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Aspekty genové regulace CYP3A4 v jaterní tkáni.

Dizertační práce

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

Lucie Krausová

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Seznam zkratek

Ag	antigen
C/EBP	CCAAT/enhancer binding protein
CAR	constitutive androstane receptor
CCRP	CAR cytoplasmic retention protein
CITCO	((6-(4-chlorfenyl)imidazo[2,1-b][1,3]thiazol-5-karbaldehyd O-(3,4-dichlorbenzyl)oxim)
CYP	cytochrom P450
DBD	DNA binding domain
DDI	drug-drug interaction
DR	direct repeat
dsDNA	double-stranded DNA
EMSA	electrophoretic mobility shift assay
ER	everted repeat
FXR	farnesoid X receptor
GRα	glucocorticoid receptorα
HDAC	histondeacetyláza
HepG2	human hepatocellular liver carcinoma cell line
HNF	hepatocyte nuclear factor
HSP90	heat shock protein 90
IR	inverted repeat
LBD	ligand binding domain
LETF	liver enriched transcription factor
LI	lékové interakce
MDR	multidrug resistance
MODY1	maturity onset diabetes of the young 1
mRNA	messenger ribonucleotid acid
MRP	multidrug resistance protein
OATP	organic anion transporting polypeptide
PGC-1α	peroxisome proliferator-activated receptor γ coactivator 1α
Pgp	P-glykoprotein
PP-2A	protein pohosphatase 2A
PXR	pregnane X receptor
RE	responzivní element
RT-PCR	reverse transcriptase – polymerase chain reaction
RXRα	retinoid X receptor
SRC-1	steroid receptor coactivator 1
SULT	sulfotransferáza
UGT	UDP-glukoronosyltransferáza
VDR	vitamin D receptor
VPA	valproic acid

1 Úvod

1.1 Problematika lékových interakcí

Lékové interakce (LI) představují pro současnou medicínu a farmakoterapii významnou komplikaci. Za LI považujeme ovlivnění farmakodynamických či farmakokinetických vlastností léčiva jiným léčivem, potravou nebo jinou chemickou látkou z okolního prostředí. U polymorbidních pacientů, léčených několika léčivými přípravky současně či v návaznosti, jsou LI spíše pravidlem nežli výjimkou.

Podle výsledného efektu je možno LI rozdělit na synergistické a antagonistické. Synergistické LI vedou k zesílení účinku, které může být záměrné a pro terapii příznivé, v jiných případech je však příčinou nežádoucích či toxických projevů. Naopak důsledkem antagonistické LI je snížení účinku. Příkladem žádoucí antagonistické LI je podání antidota, negativním následkem je pak neúčinná léčba.

Zatímco k farmakodynamickým LI dochází na úrovni působení léčiva na cílové struktuře, farmakokinetické LI spočívají v interferenci spolupodaných xenobiotik v průběhu jejich absorpce, distribuce, biotransformace nebo exkrece. Struktury zodpovědné za vznik tohoto typu LI jsou hlavně biotransformační enzymy a lékové transportéry. Nejčastěji dochází k LI na cytochromu P450 3A4 [1].

Manifestaci LI lze často velmi těžko rozeznat. Problematickou oblastí je samoléčba, laik často nebývá informován o interakcích volně prodejného léčiva s předepsanými léky či potravou. Ani v ambulantní péči nejsou LI dostatečně sledovány, kontakt s pacientem je časově omezen a lékař mnohdy přehlédne i duplicitní preskripci. V nemocniční praxi je prostor pro sledování LI nejvhodnější, projevy interakcí však mohou být zaměněny za rozvoj léčené choroby či její komplikace bez nalezení souvislosti s farmakoterapií. U problematických léčiv s úzkým terapeutickým oknem (např. aminoglykosidy, digoxin, amiodaron, lidokain, takrolimus, vankomycin) je doporučeno monitorování plazmatických hladin [2]. V případě výskytu LI je nutno individuálně upravit dávkování léčiv nebo zvolit jinou farmakoterapii.

1.2 CYP3A4

Enzymy cytochromu P450 (CYP) představují řadu mitochondriálních monooxygenáz obsahujících hemovou složku. Tvoří důležitý obranný systém organismu proti působení xenobiotik. Podílí se na I. fázi biotransformace 60 % všech léčiv [3].

CYP3A4 je nejvýznamnějším biotransformačním enzymem u dospělého člověka. Největší měrou je exprimován v játrech a tenkém střevě. Je zodpovědný za biotransformaci více než 30 % klinicky užívaných léčiv, a tím ovlivňuje jejich terapeutickou účinnost a bezpečnost. Kromě funkce v eliminaci léčiv byla také popsána jeho schopnost aktivovat prokancerogeny životního prostředí [4]. V rámci endogenního metabolismu katalyzuje 6β-hydroxylaci testosteronu a kortizolu [5].

CYP3A4 je příčinou výrazné interindividuální variability v odpovědi na podání léku, hladina mRNA CYP3A4 v hepatocytech se liší až 50x v populaci [6]. Za určující příčinu se považuje výrazná reakce na indukční působení řady jeho substrátů. Svou roli hrají i další faktory jako jsou věk, dieta, hormonální stav a zánětlivé procesy [7]. Význam genetického polymorfismu nebyl u CYP3A4 prokázán [8], výraznější úlohu pravděpodobně zastupuje takzvaný regulační polymorfismus, tedy genetické variace jaterních transkripčních faktorů regulujících transkripci CYP3A4 [9]. Indukce exprese CYP3A4 na úrovni genu jsou spolu s inhibicí jeho enzymové aktivity na úrovni proteinu hlavní příčinou vzniku lékových interakcí. Proto není překvapivé úsilí vědců objasnit strukturu CYP3A4 promotoru, okolnosti jeho genové exprese a regulace transkripce.

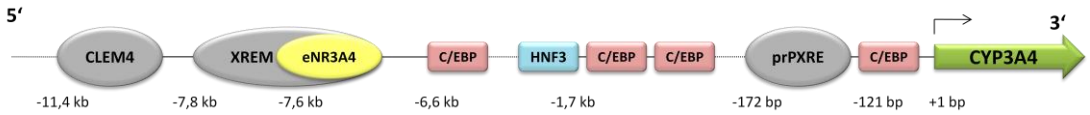
Transkripce genu pro CYP3A4 probíhá způsobem konstitutivním i indukovatelným. Konstitutivní transkripci v hepatální tkáni zajišťuje řada jaterně specifických transkripčních faktorů (LETFs, liver-enriched transcription factors) zahrnující HNF1α, HNF4α, HNF3γ (hepatocyte nuclear factor), C/EBPα a C/EBPβ (CCAAT/enhancer binding protein) a další. Podílí se na ní také hormony, jako jsou glukokortikoidy, růstový hormon a trijodthyronin (bez H) [7]. Aktivita konstitutivní transkripce se přizpůsobuje základním potřebám buňky.

Indukovatelná transkripce *CYP3A4* je vyvolána řadou chemicky odlišných látek (Tabulka 1). Jedná se zejména o up-regulaci (indukci) transkripce, tedy o regulaci pozitivní zpětnou vazbou, v jejímž důsledku dochází k dramatickému zvýšení exprese a aktivity CYP3A4 na přítomnost xenobiotika.

Významné induktory **CYP3A4**	
aminoglutetimid	lansoprazol
artemisinin	modafinil
dexametazon	nevirapin
efavirenz	omeprazol
fenobarbital	oxkarbazepin
fenytoin	primidon
hyperforin	rifampicin
karbamazepin	troglitazon

Tabulka 1: Významné induktory CYP3A4 [1].

Není to však jediný způsob, jakým xenobiotika působí na úrovni transkripce CYP3A4. Byly odhaleny látky s potenciálem down-regulace již aktivované (indukované) i konstitutivní exprese [10][11][12][13] (spojit !!). Indukovatelná transkripce se spouští aktivací nukleárních receptorů PXR (pregnane X receptor), CAR (constitutive androstane receptor), VDR (vitamin D receptor), GRα (glucocorticoid receptor) a FXR (farnesoid X receptor) v důsledku přítomnosti specifického xenobiotika či zvýšené hladiny některých endogenních látek (hormony, žlučové kyseliny). Struktura promotoru CYP3A4 s vyznačenými oblastmi pro příslušné receptory a faktory konstitutivní a indukovatelné transkripce je schematicky znázorněná na obrázku (Obrázek 1). JEŠŤĚ CHYBÍ DRE v XREMu



Obrázek 1: Struktura CYP3A4 promotoru. CYP3A4 promotor o velikosti 35,8 kb byl analyzován pouze 13 kb od transkripčního startu [9, 14, 15]. Pro vazbu PXR a CAR byly identifikovány oblasti prPXRE (obsahuje ER6) a XREM (zahrnuje sekvenci DR3 a místo pro vazbu HNF4α). Modul CLEM byl vyhodnocen jako oblast bazální aktivace transkripce, jeho součástí je sekvence pro vazbu HNF1α a HNF4α. Později byla potvrzena také indukce prostřednictvím interakce PXR a této oblasti a identifikován responzivní element ER6 [16]. Zatím nejnovější studovanou sekvenci představuje oblast eNR3A4, která byla lokalizována uvnitř XREM. Byl prokázán její podíl na PXR aktivované transkripci prostřednictvím responzivního elementu DR4 [17]. Na obrázku jsou dále vyznačeny místa vazby dalších LETFs. Popsané vztahy vysvětlují okolnosti specifické transkripce CYP3A4 v hepatální tkáni. Schéma se nezabývá oblastmi pro vazbu obecných transkripčních faktorů.

Výsledkem indukce exprese CYP3A4 je zintenzivnění biotransformace jeho substrátů, a tím snížení jejich plazmatických hladin a léčebného efektu. Pokud je metabolit léčiva nositelem farmakodynamického účinku, dochází k překročení maximálních plazmatických koncentrací a manifestaci toxického účinku.

1.3 Nukleární receptory podílející se na regulaci CYP3A4

1.3.1 Xenosenzory PXR a CAR

Pregnanový X receptor (PXR; NR1I2) a konstitutivní androstanový receptor (CAR; NR1I3) představují klíčové nukleární receptory zabezpečující indukovatelnou a do jisté míry také konstitutivní transkripci CYP3A4. Jsou aktivovány především xenobiotiky, kterým zajišťují eliminaci regulací exprese biotransformačních enzymů a lékových transportérů. Pro tuto funkci se označují také jako buněčné xenosenzory.

PXR a CAR patří spolu s dalšími receptory do podrodiny nukleárních receptorů NR1). Sdílejí společnou proteinovou strukturu, kterou tvoří vysoce rozmanitá N-terminální část, centrální oblast vážící se na DNA (DBD, DNA binding domain) a C-terminální část vážící ligand (LBD, ligand binding domain). DBD je přibližně 70 aminokyselin dlouhá a sestává se ze dvou zinkových prstů tvořených cysteinovými zbytky, které chelatují zinek. LBD čítá přibližně 250 aminokyselin a formuje se do hydrofobní kapsy vázající ligand. Její součástí jsou sekvence umožňující dimerizaci a vazbu koaktivátorů po navázání ligandu (AF-1, AF-2) [18].

I jejich signální mechanismus je podobný, po navázání ligandu tvoří heterodimer s receptorem pro 9-*cis*-retinovou kyselinu (retinoid X receptor, RXRα), v této formě se váží na responzivní elementy cílového genu, čímž způsobují uvolnění korepresorových proteinů a naopak zformování obecných transkripčních faktorů a jejich koaktivátorů. Responzivní elementy pro vazbu heterodimeru RXRα jsou tvořeny párem sekvencí hexanukleotidů (5‘-AGGTCA-3‘), které se podle vzájemné orientace označují zkratkami DR (direct repeat), ER (everted repeat) a IR (inverted repeat). Podle počtu vmezeřených nukleotidů je ke zkratce dále přiřazeno číslo 1-8 [18, 19].

1.3.1.1 *PXR*

PXR je exprimován v játrech, tenkém a tlustém střevě u člověka, králíka, potkana i myši [18] [20]. Jeho LBD se mezi druhy významně liší, což je příčinou rozdílné substrátové specificity a aktivačního profilu. Humánní PXR je nejvýznamnějším receptorem regulujícím transkripci genů pro enzymy I. a II. fáze biotransformace jako i některých lékových transportérů. PXR představuje hlavní receptor zprostředkující léčivem vyvolanou aktivaci CYP3A4 transkripce a je zodpovědný za mnohé lékové interakce, ke kterým dochází v důsledku indukce *CYP3A4*.

Krystalografická struktura LBD PXR receptoru znázorňuje velkou flexibilní hydrofobní kapsu [19]. Pro širokou substrátovou specificitu LBD je PXR aktivován řadou látek, jedná se zejména o lipofilní organické molekuly < 1000 Da. Mezi endogenní ligandy patří steroidy (5β-pregnan-3,20-dion, estradiol a kortikosteron) a žlučové kyseliny (litocholová kys. a 3-keto-litocholová kys.) [18]. Vázající se xenobiotika jsou uvedena v

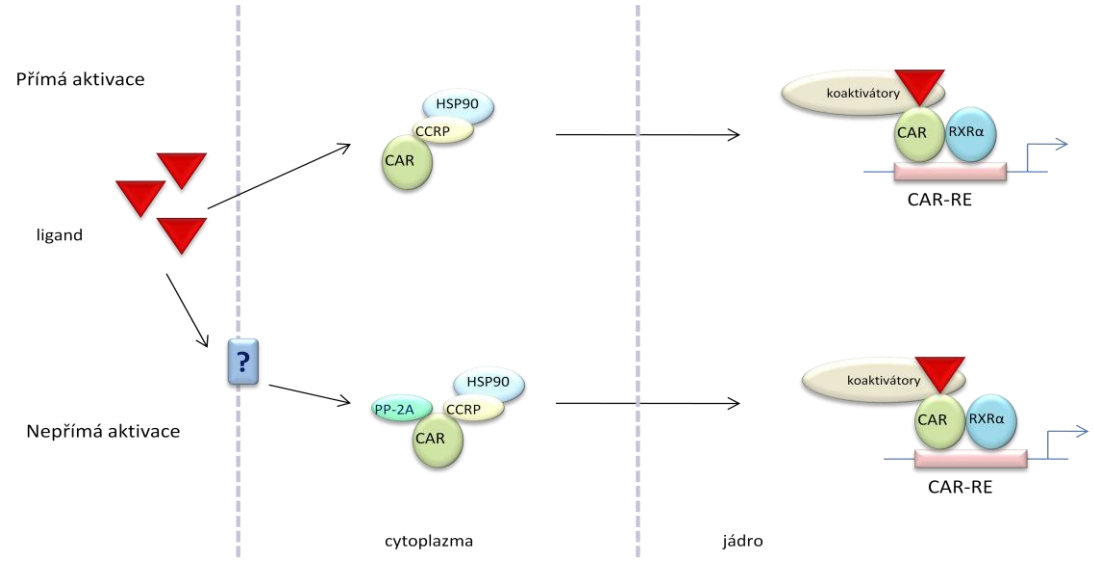
Tabulka ***2***. Ligandem aktivovaný receptor nasedá na responzivní oblasti DR-3, DR-4, ER-6 v promotorových oblastech cílových genů. Reguluje expresi enzymů CYP3A4, CYP3A5, CYP3A7, CYP2C9, CYP2C19, CYP2B6, CYP2A6, UGT1A1-4 (UDP-glukoronosyltransferáza), SULT2A1 (sulfotransferáza), MDR1 (multidrug resistance), MRP2 multidrug resistance protein), OATP1B1 (organic anion transporting polypeptide) [19]. Pro svůj plný indukční účinek na CYP3A4 promotoru vyžaduje interakci s dalšími transkripčními faktory a koaktivátory transkripce, konkrétně s HNF4α, SRC-1 (steroid receptor coactivator 1) a PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1α). Konstitutivní transkripční aktivita tohoto receptoru je dosud sporná [21] [22] sloučit.

Agonisté		Antagonisté
artemisin	kava extrakt	ketokonazol
bosentan	klotrimazol	koumestrol
dexametazon	lovastatin	sulforafan isothiokyanát
efavirenz	nifedipin	
etoposid	nikardipin	
fenobarbital	paklitaxel	
forskolin	rifampicin	
ginkgo extrakt	ritonavir	
guggulsteron	spironolakton	
hyperforin	tamoxifen	
isradipin	topiramát	
karbamazepin	topotekan	

Tabulka 2: Syntetická léčiva a přírodní produkty in vitro(proč in vitro ?) modulující aktivitu PXR [11, 23-25].

1.3.1.2 *CAR*

Hlavním místem exprese nukleárního receptoru CAR jsou játra, v menší míře je dále exprimován v ledvinách [20]. PXR a CAR jsou si velmi blízké po stránce struktury i funkce, sdílejí primární strukturu DBD a LBD ze 70 % a 50 %. Liší se ve flexibilitě LBD kapsy, substrátová specificita pro CAR je v důsledku méně přizpůsobivé struktury LBD omezenější, přesto tyto dva receptory sdílejí některé ligandy [26]. Vyšší substrátová selektivita je též kompenzována možností nepřímé aktivace receptoru, která spočívá ve stimulaci transportu CAR do jádra prostřednictvím xenobiotikem navozené fosforylace proteinů, které ho fixují v cytoplazmě (Obrázek 2) [23]. Kromě indukovatelné aktivity vykazuje CAR také významnou aktivitu konstitutivní, která může být modulována inverzními agonisty [27].



Obrázek 2: Schéma aktivace CAR přímým a nepřímým mechanismem. V inaktivním stavu je CAR fixován v cytoplazmě proteiny CCRP (CAR cytoplasmic retention protein) a HSP90 (heat shock protein 90). K aktivaci dochází přímou vazbou ligandu na LBD receptoru, nebo nepřímo, nedostatečně vysvětlenou signální kaskádou, která zahrnuje navázání PP-2A (protein phosphatase 2A) na komplex CAR s proteiny. Následkem uvolnění z vazby na proteiny se CAR translokuje do jádra, kde dimerizuje s RXRα, nasedá na svůj responzivní element (CAR-RE), váže koaktivátory transkripce a spouští přepis [28].

Endogenní ligandy zahrnují androstanol, androstenol, žlučové kyseliny a bilirubin [29]. Exogenní ligandy jsou shrnuty v

Tabulka ***3***. Aktivní CAR nasedá na responzivní elementy DR-3, DR-4 a ER-6. Spolu s PXR koregulují expresi enzymů CYP3A4, CYP2C9, CYP2B6, UGT1A1, MDR1 a MRP2, čímž vytvářejí spletitý systém regulace biotransformačních procesů [30]. Bylo však prokázáno, že na rozdíl od PXR, coby klíčového regulátoru CYP3A4, představuje CAR hlavní regulátor genu pro enzym CYP2B6 [31].

Agonisté a aktivátory		**Inverzní agonisté**
6,7-dimethyleskuletin	fenobarbital	klotrimazol
acetaminofen	fenytoin	guggulsteron
artemisin	fluvastatin	meklizin
atorvastatin	klofibrát	
cerivastatin	orfenadrin	
CITCO	pravastatin	

Tabulka 3: Syntetická léčiva a přírodní produkty in vitro modulující aktivitu CAR (CITCO; ((6-(4-chlorfenyl)imidazo[2,1-b][1,3]thiazol-5-karbaldehyd O-(3,4-dichlorbenzyl)oxim)) [32][23].

1.3.2 GRα

Glukokortikoidní receptor (GRα; NR3C1) patří do podrodiny NR3, nukleární receptory podobné estrogenu (estrogen-like). Váže glukokortikoidy a mineralokortikoidy a je tudíž zodpovědný za jejich genomický efekt na metabolismus sacharidů a lipidů, v procesech stresu a zánětu i v regulaci minerální a vodné (není nějaký odbornější termín?) homeostázy. Inaktivní GRα je lokalizován v cytoplazmě vázaný na multiproteinový komplex. Po aktivaci navázáním ligandu přestupuje do jádra, kde tvoří homodimer s dalším GRα. Dimerizace je nezbytná pro vazbu na glukokortikoidní responzivní element ve struktuře DNA, který je charakterizován sekvencí 5‘-XXTACAXXXTGTTCT-3‘, obsahující vazebná místa pro obě podjednotky dimeru [33].

Mechanismus působení GRα v regulaci transkripce CYP3A4 není přímý jako v případě výše uvedených receptorů a je závislý na přítomnosti glukokortikoidů. GRα aktivovaný glukokortikoidy přítomnými v buňce ve fyziologickém množství aktivuje transkripci a indukuje *PXR, CAR* a jejich partnera pro tvorbu dimeru *RXRα,* a tím intracelulární dispozici těchto receptorů [34].

1.3.3 HNF4α

Hepatocytární nukleární faktor 4α (HNF4α; NR2A1) je součástí podrodiny receptorů NR2, nukleární receptory podobné HNF4 (HNF4-like). Vyskytuje se hlavně v játrech, v menší míře v ledvinách, tenkém střevě, kolonu a B-buňkách pankreatu. Řadí se do skupiny jaterně specifických transkripčních faktorů, reguluje konstitutivní expresi mnoha genů pro enzymy, transportéry a další nukleární receptory, a tak se podílí na kontrole metabolismu glukózy, cholesterolu a mastných kyselin, syntéze koagulačních faktorů a diferenciaci jaterní tkáně. Mutace genu pro HNF4α jsou spojovány s diabetem prvního typu. HNF4α je také označován jako sirotčí receptor (orphan receptor), tedy receptor bez známých ligandů. Přestože se prokázala jeho schopnost vázat estery acyl-CoA, není jasné, jestli tato vazba ovlivňuje jeho aktivitu. HNF4α je lokalizován přímo v jádře buňky, na promotory kontrolovaných genů nasedá ve formě homodimeru a tvoří vazbu s responzivním elementem DR1 či DR2 [35][36].

Aktivace transkripce CYP3A4 prostřednictvím PXR a CAR vyžaduje přítomnost HNF4α [37], předpokládá se, že umožňuje vazbu dalších faktorů transkripce (SRC-1, PGC-1α) k formujícímu se komplexu [38].

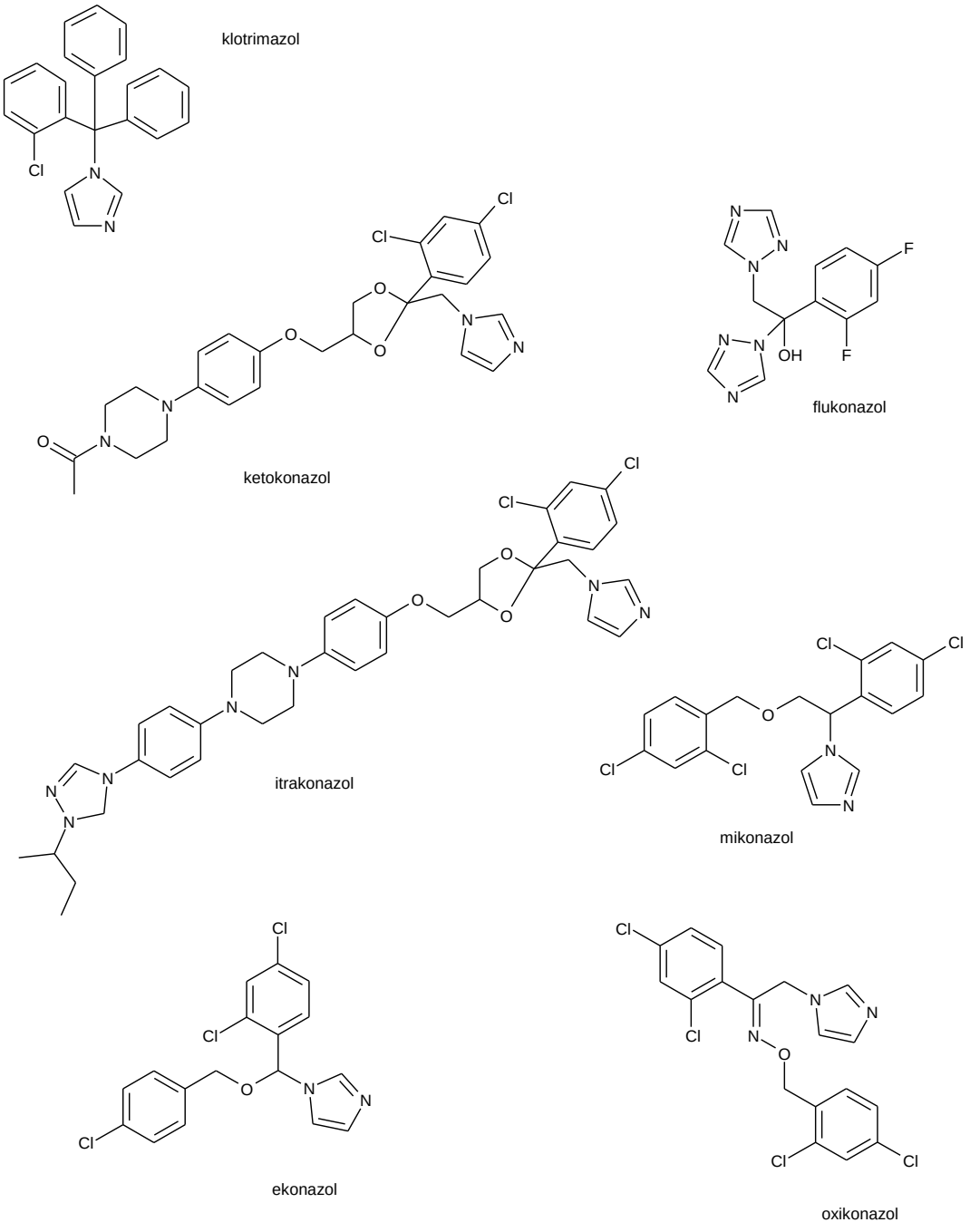
1.4 Studovaná léčiva

1.4.1 Azolová antimykotika

Azolová antimykotika (Obrázek 3) tvoří skupinu léčiv používaných k terapii lokálních i systémových fungálních infekcí. Jejich preskripce stále stoupá v souladu se zvyšující se incidencí systémových mykóz u vážně nemocných pacientů. Jsou to však látky s významným vlivem na aktivitu CYP3A4 a jejich charakteristiky čítají řady popsaných lékových interakcí [1]. Jejich účinek na CYP3A4 se do jisté míry vysvětluje dvěma způsoby. Jednak samotným mechanismem účinku azolů, tedy inhibicí fungálního cytochromového systému, který však není dostatečně selektivní a postihuje i CYP3A4 hostitele. Další příčinou je způsob biotransformace zejména lipofilních azolů, kterou zajišťuje CYP3A4 a při níž může dojít k inhibici jeho enzymové aktivity.

Azolová antimykotika však dále vykazují rozmanitý efekt na transkripci *CYP3A4*. Klotrimazol a ketokonazol jsou jedněmi z prvních popsaných ligandů PXR. Klotrimazol výrazně aktivuje expresi *CYP3A4* prostřednictvím PXR, zatímco ketokonazol vazbou na PXR down-reguluje transkripci indukovanou rifampicinem [12]. Klotrimazol je dlouhodobě známým inverzním agonistou CAR. Dále byla popsána schopnost ketokonazolu a mikonazolu down-regulovat expresi CYP3A4 indukovanou dexametazonem prostřednictvím GRα [39].

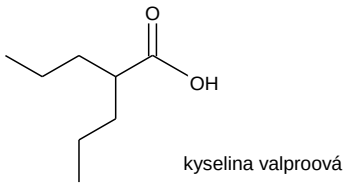
Je kupodivu, že látky se shodným farmakodynamickým působením a více či méně podobnou strukturou vykazují rozdílný efekt na úrovni exprese *CYP3A4*. Na základě těchto poznatků, je možno předpokládat, že dalším mechanismem LI s azolovými antimykotiky je ovlivnění transkripce *CYP3A4* prostřednictvím PXR.



Obrázek 3: Chemické struktury testovaných azolových antimykotik.

1.4.2 Kyselina valproová

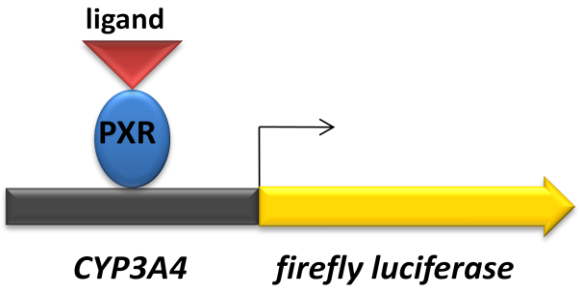
Kyselina valproová (Obrázek 4) je významným a relativně dobře snášeným terapeutikem generalizované a parciální epilepsie, dále se užívá k akutní léčbě a prevenci manické fáze u bipolárních poruch. V současnosti se sleduje její efekt na buněčný růst, diferenciaci, apoptózu a imunogenicitu nádorových buněk [40] Valproát se biotransformuje oxidací prostřednictvím mitochondriálních oxidáz a glukuronidací. Efekt valproátu na expresi genů byl v minulosti testován u potkanů, využitím metody microarray byly detekovány změny v expresi 121 genů [41]. Byla zaznamenána schopnost indukovat *Cyp2b1*, *Cyp2b2* [42] [43] a *Cyp3a2*, ortolog lidského *CYP3A4* [44]. Dále byl zjištěn *in vitro* inhibiční vliv na histondeacetylázy (HDAC), který vede k změně konformace chromatinu a aktivaci transkripce *MDR1* [44]. Inducibilita *CYP3A4* prostřednictvím valproátu nebyla již dále testována.



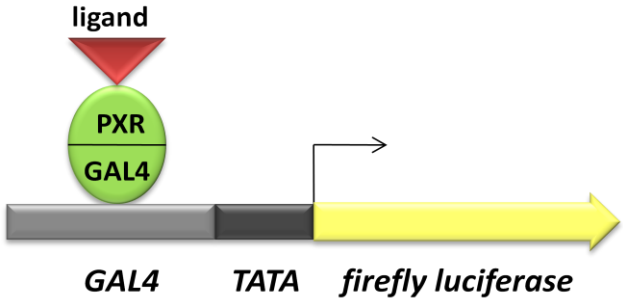
Obrázek 4: Chemická struktura kyseliny valproové.

1.5 Metodika

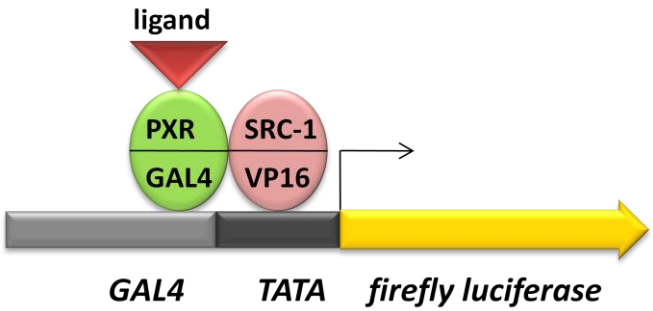
Základem experimentálních prací byly *in vitro* molekulárně biologické metody. Jedná se zejména o gene reporter assay, real time RT-PCR a EMSA (Electrophoretic Mobility Shift Assay). Gene reporter assay (Obrázek 5) a jeho variace one hybrid assay (Obrázek 6) a two hybrid assay (Obrázek 7) jsou metody umožňující testovat a kvantifikovat potenciál léčiv aktivovat daný promotor prostřednictvím sledovaného nukleárního receptoru, případně podíl dalších transkripčních faktorů na této aktivaci. Real time RT-PCR je rozšířená, vysoce citlivá metoda s širokým využitím, kterou není třeba detailněji představovat. Umožňuje relativní kvantifikaci množství mRNA sledovaného genu a změny tohoto množství v důsledku přítomnosti léčiva. EMSA (Obrázek 8) je elektroforetická metoda umožňující detekovat míru interakce DNA s nukleárními receptory v přítomnosti či nepřítomnosti léčivé látky.



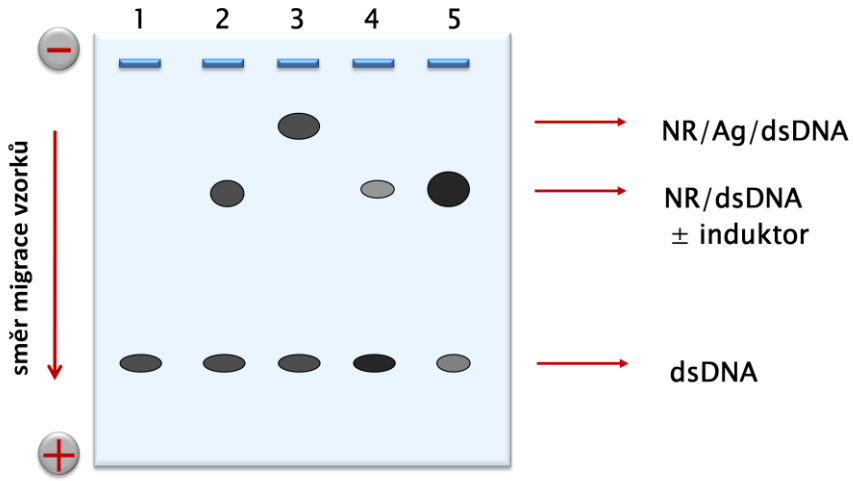
Obrázek 5: Gene reporter assay. Buňky se prostřednictvím lipozomů transfekují reportérovým plazmidem, který obsahuje promotor sledovaného genu (CYP3A4) a strukturní gen pro luciferázu (firefly luciferase). Spolu s ním je možno kotransfekovat expresní plazmid kódující sledovaný nukleární receptor (PXR) a expresní plazmid pro neinducibilní luciferázu jiného typu (renilla luciferase), který slouží ke standardizaci účinnosti transfekce, počtu a viability buněk. Transfekované buňky se poté inkubují s testovanými léčivy. Schopnost látek transaktivovat promotor prostřednictvím vazby na nukleární receptor se projeví změnou v expresi firefly luciferázy. Vyhodnocení experimentu se provádí pomocí luminometru, zaznamenává se intenzita luminiscence pro luciferázy obou typů. Porovnávají se podíly těchto hodnot získané z léčivy ovlivněných vzorků a neovlivněné kontroly.



Obrázek 6: One hybrid assay. Metoda slouží k vyhodnocení afinity sledované látky k LBD na PXR receptoru a schopnost vytvořeného komplexu ovlivnit transkripci strukturního genu. Do buněk se vnáší expresní plazmid kódující hybridní protein LBD PXR a GAL4, což je nukleární protein přítomný v kvasinkách, který zprostředkovává aktivaci určitých genů prostřednictvím galaktózy. Dále se kotransfekují reportérový plazmid obsahující promotor s responzivní oblastí pro GAL4 následovaný strukturním genem pro luciferázu (firefly luciferase) a expresní plazmid pro luciferázu typu renilla. Aktivovaný PXR zvyšuje expresi luciferázy, a tím i analyzovanou luminiscenci. Experiment se vyhodnocuje stejným způsobem jako v případě 2A.



Obrázek 7: Two hybrid assay. Metoda vyhodnocuje schopnost ligandem aktivovaného PXR tvořit komplex s koaktivátorem transkripce SRC-1. Buňky jsou transfekovány expresními plazmidy pro hybridní proteiny PXR/GAL a SRC-1/VP16. VP16 je transkripční aktivátor herpes simplex viru, který umožňuje nasednutí polymerázy typu PolII na TATA box a iniciovat transkripci. Kotransfekovaný reportérový gen obsahuje promotor s responzivní oblastí pro GAL4 a TATA boxem, dále je jeho součástí strukturní gen pro firefly luciferázu. Zvýšená schopnost aktivovaného PXR tvořit komplex se SRC-1 vede k interakci podjednotek GAL4 a VP16 s promotorem, což se projeví významným nárůstem luminiscence v důsledku zvýšené transkripce genu pro luciferázu. Vyhodnocení se provádí stejným způsobem jako v předchozích případech.



Obrázek 8: EMSA. Technika je založena na zpomalení migrace komplexu dvouřetězcové biotinylované dsDNA a nukleárního receptoru (NR) (2) oproti nevázané značené dsDNA (1) na vertikálním polyakrylamidovém gelu. Použitá dsDNA obsahuje sekvenci studovaného responzivního elementu a sleduje se její schopnost vázat nukleární receptor. Nukleární receptory je možno získat rekombinantní přípravou či izolací jaderných frakcí buněk. Otestování a porovnání obou variant umožňuje určení podílu dalších proteinů přítomných v jádře na vazbě nukleárního receptoru k responzivnímu elementu. Specificitu je možno ověřit přidáním protilátky proti nukleárnímu receptoru (Ag), která po navázání způsobí výraznější zpomalení migrace komplexu (3). Další možností je přidání několikanásobně většího množství ne-biotinylované dsDNA stejné struktury jako značená dsDNA, která vede k zeslabení intenzity bandu (4) v důsledku vytěsnění značené dsDNA. Schopnost léčiva transaktivovat vznik komplexu se projeví změnou intenzity bandu (5 jako příklad efektu induktoru). Detekce je chemiluminiscenční, kvantifikaci vizualizovaných bandů lze provést pomocí počítačových programů.

Experimenty byly realizovány s využitím kultur primárních lidských buněk a lidských buněčných linií. Používání zvířat není za tímto účelem doporučeno pro významnou mezidruhovou variabilitu jak enzymatické výbavy, tak i způsobu její genové regulace. Primární kultury lidských hepatocytů jsou považovány za standardní model s významnou výpovědní hodnotou týkající se biotransformace léčiv. Tento *in vitro* systém vykazuje podobný metabolický profil, jaký je přítomen *in vivo*. Primární hepatocyty exprimují většinu biotransformačních enzymů, které odpovídají na přítomnost induktorů [45]. Jsou izolovány z lidských jater a kultivovány po dobu několika dnů, zatímco si zachovávají dospělý fenotyp. Nevýhodou tohoto systému jsou omezené možnosti získání vhodných lidských jater, další komplikací je krátká životnost diferencovaných buněk, které nemají schopnost proliferovat. Navíc není možné hepatocyty jednoduše transfekovat prostřednictvím lipozomů.

Na rozdíl od primárních kultur buněčné linie téměř neomezeně rostou, přičemž si udržují víceméně stabilní fenotyp. Jsou snadno dostupné a standardizované mezi laboratořemi. Jedná se o zčásti dediferencovaný model, který však neexprimuje většinu jaterně specifických enzymů (

Tabulka ***4***) a transkripčních faktorů, je však snadno transfekovatelný. Proto je možné jej výhodně použít pro gene reporter assay experimenty a sledované struktury do buněk vnést pomocí plazmidů.

	CYP1A1	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Hepatocytes	100	100	100	100	100
HepG2	6,99	0,01	0,05	1,57	0,03

Tabulka 4: Srovnání relativní exprese mRNA jednotlivých cytochromů v primární kultuře hepatocytů a lidské hepatomové HepG2 linii [46].

1.6 Cíle disertační práce

Jak již bylo řečeno v příslušné kapitole, CYP3A4 je biotransformační enzym většiny metabolizovaných xenobiotik. Je také hlavní příčinou lékových interakcí. Odhlédneme-li od funkční inhibice tohoto enzymu, dalším významným mechanismem ovlivnění jeho aktivity je regulace na úrovni transkripce *CYP3A4* genu. Již několik let se vědci detailně zabývají okolnostmi této regulace, strukturou *CYP3A4* promotoru, určením typu a umístění responzivních oblastí pro nukleární receptory, spoluúčastí transkripčních faktorů a v neposlední řadě efekty léčiv na transkripci. Jedná se o širokou problematiku, která stále skýtá řadu otázek. Tato práce je výsledkem mého podílu na několika projektech, kterými se naše pracovní skupina zabývá, a je zaměřena na okolnosti hepatální genové regulace CYP3A4.

Dílčí cíle této práce byly:

1. Vliv vybraných azolových antimykotik na genovou expresi CYP3A4 prostřednictvím PXR. Objasnění povahy sledovaných efektů na molekulární úrovni (2.1).

2. Účinek kyseliny valproové na genovou expresi CYP3A4 prostřednictvím CAR. Zavedení metody EMSA na naše pracoviště za účelem testování interakcí adekvátních responzivních elementů v přítomnosti či nepřítomnosti kyseliny valproové (2.2).

3. Objasnění role HNF4α při aktivaci CYP3A4 promotoru prostřednictvím GRα. Vysvětlení jaterní specificity této aktivace (2.3).

Tato dizertační práce je koncipována jako soubor publikací, uveřejněných v odborných časopisech, které se bezprostředně týkají tématu práce.

1.7 Použitá literatura

1. Suchopár, J., Buršík, J., Mach, R., Prokeš, M., *Kompendium lékových interakcí Infopharm^{R} 2005*. 2004: Infopharm.

2. Martínková, J., *Farmakologie (pro studenty zdravotnických oborů)*. 2007: Grada. 380.

3. Suchopár, J., *Metabolismus léčiv, genetický polymorfismus a důsledky na dávkování a lékové interakce.* PACE news, 2008(1): p. 12-16.

4. Shimada, T., et al., *Roles of individual human cytochrome P-450 enzymes in the bioactivation of benzo(a)pyrene, 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons.* Cancer Res, 1989. **49**(22): p. 6304-12.

5. Martinez-Jimenez, C.P., et al., *Transcriptional regulation and expression of CYP3A4 in hepatocytes.* Curr Drug Metab, 2007. **8**(2): p. 185-94.

6. Eichelbaum, M. and O. Burk, *CYP3A genetics in drug metabolism.* Nat Med, 2001. **7**(3): p. 285-7.

7. Jover, R., et al., *Down-regulation of human CYP3A4 by the inflammatory signal interleukin-6: molecular mechanism and transcription factors involved.* Faseb J, 2002. **16**(13): p. 1799-801.

8. Mičuda, S., Martínková, J., Chládek, J., Anzenbacher P., *Význam polymorfismu metabolismu léčiv v moderní farmakoterapii.* Remedia, 1998. **8**(4): p. 226-236.

9. Schuetz, E.G., *Lessons from the CYP3A4 promoter.* Mol Pharmacol, 2004. **65**(2): p. 279-81.

10. Moore, L.B., et al., *Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors.* Mol Endocrinol, 2002. **16**(5): p. 977-86.

11. Zhou, C., et al., *The dietary isothiocyanate sulforaphane is an antagonist of the human steroid and xenobiotic nuclear receptor.* Mol Pharmacol, 2007. **71**(1): p. 220-9.

12. Huang, H., et al., *Inhibition of drug metabolism by blocking the activation of nuclear receptors by ketoconazole.* Oncogene, 2007. **26**(2): p. 258-68.

13. Healan-Greenberg, C., et al., *A human immunodeficiency virus protease inhibitor is a novel functional inhibitor of human pregnane X receptor.* Drug Metab Dispos, 2008. **36**(3): p. 500-7.

14. Goodwin, B., E. Hodgson, and C. Liddle, *The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module.* Mol Pharmacol, 1999. **56**(6): p. 1329-39.

15. Robertson, G.R., et al., *Transgenic mouse models of human CYP3A4 gene regulation.* Mol Pharmacol, 2003. **64**(1): p. 42-50.

16. Liu, F.J., et al., *The far and distal enhancers in the CYP3A4 gene co-ordinate the proximal promoter in responding similarly to the pregnane X receptor but differentially to hepatocyte nuclear factor-4alpha.* Biochem J, 2008. **409**(1): p. 243-50.

17. Toriyabe, T., et al., *Unveiling a new essential cis element for the transactivation of the CYP3A4 gene by xenobiotics.* Mol Pharmacol, 2009. **75**(3): p. 677-84.

18. Kliewer, S.A., B. Goodwin, and T.M. Willson, *The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism.* Endocr Rev, 2002. **23**(5): p. 687-702.

19. Tirona, R.G. and R.B. Kim, *Nuclear receptors and drug disposition gene regulation.* J Pharm Sci, 2005. **94**(6): p. 1169-86.

20. Nishimura, M., S. Naito, and T. Yokoi, *Tissue-specific mRNA expression profiles of human nuclear receptor subfamilies.* Drug Metab Pharmacokinet, 2004. **19**(2): p. 135-49.

21. Pascussi, J.M., et al., *Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor.* Eur J Biochem, 2001. **268**(24): p. 6346-58.

22. Wolbold, R., et al., *Sex is a major determinant of CYP3A4 expression in human liver.* Hepatology, 2003. **38**(4): p. 978-88.

23. Chang, T.K. and D.J. Waxman, *Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR).* Drug Metab Rev, 2006. **38**(1-2): p. 51-73.

24. Wang, H., et al., *The phytoestrogen coumestrol is a naturally occurring antagonist of the human pregnane X receptor.* Mol Endocrinol, 2008. **22**(4): p. 838-57.

25. Yeung, E.Y., et al., *Identification of Ginkgo biloba as a novel activator of pregnane X receptor.* Drug Metab Dispos, 2008. **36**(11): p. 2270-6.

26. Moore, L.B., et al., *Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands.* J Biol Chem, 2000. **275**(20): p. 15122-7.

27. Qatanani, M. and D.D. Moore, *CAR, the continuously advancing receptor, in drug metabolism and disease.* Curr Drug Metab, 2005. **6**(4): p. 329-39.

28. Goodwin, B. and J.T. Moore, *CAR: detailing new models.* Trends Pharmacol Sci, 2004. **25**(8): p. 437-41.

29. Moore, D.D., et al., *International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor.* Pharmacol Rev, 2006. **58**(4): p. 742-59.

30. Urquhart, B.L., R.G. Tirona, and R.B. Kim, *Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs.* J Clin Pharmacol, 2007. **47**(5): p. 566-78.

31. Faucette, S.R., et al., *Differential regulation of hepatic CYP2B6 and CYP3A4 genes by constitutive androstane receptor but not pregnane X receptor.* J Pharmacol Exp Ther, 2006. **317**(3): p. 1200-9.

32. Maglich, J.M., et al., *Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes.* J Biol Chem, 2003. **278**(19): p. 17277-83.

33. Czock, D., et al., *Pharmacokinetics and pharmacodynamics of systemically administered glucocorticoids.* Clin Pharmacokinet, 2005. **44**(1): p. 61-98.

34. Pascussi, J.M., et al., *The expression of CYP2B6, CYP2C9 and CYP3A4 genes: a tangle of networks of nuclear and steroid receptors.* Biochim Biophys Acta, 2003. **1619**(3): p. 243-53.

35. Schrem, H., J. Klempnauer, and J. Borlak, *Liver-enriched transcription factors in liver function and development. Part I: the hepatocyte nuclear factor network and liver-specific gene expression.* Pharmacol Rev, 2002. **54**(1): p. 129-58.

36. Gonzalez, F.J., *Regulation of hepatocyte nuclear factor 4 alpha-mediated transcription.* Drug Metab Pharmacokinet, 2008. **23**(1): p. 2-7.

37. Tirona, R.G., et al., *The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4.* Nat Med, 2003. **9**(2): p. 220-4.

38. Li, T. and J.Y. Chiang, *Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression.* Drug Metab Dispos, 2006. **34**(5): p. 756-64.

39. Duret, C., et al., *Ketoconazole and miconazole are antagonists of the human glucocorticoid receptor: consequences on the expression and function of the constitutive androstane receptor and the pregnane X receptor.* Mol Pharmacol, 2006. **70**(1): p. 329-39.

40. Kostrouchova M., K.Z., Kostrouchová M., *Valproic acid, a molecular lead to multiple regulatory pathways.* Folia Biologica, 2007(53): p. 37-49.

41. Bosetti, F., J.M. Bell, and P. Manickam, *Microarray analysis of rat brain gene expression after chronic administration of sodium valproate.* Brain Res Bull, 2005. **65**(4): p. 331-8.

42. Rogiers, V., et al., *Effects of valproate on xenobiotic biotransformation in rat liver. In vivo and in vitro experiments.* Pharm Weekbl Sci, 1992. **14**(3A): p. 127-31.

43. Rogiers, V., et al., *Effects of the anticonvulsant, valproate, on the expression of components of the cytochrome-P-450-mediated monooxygenase system and glutathione S-transferases.* Eur J Biochem, 1995. **231**(2): p. 337-43.

44. Eyal, S., et al., *The antiepileptic and anticancer agent, valproic acid, induces P-glycoprotein in human tumour cell lines and in rat liver.* Br J Pharmacol, 2006. **149**(3): p. 250-60.

45. Castell, J.V., et al., *Hepatocyte cell lines: their use, scope and limitations in drug metabolism studies.* Expert Opin Drug Metab Toxicol, 2006. **2**(2): p. 183-212.

46. Donato, M.T., et al., *Cell lines: a tool for in vitro drug metabolism studies.* Curr Drug Metab, 2008. **9**(1): p. 1-11.

2 Publikované práce

2.1 Azole antimycotics differentially affect rifampicin-induced pregnane X receptor-mediated *CYP3A4* gene expression.

Svecova L, Vrzal R, Burysek L, Anzenbacherova E, Cerveny L, Grim J, Trejtnar F, Kunes J, Pour M, Staud F, Anzenbacher P, Dvorak Z, Pavek P. *Azole antimycotics differentially affect rifampicin-induced pregnane X receptor-mediated CYP3A4 gene expression.* Dug Metabolism and Disposition36:339–348, 2008. **(IF 3,907$_{2007}$)**

Abstrakt:

Jak již bylo dříve prokázáno, azolové antimykotikum ketokonazol down-reguluje transkripci *CYP3A4* genu indukovanou aktivovaným PXR receptorem, a to narušením interakce PXR s koaktivátorem transkripce SRC-1. Naopak některá (která-jen klotrimazol ne?) azolová antimykotika, která mají svou strukturu odvozenou od klotrimazolu, jsou známa svou schopností vázat se na PXR, a tím významně indukovat *CYP3A4*. V této studii jsme se zabývali účinky azolových antimykotik klotrimazolu, ketokonazolu, ekonazolu, oxikonazolu, mikonazolu, flukonazolu a itrakonazolu na expresi *CYP3A4* zprostředkovanou PXR. Otestovali jsme účinky jednotlivých azolů na bazální i rifampicinem indukovanou expresi *CYP3A4* v buněčné linii LS174T a v kultuře primárních lidských hepatocytů, jejich interakce s LBD PXR receptoru a jejich vliv na interakci SRC-1 s PXR. Dále jsme s využitím dose-response analýz popsali povahu možných interakcí testovaných azolů se známým PXR ligandem rifampicinem a jejich vliv na transaktivaci *CYP3A4* genu. Zaznamenali jsme aditivní a antagonistické interakce mezi azolovými antimykotiky a rifampicinem na PXR receptoru. Demonstrujeme schopnost oxikonazolu efektivně indukovat *CYP3A4*, která může být kompetitivně antagonizována rifampicinem. Dále ukazujeme, že aktivace *CYP3A4* promotoru je komplexním procesem, který není určován výhradně navázáním azolů na PXR, ale také vlivem některých sledovaných látek na tvorbu komplexu SRC-1/PXR. Jedná se o další mechanismus, který se podílí na spletitém procesu transaktivace *CYP3A4* v přítomnosti či nepřítomnost ligandu PXR receptoru.

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Azole Antimycotics Differentially Affect Rifampicin-Induced Pregnane X Receptor-Mediated *CYP3A4* Gene Expression

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ABSTRACT:

Azole antifungal drug ketoconazole has recently been demonstrated as an inhibitor of a ligand-induced pregnane X receptor (PXR)-mediated transcriptional regulation of the *CYP3A4* gene through disruption of PXR interaction with steroid receptor coactivator (SRC)-1. In contrast, other clotrimazole-derived antifungal agents are known as potent inducers of *CYP3A4* through PXR. In the present study, we examined effects of azole antimycotics clotrimazole, ketoconazole, econazole, oxiconazole, miconazole, fluconazole, and itraconazole on PXR-mediated expression of *CYP3A4*. We investigated individual effects of the tested azoles as well as their action on rifampicin-induced PXR-mediated transactivation and expression of *CYP3A4* in LS174T cell line and primary human hepatocytes, their interactions with PXR ligand-binding domain, and azole-mediated recruitment of SRC-1 to PXR. In addition, applying the pharmacodynamic approach and dose-response analysis, we aimed to describe the nature of potential interactions of tested azole antimycotics coadministered with a prototypical PXR ligand rifampicin in transactivation of *CYP3A4* gene. We describe additive and antagonistic interactions of partial and full agonists of PXR nuclear receptor in the therapeutic group of azole antimycotics in rifampicin-mediated transactivation of *CYP3A4*. We show that oxiconazole is a highly efficacious activator of *CYP3A4* transactivation, which could be antagonized by rifampicin in a competitive manner. In addition, we show that activation of the *CYP3A4* promoter is a complex process, which is not exclusively determined by azole-PXR interactions, and we suggest that the ability of some azoles to affect recruitment of SRC-1 to PXR modulates their net effects in transactivation of *CYP3A4* both in the absence or presence of rifampicin.

Drug-induced expression of *CYP3A4* gene is predominantly regulated through the pregnane X receptor (PXR, steroid and xenobiotic receptor, hPXR, nuclear receptor 1I2), a member of the nuclear receptor family. PXR, an important component of the body's adaptive defense mechanism against xenobiotics, regulates at the transcriptional level a number of genes involved in clearance of xenobiotics (Kliewer et al., 1998, 2002; Synold et al., 2001). Its activation by a variety of prescription drugs leads to drug-drug interactions (DDIs) (Lin, 2006; Urquhart et al., 2007). PXR signaling mechanism involves ligand binding to the receptor, heterodimerization with the 9-cis retinoic acid receptor α (RXRα), binding of the PXR/RXRα heterodimer to response elements (REs) of target genes, release of corepressor proteins, and recruitment of coactivators and the general transcription machinery (Kliewer et al., 1998, 2002; Lehmann et al., 1998; Synold et al., 2001; Kliewer, 2003).

Interactions of several azole antifungals with human PXR have recently been investigated. Clotrimazole, a known inducer of *CYP3A4* expression, and its analogs have been found as highly potent ligands of human PXR, which promotes interaction of PXR with steroid receptor coactivator (SRC)-1 (NCOA1) (Bertilsson et al., 1998; Lehmann et al., 1998; El-Sankary et al., 2001; Ekins et al., 2007; and others). Itraconazole and ketoconazole are weak inducers of *CYP3A4* via PXR (Sinz et al., 2006; Huang et al., 2007). However, ketoconazole was reported to inhibit ligand-induced PXR-mediated transcriptional regulation of *CYP3A4* and *MDR1* genes (Takeshita et al., 2002;

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ABBREVIATIONS: PXR, pregnane X receptor; DDI, drug-drug interaction; RXRα, retinoid X receptor α (9-*cis* retinoic acid receptor-α); RE, response element; SRC, steroid receptor coactivator; RT, reverse transcriptase; PCR, polymerase chain reaction; LBD, ligand-binding domain; FBS, fetal bovine serum; SR12813, tetraethyl 2-(3,5-di-*tert*-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; DMSO, dimethyl sulfoxide; ER, everted repeat; CAR, constitutive androstane receptor; HNF, hepatocyte nuclear factor; GR, glucocorticoid receptor; DR, direct repeat; RID, receptor-interacting domain; VDR, vitamin D receptor; CYP, cytochrome P450; GAL4, yeast transcriptional activator of galactose-metabolizing enzymes.

Duret et al., 2006; Huang et al., 2007; Wang et al., 2007). It has been suggested that the molecular mechanism underlying the inhibition proceeds through specific disruption of PXR interaction with SRC-1 at the AF-2 coactivator binding site (Takeshita et al., 2002; Huang et al., 2007; Wang et al., 2007). Recently, oxiconazole, miconazole, and fluconazole were reported to suppress basal and rifampicin-activated PXR-mediated activation of *CYP3A4* (−10,466 to +53) luciferase reporter plasmid in transient transfection assays (Wang et al., 2007).

In the present study, we focused on effects of azole antimycotics clotrimazole, ketoconazole, econazole, oxiconazole, miconazole, fluconazole, and itraconazole on PXR-mediated expression of *CYP3A4*. We investigated potency of the individual azole antimycotics to regulate levels of CYP3A4 mRNA in LS174T cells and primary human hepatocytes employing real-time RT-PCR, transactivate *CYP3A4* promoter in gene reporter assays, bind to the PXR ligand-binding domain (LBD), and recruit SRC-1 coactivator to PXR in mammalian two-hybrid assays. Furthermore, we examined effects of tested azoles on rifampicin-induced *CYP3A4* transactivation through PXR. Finally, we aimed to describe the nature of potential pharmacodynamic interactions of tested azole antimycotics coadministered with a prototypical PXR ligand rifampicin in transactivation of the *CYP3A4* gene.

We reveal differential effects of several azole antimycotics on PXR-mediated transactivation of *CYP3A4*, binding to PXR LBD, and recruitment of SRC-1 to PXR. We show additive and antagonistic effects of azole antimycotics on rifampicin-induced PXR-mediated transactivation of *CYP3A4*. We demonstrate that oxiconazole is one of the most potent inducers of *CYP3A4* gene expression via PXR described so far, whose effect in transactivation of *CYP3A4* could be competitively antagonized by rifampicin. We also show that ability of some azoles to affect recruitment of SRC-1 to PXR modulates their net effects in transactivation of *CYP3A4*, both in the absence or presence of rifampicin. We thus suggest that PXR-mediated activation of *CYP3A4* promoter is a complex process, whose magnitude is not exclusively determined by binding characteristics of ligands to PXR LBD, and indicate important modulatory role of SRC-1 in PXR-mediated transactivation of *CYP3A4*.

Materials and Methods

Cell Lines. The human Caucasian hepatocyte carcinoma (HepG2), human colon adenocarcinoma (LS174T), and African Green monkey kidney fibroblast (CV-1) cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK) and were used within 20 passages after delivery and maintained in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). In addition, medium of HepG2 cells was supplemented with 1% sodium pyruvate and 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO).

LS174T human colonic epithelial tumor cell line is one of very few human-derived cell lines that have inducible the *CYP3A4* gene and relatively high expression of PXR (Burk et al., 2005; Cerveny et al., 2007). CV-1 cell line was used since it does not express any hepatocyte-specific transcriptional factors.

Chemicals. Rifampicin, charcoal, SR12813, and cell culture media were purchased from Sigma-Aldrich. Phenol red-free media were purchased from Invitrogen (Carlsbad, CA). FBS was purchased from PAA (Pasching, Austria). Azole antimycotics were obtained in high-grade pharmaceutical quality from Sigma-Aldrich (clotrimazole); Janssen Pharmaceutica N.V., Beerse, Belgium (ketoconazole, batch E3401; miconazole nitrate, batch I3201); Janssen Research Foundation (itraconazole, batch A2201); Hoffmann La Roche, Basel, Switzerland (oxiconazole nitrate, LOT 481812); Cilag AG, Schaffhausen, Switzerland (econazole nitrate, batch 94P4279); and Zentiva, Prague, Czech Republic (fluconazole). Stock solutions (1000× or 3000×) were prepared in DMSO (Sigma-Aldrich). The final concentration of DMSO in culture media was 0.1% (v/v) in all experiments.

Plasmids. A chimeric p3A4-luc reporter construct containing the basal promoter (−362/+53) with the proximal PXR response element (ER6) and the distal xenobiotic responsive enhancer module (−7836/−7208) of the *CYP3A4* gene 5′-flanking region inserted to pGL3-Basic reporter vector (Promega, Madison, WI) was described by Goodwin et al. (1999). The expression plasmid for PXR receptor, pSG5-hPXR, was kindly provided by Dr. S. Kliewer (University of Texas, Dallas, TX). The human CAR expression plasmid pCR3-hCAR was kindly provided by Dr. M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC). The expression plasmid pSG5-hRXRα encoding human RXRα cDNA was a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland). pcDNA3-HNF4α2 expression plasmid was kindly donated by Dr. B. Laine (INSERM Unit 459, Lille, France). The expression plasmid encoding human GRα (pSG5-hGRα) was a generous gift from Dr. J. Palvimo (University of Helsinki, Helsinki, Finland). pRL-TK was purchased from Promega. To construct pDR3-luc and pER6-luc plasmids, we synthesized complementary pairs of oligonucleotides containing three tandem copies of either DR3 or ER6 REs of the *CYP3A4* promoter (5′-CTAGCGAA-TGAACTTGCTGACCCTCTGATGGAATGAACTTGCTGACCCTCT-GATGGATGAACTTGCTGACCCTCTA-′3 to produce pDR3-luc and 5′-CTAGCATATGAACTCAAAGGAGGTCAGTGGATGGAATGAACT-CAAAGGAGGTCAGTGGATGGAATGAACTCAAAGGAGGTCAG-TGA-′3 to produce pER6-luc). The underlined sequence indicates DR3 and ER6 REs. Oligonucleotides were annealed and cloned into the NheI- and BglII-digested sites of the pGL4.23 vector containing a minimal promoter (Promega).

Transient Transfection and Luciferase Gene Reporter Assays. Transient transfection assays were carried out using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's instruction. HepG2 cells (2×10^5) were seeded into 48-well plates and transfected with a luciferase reporter construct (200 or 300 ng/well), the expression plasmid encoding PXR (50 ng/well), and *Renilla reniformis* luciferase transfection control plasmid (pRL-TK) 24 h later (30 ng/well). Cells were maintained in phenol red-free medium (200 μl) supplemented with 10% charcoal/dextran-stripped FBS with azole antimycotics or combinations of drugs for 8 or 24 h.

In the case of transient transfection experiments with pDR3-luc and pER6-luc reporter plasmids, HepG2 cells were cotransfected with 200 ng/well of a reporter plasmid, the expression plasmid encoding PXR (200 ng/well), and pRL-TK (30 ng/well). Subsequently, cells were maintained in phenol red-free medium (200 μl) supplemented with 10% charcoal/dextran-stripped FBS with azole antimycotics (10 μM) for 48 h.

For mammalian two-hybrid assays, we used the mammalian two-hybrid fusion plasmids GAL4-PXR-LBD and VP16-SRC-1-receptor-interacting domain (RID), which were a generous gift from Dr. A. Takeshita (Tonnomon Hospital, Tokyo, Japan; Takeshita et al., 2002). The GAL4 fusion plasmid contains the LBD of human PXR (107–434 amino acids) fused to GAL4-DBD (yeast DNA-binding domain), and the VP16 fusion plasmid contains the RID (595–780 amino acids) of SRC-1 fused to *Herpes simplex* virus VP16 activation domain. The pG5luc reporter vector, which contains five GAL4 binding sites upstream of a minimal TATA box and the firefly luciferase gene, was purchased from Promega. The pGAL4 (20 ng/well) and pVP16 (50 ng/well) fusion constructs were transfected along with pG5luc vector (120 ng/well) and pRL-TK (20 ng/well) into HepG2 cells. One day after transfection, azole antimycotics (20 μM) or their combinations with rifampicin (10 μM) were added into media for 8 or 24 h.

In the case of reporter experiments with pG5luc and GAL4-PXR-LBD plasmids (one-hybrid trans-activation assay) in CV-1 cells, 150 ng/well pG5luc plasmid and 100 ng/well GAL4-PXR-LBD plasmid were used together with 30 ng of pRL-TK. In the assay, an agonist binding to PXR-LBD is detectable through GAL4 activation of the pG5luc reporter vector. Cells were then exposed to azole antimycotics 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 (Dual Luciferase Reporter Assay Kit; Promega).

Real-Time RT-PCR Analysis. Total RNA was isolated from LS174T cells and primary human hepatocytes treated with tested azole antimycotics or their combinations with rifampicin for 24 or 48 h. Total RNA isolation and real-time RT-PCR for *CYP3A4* gene were performed as described before (Cerveny et al., 2007). The primer sequences for *PXR* expression analysis were the following: reverse primer, 5′-CTG TGA TGC CGA ACA ACT CC-3′; and forward

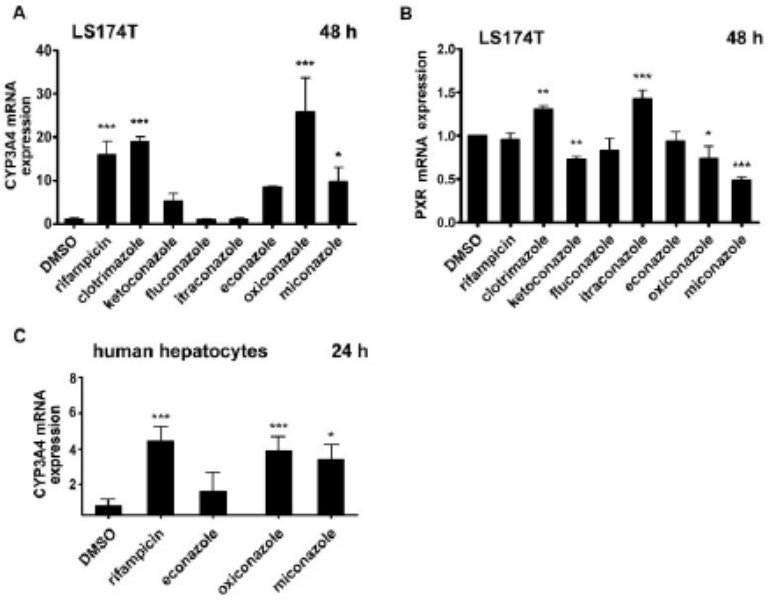

FIG. 1. Effects of azole antimycotics on expression of CYP3A4 and PXR mRNAs in LS174T cells and in primary human hepatocytes. LS174T cells were treated with 10 μM tested azoles or rifampicin for 48 h. Total RNA was isolated, and mRNA levels were analyzed by real-time RT-PCR with specific primers. Expression of tested genes was normalized to the hypoxanthine-guanine phosphoribosyl transferase housekeeping gene. The effect of tested azole antimycotics on CYP3A4 (A) and PXR (B) mRNA levels is presented as -fold mRNA expression to control vehicle-treated cells (set to 1). Data represented the means of three independent experiments ± S.D. ($n = 3$) performed in triplicate. C, long-term human hepatocytes in monolayer (batch HH 220221) were treated with 10 μM tested azoles, rifampicin (10 μM), or vehicle (DMSO; 0.1%) for 24 h. Total RNA was isolated, and mRNA levels were analyzed by real-time RT-PCR with specific primers. Values represent the means ± S.D. of triplicate. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ as compared with control cells (analysis of variance with Dunnett's test).

primer, 5′-CCC AGC CTG CTC ATA GGT TC-3′. The hypoxanthine-guanine phosphoribosyl transferase gene was used as a housekeeping gene (Cerveny et al., 2007). PCR conditions specific for each gene were optimized with respect to $MgCl_2$ concentration and annealing temperature. All samples were run in triplicate simultaneously with negative controls. Melting curve analyses were performed in each real-time RT-PCR experiment. Pfaffl's (2001) method was applied for relative quantification of gene expression normalized to a housekeeping gene. The results are expressed as -fold induction versus control vehicle-treated cells.

Primary Cultures of Human Hepatocytes. Hepatocytes were prepared from lobectomy segments, resected from adult patients for medical reasons unrelated to our research program. Human tissue was obtained according to protocols complying with the current Czech legislation. Human liver samples used in this study were obtained from two patients: LH 18 (woman, 69 years) and LH 19 (woman, 46 years) (Cerveny et al., 2007). Hepatocytes were isolated and cultured as previously described (Cerveny et al., 2007). Following isolation, the cells were plated on collagen-coated culture dishes at a density 1.4×10^5 cells/cm^2. In addition, two cultures of the long-term human hepatocytes in monolayer batch HEP220216 (77-year-old Caucasian female with hepatic lesion from adenocarcinoma, nonsmoker) and HEP220221 (73-year-old Caucasian male with hepatocellular carcinoma, nonsmoker) purchased from Biopredict International (Rennes, France) were used (Meneses-Lorente et al., 2007). The medium was exchanged for serum-free medium the day after delivery, and the cultures were allowed to stabilize for an additional 48 to 72 h prior to treatments. Cultures were maintained at 37°C and 5% CO_2 in a humidified incubator. The level of CYP3A4 mRNA expression was analyzed using real-time RT-PCR according to the protocol mentioned above after 24-h treatment with indicated compounds or their combinations.

Cell Viability Test. Cytotoxicity of azole antimycotics after 24, 48, and 72 h of incubation with tested drugs was tested by a colorimetric tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay according to the standard protocol (Roche Diagnostics, Basel, Switzerland). Simultaneously, morphology of cells in culture was microscopically assessed.

Statistical Analyses. One-way analysis of variance followed by Dunnett's multiple comparison post hoc test or Student's *t* test was used for statistical analysis of differences between two experimental groups using GraphPad Prism software (GraphPad Software Inc., San Diego, CA). EC_{50} (xenobiotic concentration required to achieve half-maximum promoter activation) and I_{max} (representing the overall maximal calculated induction produced by tested compound; maximal responding capacity; maximal efficacy) values were determined according to Hill's equation by nonlinear regression analysis using GraphPad Prism Software (GraphPad Software Inc.) from at least seven-point curves performed in triplicate. Linear regression analyses, slope, and the *p* value (*F* test) calculation were also performed employing GraphPad Prism Software.

Results

Induction of *CYP3A4* and *PXR* Gene Expression by Tested Azole Antimycotics in LS174T Cell Line and Primary Human Hepatocytes. First, we tested effects of selected azole antifungals on expression of CYP3A4 mRNA in human intestinal LS174T cells and primary human hepatocytes employing real-time RT-PCR analysis. Transcriptional regulation of the target gene via PXR is well documented (Lehmann et al., 1998; Goodwin et al., 1999). In parallel with CYP3A4 mRNA level, expression of PXR mRNA was also examined.

As shown in Fig. 1A, CYP3A4 mRNA levels were strongly induced by oxiconazole (25-fold), clotrimazole (19-fold), rifampicin (16-fold), miconazole (9-fold), and econazole (9-fold) when compared with control in LS174T cells ($n = 3$). Interestingly, ketoconazole, oxiconazole, and miconazole significantly ($p < 0.05$) downregulated PXR mRNA in LS174T cells (Fig. 1A).

In agreement, we found that oxiconazole, miconazole, and econazole are inducers of CYP3A4 mRNA in primary human hepatocytes (Fig. 1B). Contrary to results in LS174T cells, oxiconazole and rifampicin elicited comparable potency to induce CYP3A4 mRNA.

Transactivation of the *CYP3A4* Promoter by Azole Antimycotics. Potency of selected azoles to transactivate *CYP3A4* gene reporter construct by full-length PXR was assayed in transient transfection experiments in HepG2 cells. We found that oxiconazole, clotrimazole, miconazole, and econazole efficiently activate *CYP3A4* promoter through PXR after 8-h incubation in HepG2 cells (Figs. 2A and 3A).

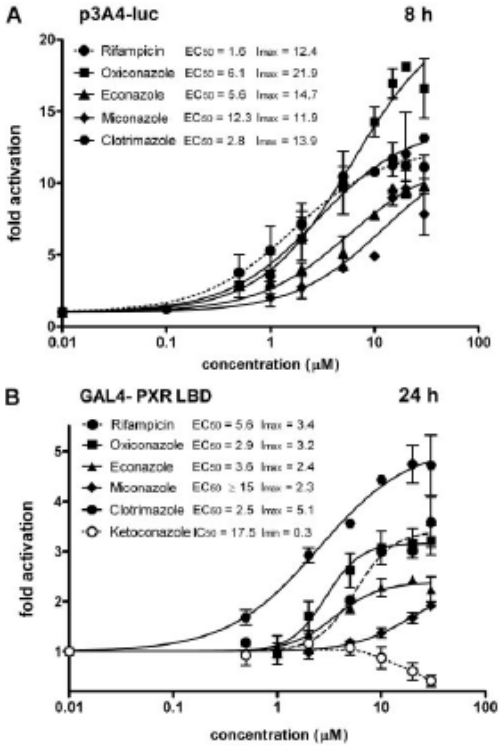

FIG. 2. Oxiconazole, miconazole and econazole transcriptionally activate *CYP3A4* promoter through PXR. A, concentration-dependent activation of p3A4-luc via PXR by tested azole antimycotics. HepG2 cells were transiently transfected with full-length PXR expression plasmid (50 ng/well) together with p3A4-luc reporter plasmid (300 ng/well) and pRL-TK control plasmid for transfection normalization (30 ng/well). After transfection, cells were treated with the indicated concentrations of azoles or rifampicin for 8 h. B, CV-1 cells were transiently transfected with a GAL4 reporter plasmid with firefly luciferase reporter gene (pG5luc, 150 ng/well), fusion GAL4-PXR ligand-binding domain expression plasmid (GAL4-PXR LBD, 100 ng/well), and pRL-TK (30 ng/well). Cells were then treated with the indicated concentrations of azoles for 24 h. After incubation with tested compounds, cells were lysed and analyzed for both firefly and *Renilla* luciferase activities. Data represent the mean of three independent experiments and are shown as -fold induction of normalized luciferase activity relative to solvent (0.1% DMSO) controls. Error bars, S.D. EC_{50}/IC_{50}, concentration required to achieve half-maximum/minimum gene reporter plasmid activation. I_{max}/I_{min}, the maximal/minimal calculated activation produced by tested compound.

Dose-response analysis using nonlinear regression analysis revealed that the xenobiotic concentration required to achieve half-maximum activation of *CYP3A4* reporter plasmid (EC_{50}) for oxiconazole was approximately 6.1 μM and calculated maximal -fold activation value (I_{max}) was 21.9. Rifampicin activated the reported construct with the EC_{50} value of 1.6 μM and I_{max} of 12.4. Thus, dose-response analysis showed that rifampicin is not able to produce full activation of *CYP3A4* reporter plasmid under the used experimental conditions. Oxiconazole was also more potent to activate p3A4-luc reporter after 24-h exposure in HepG2 cells in comparison with rifampicin (data in Fig. 4) or another prototypical PXR ligand SR12813 at an equimolar concentration of 10 μM (76.8- and 53.7-fold activation to control, respectively) under the same experimental conditions with 300 ng of p3A4-luc and 50 ng of pSG5-PXR cotransfected per well.

Estimated I_{max} for econazole (14.7) was higher than in the case of rifampicin (12.4), clotrimazole (13.9), and miconazole (11.9). EC_{50} values of econazole and miconazole were higher in comparison with rifampicin (5.6, 12.3, and 1.6 μM, respectively) (Fig. 2A). Ketoconazole had a minor effect on *CYP3A4* promoter activation; itraconazole and fluconazole did not yield any significant effect after 8 or 24 h of incubation in HepG2 cells (Fig. 3A, dose-response curves are not presented).

Identification of Oxiconazole, Miconazole, and Econazole as PXR Ligands. In next experiments, we investigated interactions of tested azole antimycotics with PXR ligand-binding domain. Consistent with the results obtained using the full-length PXR and p3A4-luc reporter plasmid, clotrimazole, oxiconazole, econazole, and miconazole efficiently activated GAL4 reporter plasmid through GAL4 PXR LBD fusion construct (Fig. 2B). Interestingly, we observed the opposite pattern of activation in the case of rifampicin and oxiconazole in the experiments. Oxiconazole activated the reporter plasmid with higher affinity to PXR LBD than rifampicin (EC_{50} = 2.9 and 5.6 μM, respectively), whereas maximal activation I_{max} did not differ significantly between these two compounds (3.2 versus 3.4). Econazole and miconazole activated GAL4 reporter plasmid through GAL4 PXR LBD with the EC_{50} values of 3.6 and $\geq$15 μM and maximal efficacy I_{max} values of 2.4 and 2.3, respectively (Fig. 2B). EC_{50} for miconazole is presented as estimate because miconazole did not reach plateau in the experiments. Clotrimazole was the most potent ligand of PXR in our experiments (EC_{50} = 2.5 μM, I_{max} = 5.1) (Fig. 2B). Surprisingly, we found that ketoconazole dose dependently inhibited the GAL4 reporter plasmid activation through GAL4 PXR LBD construct with an IC_{50} of 17.9 μM and I_{min} of 0.25 (Fig. 2B). Fluconazole and itraconazole did not activate the reporter plasmid, suggesting no interaction of the compound with PXR LBD at the tested concentrations (dose-response data not shown; Fig. 3B).

Hence, we conclude that oxiconazole, clotrimazole, miconazole, and econazole, but not ketoconazole, itraconazole, and fluconazole efficiently activate *CYP3A4* promoter through interaction with PXR LBD (Fig. 2A). We suggest that clotrimazole is a full agonist of PXR, which is able to produce maximal effect in interaction with GAL4-PXR LBD (Fig. 2B). On the other hand, rifampicin, oxiconazole, econazole, and miconazole are partial agonists on PXR LBD, which interact with GAL4-PXR LBD with substantial affinity; however, they do not produce maximal activation (Fig. 2B). Interestingly, we confirmed that ketoconazole induces CYP3A4 mRNA and transactivates *CYP3A4* promoter, although it is not an agonist of PXR (Huang et al., 2007).

In the next experiments, we addressed two questions arising from the data. First, we studied how azole antimycotics affect rifampicin-induced activation of *CYP3A4* promoter. We supposed to observe additive, synergistic, or antagonistic pharmacodynamic interactions due to different characteristics of tested azoles to transactivate the *CYP3A4* promoter via PXR and differential effects of the compounds on SCR-1 recruitment to PXR. Second, we aimed to explain why oxiconazole activates the *CYP3A4* promoter with higher efficacy in comparison with rifampicin, although they have comparable I_{max} in experiments with the GAL4-PXR LBD plasmid.

Transactivation of the *CYP3A4* Promoter by Combinations of Azole Antimycotics with Rifampicin. In the next transient transfection experiments with the p3A4-luc reporter plasmid, we observed that the effect of oxiconazole to transactivate the p3A4-luc reporter plasmid was significantly ($p < 0.05$) reduced by rifampicin at concentration of 5 μM or higher after 8-h coincubation in HepG2 cells (Figs. 3A and 4). We suppose that rifampicin attenuates competitively the effect of oxiconazole, which has higher maximal efficacy (I_{max})

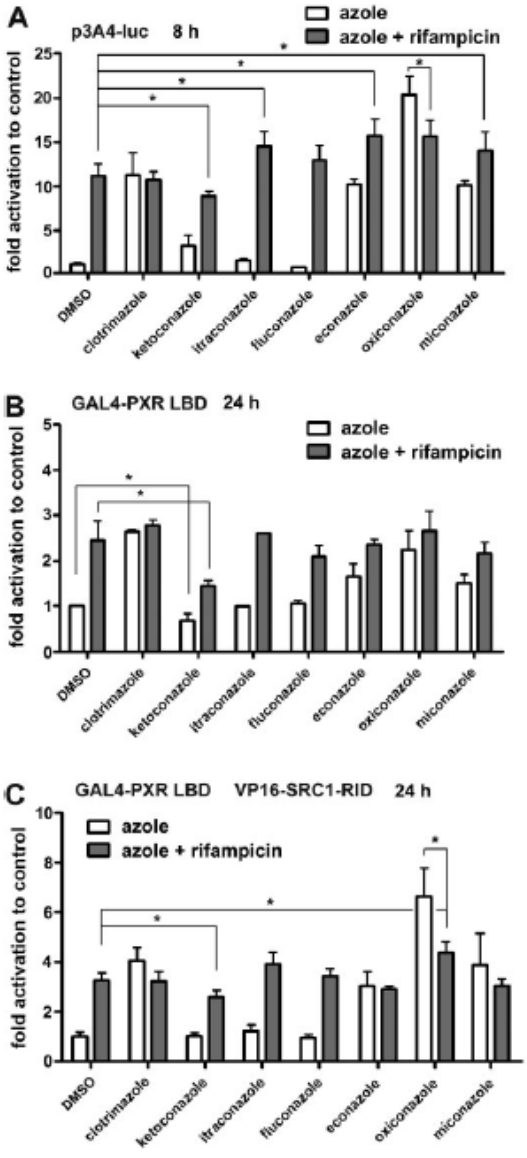

FIG. 3. Effects of azole antimycotics in combination with rifampicin on activation of *CYP3A4* promoter, interaction with PXR ligand-binding domain, and recruitment of SRC-1 coactivator. A, transient transfection experiments in HepG2 cells were performed with full-length PXR expression plasmid (50 ng/well) together with p3A4-luc reporter plasmid (300 ng/well) and pRL-TK (30 ng/well). HepG2 cells were treated with the indicated combinations of azoles antimycotics (20 μM), rifampicin (10 μM), or vehicle (control, 0.1% DMSO) for 8 h. B, CV-1 cells were transiently transfected with pG5luc GAL4 luciferase reporter plasmid (150 ng/well), expression vector GAL4-PXR LBD (100 ng/well), and pRL-TK transfection control plasmid (30 ng/well). Cells were then treated with 20 μM azole antimycotics in the presence or absence of rifampicin at the concentration of 10 μM for 24 h. C, two-hybrid assay was performed in HepG2 cells transfected with a pG5luc GAL4 reporter plasmid, GAL4-PXR LBD, VP16-SRC-1-RID, and pRL-TK plasmids. Cells were then treated with 20 μM azole antimycotics in the presence or absence of rifampicin at the concentration of 10 μM for 24 h. Data are expressed as mean ± S.D. of a representative experiment performed in triplicate and are shown as -fold induction of normalized luciferase activity relative to solvent (0.1% DMSO) control. Similar profiles were observed in three independent experiments. *, $p < 0.05$.

and EC_{50} value to transactivate the p3A4-luc reporter plasmid (Fig. 2A). Consistently with published data (Huang et al., 2007; Wang et al., 2007), we found that ketoconazole significantly ($p < 0.05$) decreased rifampicin-mediated activation of *CYP3A4* promoter (Fig. 3A). The inhibitory effect of ketoconazole was more pronounced after 24-h coincubation of ketoconazole (30 μM) with 10 μM rifampicin (more than 55% reduction of activation, data not shown). Of note, we observed indication of additive interactions in activation of the reporter plasmid by combinations of econazole and miconazole with rifampicin, but not in the case of clotrimazole (Fig. 3A). Fluconazole had no effect on both basal and rifampicin-induced PXR-mediated activation of p3A4-luc (Fig. 3A). Finally, we observed statistically significant ($p < 0.05$) stimulation of rifampicin-mediated activation of p3A4-luc by itraconazole after 8- and 24-h incubation (Fig. 3A, data for 24 h not shown).

In parallel, we performed gene reporter experiments with empty pGL3-basic reporter plasmid under the same experimental conditions with all tested compounds. We observed a slight (~20%) increase of reporter activity after treatment of HepG2 cells with oxiconazole and miconazole for 8 h. Because the activation was not statistically significant, we did not subtract the nonspecific activation in final calculation of p3A4-luc -fold activation. Other tested compounds did not significantly activated pGL3-Basic reporter plasmid after 8-h treatment (data not shown).

Interactions of Azole Antimycotics with the Ligand-Binding Domain of PXR. Next, we investigated binding of azole antimycotics in combination with rifampicin on PXR ligand-binding domain using the GAL4-PXR LBD fusion construct and pG5luc GAL4 reporter vector. We did not observe any statistically significant additive or antagonistic interactions of azole antimycotics in combination with rifampicin on the activation of the pG5luc reporter vector through pGAL4-PXR LBD except ketoconazole (Fig. 3B). In the presence of ketoconazole, we observed significant ($p < 0.05$) suppression of pG5luc reporter vector activity in CV-1 cells either in the absence or presence of rifampicin (Fig. 3B). These findings contradict to the hypothesis by Huang et al. (2007), who suggested that ketoconazole unlikely competes with ligands in PXR LBD. Itraconazole and fluconazole neither bound nor affected significantly binding of rifampicin to PXR LBD.

Azole Antimycotics Promote PXR-SRC-1 Coactivator Interaction. Interaction of PXR with coactivators is a critical part of the nuclear receptor signaling (Rosenfeld et al., 2006). Recently, ketoconazole was shown to disrupt the interaction of PXR with SRC-1 resulting in suppression of ligand-induced PXR-mediated induction of *CYP3A4* and *MDR1* genes (Takeshita et al., 2002; Huang et al., 2007; Wang et al., 2007).

We used the mammalian two-hybrid assay to evaluate whether tested azoles individually or in combination with rifampicin (10 μM) affect interaction of PXR with SRC-1. Consistent with previous reports (Lehmann et al., 1998; Synold et al., 2001; Takeshita et al., 2002; Huang et al., 2007, and others), rifampicin and clotrimazole significantly ($p < 0.01$) promoted the specific interaction of SRC-1 with PXR after 24-h incubation in HepG2 cells (Fig. 3C). In agreement with gene reporter data (Fig. 2A), the effect of rifampicin plateaued from the concentration of 10 μM (data not shown). Oxiconazole and clotrimazole had stronger effects on interaction of PXR

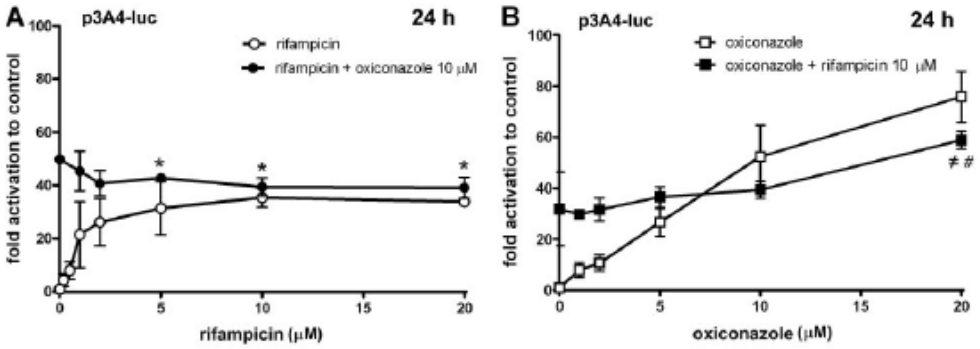

FIG. 4. Competition of rifampicin and oxiconazole in PXR-mediated transactivation of p3A4-luc reporter plasmid. Transient transfection experiments in HepG2 cells were performed with full-length PXR expression plasmid (50 ng/well) together with p3A4-luc reporter plasmid (200 ng/well) and pRL-TK (30 ng/well). A, cells were simultaneously treated with the fixed concentration of oxiconazole (10 μM) together with rifampicin at the concentrations from 0.5 to 20 μM for 24 h. B, cells were treated with the fixed concentration of rifampicin (10 μM) together with oxiconazole at the concentrations from 0.5 to 20 μM. Cells were incubated with the compounds for 24 h, harvested, and assayed for luciferase activities. Data are expressed as mean ± S.D. and are shown as -fold induction of normalized luciferase (firefly/*Renilla*) activity relative to control (0.1% DMSO). *, $p < 0.05$, as compared with cells treated with only 10 μM oxiconazole. #, $p < 0.05$, as compared with cells treated with only 10 μM rifampicin; ≠, $p < 0.05$, as compared with cells treated with 20 μM oxiconazole.

with SRC-1 in comparison with the effect of rifampicin after 24 h of incubation (Fig. 3C). Econazole and miconazole also yield significant recruitment of SRC-1 to PXR ($p < 0.05$). In agreement with reporter experiments with the p3A4-luc plasmid (Fig. 3A), rifampicin significantly ($p < 0.05$) suppressed oxiconazole-mediated interaction of PXR with SRC-1 (Fig. 3C). Fluconazole and itraconazole had no effects on recruitment of SRC-1 to PXR in the absence of rifampicin (Fig. 3C). However, we observed that itraconazole augmented rifampicin-mediated recruitment of SRC-1 to PXR after 8- and 24-h coincubation in HepG2 cells. The simulative effect of itraconazole (20 μM) was statistically significant ($p < 0.01$) after 8-h coincubation (7.7- ± 0.4-fold activation in comparison with the effect of rifampicin alone, −4.3- ± 0.1-fold activation), but not after 24-h cotreatment ($p < 0.07$) (data for 24 h in Fig. 3C). Ketoconazole (20 μM) significantly inhibited rifampicin-induced interaction of PXR and SRC-1 after 24-h incubation, which correlates with recent reports (Takeshita et al., 2002; Huang et al., 2007). Interestingly, we did not see additive effects of econazole and miconazole in combination with rifampicin on SRC-1 recruitment (Fig. 3, A and C). We thus hypothesize that additional factor (coactivator/corepressor) might underlie the interactions of the azoles with rifampicin in transactivation of *CYP3A4*.

Based on the data in Fig. 3, we can conclude that oxiconazole, clotrimazole, miconazole, and econazole are agonists of PXR, which transactivate *CYP3A4* promoter and promote SRC-1 coactivator recruitment. In addition, we indicate that oxiconazole promotes recruitment of SRC-1 to PXR more efficiently than rifampicin and clotrimazole, which consequently results in greater transactivation of CYP3A4 promoter by the azole antimycotic. Itraconazole stimulated activation of CYP3A4 promoter and recruitment of SRC-1 to PXR, although it did not interact with PXR LBD. Ketoconazole suppresses rifampicin-mediated *CYP3A4* transactivation, SRC-1 recruitment to PXR as well as interaction of rifampicin with PXR LDB. Fluconazole did not bind to PXR LBD and had no effect on transactivation of CYP3A4 promoter.

Oxiconazole Competes with Rifampicin in Activation of the *CYP3A4* Promoter in Transient Transfection Experiments. To study interaction of rifampicin and oxiconazole in activation of the *CYP3A4* promoter, we performed transient transfection experiments in HepG2 cells cultivated for 24 h with increasing concentration of oxiconazole and fixed concentration of rifampicin (and vice versa). As shown in Fig. 4A, rifampicin activated p3A4-luc in a dose-dependent manner and plateaued with the -fold activation at about 35 (calculated I_{max} = 37.6 for 24-h incubation). Combination of oxiconazole (10 μM) with increasing concentrations of rifampicin resulted in statistically significant ($p < 0.05$) suppression of oxiconazole-mediated activation of the p3A4-luc reporter plasmid (Fig. 4A).

In the next experiments, we assayed the effects of increasing concentrations of oxiconazole on activation of p3A4-luc in HepG2 cells exposed to a fixed concentration of rifampicin (10 μM) (Fig. 4B). Oxiconazole alone activated p3A4-luc in a dose-dependent manner, with the maximal activation at the concentration of 20 μM after 24-h treatment (Fig. 4B). Combination of 10 μM rifampicin with oxiconazole (20 μM) resulted in significant ($p < 0.05$) additive increase of p3A4-luc activation in comparison with the individual effect of 10 μM rifampicin (Fig. 4B). However, the activation by the combination of oxiconazole and rifampicin was significantly lower ($p < 0.05$) than the activation mediated by 20 μM oxiconazole alone (Fig. 4B). Thus, we demonstrate antagonistic effect of rifampicin on oxiconazole-mediated activation of *CYP3A4* promoter. These results confirm our hypothesis that rifampicin and oxiconazole compete for activation of the *CYP3A4* promoter.

Additive Interactions of Econazole and Miconazole with Rifampicin in Activation of the *CYP3A4* Promoter in Transient Transfection Experiments. To study in detail interactions of rifampicin with econazole and miconazole (see Fig. 3A), we performed transient transfection experiments in HepG2 cells treated for 8 h with increasing concentration of rifampicin and fixed concentration of econazole and miconazole (20 μM). We expected that concentrations closed to an EC_{50} of rifampicin (about 1.6 μM) might reduce *CYP3A4* promoter activation caused by econazole and miconazole due to different EC_{50} of the compounds (Fig. 2A). In a higher concentration (>5 μM), we supposed additive interactions of rifampicin in combination with econazole or miconazole since these drugs individually activate p3A4-luc reporter plasmid (Fig. 2A).

We found additive effects of both econazole and miconazole in combination with rifampicin in the range of concentrations from 0.5 up to 10 μM (Fig. 5). The effect was statistically significant ($p <$ 0.05) from the 5 μM concentration of rifampicin. We did not observe

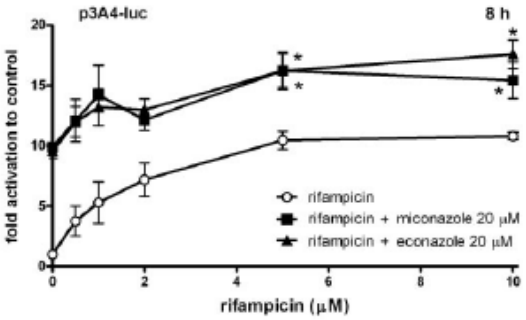

FIG. 5. Additive interactions of econazole and miconazole with rifampicin in PXR-mediated transactivation of CYP3A4 promoter. Transient transfections in HepG2 cells were performed as described in Fig. 4. Cells were simultaneously treated with the fixed concentration of econazole or miconazole (20 μM) together with rifampicin at the concentrations from 0.5 to 10 μM. After 8-h incubation with tested compounds, cells were lysed and analyzed for firefly luciferase normalized to *Renilla* luciferase activity. Data are expressed as mean ± S.D. and are shown as -fold induction of normalized luciferase activity relative to control (0.1% DMSO). *, $p < 0.05$, statistically different from cells treated with only econazole or miconazole (20 μM).

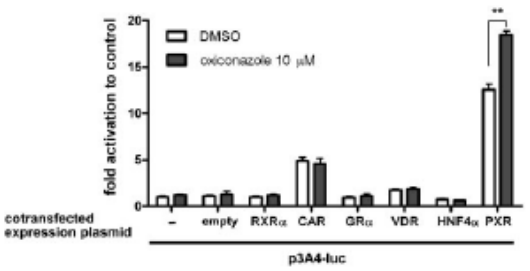

FIG. 6. Oxiconazole activates *CYP3A4* promoter specifically through PXR. Transient transfection experiments in CV-1 cells were performed with p3A4-luc reporter plasmid (200 ng/well), expression plasmids for PXR, VDR, CAR, RXRα, GRα, HNF4α, or the empty expression plasmid pcDNA3 (100 ng/well), and pRL-TK (30 ng/well). –, transfection of CV-1 cells with only p3A4-luc and pRL-TK plasmids without cotransfection with additional expression plasmid. Cells were incubated with oxiconazole at the concentrations of 10 μM or vehicle (control, 0.1% DMSO) for 24 h. After incubation, cells were lysed and analyzed for firefly luciferase normalized to *Renilla* luciferase activity. Data are expressed as mean ± S.D. of a representative experiment performed in triplicate and are shown as -fold induction of normalized luciferase activity relative to control cells (0.1% DMSO) (set to 1). **, $p < 0.01$.

any clear competitive antagonism by rifampicin in azoles-meditated transactivation of the CYP3A4 reporter plasmid.

Induction of *CYP3A4* mRNA by Combinations of Azole Antimycotics with Rifampicin in LS174T Cells and Primary Human Hepatocytes. Next, we tested effects of oxiconazole and itraconazole in combination with rifampicin on CYP3A4 mRNA expression employing real-time RT-PCR in LS174T cell line and/or in primary human hepatocytes. We did not detect any statistically significant effect of oxiconazole (10 μM) on rifampicin-mediated (10 μM) up-regulation of CYP3A4 mRNA in LS174T cells after 48 h of coincubation (22.77 ± 9.22 for rifampicin versus 26.29 ± 12.82 for rifampicin-oxiconazole combination; three independent experiments performed in triplicate). Similarly, we did not find out significantly different induction of CYP3A4 mRNA after treatment of commercial primary human hepatocytes (batch 220221) with combination of oxiconazole and rifampicin from induction mediated by oxiconazole alone (10 μM) after 24-h exposure (5.79 ± 0.91 for combination versus 4.92 ± 0.51 for oxiconazole alone).

We also examined the hypothesis that itraconazole (5 and 10 μM) stimulates rifampicin-mediated induction of CYP3A4 mRNA in primary human hepatocytes after 48-h coincubation with 10 μM rifampicin as suggested in Fig. 3A. We noted variable effect of itraconazole on rifampicin-mediated induction of CYP3A4 in primary human hepatocytes from three donors (data not shown), which at the moment does not support the hypothesis. Ongoing studies with extensive set of primary hepatocytes cultures should elucidate the effects of the azoles on rifampicin-mediated induction of CYP3A4 mRNA.

Oxiconazole Activates the *CYP3A4* Promoter Specifically through PXR. To test whether additional nuclear receptors are involved in strong activation of *CYP3A4* promoter by oxiconazole, we performed gene reporter experiments with the p3A4-luc reporter plasmid and expression vectors encoding human CAR, VDR, GRα, HNF4α, RXRα, and PXR nuclear receptors, which *trans*-activate *CYP3A4* (Martínez-Jiménez et al., 2007). We observed that oxiconazole (10 μM) stimulated significantly ($p < 0.01$) the reporter activity only in cotransfection with PXR expression plasmid (Fig. 6). This activation was relatively weak in CV-1 in comparison with hepatoma HepG2 cells (Figs. 2A, 3A, and 4). This finding corresponds with the absence of HNF4α and possibly additional hepatocyte-specific factors in CV-1 cells, which are essential for maximal transactivation of *CYP3A4* promoter (Tirona et al., 2003b; Li and Chiang, 2006). Cotransfection of CV-1 cells with PXR, CAR, and VDR expression plasmids also yielded activation of CYP3A4 promoter plasmid in the absence of an exogenous ligand (Fig. 6). This well known phenomenon is likely caused by an endogenous ligand or a ligand-independent activation of *CYP3A4* promoter reporter plasmid. Luciferase activity of p3A4-luc was not significantly increased in the absence or presence of oxiconazole in cotransfection with RXRα, GRα, and HNF4α expression plasmids or their empty expression plasmids pcDNA3 (Fig. 6) or pSG5 (data not shown). Interaction of oxiconazole with GRα and CAR has been also examined using $pGRE_3$-luc and p2B6(PBREM)-SV40-luc reporter plasmids with appropriate REs for tested nuclear receptors (Cerveny et al., 2007). These experiments confirm no statistically significant agonistic effect of oxiconazole on GRα and CAR (data not shown). Hence, we can conclude that oxiconazole at the 10 μM concentration had negligible effect on activation of p3A4-luc through CAR, VDR, GRα, HNF4α, and RXRα nuclear receptors.

Activation of ER6 and DR3 Response Elements by Tested Azole Antimycotics Correlate with Activation of the *CYP3A4* Promoter. Finally we tested activation of chimera reporter plasmids with replicate DR3 and ER6 response elements of *CYP3A4* promoter by tested azoles. The ligand-activated PXR forms a heterodimer with RXRs and binds to two central REs of *CYP3A4* promoter: the everted repeat separated by six bases (ER6) located in the proximal promoter and the direct repeat spaced by three bases (DR3) in the distal xenobiotic responsive enhancer module (Bertilsson et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999). DR3 and ER6 REs synergistically transactivate the *CYP3A4* gene via PXR, and disruption of the REs destroys 80 to 90% of PXR-mediated responsiveness of *CYP3A4* promoter (Goodwin et al., 1999). We supposed that activation of the pDR3-luc and pER6-luc reporter plasmids would correlate with activation of p3A4-luc promoter only on the assumption that no additional *cis*-acting elements are involved in azole-mediated activation of the *CYP3A4* promoter.

We found that activation of the ER6 response element with tested azole antimycotics well correlated with activation of the p3A4-luc reporter ($r^2 = 0.90$; $p = 0.0003$) (Fig. 7A). Similarly, we found

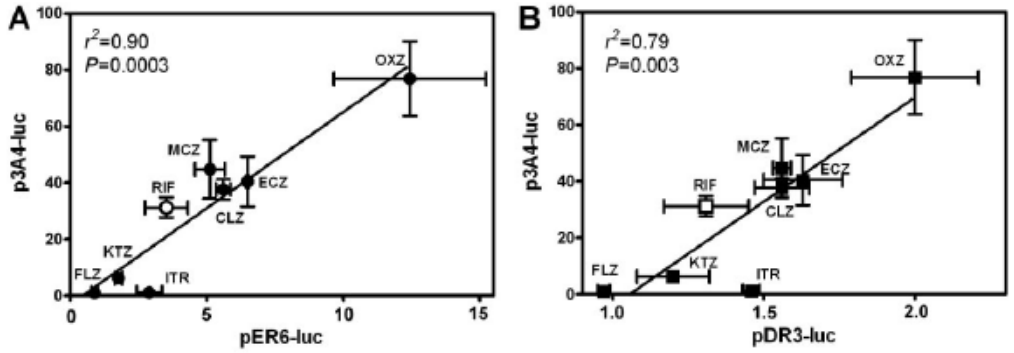

FIG. 7. Activation of ER6 and DR3 response elements by tested azole antimycotics correlate with activation of *CYP3A4* promoter. Effects of tested azole antimycotics on PXR-mediated transactivation of luciferase reporter gene plasmids containing DR3 and ER6 response elements. HepG2 cells were transfected with p3A4-luc firefly luciferase reporter construct and pSG5-hPXR expression plasmid or with pER6-luc (A) or pDR3-luc (B) and pSG5-hPXR expression plasmid as described in detail under *Materials and Methods*. Following 12-h exposure to transfection complexes, cells were treated with vehicle (control, 0.1% DMSO), 10 μM rifampicin (RIF) as a positive control, and azole antimycotics (10 μM) for 24 h (p3A4-luc) or 48 h (pDR3-luc and pER6-luc). Normalized p3A4-luc (*y*-axes) or pDR3-luc and pER6-luc (*x*-axes) gene reporter activities represent the mean ± S.D. of three independent transfections and are expressed as -fold activation to controls. CLZ, clotrimazole; ECZ, econazole; MCZ, miconazole; ITR, itraconazole; KTZ, ketoconazole; FLZ, fluconazole; OXZ, oxiconazole.

correlation between pDR3-luc and p3A4-luc activation with tested azole antimycotics ($r^2 = 0.79$; $p = 0.003$; F test), with the exception of itraconazole, which activated pDR3-luc and pER6-luc, but not the p3A4-luc reporter plasmid (Fig. 7B). Rifampicin was a less efficacious activator of both pDR3-luc and pER6-luc reporter plasmids than oxiconazole, miconazole, econazole, and clotrimazole at the concentration of 10 μM under the experimental conditions. Thus, we suggest that oxiconazole activates the *CYP3A4* promoter via PXR specifically through binding to DR3 and ER6 REs.

Cell Viability Testing after Treatment with Azole Antimycotics. Several azole antimycotics have been shown to be toxic to HepG2 cells or rat hepatocytes (Somchit et al., 2004; Sinz et al., 2006). Therefore, the cytotoxic potential of all compounds was tested employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay in HepG2 and LS174T cell lines. We found indications of lower viability of HepG2 cells in the case of itraconazole (60%) > oxiconazole (70%) = ketoconazole after 72-h incubation at the 10 μM concentration. Incubation of HepG2 with tested azoles for 24 or 48 h did not affect cellular cultures in comparison with the control. In LS174T cells, we did not observe any changes in cell viability after 24-h incubation, whereas the 48-h incubation with 10 μM itraconazole decreased formation of tetrazolium salt by about 30%. No microscopic changes in cell morphology were observed in HepG2 or LS174T cell lines after 24- and 48-h treatment with any tested compound.

Discussion

In the current paper, we reveal differential effects of several azole antimycotics on rifampicin-induced PXR-mediated transactivation of *CYP3A4*. We describe additive and antagonistic interactions of selected azole antimycotics with rifampicin in the process. We demonstrate that oxiconazole is one of the most potent inducers of *CYP3A4* gene expression via PXR described so far, whose effect in transactivation of CYP3A4 could be competitively inhibited by rifampicin. We also found that econazole and miconazole are potent PXR ligands and inducers of *CYP3A4*, which in coadministration with rifampicin activate *CYP3A4* promoter in an additive manner. Itraconazole stimulated activation of the *CYP3A4* promoter and recruitment of SRC-1 to PXR, although it did not interact with PXR LBD. Clotrimazole, a highly potent PXR ligand, neither affected rifampicin-induced transactivation of *CYP3A4* nor produced any interaction with rifampicin on PXR LBD. We also show that ketoconazole suppressed rifampicin-mediated *CYP3A4* transactivation, SRC-1 recruitment to PXR, as well as interaction of rifampicin with PXR LDB. On the other hand, ketoconazole induced CYP3A4 mRNA through CYP3A4 promoter REs, albeit it is not an agonist of PXR. Fluconazole did not bind to PXR LBD and had no effect on transactivation of CYP3A4 promoter. We thus show differential effects of tested azoles on PXR-controlled CYP3A4 transactivation and suggest that its magnitude is not solely determined by binding characteristics of azoles (or their combinations with rifampicin) to PXR LBD.

The *CYP3A4* gene regulation is a complex process mediated by numerous transcription factors (PXR, CAR, CCAAT/enhancer binding protein α, CCAAT/enhancer binding protein β, GRα, HNF4α, and HNF3α) and multiple promoter/enhancer elements (Martínez-Jiménez et al., 2007). Although PXR is critical determinant of xenobiotics-induced *CYP3A4* expression, other *trans*-acting factors such as HNF4α, SRC-1, SHR, and peroxisome proliferator-activated receptor γ, coactivator 1α are essential for maximal transactivation of the *CYP3A4* gene (Tirona et al., 2003b; Li and Chiang, 2006). For most tested compounds so far, a good correlation has been observed between transactivation of the *CYP3A4* promoter and ligand-PXR binding assay data (Zhu et al., 2004). However, discrepancies were found with some compounds showing high binding affinity in the ligand-binding assay, but with low efficacy to transactivate *CYP3A4* (Zhu et al., 2004). In the present study, employing dose-response analysis, we found that oxiconazole produced greater maximal responding capacity (I_{max}) to activate *CYP3A4* luciferase reporter plasmid in comparison with rifampicin (I_{max} = 21.9 versus 12.4; Fig. 2A). Rifampicin activated the *CYP3A4* promoter with lower EC_{50} than oxiconazole (1.6 versus 6.1 μM, respectively; Fig. 2A). In contrast, employing the transactivation assay with GAL4-PXR LBD and pG5luc plasmids, oxiconazole activated the reporter plasmid with higher affinity to PXR LBD than rifampicin (EC_{50} = 2.9 and 5.6 μM, respectively), whereas maximal activation did not differ significantly between these two compounds (I_{max}, 3.2 versus 3.4). This discrepancy clearly indicates that interaction with PXR LBD does not directly determine the magnitude and character of *CYP3A4* promoter transactivation by oxicon-

azole and rifampicin (Fig. 3). We hypothesize that additional factors such as recruitment of coactivators, release of corepressors from unliganded PXR, suppression of short/small heterodimer partner gene expression, presence of a liver-enriched transcriptional factor, histone-deacetylase activity, or binding to promoter DNA determine the net effect of PXR ligands to transactivate the *CYP3A4* promoter.

Dose-response relationships presented in Fig. 2 suggest that rifampicin is not able to produce full activation of p3A4-luc and pG5luc reporter plasmids under the used experimental conditions in comparison with other potent PXR ligands, clotrimazole and oxiconazole (Fig. 2A). In agreement with our data, PXR ligands such as hyperforin, nifedipine, SR12813, reserpin, *o,p′*-DDT (2,4′-DDT), fenvalerate, pesticide oxadiazon, herbicide pretilachlor, steroid 3α-hydroxy-5β-pregnane-11,20-dione methanesulfonate, and several other compounds have been shown to be by about 20 to 70% more potent at equimolar concentrations than rifampicin in transient transfection assays with different reporter plasmids of CYP3A4 gene or in transactivation assays with GAL4-PXR LBD (Bertilsson et al., 1998; Moore et al., 2002; Sinz et al., 2006; Lemaire et al., 2007; and others). In our experiments, oxiconazole was by about 60% more potent than rifampicin after 8-h treatment and almost 2-fold more potent after 24-h treatment at the concentration of 20 μM in the transient transfection experiment with p3A4-luc, and calculated I_{max} for oxiconazole was higher by 77% than I_{max} for rifampicin (Fig. 2A), which rates oxiconazole among the most potent inducer of CYP3A4 via PXR. A drug that produces maximal possible effect through a receptor is referred to as a full agonist, and a drug that displays submaximal effectiveness is referred to as a partial agonist. In pharmacodynamic theory, a partial agonist acts also as an antagonist in the presence of a full agonist. When it binds to the receptor, it also occupies the drug-binding site competitively with respect to a full agonist. A higher concentration of a full agonist will be required to produce a maximal effect. We observed this phenomenon in the case of coadministration of oxiconazole with rifampicin (Figs. 3B and 4). We also demonstrate that econazole and miconazole are potent ligands of PXR, activators of the *CYP3A4* promoter, and inducers of CYP3A4 mRNA. Their combinations with rifampicin activate the *CYP3A4* promoter in an additive manner since we observed partial summation of their individual effects (Figs. 3A and 5). Interestingly, another potent PXR ligand clotrimazole did not elicit any additive effect with rifampicin on *CYP3A4* transactivation or recruitment of SRC-1 to PXR (Fig. 3).

In the current paper, we used HepG2 and CV-1 cell lines, which express no or very low mRNA levels for major uptake and efflux drug transporters, which determine intracellular concentration of drugs and thus their activity in interaction with transcriptional factors (Hilgendorf et al., 2007). In contrast, in hepatocytes expressing numerous drug transporters, cellular concentration of a PXR ligand may be affected (Tirona et al., 2003a). Interface of drug transporters and nuclear receptors thus should be considered in the final effect of an inducer in hepatocytes.

During preparation of the paper, Wang et al. (2007) published their data on activation of CYP3A4 (−10,466 to +53)-luc reporter plasmid by oxiconazole, miconazole, and fluconazole in HepG2 cells. These authors suggested that the compounds suppress basal and rifampicin-activated PXR-mediated activation of the plasmid after 48-h incubation. In contrast, in our experiments with highly inducible *CYP3A4* reporter plasmid and employing chimera reporter plasmids with ER6 and DR3 REs normalized to pRL-TK *R. reniformis* control vector, oxiconazole and miconazole appeared to be highly efficacious activators of *CYP3A4* promoter after 8-, 24-, or 48-h incubation. In addition, oxiconazole and miconazole promoted recruitment of SRC-1 to PXR in two-hybrid assay and significantly induced CYP3A4 mRNA in LS174T cells and in primary human hepatocytes. Explanation of the discrepancy in not apparent now because both CYP3A4 reporter plasmids and protocols of the transient transfection have been validated in past. Other experimental conditions and approaches should be used to elucidate the conflicting observations.

We suppose that cytotoxicity is another important factor, which can lead to false negative or positive results. Therefore, to minimize any cytotoxicity of selected azole compounds, we designed some transient transfection experiments for 8-h incubation, and we used concentrations of tested drugs up to 20 μM. A shorter incubation period also eliminates potential biotransformation of tested compound, although it is very low in HepG2 cells (Rodriguez-Antona et al., 2002). In addition, 8-h incubation is too short to up-/down-regulate any transcriptional factor or nuclear receptors.

CYP-mediated DDIs are one of the most alarming problems in clinical practice and in the pharmaceutical industry (Lin, 2006). Treatment of serious mycotic infections by systemic azole antifungal agents (itraconazole, fluconazole, ketoconazole, voriconazole) in multimorbid patients is associated with the number of severe DDIs. Recent estimates suggest that as many as 95% of hospitalized patients treated with azole antifungals may receive medications capable of producing major or moderate pharmacokinetic interactions (Bates and Yu, 2003).

It is generally known that CYP inhibition is the basis of DDIs mediated by azole antimycotics (Venkatakrishnan et al., 2000). The inhibitory capability of azoles stems from their mechanism of action, which is inhibition of fungal CYP-mediated synthesis of ergosterol. As a result, the azole antifungals interact also with human cytochrome P450 and cause DDIs with a large number of drug classes, including antineoplastics, steroids, antimicrobials, antiretrovirals, cardiovascular agents, psychotropics, and oral contraceptives (Venkatakrishnan et al., 2000; Shakeri-Nejad and Stahlmann, 2006). Our results indicate that several azole antimycotics up-regulate *CYP3A4* gene expression. Therefore, it is urgent to consider potency of azole drugs to affect gene expression in pharmacotherapy. This could prevent unintended consequences in terms of DDIs but also in metabolism of endogenous compounds. Our current data imply that pharmacodynamic interactions on PXR in transactivation of *CYP3A4* gene may result in DDIs at the level of gene regulation. Further studies should elucidate additive and antagonistic interactions of coadministered PXR ligands in *CYP3A4* transactivation and study dose-response relationships of clinically relevant PXR ligands.

In conclusion, we describe agonistic and antagonistic pharmacodynamic interactions of partial and full agonists on PXR nuclear receptor in transactivation of the *CYP3A4* gene in the therapeutic group of azole antimycotics. We identify oxiconazole as a highly potent activator of *CYP3A4* promoter via PXR and an efficacious inducer of CYP3A4 mRNA. On the contrary, we established rifampicin as a partial agonist of PXR-mediated transactivation of *CYP3A4* in transient transfection gene reporter experiments. In addition, we show that activation of *CYP3A4* promoter is a complex process determined not solely by azole-PXR LBD interactions and suggest an important modulatory role of SRC-1 coactivator in some azole-mediated *CYP3A4* transactivation.

References

Bates DW and Yu DT (2003) Clinical impact of drug-drug interactions with systemic azole antifungals. *Drugs Today (Barc)* **39**:801–813.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Bäckman M, Ohlsson R, Postlind H, Blomquist P, and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A* **95**:12208–12213.

Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF, and Eichelbaum M (2005) Antimalarial artemisinin drugs induce cytochrome P450 and MDR1

expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Mol Pharmacol* 67:1954–1965.
Cerveny L, Svecova L, Anzenbacherova E, Vrzal R, Staud F, Dvorak Z, Ulrichova J, Anzenbacher P, and Pavek P (2007) Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. *Drug Metab Dispos* 35:1032–1041.
Duret C, Daujat-Chavanieu M, Pascussi JM, Pichard-Garcia L, Balaguer P, Fabre JM, Vilarem MJ, Maurel P, and Gerbal-Chaloin S (2006) Ketoconazole and miconazole are antagonists of the human glucocorticoid receptor: consequences on the expression and function of the constitutive androstane receptor and the pregnane X receptor. *Mol Pharmacol* 70:329–339.
Ekins S, Chang C, Mani S, Krasowski MD, Reschly EJ, Iyer M, Kholodovych V, Ai N, Welsh WJ, Sinz M, et al. (2007) Human pregnane x receptor antagonists and agonists define molecular requirements for different binding sites. *Mol Pharmacol* 72:592–603.
El-Sankary W, Gibson GG, Ayrton A, and Plant N (2001) Use of a reporter gene assay to predict and rank the potency and efficacy of CYP3A4 inducers. *Drug Metab Dispos* 29:1499–1504.
Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 56:1329–1339.
Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell AL, and Karlsson J (2007) Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. *Drug Metab Dispos* 35:1333–1340.
Huang H, Wang H, Sinz M, Zoeckler M, Staudinger J, Redinbo MR, Teotico DG, Locker J, Kalpana GV, and Mani S (2007) Inhibition of drug metabolism by blocking the activation of nuclear receptors by ketoconazole. *Oncogene* 26:258–268.
Kliewer SA (2003) The nuclear pregnane X receptor regulates xenobiotic detoxification. *J Nut* 133:2444S–2447S.
Kliewer SA, Goodwin B, and Willson TM (2002) The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocr Rev* 23:687–702.
Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterström RH, et al. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 92:73–82.
Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, and Kliewer SA (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* 102:1016–1023.
Lemaire G, Benod C, Nahoum V, Pillon A, Boussioux AM, Guichou JF, Subra G, Pascussi JM, Bourguet W, Chavanieux A, et al. (2007) Discovery of a highly active ligand of human pregnane X receptor: a case study from pharmacophore modeling and virtual screening to "in vivo" biological activity. *Mol Pharmacol* 72:572–581.
Li T and Chiang JY (2006) Rifampicin induction of CYP3A4 requires pregnane X receptor cross talk with hepatocyte nuclear factor 4alpha and coactivators, and suppression of small heterodimer partner gene expression. *Drug Metab Dispos* 34:756–764.
Lin JH (2006) CYP induction-mediated drug interactions: in vitro assessment and clinical implications. *Pharmacol Res* 23:1089–1116.
Martínez-Jiménez CP, Jover R, Donato MT, Castell JV, and Gomez-Lechon MJ (2007) Transcriptional regulation and expression of CYP3A4 in hepatocytes. *Curr Drug Metab* 8:185–194.
Meneses-Lorente G, Pattison C, Guyomard C, Chesné C, Heavens R, Watt AP, and Sohal B (2007) Utility of long-term cultured human hepatocytes as an in vitro model for cytochrome p450 induction. *Drug Metab Dispos* 35:215–220.
Moore LB, Maglich JM, McKee DD, Wisely B, Willson TM, Kliewer SA, Lambert MH, and Moore JT (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* 16:977–986.
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.
Rodriguez-Antona C, Donato MT, Boobis A, Edwards RJ, Watts PS, Castell JV, and Gomez-Lechon MJ (2002) Cytochrome P450 expression in human hepatocytes and hepatoma cell lines: molecular mechanisms that determine lower expression in cultured cells. *Xenobiotica* 32:505–520.
Rosenfeld MG, Lunyak VV, and Glass CK (2006) Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. *Genes Dev* 20:1405–1428.
Shakeri-Nejad K, and Stahlmann R (2006) Drug interactions during therapy with three major groups of antimicrobial agents. *Expert Opin Pharmacother* 7:639–651.
Sinz M, Kim S, Zhu Z, Chen T, Anthony M, Dickinson K, and Rodrigues AD (2006) Evaluation of 170 xenobiotics as transactivators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. *Curr Drug Metab* 7:375–388.
Somchit N, Norshahida AR, Hasiah AH, Zuraini A, Sulaiman MR, and Noordin MM (2004) Hepatotoxicity induced by antifungal drugs itraconazole and fluconazole in rats: a comparative in vivo study. *Hum Exp Toxicol* 23:519–525.
Synold TW, Dussault I, and Forman BM (2001) The orphan nuclear receptor SXR coordinately regulates drug metabolism and efflux. *Nat Med* 7:584–590.
Takeshita A, Taguchi M, Koibuchi N, and Ozawa Y (2002) Putative role of the orphan nuclear receptor SXR (steroid and xenobiotic receptor) in the mechanism of CYP3A4 inhibition by xenobiotics. *J Biol Chem* 277:32453–32458.
Tirona RG, Leake BF, Wolkoff AW, and Kim RB (2003a) Human organic anion transporting polypeptide-C (SLC21A6) is a major determinant of rifampin-mediated pregnane X receptor activation. *J Pharmacol Exp Ther* 304:223–228.
Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez FJ, et al. (2003b) The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. *Nat Med* 9:220–224.
Urquhart BL, Tirona RG, and Kim RB (2007) Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *J Clin Pharmacol* 47:566–578.
Venkatakrishnan K, von Moltke LL, and Greenblatt DJ (2000) Effects of the antifungal agents on oxidative drug metabolism: clinical relevance. *Clin Pharmacokinet* 38:111–180.
Wang H, Huang H, Li H, Teotico DG, Sinz M, Baker SD, Staudinger J, Kalpana G, Redinbo MR, and Mani S (2007) Activated pregnenolone X-receptor is a target for ketoconazole and its analogs. *Clin Cancer Res* 13:2488–2495.
Zhu Z, Kim S, Chen T, Lin JH, Bell A, Bryson J, Dubaquie Y, Yan N, Yanchunas J, Xie D, et al. (2004) Correlation of high-throughput pregnane X receptor (PXR) transactivation and binding assays. *J Biomol Screen* 9:533–540.

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

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Abstrakt:

Tato studie testuje hypotézu, zda je kyselina valproová (VPA) ve svém terapeutickém rozmezí schopna ovlivnit expresi CYP3A4 a Pgp prostřednictvím receptorů CAR a PXR. Interakce VPA s těmito nukleárními receptory byly studovány s využitím metod gene reporter assay, real time RT-PCR, EMSA a analýzou katalytické aktivity CYP3A4. Použitím gene reporter assay v buněčné linii HepG2 byla zaznamenána schopnost VPA aktivovat *CYP3A4* promotor přes CAR i PXR. Signifikantní efekt VPA na aktivaci *MDR1* promotoru byl pozorován pouze u HepG2 kontransfekovaných CAR. Tato data korelují s up-regulací mRNA *CYP3A4* a *MDR1* v buňkách transfekovaných expresními plazmidy pro CAR či PXR a ovlivněných VPA. Dále VPA významně up-reguluje *CYP3A4* mRNA v primárních hepatocytech a signifikantně zesiluje efekt rifampicinu. EMSA experimenty ukazují schopnost VPA podpořit vazbu komplexu CAR/RXRα na DR3 a DR4 responzivní elementy genů *CYP3A4*, resp. *MDR1*. Analýzou specifické katalytické aktivity CYP3A4 jsme potvrdili signifikantní vzestup jeho aktivity v LS174T linii transfekované PXR a inkubované s VPA. Dále ukazujeme, že VPA synergisticky zvyšuje efekt rifampicinu na transaktivaci *CYP3A4* v primárních lidských hepatocytech. Naše výsledky demonstrují schopnost VPA up-regulovat *CYP3A4* a *MDR1* prostřednictvím receptorů CAR a PXR, ačkoli nemůžeme vyloučit i další mechanizmy, kterými by VPA ovlivňovala transaktivaci *CYP3A4* a *MDR1* (např. inhibicí histon deacetyláz a aktivací některých signálních kaskád).

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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/cm². 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.

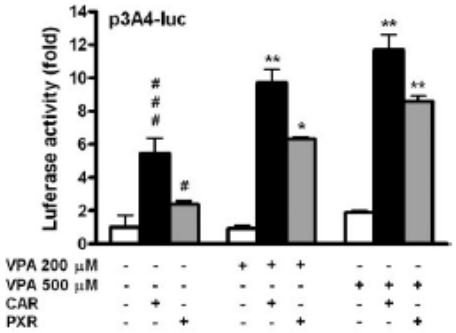

FIG. 1. Effect of VPA on CAR- and PXR-mediated transactivation of CYP3A4 promoter. HepG2 cells were transiently transfected with *p3A4-luc* reporter construct containing the basal promoter (−362/+53) with proximal PXR response element and the distal xenobiotic responsive enhancer module (−7836/−7208) 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

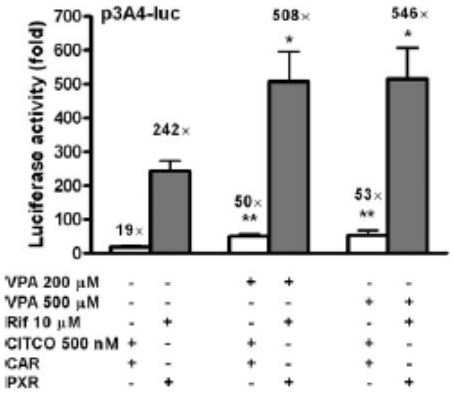

FIG. 2. Transactivation of CYP3A4 promoter by VPA in combination with CITCO or rifampicin. HepG2 cells were transiently transfected with *p3A4-luc* reporter construct (0.4 μg/well) and *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) using Lipofectamine2000 transfection reagent according to the manufacturer's 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 $p < 0.01$)

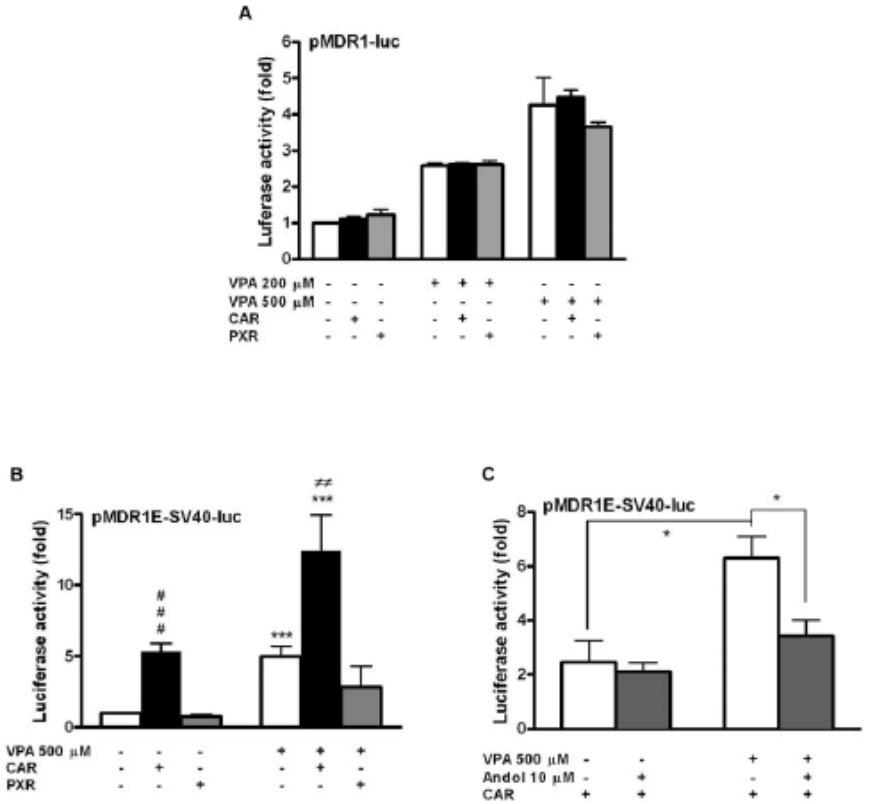

FIG. 3. Effect of VPA on CAR- and PXR-mediated activation of reporter constructs of *MDR1* gene. HepG2 cells were transiently transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) and appropriate reporter construct (0.4 μg/well) using Lipofectamine2000 transfection reagent according to 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-

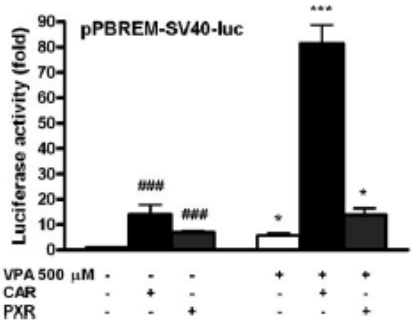

FIG. 4. Effect of VPA on CAR- and PXR-mediated activation of the PBREM region of the CYP2B6 promoter. HepG2 cells were transiently transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmid (50 ng) and *pPBREM-SV40-luc* reporter construct containing two DR4-type motifs of CYP2B6 PBREM (0.4 μg/well) 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

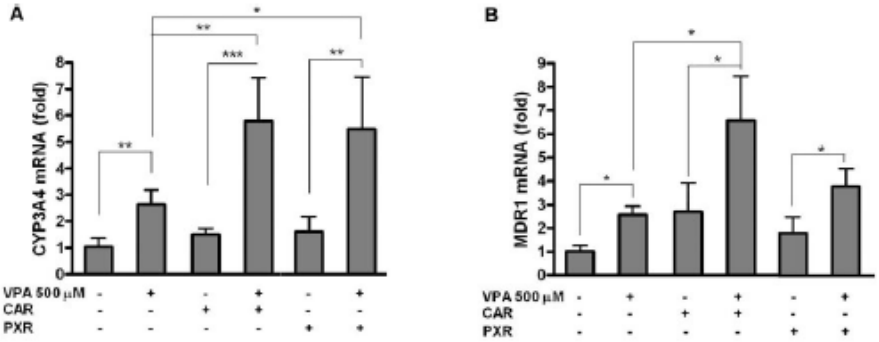

FIG. 5. Analysis of VPA-mediated up-regulation of CYP3A4 and MDR1 mRNAs. LS174T cells were transfected with *pCR3-CAR* or *pSG5-PXR* expression plasmids (400 ng/well) using Lipofectamine2000 transfection reagent according to the manufacturer's instructions and exposed to VPA (500 μM) for 48 h. mRNA expression 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.

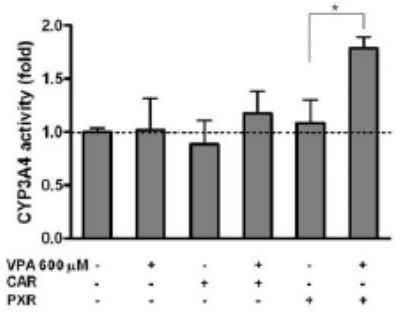

FIG. 6. Determination of CYP3A4 catalytic activity. LS174T cells were transfected with expression plasmids encoding CAR or PXR. Subsequently, cells were treated with VPA (600 μM) and specific CYP3A4-mediated testosterone 6β-hydroxylation activity was detected after 48 h. The concentration of the 6β-testosterone 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

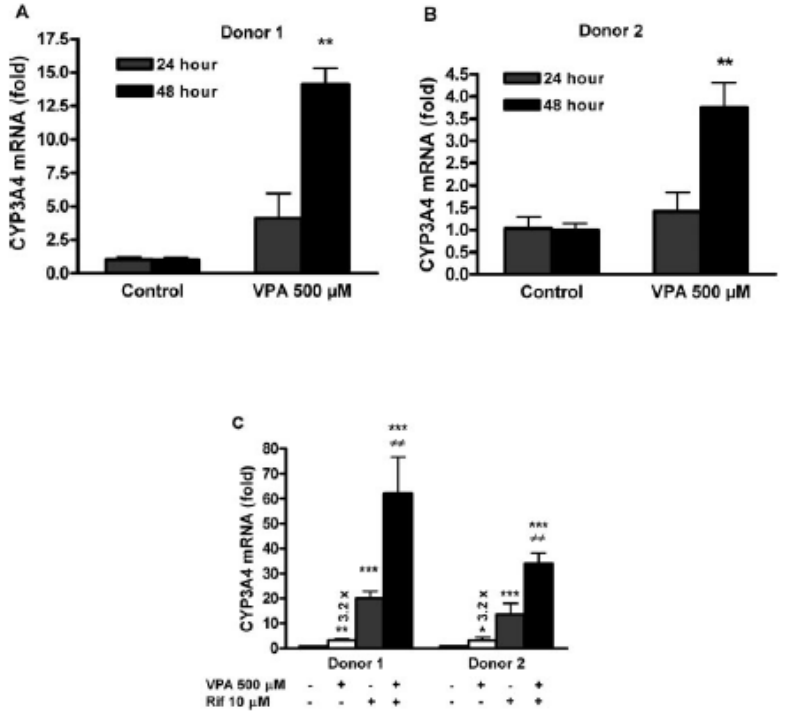

FIG. 7. VPA synergizes with rifampicin (Rif) in induction of CYP3A4 mRNA in primary human hepatocytes. Primary human hepatocyte cultures were cultivated together with VPA and/or rifampicin either individually or in combination, and then assayed using real-time RT-PCR. CYP3A4 mRNA expression is normalized 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).

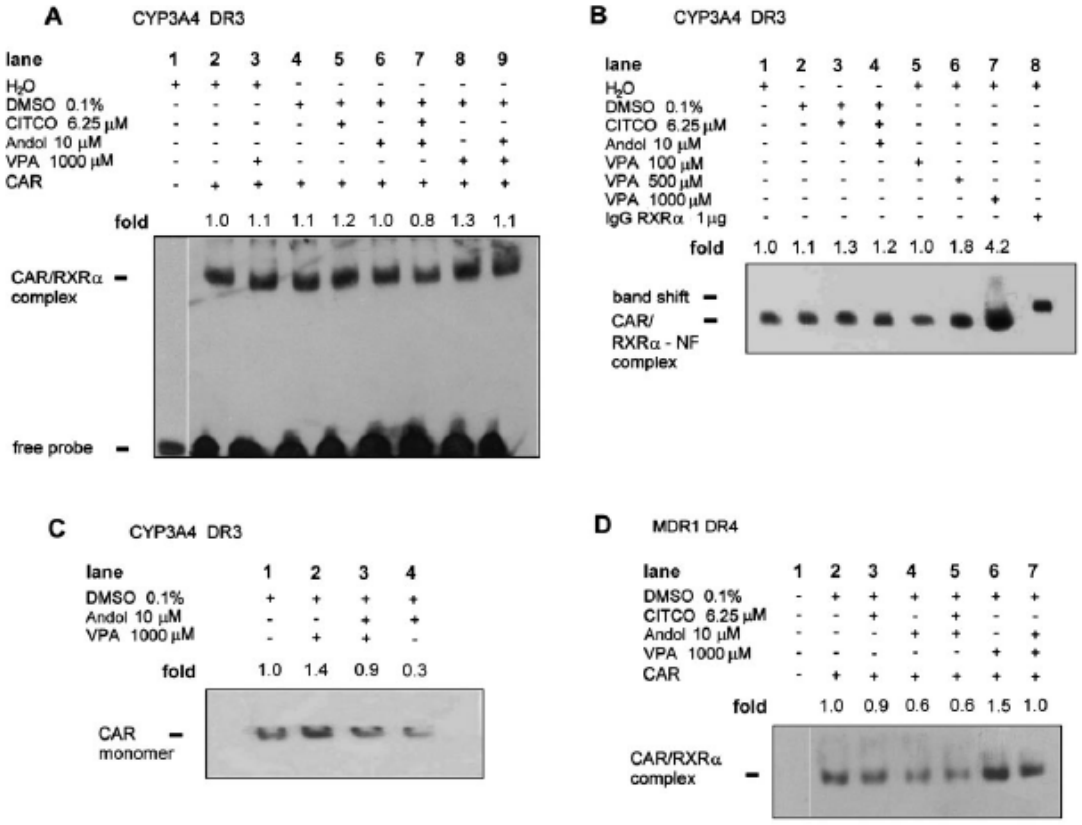

FIG. 8. The ability of CAR/RXRα heterodimer to bind CAR response elements of CYP3A4 (DR3, ER6) and MDR1 (DR4) in the presence of VPA was investigated using EMSA as described under *Materials and Methods*. A, the complex formation of CYP3A4 DR3 with CAR/RXRα was examined with in vitro translated wild-type 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.

References

Allen MH, Hirschfeld RM, Wozniak PJ, Baker JD, and Bowden CL (2006) Linear relationship of valproate serum concentration to response and optimal serum levels for acute mania. *Am J Psychiatry* 163:272–275.

Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, and Berkenstam A (1998) Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A* 95:12208–12213.

Blaheta RA, Michaelis M, Driever PH, and Cinatl J Jr (2005) Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. *Med Res Rev* 25:383–397.

Blaheta RA, Nau H, Michaelis M, and Cinatl J Jr (2002) Valproate and valproate-analogues: potent tools to fight against cancer. *Curr Med Chem* 9:1417–1433.

Bosetti F, Bell JM, and Manckam P (2005) Microarray analysis of rat brain gene expression after chronic administration of sodium valproate. *Brain Res Bull* 65:331–338.

Burk O, Arnold KA, Geick A, Tegude H, and Eichelbaum M (2005a) A role for constitutive androstane receptor in the regulation of human intestinal MDR1 expression. *Biol Chem* 386:503–513.

Burk O, Arnold KA, Nussler AK, Schaeffeler E, Efimova E, Avery BA, Avery MA, Fromm MF,

and Eichelbaum M (2005b) Antimalarial artemisinin drugs induce cytochrome P450 and MDR1 expression by activation of xenosensors pregnane X receptor and constitutive androstane receptor. *Mol Pharmacol* 67:1954–1965.
Ceniccrino F, Kelleher JP, Berry JM, Salvatore P, Eskin M, Fogarty KV, Feliman V, and Baldessarini RJ (2003) Pilot comparison of extended-release and standard preparations of divalproex sodium in patients with bipolar and schizoaffective disorders. *Am J Psychiatry* 160:1348–1350.
Chen G, Huang LD, Jiang YM, and Manji HK (1999) The mood-stabilizing agent valproate inhibits the activity of glycogen synthase kinase-3. *J Neurochem* 72:1327–1330.
Davis R, Peters DH, and McTavish D (1994) Valproic acid. A reappraisal of its pharmacological properties and clinical efficacy in epilepsy. *Drugs* 47:332–372.
Eyal S, Lamb JG, Smith-Yockman M, Yagen B, Fibach E, Altschuler Y, White HS, and Bialer M (2006) The antiepileptic and anticancer agent, valproic acid, induces P-glycoprotein in human tumour cell lines and in rat liver. *Br J Pharmacol* 149:250–260.
Faucette SR, Zhang TC, Moore R, Sueyoshi T, Omiecinski CJ, LeCluyse EL, Negishi M, and Wang H (2007) Relative activation of human pregnane X receptor versus constitutive androstane receptor defines distinct classes of CYP2B6 and CYP3A4 inducers. *J Pharmacol Exp Ther* 320:72–80.
Fisher JE, Nau H, and Loscher W (1991) Alterations in the renal excretion of valproate and its metabolites after chronic treatment. *Epilepsia* 32:146–150.
Forman BM, Tzameli I, Choi HS, Chen J, Simha D, Seol W, Evans RM, and Moore DD (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR-beta. *Nature* 395: 612–615.
Frank C, Gonzalez MM, Oinonen C, Dunlop TW, and Carlberg C (2003) Characterization of DNA complexes formed by the nuclear receptor constitutive androstane receptor. *J Biol Chem* 278:43299–43310.
Frank C, Molnar F, Matilainen M, Lempiainen H, and Carlberg C (2004) Agonist-dependent and agonist-independent transactivations of the human constitutive androstane receptor are modulated by specific amino acid pairs. *J Biol Chem* 279:33558–33566.
Geick A, Eichelbaum M, and Burk O (2001) Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* 276:14581–14587.
Goodwin B, Hodgson E, D'Costa DJ, Robertson GR, and Liddle C (2002) Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Mol Pharmacol* 62:359–365.
Goodwin B, Hodgson E, and Liddle C (1999) The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 56:1329–1339.
Goodwin B and Moore JT (2004) CAR: detailing new models. *Trends Pharmacol Sci* 25:437–441.
Goodwin B, Moore LB, Stoltz CA, McKee DD, and Kliewer SA (2001) Regulation of the human CYP2B6 gene by the nuclear pregnane X receptor. *Mol Pharmacol* 60:427–431.
Göttlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, Giavara S, Sleeman JP, Lo Coco F, Nervi C, Pelicci PG, et al. (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. *EMBO J* 20:6969–6978.
Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T, and Waxman DJ (1986) Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J Biol Chem* 261:5051–5060.
Honkakoski P, Zelko I, Sueyoshi T, and Negishi M (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol* 18:5652–5658.
Isom HC, Secott T, Georgoff I, Woodworth C, and Mummaw J (1985) Maintenance of differentiated rat hepatocytes in primary culture. *Proc Natl Acad Sci U S A* 82:3252–3256.
Jin S and Scotto KV (1998) Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Mol Cell Biol* 18:4377–4384.
Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, et al. (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* 14:27–39.
Kawamoto T, Sueyoshi T, Zelko I, Moore R, Washburn K, and Negishi M (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol Cell Biol* 19:6318–6322.
Kramer OH, Zhu P, Ostendorff HP, Golebiewski M, Tiefenbach J, Peters MA, Brill B, Groner B, Bach I, Heinzel T, et al. (2003) The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *EMBO J* 22:3411–3420.
Maglich JM, Parks DJ, Moore LB, Collins JL, Goodwin B, Billin AN, Stoltz CA, Kliewer SA, Lambert MH, Willson TM, et al. (2003) Identification of a novel human constitutive androstane receptor (CAR) agonist and its use in the identification of CAR target genes. *J Biol Chem* 278:17277–17283.
Masuyama H, Suwaki N, Tateishi Y, Nakatsukasa H, Segawa T, and Hiramatsu Y (2005) The pregnane X receptor regulates gene expression in a ligand- and promoter-selective fashion. *Mol Endocrinol* 19:1170–1180.
Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, and Kliewer SA (2000a) St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci U S A* 97:7500–7502.
Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, et al. (2000b) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* 275: 15122–15127.
Morrow CS and Nakagawa M (1994) Reversible transcriptional activation of mdr1 by sodium butyrate treatment of human colon cancer cells. *J Biol Chem* 269:10739–10746.
Pascussi JM, Drocourt L, Fabre JM, Maurel P, and Vilarem MJ (2000) Dexamethasone induces pregnane X receptor and retinoid X receptor-alpha expression in human hepatocytes: synergistic increase of CYP3A4 induction by pregnane X receptor activators. *Mol Pharmacol* 58:361–372.
Perucca E (2006) Clinically relevant drug interactions with antiepileptic drugs. *Br J Clin Pharmacol* 61:246–255.
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.
Phiel CJ, Zhang F, Huang EY, Guenther MG, Lazar MA, and Klein PS (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. *J Biol Chem* 276:36734–36741.
Pichard-Garcia L, Gerbal-Chaloin S, Ferrini JB, Fabre JM, and Maurel P (2002) Use of long-term cultures of human hepatocytes to study cytochrome P450 gene expression. *Methods Enzymol* 357:311–321.
Raffoux E, Chaibi P, Dombret H, and Degos L (2005) Valproic acid and all-trans retinoic acid for the treatment of elderly patients with acute myeloid leukemia. *Haematologica* 90:986–988.
Rodriguez-Antona C, Bort R, Jover R, Tindberg N, Ingelman-Sundberg M, Gómez-Lechón M, and Castell JV (2003) Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein. *Mol Pharmacol* 63:1180–1189.
Rogiers V, Akrawi M, Vercruysse A, Phillips IR, and Shephard EA (1995) Effects of the anticonvulsant, valproate, on the expression of components of the cytochrome-P-450-mediated monooxygenase system and glutathione S-transferases. *Eur J Biochem* 231:337–343.
Rogiers V, Callaerts A, Vercruysse A, Akrawi M, Shephard E, and Phillips I (1992) Effects of valproate on xenobiotic biotransformation in rat liver. In vivo and in vitro experiments. *Pharm Weekbl Sci* 14:127–131.
Scotto KV (2003) Transcriptional regulation of ABC drug transporters. *Oncogene* 22:7496–7511.
Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, et al. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* 98:3369–3374.
Tanaka E (1999) Clinically significant pharmacokinetic drug interactions between antiepileptic drugs. *J Clin Pharm Ther* 24:87–92.
Wen X, Wang JS, Kivisto KT, Neuvonen PJ, and Backman JT (2001) In vitro evaluation of valproic acid as an inhibitor of human cytochrome P450 isoforms: preferential inhibition of cytochrome P450 2C9 (CYP2C9). *Br J Clin Pharmacol* 52:547–553.
Werling U, Siehler S, Lüdin M, Nau H, and Gottlicher M (2001) Induction of differentiation in P9 cells and activation of peroxisome proliferator-activated receptor delta by valproic acid and its teratogenic derivatives. *Mol Pharmacol* 59:1269–1276.
Xiao JJ and Huang Y (2005) Chemoresistance to depsipeptide FK228 [(E)-(1S,4S,10S,21R)-7-[(Z)-ethylidene]-4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8,7,6]-tricos-16-ene-3,6,9,22-pentanone] is mediated by reversible MDR1 induction in human cancer cell lines. *J Pharmacol Exp Ther* 314:467–475.
Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* 98:3375–3380.

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2.3 Examination of glucocorticoid receptor α-mediated transcriptional regulation of P-glycoprotein, *CYP3A4*, nad *CYP2C9* genes in placental trofoblast cell lines.

Pavek P, Cerveny L, **Svecova L**, Brysch M, Libra A, Vrzal R, Nachtigal P, Staud F, Ulrichova J,Fendrich Z, Dvorak Z. *Examination of glucocorticoid receptor α-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines.* Placenta 28:1004-1011, 2007. **(IF 3,238$_{2007}$)**

Abstrakt:

Placentární trofoblast exprimuje v závislosti na stádiu těhotenství některé lékové transportéry, biotransformační enzymy a navíc nukleární receptory, které regulují jejich inducibilní transkripci. Nukleární receptor GRα je exprimován jak placentárním syncytiotrofoblastem, tak cytotrofoblastem. Jak je již známo, GRα reguluje inducibilní expresi některých enzymů CYP a lékového transportéru Pgp v játrech. Regulace transkripce lékových transportérů a CYP prostřednictvím GRα však dosud nebyla zkoumána v placentárním trofoblastu. V této práci se zabýváme expresí GRα a jeho vlivem na regulaci transkripce Pgp, CYP3A4 a CYP2C9 v buněčných liniích placentárního trofoblastu. S využitím metod real time RT-PCR, western blotu a gene reporter assay jsme detekovali expresi a aktivitu GRα v liniích JEG3 a BeWo. Up-regulaci exprese vlivem dexametazonu v placentárních liniích jsme však pozorovali pouze v případě MDR1 mRNA. Také v gene reporter assay experimentech byl v placentárních buňkách dexametazonem aktivován pouze promotor MDR1 genu a tato aktivace byla blokována přítomností RU486, známého antagonisty GRα. Promotory genů CYP3A4 a CYP2C9 byly v placentárních liniích aktivovány pouze po kotransfekci HNF4α, což naznačuje jaterně specifických charakter regulace těchto genů prostřednictvím GRα. Kotransfekce HNF4α však neměla žádný efekt na aktivaci MDR1 promotoru, což poukazuje na HNF4α independentní regulaci přes GRα. Ze získaných dat vyvozujeme, že GRα se může podílet na regulaci exprese Pgp v placentárním trofoblastu. Dále naznačujeme, že geny CYP3A4 a CYP2C9 nejsou v placentárních buňkách inducibilní prostřednictvím GRα v důsledku nepřítomnosti HNF4α a pravděpodobně též dalších jaterně specifických transkripčních faktorů.

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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.

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 SYBR Green 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

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

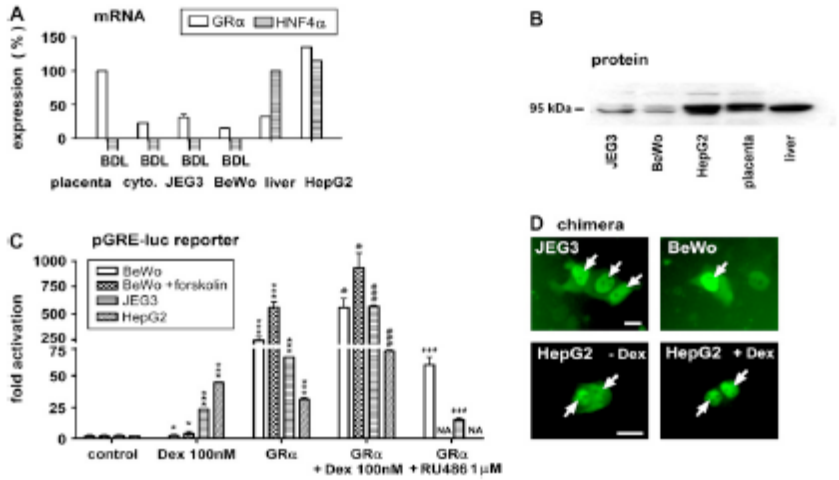

Fig. 1. Activity and expression of GRα in the placenta, isolated cytotrophoblasts and placental BeWo and JEG3 cell lines in comparison with the liver and HepG2 cell lines. (A) Relative expression of GRα and HNF4α mRNAs analysed by RT–PCR. Expression of GRα was related to the pooled placental sample (100%) 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, $^{\#\#\#}p < 0.001$ (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

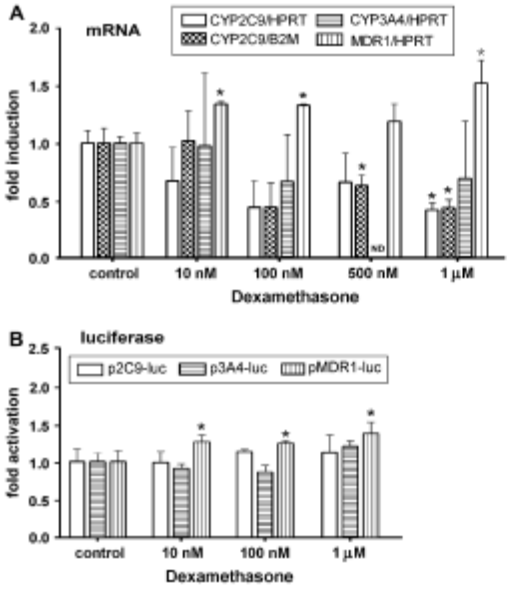

Fig. 2. Effect of dexamethasone on expression of *MDR1*, CYP2C9, and CYP3A4 mRNAs and transactivation of their luciferase reporter vectors in JEG3 cells. (A) RT–PCR analysis of mRNA expression of tested genes in JEG3 cells. Twenty-four hours after seeding on plates, cells were exposed to dexamethasone 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;

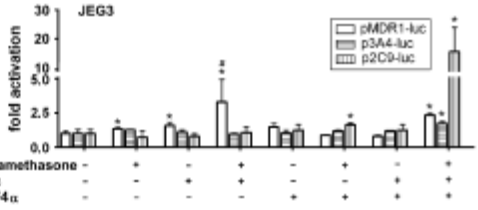

Fig. 3. Involvement of HNF4α in GRα-mediated activation of pMDR1-luc, p2C9-luc, and p3A4-luc reporter constructs in JEG3 cells. Reporter constructs were transiently transfected into JEG3 cells along with expression vectors for GRα and HNF4α either individually or in combination. Co-transfections were 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

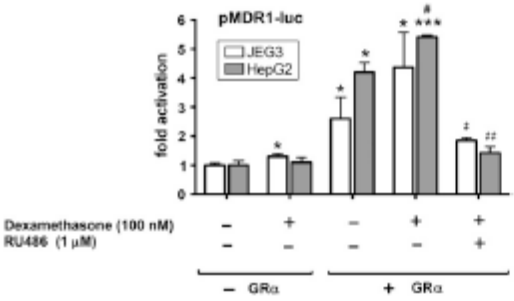

Fig. 4. GRα-mediated activation of MDR1 promoter in JEG3 and HepG2 cell lines. Cells were co-transfected with pMDR1-luc reporter construct (500 ng) and pSG5-hGRα (or empty pSG5) expression vector (50 ng). After 24 h of cultivation, cells were exposed to 100 nM dexamethasone (Dex) or a combination of dexamethasone (100 nM) and RU486 (1 μM) for 48 h before determination of firefly and renilla lucifernse 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).

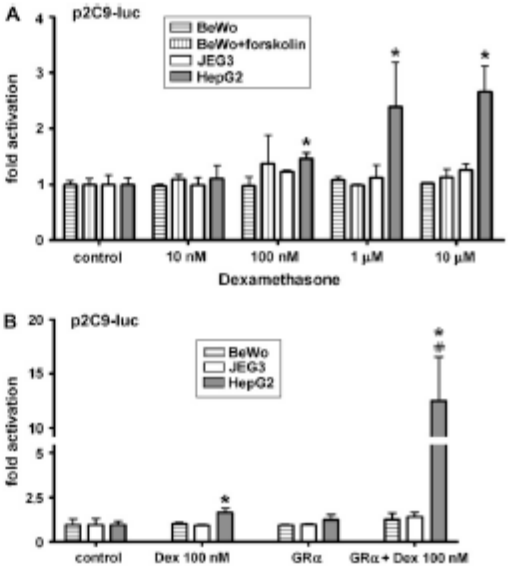

Fig. 5. GRα-mediated activation of p2C9-luc reporter plasmid in placental and HepG2 cell lines. (A) Dose-dependent activation of p2C9-luc reporter plasmid in placental and HepG2 cell lines by dexamethasone. Twenty-four hours after transfection of p2C9-luc into cells, dexamethasone was added to medium 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 lucifernse 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

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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References

[1] Hakkola J, Pelkonen O, Pasanen M, Raunio H. Xenobiotic-metabolizing cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity. Crit Rev Toxicol 1998;28:35–72.
[2] Syme MR, Paxton JW, Keelan JA. Drug transfer and metabolism by the human placenta. Clin Pharmacokinet 2004;43:487–514.
[3] Myllynen P, Pasanen M, Pelkonen O. Human placenta: a human organ for developmental toxicology research and biomonitoring. Placenta 2005;26:361–71.
[4] Hakkola J, Pasanen M, Hukkanen J, Pelkonen O, Maenpaa J, Edwards RJ, et al. Expression of xenobiotic-metabolizing cytochrome P450 forms in human full-term placenta. Biochem Pharmacol 1996;51:403–11.
[5] Hakkola J, Raunio H, Purkunen R, Pelkonen O, Saarikoski S, Cresteil T, et al. Detection of cytochrome P450 gene expression in human placenta in first trimester of pregnancy. Biochem Pharmacol 1996;52:379–83.
[6] Mathias AA, Hitti J, Unadkat JD. P-glycoprotein and breast cancer resistance protein expression in human placentae of various gestational ages. Am J Physiol Regul Integr Comp Physiol 2005;289:R963–9.
[7] Gil S, Saura R, Forestier F, Farinotti R. P-glycoprotein expression of the human placenta during pregnancy. Placenta 2005;26:268–70.
[8] Sun M, Kingdom J, Baczyk D, Lye SJ, Matthews SG, Gibb W. Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation. Placenta 2006;27:602–29.
[9] Lankas GR, Wise LD, Cartwright ME, Pippert T, Umbenhauer DR. Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice. Reprod Toxicol 1998;12:457–63.
[10] Pavek P, Fendrich Z, Staud F, Malakova J, Brozmanova H, Lazicek M, et al. Influence of P-glycoprotein on the transplacental passage of cyclosporine. J Pharm Sci 2001;90:1583–92.
[11] Pavek P, Staud F, Fendrich Z, Sklenarova H, Libra A, Novotna M, et al. Examination of the functional activity of P-glycoprotein in the rat placental barrier using rhodamine 123. J Pharmacol Exp Ther 2003;305:1239–50.
[12] Molsa M, Heikkinen T, Hakkola J, Hakala K, Wallerman O, Wadelius M, et al. Functional role of P-glycoprotein in the human blood-placental barrier. Clin Pharmacol Ther 2005;78:123–31.

[13] Cecková-Novotna M, Pavek P, Staud F. P-glycoprotein in the placenta: Expression, localization, regulation and function. Reprod Toxicol 2006;22:400–10.
[14] Mark PJ, Waddell BJ. P-glycoprotein restricts access of cortisol and dexamethasone to the glucocorticoid receptor in placental BeWo cells. Endocrinology 2006;147:5147–52.
[15] Tirona RG, Kim RB. Nuclear receptors and drug disposition gene regulation. J Pharm Sci 2005;94:1169–86.
[16] Paakki P, Kirkinen P, Helin H, Pelkonen O, Raunio H, Pasanen M. Antepartum glucocorticoid therapy suppresses human placental xenobiotic and steroid metabolizing enzymes. Placenta 2000;21:241–6.
[17] Lee MJ, Wang Z, Yee H, Ma Y, Swenson N, Yang L, et al. Expression and regulation of glucocorticoid receptor in human placental villous fibroblasts. Endocrinology 2005;146:4619–26.
[18] Waddell BJ, Benediktsson R, Brown RW, Seckl JR. Tissue-specific messenger ribonucleic acid expression of 11beta-hydroxysteroid dehydrogenase types 1 and 2 and the glucocorticoid receptor within rat placenta suggests exquisite local control of glucocorticoid action. Endocrinology 1998;139:1517–23.
[19] Schuetz EG, Schmid W, Schutz G, Brimer C, Yasuda K, Kamataki T, et al. The glucocorticoid receptor is essential for induction of cytochrome P-4502B by steroids but not for drug or steroid induction of CYP3A or P-450 reductase in mouse liver. Drug Metab Dispos 2000; 28:268–78.
[20] Pascussi JM, Gerbal-Chaloin S, Fabre JM, Maurel P, Vilarem MJ. Dexamethasone enhances constitutive androstane receptor expression in human hepatocytes: consequences on cytochrome P450 gene regulation. Mol Pharmacol 2000;58:1441–50.
[21] Gerbal-Chaloin S, Daujat M, Pascussi JM, Pichard-Garcia L, Vilarem MJ, Maurel P. Transcriptional regulation of CYP2C9 gene. Role of glucocorticoid receptor and constitutive androstane receptor. J Biol Chem 2002;277:209–17.
[22] El-Sankary W, Bomball V, Gibson GG, Plant N. Glucocorticoid-mediated induction of CYP3A4 is decreased by disruption of a protein: DNA interaction distinct from the pregnane X receptor response element. Drug Metab Dispos 2002;30:1029–34.
[23] Scotto KW. Transcriptional regulation of ABC drug transporters. Oncogene 2003;22:7496–511.
[24] Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, et al. The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nat Med 2003;9:220–4.
[25] Chen Y, Kissling G, Negishi M, Goldstein JA. The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4alpha to synergistically activate the human CYP2C9 promoter. J Pharmacol Exp Ther 2005;314:1125–33.
[26] Goodwin B, Hodgson E, Liddle C. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. Mol Pharmacol 1999;56:1329–39.
[27] Geick A, Eichelbaum M, Burk O. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. J Biol Chem 2001;276:14581–7.
[28] Galigniana MD, Scruggs JL, Herrington J, Welsh MJ, Carter-Su C, Housley PR, et al. Heat shock protein 90-dependent (geldanamycin-inhibited) movement of the glucocorticoid receptor through the cytoplasm to the nucleus requires intact cytoskeleton. Mol Endocrinol 1998;12: 1903–13.
[29] Liu F, Soares MJ, Audus KL. Permeability properties of monolayers of the human trophoblast cell line BeWo. Am J Physiol 1997;273: C1596–604.
[30] Cecková M, Libra A, Pavek P, Nachtigal P, Brabec M, Fuchs R, et al. Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo. Clin Exp Pharmacol Physiol 2006;33:58–65.
[31] Novotna M, Libra A, Kopecky M, Pavek P, Fendrich Z, Semecky V. P-glycoprotein expression and distribution in the rat placenta during pregnancy. Reprod Toxicol 2004;18:785–92.
[32] Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 2001;29:e45.
[33] Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss 3rd JF. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology 1986;118:1567–82.
[34] Demey-Ponsart E, Foidart JM, Sulon J, Sodoyez JC. Serum CBG, free and total cortisol and circadian patterns of adrenal function in normal pregnancy. J Steroid Biochem 1982;16:165–9.
[35] Speeg Jr KV, Harrison RW. The ontogeny of the human placental glucocorticoid receptor and inducibility of heat-stable alkaline phosphatase. Endocrinology 1979;104:1364–8.
[36] Jobe AH, Soll RF. Choice and dose of corticosteroid for antenatal treatments. Am J Obstet Gynecol 2004;190:878–81.
[37] Van Runnard Heimel PJ, Franx A, Schobben AF, Huisjes AJ, Derks JB, Bruinse HW. Corticosteroids, pregnancy, and HELLP syndrome: a review. Obstet Gynecol Surv 2005;60:57–70.
[38] Pascussi JM, Drocourt L, Gerbal-Chaloin S, Fabre JM, Maurel P, Vilarem MJ. Dual effect of dexamethasone on *CYP3A4* gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. Eur J Biochem 2001;268:6346–58.

2.4 Podíl na jednotlivých publikacích

Předkladatelka dizertační práce je prvním autorem publikace 2.1, tedy sepsala příslušný rukopis. S laskavou pomocí Doc. PharmDr. Petra Pávka, Ph.D. provedla gene reporter assay experimenty a real time PCR analýzu všech vzorků. Kultivaci primárních hepatocytů a přípravu vzorků pro izolaci RNA zajistili Ing. Radim Vrzal, Ph.D. a Doc. RNDr. Zdeněk Dvořák, Ph.D. z Katedry buněčné biologie a genetiky Přírodovědecké fakulty Univerzity Palackého v Olomouci.

V případě publikace 2.2 je předkladatelka druhým a v případě 2.3 třetím autorem. Podílela se na částech experimentálních prací souvisejících s tématem disertační práce a úpravách textu. Zavedla metodu EMSA spolu s přípravou rekombinačních proteinů pro tuto metodu. Realizovala EMSA experimenty uvedené ve 2.2 kapitole a zčásti spolupracovala na gene reporter assay studiích, které jsou zahrnuty do 2.3 kapitoly.

Témata řešených projektů byla navržena Doc. PharmDr. Petrem Pávkem, Ph.D., který důsledně zastával roli školitele specialisty a dohlížel na správnou realizaci všech experimentů jako i na zpracování výsledků. Projekty byly schváleny a financovány grantovými agenturami IGA MZ a GAČR.

3 Závěr

Regulace exprese biotransformačního enzymu CYP3A4 je předmětem zájmu mnoha vědeckých pracovišť na celém světě. Očekává se, že objasnění mechanismů jaterně specifické konstitutivní a indukovatelné transkripce jakožto i otestování účinků klinicky užívaných látek na regulaci exprese *CYP3A4* napomůže důkladnějšímu porozumění funkce nejvýznamnějšího lidského detoxifikačního systému, osvětlí vznik některých LI a usnadní jejich predikci.

Tato práce je reflexí aktuálního stavu problematiky genové regulace CYP3A4. S využitím v současné době upřednostňovaných molekulárně biologických metod a experimentálních modelů se nám podařilo splnit vytýčené cíle (2.6), výsledky experimentů byly publikovány v impaktovaných časopisech, prezentovány ústně či ve formě posterů na vědeckých konferencích a přispívají k rozšíření znalostí v řešené oblasti.

4 Publikované vědecké a odborné práce

Publikace v impaktovaných časopisech

1. **Svecova L**, Vrzal R, Burysek L, Anzenbacherova E, Cerveny L, Grim J, Trejtnar F, Kunes J, Pour M, Staud F, Anzenbacher P, Dvorak Z, Pavek P. *Azole antimycotics differentially affect rifampicin-induced pregnane X receptor-mediated CYP3A4 gene expression.* DMD 36:339–348, 2008. IF dopsat Počet citací: 1.
2. Cerveny l, **Svecova L**, Anzenbacherova E, Vrzal R, Staud F, Dvorak Z, Ulrichova J, Anzenbacher P, Pavek P. *Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways.* DMD 35:1032–1041, 2007. IF Počet citací: 11.
3. Pavek P, Cerveny L, **Svecova L**, Brysch M, Libra A, Vrzal R, Nachtigal P, Staud F, Ulrichova J, Fendrich Z, Dvorak Z. *Examination of glucocorticoid receptor α-mediated transcriptional regulation of P-glycoprotein, CYP3A4, and CYP2C9 genes in placental trophoblast cell lines.* Placenta 28:1004-1011, 2007.IF Počet citací 3 nebo 4 nevím
4. Dvorak Z, Vrzal R, Henklova P, Jancova P, Anzenbacherova E, Maurel P, **Svecova L**, Pavek P, Ehrmann J, Havlik R, Bednar P, Lemr K, Ulrichova J. *JNK inhibitor SP600125 is a partial agonist of human aryl hydrocarbon receptor and induces CYP1A1 and CYP1A2 genes in primary human hepatocytes.* Biochem Pharmacol. 2:580-8, 2008. **(IF 4.006$_{2007}$)**Počet citací: 9.

Ústní prezentace

1. *Novel insights in gene regulation of CYP3A4 enzyme via pregnane X nuclear receptor*. The 5th international conference of postgraduate medical students, 27. – 29. 11. 2008, Hradec Králové.
2. *Activation of CYP3A4 promoter by selected azole antifungal drugs.* International medical students´ congress in Novi Sad, 27. – 30. 7. 2006, Nový Sad, Srbsko.

Posterové prezentace a abstrakty publikované ve sbornících

1. **Svecova L**, Pavek P. *Transactivation of PXR-mediated CYP3A4 expression by selected azole antimycotics: comparison with rifampicin.* ELSO 2007, 1. – 4. 9. 2007, Drážďany, Německo.
2. Bitman M, Vrzal R, Pospechova K, **Svecova L**, Stejskalova L, Dvorak Z, Pavek P. *Effect of valproic acid on extracellular mitogen-activated protein kinases (ERK) pathway and major transcriptional factors in hepatoma cell lines and primary human hepatocytes.* 11th European Regional ISSX Meeting, 17. – 20. 5. 2009, Lisabon, Portugalsko.
3. Bitman M, Stejskalova L, Pospechova K, **Svecova L**, Vrzal R, Cerveny L, Dvorak Z, Pavek P. *Role of extracellular-signal regulated kinase (ERK) pathway in PXR-mediated valproic acid-induced activation of CYP3A4 expression.* 58. Farmakologické dny, 3. – 5. 9. 2008, Praha.
4. Pavek P, Pospechova K, **Svecova L**, Syrova Z, Bitman M, Stejskalova L, Blahos J. *Cooperation of three distinct promoter regulatory elements of PXR, CAR and VDR nuclear receptors in transcriptional regulation of CYP3A4 gene.* RNA Club ÚMG AV ČR, 28. 11. 2008, Praha.
5. Stejskalova L, Pospechova K, **Svecova L**, Bitman M, Dvorak Z, Pavek P. *Evidence of cross-talk between aryl hydrocarbon receptor and glucocorticoid receptor in placental trophoblast JEG3 cells.* 58. Farmakologické dny, 3. – 5. 9. 2008, Praha.
6. Pavek P, Cerveny L, **Svecova L**, Vrzal R, Bitman M, Dvorak Z. *Valproic acid induces CYP3A4 expression through constitutive androstane (CAR) nuclear receptor pathway.* Nuclear Receptors**:** Orphan Brothers (Z1). 30. 3. – 4. 4. 2008, Whistler, Kanada.
7. Henklova P, Vrzal R, Ulrichova J, Pavek P, **Svecova L**, Maurel P, Dvorak Z. *Activation of human aryl hydrocarbon receptor in primary human hepatocytes and HepG2 cells by c-Jun-N-Terminal kinase inhibitor SP600125.* XII. Setkání biochemiků a molekulárních biologů, 6. – 7. 2. 2008, Brno.

8. Cerveny L, **Svecova L**, Vrzal R, Bitman M, Dvorak Z, Pavek P. *Valproic acid induces CYP3A4 expression through pregnane X (PXR) and constitutive androstane (CAR) nuclear receptor pathways.* XII. Setkání biochemiků a molekulárních biologů, 6. – 7. 2. 2008, Brno.

5 Souhrn

CYP3A4 je významným enzymem podílejícím se na eliminaci většiny biotransformovaných xenobiotik. Hraje zásadní úlohu v detoxifikačním systému lidského těla, čímž je také zodpovědný za vznik řady lékových interakcí. Tyto interakce představují výraznou komplikaci současné farmakoterapie, neboť v krajních případech mohou ústit až k selhání léčby či život ohrožujícímu vystupňování toxických účinků.

LI jsou důsledkem změn aktivity CYP3A4, která je mezi jednotlivci velice variabilní. Důležitým mechanismem ovlivnění aktivity CYP3A4 je regulace indukovatelné transkripce prostřednictvím xenobiotiky aktivovaných nukleárních receptorů. Jedná se zejména o receptory PXR, CAR a GR. Intenzivní studium struktury promotoru *CYP3A4* a mechanismů regulace jeho transkripce v posledních letech ještě zdaleka není u konce, vztahy mezi jednotlivými receptory a kofaktory, stejně jako schopnost léčiv zasahovat do exprese CYP3A4 je doposud odkryta jen zčásti.

Tato práce přispívá k objasnění některých otázek týkajících se účinků azolových antimykotik na aktivitu transkripce *CYP3A4* zprostředkovanou PXR, schopnosti valproátu aktivovat PXR a CAR či okolností placentární exprese CYP3A4 prostřednictvím GR. K experimentům byly využity moderní molekulárně biologické metody a probíhaly *in vitro* v kulturách primárních hepatocytů a buněčných linií.

K jednotlivým cílům dizertační práce:

1. Vliv vybraných azolových antimykotik na genovou expresi *CYP3A4* prostřednictvím PXR. Objasnění povahy sledovaných efektů na molekulární úrovni (2.1).

Otestovali jsme účinky vybraných azolových antimykotik (klotrimazolu, ketokonazolu, itrakonazolu, flukonazolu, oxikonazolu, ekonazolu a mikonazolu) na genovou expresi *CYP3A4* prostřednictvím PXR v primární kultuře lidských hepatocytů a buněčných linií HepG2, LS174T a CV-1. S využitím metod real time RT-PCR, gene reporter assay, one hybrid assay a two hybrid assay jsme sledovali schopnost azolů transaktivovat promotor *CYP3A4* nejen individuálně, ale také v přítomnosti rifampicinu, standardního induktoru CYP3A4 přes PXR. Zaznamenali jsme významné odlišnosti mezi jednotlivými azoly, identifikovali jsme potentní induktor oxikonazol a do jisté míry objasnili povahu sledovaných efektů na úrovni interakcí azolů s LBD PXR či na úrovni jejich schopností ovlivnit tvorbu komplexu PXR/SRC-1. Na základě dose-response analýz jsme zkonstatovali, že rifampicin není schopen plné aktivace PXR a ve vztahu k oxikonazolu se chová jako parciální agonista. Naopak rifampicin v přítomnosti ekonazolu nebo mikonazolu vykazuje aditivní efekt na aktivaci PXR. Pozorované účinky vedou k závěru, že schopnost azolů ovlivnit expresi CYP3A4 je pro každou strukturu individuální a že změny, které mohou v důsledku aktivace PXR zapříčinit, se neomezují pouze na studovaný enzym.

2. Účinek kyseliny valproové na genovou expresi *CYP3A4* prostřednictvím CAR. Zavedení metody EMSA na naše pracoviště za účelem testování interakcí adekvátních responzivních elementů v přítomnosti či nepřítomnosti kyseliny valproové (2.2).

S využitím real time RT-PCR a gene reporter assay jsme popsali indukční účinek kyseliny valproové na genovou expresi *CYP3A4* prostřednictvím CAR a PXR, a to v lidských hepatocytech a liniích HepG2 a LS174T. Po zavedení metody EMSA jsme pozorovali zesílení vazby komplexu CAR/RXRα na responzivní elementy DR3, DR4 a ER6 v přítomnosti valproátu. Dále jsme zaznamenali účinkem valproátu zvýšenou katalytickou aktivitu CYP3A4 v buňkách LS174T transfekovaných PXR. Kyselina valproová ovlivňuje expresi řady genů mechanismy, které zatím nejsou uspokojivě popsány. Tyto výsledky poprvé potvrzují schopnost valproátu aktivovat transkripci *CYP3A4* prostřednictvím CAR a PXR (Naznačují také na možný vliv epigenetické regulace CYP3A4 a na možnost farmakologického zásahu do tohoto typu regulace.

3. Objasnění role HNF4α při aktivaci *CYP3A4* promotoru prostřednictvím GRα. Vysvětlení jaterní specificity této aktivace (2.3).

Metodou gene reporter assay jsme testovali schopnost buněk trofoblastu JEG3 kotransfekovaných GRα a HNF4α exprimovat CYP3A4, a to v přítomnosti či nepřítomnosti dexametazonu. Vnesení HNF4α do buněk nemělo na expresi žádný vliv, pozorovali jsme pouze mírné zvýšení exprese po inkubaci s dexametazonem. Tato pozorování jsou v souladu se způsobem regulace transkripce *CYP3A4* prostřednictvím GRα, která se realizuje nepřímo zvýšením exprese *PXR*, *CAR* a *RXRα*. Tyto receptory se v trofoblastu nevyskytují, tudíž nedošlo k aktivaci transkripce *CYP3A4*. Zajímavý je ovšem signifikantně indukční účinek dexametazonu v přítomnosti HNF4α, který naznačuje důležitou úlohu tohoto jaterně specifického receptoru při indukovatelné transkripci některých genů.

Závěrem je možno konstatovat, že vytýčené cíle byly naplněny. Experimentální práce přinesly zajímavé poznatky obohacující dosavadní znalosti ohledně aspektů genové regulace CYP3A4. Výsledky byly publikovány formou článků v impaktovaných časopisech a jako ústní i posterové prezentace na vědeckých konferencích.

6 Summary

CYP3A4 is an important enzyme involved in elimination of majority of metabolized xenobiotics. It plays a major role in the detoxification system of the human body, therefore it is responsible for many drug-drug interactions (DDIs). DDI present a complication of current pharmacotherapy, in the extreme they can lead in failure of therapy or in life-threatening toxic effects.

DDIs are caused by changes in enzymatic activity of CYP3A4, which is highly variable among individuals. An important mechanism of modulating CAP3A4 activity is the regulation of inducible transcription by nuclear receptors, especially PXR, CAR and GR. The structure of *CYP3A4* promoter and mechanisms of transcriptional regulation has been studding intensively for many years, but the research of relationship of nuclear receptors and transcriptional cofactors in CYP3A4 transactivation is still incomplete.

Present work contributes to elucidation of some questions concerning the effects of azole antimycotics on CYP3A4 transcription via PXR, potency of valproic acid to activate PXR and CAR or determinants of CYP3A4 expression via GR in placental cells. The experiments were performed with up-to-date molecular biology methods and using *in vitro*models of the primary human hepatocytes and hepatoma cell lines.

To the aims of the doctoral thesis:

1. The effects of selected azole antimycotics on CYP3A4 gene expression via PXR. Elucidation of nature of observed effects in the molecular level (2.1).

We tested effects of selected azole antimycotics (clotrimazole, ketoconazole, itraconazole, fluconazole, oxiconazole, econazole and miconazole) on CYP3A4 gene expression via PXR in primary culture of human hepatocytes and cell lines HepG2, LS174T and CV-1. Using real time RT-PCR, gene reporter assay, one hybrid assay and two hybrid assay we investigated potency of each azole to transactivate CYP3A4 promoter not only in monotherapy but also in co-treatment with rifampicin, the known CYP3A4 inductor via PXR. We noted significant differences among azoles, identified a potent inductor oxiconazole and illustrated nature of CHibí !!!