated in vitro using TNT Quick Coupled Transcription/Translation Systems (Promega, Southampton, UK). The nuclear fraction was isolated from HepG2 cells transfected with hRXRα expression vector using CelLytic NuCLEAR Extraction Kit (Sigma). The following double-stranded 5′-biotinylated oligonucleotides of specific response elements of both CYP3A4 and MDR1 promoters were used as probes: CYP3A4 DR3 sense, 5′-GAATGAACTTGCTGACCCTCT-3′; CYP3A4 DR3 antisense, 5′-AGAGGGTCAGCAAGTTCATTC-3′; CYP3A4 ER6 sense, 5′-ATATGAACTCAAAGGAGGTCAGTG-3′; CYP3A4 ER6 antisense, 5′-CACTGACCTCCTTTGAGTTCATAT-3′; MDR1 DR4 sense, 5′-CATTGAACTAACTTGACCTTGC-3′; and MDR1 DR4 antisense, 5′-GCAAGGTCAAGTTAGTTCAATG-3′. The oligonucleotides were synthesized at Generi-Biotech.

The EMSA was performed according to the protocol published previously with slight modifications (Frank et al., 2003). The binding reactions were performed in a total volume of 20 μl and contained equal amounts (~3 μg) of CAR and RXRα, 10× binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol) (Pierce), poly(dI · dC) Double Strand (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and 1% Nonidet P-40. VPA was tested at final concentrations of 100, 500, and 1000 μM. CITCO, an agonist of human CAR, was used at the concentration of 6.25 μM and androstenol at the concentration of 10 μM. DMSO was used as a solvent of the compounds at maximal final concentration of 0.1%. For supershift experiments, 1 μg of the anti-RXRα rabbit polyclonal IgG antibody was added to the reaction mixture (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Reactions were preincubated on ice for 10 min before the addition of oligonucleotide probe (20 fmol). Samples were maintained at room temperature for an additional 20 min, and then protein/DNA complexes were resolved on a 5% (w/v) nondenaturing polyacrylamide gel (acrylamide/bisacrylamide 29:1 w/v) in 0.5× Tris borate-EDTA buffer (450 mM Tris, 450 mM boric acid, 10 mM EDTA). The gels were electrophoresed at 100 V for an hour at room temperature. Next, the gels were blotted at 380 mA for 30 min at 4°C and then analyzed by the Chemiluminescent Nucleic Acid Detection Module (Pierce).

EMSA Quantification. EMSA reactions were exposed to X-ray film (Foma Bohemia a.s., Hradec Králové, Czech Republic) and bands were quantified using computerized densitometry using LabImage densitometry analysis software (Kapelan Bio-Imaging Solutions, Halle, Gemany).

Statistics. One-way ANOVA followed by Dunnett's multiple comparison post hoc test or unpaired Student's *t* test was used for statistical analysis of data using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Two-way ANOVA with interaction was used to analyze the synergistic effect of VPA and rifampicin.

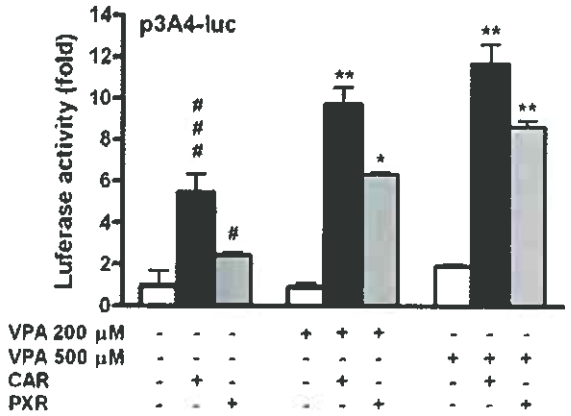


FIG. 1. Effect of VPA on CAR- and PXR-mediated transactivation of CYP3A4 promoter. HepG2 cells were transiently transfected with *p3A4-luc* reporter construct containing the basal promoter (−362/+53) with proximal PXR response element and the distal xenobiotic responsive enhancer module (−7836/−7208) of CYP3A4 (0.4 μg/well) and either *pCR3-CAR* or *pSG5-PXR* expression vector (50 ng) using Lipofectamine2000 transfection reagent according to the manufacturer's instructions. Transfected HepG2 cells were maintained in medium containing VPA at the indicated concentrations for 24 h. Luciferase activities are normalized to protein concentration and expressed as -fold activation of nontreated cells transfected with *p3A4-luc*. All means ± S.D. were calculated from quadruplicates of a representative experiment and analyzed using ANOVA followed by Dunnett's test. #, $p < 0.05$; ###, $p < 0.001$: statistically different from nontreated cells transfected with *p3A4-luc*. *, $p < 0.05$; **, $p < 0.01$: statistically different from VPA-nontreated cells cotransfected with either *pCR3-CAR* or *pSG5-PXR*.

Results

Effect of VPA on CAR- and PXR-Mediated Activation of CYP3A4 Promoter in HepG2 Cells Transiently Transfected with *p3A4-luc* Luciferase Reporter Construct. First, we examined whether VPA affects activation of *p3A4-luc* reporter construct through CAR and PXR. We observed statistically significant concentration-dependent increase in CAR- and PXR-mediated transcriptional activation of *p3A4-luc* in HepG2 cells after 24 h exposure to VPA (Fig. 1). VPA at the concentration of 500 μM doubled the activation

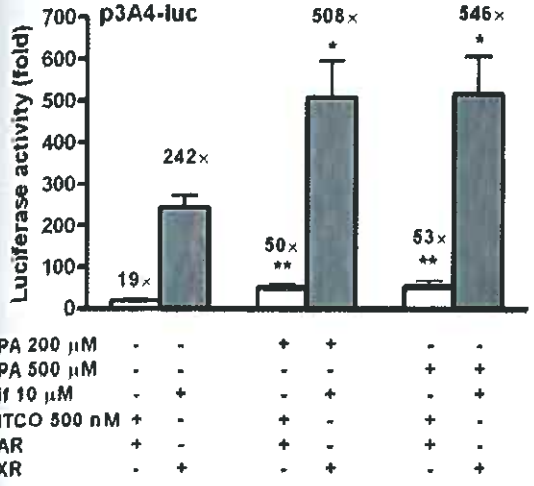


FIG. 2. Transactivation of CYP3A4 promoter by VPA in combination with CITCO or rifampicin. HepG2 cells were transiently transfected with *p3A4-luc* reporter construct (0.4 μg/well) and *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) using Lipofectamine2000 transfection reagent according to the manufacturer's instructions and subsequently exposed to VPA in combination with CITCO (500 nM) or rifampicin (Rif; 10 μM), prototype ligands of CAR and PXR, respectively, for 24 h. All means ± S.D. were calculated from quadruplicates of a representative experiment and analyzed using ANOVA followed by Dunnett's test. Data are presented as -fold activation of nontreated cells transfected only with *p3A4-luc*. *, $p < 0.05$; **, $p < 0.001$: statistically different from cells cotransfected with either *pCR3-CAR* or *pSG5-PXR* and treated with either CITCO or rifampicin.

of *p3A4-luc* in cells cotransfected with CAR expression vector (Fig. 1). In HepG2 cells cotransfected with PXR, we observed even a 4-fold increase in activation of *p3A4-luc* after treatment with VPA (500 μM) (Fig. 1). In contrast, VPA did not significantly affect transcriptional activation of *p3A4-luc* in HepG2 cells, which were not cotransfected with either CAR or PXR (Fig. 1). We observed that expression of both CAR and PXR in HepG2 cells in the absence of an exogenous ligand resulted in statistically significant activation of *p3A4-luc* reporter, which is in agreement with published data and indicates involvement of endogenous activators of the nuclear receptors in HepG2 cells (Fig. 1) (Goodwin et al., 1999, 2002).

Transactivation of CYP3A4 Promoter by VPA in Combination with CITCO or Rifampicin. In these experiments, we examined whether VPA has the ability to affect CAR- and PXR-mediated activation of *p3A4-luc* construct caused by prototypic ligands CITCO (500 nM) in CAR-expressing cells and rifampicin (10 μM) in PXR-expressing cells. VPA at tested concentrations in combination with CITCO (500 nM) significantly augmented CAR-mediated activation of *p3A4-luc* compared with CAR-expressing cells treated with CITCO ($p < 0.01$) (Fig. 2). Similarly, we observed a more pronounced effect of VPA in combination with rifampicin (10 μM) on transcriptional activation of *p3A4-luc* via PXR compared with the effect of rifampicin alone ($p < 0.05$) (Fig. 2).

Effect of VPA on CAR- and PXR-Mediated Activation of MDR1 Promoter in HepG2 Cells Transiently Transfected with *pMDR1-luc* or Heterologous *pMDR1E-SV40-luc* Luciferase Reporter Constructs. Regarding MDR1, two different luciferase reporter constructs were used in our study. First, we used *pMDR1-luc* construct containing the distal enhancer region with a DR4 responsive motif common to both PXR- and CAR-mediated transactivation of *MDR1* gene, and the native basal promoter from −1803 to +281. Using this reporter construct, we observed a similar effect of VPA on activation of MDR1 promoter in cells cotransfected with PXR or CAR in comparison with mock-transfected HepG2 cells (Fig. 3A). On the other hand, rifampicin (10 μM) and CITCO (1 μM) significantly activated (~2-fold, $p < 0.05$) the *pMDR1-luc* construct in HepG2 cells cotransfected with PXR or CAR expression plasmids under the experimental conditions used. This indicates that VPA probably has potential to transactivate this luciferase construct independently on PXR and CAR pathways through a nonspecific mechanism, which is likely related to activation of MDR1 basal promoter (Morrow and Nakagawa, 1994).

In light of our previous observations demonstrating that heterologous *pMDR1E-SV40-luc* is more responsive to both PXR- and CAR-mediated activation than *pMDR1-luc* reporter, we performed a transfection assay with *pMDR1E-SV40-luc* reporter construct containing the MDR1 enhancer upstream of viral SV-40 promoter. Using the plasmid, we observed that VPA at a concentration of 500 μM activates significantly only CAR-mediated transcription of *pMDR1E-SV40-luc* reporter construct ($p < 0.001$) (Fig. 3B). The effect of VPA on activation of *pMDR1E-SV40-luc* plasmid was comparable to the effect of CITCO (1 μM, 2-fold activation) and rifampicin (25 μM, 2.3-fold activation, $p < 0.05$) in nuclear receptor-transfected cells.

Finally, we used androstenol, an inverse agonist of CAR, to confirm that VPA activates *pMDR1E-SV40-luc* through CAR. Androstenol (10 μM) significantly repressed CAR-mediated transcriptional activation of *pMDR1E-SV40-luc* in cells exposed to VPA at a concentration of 500 μM ($p < 0.05$) (Fig. 3C). In contrast, we observed no effect of androstenol on *pGL3-Basic* or *pGL3-Promoter* construct activities in cells cotransfected with CAR expression vector (data not shown).

Effect of VPA on CAR- and PXR-Mediated Activation of CYP2B6 PBREM in HepG2 Cells Transiently Transfected with *pPBREM-SV40-luc* Luciferase Reporter Construct. To elucidate the discrepancy between CAR- and PXR-mediated activation of MDR1 promoter by VPA, we analyzed luciferase activity of *pPBREM-SV40-luc* construct in cells transiently transfected with CAR or PXR expression vectors and exposed to VPA (500 μM). *pPBREM-SV40-luc* construct contains two DR4-type responsive elements of the PBREM of the *CYP2B6* gene, which has high ability to interact with both CAR and PXR (Goodwin et al., 2001; Faucette et al., 2007). We detected significant up-regulation of *pPBREM-SV40-luc* activity in HepG2 cells cotransfected with both CAR and PXR expression plasmids ($p < 0.001$; Fig. 4). Moreover, the activation of the construct was further significantly augmented in cells coexpressing CAR and exposed to VPA (5.8-fold increase, $p < 0.001$; Fig. 4). Similarly, VPA increased significantly activation of *pPBREM-SV40-luc* in PXR-expressing cells (2-fold, $p < 0.05$). Under the same experimental conditions, CITCO (1 μM) increased activation of the plasmid 2.1-fold in the cells expressing CAR and rifampicin (10 μM) 2.4-fold in PXR-transfected cells. Nevertheless, we also detected comparable activation of the luciferase construct by VPA in cells with no exogenous PXR ($p < 0.05$) (Fig. 4), which documents that exogenous PXR has minor or no effect on activation of *pPBREM-SV40-luc* by VPA. Thus, we observed a similar pattern in activation of *pPBREM-SV40-luc* and *pMDR1E-SV40-luc* by VPA (Figs. 3B and 4). In both cases, we demonstrated that VPA increases CAR-mediated, but not PXR-mediated transactivation of the constructs.

Analysis of VPA-Mediated Up-Regulation of CYP3A4 and MDR1 mRNAs in LS174T Cells. To evaluate the ability of VPA to induce CYP3A4 and MDR1 mRNA levels by means of CAR and PXR activation, real-time RT-PCR was used. CYP3A4 and MDR1 mRNAs were quantified in samples of LS174T cells transfected with expression plasmids encoding studied nuclear receptors and exposed to VPA for 48 h. Significant effect of VPA on CYP3A4 mRNA level was observed in cells transfected with both CAR and PXR nuclear receptors yielding a 4.2-fold and 3.8-fold ($p < 0.001$ and $p < 0.01$)

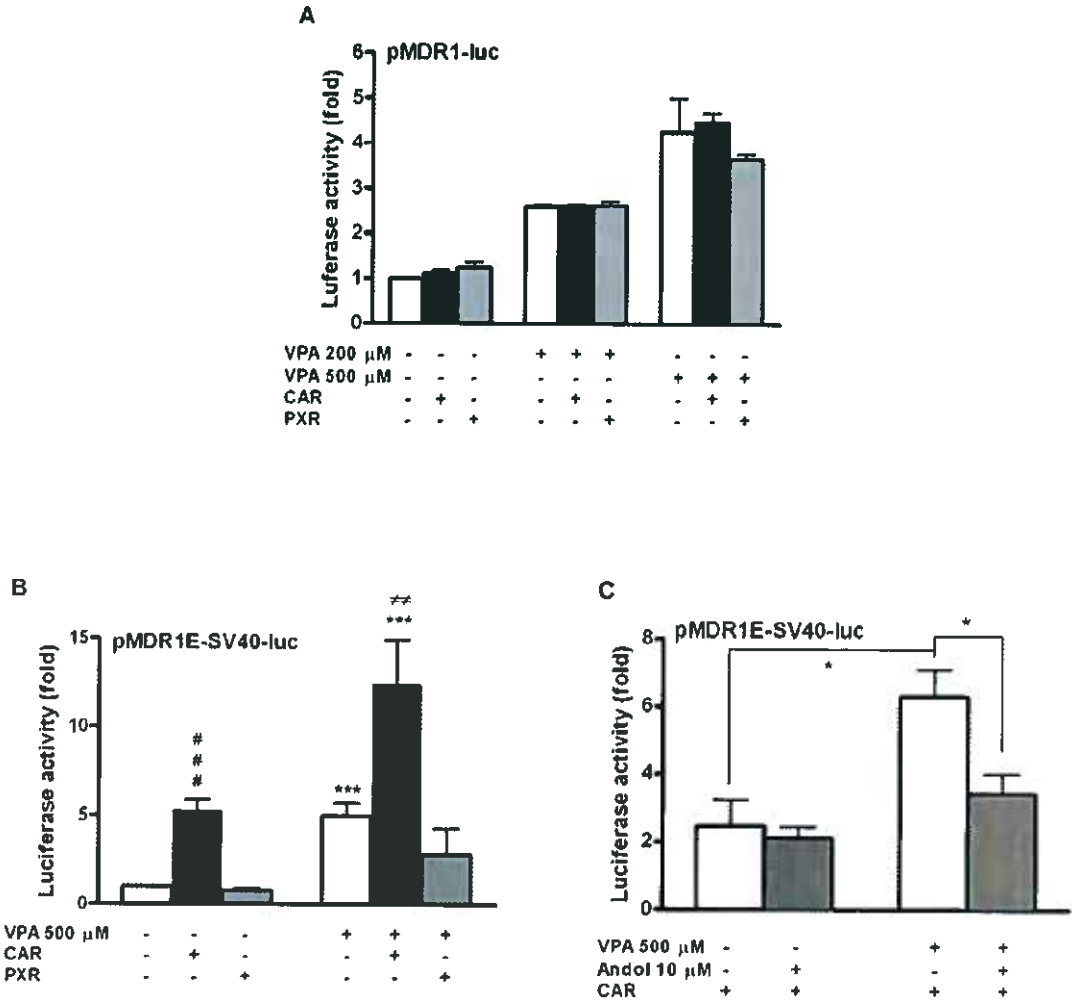


FIG. 3. Effect of VPA on CAR- and PXR-mediated activation of reporter constructs of *MDR1* gene. HepG2 cells were transiently transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) and appropriate reporter construct (0.4 μg/well) using Lipofectamine2000 transfection reagent according to the manufacturer's instructions. Transfected HepG2 cells were maintained in medium containing the tested compounds at the indicated concentrations for 24 h. A, effect of VPA on CAR- and PXR-mediated transactivation of *pMDR1-luc* construct containing the enhancer region of *MDR1* gene (−7975/−7013) and the basal promoter from −1803 to +231. B, VPA-mediated transcriptional activation of *pMDR1E-SV40-luc* construct containing the enhancer region of *MDR1* gene and viral promoter SV40 in cells coexpressing exogenous CAR or PXR. C, influence of androstenol (Andol), an inverse agonist of CAR, on CAR-mediated transcriptional activation of *pMDR1E-SV40-luc* in HepG2 cells treated with VPA. HepG2 cells were transiently transfected with *pCR3-CAR* (40 ng) and *pMDR1E-SV40-luc* construct (0.4 μg/well) using Lipofectamine2000. Control and VPA-treated cells were exposed to DMSO (0.1%) to eliminate the influence of the androstenol solvent. Data are presented as -fold activation of nontreated cells transfected only with the appropriate reporter construct, *pMDR1E-luc* or *pMDR1E-SV40-luc*. All means ± S.D. were calculated from quadruplicates of a representative experiment. ###, $p < 0.001$: statistically different from nontreated cells transfected only with reporter construct. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$: statistically different from VPA-nontreated cells cotransfected with either *pCR3-CAR* or *pSG5-PXR* expression vectors. ≠≠, $p < 0.01$: statistically different from cells transfected with reporter plasmid and exposed to VPA.

induction, respectively (Fig. 5A). VPA also significantly up-regulated CYP3A4 mRNA in LS174T cells, which were not cotransfected with any nuclear receptor ($p < 0.01$) (Fig. 5A). This might be in agreement with high expression of endogenous PXR in LS174T cells, which could at least partly participate in up-regulation of CYP3A4 mRNA by VPA (data in Fig. 1) (Burk et al., 2005b). In the case of MDR1, we observed a similar profile of VPA-mediated MDR1 mRNA up-regulation. Contrary to transfection assay results, we detected a statistically significant effect of VPA on MDR1 mRNA expression in PXR-transfected LS174T cells (2.0-fold increase, $p < 0.05$). However, the MDR1 mRNA level was not statistically significantly different from the level in VPA-treated cells (Fig. 5B). Thus, we suppose that the increase in MDR1 mRNA level after treatment with VPA is caused mostly by the mechanism of HDAC inhibition described previously rather than through activation of the PXR pathway by VPA (Morrow and Nakagawa, 1994; Jin and Scotto, 1998; Xiao and Huang, 2005). It is noteworthy that we observed statistically significant ($p < 0.05$) up-regulation of MDR1 mRNA after cotransfection of LS174T cells with CAR expression vector and treatment with VPA (Fig. 5B). On the other hand, cotransfection of LS174T cells with expression plasmid for either PXR or CAR without exposure to VPA did not result in statistically significant up-regulation of CYP3A4 and MDR1 mRNAs (Fig. 5, A and B). Under the same experimental conditions, treatment of LS174T cells with rifampicin (10 μM) resulted in a 12-fold increase in CYP3A4 mRNA and a 7-fold increase in MDR1 mRNA.

Determination of CYP3A4 Catalytic Activity in LS174T Cells Exposed to VPA (600 μM). CYP3A4 induction was subsequently investigated employing functional assay based on determination of specific CYP3A4-mediated testosterone 6β-hydroxylation activity measured in LS174T cell lysate. As shown in Fig. 6, only cells transfected with plasmid encoding PXR yielded statistically signifi-

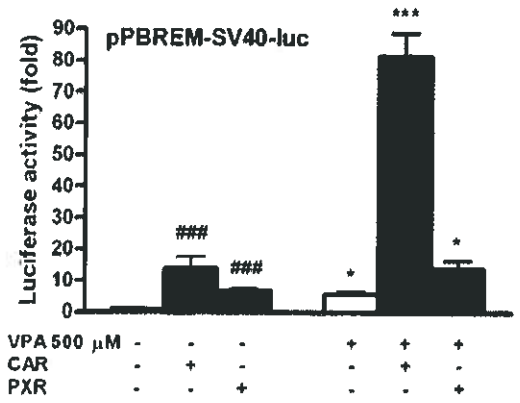


FIG. 4. Effect of VPA on CAR- and PXR-mediated activation of the PBREM region of the CYP2B6 promoter. HepG2 cells were transiently transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) and *pPBREM-SV40-luc* reporter construct containing two DR4-type motifs of CYP2B6 PBREM (0.4 μg/well) using Lipofectamine2000 transfection reagent according to the manufacturer's instructions. Transfected HepG2 cells were maintained in medium containing VPA (500 μM) for 24 h. ###, $p < 0.001$: statistically different from nontreated cells transfected only with reporter construct; *, $p < 0.05$; ***, $p < 0.001$: statistically different from VPA-nontreated cells cotransfected with either *pCR3-CAR* or *pSG5-PXR* expression vectors.

cant increase in CYP3A4 catalytic activity after 48 h exposure to VPA at a concentration of 600 μM ($p < 0.05$).

VPA Induces CYP3A4 mRNA in Primary Human Hepatocytes and Synergizes with the Effect of Rifampicin. To determine whether VPA induces CYP3A4 mRNA and whether it influences the induction by rifampicin, primary cultures of human hepatocytes were treated individually or in combination with VPA (500 μM) and/or rifampicin (10 μM). CYP3A4 mRNA was significantly up-regulated in the hepatocytes exposed to VPA for 48 h taken from both donor 1 (14.1-fold, $p < 0.01$) (Fig. 7A) and donor 2 (3.8-fold, $p < 0.01$) (Fig. 7B). As shown in Fig. 7C, by using the unpaired Student's *t* test, we revealed a statistically significant increase of CYP3A4 mRNA compared with control treated with DMSO (0.1%) in cell samples treated with rifampicin (10 μM) for 24 h (donor 1, 20.1-fold and donor 2, 13.6-fold, respectively; $p < 0.001$). In cells cultivated in medium containing VPA (500 μM) and vehicle (DMSO, 0.1%) used for elimination of rifampicin solvent effect, we also detected significant induction of CYP3A4 mRNA relative to control (DMSO, 0.1%) ($p < 0.01$ and $p < 0.05$, respectively). Furthermore, when VPA (500 μM) and rifampicin (10 μM) were added simultaneously into medium and hepatocytes were cultivated for 24 h, up-regulation of CYP3A4 was synergistic rather than additive (62- and 34-fold). Using two-way ANOVA, this synergy was found to be statistically significant at the level of $p < 0.01$ (Fig. 7C).

Examination of Interactions between CAR/RXRα Heterodimer or CAR Monomer and Responsive Elements of CYP3A4 and MDR1 Promoters in the Presence of VPA using a Ligand-Dependent EMSA. The interactions between CAR/RXRα heterodimers and nuclear receptor-binding motifs of CYP3A4 (DR3, ER6) and MDR1 enhancer (DR4) in the presence of VPA were examined via EMSA using in vitro translated CAR and RXRα proteins. CAR forms a complex with RXRα, which binds DR3, DR4, and ER6 responsive elements of CYP3A4 and MDR1 even in the absence of a ligand (Goodwin et al., 1999, 2002; Geick et al., 2001; Burk et al., 2005a). This complex formation can be enhanced by ligand binding to CAR, which can be revealed using EMSA (Frank et al., 2004). We detected that VPA at the tested concentrations of 500 and 1000 μM increased the formation of CAR/RXRα complex with the DR3 responsive element of CYP3A4 promoter (Fig. 8, A, lane 8; and B, lanes 6 and 7). Figure 8A shows that using recombinant CAR and RXRα proteins, VPA moderately (by ~30%) augmented the CAR/RXRα/DR3 complex in EMSA experiments (Fig. 8A, lane 8). In the next experiments, we used nuclear fraction from HepG2 cells transfected with expression vector encoding RXRα. VPA at the concentration of 500 μM augmented the complex 1.8-fold and at the concentration of 1000 μM increased 4.2-fold the binding of CAR/RXRα to DR3 (Fig. 8B, lanes 6 and 7). Similarly, as shown in Fig. 8D (lane 6), we detected increased (~1.5-fold) binding of CAR/RXRα heterodimer to oligonucleotide containing the DR4 motif of MDR1 enhancer in the presence of VPA (1000 μM). Consistently, CITCO, an agonist of human CAR, augmented binding of CAR/RXRα complex to DR3 response elements (Fig. 8, A, lane 5; and B, lane 3) (Maglich et al., 2003). The inverse agonist of CAR, androstenol (10 μM), decreased formation of CAR/RXRα complex with response element DR3 in samples treated with CITCO (500 nM) or VPA (500 and 1000 μM) (Fig. 8, A–D). Interestingly, we observed that VPA at tested concentrations augments interaction of recombinant CAR monomer with the DR3 motif of CYP3A4 promoter (Fig. 8C, lane 2). On the other hand, we did not detect any significant and reproducible effect of VPA on interaction of CAR/RXRα heterodimer with the ER6 response element of the *CYP3A4* gene (data not shown). RXRα alone did not reveal any

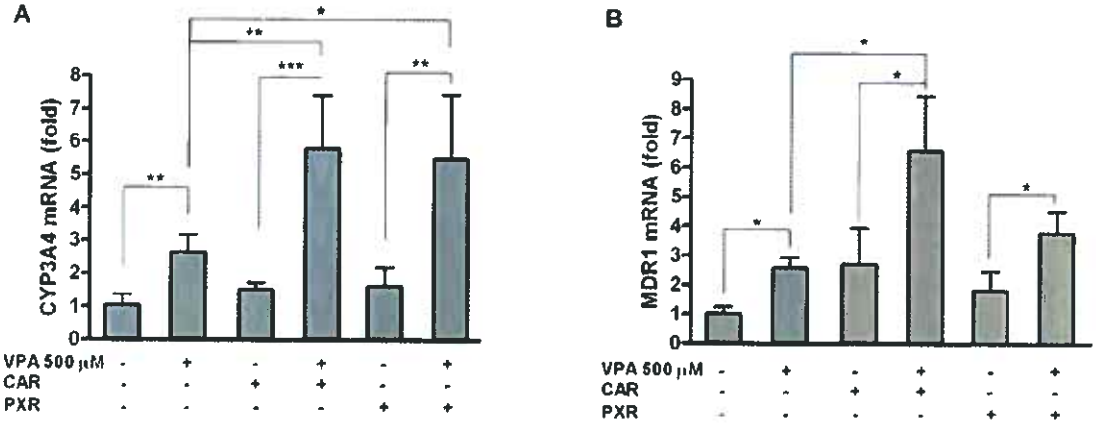


FIG. 5. Analysis of VPA-mediated up-regulation of CYP3A4 and MDR1 mRNAs. LS174T cells were transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmids (400 ng/well) using Lipofectamine2000 transfection reagent according to the manufacturer's instructions and exposed to VPA (500 μM) for 48 h. mRNA expression of tested genes was determined using real-time RT-PCR and normalized to *HPRT* housekeeping gene. The effect of VPA (500 μM) on CYP3A4 (A) and MDR1 (B) mRNA expression is presented as -fold increase to control nontreated cells. Data are the means ± S.D. of three individual cell samples. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

interaction with tested response elements (data not shown). Similarly, we did not detect any complex after incubation of nuclear fraction of HepG2 cells and recombinant RXRα with DR3 oligonucleotide under the conditions used (data not shown). These data suggest that VPA could directly interact with CAR/RXRα heterodimer and CAR monomer in binding with DR3 and DR4 response elements of CYP3A4 and MDR1.

Unfortunately, there is no report in the literature that introduces reliable ligand-dependent EMSA assay with recombinant PXR at present. Therefore, we could not analyze the interaction of VPA with PXR/RXRα complex at present, using the EMSA method.

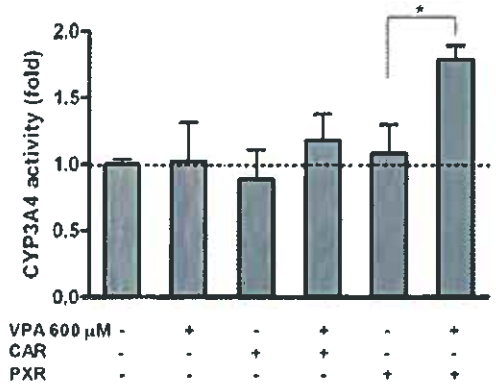
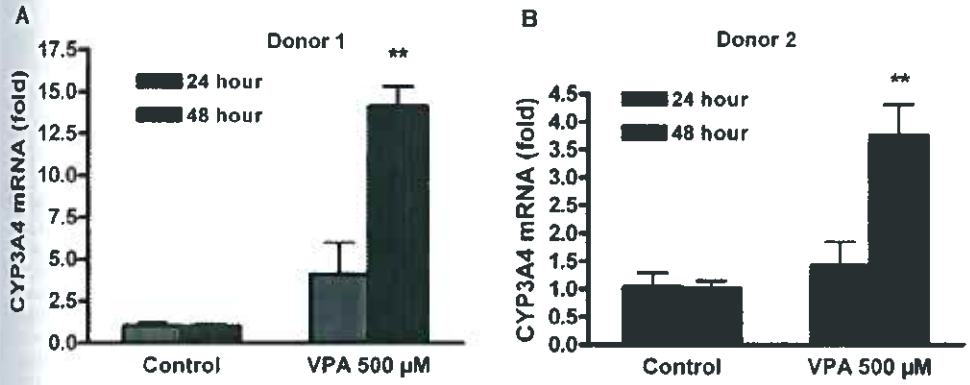


FIG. 6. Determination of CYP3A4 catalytic activity. LS174T cells were transfected with expression plasmids encoding CAR or PXR. Subsequently, cells were treated with VPA (600 μM) and specific CYP3A4-mediated testosterone 6β-hydroxylation activity was detected after 48 h. The concentration of the 6β-testosterone metabolite was determined using the HPLC system in cell lysate. Final activity was normalized to sample protein concentration. Bars indicate the means ± S.D. calculated from three samples. *, $p < 0.05$.

Discussion

VPA, an effective broad-spectrum anticonvulsant, has been shown to affect expression of a large number of genes (Bosetti et al., 2005). Some of these recognized alterations in gene expression have been attributed to HDAC inhibitory activity of VPA (Chen et al., 1999; Phiel et al., 2001; Werling et al., 2001; Eyal et al., 2006). In this study, we show for the first time (to our knowledge) that VPA is capable, in a clinically relevant range of concentrations <1000 μM (Davis et al., 1994; Wen et al., 2001; Centorrino et al., 2003; Allen et al., 2006), of up-regulating *CYP3A4* and *MDR1* gene expression also, by a different molecular mechanism, via direct activation of the CAR pathway. Moreover, we demonstrate that VPA can increase *CYP3A4* gene expression and activity through activation of PXR nuclear receptor as well.

Ligand-activated nuclear receptors CAR and PXR up-regulate expression of target genes at the transcriptional level through interaction with specific promoter response elements. Therefore, to examine the potential effect of VPA on CAR- and PXR-mediated transcriptional regulation of *CYP3A4* and *MDR1* genes, we first used transient transfection experiments with luciferase reporter constructs containing a relevant regulatory promoter sequence of tested genes (Goodwin et al., 1999, 2001, 2002; Burk et al., 2005a). Using transient transfection, we established that the reporter constructs appeared in cells separately from the chromosomal DNA and nucleosome structure, which guaranteed that the detected effect of VPA on activation of



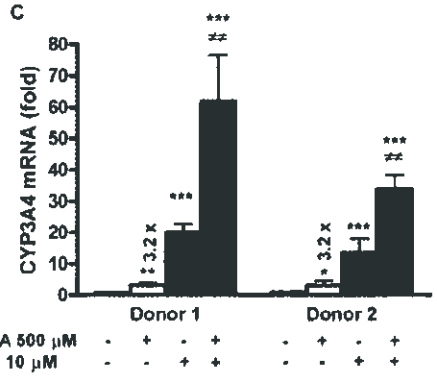


FIG. 7. VPA synergizes with rifampicin (Rif) in induction of CYP3A4 mRNA in primary human hepatocytes. Primary human hepatocyte cultures were cultivated together with VPA and/or rifampicin either individually or in combination, and then assayed using real-time RT-PCR. CYP3A4 mRNA expression is normalized to *HPRT* housekeeping gene and presented as -fold increase to control nontreated cells. Values represent the means ± S.D. of three independent wells. A and B, 48-h exposure of primary hepatocytes to VPA (500 μM) up-regulates CYP3A4 mRNA ($p < 0.01$). This effect of VPA was analyzed using unpaired Student's *t* test. C, rifampicin, VPA or a combination of these two drugs significantly up-regulates CYP3A4 mRNA to control (0.1% DMSO-treated cells) with *p* values of * $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (unpaired Student's *t* test) after 24 h of treatment. When given in combination, the induction is in both cases statistically synergistic rather than additive, at ≠≠, $p < 0.01$ (analyzed by two-way ANOVA with interaction).

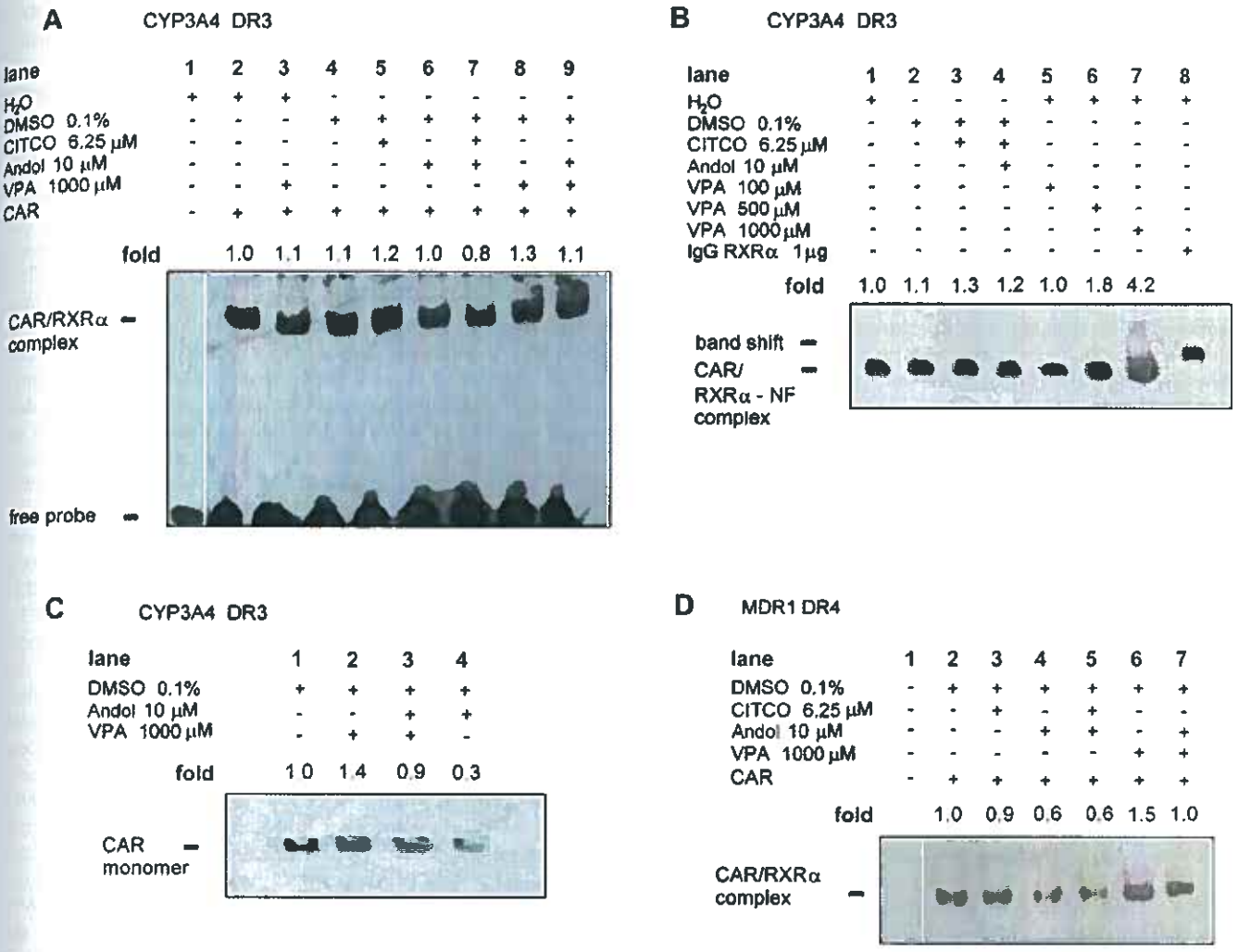


FIG. 8. The ability of CAR/RXRα heterodimer to bind CAR response elements of CYP3A4 (DR3, ER6) and MDR1 (DR4) in the presence of VPA was investigated using EMSA as described under *Materials and Methods*. A, the complex formation of CYP3A4 DR3 with CAR/RXRα was examined with in vitro translated wild-type CAR and RXRα proteins in the presence of solvent (water or DMSO 0.1% where indicated), the CAR agonist CITCO (6.25 μM; lanes 5 and 7), the inverse agonist of CAR, androstenol (Andol) (10 μM; lanes 6, 7, and 9), VPA (1000 μM; lanes 3, 8, and 9), or CITCO-androstenol and VPA-androstenol combinations (lanes 7 and 9). B, combined gel shift/supershift experiments were performed using in vitro translated wild-type CAR protein and the nuclear fraction of HepG2 cells transfected with RXRα expression vector. The concentration-dependent effect of VPA (100, 500, or 1000 μM; lanes 5–7) on CAR/RXRα complex was studied. The band supershift was performed with anti-RXRα antibody (1 μg; lane 8). C, effect of VPA on CAR monomer interaction with CYP3A4 DR3. The experiments were performed using in vitro translated CAR without RXRα and with VPA (1000 μM) and androstenol (10 μM) as an inverse agonist of CAR. D, the capability of VPA (1000 μM; lane 6) to affect CAR/RXRα heterodimer interaction with MDR1 DR4 response element was tested using in vitro translated wild-type CAR and RXRα proteins. CITCO (6.25 μM) and androstenol (10 μM) were used as a known ligand and inverse agonist of CAR, respectively. Representative gels are shown. Band signal intensity was quantitated by densitometry and data are expressed as -fold increase (fold) relative to their vehicle controls (DMSO or water).

luciferase constructs is independent of chromatin remodeling caused by VPA-mediated HDAC inhibition.

We demonstrate that VPA induces CAR- and PXR-mediated transactivation of *p3A4-luc* construct containing both DR3 and ER6 responsive elements of *CYP3A4* required for CAR- and PXR-mediated regulation of the gene (Goodwin et al., 1999) (Fig. 1). Interestingly, we found out that the combination of VPA with prototypic ligands causes far more pronounced transactivation of the *p3A4-luc* construct (Fig. 2). These data indicate a synergistic effect of VPA and rifampicin on transactivation of CYP3A4 via PXR, which could play an important role during cotreatment with these drugs.

In additional experiments, we examined activation of two different luciferase reporter constructs containing MDR1 enhancer and either native or viral SV40 basal promoter (Burk et al., 2005a). We did not detect any significant CAR- or PXR-dependent effect of VPA on activation of *MDR1* gene promoter in HepG2 cells transfected with *pMDR1-luc* reporter construct, although an increase in transcriptional activation of this reporter construct was observed in cells exposed to VPA (Fig. 3A). On the other hand, using *pMDR1E-SV40-luc* reporter construct containing the MDR1 enhancer upstream of the SV40 viral promoter, VPA was identified as an activator of the MDR1 enhancer via the CAR pathway (Fig. 3B). Thus, by using *pMDR1E-SV40-luc* construct, we eliminated the potential interference of additional transcriptional factors that bind relevant sites in the basal promoter of *MDR1* gene such as NF-Y, Sp1, AP-1, NK-κB, C/EBPβ, and so on (Scotto, 2003), and analyzed specific CAR-/PXR-mediated activation of the MDR1 enhancer by VPA. Moreover, the reporter plasmid lacks the inverted CCAAT motif (Y-box), which was shown to be essential for activation of MDR1 promoter in transient transfection reporter assays by several other HDAC inhibitors (Jin and Scotto, 1998). Despite this fact, significant activation of *pMDR1E-SV40-luc* reporter construct by VPA was observed also in the absence of cotransfected

CAR or PXR, which indicates that VPA can also activate MDR1 enhancer of *pMDR1E-SV40-luc* reporter construct (Fig. 3B). We suppose that endogenous CAR or PXR is not involved in the phenomenon because their functional expression in HepG2 cells is very low. However, we cannot exclude potential up-regulation of various transcriptional factors by VPA treatment, which could lead to transactivation of the reporter construct.

Subsequently, interaction of VPA with PXR transactivation pathway has been studied in more detail using another gene reporter construct, *pPBREM-SV40-luc*, containing two DR4-type motifs of the CYP2B6 PBREM region upstream of SV40 viral promoter, which were recently demonstrated to have a high affinity to both PXR and CAR (Faucette et al., 2007). These experiments have revealed significant CAR-mediated, but no PXR-mediated, activation of *pPBREM-SV40-luc* reporter construct by VPA, which is in agreement with the findings obtained using *pMDR1-SV40-luc* reporter construct. Thus, we suggest a different effect of VPA on CAR- and PXR-mediated transcriptional activation of heterologous reporter constructs *pPBREM-SV40-luc* and *pMDR1-SV40-luc* in comparison with *p3A4-luc* construct (Figs. 1, 3B, and 4). We hypothesize that this discrepancy can be explained considering the report of Masuyama et al. (2005), who demonstrated a ligand- and promoter-specific fashion of PXR-mediated transcription of *CYP3A4* and *MDR1* genes.

CAR- and PXR-mediated regulation of *CYP3A4* and *MDR1* gene expression was subsequently analyzed by real-time RT-PCR in samples of LS174T cells (Fig. 5, A and B). mRNA expression of the studied genes was normalized to both *HPRT* and *B2M* housekeeping genes to eliminate a false interpretation potentially caused by nonspecific VPA-mediated up-/down-regulation of these housekeeping genes. We observed the effect of coexpressed CAR and PXR on VPA-mediated up-regulation of CYP3A4 mRNA in LS174T cells (Fig. 5A), which well correlates with the reporter experiments. In the case of MDR1, the mRNA level was significantly up-regulated in LS174T cells transfected with CAR expression vector, whereas the VPA-mediated increase of MDR1 mRNA in PXR-expressing cells was not statistically different from that of nontransfected cells exposed to VPA (Fig. 5B). Considering data from gene reporter assay with *pMDR1E-SV40-luc* (Fig. 3A) and *pPBREM-SV40-luc* (Fig. 4), we assume that the observed influence of VPA on MDR1 mRNA expression in LS174T cells transfected with PXR should instead be attributed to HDAC inhibitory activity of VPA, which was previously demonstrated to cause *MDR1* gene up-regulation (Eyal et al., 2006). Thus, we hypothesize that the total induction of MDR1 mRNA expression in LS174T cells transfected with CAR and PXR might be a sum of several VPA effects such as inhibition of HDAC (Eyal et al., 2006) and interaction of VPA with CAR or PXR. On the other hand, we suppose that the effect of VPA on CYP3A4 mRNA up-regulation is mediated by a transcription factor(s), since HDAC inhibition did not result in induction of human CYP3A4 or its rat homolog Cyp3a2 mRNA (Rodríguez-Antona et al., 2003; Eyal et al., 2006). Nevertheless, we hypothesize that HDAC inhibitory activity of VPA synergistically augments the effect of rifampicin in PXR-mediated up-regulation of CYP3A4 mRNA in primary human hepatocytes (Fig. 7C).

The interaction of VPA with CAR was investigated in more detail using EMSA, in which interaction of VPA with CAR/RXRα heterodimer or CAR monomer (Frank et al., 2003) and responsive elements of both CYP3A4 and MDR1 promoters were examined. In the presence of VPA, we detected augmented binding of CAR/RXRα heterodimer to the DR3 responsive element of CYP3A4 and to the DR4 responsive element of MDR1 (Fig. 7, A–D). Binding of CAR/RXRα to the ER6 element of CYP3A4 was not affected by VPA (data not shown), which might correlate with low affinity of CAR/RXRα complex to bind the ER6 motif and with minor role of ER6 in CAR-mediated *CYP3A4* gene transactivation (Goodwin et al., 2002). Interestingly, we also observed increased binding of the CAR monomer to the DR3 motif of *CYP3A4* promoter in the presence of VPA (Fig. 7C, lane 2). Androstenol decreased the binding of CAR/RXRα heterodimer and CAR monomer to DR3 and CAR/RXRα heterodimer to DR4 in samples containing VPA (Fig. 7, A, C, and D), which provides us additional evidence that VPA transactivates CYP3A4 and MDR1 through CAR. Interestingly, we observed a difference in the VPA-activated CAR/RXRα heterodimer binding to the DR3 responsive element in EMSA experiments performed using recombinant CAR and RXRα proteins (Fig. 7A, lane 8) in comparison with the experiments in which recombinant RXRα was replaced with nuclear extract from HepG2 cells transfected with expression vector encoding RXRα (Fig. 7B, lane 7). Binding of CAR/RXRα complex with recombinant in vitro translated RXRα to the DR3 motif was only slightly augmented in the presence of VPA (~30%); however, using a nuclear fraction of HepG2 cells transfected with RXRα, we observed a more than 4-fold effect of VPA on the CAR/RXRα/DR3 complex formation (Fig. 7B, lane 7). Based on this finding, we hypothesize that the nuclear fraction of HepG2 cells could contain another transcription cofactor involved in regulation of CAR/RXRα binding to DR3 in the presence of VPA.

Finally, we analyzed CYP3A4 enzymatic activity in LS174T cells exposed to VPA (600 μM) for 48 h. As shown in Fig. 6, significant increase of CYP3A4 activity was detected only in VPA-treated LS174T cells transfected with PXR expression vector ($p < 0.05$). We did not detect any alteration of CYP3A4 enzymatic activity in nontransfected LS174T cells, although LS174T cells express PXR (our unpublished data; Burk et al., 2005b). With respect to this fact, we suppose that CYP3A4 catalytic activity can be increased only in cells rich in PXR protein.

In conclusion, we show that VPA mediates transactivation of CYP3A4 and MDR1 genes via direct interaction with CAR nuclear receptor. Furthermore, we present that VPA up-regulates CYP3A4 and its catalytic activity through PXR. With respect to the published data on the effect of VPA in regulation of *MDR1* gene expression, we suggest that interaction of VPA with the CAR signaling pathway together with VPA-mediated HDAC inhibition is the mechanism involved in up-regulation *MDR1* gene. Because MDR1 plays an important role in the multidrug resistance (MDR) phenomenon, we assume that administration of VPA could lead to an increase in tumor resistance against many anticancer drugs, which are transported by P-glycoprotein. Finally, our data indicate that VPA in clinically relevant concentrations during treatment of schizoaffective disorders, acute mania, and refractory epilepsy, when VPA plasma concentrations range within 500 to 1000 μM, could cause drug-drug interactions with coadministered drugs that are metabolized by CYP3A4.

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4. Examination of Glucocorticoid Receptor alpha-Mediated Transcriptional Regulation of P-glycoprotein, CYP3A4, and CYP2C9 Genes in Placental Trophoblast Cell Lines.

Pávek P, **Červený L**, Švecová L, Brysch M, Libra A, Vrzal R, Nachtigal P, Štaud F, Ulrichová J, Fendrich Z and Dvořák Z (2007) Examination of glucocorticoid receptor alpha-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines. *Placenta* **28:**1004-1011. [IF=2.8]

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Examination of Glucocorticoid Receptor α-Mediated Transcriptional Regulation of P-glycoprotein, *CYP3A4*, and *CYP2C9* Genes in Placental Trophoblast Cell Lines

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Abstract

The placental trophoblast at different stages of pregnancy contains some drug transporters and xenobiotic-metabolising enzymes, as well as ligand-activated nuclear receptors, which control their inducible transcriptional regulation. Glucocorticoid receptor α (GRα) is expressed in both placental syncytiotrophoblast and cytotrophoblast. GRα was shown to control inducible expression of several enzymes of the cytochrome P-450 family (CYP) and the drug transporter P-glycoprotein in the liver. However, GRα-mediated transcriptional regulation of drug transporters and CYPs has not been studied in the placental trophoblast. In this study, we examined the expression and activity of GRα in the transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 in placental trophoblast cell lines. Employing RT–PCR, Western blotting, and luciferase gene reporter assay, we detected the expression and activity of GRα in JEG3 and BeWo cell lines. However, we observed that only *MDR1* mRNA was up-regulated after treatment of placental cells with dexamethasone. Accordingly, only the promoter of the *MDR1* gene was activated by dexamethasone in gene reporter assays in placental cells and the activation was abolished by RU486, an antagonist of GRα. CYP3A4 and CYP2C9 promoters were activated in placental cells only after co-transfection with hepatocyte nuclear factor 4α (HNF4α), which indicates the hepatocyte-specific character of GRα-mediated regulation of the genes. On the other hand, coexpression of HNF4α had no effect on the activation of the *MDR1* gene promoter, suggesting HNF4α-independent regulation via GRα. We conclude that GRα may be involved in the transcriptional regulation of P-glycoprotein in the placental trophoblast. We also indicate that the *CYP3A4* and *CYP2C9* genes are not inducible through GRα in placental cell lines, due to the lack of HNF4α expression and possibly some additional hepatocyte-specific transcriptional factors.
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Keywords: Glucocorticoid receptor; Transcriptional regulation; P-glycoprotein; CYP3A4; Placenta; Cell lines; Placental barrier

1. Introduction

Throughout pregnancy, the placental trophoblast contains some drug transporters and enzymes of both phase I and II of biotransformation [1–3]. Several enzymes of the cytochrome P-450 family (CYP) such as CYP1A1, CYP2C, 2E1, 3A4-7, and 4B1 are expressed in the placental trophoblast at different stages of pregnancy, although only few of them have the functional catalytic activity to metabolise xenobiotics [1–5]. P-glycoprotein (P-gp), a membrane efflux transporter encoded by the *ABCB1* gene (*MDR1*), is highly expressed in the placental syncytiotrophoblast at different stages of pregnancy [6–8]. P-gp contributes to both xenobiotic [9–14] and glucocorticoid [14] barriers in the chorioallantoic placenta. CYP3A4 and CYP2C9 are major CYP enzymes involved in the hepatic metabolism of xenobiotics and the biotransformation of steroid hormones such as estrogens and testosterone [1].

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Significant progress has been made over the past few years in unravelling hormone/xenobiotics-mediated induction mechanisms through nuclear receptors and transcriptional factors for most of the CYPs and drug transporters in the liver [15]. However, little attention was paid to the study of the induction processes in the placental trophoblast [2,3,13,16].

Glucocorticoid receptor α (GRα) is a ubiquitous nuclear receptor, which transcriptionally regulates numerous genes through its binding to GRα response elements (GREs) in promoter regions of target genes. Expression of GRα was detected both in the syncytiotrophoblast and cytotrophoblast of human [17] and rodent placentas [18]; however, GRα-mediated transcriptional regulation of any drug transporter or GRα-regulated enzyme of the CYP family has not yet been systematically studied in the placental trophoblast [16].

The objectives of the study were to examine the expression and activity of GRα in two placental trophoblast cell lines in the transcriptional regulation of the *MDR1*, *CYP3A4*, and *CYP2C9* genes. These major genes involved in xenobiotic and steroid hormone disposition are well characterised as GRα-regulated genes in hepatocytes [19–23]. Herein, we hypothesised that GRα could up-regulate these genes at the transcriptional level in the placenta. In addition, we examined whether the placental absence of hepatocyte nuclear factor 4α (HNF4α, NR2A1) is a limitation of the GRα-mediated transcriptional regulation of the tested genes. The hepatocyte-specific transcriptional factor has been recently identified as the critical cofactor in the pregnane X receptor (*h*PXR, SXR)- and constitutive androstane receptor (CAR)-mediated transcriptional up-regulation of CYP3A4 and CYP2C9 in hepatic cells [24,25]. However, the mechanisms of GRα-mediated regulation in the placental trophoblast or other extrahepatic cells and the contribution of HNF4α to the GRα-mediated transactivation of these CYPs and P-gp have not been investigated so far.

2. Methods

2.1. Chemicals

Dexamethasone, mifepristone (RU486), forskolin, charcoal, and cell culture media were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Plasmids

In order to evaluate the comprehensively transcriptional activation of promoters for the tested genes in placental cell lines, we used luciferase reporter constructs with full-range basal and enhancer promoter sequences of *CYP3A4*, *MDR1*, and *CYP2C9* genes [24–27]. A chimeric *p3A4-luc* luciferase reporter plasmid containing the basal promoter (−362/+53) and the distal xenobiotic-responsive enhancer module (XREM) (−7836/−7208) of the *CYP3A4* gene 5′-flanking region was described by Goodwin et al. [26]. The plasmid contains an HNF4α binding site at −7783 to −7771 [24]. The reporter plasmid p-7975(Δ7012–1804) of the *MDR1* gene (*pMDR1-luc*) has been described before [27]. The reporter plasmid p2088 (−2088/+21)-luc (herein called *p2C9-luc*) was kindly provided by Dr. Gerbal-Chaloin (INSERM U672, Montpellier, France) [21]. Constructed plasmids were sequenced using a Big Dye Terminator Cycle Sequencing method (Applied Biosystems, Rockville, MD, USA). *pGRE-luc* plasmid harbouring four copies of the consensus GRE upstream of TATA box was purchased from Stratagene (La Jolla, CA, USA). The expression plasmid encoding human GRα (pSG5-hGRα) was a generous gift from Dr. J. Palvimo (University of Helsinki, Helsinki, Finland). pcDNA3-HNF4α2 expression plasmid was kindly donated by Dr. B. Laine (INSERM Unit 459, Lille, France). A chimaera mouse GRα-GFP expression plasmid was a kind gift from Dr. W.B. Pratt [28].

2.3. Cell lines and transient transfection assays

The human choriocarcinoma cytotrophoblast cell line JEG-3 was cultured in MEM medium supplemented with 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate, and 10% FBS (PAA, Pasching, Austria). BeWo, a trophoblast cell line derived from human choriocarcinoma, was maintained in Ham's F12 medium supplemented with 10% FBS. HepG2 human Caucasian hepatocyte carcinoma cells were cultured in DMEM medium supplemented with 10% FBS and 1% NEAA. All cell lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and were used within 25 passages after delivery. Cell lines were cultivated without antibiotics and experiments were performed in phenol red-free media (Invitrogen/Gibco, Carlsbad, CA, USA) with charcoal/dextran-stripped FBS. In order to promote fusion of cytotrophoblast BeWo cells into the syncytiotrophoblast, BeWo cells were treated in separate experiments with 100 μM forskolin for 48 h [29].

Cells were seeded (7×10^4 per well for BeWo and JEG3; 2×10^5 per well for HepG2) in 48-well plates and transfected 24 h later with jetPEI transfection reagent (Polyplus-Transfection, Illkirch, France). Usually, 0.5 μg of a reporter plasmid, 30 ng of pRL-TK encoding renilla luciferase (Promega, Madison, WI, USA), 50 ng of an expression plasmid and/or appropriate empty expression vector (to normalise DNA/transfection reagent ratio) were used for the transfection of cells in one well. After 24 h preincubation in media with 10% charcoal/dextran-stripped FBS, the medium was replaced and tested compounds or DMSO (vehicle, 0.1%) were added to the cells. The cells were maintained for a further 24 or 48 h in the presence of tested compounds in medium supplemented with 10% charcoal/dextran-stripped FBS. Dual luciferase assays were performed according to manufacturer's instructions (Promega) using a Tecan luminometer (Tecan Group, Mannedorf, Switzerland).

2.4. Isolation of total RNA and quantitative real-time RT–PCR analysis

Total RNA isolation, reverse transcription and *TaqMan* RT–PCR were performed as described previously [30]. RT–PCR with SYBRGreen chemistry was performed as described [31] under conditions common for all genes (annealing temperature of 60 °C, 3 mM $MgCl_2$). Primers and probes designed to bridge exon–exon junctions are specified in Table 1. Pfaffl's method was applied for the relative quantification of gene expression normalised to endogenous control (housekeeping) genes [32]. The delta–delta method with validated PCR efficiency for both genes was used for comparison of GRα and HNF4α expression in different cell lines and tissues [32].

2.5. Human cytotrophoblast isolation and preparation of tissue samples

Human tissue samples were obtained according to protocols approved by the Ethics Committees of the Charles University in Prague and the Palacky University in Olomouc, complying with current Czech legislation.

The method described by Kliman et al. [33] was used for the preparation of isolated cytotrophoblast from term human placenta.

For the preparation of pooled human liver samples of total RNA and whole-cell protein fraction, 300-mg pieces of frozen (−70 °C) liver tissue from five Caucasian male donors were pooled. Similarly, the pooled placental sample was prepared from five term placentas of Caucasian non-smoking women. Tissue samples were either homogenised for total RNA isolation with Trizol® reagent according to the manufacturer's instructions (Invitrogen) with a pestle homogeniser or homogenised with a pestle homogeniser in ice-cold RIPA buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× Roche inhibitor cocktail, pH 7.4). Whole-cell lysate was spun down at 10,000 × *g* for 10 min at 4 °C and the supernatant was stored

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Table 1
Sequences of primers and probes used for RT–PCR

Gene	Primers and probes
GRα	Forward 5′-AAACCTTACTGCTTCTCTCTTCA-3′
	Reverse 5′-GTTAAGGAGATTTTCAACCACTTC-3′
HNF4α	Forward 5′-GGGTGTCCATACGCATCCTT-3′
	Reverse 5′-GCGGTCGTTGATGTAGTCCT-3′
CYP3A4	Forward 5′-TTCAGCAAGAAGAACAAGGACAA-3′
	Reverse 5′-GGTTGAAGAAGTCCTCCTAAGC-3′
CYP2C9	Forward 5′-GGACATGAACAACCCTCAGG-3′
	Reverse 5′-TGCTTGTCGTCTCTGTCCCA-3′
	Probe TAMRA[a] 5′-AAAACACTGCAGTTGACTTGTTTGGAGC-3′ BHQ1[b]
MDR1	Forward 5′-TGCTCAGACAGGATGTGAGTTG-3′
	Reverse 5′-AATTACAGCAAGCCTGGAACC-3′
	TaqMan probe TAMRA 5′-TAACTTGAGCAGCATCATTGGCGAGCCT-3′ BHQ1
β2-microglobulin (B2M)	Forward primer: 5′-CGTGTGAACCATGTGACTTTGTC-3′
	Reverse primer: 5′-CATCTTCAAACCTCCATGATGC-3′
Hypoxanthine-guanine phosphoribosyl transferase (HPRT)	Forward 5′-CTGGAAAGAATGTCTTGATTGTGG-3′
	Reverse 5′-TTTGGATTATACTGCCTGACCAAG-3′
	TaqMan probe TAMRA 5′-AATTGACACTGGCAAAACAATGCAGACTTTG-3′ BHQ1

[a] TAMRA, tetramethylrhodamine.
[b] BHQ1, black hole quencher 1.

at −70 °C. Homogenisation and RNA isolation from cells was performed with Trizol reagent according to the manufacturer's instructions.

2.6. Western blots

Immunodetection of GRα in total cellular lysates was performed using the protocol described previously [30]. Blots were probed with the primary rabbit antibody GR(E-20)X (sc-1003) at a dilution 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Chemiluminescence detection was performed using an HRP-conjugated secondary antibody and an ECL kit (GE Healthcare, Little Chalfont, UK).

2.7. Microscopy

Cells plated in 6- or 12-well plates were transfected with the GR-GFP fusion expression plasmid (320 ng DNA/cm^2) encoding a chimaera of the green fluorescence protein (GFP) and GR [28]. GFP fluorescence was visualised from the same living cells on a Nikon Eclipse microscope with a FITC filter 24 h after transfection in the presence or absence of 1 μM dexamethasone in medium.

2.8. Statistical analyses

All bars indicate the means ± standard deviations (SD). One- or two-way ANOVA with Bonferroni or Dunnett's tests was used for data analysis, where appropriate.

3. Results

3.1. Characterisation of expression and activity of GRα in placental JEG3 and BeWo cell lines in comparison with hepatoma HepG2 cell line

Employing RT–PCR, we detected the highest expression of GRα mRNA in HepG2 cells and in the pooled placental tissue sample; expression of GRα mRNA in additional samples decreased in the following order: liver > JEG3 > isolated cytotrophoblast > BeWo (Fig. 1A). HNF4α mRNA levels were below the detection limit in placental samples or placental cell lines, but was abundant in HepG2 cells (Fig. 1A).

In Western blot experiments, we detected the highest level of GRα protein in HepG2, pooled liver, and placental samples; levels of GRα protein in JEG3 and BeWo cells were lower (Fig. 1B).

In order to assess GRα activity in BeWo and JEG3 human choriocarcinoma trophoblast cell lines, we examined the capability of endogenous GRα to transactivate pGRE-luc reporter plasmid transiently transfected into cells that were later exposed to dexamethasone (100 nM) for 24 h. Dexamethasone dramatically activated the reporter construct in JEG3 and HepG2 cells (22- and 45-fold respectively), which indicates high activity of endogenous GRα in these cell lines (Fig. 1C). On the other hand, treatment of BeWo cells with dexamethasone resulted in weak (about 2-fold, $p < 0.05$) activation of pGRE-luc (Fig. 1C) and forskolin (100 μM) had no statistically significant effect on the activation (Fig. 1C). Surprisingly, we observed a dramatic increase ($p < 0.001$) in the activation of pGRE-luc in BeWo and JEG3 cells co-transfected with a GRα expression vector in the absence of dexamethasone in media (Fig. 1C). In order to explain this phenomenon we used RU486, which is a potent antagonist of GRα. We observed that RU486 significantly repressed the activation of pGRE-luc in BeWo and JEG3 cells co-transfected with GRα ($p < 0.001$; Fig. 1C), which may document the activation of GRα by a putative intracellular ligand in BeWo and JEG3 cells.

In order to support the hypothesis, we used GR-GFP expression plasmid encoding the fusion protein of GFP and mouse GR. In the case of JEG3 and BeWo cells cultured without dexamethasone, fluorescence of the GR-GFP chimaera protein was localised mostly in the nucleus, which indicates ligand-mediated translocation of GR-GFP to the nucleus (Fig. 1D). In HepG2 cells, we observed comparable GR-GFP fluorescence in both the cytoplasm and the nucleus and fast translocation of the chimaera to the nucleus after addition of 1 μM dexamethasone to

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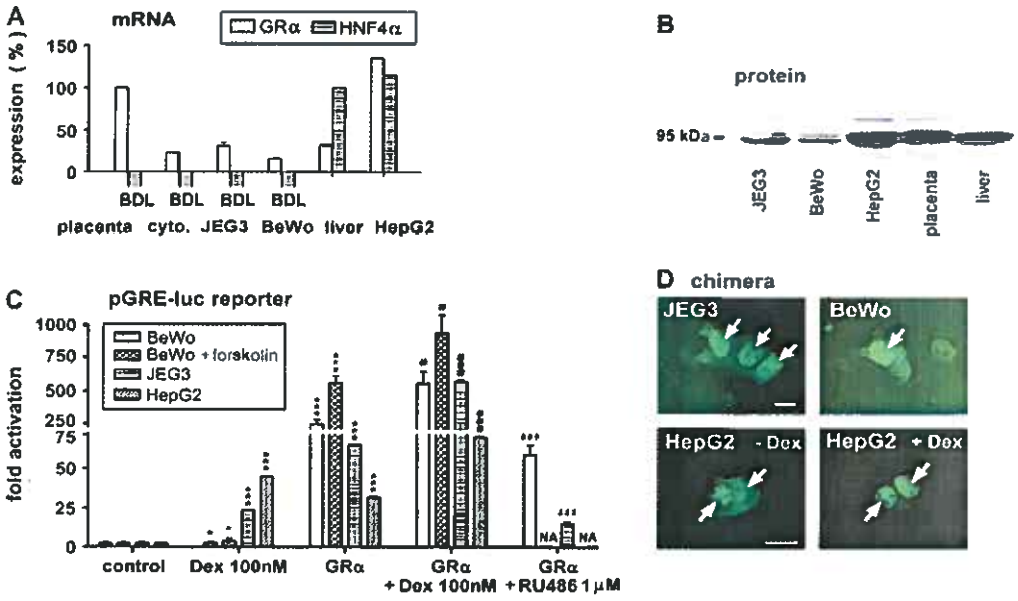

Fig. 1. Activity and expression of GRα in the placenta, isolated cytotrophoblasts and placental BeWo and JEG3 cell lines in comparison with the liver and HepG2 cell lines. (A) Relative expression of GRα and HNF4α mRNAs analysed by RT–PCR. Expression of GRα was related to the pooled placental sample (100%) and expression of HNF4α to the liver sample. Cyto, isolated cytotrophoblasts; BDL, below detection limit. (B) Western blot analysis of GRα protein. GRα was analysed in the total cellular lysates using primary rabbit antibody as described in the section Methods. (C) Transactivation of pGRE-luc reporter plasmid by dexamethasone in placental BeWo, JEG3, and HepG2 cell lines. The reporter plasmid containing four copies of the consensus GRE sequence was co-transfected (500 ng) into cells along with 50 ng of GRα expression vector pSG5-hGRα or an empty expression vector and renilla luciferase expression vector pRL-TK for transfection normalisation. Cells were treated with 100 nM dexamethasone (Dex) for 24 h. Cell lysates were analysed for firefly luciferase activity normalised to renilla luciferase. Relative activation of the reporter construct is presented as -fold activation relative to control (vehicle-treated cells). Bars show the means ± SD of a representative experiment performed in triplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$: statistically different from control, or $^{\#\#}p < 0.05$, $^{\#\#\#}p < 0.001$ from GRα-transfected cells. RU486 significantly repressed activation of pGRE-luc in cells co-transfected with pSG5-hGRα, $^{\neq\neq\neq}p < 0.001$ (ANOVA with Bonferroni); NA, not analysed. (D) Expression of chimaeric GR-GFP protein. Cells were transfected with the chimaera GR-GFP expression vector as described in Methods. Fluorescence was visualised in living cells 24 h after transfection employing fluorescent microscopy. The same HepG2 cells were photographed before (−Dex) and after 15 min of incubation in the presence of 1 μM dexamethasone (+Dex) (lower panels). Arrows indicate nuclei. Bars represent 10 μm.

the medium (Fig. 1D). Thus, we suppose that the nuclear localisation of the chimaera and activation of pGRE-luc in the absence of dexamethasone likely indicate the intracellular presence of (a) GRα ligand(s) in the trophoblast cell lines studied.

3.2. Effect of dexamethasone on expression of MDR1, CYP3A4, and CYP2C9 mRNAs and activation of their promoters in JEG3 cells

As shown in Fig. 2A, *MDR1* mRNA was modestly, but significantly ($p < 0.05$) up-regulated by dexamethasone in JEG3 cells. Contrary to *MDR1*, we observed down-regulation of CYP2C9 mRNA related to the HPRT and B2M housekeeping genes (Fig. 2A). Expression of CYP3A4 mRNA was not changed significantly after treatment of JEG3 cells with indicated concentrations of dexamethasone (Fig. 2A).

In other experiments, we observed statistically significant ($p < 0.05$) activation of pMDR1-luc reporter plasmid in placental JEG3 cells after 24- or 48-h exposure to dexamethasone (Figs. 2B, 3, 4). We did not detect any significant activation of either p2C9-luc or p3A4-luc reporter constructs in JEG3 cells after treatment with dexamethasone for 24 or 48 h (Figs. 2B, 3, 48-h data not shown).

3.3. Involvement of GRα and HNF4α in transcriptional activation of MDR1, CYP3A4, and CYP2C9 promoters in transient transfection assays

In the next experiments, we tested whether GRα is involved in the transcriptional activation of the *MDR1* promoter. In parallel, we examined whether HNF4α is essential for GRα-mediated transactivation of the *MDR1*, *CYP2C9*, and *CYP3A4* genes. Therefore, luciferase reporter constructs were co-transfected into JEG3 cells together with GRα and HNF4α expression plasmids either individually or in combination. As shown in Fig. 3, dexamethasone (100 nM) significantly up-regulated pMDR1-luc luciferase activity in JEG3 cells co-transfected with GRα in comparison with control cells (3.5-fold activation, $p < 0.05$). Co-transfection with GRα expression plasmid in the absence of dexamethasone also yielded statistically significant ($p < 0.05$) activation of the *MDR1* promoter (Figs. 3 and 4). On the other hand, expression of HNF4α in JEG3 cells had no effect on the activation of the pMDR1-luc construct (Fig. 3).

Next, we examined the transcriptional activation of the CYP2C9 promoter using p2C9-luc luciferase reporter construct in JEG3 cells co-transfected with GRα and/or HNF4α. Dexamethasone significantly up-regulated p2C9-luc reporter

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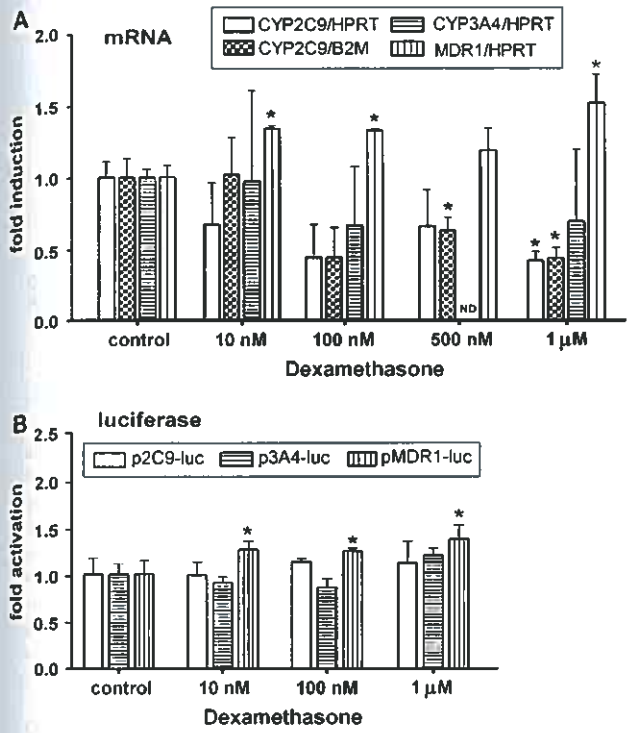

Fig. 2. Effect of dexamethasone on expression of *MDR1*, CYP2C9, and CYP3A4 mRNAs and transactivation of their luciferase reporter vectors in JEG3 cells. (A) RT–PCR analysis of mRNA expression of tested genes in JEG3 cells. Twenty-four hours after seeding on plates, cells were exposed to dexamethasone at the concentrations indicated for 48 h. The relative expression of *MDR1*, CYP2C9, and CYP3A4 mRNAs was normalised to the expression of either *HPRT* or *B2M* housekeeping genes. Data are plotted as fold induction relative to control. Values are derived from at least three independent experiments ($n \geq 3$) and are presented as the means ± SD. (B) Activation of luciferase reporter vectors with responsive promoter regions of *MDR1*, *CYP3A4*, and *CYP2C9* genes by dexamethasone in JEG3 cell lines. Twenty-four hours after transfection with luciferase reporter plasmids, cells were exposed to dexamethasone at the concentrations indicated for a further 24 h before determination of firefly and renilla luciferase activities. Data of three independent experiments are plotted as fold activation relative to control (vehicle-treated cells). $^{*}p < 0.05$: significantly different from control (ANOVA with Dunnett's test). ND, not determined.

luciferase activity in JEG3 cells co-transfected with a combination of HNF4α and GRα (16-fold increase, $p < 0.05$). In addition, p2C9-luc was activated by dexamethasone in JEG3 cells co-transfected individually with HNF4α (1.8-fold, $p < 0.05$; Fig. 3). We did not observe any statistically significant activation of the p2C9-luc construct in JEG3 cells co-transfected with the GRα expression vector alone and treated with dexamethasone.

In the case of CYP3A4, we observed a 1.9-fold ($p < 0.05$) activation of p3A4-luc reporter in JEG3 cells co-transfected simultaneously with both GRα and HNF4α expression plasmids and treated with dexamethasone (Fig. 3). Individual expression of either GRα or HNF4α and treatment with dexamethasone had no significant effect on the activation of p3A4-luc in JEG3 cells (Fig. 3). Response to dexamethasone treatment and co-transfection with expression plasmids differed significantly among the reporter plasmids studied (two-way ANOVA, $p < 0.001$). In HepG2 cells, we observed a slight (2.0-fold;

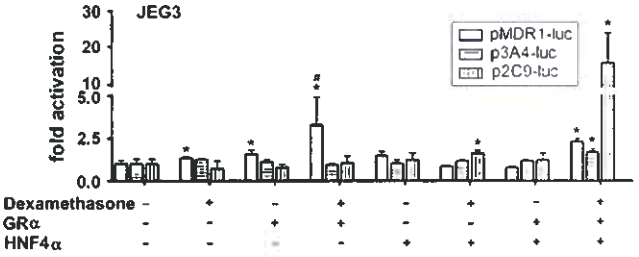

Fig. 3. Involvement of HNF4α in GRα-mediated activation of pMDR1-luc, p2C9-luc, and p3A4-luc reporter constructs in JEG3 cells. Reporter constructs were transiently transfected into JEG3 cells along with expression vectors for GRα and HNF4α either individually or in combination. Co-transfections were performed with 50 ng of each expression plasmid per well and/or with an empty expression vector to retain the DNA/transfection reagent ratio. Cells were treated with 100 nM dexamethasone for 24 h before determination of firefly and renilla luciferase activities. Data are shown as the means ± SD of three independent experiments and plotted as fold activation relative to control (DMSO-treated cells). $^{*}p < 0.05$: statistically different from control (ANOVA with Bonferroni). Activation of pMDR1-luc in GRα-transfected cells is significantly augmented by dexamethasone, $^{\#}p < 0.05$ (ANOVA with Bonferroni).

$p < 0.05$) activation of p3A4-luc reporter construct co-transfected with a GRα expression vector into cells exposed to dexamethasone and a 2.2-fold activation in cells simultaneously co-transfected with both GRα and HNF4α and treated with dexamethasone (data not shown). Empty reporter vectors pGL3-Basic and pRL-TK were not significantly affected by GRα and/or HNF4α and treatment with dexamethasone in our experiments.

3.4. HNF4α-independent activation of pMDR1-luc via GRα in placental JEG3 and HepG2 cell lines

To further demonstrate the role of GRα in the transactivation of *MDR1* promoter in both placental and HepG2 cells, we used RU486, a potent antagonist of the GRα receptor. As expected, RU486 efficiently abrogated the dexamethasone-induced activation of pMDR1-luc (Fig. 4). In addition, transfection of cells with a GRα expression vector also resulted in significant ($p < 0.05$) activation of pMDR1-luc in both JEG3 and HepG2 cells (Fig. 4).

Surprisingly, dexamethasone did not significantly activate pMDR1-luc in HepG2 cells, although the cell line expresses abundant endogenous GRα. This would indicate that the level of endogenous GRα is not sufficient to transactivate pMDR1-luc in HepG2 cells in relation to the balance of other transcriptional factors that probably suppress GRα-mediated activation of the construct in HepG2 cells. Alternatively, we can speculate that an efflux transporter such as P-gp restricts the entry of dexamethasone into HepG2 cells. Consistently, two-way ANOVA analysis confirms that profiles of response in JEG3 and HepG2 cell lines differ significantly ($p < 0.01$).

3.5. Activation of CYP2C9 promoter in placental and HepG2 cells

In order to implicate the absence of placental HNF4α expression as the cause of CYP2C9 non-responsiveness to

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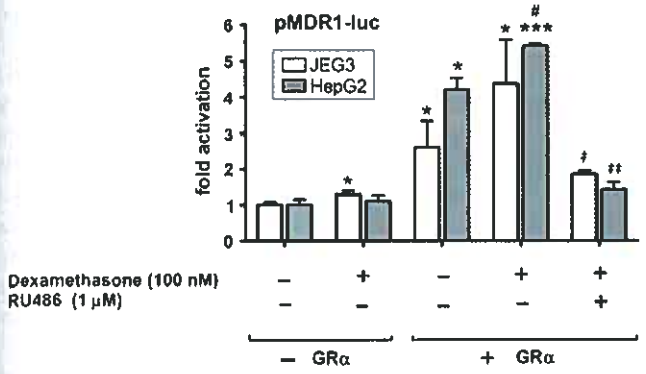

Fig. 4. GRα-mediated activation of MDR1 promoter in JEG3 and HepG2 cell lines. Cells were co-transfected with pMDR1-luc reporter construct (500 ng) and pSG5-hGRα (or empty pSG5) expression vector (50 ng). After 24 h of cultivation, cells were exposed to 100 nM dexamethasone (Dex) or a combination of dexamethasone (100 nM) and RU486 (1 μM) for 48 h before determination of firefly and renilla luciferase activities. Data are presented as the means ± SD of three independent experiments and plotted as fold activation relative to control. $^{*}p < 0.05$ and $^{***}p < 0.001$: statistically different from control: $^{\#}p < 0.05$: statistically different from GRα-transfected cells; RU486 significantly decreased activation of pMDR1-luc by dexamethasone in GRα-transfected cells at $^{\ddagger}p < 0.05$ and $^{\ddagger\ddagger}p < 0.01$ (ANOVA with Bonferroni).

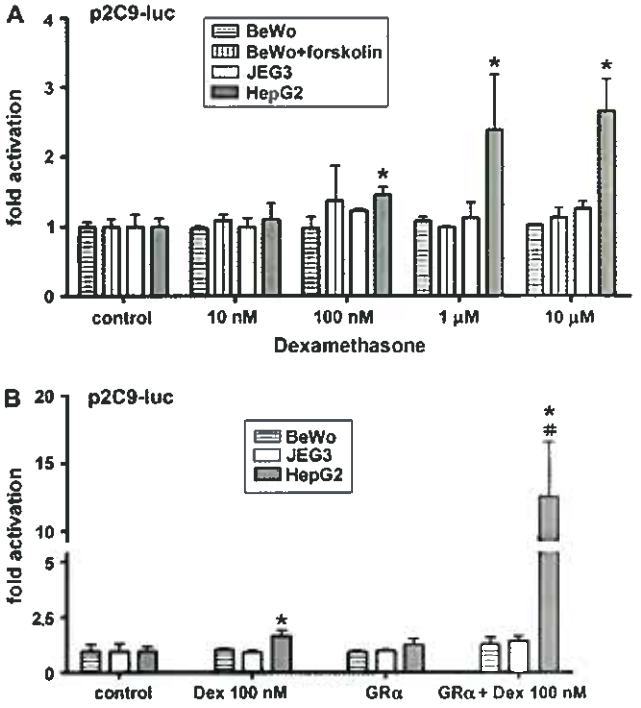

Fig. 5. GRα-mediated activation of p2C9-luc reporter plasmid in placental and HepG2 cell lines. (A) Dose-dependent activation of p2C9-luc reporter plasmid in placental and HepG2 cell lines by dexamethasone. Twenty-four hours after transfection of p2C9-luc into cells, dexamethasone was added to medium at the indicated concentrations for 24 h. Forskolin (100 μM) was used to promote differentiation of BeWo cells to syncytium, $^{*}p < 0.05$ (ANOVA with Dunnett's test). (B) GRα-mediated transactivation of the p2C9-luc reporter construct in placental cell lines in comparison with HepG2 cell lines. Cells were co-transfected with the p2C9-luc reporter (500 ng) and pSG5-hGRα expression vector (50 ng). After 24 h of cultivation, cells were exposed to 100 nM dexamethasone (Dex) for 24 h before determination of firefly and renilla luciferase activities. Data of three independent experiments ($n = 3$) are presented as the means ± SD. $^{*}p < 0.05$: statistically different from control (DMSO 0.1%); $^{\#}p < 0.05$: statistically different from GRα-transfected cells (ANOVA with Bonferroni).

dexamethasone (Fig. 3), we performed comparative experiments with p2C9-luc in hepatoma HepG2 (HNF4α expressing) versus placental BeWo and JEG3 (HNF4α lacking) cells. We observed that p2C9-luc was significantly activated by dexamethasone at concentrations of 100 nM and was higher in HepG2 cells, but not in placental cell lines (Fig. 5A).

In additional experiments, the p2C9-luc reporter construct was co-transfected into both JEG3 and HepG2 cells with a GRα expression vector. Fig. 5B shows that the p2C9-luc reporter construct was significantly activated ($p < 0.05$) by dexamethasone in the presence of overexpressed GRα in HepG2 cells. By contrast, we did not considerably activate the reporter construct by dexamethasone in any placental cells co-transfected with GRα (Fig. 5B).

In parallel experiments, we did not detect any significant effect of syncytialisation of BeWo cells after treatment with forskolin (100 μM) on the activation of either pGRE-luc (Fig. 1C) or p2C9-luc (Fig. 5A) plasmids in BeWo cells.

4. Discussion

Understanding placental transfer and metabolism of xenobiotics has significant clinical relevance for both maternal and fetal welfare. Drug transporters and xenobiotic-metabolising enzymes are important factors that determine placental transfer of some drugs, toxins, and endogenous steroid hormones across the placenta [1–3]. However, little is known about their transcriptional regulation via steroid hormones/xenobiotic-activated transcriptional factors in the placenta.

In the present study, we demonstrate high activity of the GRα pathway and up-regulation of the major placental drug transporter P-gp via GRα in trophoblast-derived placental cells. We also show the distinct lack of GRα-mediated transcriptional regulation for CYP2C9 and CYP3A4 in placental versus hepatic cells, consistent with the crucial role of HNF4α in the hepatic GRα-mediated transcriptional regulation of the genes.

Our *in vitro* data also indicate that GRα is localised in nuclei of placental trophoblast cell lines and activates pGRE-luc in the absence of dexamethasone in media. Accordingly, Lee and coworkers described abundant nuclear localisation of serine-211 phosphorylated GRα, a ligand-activated form of GRα, in the placental trophoblast [17]. We hypothesise that this phenomenon might be caused by endogenous steroid intermediates synthesised in the placental trophoblast. Nevertheless, we cannot rule out a residual effect of steroid hormones from cultivation media in placental cell lines in our experiments. In addition, we show that fusion of BeWo cells into differentiated syncytium should not have any significant effect on GRα signalling in the cell line (Fig. 1C) [28].

Recently, we have demonstrated the function of rat P-gp orthologues in both maternal-fetal and fetal-maternal

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pharmacokinetics of their substrates across the rat placenta and regulation of their expression within pregnancy [11,12,31]. However, transcriptional regulation of P-gp through GRα has not been systematically studied so far in the placental trophoblast. We show herein activation of the *MDR1* reporter construct and induction of *MDR1* mRNA in placental JEG3 cells treated with dexamethasone and/or transfected with a GRα expression vector (Figs. 2–4). RU486, a prototypical GRα antagonist, abolished GRα-mediated activation of the *MDR1* reporter construct both in JEG3 and HepG2 cells (Fig. 4). Our data thus demonstrate for the first time that *MDR1* is regulated through GRα at the transcriptional level. Nevertheless, the observed activation of the *MDR1* promoter was modest (about 5-fold) even in GRα-overexpressing cells, which would indicate that other factor(s) would likely be involved in the maximal activation of *MDR1* promoter. Since no functional GRE sequence has been described in the human *MDR1* promoter so far, we can only speculate about an indirect regulation of the *MDR1* gene by glucocorticoids via a basal transcriptional factor [23].

Of note, there is negative correlation between the rising level of cortisol in maternal blood [34] and increasing expression of placental GRα throughout pregnancy [35] in comparison with diminishing expression of P-gp in the placental trophoblast toward term [6–8], suggesting that GRα is unlikely to play a major role in the basal expression of placental P-gp during pregnancy. However, the current study indicates that regulation of the *MDR1* gene may be relevant by exogenous glucocorticoids antenatally administered to pregnant women in the treatment of a range of disorders [36,37] resulting in elevated maternal blood glucocorticoid activity.

In co-transfection experiments with GRα and HNF4α expression vectors, we show that the *MDR1* promoter is activated via GRα independently on HNF4α (Figs. 3 and 4). Thus, we can speculate that *MDR1* might also be inducible by glucocorticoids in extrahepatic tissues with a low expression of HNF4α.

In additional experiments, we focused on the GRα-mediated regulation of CYP3A4 in placental cell lines. Several mechanisms of GRα-mediated transcriptional regulation have been proposed for the principal CYP enzyme in hepatic cells [19,22,38]. We detected only minor activation of the CYP3A4 promoter in JEG3 cell lines exposed to dexamethasone and co-transfected with a combination of GRα and HNF4α (Fig. 3). Thus, our data are consistent with a role of HNF4α and other hepatocyte-specific transcriptional factor(s) in the GRα-mediated regulation of CYP3A4 in the liver.

CYP2C9 is dominantly expressed in the adult human liver; expression in the first term placenta was detected only at the level of mRNA [5]. CYP2C9 is transcriptionally up-regulated by the functional GRE in hepatocytes [21]. Recently, Chen and co-workers suggested that HNF4α is not required for the GRα-mediated activation of the CYP2C9 promoter [25]. In our experiments, however, the CYP2C9 reporter construct was substantially activated by dexamethasone only in placental trophoblast cells co-transfected simultaneously with GRα and HNF4α (Fig. 3) or in HepG2 cells expressing endogenous HNF4α (Fig. 5). These data suggest that the absence of HNF4α is one possible cause of CYP2C9 reporter plasmid non-responsiveness to dexamethasone in placental cells. In addition to this, we show that CYP2C9 mRNA was down-regulated after treatment of JEG3 cells with dexamethasone (Fig. 2A), which is possibly due to a decrease in CYP2C9 mRNA stability in the cell line, although this is yet to be established.

In conclusion, we demonstrate an ability of GRα to up-regulate P-gp mRNA in placental trophoblast cells. We show for the first time that the human *MDR1* is transcriptionally activated through GRα in placental and hepatic cells and that the process is HNF4α-independent. In addition, we have identified the absence of HNF4α in placental cell lines as the cause of CYP3A4 and CYP2C9 promoter non-responsiveness to glucocorticoids. Taken together, these findings will give a better understanding of the regulation of placental drug transporters and CYPs.

Acknowledgements

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5. Nukleární receptory: xenosenzory zprostředkující odpověď organismu na xenobiotika a příčina některých lékových interakcí

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Nukleární receptory: xenosenzory zprostředkující odpověď organismu na xenobiotika a příčina některých lékových interakcí

Pregnanový X receptor (PXR) a konstitutivní androstanový receptor (CAR)

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Souhrn

Pávek P., Červený L., Mičuda S., Štaud F., Novotná-Čečková M., Fendrich Z. ***Nukleární receptory.*** *Remedia 2005; 15: 406–409.*

Nukleární receptory PXR a CAR regulují na základě interakce s xenobiotiky expresi enzymů I. a II. fáze biotransformace a některých detoxifikačních lékových transportérů. Ve většině případů se jedná o indukci exprese (up-regulaci) cílových genů, které se přímo podílejí na biotransformaci nebo exkreci xenobiotik. Tímto mechanismem mohou nukleární receptory výrazně modifikovat expozici organismu xenobiotiku, proto jsou často označovány jako xenosenzory. Je-li xenobiotikem léčivo – může být aktivace receptorů PXR nebo CAR příčinou farmakokinetických lékových interakcí, při kterých léčivo zvyšuje aktivitu biotransformačních enzymů nebo transportérů podílejících se na eliminaci jiného spolupodaného léčiva, a mění tak jeho farmakokinetické a případně i farmakodynamické vlastnosti. Je-li xenobiotikem toxická látka, pak nukleárními receptory zprostředkované urychlení eliminace představuje jeden ze základních homeostatických obranných mechanismů. Cílem tohoto přehledového článku je přiblížit nukleární receptory PXR a CAR jako důležité faktory farmakokinetických lékových interakcí.

Klíčová slova: *lékové interakce – nukleární receptory – pregnanový X receptor (PXR) – konstitutivní androstanový receptor (CAR).*

Summary

Pávek P., Červený L., Mičuda S., Štaud F., Novotná-Čečková M., Fendrich Z. ***Nukleární receptory.*** *Remedia 2005; 15: 406–409.*

Based on interaction with xenobiotics, PXR and CAR nuclear receptors regulate expression of phase I and II biotransformation enzymes and some detoxication drug transporters. In most cases, expression (up-regulation) of target genes directly involved in biotransformation or excretion of xenobiotics is induced. By this mechanism, nuclear receptors markedly modify exposure of the organism to the xenobiotic, and that is why they are often called xenosensors. If the xenobiotic is a drug, then activation of PXR and CAR receptors can cause pharmacokinetic drug interactions with the drug enhancing activity of biotransformation enzymes or transporters involved in elimination of another co-administered drug which may lead in turn to changes in pharmacokinetic and possibly also pharmacodynamic properties of the latter. If the xenobiotic is a toxic substance, then the nuclear receptor mediated enhancement of elimination is one of the major homeostatic protective mechanisms. The objective of this review article is to present the PXR and CAR nuclear receptors as important factors in pharmacokinetic drug interactions.

Key words: *drug interactions – nuclear receptors – pregnane X receptor (PXR) – constitutive androstane receptor (CAR).*

Úvod

Indukční účinky léčiv i hormonální účinek endogenních steroidů jsou založeny na ovlivnění exprese cílových genů prostřednictvím aktivace tzv. nukleárních receptorů. Tento proces umožňuje transkripční regulaci konkrétních genů podle aktuálních potřeb organismu. V tomto ohledu jsou významné mimo jiné změny v expresi genů biotransformačních enzymů a transportních proteinů, které představují základní eliminační mechanismus bránící kumulaci některých endogenních látek nebo xenobiotik. Nejnovější literární údaje ukazují, že klíčovou roli v transkripční regulaci těchto detoxikačních procesů mají dva nukleární receptory – pregnanový X receptor a konstitutivní androstanový receptor.

Pregnanový X receptor (Pregnane X receptor, PXR, NR1I2, syn. steroid X receptor, SXR) a konstitutivní androstanový receptor (Constitutive androstane receptor, CAR, NR1I3) náleží do „ligandem aktivované" rodiny transkripčních faktorů a jsou označovány jako tzv. nukleární receptory. Společně s PXR a CAR do této rodiny náleží také receptor pro vitamin D (VDR), glukokortikoidní receptor (GRα), estrogenní receptor (ER) a řada dalších [1]. Receptory PXR a CAR se také někdy označují jako *sirotčí* (orphan) nukleární receptory, protože na rozdíl od dalších zástupců nukleárních receptorů nebyl dosud identifikován fyziologický ligand, který by primárně působil prostřednictvím PXR nebo CAR [1–4]. Rozvoj molekulárněbiologických metod umožnil identifikaci exogenních ligandů (viz dále), které v naprosté většině vedou k aktivaci transkripce a indukci biotransformačních enzymů a transportérů s následným urychlením eliminace xenobiotik. Proto jsou PXR a CAR někdy označovány jako **xenosenzory.** Zajímavým faktem svědčícím o tom, že PXR a CAR jsou primárně defenzivním mechanismem organismu regulujícím spíše dispozici xenobiotik, a nikoli účinek specifického endogenního ligandu nebo hormonu, je, že geneticky manipulované myši postrádající geny pro Pxr a Car jsou normálně životaschopné bez abnormalit [5].

Struktura nukleárních receptorů

Protein nukleárního receptoru je složen ze čtyř částí: z modulátorové domény interagující s modulačními proteiny (koaktivátory), domény rozpoznávající specifické sekvence promotorové DNA (DBD – DNA binding domain), spojovací struktury a domény vázající ligand (LBD – ligand binding domain). DBD svojí specifitou k promotorové sekvenci konkrétních genů určuje škálu genů, které jsou regulovány. LBD tvoří jakousi kapsu, ve které ligandy neko-

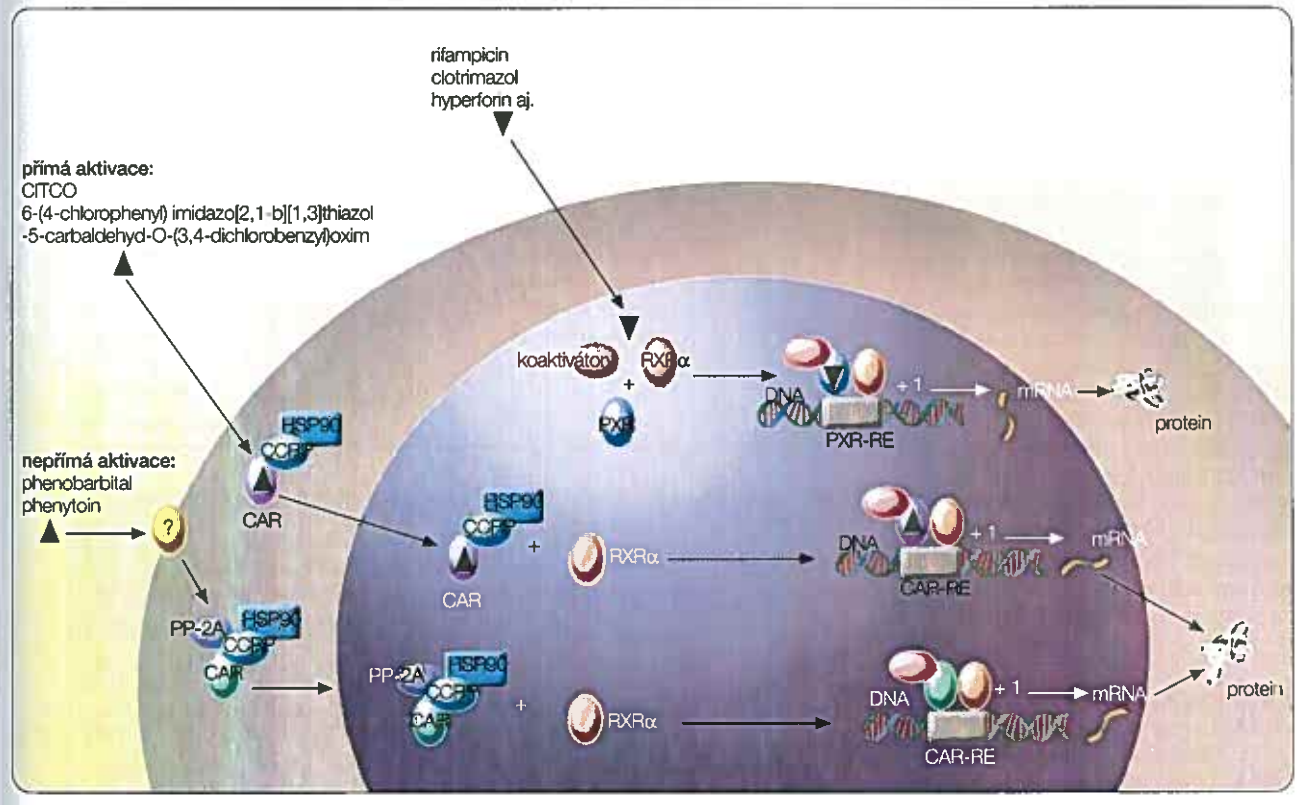
valentně interagují s aminokyselinovými skupinami LBD domény na základě jejich chemické sterické struktury [1, 4]. Mechanismus aktivace nukleárních receptorů PXR a CAR viz **obr. 1**. Tento proces je velmi komplikovaný, spoluúčastní se ho dále represorové proteiny, řada koaktivátorů i proteiny s enzymovou aktivitou ovlivňující chromatinovou strukturu DNA [1–4].

Pregnanový X receptor (PXR)

Přestože je PXR lokalizován především v jádře, byla popsána také cytoplazmatická lokalizace a translokace receptoru PXR z cytoplazmy do jádra během aktivace. V těle je PXR ve velké míře přítomen především v játrech, méně v tenkém a tlustém střevě, v žaludku a v ledvinách. Tato lokalizace významně koreluje s expresí cytochromu CYP3A4 i P-glykoproteinového transportéru (viz dále) [2, 4, 5]. Aktivace PXR probíhá přímou vazbou ligandu v jádře. PXR tvoří dimer s retinoidním receptorem RXRα a společně rozpoznávají specifické sekvence promotorové DNA regulovaných genů označované jako responzivní sekvence PXR-RE (PXR response element). V případě nejdůležitějšího biotransformačního cytochromu P-450 CYP3A4 byla identifikována dvě vazebná místa označovaná jako proximální a distální, která pro maximální indukční účinek spolupracují [7]. PXR-RE různých genů jsou charakteristické velkou sekvenční homologií, i když se v jednotlivých nukleotidech odlišují [2, 3].

K ligandům PXR náleží farmaka z řady terapeutických skupin, některé toxiny i endogenní steroidy (**tab. 1**). Ligandy PXR mají velice různorodou chemickou strukturu a fyzikálně-chemické vlastnosti molekul (**tab. 1**). Tím se PXR odlišuje od ostatních nukleárních receptorů, např. estrogenního (ER), receptoru pro vitamin D (VDR) nebo receptoru pro hormony štítné žlázy (TR). Díky této široké specifitě ligandů i množství regulovaných biotransformačních enzymů a lékových transportérů je PXR receptor považován za jeden z nejdůležitějších faktorů ovlivňujících prostřednictvím eliminačních procesů farmakokinetiku mnoha léčiv [3]. Řada léčiv (např. phenobarbital) navíc aktivuje současně receptory PXR i CAR, což svědčí o určitém překryvu specifity ligandů/aktivátorů obou sirotčích receptorů [4]. Typickým jevem je i tzv. **autoindukce**, tzn. léčivo indukuje prostřednictvím PXR nebo CAR biotransformační enzymy nebo transportéry, pro které je samo substrátem, a tím urychlí svou vlastní eliminaci (např. carbamazepin). Funkce biotransformačních enzymů, lékových transportérů a xenosenzorů nukleárních receptorů tak společně tvoří plastický a důmyslný detoxikační systém lidského těla.

PXR společně s CAR regulují celou řadu genů pro enzymy I. a II. fáze biotransformace a transportérů (**tab. 2**). K nejdůležitějším patří hlavní izoforma cytochromu P-450 zapojená do metabolismu léčiv, CYP3A4, který se podílí na biotransformaci více než 50 % léčiv používaných v humánní medicíně. Nicméně v lidských játrech byla sledována až 90násobně rozdílná exprese této izoformy. Podobně byly zjištěny až 30násobné rozdíly v clearance modelových substrátů CYP3A4, což výrazným způsobem modifikuje expozici a celkovou farmakokinetiku léčiv [7]. Interindi-



Obr. 1 *Schematické znázornění mechanismu transkripční regulace exprese genů řízené nukleárními receptory PXR a CAR*

Pregnanový X receptor (PXR) je aktivován přímou vazbou ligandu na protein PXR lokalizovaný v jádře. Po navázání ligandu na PXR se vytváří komplex PXR s retinoidním receptorem RXRα, který rozpozná responzivní místo pro PXR (response element, PXR-RE) regulovaných genů. Komplex se dále spojuje s dalšími koaktivátory a RNA-polymerázou II, která zahajuje transkripci z transkripčního startu označovaného jako +1. CAR receptor je lokalizován v cytoplazmě v neaktivním stavu v komplexu s CAR cytoplasmic retention proteinem (CCRP) a heat shock proteinem 90 (HSP90). CAR může být aktivován dvěma cestami. Při přímé aktivaci se ligand váže na CAR v cytoplazmě a komplex vstupuje do jádra. Nepřímá aktivace zahrnuje ne příliš prostudovanou kaskádu, při níž se tvoří komplex fosfatázy 2A (PP-2A), CCRP, CARu a HSP90, který přestupuje do jádra a iniciuje transaktivaci genů bez vazby ligandu na receptor CAR. V jádře se komplex CAR receptoru váže na responzivní sekvenci (CAR-RE) v promotorové oblasti regulovaných genů, ke komplexu se přidává RNA polymeráza II a spouští se transkripce.

vidуální variabilita metabolismu léčiv je všeobecně spojována s fenoménem genetického polymorfismu daného přítomností bodových mutací DNA (tzv. SNP, Single Nucleotide Polymorphism), které modifikují finální aktivitu enzymů. U CYP3A4 je však tato asociace nejasná a ukazuje se, že SNP v kódující nebo regulační oblasti genu CYP3A4 nejsou hlavní příčinou této variability [3]. Polymorfismus PXR, který přímo reguluje expresi CYP3A4, se proto nabízí jako nejpravděpodobnější vysvětlení těchto fenoménů. V promotorové i kódující oblasti genu PXR bylo identifikováno kolem 40 SNP, a to včetně sekvencí, které kódují ligand vázající doménu PXR [8]. Byly publikovány studie, které prokázaly souvislost mezi některými SNP a expresí CYP3A4 v játrech. Rovněž byly publikovány studie, ve kterých byla popsána silná korelace mezi expresí PXR a CYP3A4 [9]. Přesto se předpokládá, že exprese a genotyp PXR nejsou jedinými výhradními faktory interindividuální variability funkce CYP3A4 v populaci [3].

Konstitutivní androstanový receptor (CAR)

Konstitutivní androstanový receptor (Constitutive Androstane Receptor, CAR, NR1I3) je blízký příbuzný receptoru PXR. Na rozdíl od PXR je CAR intracelulárně přítomen v cytoplazmě, odkud se dostává do jádra tzv. translokací [1, 6]. Kromě genů pro enzymy I. a II. fáze biotransformace a transportérů léčiv (**tab. 2**) řídí CAR i transkripci důležitých genů podílejících se na metabolismu endogenních látek, jako jsou bilirubin, žlučové kyseliny, hormony štítné žlázy, steroidní hormony a mastné kyseliny [6].

Aktivace CAR probíhá dvěma způsoby: přímou aktivací nukleárního receptoru ligandem, tj. vazbou ligandu na nukleární receptor lokalizovaný v cytoplazmě, a následnou translokací komplexu nukleárního receptoru, ligandu a koaktivujících proteinů (HSP90 aj.) do jádra. Tímto způsobem aktivují transkripci modelové látky CITCO (6-(4-chlorophenyl) imidazo[2,1-b][1,3] thiazole-5-carbaldehyd-O-(3,4-dichlorobenzyl)oxim) nebo v případě myšího Car receptoru karcinogen TCPOBOP (1,4-bis [2-(3,5-dichloropyridyloxy)]benzen). Phenobarbital a phenytoin, nejznámější induktory kooperující s receptorem CAR, aktivují transkripci regulovaných genů prostřednictvím tohoto nukleárního receptoru nepřímo bez vazby na nukleární receptor (**obr. 1**) [6]. Po translokaci komplexu CAR a koaktivátorů do jádra se komplex proteinů CAR a RXRα váže na CAR-RE responzivní sekvence v promotorové oblasti regulovaných genů. Transkripční aktivace pak probíhá podobně jako v případě receptoru PXR.

Kromě aktivace receptoru CAR xenobiotiky mohou tuto transaktivační kaskádu spustit i další mechanismy při některých fyziologických nebo patologických stavech. Například vysoké intracelulární koncentrace cAMP při hladovění indukují prostřednictvím CAR expresi genů, které se podílejí na odpovědi při nutričním stresu. Podobně je CAR nepřímo aktivován (**obr. 1**) bilirubinem při hyperbilirubinémii a spouští adaptační mechanismy, které aktivují biotransformační enzymy a transportéry zapojené do metabolismu bilirubinu a jeho konjugátů (např. GSTA1/A2, UGT1A1, MRP2 aj.) [6]. CAR a PXR společně chrání játra před toxickým působením kyseliny lithocholové (LCA) prostřednictvím indukce enzymů účastnících se metabolismu LCA. CAR například aktivuje SULT2A9 podílející se na sulfátové konjugaci LCA, myší cytochrom C/p3A11 a Cyp7a1 a transportéry Mrp3 a Oatp2 [5, 6].

Tab. 1 NĚKTERÁ LÉČIVA, XENOBIOTIKA A ENDOGENNÍ STEROIDY INTERAGUJÍCÍ S PREGNANOVÝM X (PXR) A KONSTITUTIVNÍM ANDROSTANOVÝM (CAR) NUKLEÁRNÍM RECEPTOREM

PXR	CAR
PCN (pregnenolon 16α-carbonitril)	phenobarbital
rifampicin	phenytoin
troglitazon	chlorpromazin ?
dexamethason – ve vysokých koncentracích	cerivastatin
clotrimazol	simvastatin
mifepriston (RU-486)	fluvastatin
hyperforin	atorvastatin
nifedipin	
paclitaxel	CITCO 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thi-
ritonavir	azol-5-carbaldehyd-O-(3,4-dichlorobenzyl)oxim
gemfibrozil	
spironolacton	*Inhibitory*:
tamoxifen	androstanol
cyproteron	androstenol
glutethimid	clotrimazol
lovastatin	3α, 5α-androstanol
metyrapon	
phenobarbital	
5β-pregnan-3,20-dion	

Tab. 2 GENY BIOTRANSFORMAČNÍCH ENZYMŮ I. A II. FÁZE BIOTRANSFORMACE A LÉKOVÝCH TRANSPORTÉRŮ REGULOVANÉ PREGNANOVÝM RECEPTOREM X (PXR) A KONSTITUTIVNÍM ANDROSTANOVÝM NUKLEÁRNÍM RECEPTOREM (CAR)

PXR	CAR
izoenzymy cytochromu P-450 – CYP3A4, CYP2C9, CYP2C8, CYP2C19, CYP2B6	izoenzymy cytochromu P-450 – CYP2B6, CYP3A4, CYP2C9
glutathion S-transferázy (GST) – GSTA2	glutathion S-transferázy (GST)-GSTA1/A2
sulfotransferázy (SULT) – *Sult1B1, St-20/21, St-40/41 a St-60*	sulfotransferázy (SULT)-*Sult1a1, 1d1, 2a1, Sult2a9*
karboxylesterázy – *Rl2*	UDP-glukuronosyltransferázy (UGT) – UGT1A1
transportéry – P-glykoprotein, MRP2, OATP2, BSEP	transportéry – MRP2, OATP2, P-glykoprotein
cytochrom CYP7A (cholesterol 7α-hydroxyláza)	

Kurzívou a malými písmeny jsou označeny myší, případně potkaní geny.

PXR a CAR jako prostředník závažných klinických lékových interakcí

Nukleární receptory PXR a CAR společně představují jeden z hlavních regulačních mechanismů určujících finální aktivitu základních enzymů a transportních proteinů zapojených do eliminace léčiv (**tab. 2**). V oblasti metabolismu to jsou především izoformy (enzymy) cytochromu P-450, CYP3A4, CYP2C9 a CYP2C19, které tvoří až 80 % obsahu P-450 v játrech a střevě a zprostředkují většinu reakcí

I. fáze biotransformace léčiv [2–4]. Další izoforma cytochromu P-450, CYP2B6, je charakteristická svojí indukovatelností především prostřednictvím receptoru CAR [3, 6]. Z transportních proteinů se pozornost soustřeďuje na P-glykoprotein (P-gp, MDR1) a Multidrug resistance-associated protein 2 (MRP2), které transportují desítky farmakoterapeuticky důležitých léčiv, jejich metabolitů a konjugátů [11]. Ovlivnění PXR a CAR při současné aplikaci několika léčiv je proto významným mechanismem farmakokinetických lékových interakcí. To dokládá i skutečnost, že většina dosud klinicky popsaných lékových interakcí, při kterých dochází k indukci CYP3A4, je zprostředkována PXR nukleárním receptorem [2]. S ohledem na spektrum ligandů PXR a CAR a jejich používání v klinické praxi je vhodné zdůraznit příklady nejčastějších případů. Dobře dokumentované jsou především interakce antiepileptik phenobarbitalu, carbamazepinu a phenytoinu a antituberkulotika rifampicinu, které prostřednictvím PXR a CAR indukují izoformy cytochromu P-450 (např. CYP3A4, 2C9, 2C19, 2B6), což následně vede k urychlení eliminace jejich substrátů, poklesu plazmatických koncentrací a vážným klinickým interakcím [12]. Obzvláště závažné jsou klinické interakce phenobarbitalu, carbamazepinu a antituberkulotika rifampicinu s antikoagulanciem warfarinem. Indukce biotransformace warfarinu v těchto případech může vést až k fatálnímu snížení antikoagulační aktivity warfarinu [13].

Rifampicin je jedním z nejsilnějších dosud známých ligandů PXR a induktorů biotransformačních enzymů CYP3A4, CYP2B6, CYP2C9 a transportérů P-glykoproteinu v játrech i ve střevě. Plný projev indukce biotransformačních enzymů a transportérů se během terapie rifampicinem projeví přibližně po 7 dnech. Podobně k regresi změn dochází po ukončení podávání přibližně za stejnou dobu. Klinicky významné interakce rifampicinu s léčivy prostřednictvím vazby na PXR a následné indukce enzymů a transportérů uvádí tab. 3 [13–15].

Tab. 3 LÉČIVA, U NICHŽ BYLY DOKUMENTOVÁNY KLINICKY ZÁVAŽNÉ LÉKOVÉ INTERAKCE S RIFAMPICINEM, LIGANDEM PXR RECEPTORU

chinidin	imatinib	ondasetron
ciklosporin A	ifosfamid	propafenon
dapson	itraconazol	ritonavir
delavirdin	carvedilol	saquinavir
dextromethorfan	ketoconazol	simvastatin
disopyramid	clarithromycin	sirolimus
dolasetron	codein	tacrolimus
ethinylestradiol	mefloquin	tamoxifen
etoricoxib	methadon	tolbutamid
everolimus	midazolam	toremifen
fenprokuron	morphin	triazolam
gliclazid	nateglinid	verapamil
glimepirid	nelfinavir	voriconazol
glipizid	nifedipin	warfarin
haloperidol	nilvadipin	zopiclon
indinavir	norethisteron	

podle [12,13] – Suchopár, Niemi, et al., 2003, 2005

Známé jsou také klinicky významné interakce přírodního sedativa hyperforinu obsaženého v extraktu třezalky tečkované (Hypericum perforatum) s některými kontraceptivy, warfarinem, imunosupresivem ciklosporinem A, HIV proteázovým inhibitorem indinavirem a digoxinem [2]. Hyperforin má vysokou afinitu k receptoru PXR a v nízkých koncentracích významně indukuje jaterní i střevní CYP3A4 a CYP2C9, které primárně biotransformují výše zmíněná farmaka.

Závěr

Nukleární receptory řídí expresi enzymů I. a II. fáze biotransformace a detoxikačních lékových transportérů jako odpověď organismu na přítomnost xenobiotika. Po ukončení eliminace xenobiotika z organismu ustává aktivace nukleárních receptorů a následně i up-regulace cílových genů. Tento jednoduchý model je však mnohem komplikovanější a vstupují do něj další faktory. Exprese nukleárních receptorů PXR a CAR je sama regulována některými nukleárními receptory a transkripčními faktory. Příkladem jsou syntetické a endogenní glukokortikoidy, které indukují expresi PXR i CAR prostřednictvím glukokortikoidního receptoru GRα. Zvýšení exprese transportéru OATP2 prostřednictvím PXR může navíc urychlit vstup potenciálních induktorů do jádra. Naopak indukce efluxních lékových transportérů P-glykoproteinu a MRP2 prostřednictvím PXR může zamezit vstupu induktorů do buněk a snížit aktivaci nukleárních receptorů. Celý systém detoxikace a biotransformace xenobiotik tak nabývá na komplexnosti a porozumění tomuto systému a nalezení klinických implikací je úkolem moderní experimentální a klinické farmakologie.

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8. Souhrn

Epilepsie je chronické onemocnění mozku různé etiologie, které postihuje 1 – 2 % populace. Pro toto onemocnění jsou charakteristické opakující se záchvaty nekontrolovaného dráždění neuronů mozku. Antiepileptika snižují výskyt motorických a psychických projevů epilepsie tím, že redukují křečovou pohotovost organismu. Základní mechanizmus účinku antiepileptik tkví ve snížení dráždivosti nervových buněk tvořících paroxysmální výboje stabilizací jejich klidového membránového potenciálu. Cílem antiepileptické léčby je dosáhnout stavu bez záchvatů a umožnit tím pacientovi plnohodnotný život.

Přibližně 30 % pacientů však tvoří skupinu s **farmakorezistentní** (refrakterní) **epilepsií.** Ta je definována jako selhání kontroly epileptických záchvatů navzdory užívání kombinace tří nebo více antiepileptik v maximálních tolerovaných dávkách a představuje vážný klinický problém, který je spojený s vysilujícími psychosociálními potížemi a se zvýšeným rizikem úmrtí pacienta.

Většina pacientů trpících refrakterní epilepsií je rezistentní k téměř všem klinicky používaným léčiva, přestože antiepileptikům působí rozdílnými mechanismy. Tento fakt popírá teorii patologických změn specifických vazebných míst pro antiepileptika a podporuje teorii, že se jedná o nespecifický, pravděpodobně adaptabilní mechanismus jako je snížený up-take antiepileptik do mozku. Možnou příčinou tohoto fenoménu by tedy mohla být mezi jinými i up-regulace a ektopická exprese některých efluxních lékových ABC (ATP-binding cassette) transportérů v hematoencefalické bariéře (HEB).

Užíváni antiepileptik pacienty s sebou často nese riziko více či méně vyjádřených nežádoucích účinků. Příkladem nežádoucích účinků antiepileptik jsou sedace, zmatenost, ataxie, hepatotoxicita či teratogenní efekt. Dalšími nežádoucími účinky jsou **farmakokinetické interakce** antiepileptik s léčivy podávanými v průběhu terapie souběžně probíhajícího onemocnění. Tyto interakce se mohou projevit zvýšením nebo snížením účinku antiepileptika, stejně tak jako souběžně podávaného léčiva. Zvýšení účinku antiepileptik s sebou nese riziko většího projevu nežádoucích účinků a naopak snížení se může projevit epileptickými záchvaty. Příčinou těchto problémů často bývá léčbou podmíněná modulace aktivity a exprese některých efluxních lékových ABC transportérů a metabolických enzymů z rodiny cytochromu P450.

V poslední době je úsilí molekulárních farmakologů směřováno ke studiu mechanismů transkripční regulace genů kódujících cytochromy P450 a efluxních lékových ABC

transportérů. Současné studie ukázaly, že transkripční aktivace těchto genů může být kromě jiného řízena i dvěma důležitými nukleárními receptory, konkrétně konstitutivním androstanovým receptorrem (CAR, NR1I3) a pregnanovým X receptorem (PXR; SXR, NR1I2).

Širokospektré antiepiletikum kyselina valproová má schopnost ovlivnit expresi řady genů na příklad inhibicí histonových deacetyláz (HDAC) . Na základě dosud publikovaných dat o účincích kyseliny valproové jsme vytvořili hypotézu, že v některých případech transkripční aktivace (např. *CYP3A4* a *MDR1*) existuje i selektivnější molekulární mechanismus než je inhibice HDAC.

Předkládaná dizertační práce se zabývá :

- Interakcí vybraných antiepileptik s efluxními lékovými ABC transportéry a rolí studovaných transportérů ve vzniku rezistence vůči antiepileptické léčbě.
- Interakcí kyseliny valproové s konstitutivním androstanovým receptorem (CAR) a pregnanovým X receptorem (PXR) a vlivem této interakce na expresi *CYP3A4* a *MDR1*.

1. Interakce vybraných antiepileptik s efluxními lékovými ABC transportéry; role studovaných transportérů ve vzniku rezistence vůči antiepileptické léčbě

Prvním efluxním lékovým ABC transportérem objeveným v endoteliálních buňkách HEB byl P-glykoprotein (P-gp). Dále byly ze skupiny efluxních lékových ABC transportérů v HEB detekovány a lokalizovány MRP2 (multidrug resistance-associated protein 2) a BCRP (breast cancer resistance protein).

Zvýšená exprese těchto ABC transportérů v HEB pacientů trpících farmakorezistentní epilepsií, analogicky s vícečetnou lékovou rezistencí nádorů, je považována za jednu z příčin selhání léčby antiepileptiky. Tato teorie byla navíc podpořena faktem, že některá antiepileptika (např. fenytoin nebo levetiracetam) jsou prokázanými substráty P-gp.

Hlavním cílem naší studie bylo *in vitro* testování interakcí vybraných antiepiletik s lidským BCRP transportérem. Studie byly provedeny pomocí i) transportních experimentů a ii) akumulačních studií. Testováni byli zástupci všech tří generací antiepileptik: fenytoin, fenobarbital, primidon a ethosuximid (1. generace), kyselina valproová, karbamazepin a klonazepam (2. generace) a lamotrigin (3. generace).

ad i) Pomocí transportních experimentů na monovrstvách tvořených polarizovanými MDCKII buňkami (Madine-Darby Canine Kidney Cells) trvale transfekovanými cDNA lidského BCRP (MDCKII-BCRP) jsme v rámci skupiny vybraných antiepileptik neidentifikovali žádný substrát BCRP.

ad ii) Akumulační experimenty provedené v MEF3.8 buňkách trvale transfekovaných cDNA humánního BCRP (MEF3.8-BCRP) odhalily pouze mírný efekt kyseliny valproové (500 μM) na buněčnou akumulaci fluorescenčního substrátu BCRP BODIPY FL prazosinu. Tento inhibiční efekt ovšem nebyl potvrzen pomocí druhého přístupu, v kterém bylo využito jiného fluorescenčního substrátu BCRP cytostatika mitoxantronu. Fluorescence BODIPY FL prazosinu byla stanovována fluorimetrem v buněčném lyzátu a fluorescence mitoxantronu byla měřena průtokovým cytometrem v intaktních buňkách. Ostatní studovaná antiepileptika při testovaných koncentracích neměla žádný modulační vliv na aktivitu transportu BODIPY FL prazosinu ani mitoxantronu zprostředkovaného BCRP transportérem.

Ze získaných dat usuzujeme, že se BCRP pravděpodobně nepodílí na vzniku rezistence pacientů vůči vybraným antiepileptikům. Navíc, testovaná antiepileptika v terapeuticky relevantních koncentracích zřejmě nemají inhibiční vliv na BCRP-zprostředkovaný transport ani v hematoencefalické bariéře ani v jiných tkáních exprimujících BCRP (střevo, placenta aj.). Pozorovaný inhibiční efekt kyseliny valproové nebyl našimi experimenty plně potvrzen, proto můžeme pouze spekulovat, zda je kyselina valproová inhibitorem BCRP-zprostředkovávaného transportu.

2. Interakce kyseliny valproové s konstitutivním androstanovým receptorem (CAR) a pregnanovým X receptorem (PXR); vliv této interakce na expresi *CYP3A4* a *MDR1*

PXR a CAR jsou nukleární receptory regulující míru transkripční aktivity mnoha genů kódující cytochrom P450 (např. *CYP3A4, CYP2C9, CYP2C19*) a ABC transportéry (*MDR1, Mrp2*). Aktivovaná forma nukleárního receptoru CAR nebo PXR vytváří ve většině případů heterodimer s RXRα (receptorem X pro 9-*cis* retinovou kyselinu α), který se následně váže k responzivnímu elementu v promotorové oblasti cíleného genu a spouští jeho transkripci.

Kyselina valproová je vysoce účinným a dobře snášeným léčivem s antikonvulzivními vlastnostmi, které je řazeno mezi antiepileptika druhé generace. Je používáno hlavně k léčbě generalizovaných tonicko-klonických záchvatů, absencí a parciálních záchvatů.

Cílem naší studie bylo zjistit, zda potenciální interakce kyseliny valproové s CAR a PXR nemůže být dalším možným mechanismem, jímž kyselina valproová ovlivňuje expresi genů. Konkrétně jsme se zaměřili na geny *CYP3A4* a *MDR1*.

Při řešení této problematiky jsme použili několik soudobých metod molekulární farmakologie využitelných pro analýzu i) transkripční aktivity (genové reportérové studie), ii) genové exprese na úrovni mRNA, iii) enzymatické aktivity a iv) interakce komplexu ligand/nukleární receptor s responzivním elementem v promotoru cíleného genu.

ad i) Genové reportérové studie v HepG2 buněčné linii nám poskytly informace o transkripční aktivaci luciferázových reporterových plazmidů obsahujících v promotorové oblasti důležité responzivní elementy (DR3, DR4 a ER6) testovaných genů *CYP3A4* a *MDR1*.

ad ii) Expresi genů *CYP3A4* a *MDR1* na úrovni mRNA jsme analyzovali v LS174T buňkách a kultuře primárních hepatocytů pomocí real time RT-PCR.

ad iii) Enzymatická aktivita CYP3A4 byla detekována v LS174T buňkách užitím chemické analýzy specifického metabolitu 6β-hydroxytestosteronu vznikajícího při CYP3A4-zprostředkovaného testosteronu.

ad iv) Interakce komplexu CAR/RXRα s responzivními elementy genů *CYP3A4* a *MDR1* (DR3, DR4 a ER6) byla studována pomocí „Electrophoretic mobility shift assay" (EMSA).

Kyselina valproová jevila v genových reporterových studiích provedených v HepG2 buněčné linii signifikantní hodnoty aktivace CAR-řízené transkripce *CYP3A4* i *MDR1*. Kyselinou valproovou podmíněná aktivace PXR měla indukční efekt pouze na transkripci *CYP3A4*. Data získaná pomocí real time RT-PCR analýzy exprese *CYP3A4* a *MDR1* mRNA v LS174T a primární kultuře lidských hepatocytů korelovala s daty z genových reportérových studií. Pomocí metody EMSA bylo potvrzeno, že kyselina valproová zesiluje vazbu CAR/RXRα na responzivní element (DR3, DR4 a ER6). Navíc v LS174T transfekovaných expresním vektorem pro PXR byla detekována zvýšená katalytická aktivita CYP3A4.

Naše studie prokázala, že kyselina valproová může aktivací CAR a PXR zvyšovat expresi *CYP3A4* i *MDR1*. Popis této interakce na molekulární úrovni může napomoci k vysvětlení různých disproporcí v účincích kyseliny valproové na genovou expresi, a to nejen cytochromů

P450 a efluxních lékových ABC transportérů. V neposlední řadě kyselina valproová, použitá jako adjuvantní léčivo při léčbě malignit, může v neposlední řadě způsobit skrze zvýšenou transkripční aktivitu *MDR1* prohloubení rezistence nádorů vůči terapii těmi cytostatiky, která jsou substráty P-gp.

9. Summary

Epilepsy is a common neurological disorder of the brain with various etiology affecting 1 - 2 % of the population. Epilepsy in general is characterized by epileptic seizures (with motoric or psychic manifestation) that are caused by attacks of uncontrolled excitation of neurons in the brain. Antiepileptic drugs reduce excitability of the neurons and block fast spread of paroxysmal discharges within the brain.

Pharmacoresistant (refractory) epilepsy occurs in about one third of cases overall. It is a serious clinical problem associated with exhausting psycho-sociological complaints and increased risk of patient death. Resistance in epilepsy has probably a multifactorial and drug-nonspecific origin. Patients with refractory epilepsy are resistant to most of antiepileptic drugs, even though these drugs act by different mechanisms. This argues against epilepsy-induced alterations in specific drug targets as a major cause of refractory epilepsy and supports the hypothesis that a nonspecific mechanism, such as decreased drug up-take into the brain, is involved.

Treatment with antiepileptic drugs is usually a long time process, which is often terminated when patient dies. Depending on type of antiepileptic drugs administered to organism, a lot of adverse effects such as sedation, confusion, ataxia, hepatotoxicity or teratogenicity can be expected.

During life of people suffering from epilepsy there is often a need to medicate common disorders by co-administered drugs. In many cases there is a high risk of drug-drug interaction between antiepileptic and co-administered drug. Interaction at the level of expression and activity of cytochrome P450 and efflux drug ABC transporters can be introduced into high or low concentration of antiepileptic drug in epileptogenic centers. High concentration of antiepileptic drug is often associated with more expressed manifestation of adverse affects of antiepileptic drug and low concentration can be the cause of epileptic seizures occurrence.

Recently, effort of molecular pharmacologists is aimed at description of molecular mechanisms of transcriptional regulation of genes encoding cytochrome P450 and efflux drug ABC transporters. Some studies showed that activation of nuclear receptors such as constitutive androstane receptor (CAR, NR1I3) and pregnane X receptor (PXR, SXR, NR1I2) can be one of the mechanisms involved in transcriptional regulation.

Broad-spectrum antiepileptic drug valproic acid is able to affect gene expression by mechanism of histone deacetylases (HDAC) inhibition. Based on so far published data we

supposed that in many cases an undocumented and more selective mechanism has to be employed. We suggested interaction of valproic acid with CAR and PXR to be additional mechanism that affects expression of some genes.

This dissertation thesis concerns with:

- Interaction of selected antiepileptic drugs with efflux drug ABC transporters and role of studied ABC transporters in development of resistance against antiepileptic treatment.
- Interaction of valproic acid with constitutive androstane receptor (CAR) and pregnane X receptor (PXR) and effect of this interaction on *CYP3A4* and *MDR1* gene expression.

1. Interaction of selected antiepileptic drugs with efflux drug ABC transporters; role of studied ABC transporters in development of resistance against antiepileptic treatment

P-glycoprotein (P-gp) was the first efflux drug ABC transporter (ATP-binding cassette) discovered in endothelial cells of the blood brain barrier (BBB). Further efflux drug ABC transporters detected and localized in the BBB were MRP2 (multidrug resistance-associated protein 2) and BCRP (breast cancer resistance protein). Up-regulation of efflux drug ABC transporters in the BBB is considered to be, analogically to multidrug resistance of tumor against treatment with cytostatic drug, the cause of lower up-take of antiepileptic drugs to epileptogenic centers in the brain. Additionally, this hypothesis is supported by recent studies that revealed some of antiepileptic drugs to be substrate of efflux drug ABC transporters.

In our study, we focused on *in vitro* testing of interaction between BCRP and selected antiepileptic agents. The study was performed employing i) transport experiments and ii) accumulation studies. Representatives of all three generations of antiepileptic drugs; in particular: phenytoin, phenobarbital, primidone, and ethosuximide (1. generation), valproic acid, carbamazepine, and clonazepam (2. generation) and finally lamotrigin (3. generation), were examined at relevant therapeutic concentrations.

re i) Using transport experiments in MDCKII cells transfected with cDNA of human BCRP, none of selected antiepileptic drug was identified as a substrate of BCRP.

re ii) Accumulation studies performed in MEF3.8 cells transfected with cDNA of human BCRP revealed slight but significant inhibition effect of valproic acid (500 μM) on BCRP-mediated transport of BODIPY FL prazosin. However, this

effect was not confirmed employing the second approach – the accumulation of mitoxantrone. The other tested antiepileptic drugs did not affect BCRP-mediated transport.

Our data demonstrate that the selected antiepileptic drugs at tested concentrations are neither substrates nor inhibitors of BCRP transporter. Thus, we conclude that up-regulated BCRP in the blood-brain barrier is not involved in the development of drug resistance to the tested antiepileptic drugs. Moreover, we suppose that tested AEDs do not affect BCRP-mediated transport in the blood-brain barrier or in other tissues expressing BCRP, such as the intestine and placenta.

2. Interaction of valproic acid with constitutive androstane receptor (CAR) and pregnane X receptor (PXR); effect of this interaction on *CYP3A4* and *MDR1* gene expression

CAR and PXR are ligand-activated nuclear receptors that act as heterodimers with retinoid X receptor α (RXRα) and modulate transcriptional activation of their target genes including genes encoding cytochrome P450 (e.g. *CYP3A4, CYP2C9,* and *CYP2C19*) and ABC transporters (*MDR1* and *Mrp2*) by interaction with specific promoter-binding motifs (responsive elements).

Valproic acid is an effective broad-spectrum anticonvulsant (2. generation) used in the treatment of primary generalized tonic-clonic, absence, and partial seizures. Moreover, valproic acid has recently been identified as an inhibitor of histone deacetylase (HDAC) with potential antitumorous activity that has been studied in several clinical trials.

The aim of this study was to examine whether valproate has an ability to activate CAR- and/or PXR- mediated transcription of *CYP3A4* and *MDR1* genes. We employed several advanced techniques of molecular pharmacology to analyze: i) gene transcriptional activity, ii) gene expression iii) catalytic activity iv) interaction between complex ligand/nuclear receptor and responsive element in promoter sequence of target genes.

re i) Gene reporter assays in HepG2 cells were used to study transcriptional activation of reporter luciferase plasmids containing responsive elements (DR3, DR4, and ER6) in gene promoter of studied genes *CYP3A4* and *MDR1*.

re ii) Real time RT-PCR enabled us to determine alterations in *CYP3A4* and *MDR1* gene expression in LS174T cells and culture of primary human hepatocytes.

re iii) Analysis of catalytic activity was based on HPLC determination of specific product of CYP3A4-mediated biodegradation of testosterone.

re iv) Using electrophoretic mobility shift assay (EMSA) we detected interaction between ligand/CAR complex and responsive elements DR3, DR4, ER6 in promoter of *CYP3A4*.

Using transient transfection reporter assays in HepG2 cells, valproic acid was recognized to activate *CYP3A4* promoter via CAR and PXR pathways. By contrast, a significant effect of valproic acid on *MDR1* promoter activation was observed only in CAR-cotransfected HepG2 cells. These data well correlated with up-regulation of CYP3A4 and MDR1 mRNAs analyzed by real-time RT-PCR in cells transfected with expression vectors encoding CAR or PXR and treated with valproic acid. In addition, valproic acid significantly up-regulated CYP3A4 mRNA in primary hepatocytes and augmented the effect of rifampicin. EMSA experiments showed valproic acid-mediated augmentation of CAR/RXRα binding to DR3 and DR4 responsive elements of *CYP3A4* and *MDR1* genes, respectively. Finally, the analysis of specific CYP3A4 catalytic activity revealed its significant increase in valproic acid-treated LS174T cells transfected with PXR.

In conclusion, we provide novel insight into the mechanism by which valproic acid affects gene expression of *CYP3A4* and *MDR1* genes. Our results demonstrate that valproic acid has a potential to up-regulate *CYP3A4* and *MDR1* through direct activation of CAR and/or PXR pathways. We believe that description of this interaction at the molecular level can help to explain various discrepancies in valproic acid effects on expression of genes containing in its promoter specific binding motives for CAR and PXR. Moreover, we suggest valproic acid might cause an increase of some solid tumors drug resistance against therapy with P-gp substrates such as paclitaxel and doxorubicin.