UNIVERZITA KARLOVA

FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

Katedra farmakologie a toxikologie

NOVÉ ASPEKTY FUNKCE A REGULACE PREGNANOVÉHO X RECEPTORU

DISERTAČNÍ PRÁCE

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Hradec Králové, 2018

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Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně pod vedením svého školitele doc. PharmDr. Františka Trejtnara, CSc. a konzultanta prof. PharmDr. Petra Pávka, Ph.D. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

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Mgr. Lucie Hyršová

Poděkování

Tímto bych ráda poděkovala všem mým kolegům, spolužákům, přátelům a vedoucím, s jejichž pomocí jsem mohla svou disertační práci úspěšně dokončit.

Děkuji svému školiteli doc. PharmDr. Františku Trejtnarovi, CSc. za veškerou poskytnutou pomoc i cenné rady jak při provádění experimentů, tak i při sepisování odborných vědeckých publikací. Velké díky patří mému konzultantovi prof. PharmDr. Petru Pávkovi, PhD. za možnost stát se členem jeho pracovního týmu. Děkuji za inspirativní konzultace, za jeho neuvěřitelnou trpělivost, ochotu, vstřícný přístup i za neobyčejné úsilí vyvinuté v průběhu celého mého studia.

Dále děkuji všem mým spolupracovníkům z oddělení Radiofarmak na katedře farmakologie a toxikologie, se kterými jsem měla tu čest se setkat. Jmenovitě děkuji Evě Teichmanové, Jarmile Hoderové, Mgr. Pavlu Bártovi, PhD., PharmDr. Janě Ramos Mandíkové, PhD. a prof. PharmDr. Ing. Milanu Lázníčkovi, CSc. za přátelské pracovní prostředí i ochotu s čímkoliv poradit. Speciální poděkování patří také RNDr. Janě Maixnerové, PhD. za spoustu cenných rad a vřelou pomoc při sepisování disertační práce.

Mé díky patří rovněž Sabine Gerbal-Chaloine, PhD. za veškerou její pomoc a podporu i všem ostatním pracovníkům z Institutu regenerativní medicíny a bioterapie v Montpellier za příjemný čas strávený ve Francii.

V neposlední řadě děkuji mým rodičům, prarodičům a přátelům, že mi byli silnou oporou po celou dobu mého doktorského studia. Děkuji také Tomovi, že se mnou snášel veškeré strasti i radosti.

Závěrem bych chtěla rovněž poděkovat za finanční podporu poskytnutou Grantovou agenturou Univerzity Karlovy (GAUK 338315), Grantovou agenturou České republiky (GAČR 303/12G163) a fondem Specifického vysokoškolského výzkumu SVV 260 414.

Abstrakt

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Název disertační práce:	Nové aspekty funkce a regulace pregnanového X receptoru

Pregnanový X receptor je receptor náležející do nadrodiny nukleárních receptorů, které působí jako na ligandu závislé transkripční faktory řídící expresi cílových genů. PXR je v posledních dvou desetiletích extenzivně studován jakožto tzv. xenosenzor, tj. receptor schopný vázat xenobiotika včetně řady léčiv, a regulovat jejich metabolismus indukcí biotransformačních enzymů I. i II. fáze biotransformace. Indukce, tj. transkripční stimulace exprese nejvýznamnějších enzymů cytochromu P450 ligandy PXR byla detailně popsána na mnoha úrovních.

V této disertační práci se věnuji aspektům regulace prostřednictvím PXR, které rozšiřují naše klasické chápání PXR jakožto receptoru, jehož výlučnou funkcí je indukovat biotransformační enzymy prostřednictvím svých agonistů.

V první projektu jsem studovala regulaci transportéru OCT1 v hepatálních buňkách a ukázala, že PXR nikoli indukuje, ale potlačuje expresi tohoto významného transportéru léčiv i endogenních látek. Tato práce je první detailní studie ukazující mechanismus potlačení exprese (tj. *down-regulace)* transportéru léčiv skrze PXR.

Ve druhé práci jsem studovala resveratrol a strukturálně podobné látky se stilbenoidní strukturou při regulaci cílových genů PXR. Resveratrol byl popsán jako jeden

z několika málo inhibitorů PXR, avšak práce na toto téma doposud publikované byly ve svých zjištěních rozporuplné.

Ve třetím výzkumném směru jsem se podílela na studiu interakcí endogenních žlučových kyselin a jejich předpokládaných intermediátů s PXR. Cílem bylo odhalit, zdali metabolity žlučových kyselin nejsou fyziologickými endogenními ligandy PXR.

Celkově lze konstatovat, že v této práci jsem rozšířila obecné znalosti o PXR z pohledu down-regulace OCT1 nebo jeho potenciální inhibice látkami strukturálně příbuznými s resveratrolem.

Abstract

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Title of Dissertation Thesis:	New aspects of pregnane X receptor function and regulation

The pregnane X receptor belongs to the superfamily of nuclear receptors; it is a ligand dependent transcription factor regulating expression of its target genes. During last two decades, PXR was extensively studied as a xenosensor, i.e. the receptor, which is able to bind xenobiotics including many drugs and to regulate their metabolism by induction of the most important metabolizing enzymes of both phase I. and phase II. Induction, i.e. transcriptional stimulation of expression of the most important cytochrome P450 enzymes, by PXR ligands was described in details at many levels.

Within this dissertation thesis, I am dealing with aspects of regulation via PXR, which extend the common understanding of PXR as a receptor whose exclusive function is to up-regulate drug metabolizing enzymes mediated by its agonists.

Within the first, project I studied regulation of OCT1 transporter in hepatic cell models, I shown that PXR did not induce, but it rather suppressed the expression of this important transporter of drugs and endogenous substances. This work is the first detailed study presenting mechanism of down-regulation of drug transporter via PXR.

Within the second work, I studied resveratrol and structurally related stilbenoid substances to regulate expression of PXR target genes. Resveratrol was described as one

of the PXR inhibitors, however studies published on this issue were contradictory in their findings.

Within the third research field, I participated on the study of interaction of endogenous bile acids and their hypothetical metabolites with PXR. The aim of the study was to elucidate if metabolites of bile acids can act as endogenous ligands of PXR.

Overall, my work extended our general understanding of PXR from the perspective of down-regulation of OCT1 or the potential inhibition of PXR by substances structurally related to resveratrol.

Obsah

1 Úvod

Nukleární, neboli jaderné receptory (NR), jsou receptory, které působí v jádře buňky (Mangelsdorf and Evans, 1995). Nadrodinu nukleárních receptorů tvoří více než čtyři desítky členů. Jedná se o transkripční faktory, které ovlivňují transkripci cílových genů (da Silva and Burbach, 1995; Mani et al., 2013; Wu et al., 2016). Obecně lze konstatovat, že NR regulují veškeré biologické pochody, včetně diferenciace a embryogeneze, mohou ovšem hrát také roli při různých patologických procesech, včetně nádorových onemocnění (Baek and Kim, 2014; Chen et al., 2012; Safe et al., 2014; Wu et al., 2016).

Některé NR se také významnou měrou podílejí na regulaci metabolismu a detoxikaci organismu prostřednictvím koordinace exprese biotransformačních enzymů a transportních proteinů (Chai et al., 2016).

Mezi nukleární receptory náleží i tzv. xenosenzory jako je pregnanový X receptor (PXR), konstitutivní androstanový receptor (CAR) nebo arylhydrokarbonový receptor (AhR) (Hakkola et al., 2018). Tyto receptory regulují transkripci enzymů I. a II. fáze metabolismu a transportérů, které přenáší xenobiotika přes membrány (di Masi et al., 2009). Nejdůležitějšími enzymy I. fáze, které jsou regulované pomocí těchto xenosenzorů, jsou jednoznačně enzymy cytochromu P450 (CYP450). Uvádí se, že jen izoenzym CYP3A4, jehož exprese je řízena zejména PXR, metabolizuje více než 50 % používaných léčiv (Xie and Evans, 2001).

Xenosenzory se neuplatňují pouze při regulování biotransformace, ale všech farmakokinetických fází, kterými podané léčivo prochází (di Masi et al., 2009). Řídí totiž také expresi řady transportérů, které se uplatňují při přenosu látek, jejichž fyzikálně chemické vlastnosti neumožňují jejich transport prostou difúzí biologickými bariérami (di Masi et al., 2009; Klaassen and Aleksunes, 2010; Nies et al., 2009). Tímto způsobem je umožněna absorpce, distribuce i exkrece např. malých organických kationtů nebo aniontů (Klaassen and Aleksunes, 2010).

Obecně má nejvyšší podíl na regulaci exprese lidských biotransformačních enzymů a transportních systémů jaderný receptor PXR (Bertilsson et al., 1998; Willson and Kliewer, 2002).

Změněné množství biotransformačních enzymů a transportérů může mít i závažné klinické důsledky kvůli vzniklým lékovým interakcím. Tyto interakce vznikají na úrovni eliminace, vlivem zesílení first pass efektu a také snížením biodostupnosti. Následkem aktivace metabolismu dochází zejména ke snížení účinnosti dalšího současně podávaného léčiva. Na druhé straně však může docházet ke zvýšení toxicity léčiva v důsledku zvýšené produkce a akumulace toxických metabolitů (Chai et al., 2016; Ma et al., 2008).

2 Pregnanový X receptor v regulaci farmakokinetických procesů

2.1 Nukleární receptory

V rámci klasifikace NR rozlišujeme tři menší podskupiny: hormonální receptory (nebo také klasické receptory), adoptované sirotčí receptory a sirotčí receptory. První skupina receptorů je charakterizována vysokou afinitou k lipofilním hormonům. Řadíme sem např. estrogenový (ER), glukokortikoidní (GR) nebo mineralokortikoidní receptor (MR) a receptor pro vitamin D (VDR). Na druhou stranu, skupina sirotčích receptorů je tvořena receptory, pro které nejsou doposud známy žádné fyziologické ligandy nebo jejich transaktivace není na přítomnosti ligandu závislá. Sirotčí receptory jsou zastoupeny např. hepatocytárním nukleárním faktorem 4 alfa (HNF4α). Poslední skupina, adoptované sirotčí receptory, jsou tvořeny receptory, které byly původně označovány jako sirotčí, ale později byly identifikovány fyziologické ligandy, avšak s nižší afinitou. Mezi významné členy skupiny adoptovaných sirotčích receptorů zahrnujeme PXR, CAR, farnesoidní X receptor (FXR), retinoidní X receptor (RXR) a řada dalších NR (da Silva and Burbach, 1995; Safe et al., 2014; Wu et al., 2016).

NR sdílí společnou evoluční historii, což je patrné v jejich struktuře, kde můžeme nalézt některé společné rysy, a ve vysokém stupni zachování sekvence v průběhu evoluce. Mezi konkrétní společné strukturní rysy NR patří:

- na ligandu nezávislá aktivační funkce 1 (AF1), která se nachází na N-konci DNA vázající domény,
- DNA vázající doména (DBD, DNA-binding domain),
- ligand vázající doména (LBD),
- na ligandu závislá aktivační funkce 2 (AF2), kterou můžeme najít spolu s ligand vázající kapsou v LBD, konkrétně na C-konci LBD (di Masi et al., 2009; Mani et al., 2013).

Obecně platí, že po navázání ligandu do LBD dochází ke změně konformace AF2 oblasti a disociaci korepresorů, což ve výsledku vede k navázání koaktivátorů na

nukleární receptor. Po aktivaci ligandem se NR vážou svou DBD na specifické sekvence nukleotidů tzv. responsivní oblasti v promotorech svých specifických cílových genů a spouští jejich transkripci (Bourguet et al., 1995; da Silva and Burbach, 1995; Mani et al., 2013; Wu et al., 2016).

Ligand vázající doména některých jaderných receptorů a především receptoru PXR je značně flexibilní, z hlediska velikosti i tvaru, což umožňuje navázání ligandů, které jsou strukturálně velmi odlišné. Tato vlastnost xenosenzorů je důležitá při regulaci exprese biotransformačních enzymů, které se uplatňují při metabolismu široké škály endogenních látek i xenobiotik. NR tak vykonávají funkci metabolických senzorů, schopných reagovat na přítomnost široké palety látek včetně látek tělu vlastních, léčiv, insekticidů, pesticidů nebo různých složek potravy (di Masi et al., 2009; Chen et al., 2012). Schopnost interakce s širokým spektrem ligandů však neplatí pro jaderné receptory obecně, neboť např. receptor pro tyroidní hormon nebo retinoidní receptor vykazují značnou substrátovou specifitu (Watkins et al., 2001). Oproti tomu PXR disponuje zřejmě největší LBD v rámci nadrodiny NR, což umožňuje navázání pestré škály ligandů (Mackowiak et al., 2018).

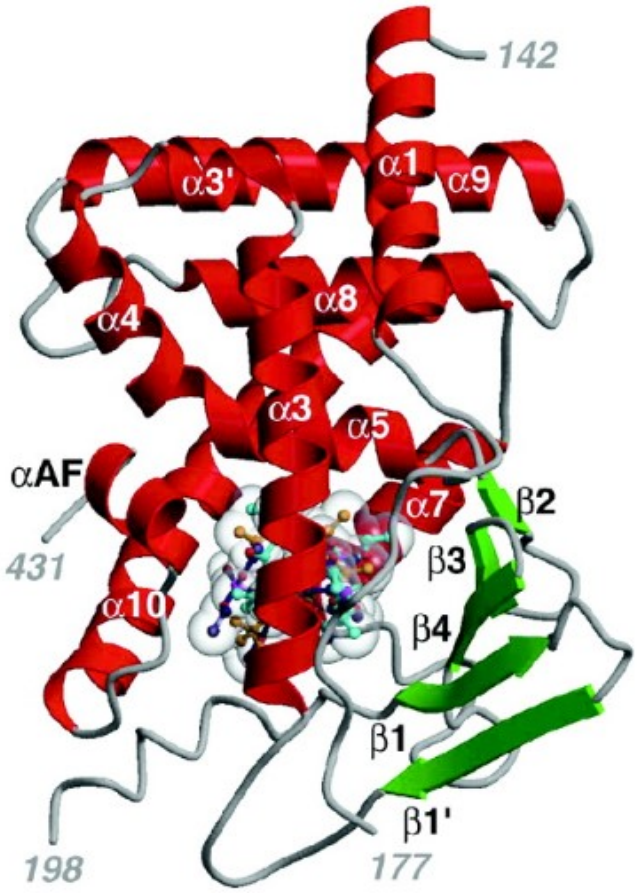
Jednotlivé NR se do svých responzivních oblastí mohou vázat buď jako monomery (např. stroidogenní faktor 1, SF1), homodimery, jak je tomu v případě GR nebo HNF4α receptoru, nebo jako heterodimery. PXR a CAR patří mezi jaderné receptory, které tvoří heterodimerní komplexy s retinoidním X receptorem α (RXRα) (Chen et al., 2012; Mangelsdorf and Evans, 1995; Tremblay and Viger, 2003).

2.1.1 Pregnanový X receptor

Pregnanový X receptor (PXR, NR1I2) je znám také jako steroidní X receptor (SXR) nebo pregnanem aktivovaný receptor (PAR). Jedná se o jaderný receptor, na ligandu závislý transkripční faktor, který náleží do podrodiny NR1I receptorů spolu s CAR a VDR. PXR se dále řadí do skupiny adoptovaných sirotčích receptorů, tedy do skupiny receptorů, jejichž přirozený ligand byl objeven až později, v tomto případě v roce 1998. Díky těmto ligandům - pregnanovým steroidním sloučeninám získal své nové označení, které se v současné době používá téměř výlučně (Carnahan and Redinbo, 2005; Chen et al., 2012; Mackowiak et al., 2018; Mani et al., 2013).

NR1I2 gen, tedy gen kódují PXR, se nachází na chromozomu 3, konkrétně v místech 3q12-q13.3. NR1I2 čítá přibližně 38 000 párů bází, které jsou organizovány do 10 exonů a 9 intronů. Existuje 9 různých variant PXR mRNA a proteinu vzniklých alternativním sestřihem (splicingem) nebo transkripcí (Brewer and Chen, 2016; di Masi et al., 2009).

Protein PXR receptoru se skládá ze 434 aminokyselin. V rámci krystalické struktury PXR proteinu, jak již bylo zmíněno výše, byly identifikovány dvě důležité domény: DNA vázající domény (DBD) a ligand vázající domény (LBD), která je znázorněna na obr. 1. Obě tyto domény jsou vzájemně propojeny oblastí H (hinge region) (Brewer and Chen, 2016; di Masi et al., 2009).



Obr. 1 Krystalická struktura ligand vázající domény pregnanového X receptoru je vyobrazena spolu s navázaným modelovým ligandem SR12813. Převzato z Watkins et al., 2001.

PXR je exprimován v několika tkáních lidského organismu, avšak dominantní je jeho exprese v játrech. V menší míře je exprimován i v tlustém a tenkém střevě a ledvinách, tedy v místech, které jsou spojeny s detekcí a „neutralizací" potenciálně toxických látek jak endogenního, tak i exogenního původu (Carnahan and Redinbo, 2005; di Masi et al., 2009; Lehmann et al., 1998). V minoritním množství byl PXR detekován i v žaludku, plicích, srdci, některých částech mozku, hematoencefalické bariéře, míše, kostní dřeni, monocytech, osteoklastech, placentě, prsu, vaječnících, děloze nebo v nadledvinách. Tento velmi široký výskyt ukazuje, že PXR je součástí protektivního systému organismu, jenž má chránit citlivé oblasti před působením potenciálně škodlivých látek (di Masi et al., 2009; Lamba et al., 2004).

Funkce PXR

PXR hraje zásadní roli v regulaci metabolismu xenobiotik i endogenních látek. Jak již bylo výše zmíněno, po aktivaci PXR dochází k jeho nasednutí do PXR responzivních oblastí promotorů a spuštění transkripce příslušných genů. Heterodimer PXR/RXR se váže do dvou hexamerických míst na molekule DNA, které jsou buď sekvenčně shodné (direct repeat, DR) nebo je jejich sekvence převrácená (everted repeat, ER). Tyto hexametrické úseky DNA jsou od sebe odděleny buď 3 (DR-3) nebo 6 (ER-6) dalšími bázemi (di Masi et al., 2009; Mani et al., 2013).

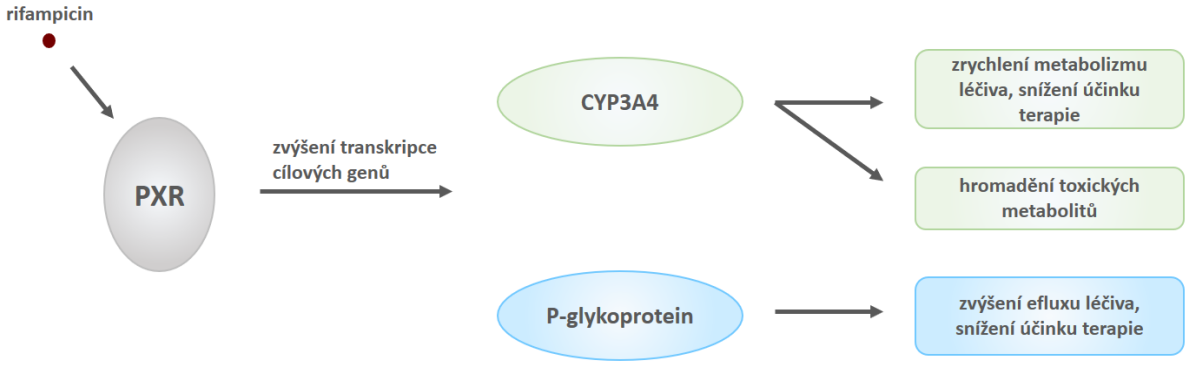
V rámci endogenního metabolismu se PXR uplatňuje při metabolismu steroidních hormonů testosteronu, progesteronu kortizolu nebo aldosteronu. Podílí se i na regulaci metabolismu glukózy a lipidů, kde potlačuje glukoneogenezi snižováním exprese genů pro fosfoenolpyruvátkarboxykinázu (PEPCK1) a glukóza-6-fosfatázu (G6Páza), které jsou stěžejní pro glukoneogenezi, a tím snižuje hladinu glukózy a naopak podporuje syntézu triglyceridů a snižuje metabolismus lipidů (di Masi et al., 2009; Mackowiak et al., 2018). PXR se podílí i na regulaci homeostázy cholesterolu a žlučových kyselin, kde dochází ke snížení exprese cholesterol 7α-hydroxylázy (CYP7A1) a tím ke zpomalení biotransformace cholesterolu na žlučové kyseliny, dále byla pozorována snížená hladina i u dalších genů účastnících se metabolismu a transportu žlučových kyselin včetně sulfotransferáz, CYP3A4 nebo transportéru OATP2 (Ma et al., 2008; Staudinger et al., 2001a; Staudinger et al., 2001b).

PXR má vliv také na udržování homeostázy vitaminu D_3 (Ma et al., 2008). PXR byl také popsán jako důležitý faktor při rozvoji některých druhů nádorového bujení, kvůli svému vlivu na inhibici apoptózy (Carnahan and Redinbo, 2005; Mackowiak et al., 2018). PXR se podílí i na regulaci buněčné proliferace, vlivem aktivace PXR dochází k tumor-supresivnímu působení na některé druhy nádorů. Navíc byla popsána i regenerace jater vlivem působení PXR na proliferaci hepatocytů (Mackowiak et al., 2018).

Jednou z hlavních fyziologických funkcí PXR je kontrola exprese řady biotransformačních enzymů, které jsou zapojeny jak do první fáze metabolismu, včetně cytochromu P450, tak i do druhé fáze biotransformace (Carnahan and Redinbo, 2005; Mani et al., 2013) (tab. 1). PXR reguluje i expresi mnoha transportních proteinů (tab. 1). Tímto způsobem se předpokládá, že PXR koordinuje detoxikaci a zajišťuje organismu

ochranu před mnoha potenciálně toxickými látkami endogenního i exogenního původu (Hyrsova et al., 2016a; Chen et al., 2012; Synold et al., 2001).

Avšak tato vlastnost PXR má i své negativní rysy, které se projeví při nechtěné aktivaci PXR xenobiotikem podávaným v průběhu farmakoterapie (obr. 2). Může docházet k nepříznivé lékové interakci, kdy v důsledku extenzivního metabolismu dojde k rychlejšímu poklesu hladiny léčiva pod terapeutickou koncentraci nebo k rychlejší tvorbě toxických metabolitů, což může vyústit až ve zvýšenou hepatotoxicitu. Dále v důsledku zvýšené transkripce efluxních transportérů hrozí riziko vzniku rezistence na současně podávanou léčivou látku (Chai et al., 2016).



Obr. 2 Schématické znázornění lékových interakcí zprostředkovaných PXR.

PXR reguluje expresi mnoha enzymů zapojených do I. fáze metabolismu. Mezi nejdůležitější enzymy regulované PXR patří celá řada enzymů cytochromu P450, kde se spektrum regulovaných genů nezřídka překrývá s dalším jaderným receptorem CAR, jako je tomu v případě regulace CYP2B6. PXR hraje stěžejní roli zejména při zvyšování jaterní a střevní exprese enzymů skupiny CYP3A, především pak CYP3A4, který se podílí na metabolismu mnoha léčiv (je uváděno, že více než 50 % používaných léčiv je biotransformováno CYP3A4) a může proto být místem řady lékových interakcí (Faucette et al., 2007; Chen et al., 2012; Kliewer et al., 1998). Aktivace PXR indukuje také expresi enzymů skupiny CYP2C, konkrétně CYP2C8 a CYP2C9 (Ferguson et al., 2005; Chen et al., 2004). Na druhou stranu PXR tlumí expresi CYP7A1, i když nepřímým způsobem. Bylo popsáno, že jaterní exprese CYP7A1 je řízena především hepatocytárním nukleárním faktorem 4α (HNF4α), který jako svůj koaktivátor využívá PGC1α stejně jako PXR. Tím dochází ke kompetici mezi těmito dvěma jadernými receptory, přičemž převáží

efekt PXR a nedochází tak k aktivaci transkripce CYP7A1. Stejný fenomén byl popsán i pro CYP8A1 (Pavek, 2016).

PXR se navíc podílí i na regulaci metabolických reakcí I. fáze, do kterých jsou zapojeny dehydrogenázy a karboxylesterázy (CES) účastnící se jak metabolismu endogenních látek, tak i aktivace proléčiv na účinné formy (Rosenfeld et al., 2003). PXR zvyšuje expresi aldehyddehydrogenázy ALDH1A7 nebo ALDH1A1 (Maglich et al., 2002).

Jak již bylo zmíněno, PXR svým působením ovlivňuje rovněž konjugační enzymy II. fáze metabolismu. Po aktivaci PXR je ve vyšší míře transkribován enzym GSTA4 ze skupiny glutathion-S-transferáz (GST), který katalyzuje konjugaci metabolizovaných látek s glutathionem (Glass and Rosenfeld, 2000). PXR up-reguluje také některé UDP-glukuronosyltransferázy (UGT), což jsou enzymy zprostředkovávající glukuronidační reakce, konkrétně jde např. o enzymy UGT1A4 nebo UGT1A1 (Moscovitz et al., 2018). Další skupinou konjugačních enzymů, jejichž exprese je regulována pomocí PXR, jsou sulfotransferázy (SULT), které katalyzují vznik konjugátu mezi sulfátovým zbytkem a metabolitem vzniklým v průběhu I. fáze metabolismu. Jako první enzymy této skupiny, jejichž exprese je kontrolována PXR, byly identifikovány SULT1A1 a SULT2A1, v obou případech se jedná o stimulaci exprese a tedy urychlení eliminace látek z organismu (Duanmu et al., 2002). Nicméně v nedávno publikované práci byl u primárních lidských hepatocytů po aplikaci rifampicinu pozorován opačný efekt - tedy snížení exprese zhruba o 25 % v případě enzymu SULT1E1 (Moscovitz et al., 2018).

Transportní proteiny svým působením ovlivňují jednak absorpci a distribuci látek, ale zprostředkovávají i transport jejich metabolitů z hepatocytů do žluče nebo zpět do krve. V případě enterocytů mohou ovlivňovat vstřebávání perorálně podaných látek. Z toho tedy vyplývá, že zvyšování či snižování jejich hladin v organismu může mít zásadní vliv na účinnost terapie (Chen et al., 2012). Jedním z nejdůležitějších transportérů, které zprostředkovávají eflux látek z buňky je P-glykoprotein (P-gp, ABCB1), jiným názvem multidrug resistance 1 (MDR1). Exprese ABCB1 aktivovaná PXR je spojována s řadou lékových interakcí a s lékovou rezistencí. Dalšími efluxními transportéry, jejichž hladina je zvýšená po aktivaci PXR jsou proteiny MRP 2-6 (multidrug resistance-associated protein 2-6, ABCC2-6) (Jigorel et al., 2006; Kast et al.,

2002; Moscovitz et al., 2018). Na druhou stranu byla popsána i suprese efluxního transportéru pro žlučové kyseliny BSEP (bile salt export pump, ABCB11) v primárních lidských hepatocytech působením rifampicinu o téměř polovinu (Moscovitz et al., 2018). Další transportéry, jejichž exprese je ovlivňována PXR, jsou přehledně shrnuty společně s enzymy I. a II. fáze metabolismu v tab. 1.

Tab. 1. Přehled genů ze skupiny biotransformačních enzymů a transportních proteinů regulovaných PXR. Geny jsou seřazeny v abecedním pořadí, v závorce jsou uvedeny jimi kódované proteiny. Regulované geny jsou rozděleny do 3 skupin podle své funkce na transportní proteiny, enzymy účastnící se I. a II. fáze metabolismu. Současně je uvedeno i relativní ovlivnění genové exprese ve smyslu jejího snížení (↓) nebo zvýšení (↑). Vysvětlení jednotlivých zkratek je uvedeno v kapitole 12 Seznam zkratek.

Funkční skupina	Geny regulované PXR (proteiny)	Vliv na expresi	Odkaz
Transportéry	ABCB1 (MDR1, P-gp)	↑↑	Synold et al., 2001
	ABCB4 (MDR3)	↑	Moscovitz et al., 2018
	ABCB11 (BSEP)	↓	Moscovitz et al., 2018
	ABCC2 (MRP2)	↑	Kast et al., 2002
	ABCC3 (MRP3)	↑	Jigorel et al., 2006
	ABCC4 (MRP4)	↑	Moscovitz et al., 2018
	ABCC5 (MRP5)	↑	Moscovitz et al., 2018
	ABCC6 (MRP6, ARA)	↑	Moscovitz et al., 2018
	SLC10A1 (NTCP1)	↑	Moscovitz et al., 2018
	SLC22A1 (OCT1)	↓	Hyrsova et al., 2016a

	SLC22A7 (OAT2)	↓	Moscovitz et al., 2018
	SLC47A1 (MATE1)	↑	Moscovitz et al., 2018
	SLCO1B1 (OATP2)	↑	Moscovitz et al., 2018
	SLCO1B3 (OATP8)	↓	Moscovitz et al., 2018
	SLCO2B1 (OATP2B1)	↑	Moscovitz et al., 2018
Enzymy I. fáze metabolismu	ALDH1A1	↑	Maglich et al., 2002
	ALDH1A7	↑	Rosenfeld et al., 2003
	CES1	↑	Moscovitz et al., 2018
	CES2	↑	Rosenfeld et al., 2003
	CYP2A6	↑	Itoh et al., 2006
	CYP2B6	↑↑	Faucette et al., 2007
	CYP2C8	↑	Ferguson et al., 2005
	CYP2C9	↑↑	Chen et al., 2004
	CYP2C19	↑	Chen et al., 2003
	CYP3A4	↑↑↑	Faucette et al., 2007
	CYP3A7	↑	Pascussi et al., 1999
	CYP3A23	↑	Huss and Kasper, 2000
	CYP4F12	↑	Hariparsad et al., 2009
	CYP7A1	↓↓	Li and Chiang, 2005

	CYP8B1	↓	Bhalla et al., 2004
Enzymy II. fáze metabolismu	GSTA1	↑	Moscovitz et al., 2018
	GSTA4	↑	Rosenfeld et al., 2003
	GSTP1	↑	Moscovitz et al., 2018
	SULT1A1	↑	Duanmu et al., 2002
	SULT1E1	↓	Moscovitz et al., 2018
	SULT2A1	↑	Duanmu et al., 2002
	UGT1A1	↑	Moscovitz et al., 2018
	UGT1A4	↑	Moscovitz et al., 2018
	UGT1A6	↑	Moscovitz et al., 2018
	UGT1A9	↑	Moscovitz et al., 2018
	UGT2B4	↑	Moscovitz et al., 2018

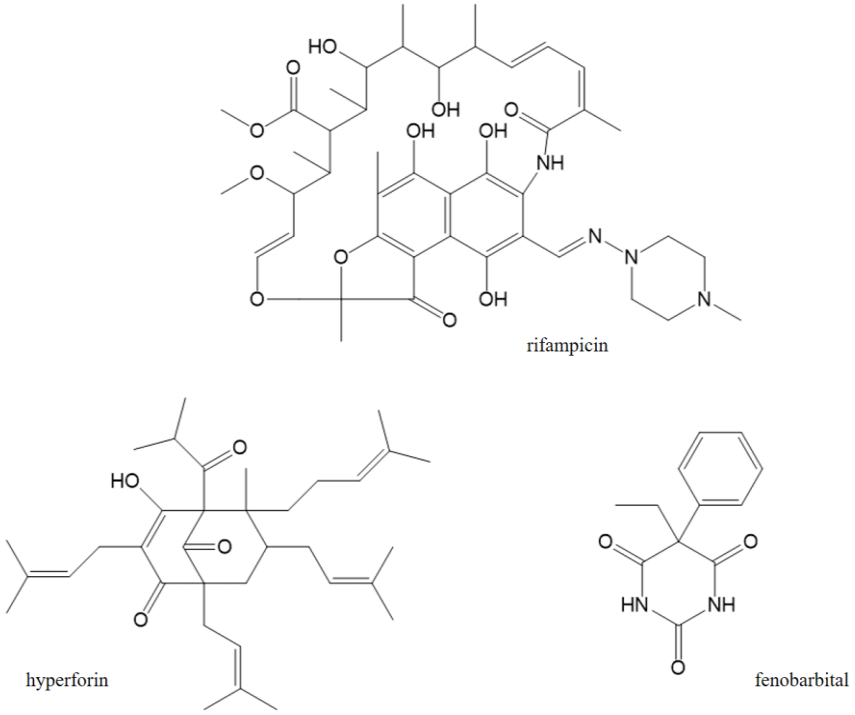
Mechanismus aktivace a agonisté PXR

O PXR se hovoří jako o promiskuitním receptoru kvůli jeho objemné a flexibilní ligand vázající kapse, kam se může navázat velké množství zpravidla lipofilních ligandů. Tato kapsa je obklopena třemi vrstvami α-helikálních smyček, které jsou uspořádány do tzv. „α-helikálního sendviče“ (Carnahan and Redinbo, 2005; Ekins et al., 2009; Jones et al., 2000; Mackowiak et al., 2018; Watkins et al., 2001). Právě díky své schopnosti rozpoznávat široké spektrum ligandů PXR funguje jako senzor xenobiotik, který následně reguluje jejich metabolismus a exkreci. Na této úrovni může docházet k interakcím mezi současně podanými léčivy, doplňky stravy nebo potravou (Ekins and Erickson, 2002; Chen et al., 2012).

Jak již bylo naznačeno, aktivace PXR agonisty má za následek jeho vazbu pomocí DBD na určité oblasti v promotorech cílových genů PXR, které jsou označovány jako responzivní elementy. Interakce mezi ligandem a PXR probíhá v cytoplazmě buněk, teprve až po vazbě na PXR dochází k jeho translokaci z cytoplazmy do buněčného jádra a začíná proces transkripce (Carnahan and Redinbo, 2005; Chen et al., 2012; Mani et al., 2013; Squires et al., 2004). Vazba ligandu do LBD vyvolá konformační změny PXR, díky čemuž se odpojí korepresory (např. NCoR nebo SMRT), které jsou na receptor vázány v přítomnosti antagonisty, případně při absenci agonisty. Dále dochází k navázání koaktivátorů (např. SRC-1 nebo PGC1α) a vytvoření heterodimerního komplexu s RXRα a spuštění transkripce cílových genů PXR (Carnahan and Redinbo, 2005; Glass and Rosenfeld, 2000).

Tento receptor je aktivován širokým spektrem strukturně různorodých látek, ať už látkami endogenního původu, léčivy, různými složkami potravy případně i potravinovými doplňky nebo kontaminanty životního prostředí (Blumberg and Evans, 1998; Kliewer et al., 1998; Watkins et al., 2001). Z prototypových agonistů PXR (obr. 3) se může jednat jak o látky s relativně malou molekulovou hmotností mírně přesahující 230 Da, jako je např. antiepileptikum fenobarbital, sekundární metabolit izolovaný z třezalky tečkované (*Hypericum perforatum*) hyperforin o molekulové hmotnosti 537 Da, nebo značně větší, jako třeba antituberkulotikum rifampicin, jehož molekulová hmotnost překračuje 800 Da (Carnahan and Redinbo, 2005; Mani et al., 2013; Moore et al., 2000).

Mezi další více či méně významné agonisty PXR z řady léčiv patří např. antimykotikum klotrimazol, diuretikum spironolakton, glukokortikoid dexametazon, hypolipidemicky působící léčiva lovastatin a SR12813, inhibitor HIV-proteáz ritonavir nebo blokátor vápníkových kanálů nifedipin. I přirozené látky jako je cholesterol, kyselina litocholová, α-, β-, γ- a δ-tokotrienoly patřící do skupiny vitaminu E, vitamin K_2 nebo přirozené steroidní hormony fungují jako agonisté PXR, z těch nejsilněji aktivujících lze jmenovat 5β-pregnan-3,20-dion, 17β-estradiol, progesteron nebo kortikosteron (Bertilsson et al., 1998; Carnahan and Redinbo, 2005; Ekins and Erickson, 2002; Jones et al., 2000; Ma et al., 2008; Staudinger et al., 2001b; Zhou et al., 2004).



Obr. 3 Strukturní různorodost vybraných ligandů pregnanového X receptoru.

Z rostlinných látek můžeme mezi agonisty PXR zařadit kromě již zmiňovaného hyperforinu také např. látky obsažené v extraktu izolovaného z jinanu dvoulaločného (*Ginkgo biloba*), pepřovníku opojného (*Piper methysticum*) známého také pod názvem kava kava nebo z klanoprašky čínské (*Schisandra sinensis*) (Chang, 2009).

Inhibice PXR

Na rozdíl od téměř nepřeberného množství ligandů byl zatím identifikován pouze omezený počet antagonistů PXR, mezi něž patří např. antimykotika ketokonazol (pouze v koncentracích, které převyšují terapeutickou hladinu léčiva v krvi) a flukonazol, přírodní látky sulforafan, fukoxantin nebo sesamin, široce používané antidiabetikum metformin nebo inhibitor HIV proteáz A792611 (Carnahan and Redinbo, 2005; Mani et al., 2013). Nedávno byla popsána látka označovaná SPA70, jež vykazuje silné inhibiční působení na PXR, avšak i velmi mírné inverzně agonistické účinky na CAR (Lin et al., 2017).

Inhibice může probíhat dvěma základními mechanismy:

- na ligandu závislým mechanismem, kdy se nejprve musí stabilizovat AF-2 oblast navázáním ligandu a až poté může dojít k navázání inhibitoru, který následně znemožní navázání koaktivátorů na PXR, nebo
- na ligandu nezávislým mechanismem, kdy inhibitor alostericky znemožňuje navázání koaktivátorů (Mani et al., 2013).

Všichni antagonisté PXR mají omezený efekt blokovat PXR a doposud chybí účinný antagonista schopný inhibovat lidský PXR v nanomolárních koncentracích v ligand vázající doméně. Příčinou je především velmi objemná vazebná kapsa PXR.

2.1.2 Konstitutivní androstanový receptor

Konstitutivní androstanový receptor (CAR, NR1I3) patří do skupiny ligandem aktivovaných jaderných receptorů, původně byl tento receptor jakožto sirotčí označen MB67. Podobně jako PXR i CAR spadá do skupiny adoptovaných sirotčích receptorů, avšak na rozdíl od PXR, CAR vykazuje relativně vysokou bazální aktivitu, aniž by byl aktivován svým agonistou (di Masi et al., 2009; Chai et al., 2016; Chai et al., 2013; Chen et al., 2012). CAR získal své jméno díky inverzně agonistickému působení metabolitů androstanu (5α-androstan-3α-olu a 5α-androstan-16-en-3α-olu) na tento NR (Forman et al., 1998; Mackowiak et al., 2018; Wang and LeCluyse, 2003).

NR1I3 gen je lokalizovaný na chromozomu 1, přesněji v místě 1q23. Lidský NR1I3 se skládá se z 8 545 párů bází, které jsou organizovány do 9 exonů vzájemně oddělených 8 introny, což umožňuje vznik 22 alternativních sestřihových (splicingových) variant CAR, z nichž některé izoformy nekódují funkční CAR protein (di Masi et al., 2009).

CAR je exprimován především ve tkáních s vysokou metabolickou kapacitou, zejména v jaterní tkáni, nižší, ale stále významná exprese je i v tenkém střevě. Klíčovým faktorem při řízení exprese CAR je HNF4α, což je v souladu s jeho dominantní jaterní lokalizaci. V mnohem menší míře byl lokalizován i v dalších tkáních jako je srdce, kosterní svalstvo, mozek, plíce nebo ledviny (Baes et al., 1994; Molnár et al., 2013; Xu et al., 2016).

Funkce CAR

Podobně jako lokalizace i role jaderných receptorů CAR a PXR se do značné míry překrývají. CAR stejně jako PXR hraje důležitou roli při regulaci exprese genů účastnících se metabolismu xenobiotik. Analogicky jako v případě PXR, i CAR se váže do promotorových sekvencí, jež mají určitou strukturu. Heterodimer CAR/RXR se také váže do obou zmiňovaných typů sekvencí DR i ER, avšak v případě CAR se jedná o oblasti DR-4 nebo ER-8 (hexamery oddělené 4 resp. 8 bázemi) (di Masi et al., 2009; Chai et al., 2016; Chai et al., 2013).

Co se týče regulace metabolismu endogenních látek, CAR významně podílí na udržování homeostázy endogenních látek včetně cholesterolu a tyroidních a steroidních hormonů, na zvyšování biosyntézy hemu a na regulaci metabolismu glukózy, tuků a

mastných kyselin, cholesterolu a žlučových kyselin (di Masi et al., 2009; Chai et al., 2013; Jiang and Xie, 2013; Kachaylo et al., 2011; Wada et al., 2009). Účastní se také na glukuronidaci bilirubinu pomocí enzymu UGT1A1, vzniklý konjugát je následně z jater vyloučen do žluči aktivním transportem pomocí transportéru MRP2 a snižuje tak hladinu bilirubinu v organismu (di Masi et al., 2009; Chai et al., 2016). CAR také hraje roli při udržování energetické homeostázy (zřejmě kombinovanou represí genů zahrnutých v glukoneogenezi a v syntéze mastných kyselin) a v metabolických poruchách (Mackowiak et al., 2018). Mimo to endogenní funkce CAR zasahuje i do řízení buněčného cyklu, proliferace a apoptózy (Hakkola et al., 2018; Mackowiak et al., 2018)

Původně byl CAR identifikován jako jaderný receptor, který se podílí na odpovědi organismu na přítomnost látek exogenního původu. Byla popsána jeho klíčová role při regulaci exprese enzymů I. i II. fáze metabolismu a transportních proteinů. Stejně jako v případě PXR i aktivace CAR může vést k nechtěným lékovým interakcím nebo ke vzniku toxických metabolitů (Hernandez et al., 2009; Chai et al., 2016; Chai et al., 2013; Wada et al., 2009). Jelikož se CAR a PXR vážou na podobné responzivní oblasti v promotorech regulovaných genů, spektrum jejich cílových genů se do značné míry překrývá (Moscovitz et al., 2018; Xie et al., 2000).

V rámci I. fáze metabolismu je nejdůležitější schopnost CAR regulovat enzymy cytochromu P450. CAR hraje klíčovou roli v regulaci exprese CYP2B6, na níž se podílí i PXR, ale role CAR je zde dominantní. Spolu s PXR mají vliv na zvyšování hladiny skupiny CYP3A, včetně CYP3A4, zde však převažuje vliv PXR nad CAR (Faucette et al., 2007; Chen et al., 2012; Wang and LeCluyse, 2003).

Po aplikaci agonistů CAR jsou také exprimovány ve vyšší míře enzymy II. fáze metabolismu, včetně několika UDP-glukuronosyltransferáz (UGT) a glutathion-S-transferáz (GST), čímž opět dochází k urychlení vylučování léčiv z lidského organismu. Jako konkrétní příklady lze jmenovat konjugační enzymy GSTA1, sulfotransferázu 1E1 (SULT1E1) nebo UGT1A1 (Moscovitz et al., 2018; Wortham et al., 2007).

Aktivace CAR má indukční účinky na celou řadu transportérů, čímž dochází mj. ke zvyšování efluxu mnoha látek, včetně léčiv, z buněk. Podobně jako v případě PXR i CAR zvyšuje expresi efluxních jaterních transportérů MRP2-4, ale i uptake transportéru OATP2 (Kast et al., 2002; Klaassen and Slitt, 2005; Moscovitz et al., 2018; Wortham et al., 2007). Na druhou stranu bylo popsáno snižování hladiny transportérů OCT1 a OATP2

aktivací CAR receptoru, což může negativně ovlivnit transport substrátů těchto dvou transportérů do hepatocytů (Moscovitz et al., 2018). Geny pro transportéry a biotransformační enzymy I. a II. fáze regulované CAR jsou shrnuty v tab. 2.

Tab. 2 Přehled genů ze skupiny biotransformačních enzymů a transportních proteinů regulovaných CAR. Geny jsou seřazeny v abecedním pořadí, v závorce jsou uvedeny jimi kódované proteiny. Regulované geny jsou rozděleny do 3 skupin podle své funkce na transportní proteiny, enzymy účastnící se I. a II. fáze metabolismu. Současně je uvedeno i relativní ovlivnění genové exprese ve smyslu jejího snížení (↓) nebo zvýšení (↑). Vysvětlení jednotlivých zkratek je uvedeno v kapitole 12 Seznam zkratek.

Funkční skupina	Geny regulované PXR (proteiny)	Vliv na expresi	Odkaz
Transportéry	ABCB1 (MDR1, P-gp)	↑	Wang et al., 2014
	ABCB11 (BSEP)	↓	Moscovitz et al., 2018
	ABCC2 (MRP2)	↑↑↑	Kast et al., 2002
	ABCC3 (MRP3)	↑↑	Moscovitz et al., 2018
	ABCC4 (MRP4)	↑	Klaassen and Slitt, 2005
	ABCG2 (BCRP)	↑	Moscovitz et al., 2018
	SLC22A1 (OCT1)	↓	Moscovitz et al., 2018
	SLC22A7 (OAT2)	↓	Moscovitz et al., 2018
	SLCO1B1 (OATP2)	↑	Wortham et al., 2007

Enzymy I. fáze metabolismu	ADH1B	↓	Lambert et al., 2009
	CYP1A1	↑	Moscovitz et al., 2018
	CYP2A6	↑↑	Wortham et al., 2007
	CYP2B6	↑↑↑	Faucette et al., 2007
	CYP2C8	↑	Moscovitz et al., 2018
	CYP2C9	↑	Moscovitz et al., 2018
	CYP2C19	↑	Moscovitz et al., 2018
	CYP2D6	↑	Wortham et al., 2007
	CYP3A4	↑↑	Faucette et al., 2007
Enzymy II. fáze metabolismu	GSTA1	↑	Moscovitz et al., 2018
	GSTP1	↑	Moscovitz et al., 2018
	SULT1E1	↑	Moscovitz et al., 2018
	UGT1A1	↑	Wortham et al., 2007
	UGT1A4	↑	Moscovitz et al., 2018
	UGT2B4	↑	Moscovitz et al., 2018

Mechanismus aktivace a agonisté CAR

Na rozdíl od většiny ostatních jaderných receptorů CAR vykazuje silnou bazální transkripční aktivitu i bez přítomnosti svých ligandů, avšak může být též aktivován navázáním agonistů. CAR v buněčném jádře může být konstitutivně aktivní i bez stimulace ligandy, což bylo pozorováno u jaterní nádorové buněčné linie HepG2 (Wang and Negishi, 2003). Kromě tzv. wild-type varianty (CAR1), existují i dvě další často se

vyskytující se varianty CAR vzniklé alternativním splicingem – CAR2 a CAR3, které nejsou konstitutivně aktivní a lze je využít při např. k identifikaci potenciálních agonistů CAR (Auerbach et al., 2003)

Neaktivovaný CAR je lokalizován v cytoplazmě hepatocytů, kde je vázán na komplex několika proteinů. K translokaci CAR do jádra dochází až po jeho aktivaci a po uvolnění z vazby na bílkovinný komplex (Hernandez et al., 2009; Kobayashi et al., 2003; Sueyoshi et al., 2008; Wang and Negishi, 2003). V HepG2 buněčné linii je CAR akumulován přímo v jádře buněk kvůli jejich dediferenciaci, v důsledku čehož buňky ztrácí schopnost exprimovat tento komplex (Kobayashi et al., 2003). CAR může být aktivován jak na ligandu závislým mechanismem, tak i na ligandu nezávisle. Obdobně jako v případě aktivace PXR dojde po navázání ligandu CAR k disociaci korepresorů, navázání koaktivátorů, heterodimerizaci s RXRα a navázání na příslušné responsivní elementy v promotorové oblasti cílových genů (Chai et al., 2013; Chen et al., 2012). Vysoká konstitutivní aktivita CAR je způsobena jeho malou a poměrně rigidní AF-2 doménou, díky které může CAR zůstávat ve své aktivní konformaci (Hernandez et al., 2009).

V rentgenových krystalografických studiích bylo prokázáno, že ligand vázající kapsa CAR je mnohem menší ve srovnání s PXR (Hernandez et al., 2009; Chai et al., 2013). Sekvenční analýza LBD lidských receptorů PXR a CAR ukázala 45 % shodu, což naznačuje jistou podobnost v ligandovém spektru, které je však v případě CAR o poznání užší (Moore et al., 2002).

Agonistické účinky na CAR vykazuje poměrně rozsáhlá řada strukturně rozličných látek, které mohou být přírodního i syntetického původu. Z rostlinných látek je možné jako agonisty CAR jmenovat např. flavony chrysin a baikalein nebo flavonol galangin, izolovaný z galgánu lékařského (*Alpinia officinarum*) (Carazo Fernández et al., 2015). Chrysin byl izolován mj. z mučenky modré (*Passiflora caerulea*), baikalein z kořene šišáku bajkalského (*Sculletaria baicalensis*) (Bochořáková et al., 2003; Carazo Fernández et al., 2015; Mani and Natesan, 2018). Také extrakt z jinanu dvoulaločného (*Ginkgo biloba*) nebo obsahová látka pelyňku ročního (*Artemisia annua*) seskviterpenický lakton artemisinin mají aktivační účinky na CAR (Hernandez et al., 2009).

Rovněž některé syntetické látky jsou schopné aktivace CAR. Modelovým případem je fenobarbital, jehož nevýhodou je současná aktivace jaderných receptorů CAR a PXR (Carnahan and Redinbo, 2005; Zelko and Negishi, 2000). V roce 2003 byl objeven specifický agonista CAR, jedná se o syntetickou látku 6(4chlorfenyl)imidazo[2,1-b]thiazol-5-karbaldehyd O-(3,4-dichlorbenzyl)oxim (CITCO), která je schopná aktivovat CAR bez výraznější aktivace PXR (Maglich et al., 2003). Nespecifickým exogenním agonistou je např. kyselina ftalová nebo nonylfenol, což jsou látky využívané jako změkčovadla (Hernandez et al., 2009). Silně agonistické vlastnosti vykazují také některé 2-(3-methoxyfenyl)-3,4-dihydrochinazolinové deriváty (Smutny et al., 2016). Také celé řada léčiv má aktivační účinky na CAR, lze jmenovat např. látky používané v terapii epilepsie karbamazepin a fenytion, hypolipidemikum atorvastatin nebo benzodiazepinové anxiolytikum diazepam, jejich afinita je však velmi nízká a aktivují CAR v milimolárních koncentracích (Kachaylo et al., 2011).

2.2 Transportéry v lidských játrech

Membránové transportéry se podílejí na udržování homeostázy buněk i celého organismu prostřednictvím transportu živin, signálních molekul nebo toxinů přes buněčné membrány. Transportéry rovněž mohou zprostředkovávat přenos léčiv či toxinů, mohou být příčinou mnohočetné lékové rezistence, ale i být cílovými strukturami podané léčivé látky (Hacker et al., 2009).

Transportní systémy významnou měrou ovlivňují farmakokinetiku léčiv. Jedná se o speciální proteiny, které transportují léčiva přes biologické membrány a tím umožňují, aby se dostaly z místa podání na místo účinku, a posléze i do eliminačních orgánů, aby mohly být vyloučeny z organismu. Přenos pomocí transportérů je významný především u látek, které nesou elektrický náboj a jsou hydrofilní, a nemohou tak procházet přes fosfolipidovou dvojvrstvu buněčné membrány prostou difuzí (Klaassen and Aleksunes, 2010; Nigam, 2014; Oostendorp et al., 2009).

Transportéry lze rozdělit do dvou hlavních nadrodin - solute carrier (SLC) a ATP-binding cassette (ABC) transportérů. Přenašeče z nadrodiny SLC lze obecně považovat za proteiny umožňující uptake jejich substrátů, tedy transport látek z vnějšího prostředí dovnitř do buňky, i když byl popsán i fenomén, kdy SLC transportéry přenášejí látky v obou směrech. ABC přenašeče na rozdíl od nadrodiny SLC jsou efluxní, transportují látky směrem ven z buňky nebo z organely. SLC transportéry typicky využívají sekundární nebo terciární aktivní transport, kdežto pro ABC přenašeče je charakteristický primární aktivní transport (Klaassen and Aleksunes, 2010; Morris et al., 2017; Nigam, 2014).

Široká škála transportních proteinů je exprimována na bazolaterální i apikální membráně hepatocytů. Tyto molekuly mají za úkol transportovat látky z krve do hepatocytu, nebo je naopak přenášet z hepatocytu zpět do krve nebo do žluče. Můžeme zde najít přenašeče z obou nadrodin – SLC i ABC transportéry (Klaassen and Aleksunes, 2010; Nigam, 2014).

Z konkrétních jaterních transportérů z nadrodiny SLC je možné jmenovat OCT1 (SLC22A1), který přenáší malé organické kationty (Klaassen and Aleksunes, 2010). Podrobnější informace o OCT1 jsou v následující podkapitole. Dalším jaterním transportérem, který je schopný transportovat kladně nabité molekuly, je MATE1

(multidrug and toxin extrusion protein 1, SLC47A1). Ovšem, na rozdíl od většiny ostatních členů nadrodiny SLC, MATE1 funguje jako efluxní protein, který transportuje substráty (např. platinová cytostatika cisplatinu a oxaliplatinu nebo antidiabetikum metformin) z hepatocytů do žluče (Damme et al., 2011).

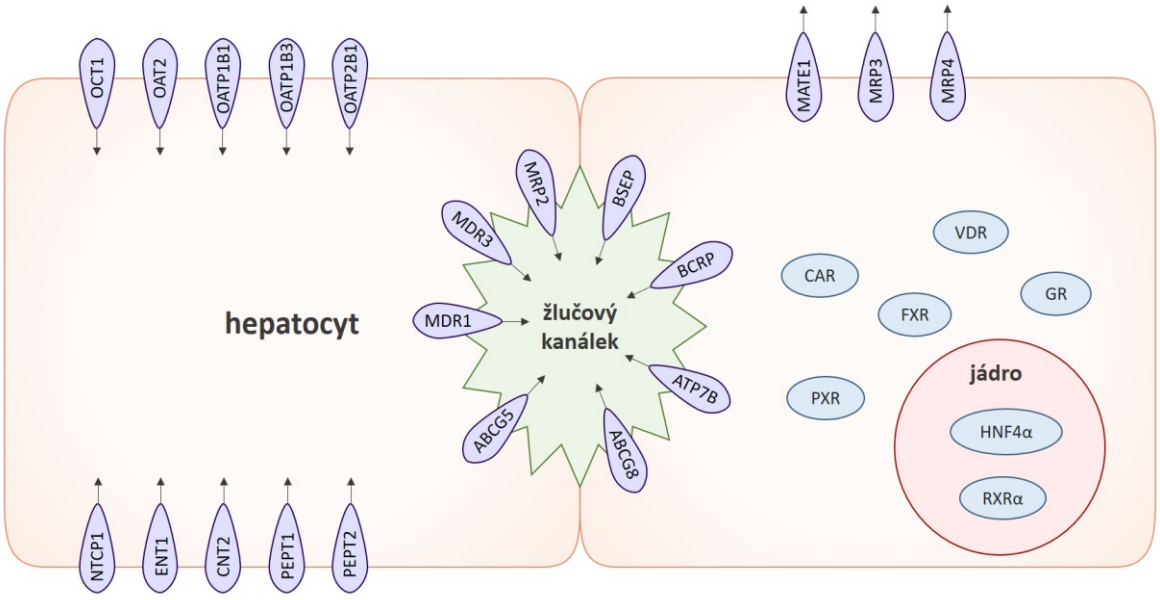
V játrech jsou též přítomny transportéry, které jsou schopné vychytávat z krve organické anionty a přenášet je do hepatocytů, jedná se jednak o skupinu polypeptidů transportujících organické anionty (OATP) a transportéry organických aniontů (OAT). OATP transportéry přenáší širokou řadu negativně nabitých látek. Z konkrétních členů je možné jmenovat OATP1B1 (SLCO1B1), který transportuje jak např. β-laktamové antibiotikum benzylpenicilin, antituberkulotikum rifampicin, hypolipidemicky působící atorvastatin, tak i látky tělu vlastní, jako je bilirubin a jeho konjugáty nebo kyselinu cholovou, čímž přispívá k transportu žlučových kyselin do hepatocytů. Také další transportér pro organické anionty OAT2 vykazuje širokou substrátovou selektivitu, do hepatocytů jeho prostřednictvím vstupují např. salicyláty, tetracyklin nebo antiepileptikum kyselina valproová (Klaassen and Aleksunes, 2010).

Látky peptidového charakteru (di- a tripeptidy) také nemohou samovolně přecházet přes hepatocytární membránu, využívají k tomu peptidové transportéry, jako je PEPT1 (SLC15A1) nebo PEPT2 (SLC15A2) (Terada and Inui, 2012; Zhang et al., 2004).

Jedním z nejdůležitějších transportérů nadrodiny ATP-binding cassette (ABC) transportérů v jaterních buňkách je bezesporu P-glykoprotein. P-gp zprostředkovává eflux široké škály látek včetně mnoha cytostatik (např. paklitaxel, daunorubicin, doxorubicin, vinblastin nebo vinkristin), antiretrovirálních látek (např. ritonavir, saquinavir), antibakteriálně účinných látek (např. sparfloxatin a tetracyklin), blokátorů vápníkového kanálu (verapamil a diltiazem), digoxinu, ondansetronu, losartanu a celé řady dalších xenobiotik z hepatocytů do žluče, což může být spojeno i se selháním terapie uvedenými léčivy (Klaassen and Aleksunes, 2010; Morris et al., 2017). Na apikální membráně hepatocytů se nachází i další efluxní transportér BCRP (breast cancer resistence protein, ABCG2), jehož substrátové spektrum se částečně překrývá s P-gp. BCRP transportuje do žluče např. fluorochinolonová antibiotika ciprofloxacin a grapafloxacin, antiretrovirální látky abakavir a lamivudin, kličkové diuretikum furosemid nebo vitamin B_2 riboflavin (Klaassen and Aleksunes, 2010; Morris et al., 2017).

Transportér MRP3 (multidrug resistence protein 3, ABCC3) také zprostředkovává eflux substrátů podobně jako výše zmíněné transportéry P-gp a BCRP. Na rozdíl od těchto transportérů je lokalizován na bazolaterální membráně hepatocytů a umožňuje zpětný transport jeho substrátů zpět do krve. MRP3 přenáší např. glukuronidové konjugáty, žlučové kyseliny a široké spektrum léčiv včetně antirevmatika methotrexátu (Klaassen and Aleksunes, 2010).

Přehled zmíněných a dalších transportérů z nadrodin SLC i ABC, které se podílejí na transportu endogenních i exogenních látek v hepatocytech, jejich buněčná lokalizace a směr transportu substrátů jsou uvedeny na obr. 4.



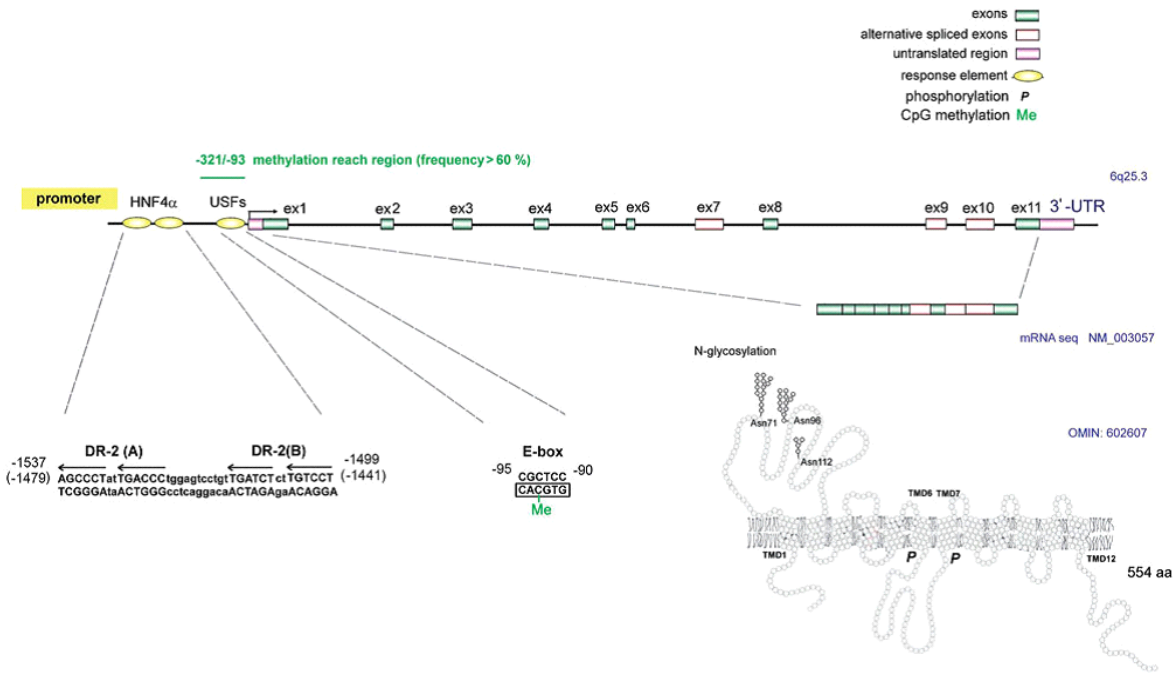
Obr. 4 Schématické znázornění hlavních transportních proteinů a vybraných nukleárních receptorů regulujících jejich činnost v lidských hepatocytech. Transportéry jsou zobrazeny podlouhlým fialovým obrazcem se šipkou, která naznačuje hlavní směr transportu látek. Nukleární receptory jsou na tomto obrázku znázorněny jako modrý ovál. Vysvětlení jednotlivých zkratek je uvedeno v kapitole 12 Seznam zkratek.

2.2.1 Transportér pro organické kationty 1 (OCT1)

Transportér pro organické kationty 1 (organic cation transporter 1, OCT1) hraje stěžejní roli při transportu široké škály malých kladně nabitých organických sloučenin (Jonker and Schinkel, 2004; Klaassen and Aleksunes, 2010; Koepsell, 2011). Vzhledem k tomu, že za fyziologického pH přibližně 40 % v současnosti používaných léčiv se vyskytuje ve formě kationtů, je řada z těchto látek potenciálními substráty OCT1 (Nies et al., 2009).

OCT1 kódovaný genem SCL22A1 patří mezi nejdůležitější členy nadrodiny SLC. Transportérová rodina SLC22 čítá 13 členů včetně 3 patřících do podskupiny SLC22A, kam se řadí i OCT1 (Koepsell, 2004; Koepsell, 2007). Lidský gen SLC22A1 byl poprvé popsán v roce 1997, avšak potkaní Slc22a1 byl naklonován ještě o 3 roky dříve a zařazen do podskupiny Slc22a jako její první člen (Gorboulev et al., 1997; Gründemann et al., 1994; Zhang et al., 1997). SLC22A1 je lokalizovaný společně s geny pro OCT2 a OCT3 (SLC22A2 resp. SLC22A3) na chromozomu 6q25-q27 (Koehler et al., 1997; Koepsell et al., 2007; Schweifer and Barlow, 1996). SLC22A1 se skládá z 11 exonů a 10 intronů, což umožňuje vznik různých alternativních variant OCT1, avšak pouze varianta označovaná číslem 1 tvoří plnohodnotný protein schopný transportu (Hayer et al., 1999; Herraez et al., 2013; Koepsell et al., 2007). Alternativní splicing je znázorněn na obr. 5, kde je patrné možné vystřižení exonu 7, 9 a 10 (Herraez et al., 2013; Kusuhara et al., 1999).

OCT1 protein se podobně jako ostatní členy SLC22 rodiny skládá z 554 aminokyselin, které tvoří celkem 12 α-helikálních transmembránových domén, přičemž karboxy- i amino- konce bílkoviny jsou umístěny uvnitř buňky (Burckhardt and Wolff, 2000; Lozano et al., 2013). Mezi transmembránovými doménami 1 a 2 se nachází velká extracelulární smyčka, ve které dochází v pozicích 71, 96 a 112 k N-glykosylaci asparaginu (Burckhardt and Wolff, 2000; Lozano et al., 2013). Další velká smyčka se nachází na vnitřní straně plazmatické membrány mezi transmembránovými doménami 6 a 7. Protein v oblasti této smyčky obsahuje fosforylační místa (Wright, 2005). Struktura OCT1 transportéru je znázorněna na obr. 5.



Obr. 5 Struktura lidského genu SCL22A1 kódujícího transportní protein OCT1 a struktura transportéru OCT1 se znázorněnými transmembránovými doménami. Na obrázku jsou vyznačeny důležité oblasti promotoru (vazebná místa pro HNF4α a USFs), které se významnou měrou podílejí na regulaci exprese SLC22A1 (převzato z Hyrsova et al., 2016b).

Funkce a lokalizace OCT1 v organismu

OCT1 je exprimován v největší míře v játrech, konkrétně na bazolaterální membráně hepatocytů a v mnohem menší míře je přítomný i v plazmatické membráně cholangiocytů (Koepsell et al., 2007; Nies et al., 2009; Nishimura and Naito, 2005). OCT1 tedy hraje významnou roli při vstřebávání látek z krve do hepatocytů, v nichž dochází k jejich metabolizaci, případně k transportu na místo jejich účinku. Významnou měrou se tedy podílejí jak na distribuci, tak i eliminaci řady látek včetně mnoha léčiv (Hyrsova et al., 2016b; Koepsell, 2011).

V jiných tkáních je OCT1 exprimován v mnohem menší míře. Poměrně významná je jeho lokalizace ve slezině, plicích, varlatech, proximálních tubulech ledvin nebo v kosterní svalovině (Nies et al., 2009; Nishimura and Naito, 2005). Dále byla OCT1 mRNA detekována i v tenkém střevě, mozku, srdci, oku, placentě nebo v buňkách imunitního systému (Lee et al., 2013; Minuesa et al., 2008; Nishimura and Naito, 2005;

Zhang et al., 2008). Avšak při porovnání kvantity exprese SLC22A1 v hepatocytech a v ostatních tkáních, je dominantní exprese v hepatocytech jasně patrná, neboť je vyšší o 3-5 řádů (Klaassen and Aleksunes, 2010; Nishimura and Naito, 2005)

OCT1 transportní protein zprostředkovává přenos substrátů přes plazmatickou membránu facilitovanou difúzí (Koepsell, 2011; 2015). Po navázání kationtu na OCT1 transportér vyvolá konformační změnu proteinu a tím dojde k translokaci přenášeného kationtu na druhou stranu plazmatické membrány (Hyrsova et al., 2016b). Tento přenos nemusí být pouze ve směru do buňky, i když je to pro OCT1 typické, ale může fungovat i jako efluxní transportér (Koepsell, 2015).

Vzhledem k široké distribuci v organismu hraje OCT1 důležitou roli ve farmakokinetice bazických léčiv. Uplatňuje se zejména při distribuci endogenních látek a léčiv, ale i při jejich absorpci a exkreci (Hyrsova et al., 2016b; Jonker and Schinkel, 2004; Nies et al., 2009). Typickým příkladem nezbytnosti správné funkce tohoto transportéru je metformin, který je z krve přenášen do hepatocytů, tedy na místo jeho účinku, primárně pomocí OCT1. OCT1 je tedy nezbytný pro výsledný farmakodynamický efekt tohoto léčiva (Becker et al., 2009; Shu et al., 2007; Wang et al., 2002). Dále OCT1 zprostředkovává transport látek z krve do mozku nebo do buněk imunitního systému (Lin et al., 2010; Minuesa et al., 2008). OCT1 hraje roli i při absorpci kladně nabitých molekul z tenkého střeva a v malé míře se zde uplatňuje i při sekreci látek (Jonker and Schinkel, 2004; Koepsell, 2015). OCT1 zprostředkovává i absorpci některých léčiv z bronchů (Ingoglia et al., 2015).

Je třeba vzít v potaz také možnou interakci na farmakokinetické úrovni v důsledku kompetice dvou substrátů o stejný transportér, jako je tomu v případě terapie metforminem. OCT1 transportérem jsou přenášeny i látky tělu vlastní, např. vitamin B_1 thiamin. Terapie metforminem, který v tomto případě působí jako kompetitivní inhibitor, tak může snižovat dostupnost thiaminu pro organismus (Chen et al., 2014; Wang et al., 2002).

Substráty a inhibitory OCT1

Jak již bylo zmíněno v úvodu podkapitoly pojednávající o OCT1, jedná se o polyspecifický transportér, jehož substrátové spektrum zahrnuje řadu různorodých látek

(Hyrsova et al., 2016b; Klaassen and Aleksunes, 2010). Právě kvůli velkému rozsahu substrátového spektra jsou v této podkapitole zmíněna pouze vybraná léčiva, přírodní, endogenní a modelové látky. Nejběžněji používaným modelovým substrátem je látka označovaná zkratkou MPP^+ (1-methyl-4-fenylpyridinium) (Koepsell et al., 2007). Z dalších modelových sloučenin lze jmenovat tetraethylamonium (TEA) nebo 4-(4-(dimethylamino)-styryl)-N-methylpyridinium (ASP^+) (Koepsell, 2013; Koepsell et al., 2007). Tyto modelové substráty se používají ke stanovení funkčnosti transportéru nebo při inhibičních studiích (Umehara et al., 2008).

Mezi významné substráty OCT1 kromě metforminu (Wang et al., 2002), patří i běžně používané léčivé látky jako např. blokátor β-adrenergních receptorů atenolol (Yin et al., 2015), dále platinová cytostatika cisplatina, oxaliplatina a pikoplatina (More et al., 2010; Yonezawa et al., 2006) nebo antivirová léčiva lamivudin (Minuesa et al., 2009), ganciclovir a aciclovir (Koepsell et al., 2007). Dalšími látkami identifikovanými jako substráty OCT1 transportéru jsou i rostlinné sekundární metabolity, např. alkaloid berberin (Nies et al., 2008), protoberberinový alkaloid jatrorhizin (Li et al., 2016) nebo flavonoid kvercetin (Glaeser et al., 2014).

Substráty OCT1 však nejsou jenom xenobiotika, transportér je schopný přenášet i látky endogenního původu včetně adrenalinu a noradrenalinu, histaminu (Koepsell, 2015), serotoninu (Boxberger et al., 2014) prostaglandinu E_2 a F_2 (Koepsell et al., 2007) nebo již zmiňovaného thiaminu (Chen et al., 2014).

Byly identifikovány i látky, které na OCT1 působí inhibičně. Jako inhibitory OCT1 působí např. antivirotikum ritonavir (Jung et al., 2008), dále blokátory β -adrenergních receptorů pindolol a propranolol (Umehara et al., 2008) nebo acetylcholin (Koepsell et al., 2007).

Exprese OCT1 a možnosti její regulace

Regulace exprese OCT1 transportéru je kontrolovaná zejména transkripčními faktory nacházejícími se ve vysoké míře v játrech (liver-enriched transcription factors, LETFs), kam patří hepatocytární nukleární faktory 1α, 3α a 4α, dále CAAT/enhancer vázající proteiny α a β (CAAT/enhancer binding protein α, β; C/EBPα, β). Tyto transkripční faktory jsou velmi důležité pro diferenciaci, udržování jaterního fenotypu a transkripční

regulaci biotransformačních enzymů a lékových transportérů (Castell et al., 2006; Kamiyama et al., 2007).

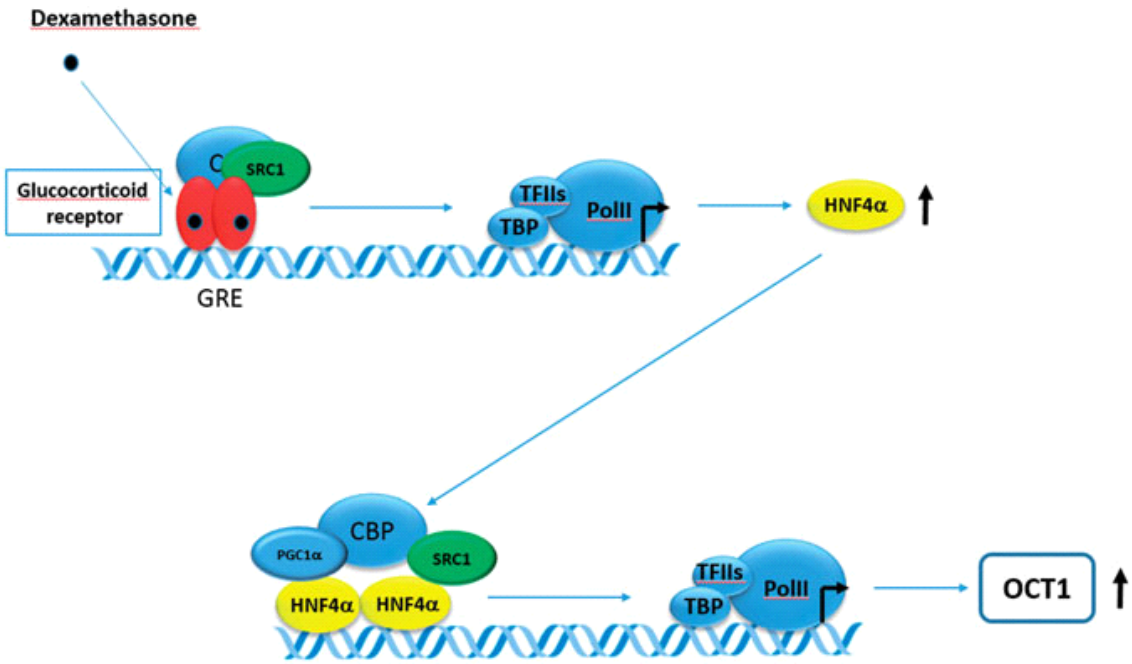
Absolutně nejvyšší podíl na regulaci exprese OCT1 má hepatocytární nukleární faktor 4α (HNF4α) (Kamiyama et al., 2007; Saborowski et al., 2006). HNF4α patří do nadrodiny nukleárních receptorů a vykazuje tak některé podobné vlastnosti. HNF4α ovlivňuje expresi cílových genů zejména v játrech, v menší míře jej můžeme detekovat i v jiných tkáních, např. ve střevě nebo slinivce. Bylo zjištěno že, HNF4α nasedá ve formě homodimeru do dvou svých responzivních oblastí na promotoru jeho cílových genů včetně SLC22A1 genu, tedy genu pro OCT1 transportér viz obr. 5 (Benoit et al., 2006; Saborowski et al., 2006; Sladek et al., 1990). Tyto oblasti jsou nazývány DR-2 a jedná se o pravidelně se opakující hexamery oddělené dvěma bázemi. Po navázání HNF4α do obou DR-2 dochází k navázání koaktivátorů a ke spuštění transkripce SLC22A1 (Saborowski et al., 2006). Důležitost tohoto nukleární receptoru v regulaci exprese OCT1 podtrhuje i studie, ve které byly primární lidské hepatocyty transfekovány siRNA (small interfering RNA) proti HNF4α. V buňkách došlo k markantnímu snížení exprese HNF4α a současně i k významnému poklesu hladiny OCT1 mRNA (Kamiyama et al., 2007).

Roli v regulaci SLC22A1 hrají i další faktory, konkrétně tzv. nadřazené (neboli upstream) stimulační faktory 1 a 2 (USF1 a USF2). Tyto USF se vážou do jiného místa v promotoru SLC22A1, konkrétně do E-box sekvence a zesilují transaktivaci vyvolanou navázáním HNF4α (Hyrsova et al., 2016a; Kajiwara et al., 2008). Dále bylo zjištěno, že i C/EBPβ patřící do skupiny LEFTs ovlivňuje expresi OCT1. Z těchto poznatků vyplývá, že vzhledem k tomu, že exprese OCT1 transportéru je regulovaná především HNF4α a C/EBPβ, lokalizace tohoto transportéru je převážně jaterní, v ostatních tkáních se OCT1 vyskytuje minimálně (Nies et al., 2009; Rulcova et al., 2013).

Exprese OCT1 je regulována i nepřímo, prostřednictvím jaderných receptorů, konkrétně byla popsána regulace glukokortikoidním a farnesoidním X receptorem. Regulace byla identifikována jako nepřímá, protože nebyla nalezena žádná responsivní oblast v promotoru SLC22A1 genu, na kterou by se výše zmíněné nukleární receptory vázaly (Rulcova et al., 2013; Saborowski et al., 2006). Mechanismus této regulace je však v případě každého výše zmíněného jaderného receptoru jiný.

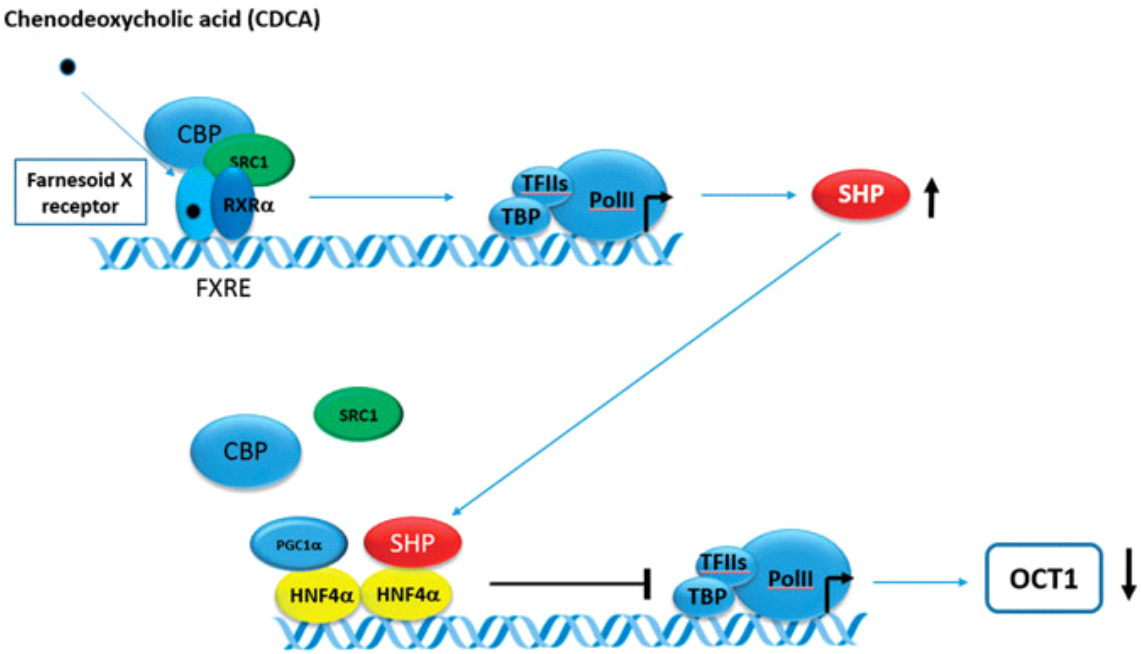
i. V nedávno publikované práci byla popsána zvýšená exprese SLC22A1 v lidských hepatocytech po přidání dexametazonu do média, což je agonista

glukokortikoidního receptoru (Rulcova et al., 2013). Po celé řadě experimentů byla vyloučena přítomnost odpovídajících oblastí (glucocorticoid receptor responsive elements, GRE) pro GR v promotoru OCT1 genu a naopak byla popsána nepřímá regulace vlivem zvýšení exprese HNF4α, který je v regulaci SLC22A1 stěžejní (Rulcova et al., 2013; Saborowski et al., 2006). Zvyšování hladiny mRNA HNF4α v primárních lidských hepatocytech prostřednictvím glukokortikoidů bylo již dříve popsáno dvěma na sobě nezávislými výzkumnými skupinami (Godoy et al., 2010; Onica et al., 2008). Pro přehlednost je mechanismus znázorněn na obr. 6.



Obr. 6 Znázornění mechanismu indukce exprese OCT1 prostřednictvím glukokortikoidního receptoru. Ten se váže do responzivní oblasti (GRE) v promotoru svých cílových genů. Po vazbě dexametazonu na glukokortikoidní receptor dojde k disociaci korepresorů a naopak k navázání koaktivátorů (SRC1 a CBP) a spuštění transkripce GR regulovaných genů, v tomto případě HNF4α. (Převzato z Hyrsova et al., 2016b). Vysvětlení jednotlivých zkratek je uvedeno v kapitole 12 Seznam zkratek.

ii. Snížení exprese OCT1 mRNA bylo popsáno v důsledku působení chenodeoxycholové kyseliny (chenodeoxycholic acid, CDCA), což je agonista farnesoidního X receptoru (Parks et al., 1999; Saborowski et al., 2006). Stejně jako v předchozích dvou případech nebyla v SLC22A1 promotoru identifikována FXR responsivních oblastí (FXRE) (Saborowski et al., 2006). Jedním z cílových genů FXR je malý heterodimerní partner (small heterodimer partner, SHP), který působí jako korepresor dalších transkripčních faktorů včetně HNF4α (Lee et al., 2000). Po aktivaci FXR tedy dojde ke zvýšení transkripce SHP, které následně působí proti transkripci OCT1 způsobené HNF4α, jak je zobrazeno na obr. 7 (Nies et al., 2009; Saborowski et al., 2006).



Obr. 7 Znázornění mechanismu snížení exprese OCT1 prostřednictvím farnesoidního X receptoru. Po aktivaci a navázání FXR do svých responzivních oblastí (FXRE) dochází ke zvýšení hladiny FXR-cílového genu pro SHP (small heterodimer partner), který potlačuje transkripci OCT1 zprostředkovanou HNF4α Hyrsova et al., 2016b). Vysvětlení jednotlivých zkratek je uvedeno v kapitole 12 Seznam zkratek.

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4 Cíle práce

- Určení mechanismu suprese OCT1 transportéru pomocí nukleárního receptoru PXR v hepatocytárních modelech.
- Porovnat zjištěný mechanismus suprese OCT1 s dalšími nukleárními receptory.
- Stanovení potenciálního antagonistického působení derivátů resveratrolu na PXR receptor.
- Určení potenciálního inhibičního vlivu vybraných derivátů žlučových kyselin na PXR.

5 Seznam publikací

Tato disertační práce je založena na následujících čtyřech publikacích s vyznačením současným impaktním faktorem:

1. **Hyrsova L**, Smutny T, Carazo A, Moravcik S, Mandikova J, Trejtnar F, Gerbal-Chaloin S and Pavek P (2016a) The pregnane X receptor down-regulates organic cation transporter 1 (SLC22A1) in human hepatocytes by competing for ("squelching") SRC-1 coactivator. *British Journal of Pharmacology* 173:1703-1715.

   (IF 2016: **5,491**)

2. **Hyrsova L**, Smutny T, Trejtnar F and Pavek P (2016b) Expression of organic cation transporter 1 (OCT1): unique patterns of indirect regulation by nuclear receptors and hepatospecific gene regulation. *Drug Metabolism Reviews* 48:139-158.

   (IF 2016/2017: **4,097**)

3. **Hyrsova L**, Vanduchova A, Dusek J, Smutny T, Carazo A, Maresova V, Trejtnar F, Barta P, Anzenbacher P, Dvorak Z and Pavek P (2018) Trans-resveratrol, but not other natural stilbenes occuring in food, carries the risk of drug-food interaction via inhibition of cytochrome P450 enzymes or interaction with xenosensor receptors. *Toxicology Letters.*
   https://doi.org/10.1016/j.toxlet.2018.10.028

   (IF 2017: **3,166**)

4. Carazo A, **Hyrsova L**, Dusek J, Chodounska H, Horvatova A, Berka K, Bazgier V, Gan-Schreier H, Chamulitrat W, Kudova E and Pavek P (2017) Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor. *Toxicology Letters* 265:86-96.

   (IF 2017: **3,166**)

6 Přispění autora k jednotlivým publikacím

Tato disertační práce je založena na výše zmíněných publikacích, které jsou označeny čísly 1-4. Kandidátka je první autorkou prvních tří publikací.

- Přispění kandidátky k publikaci označené č. 1:
  - podílení se na plánování experimentů
  - příprava, provedení a analýza experimentů týkajících se RT-PCR (obr. 1A-C, 3), genových reportérových experimentů (obr. 2, 4, 5), akumulační studie (obr. 1E), western blotting experimentů (obr. 1D) a chromatinové imunoprecipitace (obr. 6)
  - grafické zpracování výsledků výše zmíněných pokusů
  - podílení se na psaní a konečných úpravách publikace

- Přispění kandidátky k publikaci označené č. 2:
  - podílení se na celkovém psaní publikace se zaměřením na kapitoly a podkapitoly: Úvod, Exprese OCT1, Funkce OCT1, Regulace bazální jaterní exprese, Regulace zprostředkovaná na ligandu závislými jadernými receptory a Diskuse a závěry
  - vyhledávání informací obsažených v tabulkách 1 a 2
  - podílení se na kreslení obr. 1
  - podílení se na závěrečných úpravách publikace

- Přispění kandidátky k publikaci označené č. 3:
  - podílení se na celkovém sepsání publikace
  - plánování, provedení a vyhodnocení genových reportérových experimentů (obr. 3C, D, E a F)
  - podílení se na statistickém a grafickém zpracování dat
  - podílení se na konečných úpravách

- Přispění kandidátky k publikaci označené č. 4:
  - příprava, provedení a analýza experimentů týkajících se RT-PCR (obr. 4A)
  - grafické zpracování výsledků a podíl na sepsání publikace

7 Články uveřejněné v periodicích s impaktním faktorem nevztahující se k této disertační práci

Kandidátka je spoluautorkou dalších pěti publikací, které ovšem nejsou diskutovány v této disertační práci.

I. Smutny T, Nova A, Drechslerová A, Carazo A, **Hyrsova L**, Hrušková ZR, Kuneš J, Pour M, Špulák M, and Pavek P (2016) 2-(3-Methoxyphenyl)quinazoline Derivatives: A New Class of Direct Constitutive Androstane Receptor (CAR) Agonists. *Journal of Medicinal Chemistry* 59: 4601-10. (IF 2016: **6,259**)

II. Mandíková J, Volková M, Pávek P, Navrátilová L, **Hyršová L**, Janeba Z, Pavlík J, Bárta P, and Trejtnar F (2015) Entecavir Interacts with Influx Transporters hOAT1, hCNT2, hCNT3, but Not with hOCT2: The Potential for Renal Transporter-Mediated Cytotoxicity and Drug–Drug Interactions. *Frontiers in Pharmacology* 6: 304. (IF 2015: **4,418**)

III. Carazo Fernández A, Smutny T, **Hyrsová L**, Berka K, and Pavek P (2015) Chrysin, baicalein and galangin are indirect activators of the human constitutive androstane receptor (CAR). *Toxicology Letters* 233: 68-77. (IF 2015: **3,522**)

IV. Dusek J, Carazo A, Trejtnar F, **Hyrsova L**, Holas O, Smutny T, Micuda S and Pavek P (2017) Steviol, an aglycone of steviol glycoside sweeteners, interacts with the pregnane X (PXR) and aryl hydrocarbon (AHR) receptors in detoxification regulation. *Food and Chemical Toxicology* 109:130-142. (IF 2017/2018: **3,977**)

V. Carazo A, Dusek J, Holas O, Skoda J, **Hyrsova L**, Smutny T, Soukup T, Dosedel M and Pávek P (2018) Teriflunomide Is an Indirect Human Constitutive Androstane Receptor (CAR) Activator Interacting With Epidermal Growth Factor (EGF) Signaling. *Frontiers in Pharmacology* 9. (IF 2017: **3,831**)

VI. Dolezelova E, Prasnicka A, Cermanova J, Carazo A, **Hyrsova L**, Hroch M, Mokry J, Adamcova M, Mrkvicova A, Pavek P, and Micuda S (2017) Resveratrol modifies biliary secretion of cholephilic compounds in sham-operated and cholestatic rats. *World Journal of Gastroenterology* 23: 7678-92. (IF 2017: **3,300**)

8 Soubor publikací uveřejněných v časopisech s impaktním faktorem vztahující se k tématu disertační práce doplněné komentářem kandidátky

8.1 The pregnane X receptor down-regulates organic cation transporter 1 (SLC22A1) in human hepatocytes by competing for ("squelching") SRC-1 coactivator.

Hyrsova L, Smutny T, Carazo A, Moravcik S, Mandikova J, Trejtnar F, Gerbal-Chaloin S and Pavek P (2016a) The pregnane X receptor down-regulates organic cation transporter 1 (SLC22A1) in human hepatocytes by competing for ("squelching") SRC-1 coactivator. *British Journal of Pharmacology* 173:1703-1715.

(IF 2016: **5,491**)

V první publikaci zahrnuté v této disertační práci jsme se zaměřili na odhalení mechanismu potlačení exprese (down-regulace) OCT1 transportéru nukleárním receptorem PXR. Exprese obou těchto proteinů je dominantní právě v játrech. OCT1 zprostředkovává transport malých organický kationtů do hepatocytů, PXR řídí expresi hlavních biotransformačních enzymů a transportérů v lidských játrech. Exprese OCT1 je silně kontrolována jaderným receptorem HNF4α.

S využitím metod kvantitativní RT-PCR, western blotting a akumulačních studií jsme zaznamenali významné snížené množství OCT1 mRNA a proteinu, také funkce OCT1 byla významně snížena v přítomnosti agonistů PXR v primárních lidských hepatocytech nebo v diferencované hepatocytární buněčné linii HepaRG.

V dalších experimentech týkající se ověření mechanismu účinku byla provedena série genových reportérových experimentů s reportérových konstruktem obsahujícím 1,8 kb promotorové oblasti genu kódující OCT1 a konstitutivně aktivní mutant PXR T248D nebo inaktivní mutant s nefunkční AF-2 oblastí T422D, který není schopný vázat koaktivátory. T248D významně snížil aktivitu reportérového plazmidu, avšak T422D jeho aktivitu neovlivnil. Dále jsme použili řadu mutovaných reportérových konstruktů, jejichž promotor byl postupně zkracovaný nebo mutovaný v klíčových oblastech pro navázání nezbytných transkripčních faktorů, případně kombinace obojího. Z výsledků se jasně potvrdila nutná přítomnost funkčních DR-2 oblastí, což jsou vazebné oblasti HNF4α, a oblasti E-boxu, kam se vážou USF1 a 2 transkripční faktory, které zvyšují transaktivaci OCT1 zprostředkovanou HNF4α. V následných genových reportérových experimentech jsme odhalili, že se zvyšujícím se množstvím koaktivátoru SRC-1, který využívá PXR i HNF4α, dochází k postupnému snižování vlivu aktivace PXR na aktivitu OCT1 reportérového plazmidu.

Teorii kompetice o SRC-1 koaktivátor mezi PXR a HNF4α podepírají výsledky chromatinové imunoprecipitace, kdy bylo v přítomnosti rifampicinu detekováno nižší množství koaktivátoru SRC-1 v DR-2 a E-box promotorových oblastech.

Výsledky lze shrnout tak, že OCT1 je první gen ovlivňující farmakokinetiku léčiv down-regulovaný PXR s přesně a detailně popsaným mechanismem.

British Journal of Pharmacology

British Journal of Pharmacology (2016) **173** 1703–1715 1703

RESEARCH PAPER

The pregnane X receptor down-regulates organic cation transporter 1 (SLC22A1) in human hepatocytes by competing for ("squelching") SRC-1 coactivator

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Received 30 June 2015; **Revised** 29 January 2016; **Accepted** 1 February 2016

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BACKGROUND AND PURPOSE

The organic cation transporter 1 (OCT1) transports cationic drugs into hepatocytes. The high hepatic expression of OCT1 is controlled by the HNF4α and USF transcription factors. Pregnane X receptor (PXR) mediates induction of the principal xenobiotic metabolizing enzymes and transporters in the liver. Here, we have assessed the down-regulation of OCT1 expression by PXR activation.

EXPERIMENTAL APPROACH

We used primary human hepatocytes and related cell lines to measure OCT1 expression and activity, by assaying MPP^+ accumulation. Western blotting, qRT-PCR, the OCT1 promoter gene reporter constructs and chromatin immunoprecipitation assays were also used.

KEY RESULTS

OCT1 mRNA in human hepatocytes was down-regulated along with reduced [^{3}H]MPP^+ accumulation in differentiated HepaRG cells after treatment with rifampicin. Rifampicin and hyperforin as well as the constitutively active PXR mutant T248D suppressed activity of the 1.8 kb OCT1 promoter construct in gene reporter assays. Silencing of both PXR and HNF4α in HepaRG cells blocked the PXR ligand-mediated down-regulation of OCT1 expression. The mutation of HNF4α and USF1 (E-box) responsive elements reversed the PXR-mediated inhibition in gene reporter assays. Chromatin immunoprecipitation assays indicated that PXR activation sequestrates the SRC-1 coactivator from the HNF4α response element and E-box of the OCT1 promoter. Consistent with these findings, exogenous overexpression of the SRC-1, but not the PGC1α coactivator, relieved the PXR-mediated repression of OCT1 transactivation.

CONCLUSIONS AND IMPLICATIONS

PXR ligands reduced the HNF4α-mediated and USF-mediated transactivation of OCT1 gene expression by competing for SRC-1 and decreased delivery of a model OCT1 substrate into hepatocytes.

Abbreviations

CAR, constitutive androstane receptor; ChiP, chromatin immunoprecipitation assay; CML, chronic myeloid leukaemia; DR-2, direct repeat separated by two nucleotides; HNF4α, hepatocyte nuclear factor-4-α; OCT1, organic cation transporter 1; PXR, pregnane X receptor; SRC-1, steroid receptor coactivator; PGC1α, PPARγ coactivator 1α; USFs, upstream stimulating factors

DOI:10.1111/bph.13472

Tables of Links

TARGETS
Transporters[a]
OCT-1, organic cation transporter 1, SLC22A1
Nuclear hormone receptors[b]
CAR, constitutive androstane receptor
GRIP-1, NCOA2
HNF4α, hepatocyte nuclear factor-4-α, NR2A1
PGC1α, PPARGC1A
PXR, pregnane X receptor
SRC-1, steroid receptor coactivator, NCOA1

LIGANDS
Hyperforin
MPP^+, methyl-4 phenylpyridinium
Rifampicin

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

Introduction

The human organic cation transporter 1 (OCT1), encoded by the SLC22A1 gene, is responsible for the drug delivery of various cationic drugs (the antiviral drugs lamivudin, zalcitabine, ganciclovir and acyclovir; the anticancer drug oxaliplatin; the anti-hyperglycaemic drug metformin) and endogenous substrates such as dopamine, serotonin and choline into the hepatocyte from sinusoidal blood (Jonker and Schinkel, 2004; Koepsell *et al.*, 2007; Nies *et al.*, 2009; Boxberger *et al.*, 2014). Notably, hepatocytes are the pharmacological target of some OCT1 substrate drugs including metformin and lamivudine, and they are the site of the biotransformation and clearance of most drugs in general.

Studies with Oct1-null mice have clearly shown that Oct1 is the major physiological hepatic uptake system for small organic cations (Jonker *et al.*, 2003). In $Oct1^{-/-}$ mice, the blood glucose-lowering effect of metformin was completely abolished (Shu *et al.*, 2007), and the liver concentration of metformin was approximately 30 times higher in wild-type mice than in $Oct1^{-/-}$ mice (Wang *et al.*, 2002; Wang *et al.*, 2003). In addition, genetic variation rs622342 of the human SLC22A1 gene has been associated with the glucose-lowering effect of metformin in patients with diabetes mellitus (Becker *et al.*, 2009).

The hepatocyte-specific expression of OCT1 is strongly controlled by the liver-enriched transcription factor hepatocyte nuclear factor-4-α (HNF4α, NR2A1) (Saborowski *et al.*, 2006; Kamiyama *et al.*, 2007; Rulcova *et al.*, 2013). Saborowski *et al.* (2006) identified two interacting HNF4α response elements of the direct repeat (DR-2) format in the OCT1 promoter at −1479 to −1441 bp of 5′-flanking region of the OCT1 (SLC22A1) gene. In addition, a functional E-box (CACGTG) has been identified in the core proximal promoter region controlling the initiation of transcription. The E-box binds the upstream stimulating factors (USFs) USF1 and USF2 that further stimulate the HNF4α-mediated transactivation of the OCT1 gene (Kajiwara *et al.*, 2008). OCT1 mRNA and protein expression varies between 113-fold and 83-fold, respectively, in human livers population (Nies *et al.*, 2009).

Recently, however, contradictory data have also been published regarding OCT1 gene regulation by pregnane X receptors (PXR, NR1I2). PXR, the ligand-activated nuclear receptor of nuclear receptor subfamily NR1I, is the xenobiotic receptor governing the inducible expressions of a broad spectrum of target genes that encode phase-I and phase-II xenobiotic-metabolizing enzymes and drug transporters. Thus, PXR mediates a detoxification response to potentially toxic xenobiotics and also to toxic endogenous compounds (Chen *et al.*, 2012).

Cho and co-workers have reported that rifampicin increases the glucose-lowering effect of metformin, an OCT1 substrate, by 54% and that rifampicin significantly induces OCT1 mRNA in peripheral blood cells. Therefore, the authors hypothesize that rifampicin induces OCT1 in hepatocytes, which subsequently increases the uptake of metformin into hepatocytes, resulting in the increased effect of metformin (Cho *et al.*, 2011). Similarly, agonists of PXR induced OCT1 mRNA in the chronic myeloid leukaemia (CML) cell line and primary CML cells (Austin *et al.*, 2015). However, contradictory data have been also published indicating the down-regulation of OCT1 mRNA in human hepatocytes after treatment with rifampicin (Jigorel *et al.*, 2006; Badolo *et al.*, 2013). As yet, the underlying molecular mechanism of the rifampicin-mediated changes in OCT1 expression has not been determined.

Therefore, in the current work, we aimed to examine whether OCT1 is regulated by the PXR nuclear receptor and to elucidate the mechanism of this regulation in primary human hepatocytes and in hepatocyte-derived cellular models.

Methods

Cellular models

The human Caucasian hepatocellular carcinoma (HepG2) and human hepatocellular carcinoma HuH7 (D12) cell lines were purchased from the European Collection of Cell Cultures (Salisbury, UK) or Japanese Collection of Research Bioresources

Cell Bank, Osaka, Japan. Because the proliferating tumour hepatic cell lines lack appropriate hepatic phenotype of differentiated hepatocytes and do not express enough functional endogenous PXR under normal conditions, we also used differentiated HepaRG cells and primary human hepatocyte models.

Cryopreserved HepaRG™ (GIBCO®) cells and media were purchased from Life Technologies (Carlsbad, CA, USA). The HepaRG™ cell line is an immortalized and terminally differentiated hepatic cell line that retains many liver-specific characteristics of primary human hepatocytes. The HepaRG cells were initially isolated from a liver tumour of a female patient suffering from hepatocarcinoma (Gripon *et al.*, 2002). A substantial expression of OCT1 mRNAs reaching 50% of those found in primary human hepatocytes was detected in confluent and DMSO-treated HepaRG cells cultured in conditions promoting their differentiation (Le Vee *et al.*, 2006). In contrast, HepG2 and Huh7 cells express low OCT1 mRNA levels (Hilgendorf *et al.*, 2007); unpublished data of present authors).

The primary human hepatocytes were prepared from lobectomy segments resected from adult patients for medical reasons unrelated to our research programme (for details see Supporting Information). The human tissue acquisition was undertaken according to procedures complying with the current Czech and French legislation. In addition, five commercial cultures of long-term human hepatocytes, as monolayers, were used (Biopredic International, Rennes, France or Primcyt, Scwerin, Germany)(Supporting Information).

Cell lines, HepaRG and primary human hepatocyte cells cultivation protocols are in Supporting Information.

Plasmids and siRNA

To produce a 1.8 kb OCT1 reporter construct with two DR-2 HNF4*a* binding sites (Saborowski *et al.*, 2006), the promoter sequence from −1812 to +102 was synthesized and inserted into the pGL4.10 vector (Promega) using *KpnI* and *XhoI* restriction enzymes (pOCT1 1.8 kb-luc). Sequences −1649 to +102, −1458 to +102, −430 to +102 and −99 to +102 were PCR-amplified and inserted into the pGL4.10 vector (Promega) using *KpnI* and *XhoI* restriction enzymes (Rulcova *et al.*, 2013). Construct pOCT1(−1649/+102)-luc contains both DR-2 elements binding HNF4α, whereas construct pOCT1(−1458/+102)-luc lacks the first HNF4α response element critical for OCT1 promoter transactivation with HNF4α. Construct OCT1(−430/+102)-luc lacks both DR-2 elements. Generation of mutant plasmids and description of additional constructs is mentioned in Supporting Information.

Transient transfection assays

The transient transfection assays were carried out using TransFectin transfection reagent (BioRad, Hercules, CA, USA) in the case of the HepG2 cells and using JetPEI (Polyplus-transfection SA, Illkirch, France) to transfect the HuH-7 cell line according to the manufacturer's protocol as described elsewhere (Smutny *et al.*, 2014)(Supporting Information).

qRT-PCR

In these series of experiments, the HepG2 cells were seeded into 12-well plates and 24 h later were transfected with PXR expression vector (300 ng per well) or with empty control plasmid as described above. The HepaRG cells were seeded into 12-well plates without exogenous PXR transfection. Both cell lines were then maintained in cultivating medium supplemented with the tested compounds at the indicated concentrations for 24 hours. Total RNA isolation and qRT-PCR is described in Supporting Information.

Uptake studies in HepaRG cells

Uptake assays were carried out in 12-well plates as described previously (Mandikova *et al.*, 2013). The transport assays were performed in HepaRG cells differentiated for 14 days under confluent conditions and pre-treated with 10 μM rifampicin for 24 or 48 h. Before the accumulation experiment, the HepaRG cultivation medium was removed, and the cells were washed with transport solution (NaCl 130 mM, KCl 4 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, glucose 5 mM and HEPES 10 mM, pH 7.4) and pre-incubated for 10 min at 37°C. The standard radiolabelled substrate for the OCT1 transporter, [^{3}H]MPP$^+$, was used at 1 μM. After a 2 min incubation interval at 37°C, the incubation was stopped by washing the cells twice with ice-cold solution containing 137 mM NaCl and 10 mM HEPES, pH 7.4. The cells were lysed with 0.1 mL of 0.5% Triton X-100 in 100 mM NaOH for 30 min. Radioactivity of the samples in scintillation solution (Sigma-Aldrich) was measured with a β counter (Tri-Carb 2900TR; Perkin Elmer, Shelton, CT, USA).

ChIP assay

Steroid receptor coactivator (SRC)-1 recruitment to the HNF4α responsive elements (DR-2) and E-box in the OCT1 gene promoter was determined using a chromatin immunoprecipitation (ChIP) assay in the HepG2 cells, which were transfected with pSG5-hPXR and pSG5-FLAG hSRC-1 (3.2 μg in 25 cm^2 flask) expression plasmids after one day of cultivation. Cells were then treated using 10 μM rifampicin or 1‰ DMSO as control sample for 24 h. After the interval, the cells were harvested, and immunoprecipitation was conducted with the Imprint® Chromatin Immunoprecipitation Kit (Sigma-Aldrich) according to the manufacturer's protocol. Anti-FLAG M2 antibody (Sigma-Aldrich), normal mouse IgG antibody and anti-RNA polymerase II (Pol II) antibody were used for precipitation. Levels of SRC-1 recruitment to the HNF4α response elements and into the E-box were analysed by qRT-PCR with primers and probes specifically designed to amplify indicated regions (Figure 6, upper panel, arrows). Data are presented as a relative binding to control vehicle (DMSO)-treated cell samples (100%) immunoprecipitated with the same antibody. Non-specific immunoprecipitation with mouse IgG antibody was 1% lower than with anti-FLAG or the anti-Pol II antibodies. Primers for ChiP experiments are listed in Supporting Information Table S2.

Immunoblotting analysis of OCT1 protein

A Western blotting analysis of OCT1 protein expression in differentiated HepaRG cells, in primary hepatocytes LH42 (48 h treatment) or in human hepatocytes in monolayer-long term cultures (Biopredic, Rennes, France, Batch No. HEP220879, female, Caucasian, 65 years old, liver metastases, 96 h treatment) was performed as described previously (Rulcova *et al.*, 2013). To determine the protein level of OCT1 transporter, the primary hepatocyte cultures or HepaRG cells (differentiated as described before for 3 weeks) were treated with 10 μM rifampicin, 10 μM hyperforin or 1‰ DMSO (control) for 48 or

96 h. Total cellular fractions were prepared, denatured for 5 min at 95°C in 2× Laemmli sample buffer and separated (50 μg of protein per well) on 7.5% SDS/polyacrylamide gels. Membranes were then incubated for 24 h at 4°C with anti-OCT1 antibody (ab55916; Abcam, Cambridge, UK) (1:1000 dilution), anti-HNF-4-α antibody (ab139420; Abcam), anti-PXR antibody (ab85451; Abcam) or anti-GADPH polyclonal antibody (PA1-987; Thermo Fisher, Waltham, MA, USA; 1:5000 dilution). After washing with Tris-buffered saline with Tween 20 (0,1%v/v), pH 7.4, membranes were incubated with the corresponding goat anti-rabbit HRP-conjugated ab136636 (Abcam) or sc-2004 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibodies (1:5000 dilution) for 60 min at room temperature. Chemiluminescence was detected using the Image Quant 400 CCD camera (GE Healthcare, Little Chalfont, UK). Digital image densitometry analysis has been performed with LabImage 2.7 software (Kapelan, Leipzig, Germany).

Study design and statistical analysis

The study design conforms to the recent guidance on experimental design and analysis (Curtis *et al.*, 2015). In all experiments, data subjected to statistical analysis are from at least 5 independent values or samples). Groups were of equal sizes. In Figure 3A and D, data have been obtained from three independent experiments ($n = 3$) and have not been subjected to statistical analysis. Justification is given in the Results section. The order of treatment in cellular assays were randomized (i.e. vehicle-treated controls were not systematically treated first). Data analysis was blinded to the analyst who helped with statistical analysis.

The absolute effect of PXR activation on OCT1 expression is useless and we therefore, normalized these data. In all figures, control and test values were normalized to the mean value of the experimental control group in order to set the Y-axis, so the control group value is 1 or 100%. Data for parametric statistical analysis were not normalized.

All results are presented as the mean ± SD. Differences between the groups were compared using a Student's unpaired two-tailed *t*-test. One-way analysis of variance with a Dunnett's *post hoc* test was applied to the data if more than two groups were analysed, only if *F* achieved the level of significance $P < 0.05$ and no significant variance inhomogeneity was observed. A nonparametric Mann–Whitney *U*-test (also called Wilcoxon rank-sum test) was used to compare OCT1 mRNA expression in two sets of samples of primary human hepatocytes or HepaRG cells. All of the statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). A *P*-value of <0.05 was considered to be statistically significant.

Materials

Rifampicin and hyperforin, non-essential amino acids (NEAA), DMSO and DMEM medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Drug stock solutions (1000× or 5000×) were prepared in DMSO. Radio-labelled methyl-4-phenylpyridinium acetate ([^{3}H]MPP$^+$), the substrate for OCT1, was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). Ultima Gold™ LSC cocktail was purchased from Perkin Elmer (Waltham, MT, USA) and MPP$^+$ from Sigma-Aldrich.

Results

Ligand-mediated activation of PXR resulted in the down-regulation of OCT1 mRNA in hepatocyte cellular models

Firstly, we determined the effects of activation of the PXR nuclear receptor on the OCT1 transporter at mRNA and protein level in primary human hepatocytes and in differentiated HepaRG cells. In a group of 15 primary human hepatocyte preparations from different donors, rifampicin (10 μM), a model agonist of PXR, significantly ($P < 0.0054$, Mann–Whitney *U*-test) suppressed OCT1 mRNA expression after 24 h treatment. In these experiments, OCT1 mRNA levels in rifampicin-treated samples were consistently lower than in vehicle-treated samples in all cases examined, with the exception of two preparations (Figure 1A).

In HepaRG cells differentiated for 14 days in medium without 1.5% DMSO, rifampicin down-regulated OCT1 mRNA by 50% after 24 h treatment (Figure 1B). Moreover, in HepG2 cells transiently transfected with exogenous PXR, a consistent and concentration-dependent down-regulation of OCT1 mRNA level was observed after 24 h treatment with rifampicin or hyperforin, activators of PXR (Figure 1C). The suppressive effect of these PXR ligands was apparent only with exogenous PXR cDNA transfected into cells, a finding, which is in accordance with the low functional expression of PXR in hepatic HepG2 cells (Smutny *et al.*, 2014) and clearly indicates involvement of PXR in OCT1 gene down-regulation.

Using Western blotting, we confirmed down-regulation of OCT1 protein in differentiated HepaRG cells and in primary human hepatocytes by the PXR ligands, rifampicin and hyperforin (Figure 1D), after 48 and 96 h of treatment.

We also performed accumulation experiments to confirm the influence of PXR activation on OCT1 transporter activity in HepaRG cells pre-treated with rifampicin. A decreased accumulation of the model substrate [^{3}H]MPP$^+$ by the OCT1 transporter was decreased in cells pre-treated with 10 μM rifampicin for 24 or 48 h when compared with control DMSO-treated cells (Figure 1E). These data confirm that the down-regulation of OCT1 gene expression correlates with the reduced delivery of OCT1 substrates into hepatic cells.

Transactivation of OCT1 gene is suppressed by activated PXR in hepatocyte-derived cell lines

First, HepG2 and HuH7 cells were transfected with a pOCT1 1.8 kb-luc reporter construct harbouring 1.8 kb of the OCT1 promoter sequence upstream of the transcription start together with PXR and/or HNF4α expression plasmids. We observed a significant suppression of OCT1 gene reporter construct activity after co-transfection with the PXR expression construct. These observations are consistent with a degree of ligand-independent activity of PXR in HepG2 cells, a finding which is likely to be the consequence of an endogenous ligand. Increasing amounts of exogenous PXR progressively repressed OCT1 transactivation both alone and in the presence of rifampicin (data not shown). Treatment with

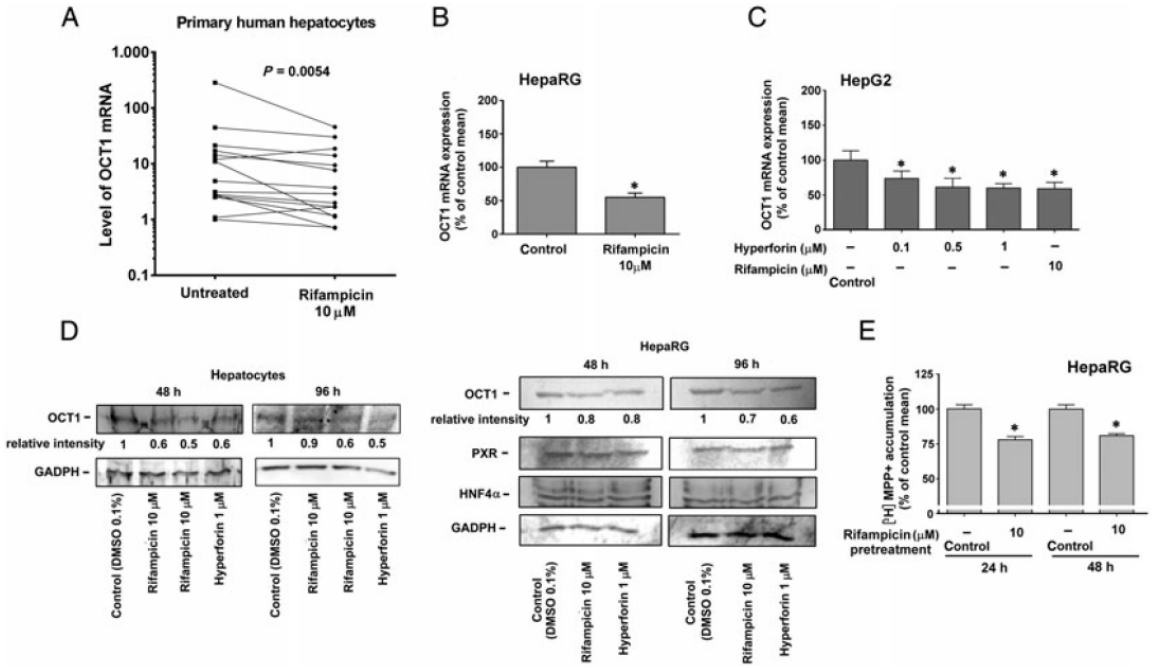

Figure 1

Rifampicin down-regulates OCT1 mRNA. (A) Rifampicin down-regulates OCT1 mRNA in a set of primary human hepatocytes. Different primary human hepatocyte preparations were isolated and cultivated according to standard protocol and then either treated with rifampicin (10 μM) for 24 h or treated with vehicle (0.1% DMSO, control). The expression of OCT1 mRNA normalized to the housekeeping gene is shown, and data have been analysed using the Mann–Whitney *U*-test. Rifampicin and hyperforin suppress the expression of OCT1 mRNA in differentiated HepaRG (B) and HepG2 (C) cells. HepG2, but not HepaRG, cells were transfected with pSG5-hPXR and treated with the indicated concentrations of PXR ligands, rifampicin and hyperforin, or with vehicle (DMSO 0.1%, control), for 24 h. After this, total RNA was isolated, and OCT1 mRNA levels were analysed employing qRT-PCR. All control and test values were normalized to the mean value of the experimental control group in order to set the Y-axis, so the control group value is 100%. The effects of compounds on the normalized OCT1 mRNA levels are presented as % of the control group's mean value ($n = 5$).*$P < 0.05$, significant effect of rifampicin or hyperforin on OCT1 mRNA expression, compared with vehicle-treated controls; ANOVA with Dunnett's *post hoc* test. (D) PXR ligands down-regulate OCT1 protein expression in differentiated primary human hepatocytes and HepaRG cells. Western blots were performed with anti-OCT1 polyclonal antibody, anti-PXR and anti-HNF4α antibodies after 48 or 96 h of treatment with rifampicin (10 μM) and hyperforin (10 μM). Total cellular lysates were used for immunoblotting experiments. Densitometry analyses were performed to evaluate relative intensity of OCT1 bands. (E) Relative accumulation of OCT1 substrate [^{3}H]MPP$^+$ in HepaRG cells. Accumulation of [^{3}H]MPP$^+$ was reduced after 24 and 48 h of treatment with 10 μM rifampicin in differentiated HepaRG cells. *$P < 0.05$. Accumulation of [^{3}H]MPP$^+$ in control cells was set as 100%, which represent about 1.1% of [^{3}H]MPP$^+$ radioactivity in the medium. All control and test values were normalized to the mean value of the experimental control group. The effects of rifampicin on the normalized [^{3}H]MPP$^+$ radioactivity levels are presented as % of the control group's mean value ($n = 5$). *$P < 0.05$, significant effect of rifampicin on [^{3}H]MPP$^+$ accumulation in HepaRG cells, compared with vehicle-treated controls; Student's unpaired two-tailed *t*-test.

rifampicin or hyperforin further potentiated PXR-mediated transcriptional repression (Figure 2A, B and C). In experiments with cells transfected with only HNF4α (without PXR), basal activity of OCT1 gene reporter was increased as expected, and rifampicin had no suppressive effect on OCT1 promoter construct activity (Figure 2A).

Finally, gene reporter assays were performed with pOCT1 1.8 kb-luc reporter vector together with a series of modified PXR expression constructs. The constitutively active PXR T248D construct, but not the inactive PXR T422D construct with a mutation in the AF2 domain, suppressed OCT1 transactivation (Figure 2C). Importantly, the pGAL4-PXR LBD construct, lacking the DNA binding domain, after rifampicin treatment and its constitutively active variant pGAL4-PXR LBD (S247W/C248W) with a mutated LBD also diminished OCT1 promoter construct activation (Figure 2C). The effects of the T248D mutant was only similar to but not stronger than, unliganded wild-type PXR, which may reflect a promoter-specific effect of the mutant, in comparison with CYP3A4 regulation (Doricakova *et al.*, 2013).

These results indicate that (i) PXR-mediated suppression of OCT1 gene transactivation is mediated by PXR-HNF4α crosstalk; (ii) the activation domain AF2 of the PXR LBD is critical for the phenomenon; (iii) the phenomenon is independent of the PXR DNA-binding domain; and (iv) the phenomenon is likely to be hepatocyte-cell dependent and HNF4α dependent.

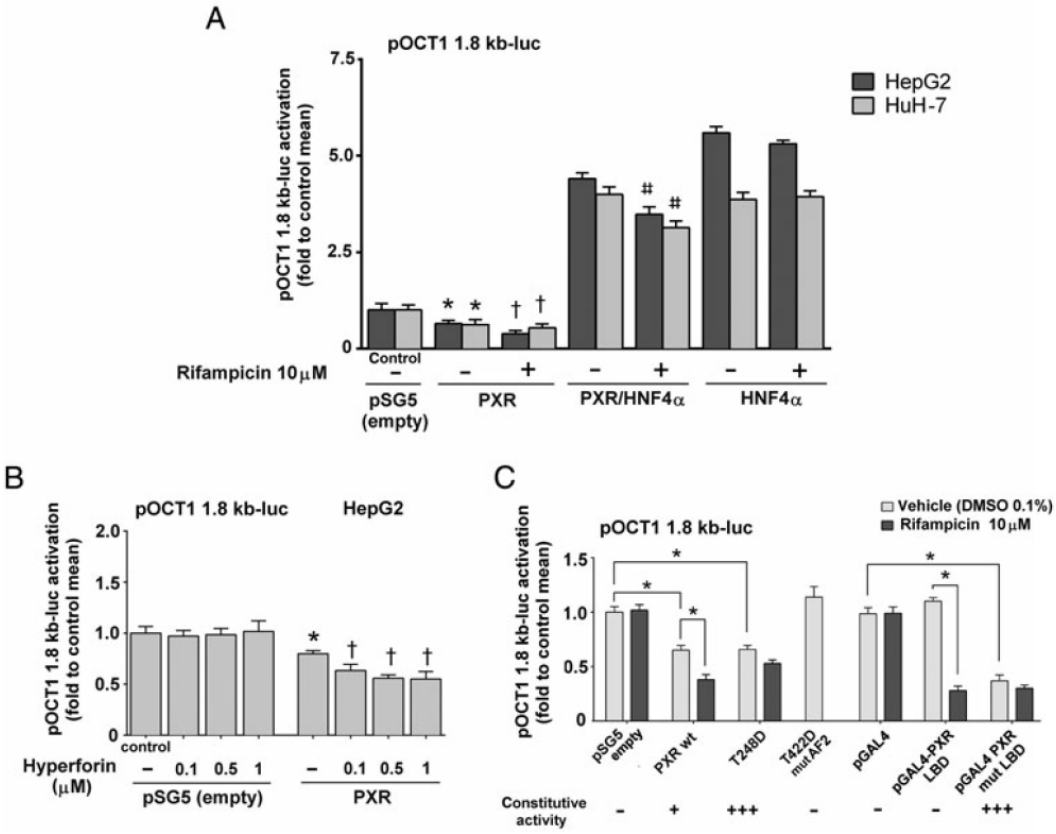

Figure 2

PXR mediates the inhibition of OCT1 promoter transactivation in gene reporter assays. Hepatic HepG2 and HuH-7 (A) were transfected with the pOCT1 1.8 kb-luc reporter construct and cotransfected with PXR, HNF4α or their combination as indicated or only with PXR expression construct. (B) Cells were then treated with rifampicin (10 μM), hyperforin (0.1, 0.5, 1 μM) or with vehicle (−) as a control for 24 h. Activity of OCT1 gene reporter construct was detected with the use of Dual-Luciferase Reporter Assay (Promega). All control and test values were normalized to the mean value of the experimental control group in order to set the Y-axis so the control group value is 1. The effects of tested combinations on the normalized pOCT1 1.8-luc activation levels are presented as fold of the control group's mean value ($n = 5$).*$P < 0.05$ indicates a statistically significant effect of exogenous PXR expression on basal OCT1 transactivation compared with the mock (empty expression vector) transfected cells. †$P < 0.05$, significant effect of a PXR ligand (rifampicin or hyperforin) on OCT1 transactivation in PXR expressing cells; ANOVA with Dunnett's *post hoc* test. #$P < 0.05$, significant effect of rifampicin on OCT1 transactivation in PXR and HNF4 expressing cells. (C.) HepG2 cells were transfected with expression constructs encoding either wild-type human PXR, constitutively active PXR T248D mutant, inactive PXR T422D mutant with mutated AF2 domain, pGAL4-PXR LBD construct, constitutively active pGAL4-PXR LBD S247W/C284W mutant or empty pSG5 or GAL4 vectors (100 ng per well), respectively, and treated with rifampicin (10 μM) or vehicle (DMSO 1 ‰, v/v) for 24 h. Activity of OCT1 gene reporter construct (150 ng per well) was detected with the use of Dual-Luciferase Reporter Assay. All control (empty expression constructs with vehicle) and test values were normalized to the mean value of the experimental control group, and the data are presented as fold of the control group's mean value ($n = 5$).*$P < 0.05$, significant effect; ANOVA with Dunnett's *post hoc* test.

Silencing of PXR and HNF4α in HepaRG cells blocks the PXR-induced suppression of OCT1 gene expression

HepaRG is the only cell line that, under differentiation conditions, has hepatic phenotype and expresses key nuclear receptors and drug-metabolizing enzymes. In our initial experiments, the mRNA levels of OCT1, HNF4α, CYP3A4 and PXR (NR1I2) genes were analysed in HepaRG cells, cultivated under the standard differentiation protocol with 1.5% DMSO. We observed that the level of OCT1 and HNF4α was decreased after DMSO addition (data not shown). Therefore, it was decided to perform a kinetic experiment in the confluent cells, 2 weeks after their seeding. For this purpose, HepaRGs were differentiated, without addition of DMSO to the regular culture medium, for another two weeks (Figure 3A). mRNA levels of OCT1, HNF4α, CYP3A4 and PXR (NR1I2) genes were assessed twice a week (on days 4, 8, 11, 15, 18, 21, 24 and 29). All four tested genes were stably expressed

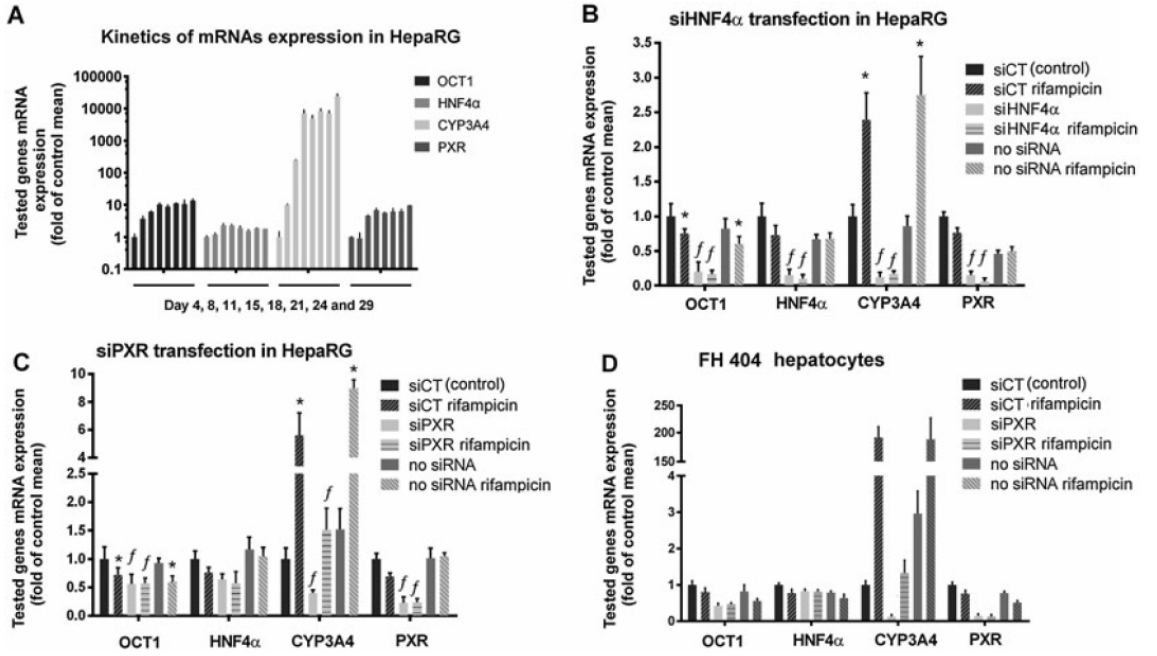

Figure 3

Silencing of PXR and HNF4α prevents PXR-mediated down-regulation of OCT1 mRNA. (A) Optimization of HepaRG cultivation and kinetics of OCT1, HNF4α, PXR and CYP3A4 mRNAs expression in HepaRG cells during 4 weeks of differentiation in DMSO-free medium. Levels of tested genes were assessed twice a week (on days 4, 8, 11, 15, 18, 21, 24 and 29, respectively); total mRNA was extracted, and tested genes mRNA were quantified using qRT-PCR. Data are presented as the mean ± SD from three experiments performed in duplicate and are expressed as fold of the control group's mean value (day 4, the control group value is 1. (B–C) HepaRG cells or representative primary human hepatocyte culture FH 404 (D) were transfected with siHNF4α (20 nM in medium) (B) or siPXR (10 nM) (C, D). Total RNA was then isolated and OCT1, HNF4α, CYP3A4 and PXR mRNA expression was analysed using qRT-PCR. All control and test values were normalized to the mean value of the experimental control group in order to set the Y-axis so the control group value is 1. The effects of tested combinations on the normalized tested genes mRNA expression are presented as fold of the control group's mean value ($n = 5$).*$P < 0.05$, significant effect of rifampicin (10 μM) on OCT1, CYP3A4 or PXR mRNA expression in samples transfected with the same siRNAs; †$P < 0.05$, significant effect of siPXR or siHNF4α on tested genes expression compared with siCT (scramble, control) transfected sample counterparts; ANOVA with Dunnett's *post hoc* test.

after 15 days of the DMSO-free differentiation period ($n = 3$, Figure 3A). Therefore, based on these preliminary experiments, we chose a 2 week (15 days) differentiation protocol without DMSO as providing the most suitable and reliable experimental conditions.

At this time, the silencing of HNF4α in HepaRG cells was tested. Transfection of siRNA targeting HNF4α was performed twice in 2 days to improve silencing efficiency. HNF4α mRNA levels and the relevant target genes OCT1, CYP3A4 and PXR were decreased (Figure 3B). Importantly, the silencing of HNF4α abolished the suppressive PXR-mediated effect on OCT1 gene expression.

In the next series of experiments, HepaRG cells were transfected three times with siRNA against PXR to obtain a greater efficiency of PXR silencing. Levels of both PXR and its main target gene CYP3A4 were significantly decreased. We observed slight suppressive effects of siPXR on OCT1 and HNF4α (Figure 3C). Also of note, siPXR again blocked the rifampicin-induced PXR-mediated suppression of OCT1 expression (Figure 3C).

Finally, the effect of PXR silencing in primary human hepatocytes (Figure 3D) was also determined. We analysed levels of OCT1, HNF4α, PXR and CYP3A4 mRNAs in three different batches of human hepatocytes ($n = 3$) after transfection with siRNA targeting PXR and treatment with 10 μM rifampicin. In concordance with our previous findings in HepaRG cells, there were both a decrease in OCT1 mRNA levels and the prevention of PXR-mediated repression of OCT1 expression, after the silencing of PXR in primary human hepatocytes (Figure 3D, a representative data from FH 404 preparation are shown). We observed similar profiles in all the primary human hepatocyte preparations we used, even though there was typically high inter-individual variation.

Interestingly, the silencing of PXR in both HepaRG and in primary human hepatocytes did not lead to up-regulation of OCT1 mRNA but to the opposite phenomenon, which was consistent with the results obtained using the inactive PXR T422D mutant (Figure 2C).

These results demonstrate that human PXR is responsible for rifampicin-mediated OCT1 repression in hepatic cells and that

HNF4α, a key regulator of OCT1 expression, is necessary for the underlying molecular mechanism. PXR is unlikely to transrepress OCT1 gene expression, because the silencing or inactivation of PXR does not up-regulate OCT1 mRNA expression.

Cotransfection of SRC-1, but not PGC1α coactivator, blocks PXR-mediated down-regulation of OCT1 gene transactivation

In subsequent experiments, we tested to see whether the forced expression of SRC-1 or the PPARγ coactivator 1α, (PGC1α) in HepG2 cells could reverse the rifampicin-induced repression of OCT1 gene expression. In transient transfection gene reporter assays with the 1.8 kb OCT1-luc construct, incremental amounts of SRC-1 elicited a progressive reduction of the PXR-mediated repression of OCT1 transactivation (Figure 4A). When the SRC-1 expression construct (400 ng per well) was co-transfected, we even observed the reversal of the phenomenon and induction of OCT1 transactivation. In other experiments, increasing amounts of SRC-1 significantly stimulated PXR-responsive p3A4-luc luciferase reporter construct with critical CYP3A4 gene promoter regulatory elements (Figure in Supporting Information). In contrast, increasing amounts of exogenous PGC1α did not reverse the suppression of OCT1 by activated PXR (Figure 4B).

These data further support the idea that the common coactivator SRC-1 is depleted from HNF4α in the OCT1 promoter, after rifampicin-treatment, by activated PXR and that this depletion results in the repression of the OCT1 gene. However, if SRC-1 is overexpressed in cells, depletion from HNF4α does not occur, nor is transactivation OCT1 expression by HNF4α substantially attenuated.

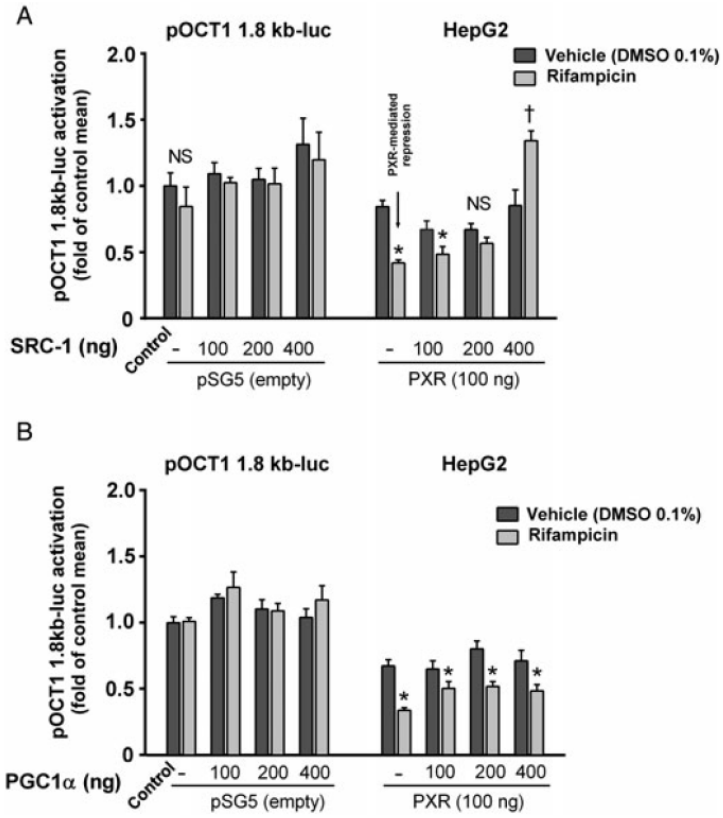

Figure 4

Cotransfection of SRC-1, but not PGC1α coactivator, blocks PXR-mediated down-regulation of OCT1 gene transactivation. HepG2 cells were co-transfected with the 1.8 kb OCT1 reporter construct, pSG5-hPXR expression construct and with increasing amount (100, 200 or 400 ng per well) of SCR-1 (A) or PGC1α (B) expression vectors. Cells were then treated with rifampicin (10 μM) or with vehicle (−) for 24 h. Activity of OCT1 gene reporter construct was detected with the use of Dual-Luciferase Reporter Assay (Promega). All vehicle-treated empty construct-transfected control, and test values were normalized to the mean value of the experimental control group in order to set the Y-axis so the control group value is 1. The effects of tested combinations on the normalized pOCT1 1.8 kb-luc activation are presented as fold of the control group's mean value ($n = 5$).*$P < 0.05$, significant suppression of OCT1 construct transactivation by rifampicin in comparison with samples transfected with the same expression constructs and treated with vehicle; †$P < 0.05$, significant augmentation of OCT1 promoter construct transactivation by rifampicin in comparison with samples transfected with the same expression constructs and treated with vehicle; ANOVA with Dunnett's *post hoc* test was used. NS – non-significant effect of rifampicin.

HNF4α and E-box response elements are involved in PXR-mediated suppression of OCT1 expression

In order to further confirm and evaluate the involvement of the HNF4α and USF factors in OCT1 down-regulation, we generated a series of OCT1 gene promoter luciferase reporter constructs (Figure 5).

The USF1 and USF2 basal transcription factors, members of the eukaryotic basic helix-loop-helix leucine zipper transcription factor family and their E-box response element act in cooperation with HNF4α, in hepatocyte-specific OCT1 expression (Kajiwara *et al.*, 2008). Importantly, USF1 has been found to be co-activated by SRC-1 (Huang *et al.*, 2007). Therefore, we generated a series of the E-box and HNF4α response elements mutants of the OCT1 promoter.

We observed statistically significant PXR-mediated suppressive effects on OCT1 reporter constructs with −1812/+102 promoter sequence (lane 1), truncated −1692/+102 sequence (lane 5) and in −1458/+102 construct, which lacks the first HNF4α response element (lane 6). However, mutation of either HNF4α response elements or USFs response element (E-box) significantly decreased the PXR-mediated suppression of the OCT1 promoter activation in these experiments.

These results indicate that both HNF4α response elements and the USFs response element (E-box) are together critical for the PXR-mediated transrepression of OCT1 promoter constructs in the gene reporter assays.

Chromatin immunoprecipitation studies to monitor SRC-1 recruitment to HNF4α and E-box elements after treatment with rifampicin

To demonstrate that competition for binding to the SRC-1 co-activator may be the underlying mechanism of the PXR-mediated suppression of OCT1 expression, chromatin immunoprecipitation experiments with probes designed to amplify both HNF4α response elements and E-box elements using qPCR were performed (Figure 6, arrows in upper panel). Rifampicin treatment resulted in the dissociation of SRC-1 from HNF4α response elements and the E-box, as indicated by a decreased amount of DNA fragments immunoprecipitated with the anti-FLAG SRC-1 antibody (Figure 6).

These results are again consistent with our hypothesis that ligand-activated PXR interacts with HNF4α (and USFs) signalling in OCT1 regulation by targeting the common coactivator SRC-1. In other words, these data suggest that after

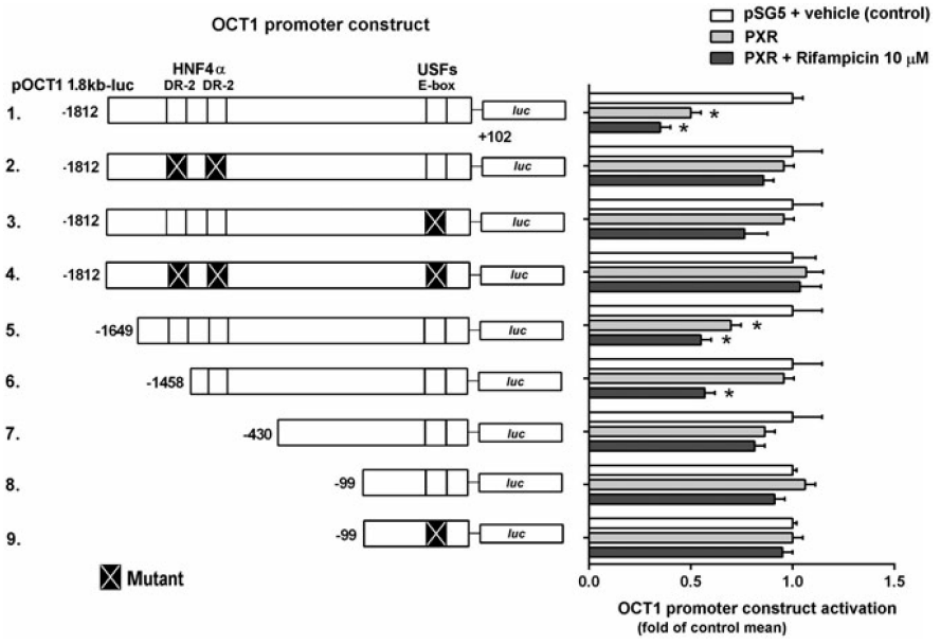

Figure 5

Involvement of HNF4α response elements (DR-2) and E-box in transcriptional regulation of the OCT1 gene through PXR nuclear receptors in the HepG2 cell line. Transactivation of the wild-type pOCT1 1.8 kb-luc luciferase reporter gene construct, its truncated forms and HNF4α RE or E-box mutants (150 ng/well) were examined in HepG2 cells transiently co-transfected with PXR or empty expression pSG5 vectors (100 ng per well). pRL-TK control plasmid was used for transfection normalization (30 ng per well). Transfected cells were maintained in a medium containing rifampicin (10 μM) for 24 h. After incubation, the cells were lysed and analysed for both firefly and *Renilla* luciferase activities. Firefly luciferase activity was normalized to *Renilla* activity. All control and test values were normalized to the mean value of the experimental control group in order to set the Y-axis so the control group value is 1. The effects of PXR on the normalized OCT1 constructs activation are presented as fold of the control group's mean value ($n = 5$).*$P < 0.05$, significantly different from the vehicle-treated cells transfected with the same luciferase reporter construct and an empty expression vector; ANOVA with Dunnett's *post hoc* test.

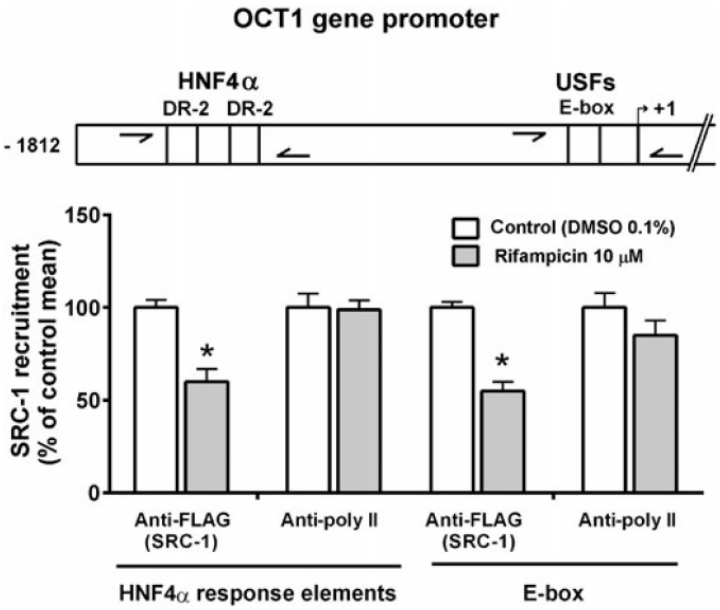

Figure 6

Recruitment of SRC-1 into the OCT1 gene promoter regions in response to rifampicin treatment in HepG2 cells. Chromatin immunoprecipitation (ChIP) assay was performed to analyse the association of SRC-1 within the HNF4α response elements (DR-2 motifs) and E-box of the OCT1 gene. HepG2 were transfected with pSG5-FLAG hSRC-1 and pSG5-hPXR constructs and then treated with rifampicin (10 μM) or vehicle (DMSO, 0.1%) for 24 h. After this, immunoprecipitated DNA fragments extracted from sonicated HepG2 cell lysates with anti-FLAG antibody (M2 antibody, Sigma-Aldrich) or anti RNA polymerase II (poly II) antibody were analysed using qPCR with specific TaqMan probes that detect the individual regions of the OCT1 gene promoter (arrows shown in upper panel). All vehicle-treated control and test values were normalized to the mean value of the experimental control group in order to set the Y-axis so the control group value is 100%. The effects of PXR activation on SRC-1 recruitment are presented as % of the control group's mean value ($n = 5$).*$P < 0.05$, significantly different from vehicle-treated cells; Student's unpaired two-tailed *t*-test.

PXR activation, PXR, HNF4α and USF all compete with each other to bind to the common co-activator SRC-1.

Discussion

Drug delivery into hepatocytes is critical for hepatic metabolism and the biliary elimination of most drugs. Drug transporters determine entry into hepatocytes via the sinusoidal (basolateral) membrane. These membrane transporters are also critical for responses to drugs, in those cases in which hepatocytes are the pharmacological target site. The OCT1 transporter ranks among the most important liver uptake carriers, which transport positively charged substances. At physiological pH, approximately 40% of drugs are organic cations or weak bases, and thus, many of them are potential substrates for OCT1 (Nies *et al.*, 2009). As variable OCT1 expression and gene regulation contributes to intra-individual and inter-individual variation in the hepatic disposition of some cationic drugs, further inquiries into OCT1 gene regulation are necessary to predict cationic drug delivery and response in hepatocytes, in the context of clinical consequences of the interaction.

In the current study, the effect of PXR activation on OCT1 expression was examined in several validated hepatocyte cellular models including primary human hepatocytes and differentiated HepaRG cells. In contrast to tumour hepatic cell lines, the differentiated HepaRG cells express functional endogenous PXR and OCT1. All our data indicate that OCT1 hepatic expression is down-regulated by PXR agonists at the transcriptional level and that the accumulation of the model OCT1 substrate MPP+ is reduced in HepaRG cells pretreated with rifampicin. Moreover, we propose the mechanism of the down-regulation and postulate that PXR reduces OCT1 transactivation by competing for the SRC-1 coactivator, with the HNF4α and USF factors which control the high constitutive hepatic expression of the OCT1 gene. Such competition for coactivators is called squelching (Cahill *et al.*, 1994; Min *et al.*, 2002) and we shall use this term from now on. For our experiments, a squelching mechanism is based on the results of experiments involving the overexpression of SRC-1 coactivator or various PXR mutants, silencing of PXR and HNF4α in HepaRG cells and primary human hepatocytes, mutagenesis of the HNF4α responsive DR-2 and E-box response elements and chromatin immunoprecipitation assays.

Recently, Cho and co-workers hypothesized that rifampicin induced the OCT1 transporter in hepatocytes and thus increased uptake of metformin (Cho *et al.*, 2011). In contrast to this speculation, two published studies showed that

rifampicin, via PXR activation, actually down-regulated OCT1 mRNA expression in primary human hepatocytes (Jigorel *et al.*, 2006; Badolo *et al.*, 2013). Consistent with the latter reports, we observed OCT1 mRNA down-regulation in most of our tested samples of 15 primary human hepatocytes derived from different donors (Figure 1A) and in differentiated HepaRG cells (Figure 1B). In addition, employing a gene reporter assay, the PXR-mediated inhibition of OCT1 gene reporter construct was observed in hepatocyte-derived HuH-7 and HepG2 cell lines (Figure 2).

The hepatocyte-specific expression of OCT1 is tightly controlled by the liver-enriched HNF4α (NR2A1) (Saborowski *et al.*, 2006; Kamiyama *et al.*, 2007; Rulcova *et al.*, 2013). HNF4α is an orphan member of the nuclear receptor superfamily, which has a critical role as a transcription factor in the regulation of a wide variety of liver-specific genes, involved in lipid and glucose metabolism, hepatocyte differentiation and the maintenance of the hepatic gene expression profile (Watt *et al.*, 2003). Two cooperating DR-2 HNF4α response elements have been identified between nucleotides −1479 and −1441 of the 5′-flanking region of the OCT1 gene upstream of the transcription initiation site, which contains directly repeated hexamers separated by two bases (Saborowski *et al.*, 2006). This regulation explains well the dominant hepatic expression of OCT1 mRNA and minimal expression in other non-hepatic tissues (Nies *et al.*, 2009). In addition, a cognate E-box interacting with USF1 and USF2 has been identified in the core proximal promoter region that co-ordinately augments the HNF4α-mediated transactivation of the OCT1 gene in an additive manner (Kajiwara *et al.*, 2008).

In contrast to the situation with hepatocytes, contradictory data has been observed in non-hepatic, blood cells. Austin *et al.* have recently published results in which OCT1 mRNA is up-regulated in presence of the PXR ligand in both primary CML cells and in the CML cell line KCL22 (Austin *et al.*, 2015). This discrepancy can be explained by the absence of HNF4α in peripheral blood cells. We observed a similar phenomenon in non-hepatic Hela cells, where rifampicin activated the OCT1 promoter by PXR activation in HNF4α-deficient HeLa cells. However, concomitant PXR and HNF4α co-expression in the HeLa cells reversed the effect of rifampicin (our unpublished data). Silencing of HNF4α in hepatocyte cells also did not lead to OCT1 mRNA up-regulation after rifampicin treatment (Figure 3B and D). Thus, the phenomenon of tissue-specific OCT1 gene regulation requires further investigation.

In the mechanistic analysis of the PXR-mediated repression of OCT1 transactivation in hepatic cells, OCT1 promoter transactivation was repressed by the ligand-activated wild-type PXR, GAL4-PXR LBD fusion chimera and constitutively active PXR LBD mutant T248D with activation domain (AF2), but not by the PXR T422D AF2 mutant that fails to bind to coactivators (Figure 2C). These data indicate that PXR coactivation is critical in the phenomenon. In the next experiments, we found that the silencing of PXR and HNF4α abolished the suppressive effect of rifampicin on OCT1 mRNA expression in HepaRG cells (Figure 3B and C) and in primary human hepatocytes (Figure 3D). These results showed that both nuclear receptors were necessary for the phenomenon of OCT1 down-regulation, a finding which suggests that competition for a common coactivator may underlie the PXR-mediated inhibition of OCT1 expression. Both PXR and HNF4α are coactivated by SRC-1 (NCOA1) and PGC1α coactivators (Wang *et al.*, 1998). In the following series of experiments, increasing amounts of SRC-1 were co-transfected in HepG2 cells together with the OCT1 reporter construct and PXR in the presence of rifampicin. Consistent with a squelching mechanism, the gradual overexpression of SRC-1 in HepG2 cells reversed the repressive effect of rifampicin on OCT1 transactivation (Figure 4A). In contrast, increasing the amount of PGC1α in the HepG2 cell line did not de-repress PXR-mediated OCT1 promoter inhibition. These data suggested that activation of PXR reduced OCT1 expression by squelching SRC-1, but not PGC1α, the coactivator in HNF4α-controlled OCT1 transactivation.

Because PXR binds to DNA motifs, including DR-3, DR4, everted ER6 and the ER8 motifs (Kliewer *et al.*, 2002), it is unlikely that PXR would inhibit HNF4α-binding to the OCT1 promoter DR-2 motifs. In addition, OCT1 gene reporter construct transactivation is attenuated by cotransfection with the GAL4-PXR LBD construct without the PXR DNA-binding domain (Figure 2 C). This evidence showed that only PXR-LBD with a functional AF2 domain binding to coactivators is necessary for the phenomenon.

In a ChIP assay, we employed two sets of DNA probes to quantify SRC-1 binding to DR-2 HNF4α response elements and to the E-box of the OCT1 gene promoter. Consistent with the other results, the decreased binding of SRC-1 to these response elements was observed after treatment with the PXR ligand rifampicin (Figure 6), confirming the squelching of SRC-1 in HNF4α and USFs transactivation.

This conclusion is supported by the results of gene reporter experiments employing the mutated or truncated OCT1 gene promoter construct. The PXR-mediated OCT1 suppression was markedly diminished when DR-2 HNF4α response elements or the E-box were mutated. In the double-mutant HNF4α/E-box OCT1 gene reporter construct, we observed no suppressive effect of unliganded PXR and a slightly stimulating effect of rifampicin on transactivation (Figure 5, line 4). These data again confirm our hypothesis.

Competition for the common coactivators PGC1α, GRIP-1 or SRC-1 has been recently reported as a putative mechanism of crosstalk between CAR and the oestrogen receptor (for GRIP-1) (Min *et al.*, 2002), PXR and CAR (for SRC-1) (Saini *et al.*, 2005) and HNF4A and CAR (for PGC1α and GRIP-1) (Miao *et al.*, 2006), as well as LXR and the retinoid-related orphan receptor α (Wada *et al.*, 2008). Competition for a common SRC-1 coactivator has been reported for PXR-CAR crosstalk (Saini *et al.*, 2005). However, for the HNF4α-CAR crosstalk (Miao *et al.*, 2006) or for CAR-LXRα interaction, the effect was not statistically significant in a CHIP assay and competition for the SRC-1 coactivator does not appear to be the crucial mechanism of the crosstalk (Zhai *et al.*, 2010).

We can conclude that, to the best of our knowledge, this is the first report showing squelching of the SRC-1 coactivator, between PXR and HNF4α, as an underlying mechanism of PXR-mediated HNF4α target gene down-regulation. Significantly, our results show for the first time the mechanism for PXR-mediated down-regulation of a drug transporter involved in xenobiotic handling or clearance. Thus, it is possible that other xenobiotic metabolizing enzymes or drug transporters dominantly regulated in the liver by HNF4α might be regulated via

similar coactivator squelching by PXR ligands including drugs. Finally, we can hypothesize that PXR-mediated squelching of coactivators in the regulation of OCT1 can be proposed as a new molecular mechanism of regulating the entry of cationic drugs into hepatocytes. Nevertheless, further experiments are needed to confirm this possibility.

Acknowledgements

This work was funded by the Czech Scientific Agency (GACR 303/12/G163 to P.P.) and by SVV project 260 293.

Author contributions

All authors substantially contributed to the conception or design of the work or the acquisition, analysis or interpretation of data for the work and critically revised the manuscript and its final version. They guarantee accuracy and integrity of the work. L.H., T.S., A.C., S.M. and J.M. mainly performed the research and analysed the data. F.T., S.G.C. and P.P. designed some experiments. P.P. designed the research study. L.H. and P.P. wrote the paper.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13472

Table S1 Primer sequences
Table S2 Primer sequences
Figure S1 Cotransfection of SRC-1 significantly stimulates PXR-mediated transactivation of CYP3A4 gene promoter p3A4-luc construct.

8.2 Expression of organic cation transporter 1 (OCT1): unique patterns of indirect regulation by nuclear receptors and hepatospecific gene regulation.

Hyrsova L, Smutny T, Trejtnar F and Pavek P (2016b) Expression of organic cation transporter 1 (OCT1): unique patterns of indirect regulation by nuclear receptors and hepatospecific gene regulation. *Drug Metabolism Reviews* 48:139-158.

(IF 2016/2017: **4,097**)

Při psaní této publikace jsme shromáždili komplexní recentní informace týkající se OCT1 transportéru, včetně co možná nejpodrobnějšího popisu OCT1 proteinu a SLC22A1 genu, tkáňové exprese a funkce transportéru. Nechybí ani podrobný souhrn známých substrátů a inhibitorů OCT1.

Tento přehledový článek je speciálně zaměřený na detailní popis regulace genové exprese genu SLC22A1 a to jak přímé, tak nepřímé. Je dobře známo, že HNF4α hraje stěžejní roli v regulaci OCT1 a má na svědomí i specificky jaterní lokalizaci tohoto transportéru. Dále jsme se věnovali různému způsobu nepřímé regulace OCT1 nukleárními receptory PXR, FXR a GR. V případě PXR se jedná o již zmiňovanou kompetici mezi PXR a HNF4α o koaktivátor SRC1, čímž dochází ke snížení exprese OCT1. FXR také zprostředkovává snížení exprese OCT1, mechanismem je však zvýšení syntézy SHP, což je cílový gen FXR a současně působí represivně na transaktivaci OCT1 zprostředkovanou HNF4α. Naopak v případě GR dochází ke zvýšení exprese OCT1 v důsledku zvýšeného množství HNF4α. Ze všech těchto případů je patrné, že nepřímé působení jaderných receptorů na OCT1 je způsobeno působením na HNF4α, který zprostředkovává další aktivační či případně inhibiční působení na transkripci SLC22A1.

Tento přehledový článek popisující spletité mechanismy genové regulace OCT1 transportéru v hepatocytech prostřednictvím nukleárních receptorů by měl sloužit především jako vzor ke studiu exprese dalších jaterně specifických transportérů nebo

biotransformačních enzymů, pro které mechanismy genové regulace nebyly detailně popsány.

Drug Metabolism Reviews

ISSN: 0360-2532 (Print) 1097-9883 (Online) Journal homepage: http://www.tandfonline.com/loi/idmr20

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To cite this article: Lucie Hyrsova, Tomas Smutny, Frantisek Trejtnar & Petr Pavek (2016) Expression of organic cation transporter 1 (OCT1): unique patterns of indirect regulation by nuclear receptors and hepatospecific gene regulation, Drug Metabolism Reviews, 48:2, 139-158, DOI: 10.1080/03602532.2016.1188936

To link to this article: http://dx.doi.org/10.1080/03602532.2016.1188936

Published online: 09 Jun 2016.

DRUG METABOLISM REVIEWS, 2016
VOL. 48, NO. 2, 139–158
http://dx.doi.org/10.1080/03602532.2016.1188936

Taylor & Francis
Taylor & Francis Group

REVIEW ARTICLE

Expression of organic cation transporter 1 (OCT1): unique patterns of indirect regulation by nuclear receptors and hepatospecific gene regulation

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ABSTRACT

The organic cation transporter 1 (OCT1) is the dominant carrier of organic cationic drugs and some positively charged endogenous compounds into hepatocytes. OCT1 has unique expression pattern. It has the highest expression among drug transporters in normal human hepatocytes with large interindividual variability, but it has negligible expression in other tissues or their tumors. Nowadays, it is clear that the regulation of *SLC22A1* gene encoding OCT1 transporter is rather complex and that transactivation with hepatocyte nuclear factor 4α (HNF4α) and CCAAT-enhancer-binding protein (C/EBPs) transcription factors as well as epigenetic regulation contribute to its unique hepatocyte-specific expression pattern. Unfortunately, species- and tissue-specific regulation of OCT1 and its orthologs as well as significant down-regulation in most immortalized cell lines hamper the study of *SLC22A1* gene regulation. In the current review, we summarize our current understanding of human OCT1 transporter hepatic gene regulation and we propose potential post-transcriptional regulation by predicted miRNAs. We also discuss in detail recent findings on indirect regulation of the transporter via farnesoid X receptor (FXR), glucocorticoid receptor and pregnane X (PXR) receptor, which point out to potential novel mechanisms of xenobiotic-transporting and drug-metabolizing proteins regulation in the human liver as well as to potentially novel drug–drug interaction mechanisms. We also propose that comprehensive understanding of mechanisms of *SLC22A1* gene regulation could direct research for other drug transporters and drug-metabolizing enzymes highly expressed in hepatocytes and controlled by HNF4α or other liver-enriched transcription factors.

Abbreviations: ABCC2: ATP-biding cassette transporter C2, MRP2; **ATP**: ATP-binding cassette family of transporters; **AhR**: aryl hydrocarbon receptor; **AMPK**: AMP-activated protein kinase; **ASP$^+$**: 4-(4-(dimethylamino)-styryl)-N-methylpyridinium; **BCRP**: breast cancer resistance protein, ABCG2; **CAR**: constitutive androstane receptor; **C/EBPα and C/EBPβ**: CCAAT/enhancer binding proteins α and β; **DR-2**: two direct repeats separated by two nucleotides; **FXR**: farnesoid X receptor; **GR**: glucocorticoid receptor; **HNF4α**: hepatocyte nuclear factor 4α; **LETF**: liver-enriched transcription factor; **MDR1**: multidrug resistance protein/P-glycoprotein; **MPP$^+$**: 1-methyl-4-phenylpyridinium; **NR**: nuclear receptor; **OAT2**: organic aniont transporter 2; **OATP**: organic anion-transporting polypeptide; **NTCP**: Na+-taurocholate cotransporting polypeptide; **MRP2**: Multidrug resistance-associated protein; **OCT1**: organic cation transporter 1; **PGC1α**: peroxisome proliferator-activated receptor gamma, coactivator 1 α (PPARGC1A); **PPAR**: peroxisome proliferator-activated receptor; **PXR**: pregnane X receptor; **SHP**: small heterodimer partner; **SLC22A1**: solute carrier family of transporter A 1; **SNP**: single nucleotide polymorphism; **SRC-1**: steroid receptor coactivator 1; **TEA**: tetraethylammonium; **TMD**: transmembrane domain; **USF**: upstream stimulating factor.

ARTICLE HISTORY
Received 27 February 2016
Revised 3 May 2016
Accepted 6 May 2016
Published online 8 June 2016

KEYWORDS
Gene regulation; hepatic uptake; liver; nuclear receptors; organic cation transporter 1; transporter

Introduction

The delivery of xenobiotics into hepatocytes via the sinusoidal (basolateral) membrane is one of the most important steps for hepatic metabolism as well as the biliary elimination of most drugs. Uptake drug transporters are crucial carriers for the entry of weak acid(ic) and base drugs into hepatocytes since these compounds are ionized which restrict their passive diffusion across lipid membranes of hepatocytes. Approximately 40% of the drugs are organic cations at physiological pH, thus many of them are potential substrates for organic cation transporters (Nies et al., 2009). Expression of these membrane transporters is critical not only for drug metabolism but also for drug response of many cationic compounds acting inside hepatocytes, e.g. widely used antidiabetic drug metformin. The organic cation

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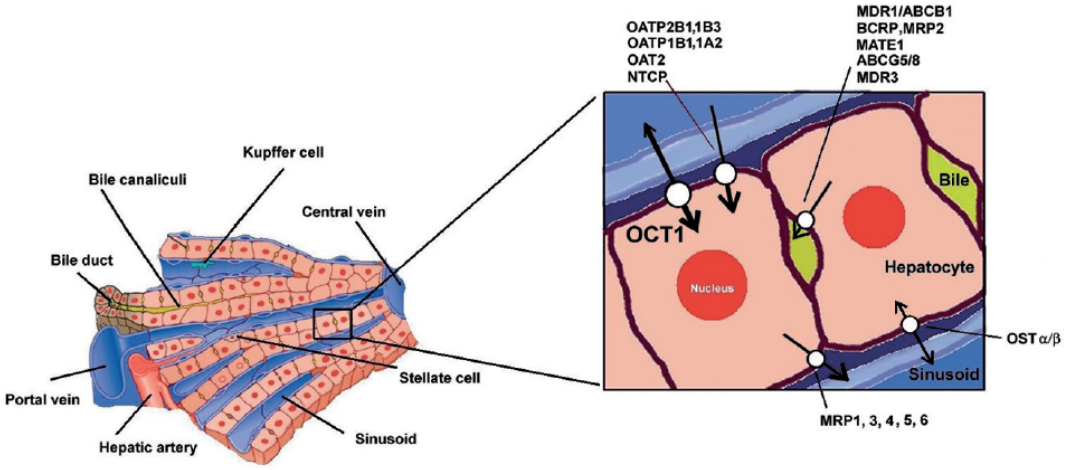

Figure 1. Schematic depiction of OCT1 localization and function in the human liver. OCT1 mediates uptake of organic cations into hepatocytes (bold black arrow) across the sinusoidal membrane. OCT1 may also mediate translocation of organic cations in the opposite direction out of hepatocytes into sinusoids. Secretion of into bile is performed by several ATP transporters including MRP2, BCRP, MDR3 and MDR1 or by MATE1 transporter. Uptake of endogenous compounds and xenobiotics into hepatocytes is also controlled by other uptake transporters (e.g. NTCP, OAT2, OATPs, etc. – narrow black arrow). Export of conjugates ito sinusoidal blood is mediated by MRP transporters.

transporter 1 belongs between most important polyspecific uptake transporters that translocate positively charged substances including numerous important drugs into hepatocytes (Figure 1). Minor expression of OCT1 can also be detected in other organs, e.g. small intestine, lungs, testes, eye, heart or placenta. As variable expressions of regulatory factors and numerous single nucleotide polymorphisms contribute to huge interindividual variation in OCT1 hepatic expression, further studies are warranted into *SLC22A1* gene regulation and expression to predict both cationic drug delivery and the response of cationic drugs in hepatocytes.

OCT1 belongs to the solute-carrier family (SLC) of membrane transport proteins. The SLC22 family contains 13 functionally characterized human plasma membrane proteins, each with 12 α-helical transmembrane domains. SLC22A subgroup contains three OCT transporter subtypes 1–3, encoded by *SLC22A1–3* genes, which are membrane carriers of organic cations, weak bases and some neutral compounds (Koepsell, 2004, 2007). The three OCT transporter subtypes have amino acid identities between 50 and 70% (Koepsell, 2015). The transport by OCT1–3 transporters is classified as facilitative diffusion with driving force supplied by the electrochemical gradient of the transported compounds independent from sodium, chloride or proton gradients. The transporters translocate their substrates in both directions across the plasma membrane and function as electrogenic uniporters or as cation exchangers (Koepsell, 2011, 2015).

This review presents an elaborate overview of the human OCT1 expression and *SLC22A1* gene regulation. We believe that comprehensive understanding of *SLC22A1* gene regulation can bring new information about physiological and detoxification functions of the OCT1 transporter that await more detailed investigations. In addition, *SLC22A1* gene regulation may encourage the study of other hepatic drug transporters and drug-metabolizing enzyme (DMEs) regulation and help us in future to predict individual activity of the genes with respect to personalized therapy.

OCT1 protein and SLC22A1 gene

In 1994, rat *Slc22a1* was cloned as the first member of SLC family (Grundemann et al., 1994). Later, human *SLC22A1* gene was described and cloned (Gorboulev et al., 1997; Zhang et al., 1997). The human OCT1 protein, product of *SLC22A1* gene, contains 554 amino acids that are organized into 12 α-helical transmembrane domains with a pseudosymmetry between transmembrane helixes 1–6 and 7–12. Both carboxy and amino termini of the transporter are localized intracellulary in cytoplasm. The first extracellular loop (between TMD1 and TMD2) is *N*-glycosylated in positions 71, 96 and 112 (asparagine). The OCT1 protein is also post-translationally phosphorylated in the site of large intracellular loop between TMD6 and TMD7

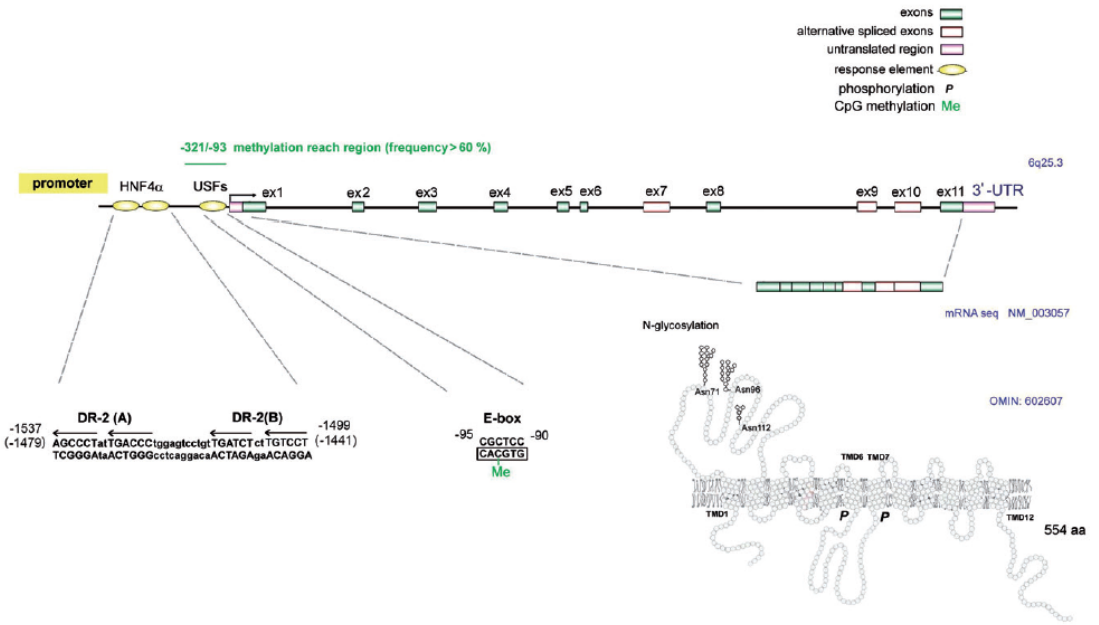

Figure 2. Schematic diagram of genomic organization of the human SLC22A1 gene, its promoter and primary structure of OCT1 protein. Promoter, exons and introns are sized to scale. Promoter bp numbering is relative to the transcription start site according to Kajiwara et al. (2008). The numbers in parentheses are according to Saborowski et al. (2006).

domains (Figure 2). Molecular weight of the transporter was determined approximately 61 kDa (Koepsell, 2007; Lozano et al., 2013) (uniport.org; tcdb.org). Cation binding to OCT1 induces conformational changes that mediate cation translocation across membrane.

In humans, genes coding OCT1, OCT2 and OCT3 are localized within a cluster on chromosome 6q25-q27, each from the three genes comprises 11 exons and 10 introns spanning approximately 37.41 kb (Koehler et al., 1997; Koepsell et al., 2007). Extensive *in silico* and *in vitro* evidence suggests that rat Oct1, rat Oct2 and human OCT2 form homo-oligomers (Keller et al., 2011; Koepsell, 2015). Only transcript variant 1 encodes full-length and functional OCT1 protein (Hayer et al., 1999) and amino acid sequence of the isoform 1 was chosen as the reference one (uniport.org). Seven splice variants of SLC22A1 have also been discovered with skipping exons 7, 9 and 10 (Herraez et al., 2013; Kusuhara et al., 1999); variant 2 is present in about 1/3 hepatocyte samples (Nies et al., 2009).

Expression of OCT1

OCT1 transporter protein is mainly expressed in liver, where it is located in the sinusoidal membrane of hepatocytes and to lesser extend in the plasma membrane of cholangiocytes (Koepsell et al., 2007; Nies et al., 2009; Nishimura & Naito, 2005) (Figure 1). High interindividual variation of OCT1 protein levels and mRNA transcripts (83- and 113-fold, respectively) has been observed in a group of 136/150 liver samples collected from the German population (Nies et al., 2009). Similar high variation (23.6- and 15.9-fold, respectively) has been observed in the Korean population also (Kim et al., 2012). OCT1 protein and transcript levels significantly correlate ($r^2 = 0.53$), which may indicate low post-transcriptional regulation of OCT1 gene expression (Nies et al., 2009). Non-genetic factors, such as age, gender, and cholestasis, were not significantly associated with OCT1 expression in livers in the Korean population (Kim et al., 2012).

The hepatic OCT1 mRNA expression is by two magnitudes higher than in adrenary glands, kidney, testis, spleen, lung, and skeletal muscles (Aoki et al., 2008; Nies et al., 2009; Nishimura & Naito, 2005; Schaeffeler et al., 2011). In human kidney, OCT1 protein is localized at luminal membranes of proximal and distal tubules (Tzvetkov et al., 2009). However, in rat, mouse, and rabbit, strong expression of Oct1 protein was localized at the basolateral membranes of S1 and S2 segments of proximal tubules employing immunohistochemistry (Koepsell et al., 2007). Human OCT1 mRNA transcript is also expressed in low levels in various additional organs, including small intestine, heart, brain, placenta,

mammary gland, eye, adipose tissue, and immune cells (Gilchrist & Alcorn, 2010; Ito et al., 2014; Lee et al., 2013; Minuesa et al., 2008; More et al., 2010; Moreno-Navarrete et al., 2011; Nishimura & Naito, 2005; Zhang et al., 2008). OCT1 protein was also localized in ileum at the brush-border luminal as well as at lateral membranes of the enterocytes (Han et al., 2013; Muller et al., 2005), at the luminal membrane of bronchial epithelial cells (Ingoglia et al., 2015; Lips et al., 2005) and in endothelial cells of brain microvessels (Lin et al., 2010). In human eye, OCT1 was detected in cornea, iris–ciliary body, and blood–retina barrier (Zhang et al., 2008) (Table 1).

In addition, OCT1 expression has been reported in various tumor cells, e.g. hepatocellular carcinoma cells, lymphoma cells or lung cancer cell lines (A549, A460, H838 or H1703) (Gupta et al., 2012; More et al., 2010; Schaeffeler et al., 2011) (Table 1). OCT1 expression is down-regulated in liver tumors such as in hepatocellular carcinoma (HCC) (Heise et al., 2012; Martinez-Becerra et al., 2012; Namisaki et al., 2014, Schaeffeler et al., 2011), in cholangiocarcinoma (CGC) (Herraez et al., 2013) and hepatoblastoma (HPB) (Martinez-Becerra et al., 2012) when compared with surrounding normal tissue and in some lung cancers (More et al., 2010). Importantly, there is minimal expression of OCT1 mRNA in hepatocellular carcinoma HepG2 and Huh7 cell lines, which are used as model lines of human hepatocytes (Hilgendorf et al., 2015; Le Vee et al., 2006) (*our observations*) or in rat hepatocarcinoma cells (Lecureur et al., 1998). On the contrary, OCT1 mRNA is significantly expressed (about 45% of primary human hepatocyte expression) in HepaRG cells differentiated by DMSO (Table 1). Differentiated HepaRG cells or 3D spheroid culture of hESC/hiPSC-derived hepatocyte-like cells are thus the only useful cellular hepatic model with sufficient endogenous OCT1 expression (Hyrsova et al., 2016; Le Vee et al., 2006; Takayama et al., 2013).

Zonal distribution patterns of Oct1 expression in rodent liver

Liver transporters were shown to be variably expressed throughout the liver revealing characteristic zonal distribution patterns spanning from periportal to pericentral regions of hepatocytes (Li et al., 2009). Protein expression of rat Oct1 was detected mainly in hepatocytes surrounding central veins however rat Oct1 mRNA was occurred in all hepatocytes indicating that translation regulation determines zonation of rat Oct1 (Meyer-Wentrup et al., 1998). In the same line, recent study also confirmed the central vein as primary expression location of Oct1 in mouse liver (Chen et al., 2014). Unfortunately, no data exist on zonal expression of human OCT1 expression.

Function of OCT1

The human OCT1 is mainly responsible for the delivery of various cationic drugs as well as endogenous substrates (Table 2) into the hepatocytes from sinusoidal blood (Figure 1) (Jonker & Schinkel, 2004; Koepsell et al., 2007; Nies et al., 2009). The carrier-mediated uptake, the first step in hepatic excretion of many drugs and the other xenobiotics, is sometimes referred to as the zero phase of drug detoxification.

In the small intestine, OCT1 is supposed to be involved in both absorption and secretion of organic cations, although the physiological role of secretion in small intestine is less obvious (Jonker & Schinkel, 2004; Koepsell, 2015). In contrast to rodents, human OCT1 might be involved in the reabsorption of ultra-filtrated cations in human kidney (Jonker & Schinkel, 2004). In human lung, OCT1 is proposed to mediate absorption of some drugs from the bronchi (Ingoglia et al., 2015). In human brain, OCT1 may facilitate passage of endogenous substrates and drugs across the blood–brain barrier (Lin et al., 2010). In human immune cells, OCT1 facilitates uptake of endogenous substrates and accelerates uptake of antiviral drugs (Minuesa et al., 2008).

Recently, OCT1 has been demonstrated as the principal transporter of thiamin into hepatocytes and metformin as competitive inhibitor of the thiamin uptake via OCT1 transporter. Thiamin deficiency enhanced the phosphorylation of AMPK and its downstream target, acetyl-CoA carboxylase. In this way, OCT1 plays a role in hepatic steatosis through modulation of AMPK pathway and acetyl-CoA carboxylase (ACC) activity (Chen et al., 2014). Oct1 might also be involved in secretory transfer of Oct1 substrates, such as acyclovir, thiamin and cimetidine into milk in mice (Ito et al., 2014; Kato et al., 2015).

Animal models

Oct1-null and *Oct1/Oct2* double knockout mice have been generated to describe the impact of this transporter on pharmacokinetics of cationic drugs in Dr. Alfred Schinkel's laboratory. Studies with *Oct1*-null mice have clearly shown that Oct1 is the major physiological hepatic uptake system for small organic cations such as metformin, tetraethylammonium, 125I-labeled anticancer drug metaiodobenzylguanidine (MIBG), neurotoxin 1-methyl-4-phenylpyridinium, cis-diammine(pyridine)chloroplatinum(II)(CDPCP) and oxaliplatin (Jonker et al., 2001; Li et al., 2011; Wang et al., 2002, 2003). Hepatic uptake of [^{14}C]TEA was reduced by

Table 1. Relative expression of OCT1 mRNA and protein in different organ and species.

Organ	Species	Relative expression[a] mRNA	Protein	Reference
Liver	Human/rodents	+++/++	+++/	(Hyrsova et al., 2016; Klaassen & Aleksunes, 2010)
Intestine	Human/rodents	−/+		(Klaassen & Aleksunes, 2010; Nishimura & Naito, 2005)
Kidney	Human/rodents	−/+++	−/++	(Klaassen & Aleksunes, 2010; Koepsell et al., 2007; Tzvetkov et al., 2009)
Tumor cells				
HCC	Human	+		(Martinez-Becerra et al., 2012; Schaeffeler et al., 2011)
CGC	Human	−		(Martinez-Becerra et al., 2012)
HPB	Human	+		(Martinez-Becerra et al., 2012)
HepaRG-differentiated hepatocyte model	Human	++	++	(Hyrsova et al., 2016)
HepG2, HuH-7 hepatocarcinoma cells	Human	−[b]	−	(Hyrsova et al., 2016), our observations
Lymphoma cells	Human	+		(Gupta et al., 2012)
Lung cancer cell lines A549/H838	Human	−/+	+/−	(More et al., 2010)
CACO-2, LST174T/R intestinal tumor cell lines	Human	−[b]		(Herraez et al., 2013)
JEG-3, BeWO placental (choriocarcinoma) cell lines	Human	−[b]		(Herraez et al., 2013)
Alexander, SK-HEP-1, TFK-1, EGI-1, WIF-B9/R liver tumor-derived cells	Human	−[b]		(Herraez et al., 2013)
HEK-293	Human	−[b]		(Herraez et al., 2013)

[a]Expression is related to the expression in liver with the highest expression (+++); no or very low expression (−).
[b]Low, but still detectable level by qRT-PCR.

85% after 20 min and by 77% after 60 min in Oct1$^{-/-}$ mice compared to *Oct1*$^{+/+}$ mice, respectively. Cumulative excretion of [^{14}C]TEA in urine was also slightly increased by 1.5-fold in the *Oct1*$^{-/-}$ mice (Jonker et al., 2001). Small intestine excretion of TEA was reduced in the case of *Oct1*-null mice, suggesting the importance of Oct1 in enterocyte uptake in the basolateral membrane and subsequent excretion through the luminal membrane. The study also revealed that Oct1 is not crucial for normal living functions of mice, since *Oct1* knockouts exhibited normal fertility, physiological functions and showed no obvious physiological defects (Jonker & Schinkel, 2004). In addition, in *Oct1*$^{-/-}$ mice, the blood glucose-lowering effect of metformin was completely abolished along with reduction in metformin-induced AMPK phosphorylation and gluconeogenesis (Shu et al., 2007) and the liver concentration of metformin was approximately 30 times lower in *Oct1*$^{-/-}$ mice than in wild-type mice (Wang et al., 2002, 2003). Consistently with this decreased hepatic accumulation, metformin-induced levels of blood lactate were significantly decreased in *Oct1*$^{-/-}$ mice, indicating connection of metformin-induced lactic acidosis with its uptake into hepatocytes (Wang et al., 2003). Distribution of metformin into the duodenum, jejunum and ileum was also decreased in *Oct1*$^{-/-}$ mice (Wang et al., 2002). In the case of *Oct1*$^{-/-}$ mice, elimination shift from hepatobiliary excretion to the renal one was reported when compared to wild-type *Oct1*$^{+/+}$ (Jonker et al., 2001; Wang et al., 2002). It was also proposed that the absence of hepatic Oct1 activity is not necessarily harmful and *Oct1*$^{-/-}$ mice can display lower hepatotoxic adverse effects of many substances, e.g. lactic acidosis after treatment with metformin and the other biguanides or after treatment with toxic antineoplastic drugs (Jonker & Schinkel, 2004; Li et al., 2011). Recently, a decreased thiamin level in hepatocytes was observed in Oct1 knockout-mice, which was associated with activation of the energy sensor AMP-activated kinase and reduced level of triglycerides (Chen et al., 2014).

Substrates and inhibitors of human OCT1

OCT1 substrates are relatively small hydrophilic molecules with a broad range of chemical structures including monovalent and divalent organic cations, some weak bases and some non-charged compounds although there are disputations whether OCTs really transport bases such as prostaglandin E2 and F2α anions (Koepsell, 2007). The relative molecular mass of most compounds that are transported by OCT1–3 transporters is below 500 and the smallest diameter of the molecules is below 4 A° (Jonker & Schinkel, 2004; Koepsell, 2007). Notably, hepatocytes are the pharmacological target of some drugs belonging to OCT1 substrates including metformin and lamivudine. Thus, the activity of OCT1 may determine the antidiabetic effect of metformin, the most widely prescribed drug for the therapy of the type 2 diabetes (Becker et al., 2009; Shu et al., 2007; Wang et al., 2002). Antivirals, aciclovir and ganciclovir; some antineoplastic drugs such as oxaliplatin and antiparasitic drugs quinine and pentamidine are another important examples of drugs transported by human OCT1.

Table 2. Substances transported by OCT1.

Type of compound	Substance	K_m [μM]	IC_{50} [μM]	Cell type (IC_{50})	Reference
Endogenous substrates	Acetylcholine	–	580	X/E	(Koepsell et al., 2007)
	Agmatine[f]	–	24 000	X/E	(Koepsell, 2007, 2015)
	Choline	100–450[e]	16 700	X/E	(Koepsell, 2007, 2015)
	Corticosterone	–	21.7[a]	HEK293	(Funk, 2008; Hayer-Zillgen et al., 2002)
	Epinephrine	–	>30 000	X/E	(Koepsell, 2007, 2015)
	Guanidine	–	–		(Grundemann et al., 1999)
	Histamine	–	>20 000	X/E	(Koepsell, 2007, 2015)
	Histidyl-proline diketopiperazine	–	–		(Koepsell, 2015)
	Norepinephrine	–	7100	X/E	(Koepsell, 2007, 2015)
	Progesterone	–	3.05[a]	HEK293	(Funk, 2008; Hayer-Zillgen et al., 2002)
	Prostaglandin E_2[f]	0.66	–		(Koepsell et al., 2007)
	Prostaglandin F_2[f]	0.48	–		(Koepsell et al., 2007)
	Putrescine	–	–		(Koepsell, 2015)
	Salsolinol	–	–		(Koepsell, 2015)
	Serotonin	197	>20 000	X/E	(Boxberger et al., 2014) (Koepsell et al., 2007)
	Spermidine	–	–		(Sala-Rabanal et al., 2013)
	Thiamine	780	–		(Chen et al., 2014)
	Tyramine	94.7	76.5	HEK293	(Dos Santos Pereira, 2015; Seitz et al., 2015)
Xenobiotics					
Antiviral	Acyclovir	151	–		(Koepsell et al., 2007)
Diuretic	Amiloride	–	57[c]	HEK293	(Ahlin et al., 2008; Koepsell, 2015)
Antiarrhytmic	Amiodarone	–	ud	HEK293	(Hendrickx et al., 2013; Umehara et al., 2008)
Antipsychotic	Amisulpride	31.3	17.2[a]	HEK293	(Dos Santos Pereira et al., 2014; Haenisch et al., 2012)
Antibiotic	Anisomycin	–	–		(Hendrickx et al., 2013)
β_2-Blocker	Atenolol	3080	ud		(Umehara et al., 2008; Yin et al., 2015)
Anticholinergic	Atropin[f]	–	1.2; 12.2[c]	X/E, HEK293	(Koepsell, 2015) (Koepsell et al., 2007; Ahlin et al., 2008)
Antibiotic	Azithromycin	–	–		(Hendrickx et al., 2013)
Antineoplastic	Bendamustine	–	9.6[c] to >100[c]	HEK293	(Huber et al., 2015; Koepsell, 2015)
Epitelial sodium channel blocker	Benzamil	–	–		(Koepsell, 2015)
Peripheral vasodilator	Buflomedil	–	–		(Hendrickx et al., 2013)
Antineoplastic	Camostat	–	–		(Hendrickx et al., 2013)
Histamin H_2-blocker	Cimetidine	2600	166	X/E	(Koepsell, 2015; Umehara et al., 2007; Koepsell et al., 2007)
Antineoplastic	cis-Diammine(pyridine) chlorplatin	–	–		(Koepsell, 2015)
Anticholinergic	Clidinium	–	–		(Koepsell, 2015)
α_2-Agonist	Clonidine	–	0.6–6.5; 22.6[c]	X/E, HEK293	(Ahlin et al., 2008; Hendrickx et al., 2013; Koepsell et al., 2007)
Antipsychotic	Clozapine	–	6.65[a]	HEK293	(Hendrickx et al., 2013; Haenisch et al., 2012)
Antitusic	Codeine	–	10.5[a]	HEK293	(Koepsell, 2015; Tzvetkov et al., 2013)
Antihypertensive	Debrisoquine	5.9	6.2[a]	HEK293	(Saadatmand et al., 2011)
Tricyclic antidepressant	Desipramine[f]	–	5.4; 56.8[c]	X/E, HEK293	(Hendrickx et al., 2013, Koepsell et al., 2007; Ahlin et al., 2008)
Calcium channel blocker	Diltiazem	–	12.4[c]	HEK293	(Koepsell, 2015; Ahlin et al., 2008)
Antiarrhytmic	Disopyramide R (−)/S (+)[f]	–	15.4[b]/29.9[b]	HeLa	(Koepsell, 2015)
Antiviral	Efavirenz	–	21.8[b]	KCL22	(Koepsell, 2015; Moss et al., 2015)
β_2-Agonist	Fenoterol	–	–		(Koepsell, 2015)
β_2-Agonist	Formoterol	–	22.3[a]	HEK293	(Koepsell, 2015; Salomon et al., 2015)
Antiparasitic	Furamidine	6.1	7.4[a]	CHO	(Ming et al., 2009)
Antiviral	Ganciclovir	516	–		(Koepsell et al., 2007)
Antineoplastic	Gefitinib	–	1.07[d]	HEK293	(Hendrickx et al., 2013;

(*continued*)

Table 2. Continued

Type of compound	Substance	K_m [μM]	IC_{50} [μM]	Cell type (IC_{50})	Reference
					Minematsu & Giacomini, 2011)
Anticholinergic	Glycopyrrolate	–	–		(Koepsell, 2015)
Antipsychotic	Haloperidol	–	12.4[a]	HEK293	(Hendrickx et al., 2013; Haenisch et al., 2012)
Antineoplastic	Imatinib[f]	–	1.47[d]	HEK293	(Hendrickx et al., 2013)
Tricyclic antidepressant	Imipramine	nd	17.1[c]	HEK293	(Hendrickx et al., 2013; Koepsell, 2004; Ahlin et al., 2008)
β_2-Agonist	Indacaterol	–	–		(Koepsell, 2015)
Bronchodilatant	Ipratropium	9	–		(Hendrickx et al., 2013)
Antineoplastic	Irinotecan	–	–		(Koepsell, 2015)
Anesthetic	Ketamine	–	–		(Hendrickx et al., 2013)
Antiviral	Lamivudine	249	17[a]	HEK293	(Koepsell, 2015; Jung et al., 2008)
Anticonvulsant	Lamotrigin	62	–		(Dickens et al., 2012)
Anti-Parkinson disease drug	Levodopa	–	–		(Becker et al., 2011)
Antidairrhetic	Loperamide	–	23.7[c]	HEK293	(Hendrickx et al., 2013; Ahlin et al., 2008)
NMDA receptor antagonist	Memantine	nd	3.7; 27.2[c]	X/E, HEK293	(Hendrickx et al., 2013; Koepsell, 2004; Koepsell et al., 2007; Ahlin et al., 2008)
Anticholinergic	Mepenzolate	–	64.9[c]	HEK293	(Koepsell, 2015; Ahlin et al., 2008)
Antineoplastic	Metaiodobenzylguanidin	–	–		(Ito et al., 2012)
Antidiabetic	Metformin	1470	2 010	X/E	(Koepsell et al., 2007)
Antiemetic	Metoclopramide	–	–		(Koepsell, 2015)
Calcium channel blocker	Mibefradil	–	–		(Hendrickx et al., 2013)
Analgetic	Morphine	3.4	4.2a,0. 28[c]	HEK293	(Tzvetkov et al., 2013; Carpaneto et al., 2010; Ahlin et al., 2008)
β_2-Blocker	Nadolol	>500	–		(Misaka et al., 2016)
Histamin H_2-blocker	Nizatidine	–	–		(Koepsell, 2015)
Metabolite of tramadol	o-Desmethyltramadol	–	172[c]	HEK293	(Tzvetkov et al., 2011)
Antiemetic	Ondansetron[f]	–	63[c]	HEK293	(Arimany-Nardi et al., 2014; Tzvetkov et al., 2012; Saadatmand, 2012)
Antineoplastic	Oxaliplatin	–	ud		(Koepsell, 2015; Yonezawa et al., 2006; Zeeh et al., 2013)
Anticholinergic	Oxyphenonium	–	–		(Hendrickx et al., 2013)
β-Blocker (non-selective)	Oxyprenolol	–	87.3[a]	HEK293	(Hendrickx et al., 2013; Umehara et al., 2008)
Antineoplastic	Paclitaxel	–	–		(Koepsell, 2015)
Antiparasitic	Pentamidine	36.4	16.4[a]	CHO	(Ming et al., 2009)
Antipsychotic	Perphenazine	–	–		(Koepsell, 2015)
Antidiabetic	Phenformin	12.5	10.153	X/E	(Sogame et al., 2009) (Koepsell et al., 2007)
Antineoplastic	Picoplatin	–	0.44[a]	HEK293	(Koepsell, 2015; More et al., 2010)
β-Blocker (non-selective)	Pindolol	–	39.1[a]	HEK293	(Hendrickx et al., 2013; Umehara et al., 2008)
α_1-Blocker	Prazosin	–	1.8; 9.9[c]	X/E, HEK293	(Hendrickx et al., 2013; Koepsell et al., 2007; Ahlin et al., 2008)
Antiarrhytmic	Procainamide	–	74	X/E	(Koepsell, 2015; Koepsell et al., 2007)
β_2-Agonist	Procaterol	–	–		(Hendrickx et al., 2013)
Antiarrhytmic	Propafenone	–	11.1[c]	HEK293	(Hendrickx et al., 2013; Ahlin et al., 2008)
β_2-Blocker	Propranolol	–	63.2[c]; 113[a]	HEK293	(Hendrickx et al., 2013; Ahlin et al., 2008; Umehara et al., 2008)
Antiarrhytmic	Quinidine	–	18; 113.8[c]	X/E, HEK293	(Hendrickx et al., 2013; Koepsell et al., 2007; Ahlin et al., 2008)

(*continued*)

Table 2. Continued

Type of compound	Substance	K_m [μM]	IC_{50} [μM]	Cell type (IC_{50})	Reference
Histamin H_2-blocker	Ranitidine	70	33[a]	XLO	(Koepsell, 2015; Bourdet et al., 2005)
Antiviral	Ritonavir	–	14[a]. 5.2[b]	HEK293, HeLa	(Funk, 2008, Jung et al., 2008[a]; Zhang et al., 2000)[b]
β_2-Agonist	Salbutamol (albuterol)	–	–		(Koepsell, 2015)
β_2-Agonist	Salmeterol	–	47.8[a]	HEK293	(Hendrickx et al., 2013; Salomon et al., 2015)
Anticholinergic	Scopolamine	–	–		(Hendrickx et al., 2013)
Anti-Parkinson disease drug	Selegiline	–	–		(Koepsell, 2015)
Antineoplastic	Sorafenib	3.8	>30[d]	HEK293	(Swift et al., 2013; Minematsu & Giacomini, 2011)
Serotonin 5-HT_{1d} agonist	Sumatripan	55.4	–		(Koepsell, 2015; Matthaei et al., 2015)
Antibiotic	Sulfaphenazole	–	–		(Hendrickx et al., 2013)
Antipsychotic	Sulpiride	259.7	–		(Dos Santos Pereira et al., 2014)
α_1-Blocker	Terazosin	–	23.7[c]	HEK293	(Koepsell, 2015; Ahlin et al., 2008)
β_2-Agonist	Terbutaline	–	–		(Koepsell, 2015)
Bronchodilatant	Tiotropium	–	–		(Koepsell, 2015; Nakanishi et al., 2011)
Analgetic	Tramadol[f]	–	30.3[c]	HEK293	(Arimany-Nardi et al., 2015; Tzvetkov et al., 2011; Saadatmand, 2012)
Diuretic	Triamterene	–	–		(Koepsell, 2015)
Antibiotic	Trimethoprim	–	56.8[c]	HEK293	(Koepsell, 2015; Ahlin et al., 2008)
Antiemetic	Tropisetron	–	8.5[c]	HEK293	(Arimany-Nardi et al., 2015; Tzvetkov et al., 2012)
Urinary antispasmodic	Trospium chloride	106	6.2[a]	CHO	(Bexten et al., 2015; Lips et al., 2007)
Anti-nicotine addiction	Varenicline	–	–		(Koepsell, 2015)
Calcium channel blocker	Verapamil	–	2.9; 6.8[c]	X/E, HEK293	(Hendrickx et al., 2013; Koepsell et al., 2007; Ahlin et al., 2008)
Anticholinergic	Vesamicol	–	–		(Hendrickx et al., 2013)
Cardiac stimulant	Xamoterol	–	–		(Hendrickx et al., 2013)
Antiviral	Zalcitabine	242	24[a]	HEK293	(Jung et al., 2008)
Antineoplastic	Zebularine	–	–		(Koepsell, 2015)
Herbal compounds					
Alkaloid	Berberine	14.8	7.28[d]	HEK293	(Koepsell, 2015; Nies et al., 2008; Kwon et al., 2015)
Protoberberine alkaloid	Berberrubine	1.27	1.26[a]	MDCK	(Li et al., 2016)
Protoberberine alkaloid	Coptisine	5.8	0.931[a]	MDCK	(Li et al., 2016)
Protoberberine alkaloid	Epiberberine	4.37	1.31[a]	MDCK	(Li et al., 2016)
Protoberberine alkaloid	Jatrorrhizine	4.46	0.932[a]	MDCK	(Li et al., 2016)
Alkaloid (Crotalaria)	Monocrotalin	25	5.52[a]	MDCK	(Tu et al., 2013)
Alkaloid (Zanthoxylum nitidum)	Nitidine chloride	0.797	1.09[a]	MDCK	(Li et al., 2014)
Flavonoid	Quercetin	2.20	–		(Glaeser et al., 2014)
Fluorescent dyes	DAPI (4′,6-diamidino-2-phenylindol)	8.94	–		(Koepsell, 2013a; Yasujima et al., 2011)
	Rhodamine 123	0.54	0.37[b]	HEK293	(Jouan et al., 2014)
Toxines	Aflatoxin B1	–	–		(Koepsell, 2013b)
	Ethidiumbromide	0.8	0.6[a]	CHO	(Koepsell, 2013a; Lee et al., 2009)
Model compounds	ASP^+	9.21			(Koepsell, 2013b)
	MPP^+	15.32			(Koepsell et al., 2007)
	Phenylthiophenecarboxamide ureas	–			(Hendrickx et al., 2013)
	TEA	229			(Koepsell et al., 2007)

CHO: Chinese hamster ovary cells overexpressing human OCT1; HeLa: human cervix epitheloid carcinoma cells overexpressing human OCT1; HEK293: human embryonal kindey cells overexpressing human OCT1; KCL22: chronic myeloid leukemia cells overexpressing human OCT1; MDCK: Madin–Darby canine kidney cells overexpressing human OCT1; XLO: *Xenopus laevis* oocytes overexpressing human OCT1; X/E: *Xenopus laevis* oocytes or epithelial cell lines overexpressing human OCT1.

[a]IC_{50} is related to MPP^+.
[b]IC_{50} is related to TEA.
[c]IC_{50} is related to ASP+.
[d]IC_{50} is related to metformin.
[e]K_m value for OCT1-3.
[f]Contradictory data on substrate identification.

Very recently, thiamine has been shown as a principal endogenous substrate of OCT1 (Chen et al., 2014). Other endogenous compounds transported by human OCT1 include choline, acetylcholine, the neuromodulators histidyl-proline diketopiperazine (cyclo(His-Pro)), the L-arginine metabolite agmatine and amino acid metabolite putrescine (Koepsell, 2015).

The most widely used prototype OCT1 substrates for evaluation of human OCT1 activity are tetraethylammonium, 1-methyl-4-phenylpyridinium and 4-(4-(dimethylamino)styryl)-N-methylpyridinium. Both endogenous and exogenous OCT1 substrates are summarized in Table 2. Although the substrate specificities of OCT1, OCT2 and OCT3 overlap significantly, there exist differences in specificity between individual subtypes of OCT transporters (Koepsell, 2007).

A large number of compounds has been identified as OCT1 inhibitors, which are able to interact with the transporters without being translocated. Among the most important inhibitors of human OCT1 are the muscarinic drug atropine, α_2 adrenoreceptor agonist clonidine, Ca^+channel blocker verapamil, selective re-uptake inhibitor citalopram, the anesthetic drug midazolam and antiviral drug ritonavir (Table 2).

Clinical significance of OCT1 transporter and its genetic variability

Interindividual variability of OCT1 activity may play an important role in different response and sensitivity of organism to the cationic drugs (Jonker & Schinkel, 2004). Nowadays, it is clear that the *SLC22A1* gene is highly ethnic-specific polymorphic in humans. In 2002, Kerb et al. described for the first time 25 polymorphic variants in Caucasians, in eight cases polymorphisms led to the amino acid substitution (Kerb et al., 2002). Since then, over 1000 single-nucleotide polymorphisms have been identified, 22 of which have clinical relevance to drugs belonging to OCT1 substrates and consequences for the treatment outcomes (Arimany-Nardi et al., 2015). Six variants (R61C, F160L, P341L, M408C, G465R and the intronic rs622342) are SNPs with allelic frequencies higher than 1% in some populations (Arimany-Nardi et al., 2015). In addition, 11 haplotypes with frequencies higher than 1% have been identified for *SLC22A1* gene (Nies et al., 2009).

Reduced therapeutic effect of the antidiabetic drug metformin has recently been clearly demonstrated in persons with alleles of OCT1 with reduced functions such as R61C, G401S, 420del, or G465R. In these individuals carrying *SLC22A1* polymorphisms, significantly higher glucose levels after metformin treatment have been associated with higher AUC and C_{max} for metformin (Shu et al., 2007, 2008). In patients with type II diabetes mellitus, only genetic variation rs622342 has been associated with the glucose-lowering effect of metformin (Becker et al., 2009), although there also exist controversial reports (Arimany-Nardi et al., 2015). Similarly, polymorphism rs622342 A > C has been shown to be associated with poorer drug response to antiparkinson drug levodopa (Becker et al., 2011). Although imatinib does not seem to be translocated by human OCT1 (Nies et al., 2014), the polymorphic variants of OCT1 have been several times reported to affect outcome in the treatment of chronic myeloid leukemia (CML). This apparent controversy between human OCT1 polymorphic variants and outcomes of treatment with imatinib has been recently again confirmed in other studies (Angelini et al., 2013; Arimany-Nardi et al., 2015; Crossman et al., 2005). Drugs such as morphine, analgesic drug tramadole metabolite O-desmethyltramadol, and antiemetic drugs tropisetron and ondasetron have also been shown in pharmacogenetic clinical studies to be affected by OCT1 variants (Arimany-Nardi et al., 2015).

Mutations in *SLC22A1* gene may not only lead to lower transporter protein expression and/or to reduction of OCT1 protein turnover. Mutations in OCT1 have also been reported to change the selectivity for transported substrates or inhibitory activity of some drugs (Ahlin et al., 2011; Choi & Song, 2012; Nies et al., 2009; Kerb et al., 2002). For summary see Table 3 in recent report by Nies et al. (2009).

Hayer and coworkers identified three alternatively spliced (hOCT1G/L506, hOCT1G483 and hOCT1G353) and one full-length (hOCT1G/L554) variants of human OCT1 transporter. Two of the alternatively spliced variants lack two last TMDs and the other (hOCT1G353) lacks the last six TMDs. All four OCT1 variants were identified in the human glioma SK-MG-1 cell line, but only hOCT1G/L544 and hOCT1G/L506 were detected in cDNA obtained from human liver. Importantly, all these variants lack significant uptake of MPP^+ (Hayer et al., 1999). Abundant expression of recently discover inactive variants R61S fs*10 and C88A fs*16 of *SLC22A1* gene have been found in hepatocellular carcinoma and cholangiocarcinoma samples (Herraez et al., 2013) or in chronic myeloid leukemia cells (Grinfeld et al., 2013).

Influence of genetic variability of OCT1 on clinical aspects of pharmacotherapy has been recently summarized in review article by Arimany-Nardi et al. (2015). In addition, drug–drug interactions at the level of OCT transporters have been recently reviewed by the "father" of OCTs (Koepsell, 2015).

Regulation of SLC22A1 gene expression

Regulation of basal hepatic expression

OCT1 is one of the highly expressed transporters in the liver parenchyma cells (hepatocytes). In contrast, only weak expression of OCT1 has been found in other tissues. Thus, it was suggested that *SLC22A1* gene expression is predominantly controlled by a transcription factor of the so-called liver-enriched transcription factors. The group is composed of hepatocyte nuclear factor 4α, other transcription factors such as CCAAT/enhancer binding proteins α and β and hepatocyte nuclear factor 1α (HNF1α) and 3γ (HNF3γ, FOXA3, forkhead box A3). LETFs play a special role in maintaining hepatic phenotype, differentiation and in the transcriptional regulation of hepatic DMEs and drug transporters (Castell et al., 2006; Kamiyama et al., 2007).

Indeed, the hepatocyte-specific expression of OCT1 is strongly controlled by the *hepatocyte nuclear factor 4α (NR2A1)*, which belongs to genes of the nuclear receptor superfamily (Kamiyama et al., 2007; Rulcova et al., 2013; Saborowski et al., 2006). In a study with primary human hepatocytes, where HNF4α was silenced using an adenovirus-mediated HNF4α-small interfering RNA, it was found that OCT1 is the drug transporter whose expression is most significantly affected by HNF4α silencing (Kamiyama et al., 2007). Two co-operating HNF4α response elements have been identified between nucleotides -1479 and -1441 of the 5′-flanking region of the *SLC22A1* gene upstream of the transcription initiation site which contain directly repeated hexamers separated by two bases (Figure 2). In electrophoretic mobility shift assay (EMSA), recombinant HNF4α directly interacts with both sites. Mutation of these sites in *SLC22A1* promoter luciferase reporter constructs abolished transactivation (Hyrsova et al., 2016; Saborowski et al., 2006). These motifs are conserved in primates, but not in rodents indicating different patterns of *SLC22A1/Slc22a1* gene regulation in these species (Saborowski et al., 2006).

HNF4α is a transcription factor that belongs to the steroid/thyroid superfamily of NRs encoded by HNF4A gene. It binds as a homodimer to regulatory promoter sequences of its target genes in the liver, to lesser extend also in gut, kidney and pancreas (Benoit et al., 2006; Sladek et al., 1990). HNF4α has been shown to constitutively bind fatty acids and some fatty acids are speculated to be true ligands of the orphan NR. Functionally, HNF4α plays a critical role in liver development, hepatocyte differentiation, xenobiotic detoxification, bile acid synthesis, serum protein production and metabolism (Watt et al., 2003). A rare loss-of-function mutation in the HNF4α gene causes a monogenic form of early-onset type 2 diabetes (maturity-onset diabetes of the young, MODY1) (Yamagata et al., 1996).

In addition, *upstream stimulating factors USF1 and USF2* have been identified to regulate basal hepatic expression of OCT1 via a cognate E-box (5′-CACGTG-3′ sequence) (Figure 2). Moreover, activation of E-box in the core proximal promoter region (-95/-90) has been shown to coordinately augment the HNF4α-mediated transactivation of the *SLC22A1* gene in an additive (Hyrsova et al., 2016; Kajiwara et al., 2008).

Our recent experiments dealing with viral transduction of MZ-Hep1 cells with the expression constructs for HNF4α, CCAAT/enhancer binding proteins β and peroxisome proliferator-activated receptor-γ coactivator 1α revealed significant roles of the transcription factors in *SLC22A1* gene regulation (Rulcova et al., 2013). In addition, we found that the expression of OCT1 mRNA in human liver significantly correlates with C/EBPβ and HNF4α mRNAs expression and that C/EBPβ co-transfection stimulates OCT1 gene reporter construct in HepG2 cells. Thus, involvement of two LETFs HNF4α and C/EBPβ well explains the unique hepatic expression of OCT1 mRNA and minimal expression in other non-hepatic tissues (Nies et al., 2009). Since the levels of OCT1 and OCT3 mRNAs weakly correlate with each other, we can suppose different mechanisms of *SLC22A1 and SLC22A3* gene regulation in the liver (Nies et al., 2009).

Ligand-dependent nuclear receptor-mediated regulation

Nuclear receptors, such as pregnane X receptor, farnesoid X receptor, constitutive androstane receptor, glucocorticoid receptor or peroxisome proliferator-activated receptor α and γ, are ligand-activated transcription factors of the NR superfamily. Their target genes are regulated after ligand binding, which triggers transcription activation or repression of gene expression.

Recently, we found that dexamethasone (DEX), a substrate of *glucocorticoid receptor*, significantly up-regulates OCT1 mRNA and protein in normal primary human hepatocytes, but not in hepatocyte-derived tumor cell lines HepG2 and MZ-Hep1. We also observed that HNF4α is induced by DEX in primary human hepatocytes, but not in hepatocyte-derived tumor cell lines. Luciferase gene reporter construct with 2 kb *SLC22A1* promoter sequence; however, was not transactivated by glucocorticoid receptor activation with DEX in hepatic cell lines indicating no direct transactivation via GR. The HNF4α mRNA induction by DEX was totally inhibited by the glucocorticoid receptor antagonist RU486 (Rulcova et al., 2013; Vrzal et al., 2009). HNF4α mRNA up-regulation by glucocorticoids was also described by others in

primary human hepatocytes (Godoy et al., 2010; Onica et al., 2008). In contrast, neither C/EBPβ nor PGC1α involved in *SLC22A1* gene regulation were up-regulated in human hepatocytes by DEX (Rulcova et al., 2013). Based on these observation, we hypothesized that GR-induced up-regulation of HNF4α by DEX indirectly induces *SLC22A1* gene expression in primary human hepatocytes (Figure 3(A)) (Rulcova et al., 2013).

In rats, treatment with lipopolysaccharides or dexamethasone resulted in down-regulation of Oct1 in the liver (Cherrington et al., 2004; Maeda et al., 2008). In case of rat Oct1, Maeda et al. proposed dexamethasone-dependent down-regulation of rat Pxr in the liver as an indirect molecular mechanism of Oct1 down-regulation (Maeda et al., 2008).

Pregnane X receptor, the ligand-activated nuclear receptor of NR subfamily NR1I, is the "xenobiotic" receptor that regulates a broad spectrum of target genes of phase I DMEs, phase II DMEs and drug transporters. PXR is activated by a huge variety of xenobiotics, drugs, some endobiotics and metabolites of intermediary or microbiome metabolism (Chen et al., 2012; Smutny et al., 2013; Venkatesh et al., 2014). Thus, PXR mediates overall detoxification of potentially toxic xenobiotics and endogenous compounds. PXR is predominantly expressed in the liver and intestine. This expression pattern correlates with the expression of major DMEs and drug transporters, many of them are regulated by PXR (Banerjee et al., 2015).

Contradictory data have been published on PXR-mediated regulation of human *SLC22A1* gene. Rifampicin, a prototype ligand of PXR, has been found to increase the glucose-lowering effect of metformin, an OCT1 substrate, and significantly induce OCT1 mRNA in peripheral blood cells. Therefore, it was hypothesized that rifampicin may induce OCT1 in hepatocytes and thus increase the uptake of metformin into hepatocytes (Cho et al., 2011). In other studies, agonists of PXR have been found to mildly induce OCT1 mRNA in the chronic myeloid leukemia (CML) cell line KCL22 and primary CML cells (Austin et al., 2015). Rat *Slc22a1* gen has been clearly demonstrated as a target of rat Pxr and pregnenolone-16α-carbonitrile (PCN), a prototype rodent Pxr ligand, significantly induced Oct1 in the rat liver (Maeda et al., 2007). However, in human hepatocytes, rifampicin was reported to down-regulate OCT1 mRNA (Badolo et al., 2013; Hyrsova et al., 2016; Jigorel et al., 2006).

Recently, we focused on the discrepancy and studied OCT1 expression and mechanism of *SLC22A1* gene transactivation in a series of hepatic cellular models and in non-hepatic Hela cells. We observed that rifampicin significantly suppressed OCT1 mRNA expression in a group of 15 cultures of primary human hepatocytes and differentiated HepaRG cells. In addition, PXR ligands rifampicin and hyperforin as well as the constitutively active PXR mutant T248D significantly suppressed transactivation of the 1.8 kb OCT1 promoter construct in gene reporter assays. The silencing of both PXR and HNF4α in HepaRG cells abrogated the PXR ligand-mediated down-regulation of *SLC22A1* gene expression and mutation of HNF4α and USF1 (E-box) responsive elements reversed the PXR-mediated inhibition in gene reporter assays. These data indicated that HNF4α and E-box factors are together critical for PXR-mediated down-regulation of OCT1 expression and that PXR itself does not trans-repress *SLC22A1* gene expression. In next experiments employing chromatin immunoprecipitation assays (ChIP), we found that PXR activation sequestrates the SRC-1 coactivator, which is common for PXR, HNF4α and USF, from the HNF4α response element and E-box of the *SLC22A1* gene promoter. Overexpression of the SCR-1, but not the PGC1α, coactivator abrogated the PXR-mediated repressive effect on *SLC22A1* gene transactivation. We therefore proposed that PXR ligands reduce the HNF4α/USF-mediated transactivation of *SLC22A1* gene expression by squelching the SRC-1 coactivator (Figure 3(B)). We also hypothesized that in this manner, PXR ligands may decrease delivery of model OCT1 substrate into hepatocyte cells as we demonstrated with MPP^+ in differentiated HepaRG cells (Hyrsova et al., 2016).

However, when we studied the effect of PXR activation on OCT1 mRNA expression in non-hepatic Hela cells (lacking HNF4α), we observed significant stimulation of OCT1 transactivation by rifampicin in these cells transfected with exogenous PXR. We can therefore explain the different effect of PXR activation on *SLC22A1* gene regulation in hepatic (HNF4α expressing) and non-hepatic (HNF4α lacking) cells based on crosstalk of PXR and HNF4α nuclear receptors that compete for common coactivator SRC-1 (Hyrsova et al., 2016).

Similar competition for a common SRC-1 coactivator has already been reported as a molecular mechanism for PXR-CAR crosstalk in case of multidrug resistance associated protein 2 (MRP2) regulation (Saini et al., 2005).

Model ligands/activators (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) and phenobarbital, respectively) of other "xenobiotic" sensors *Aryl hydrocarbon receptor* and *Constitutive androstane receptor* have no significant effect on OCT1 mRNA expression in primary human hepatocytes (Jigorel et al., 2006). Interestingly, a multivariate linear regression analysis revealed that *AhR* (and

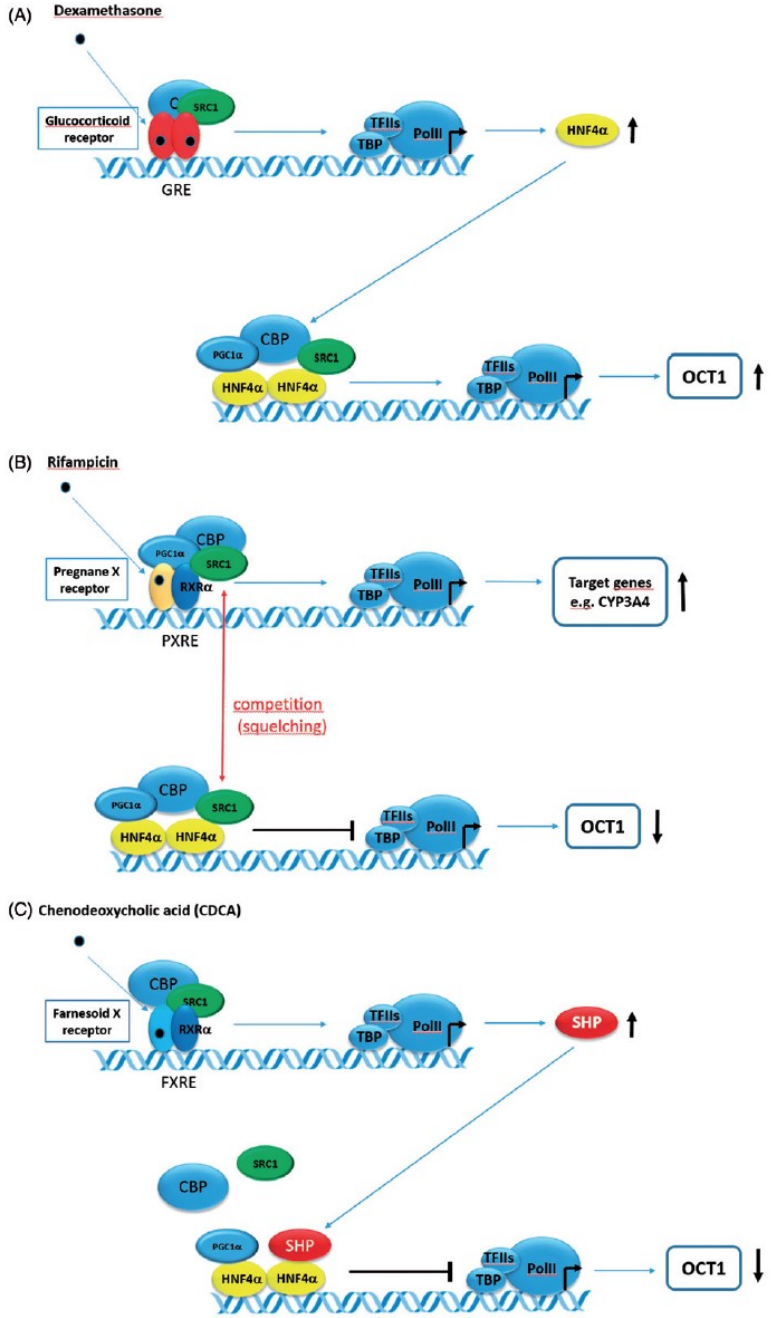

Figure 3. Schematic of glucocorticoid receptor, farnesoid X receptor and pregnane X receptor-mediated indirect regulation of human SLC22A1 (OCT1) gene in human hepatocytes. (A) Dexamethasone binds to glucocorticoid receptor which subsequently induces HNF4α expression. Up-regulated HNF4α promotes OCT1 expression in human hepatocytes. (B) PXR activation by rifampicin sequestrates the SRC-1 coactivator, which is common for PXR, HNF4α and USF, from the HNF4α response element and E-box of the OCT1 promoter. OCT1 expression is thus indirectly suppressed by PXR activation. (C) Activated farnesoid X receptor (FXR) induces small heterodimer partner (SHP), which is a corepressor of HNF4α transactivation and thus suppresses OCT1 expression.

PXR) expression might be involved in *SLC22A1* gene expression in the livers of patients with chronic hepatitis C. Nevertheless, no direct data indicate the induction of hepatic *SLC22A1* gene expression by AhR ligands (Hanada et al., 2012).

Saborovski et al. demonstrated down-regulation of *SLC22A1* gene expression by bile acid chenodeoxycholic acid (CDCA) (Saborowski et al., 2006). CDCA is a ligand of *Farnesoid X receptor*, a bile acid sensor belonging to superfamily of NRs (Makishima et al., 1999; Parks et al., 1999). *Small heterodimer partner (NR0B2)* is one of the target genes induced by FXR after activation with its ligands. SHP is NR lacking a DNA-binding domain that functions as corepressor of other numerous transcription factors including HNF4α (Lee et al., 2000; Seol et al., 1996). Thus, activation of FXR by either endogenous bile acids or exogenous ligands induces SHP expression in the liver. SHP subsequently down-regulates HNF4α-controlled *SLC22A1* gene expression as an HNF4α suppressor (Figure 3(C)). This finding documents potential molecular basis for OCT1 down-regulation in cholestatic states in humans reported recently, although also contradictory data exist in Korean population (Kim et al., 2012; Nies et al., 2009). In agreement, reduced expression of rat Oct1 and lower hepatic accumulation of intravenously injected TEA have been documented in obstructive cholestasis in rats again confirming the proposed mechanism of *SLC22A1* gene regulation via activated FXR by bile acids (Denk et al., 2004; Saborowski et al., 2006).

Similar repression of transactivation by SHP after FXR activation has been reported for other hepatic transporters human organic anion transporter 2 (hOAT2, SLC22A7) and Na^+-taurocholate cotransporting polypeptide (NTCP, SLC10A1) (Eloranta et al., 2006; Popowski et al., 2005).

In agreement with different *SLC22A1/Slc22a1* gene regulation between primates and rodents, the activation of *peroxisome proliferator-activated receptor α and PPARγ* with clofibrate and ciglitazone has been proposed to regulate murine homolog of Oct1, but the receptors ligands do not regulate human OCT1 (Nie et al., 2005; Saborowski et al., 2006). In addition, murine Oct1 was found to be up-regulated in the liver in mice fed a high-fat (HF) diet for 19 weeks compared with mice fed a control diet (Jang et al., 2010). However, we have no relevant data on humans that would confirm *SLC22A1* gene regulation in the liver under different dietary conditions.

Recently, Badolo et al. reported up-regulation of OCT1 mRNA in cryopreserved human hepatocytes by testosterone, estradiol, *cis*-androsterone and by venlafaxine and naltrexone. The molecular mechanisms behind the regulation are not known (Badolo et al., 2013).

Role of polymorphisms on OCT1 expression

Several SNPs have been identified in the regulatory promoter region of *SLC22A1* gene including -1795G > A (heterozygote frequency 23.7%), -1685G > A and -1672G > C in Caucasians (Kerb et al., 2002). However, no significant difference in the levels of OCT1 expression between the -1795 GG or GA genotypes has been observed in 65 liver samples of the Korean origin (Kim et al., 2012).

It has been demonstrated that SNPs within the E-box core motif can modulate gene expression. However, no such polymorphisms in the *SLC22A1* gene promoter region, including the E-box, have been identified in 109 individuals (Kajiwara et al., 2008).

The two variants, R61S fs*10 and C88A fs*16, were recently discovered in hepatocellular carcinoma and cholangiocarcinoma samples. These variants encoded nonfunctional proteins due to induced frameshifts resulting in truncated proteins unable to reach the plasma membrane and to transport sorafenib or uptake of tetraethylammonium. Similarly, C88R and S189L variants encode mainly intracellular proteins (Herraez et al., 2013).

Epigenetic regulation of OCT1

Differential *SLC22A1* gene expression between hepatic tumor cell lines and normal primary human hepatocytes might be due to epigenetic silencing of OCT1 in hepatocellular carcinoma, as suggested by Schaeffeler et al. (2011). The authors reported the increased CpG methylation of *SLC22A1* gene promoter in human hepatocellular carcinoma cells in comparison with normal hepatocytes. Nevertheless, direct demonstration of epigenetic regulation of *SLC22A1* gene expression has not been reported in hepatocellular carcinoma cell lines.

Methylation at the CpG site located within the E-box motif (CACpGTG) strongly inhibits the formation of the transcription factor complex and may negatively regulate gene expression (Kajiwara et al., 2008). Promoter sequence -321/-93 of SLC22A1 gene in the liver has been found to be highly methylated (Aoki et al., 2008). However, it was found that methylation status of E-box and -321/-93 bp promoter fragment is not significantly different between the liver and kidney and that renal-specific expression of *SLC22A2* gene is due to hypomethylation of E-box in kidney

in comparison with the liver expression (Aoki et al., 2008; Kajiwara et al., 2008).

Involvement of miRNAs in OCT1 regulation

Over the last couple of years, it has become obvious that post-transcriptional regulation mediated by microRNAs (miRNAs) may play an important role in the regulation of gene expression of DMEs and drug transporters (Nakajima & Yokoi, 2011; Yu et al., 2015). MiRNAs are short nucleotides ~22 nt in length of non-coding RNA preferentially binding to complementary elements of 3′-untranslated region (3′-UTR) of their target mRNAs leading to mRNA degradation or translation repression (Bartel, 2004).

Coding sequence of OCT1 transcript variant 1 (GenBank accession NM_003057) is flanked by 3′-UTR reaching 134 bp. To the best of our knowledge, no data have been reported about miRNA-dependent regulation of *SLC22A1* gene so far. Based on the extended study of 136 human liver samples, significant correlation ($r^2 = 0.53$) between OCT1 protein and transcript levels was observed, suggesting low post-transcriptional regulation of *SLC22A1* gene expression (Nies et al., 2009). The short 3′-UTR of OCT1 mRNA provides probably limited potential for miRNAs regulation, as evident from the correlation between length of 3′-UTR of mRNAs and the number of regulatory miRNAs predicted by *in silico* methods (Ramamoorthy & Skaar, 2011; Smutny et al., 2015). On the other hand, using miRDB database (ver. 5) hosting predicted miRNA targets based on mirTarget algorithm and mature miRNA sequences derived from miRBase (release 21) (Wong & Wang, 2015), we found several potential miRNAs regulating 3′-UTR of OCT1 mRNA var. 1 including hsa-miR-3169, hsa-miR-6751–5p, hsa-miR-6803–5p and hsa-miR-6806–5p. In this case, prediction score above 60 was selected as the confidence threshold for potentially functional miRNA target. Next, miRanda prediction algorithm (Betel et al., 2008) (release 2010) uses mature miRNA sequences from miRBase (release 15) identified hsa-miR-544 to target 3′-UTR of OCT1 mRNA.

Interestingly, it is well known that more than one miRNA may regulate target gene (Wilczynska & Bushell, 2015). Since the levels of both miRNAs (Sassen et al., 2008) and OCT1 change during different pathological states, e.g. cancer, we can speculate that changes in miRNAs expression could have an impact on OCT1 expression during various diseases. Taken together, further studies are needed to reveal the role of miRNAs in the regulation of *SLC22A1* gene expression and in OCT1-mediated drug disposition and response.

Post-translational modulation of OCT1 activity

It was found that post-translational modification of human OCT1 protein by either cAMP-dependent Protein Kinase (PKA), calmodulin, calmodulin-dependent kinase II, and p56(lck) tyrosine kinase inhibited or activated OCT1 function, respectively, in Chinese hamster ovary cells (CHO-hOCT1) and in human embryonic kidney cells (HEK293-hOCT1) (Ciarimboli et al., 2004). It has also been demonstrated that protein kinase C (PKC)-dependent phosphorylation of the large intracellular loop of rat Oct1 alters the affinity of some organic cations to inhibit uptake of the fluorescent cationic substrate 4-(4-(dimethylamino)styryl)N-methylpyridinium (ASP+) (Ciarimboli et al., 2005).

Discussion and conclusions

Enormous effort has been spent to identify both exogenous and endogenous substrates of OCT1 transporter to understand the physiological and toxicological significance of the polyspecific transporter. Nevertheless, understanding of its liver-dominant regulation during short-term exposure to toxicants or after long-term adaptations and under pathological conditions may also help us to understand the role of OCT1 in liver physiology. Nowadays, it is clear that the regulation of the transporter is complex and that transcription regulation, epigenetics as well as post-translational regulation of OCT1 transporter contributes to hepatospecific pattern of expression and to its huge interindividual variability in hepatic activity and expression.

Expression and transport activity of OCT1 influence the pharmacokinetics of drugs that are transported by OCT1 into the hepatocyte for metabolic deactivation, prodrug metabolic activation or to act in hepatocytes as their target tissue. Thus, therapeutic dose–response and toxicity is influenced by the functional activity of OCT1 and the activity is essential to achieve therapeutic effect for drugs targeting hepatocytes. In addition, OCT1 may be the site of clinically significant transporter-based interactions of its substrates (such as metformin) and inhibitors even though FDA guidance considers only renal OCT2 as an important transporter with this respect (FDA Guidance for Industry, Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, February 2012).

In the review, we demonstrate OCT1 transporter as unique hepatic drug-handling factor with specific basal expression gene regulation in hepatocytes through HNF4α and C/EBP transactivation. In addition, we summarize recent findings regarding indirect down-regulation mechanisms of *SLC22A1* gene expression by

farnesoid X and pregnane X receptors and indirect up-regulation mechanism by activated glucocorticoid receptor in human hepatocytes. This regulation of *SLC22A1* gene may be used as a pattern of hepatocyte-specific gene regulation in normal liver tissue for other hepatic drug transporters or DMEs. Although we have made considerable progress in our understanding of *SLC22A1* gene expression in the liver, prediction of inter-individual OCT1 activity and explanation of huge variability in OCT1 expression is still elusive.

We can conclude that further studies are needed to comprehensively describe mechanisms, transcription factors, epigenetic factors and post-transcriptional and post-translational factors regulating expression of *SLC22A1* gene in the human liver. On the other hand, we have to examine those factors that suppress the expression of *SLC22A1* gene in non-hepatic tissues and in tumor cells.

Disclosure statement

The authors report no declarations of interest. This work was funded by the Czech Scientific Agency ([GACR 303/12/G163] to P.P.) and by SVV project 260 293.

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8.3 *Trans*-resveratrol but not other natural stilbenes occuring in food, carries the risk of drug-food interaction via inhibition of cytochrome P450 enzymes or interaction with xenosensor receptors.

Hyrsova L, Vanduchova A, Dusek J, Smutny T, Carazo A, Becicova V, Trejtnar F, Barta P, Anzenbacher P, Dvorak Z and Pavek P (2018) *Trans*-resveratrol but not other natural stilbenes occuring in food, carries the risk of drug-food interaction via inhibition of cytochrome P450 enzymes or interaction with xenosensor receptors. *Toxicology Letters* https://doi.org/10.1016/j.toxlet.2018.10.028.

(IF 2017: **3,166**)

Resveratrol byl v odborné literatuře zmiňován také jako inhibitor PXR. V této práci jsme testovali antagonismus resveratrolu a dalších strukturálně podobných stilbenoidů několika novými přístupy a metodami včetně TR-FRET nebo vyhodnocení genových reportérových experimentů pomocí poměru mezi luciferázovou aktivitou lyzátu buněk s kotransfekovaným PXR a bez tohoto receptoru kvůli inhibičnímu působení stilbenoidů na světluškovou a *Renilla* luciferázu.

Testováno bylo 13 látek přírodního i syntetického původu včetně několika stereoizomerů, jmenovitě se jedná o *trans*- a *cis*-resveratrol, a,b-dihydroresveratrol, *trans*-3,4,5,4'−tetramethoxystilben, *trans*-2,4,3',5'-tetramethoxystilben, *trans*-4-methoxystilben, *trans*- a *cis*-piceatanol, oxyresveratrol, *trans*- a *cis*-trismethoxyresveratrol, pterostilben a pinostilben. Stanovovali jsme potenciál stilbenů zvyšovat expresi cílových genů PXR a CAR v primárních lidských hepatocytech, stejně tak i jejich schopnosti interagovat s rekombinantní ligand vázající doménou PXR i jejich vliv na aktivaci reportérového plazmidu v genových reportérových experimentech. V následující části projektu jsme se zabývali zkoumáním inhibičního vlivu stilbenů na lidské mikrozomální enzymy cytochromu P450.

Zjistili jsme, že přírodní látky s výjimkou pterostilbenu a *trans*-4-methoxystilbenu nejsou ligandy PXR. Ze syntetických látek jsou slabými agonisty PXR *trans*-2,4,3',5'-tetramethoxystilben a *trans*- a *cis*-trismethoxyresveratrol. V případě CAR projevil pouze *cis*-trismethoxyresveratrol slabé schopnosti interagovat s CAR LBD. Dále jsme prokázali, že přírodní stilbeny inhibují enzymatickou aktivitu několika enzymů CYP450. Ačkoliv pouze v případě *trans*-resveratrolu byla enzymová aktivita inhibována už při mikromolárních koncentracích, což může vyústit v potenciálně závažné interakce mezi potravními doplňky a léčivy.

Celkově lze shrnout, že mezi 13 testovanými látkami stilbenoidní struktury nebyl nalezen žádný antagonista PXR.

Toxicology Letters xxx (2018) xxx-xxx

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com

Trans-resveratrol, but not other natural stilbenes occurring in food, carries the risk of drug-food interaction via inhibition of cytochrome P450 enzymes or interaction with xenosensor receptors

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ARTICLE INFO

Keywords:
Stilbenes
Pregnane X receptor
Drug metabolism
Cytochrome P450
Resveratrol
Food-drug interactions

ABSTRACT

Resveratrol (RSV) is a stilbene phytochemical common in food and red wine. RSV inhibits cytochrome P450 CYP3A4 activity and interacts with the pregnane X receptor (PXR), the central regulator of drug/xenobiotic metabolizing enzyme expression. In this work, we comprehensively examined the effects of 13 stilbenes (*trans*- and *cis*-resveratrol, *trans*- and *cis*-piceatannol, oxyresveratrol, pterostilbene, pinostilbene, a,b-dihydroresveratrol, *trans*- and *cis*-trismethoxyresveratrol, *trans*-3,4,5,4′-tetramethoxystilbene, *trans*-2,4,3′,5′-tetramethoxystilbene, *trans*-4-methoxystilbene), on CYP3A4 and CYP2B6 mRNA induction, and on CYP3A4/5, CYP2C8/9/19, CYP2D6, CYP2A6, CYP2E1, CYP1A2 and CYP2B6 cytochrome P450 enzyme activities. Expression experiments in five different primary human hepatocyte preparations, reporter gene assays, and ligand binding assays with pregnane X (PXR) and constitutive androstane (CAR) receptors were performed. Inhibition of cytochrome P450 enzymes was examined in human microsomes. We found that only polymethoxylated stilbenes are prone to significantly induce CYP2B6 or CYP3A4 in primary human hepatocytes via pregnane X receptor (PXR) interaction. Natural resveratrol derivatives such as *trans*- and *cis*-RSV, oxyresveratrol, pinostilbene and pterostilbene significantly inhibit CYP3A4/5 enzymatic activities; however, only *trans*-RSV significantly inhibits CYP3A4/5 activity (both testosterone 6β-hydroxylation and midazolam 1′hydroxylation) in micromolar concentrations by a non-competitive mechanism, suggesting a potential risk of food-drug interactions with CYP3A4/5 substrates.

1. Introduction

Natural stilbenes are synthesized by dozens of plants (ISVV Polyphenols reference database, 2017; Riviere et al., 2012), with the most studied of these phytochemicals being the compound resveratrol (RSV, *trans*(E)-resveratrol, trihydroxystilbene, Fig. 1A and B). RSV is a common constituent of the average diet and is found in many dietary supplements as well. It is present in red wine, in the skin of grapes, in blueberries, in peanuts and pistachios, in grape and cranberry juices as well as in many other vegetable products (Bavaresco et al., 2016).

Inhibition of drug metabolizing enzymes by RSV have been repeatedly described, including the inhibition of the enzymes CYP1A1, CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Basheer et al., 2017; Chow et al., 2010; Piver et al., 2001; Yu et al., 2003). RSV has been reported to irreversibly (mechanism-based) inhibit the in vitro activity of the dominant drug-metabolizing enzyme CYP3A4. Importantly, RSV has been shown to affect the metabolism of the CYP3A4

Abbreviations: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; FRET, fluorescence resonance energy transfer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; MDZ, midazolam; PXR, pregnane X receptor; PXR-LBD, PXR ligand binding domain; RSV, resveratrol; TST, testosterone.

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https://doi.org/10.1016/j.toxlet.2018.10.028
Received 13 July 2018; Received in revised form 3 September 2018; Accepted 8 October 2018
Available online xxx
0378-4274/

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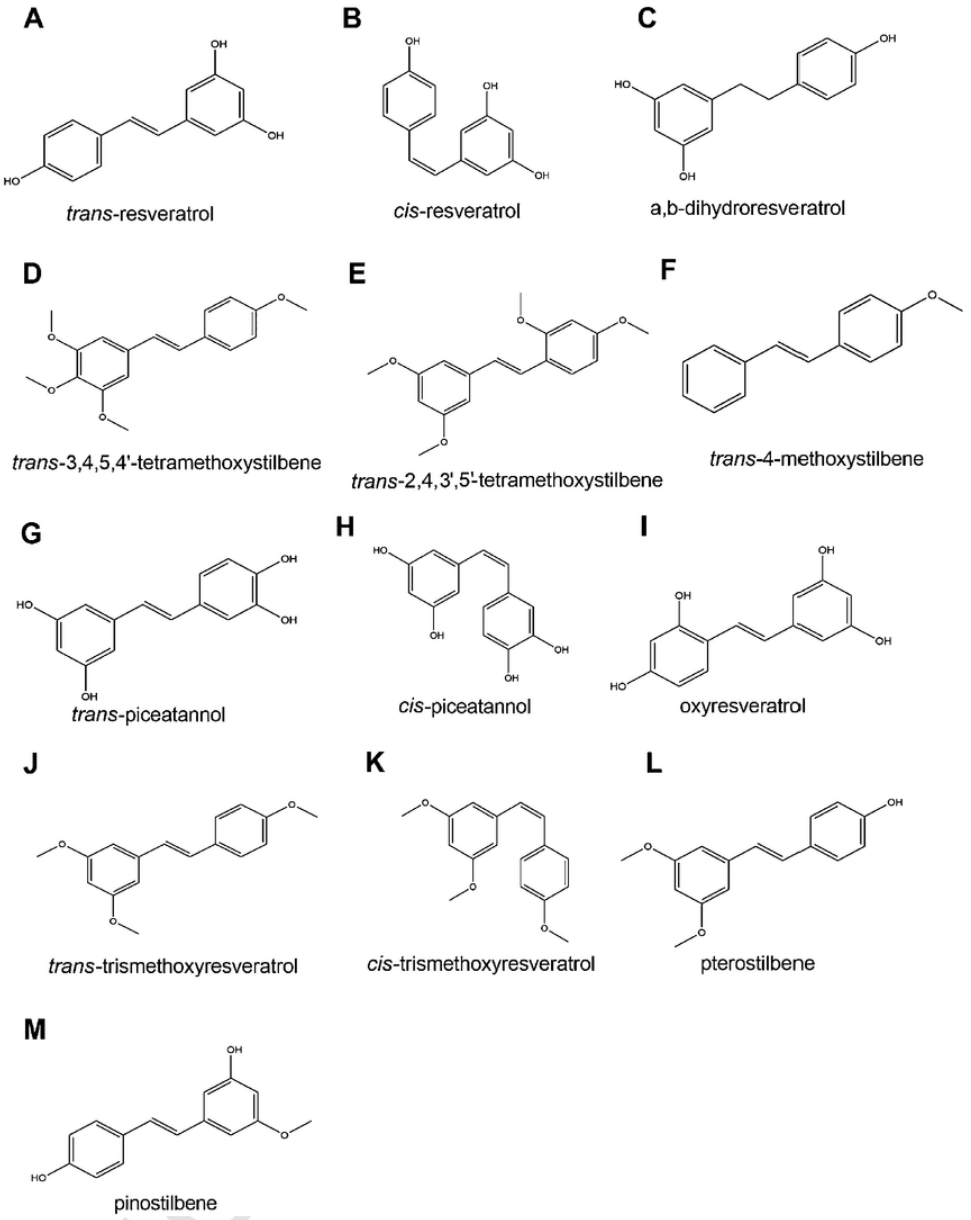

Fig. 1. Structure of the tested stilbenes.

substrates buspirone and carbamazepine in humans, even though RSV has been considered as a moderate low-affinity CYP3A4 inhibitor (IC_{50} = 1.0–6.8 μM) (Bedada and Nearati, 2015; Detampel et al., 2012; Chow et al., 2010).

In addition to *trans*-RSV, *cis*-resveratrol, *trans*-piceatannol, oxyresveratrol, pterostilbene and pinostilbene are naturally occurring stilbenoids present in food. *Cis*-resveratrol is mainly present in red wine together with its isomer; piceatannols are present in red wine and in northern highbush blueberry (Rimando and Mikstacka, 2005); and oxyresveratrol is present in mulberries and in grapes and peanuts (Song et al., 2009). Pterostilbene is present in grapes, peanuts, almonds, pine and vaccinium berries (Rimando and Mikstacka, 2005). a,b-Dihydroresveratrol is a metabolite of resveratrol (Walle, 2011). Pinostilbene is present in *Leopoldia comosa* bulbs, a traditional Italian food (Borgonovo et al., 2008). Methoxylates, stilbenes and resveratrols are naturally occurring compounds in plants (ISVV Polyphenols reference database, 2017). Notably, 2,3′,4,5′-tetramethoxystilbene (TMS) and *trans*-3,4,5,4′-tetramethoxystilbene, an analogue of resveratrol, are considered as potential anticancer drugs (Sale et al., 2004).

Several research groups focused on the interaction between the pregnane X receptor (PXR) and RSV. Some of these studies identified RSV as an agonist of PXR (Dolezelova et al., 2017; Dring et al., 2010; Jacobs et al., 2005; Kluth et al., 2007; Smutny and Pavek, 2014; Yu et al., 2011), with *trans*-RSV being a more potent PXR ligand than the *cis*-isomer (Yu et al., 2011). However, another group has recently reported an antagonistic effect of RSV on PXR activation and CYP3A4/Cyp3a11 expression (Deng et al., 2014).

Nevertheless, any systematic and comprehensive study on induction of CYPs in primary human hepatocytes treated with both natural and synthetic stilbenes has not been done. Importantly, pterostilbene was also shown to activate PXR, a finding which indicates a potential "class effect of stilbenes" on PXR activation (Dring et al., 2010). It was reported recently that 13 stilbenes might activate the aryl hydrocarbon receptor (AhR), a critical transcription factor in cytochrome P450 CYP1 subfamily gene regulation (Pastorkova et al., 2017).

In addition, there have been numerous reports in the literature on the interactions between RSV and CYPs (see review by Detampel et al., 2012); however, no comprehensive study reporting the influence of stilbenes on CYPs activities has as of yet been published. Some stilbenes were individually described as inhibitors of various CYP forms, including pterostilbene, pinostilbene and *trans*-piceatannol that were shown to be inhibitors of CYP1A1, CYP1A2 and CYP1B1 (Mikstacka et al., 2007).

These results indicate that RSV and other stilbenes may affect drug or xenobiotic metabolism if food or a high-dosage dietary supplement with these compounds are taken simultaneously with medication.

Within this work, the effects of 13 stilbenes on CYP3A4 and CYP2B6 mRNA expression in five primary cultures of human hepatocytes were tested, and the interactions of these compounds with PXR and constitutive androstane (CAR) receptor xenosensors in cell-based and cell-independent assays were evaluated. In addition, a comprehensive biochemical analysis of the interaction of these compounds with CYP3A4/5, CYP2C8/9/19, CYP2D6, CYP2A6, CYP2E1, CYP1A2 and CYP2B6 enzymes in human hepatocyte microsomes was performed.

2. Material and methods

2.1. Materials

The stilbene compounds *trans*- and *cis*-resveratrol (referred as to compounds A, B; product codes 70675 and 10004235), a,b-dihydroresveratrol (C; product code 19651), *trans*-2,4,3′,5′,-tetramethoxystilbene (E; product code 10038), *trans*-piceatannol (G, product code 10009366), oxyresveratrol (I; product code 12028), *trans*- and *cis*-trismethoxyresveratrol (J and K; product codes 10188 and 13199), pterostilbene (L; product code 13000) and pinostilbene (M; product code 14044) were purchased from the Cayman Chemical Company (Ann Arbor, MI, USA); *cis*-piceatannol (H; catalog number sc-211111) was acquired from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The stilbene compounds *trans*-3,4,5,4′,-tetramethoxystilbene (D; catalog number SML0963, DMU-212) and *trans*-4-methoxystilbene (F; catalog number S880159) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Plasmids

Expression constructs for PXR, CAR3 and luciferase reporter gene constructs with responsive promoter sequences of CYP2B6 and CYP3A4 genes have been described and used in luciferase gene reporter assays as we described in our previous reports (for details, see *Supplementary material*).

2.3. Cell cultures and primary human hepatocytes

HepG2 cells (European Collection of Authenticated Cell Cultures, Salisbury, UK) were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (NAA), 2 mM glutamine and 1 mM sodium pyruvate (all purchased from Sigma-Aldrich). The cell line was used within 25 passages after delivery.

2.4. Primary human hepatocytes

Five primary human hepatocyte cultures were used in the research. Hepatocytes were isolated from human liver resected from three adult multiorgan donors: HH65 (male, 34 years), HH66 (male, 65 years) and HH71 (male, 58 years), with the applicable preparations, cultivation and treatment having been described in a previous report (Pastorkova et al., 2017). The tissue acquisition protocol was in accordance with the requirements stated by the local ethics commission in the Czech Republic and The Code of Ethics of the World Medical Association. In addition, two commercial cultures of long-term human hepatocytes in monolayer were purchased from Biopredic International (Rennes, France). Batch No. Hep200525 was prepared from the liver biopsy of a 44-year-old male and Batch No. Hep200529 from a 24-year-old female.

2.5. mRNA determination and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated and the levels of CYP3 A4, CYP2B6 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were determined with qRT-PCR using a LightCycler® 480 Instrument II (Roche Diagnostic Corporation, Czech Republic) as described elsewhere (Kamenickova et al., 2013). mRNA determination is described in the *Supplementary materials* section in more details. Measurements were carried out in triplicate and data were processed by the Delta-Delta method.

2.6. Western blotting analyses using the Sally Sue™ system

Protocols for Western blotting experiments using the Sally Sue™ automated system is described in the *Supplementary materials* section in more details. CYP3A4 (mouse monoclonal; sc-53850, HL3) and CYP2B6 (rabbit polyclonal, sc-67224, H-110) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. β-actin (mouse monoclonal; 3700S, 8H10D10) primary antibody was obtained from Cell Signaling Technology (Danvers, Massachusetts, USA).

2.7. Effect of tested stilbenoids on firefly and Renilla luciferase activities

In the case of testing of inhibitory activities of stilbenoids on luciferase activities, HepG2 cells were seeded into 48-well plates and transfected 24 h later with the Firefly luciferase expression vector pGL3-promoter or pRL-TK vector for Renilla luciferase, respectively. Firefly and Renilla luciferase cDNAs in these constructs are under control of strong viral promoters. Then, transfected cells were lysed without treatment after 24 h, and all the substances were added to lysate 10 min before reading. The lysis of cells and luminescence measurements were performed with Dual-Luciferase® Reporter Assay System kit reagents (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol.

2.8. Transient transfection and gene reporter assays

Transient transfection experiments were performed using Lipofectamine™ 2000 after 24 h cultivation according to the protocol described in our previous reports (Hyrsova et al., 2016; Rulcova et al., 2010). Plasmids, transient transfection and gene reporter assays are described in more detail in the *Supplementary materials* section.

2.9. PXR and CAR TR-FRET ligand binding assays

The LanthaScreen™ TR-FRET PXR Competitive Binding Assay and CAR Coactivator Binding Assay (Thermo Fisher Scientific, Waltham, MA, USA) were used as described in our previous reports (Carazo and Pavek, 2015; Smutny et al., 2014). These ligand binding assays are described in more detail in the *Supplementary materials* section.

2.10. Determination of P450 activities

All activities of individual CYP forms (CYP3A4/5, CYP2C8/9/19, CYP2D6, CYP2A6, CYP2E1, CYP1A2 and CYP2B6) were determined according to established protocols (Chang and Waxman, 2006). The following microsomal CYP activities were assayed: CYP1A2, 7ethoxyresorufin Odeethylation (EROD) (Chang TKH, 1998); CYP2A6, coumarin 7-hydroxylation (Chang and Waxman, 1998); CYP2B6, 7-ethoxy4(trifluoromethyl)coumarin 7-deethylation (Morse and Lu, 1998); CYP2C8, paclitaxel 6hydroxylation (Crespi et al., 2006); CYP2C9, diclofenac 4′-hydroxylation (Crespi CL and Waxman, 1998); CYP2C19, S-mephenytoin 6hydroxylation (www.cypex.co.uk/2c19info.htm); CYP2D6, bufuralol 1′-hydroxylation; CYP2E1, chlorzoxazone 6hydroxylation (Lucas et al., 1996) and CYP3A4, testosterone 6β-hydroxylation (Guengerich et al., 1986) and midazolam 1′hydroxylation (Kronbach et al., 1989). High-performance liquid chromatography analyses have been performed as described in *Supplementary material* section.

2.11. Inhibition of P450 enzyme activities in the human liver microsomal fraction

For each P450 enzyme inhibition assay, the Michaelis constant (K_m) and maximum velocity (V_{max}) values were determined to obtain the substrate concentration suitable for the inhibition experiments. Substrate concentration was chosen in the range corresponding to the value of the K_m. Inhibition assays were routinely performed with six concentrations of stilbenoids (5; 10; 25; 50; 75; 100 μM) and stilbenoid-free control (buffer control). For details see *Supplementary material.*

2.12. Statistical analysis

All of the statistical analyses were performed using GraphPad PRISM 7 software (GraphPad Software Inc., San Diego, CA, USA). ANOVA with Dunnett's *post hoc* test was use in statistical analysis of data from luciferase gene reporter experiments and from TR-FRET assays. Differences between means of two groups (a stilbene-treated and vehicle-treated) were compared using paired Student's *t*-test (two-tailed) in induction experiment with primary human hepatocytes due to high variability in target gene expression. A P-value of <0.05 was considered to be statistically significant. All data are presented as the mean ± standard deviations (SDs) based on at least three independent experiments ($n = 3$).

3. Results

3.1. Stilbene phytochemicals increase the level of cytochromes P450 CYP3A4 and CYP2B6 mRNAs in primary human hepatocytes

At first, 13 stilbene compounds A–M (Fig. 1) were tested to up-regulate *CYP3A4* and *CYP2B6* gene mRNAs in the set of primary hepatocytes obtained from five different human donors. Among 13 stilbenes, four compounds such as *trans*-3,4,5,4′-tetramethoxystilbene (D), *trans*-2,4,3′,5′-tetramethoxystilbene (E), *trans*-trismethoxyresveratrol (J) and *cis*-trismethoxyresveratrol (K) were observed to induce CYP3A4 mRNA (Fig. 2A), but the up-regulation was not statistically significant. Six stilbenes significantly induced CYP2B6 mRNA (Fig. 2B). Namely, substances *trans*-3,4,5,4′-tetramethoxystilbene (D), *trans*-2,4,3′,5′-tetramethoxystilbene (E), *trans*-4-methoxystilbene (F), *trans*-trismethoxyresveratrol (J), *cis*-trismethoxyresveratrol (K) and pterostilbene (L) showed statistically significant up-regulation of CYP2B6 mRNA after treatment with 100 μM, compared to untreated cells (Fig. 2B). Importantly, pterostilbene (L), a compound present in food, displayed induction of CYP2B6 mRNA when analyzed at 100 μM concentration.

Consistently, higher levels of CYP3A4 protein in three hepatocyte preparations treated with compounds D, E and F were observed compared to untreated cells (Fig. 2C). Similarly, increased levels of CYP2B6 protein were observed in cells treated with substances D, E, F, J, K and L (Fig. 2D). However, none of the protein up-regulation was statistically significant (Fig. 2C and D), due to high interindividual variability in the CYPs expression, which is typical in human hepatocytes.

Trans-RSV and *cis*-RSV did not induce CYP3A4 or CYP2B6 expression in human hepatocytes questioning the significant activation of PXR (Fig. 2A and B) (Yu et al., 2011).

3.2. Interaction of selected stilbene phytochemicals with PXR and CAR nuclear receptors

Based on these results, stilbenes D, E, F, J, K and L were tested for their possible potency to interact with the PXR receptor in the following TR-FRET or luciferase reporter gene experiments. Activation of CAR and its variant CAR3 was also considered. Rifampicin, SR-12813 (SR) or CITCO were used as prototype PXR or CAR/CAR3 ligands, respectively.

Tested compounds showed significant potency to interact with PXR-LBD in the TR-FRET assay (Fig. 3A) except for of *trans*-3,4,5,4′-tetramethoxystilbene (D). In the case of CAR, only *cis*-trismethoxyresveratrol (K) significantly activated CAR-LBD by approximately 1.5-fold at 30 μM concentration in the CAR TR-FRET assay (Fig. 3B).

Next, luciferase gene reporter assays were employed to evaluate the activation of PXR and CAR by tested stilbenes in cellular assays (Fig. 3C and D). In the experiments, only compound E (*trans*-2,4,3′,5′-tetramethoxystilbene) and K (*cis*-trismethoxyresveratrol) had a significant effect on PXR (2.6 and 2.5-fold, respectively), and compound K at the same time activated CAR3 (1.5-fold) (Fig. 3C and D).

Before that the inhibitory effect of tested stilbenes on both firefly luciferase and *Renilla* activities was evaluated under our experimental conditions (Fig. 3E and F). In preliminary experiments, a significant influence of all compounds on firefly luciferase activity and the effects of J and K compounds on *Renilla* luciferase activity was observed (Fig. 3E and F). Therefore, luciferase gene reporter assays were performed according to a modified protocol to eliminate the inhibitory activities of tested stilbenes (see Material and methods section). Cell viability assays were performed, but no cytotoxicity was observed after either 24 or 48 h (*data not shown*).

These data indicate that methoxylated stilbenes are prone to activate PXR or CAR nuclear receptors and induce their target genes

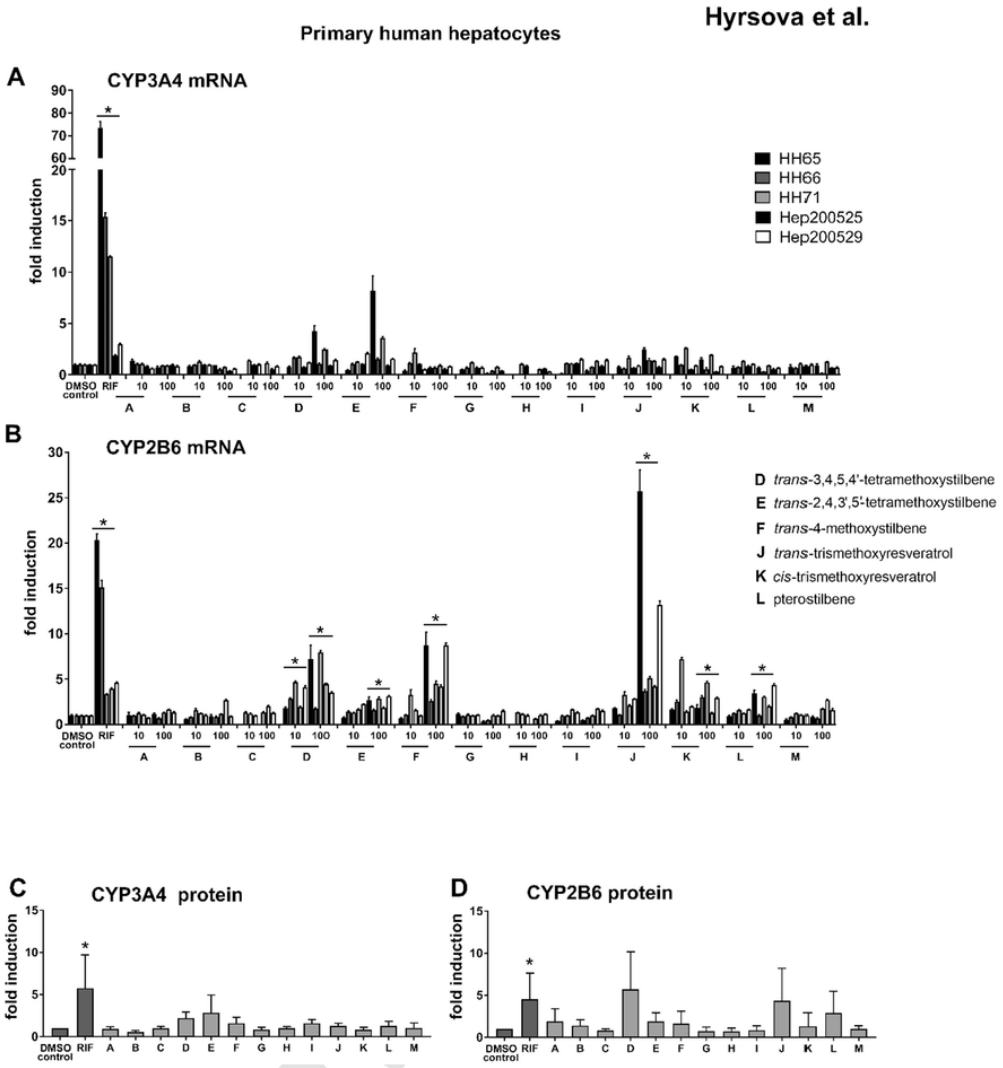

Fig. 2. Expression of *CYP3A4* mRNA (panel A) and *CYP2B6* mRNA (panel B) and proteins (panels C and D) in five different primary human hepatocyte preparations (HH65, HH66, HH71, Hep200525 and Hep200529) after treatment with the stilbenes. The cells were treated with the stilbenes at 10 μM or 100 μM concentration for 24 h. Rifampicin (RIF, 10 μM) was used as a prototype inducer and PXR ligand. qRT-PCR and Sally Sue-based western blotting were employed. The vehicle-treated control hepatocytes (DMSO control) were set as 1 in both qRT-PCR and western blotting data; all other results were normalized to this value and are expressed as fold induction to control. Significantly different compared to the vehicle-treated control, $^{*}P < 0.05$.*trans-* and *cis-*resveratrol (referred as to compounds A, B), a,b-dihydroresveratrol (C), *trans*-3,4,5,4′-tetramethoxystilbene (D), *trans*-2,4,3′,5′-tetramethoxystilbene (E), *trans*-4-methoxystilbene (F), *trans*-piceatannol (G), *cis*-piceatannol (H), oxyresveratrol (I), *trans-* and *cis*-trismethoxyresveratrol (J and K), pterostilbene (L) and pinostilbene (M).

CYP3A4 and CYP2B6 in primary human hepatocytes. The only exception was pinostilbene (3,4′-dihydroxy-5-methoxystilbene or 3-methoxyresveratrol, compound L), which neither induces CYP3A4 and CYP2B6 mRNAs nor interacts with the tested nuclear receptors (Fig. 2 and 3). Importantly, we observed that of the inducers, only *trans*-3,4,5,4′-tetramethoxystilbene (compound D, DMU-212) up-regulates CYP2B6 mRNA at lower than 10 μM concentration, which might be therapeutically relevant during potential anticancer therapy with the synthetic stilbenoid DMU-212 (Sale et al., 2004).

3.3. Effects of stilbenes on catalytic activities of the most important cytochrome P450 drug-metabolizing enzymes in human liver microsomes

Next, we studied effects of ten stilbenes on activities of nine CYP enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5). IC_{50} values and information about the mechanism of the inhibition and respective K_i values were evaluated in these experiments.

The most significant inhibition of a CYP activity was observed for CYP3A4/5 testosterone (TST) 6βhydroxylation (Fig. 4A). The enzyme activity was almost completely inhibited by all studied stilbenes at 100 μM concentration except for pterostilbene (L) and *cis-* and *trans*trismethoxyresveratrol (K and J) (Fig. 4A). In another experiment, midazolam (MDZ) was used as a CYP3A4/5 substrate (Fig. 4B). In this case, CYP3A4/5 activity was reduced by all tested compounds except for *cis-* and *trans*trismethoxyresveratrol (K and J) (Fig. 4B). However, the inhibitory effects were not as prominent compared to results with testosterone (Fig. 4A).

Next, we studied in detail the enzymatic inhibition of CYP3A4/5 activity by *trans*-RSV, which was found to be the most potent CYP3A4/5 inhibitor, with IC_{50} <20 μM (Fig. 4A and B). These assays were re-

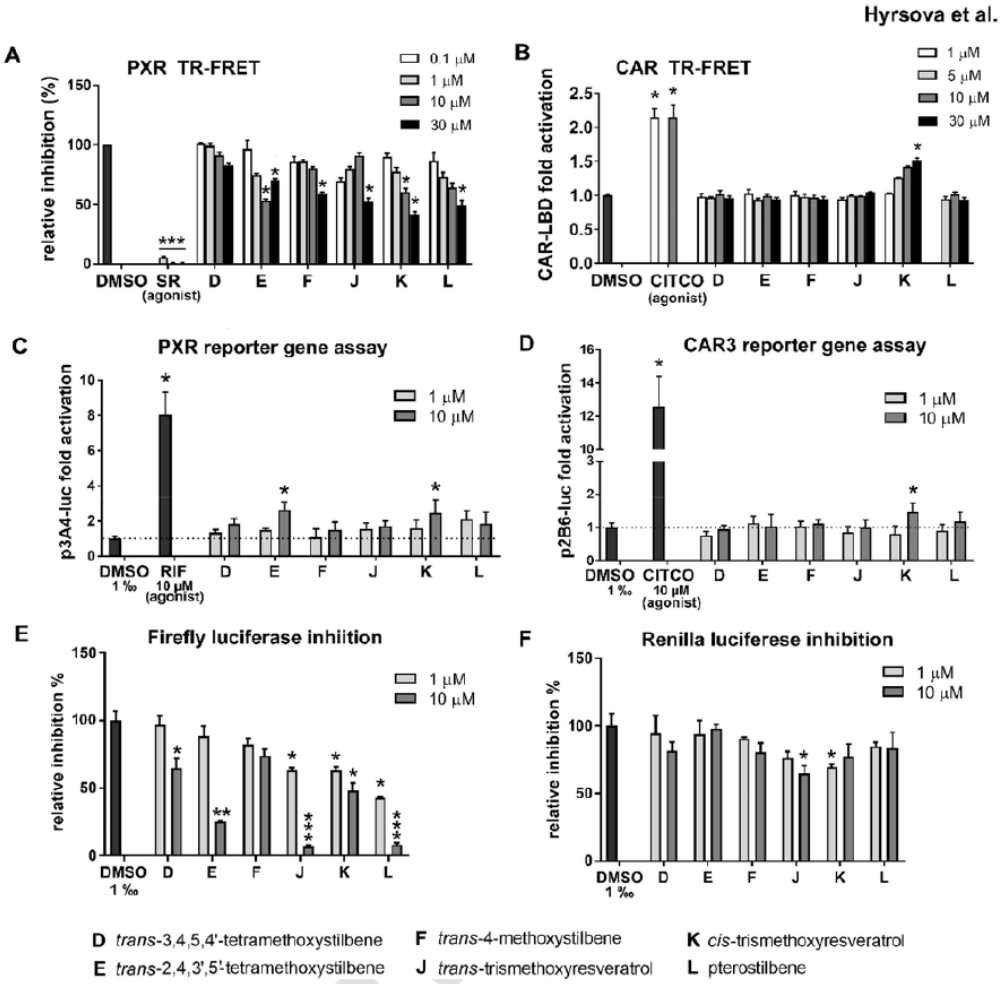

Fig. 3. Interaction of tested stilbenes with PXR and CAR were examined using Lantha™ TR-FRET assays with human PXR-LBD (panel A) and CAR-LBD (panel B). Gene reporter assays with PXR- (panel C) and CAR3- (panel D) responsive luciferase constructs were performed in transiently transfected HepG2 cells. *Trans*-3,4,5,4′-tetramethoxystilbene (D), *trans*-2,4,3′,5′-tetramethoxystilbene (E), *trans*-4-methoxystilbene (F), *trans*- and *cis*-trismethoxyresveratrol (J and K), and pterostilbene (L) were tested at the indicated concentrations and compared with the standard agonists of PXR SR12183 (SR) and rifampicin (RIF), or with the CAR3 receptor ligand CITCO. All results are related to vehicle-treated (DMSO) controls, set at 100% or 1. Data are presented as the means and S.D. of fold activation to controls or the relative inhibition calculated based on three independent experiments (n = 3). Significantly different compared to the vehicle-treated control, $*P < 0.05$, $***P < 0.001$ (ANOVA with Dunnett's *post hoc* test).Inhibition of firefly (panel E) and *Renilla* (panel F) luciferase activities by tested stilbenes. HepG2 cells were transfected with pGL3-promoter, an expression vector for firefly luciferase (panel E) and with pRL-TK, an expression vector for *Renilla* luciferase (panel F). Tested compounds were added directly to cell lysate 10 min before reading luciferase activity. All tested stilbenes were used at 1 µM or 10 µM concentrations, respectively, in three independent experiments ($n = 3$). Control (DMSO 1‰ (v/v))-treated cells were set as 100%.

peated with three concentrations of specific substrate (corresponding to ½K_m, K_m and 2K_m). Dixon and Lineweaver-Burk plots of *trans*-RSV inhibition on CYP3A4/5 enzymatic activities were fitted and indicated basic non-competitive inhibition with $K_i = 5.5$ µM for midazolam and $K_i = 1.5$ µM for substrate testosterone, respectively (Fig. 4C–F).

Inhibition of CYP2C19 enzyme activity, S-mephenytoin 6-hydroxylation, was the most prominent with pinostilbene (M) and pterostilbene (L) (with $IC_{50} = 12$ µM for pinostilbene and $IC_{50} = 13.2$ µM for pterostilbene, respectively) (Table 1 and Fig. 5). For these compounds, Dixon and Lineweaver-Burk plots fit well for the typical course of mixed type of inhibition, with $K_i = 72.7$ µM for pinostilbene and $K_i = 26.3$ µM for pterostilbene, respectively (Table 2). In most experiments, we did not observe inhibition of CYP enzymatic activities by tested stilbenes or weak inhibition in higher concentration, which did not allow us to calculate IC_{50} (see Table 1). Thus, these stilbenes does not function as inhibitors of CYPs.

In the case of 7-ethoxyresorufin O-demethylation assay for CYP1A2 activity, oxyresveratrol (I), *trans*-resveratrol (A), pterostilbene (L), pinostilbene (M) and *cis*- and *trans*-piceatannol (H and G) significantly reduced the enzymatic activity; IC_{50} values for oxyresveratrol and *trans*resveratrol were 5.9 and 7.8 µM, respectively (Table 1 and Fig. 5). Dixon and Lineweaver-Burk plots characterized mixed type of inhibition for *trans*-resveratrol with $K_i = 38.8$ µM and noncompetitive type of inhibition for oxyresveratrol with $K_i = 35.7$ µM (Table 2). Dihydroresveratrol (C) and *cis*-trismethoxyresveratrol (K) did not affect CYP1A2 activity at all.

Activity of CYP2C8 was inhibited by oxyresveratrol (I) with $IC_{50} = 19.4$ µM and by *trans*-resveratrol (A) with $IC_{50} = 17.2$ µM; the type of inhibition was characterized as competitive $K_i = 33.3$ µM and mixed $K_i = 26$ µM, respectively (Table 1 and 2).

All compounds studied exhibited weak inhibitory effects on CYP2B6 enzyme activity. Decreased activity (approximately 50%) of inhibitor-

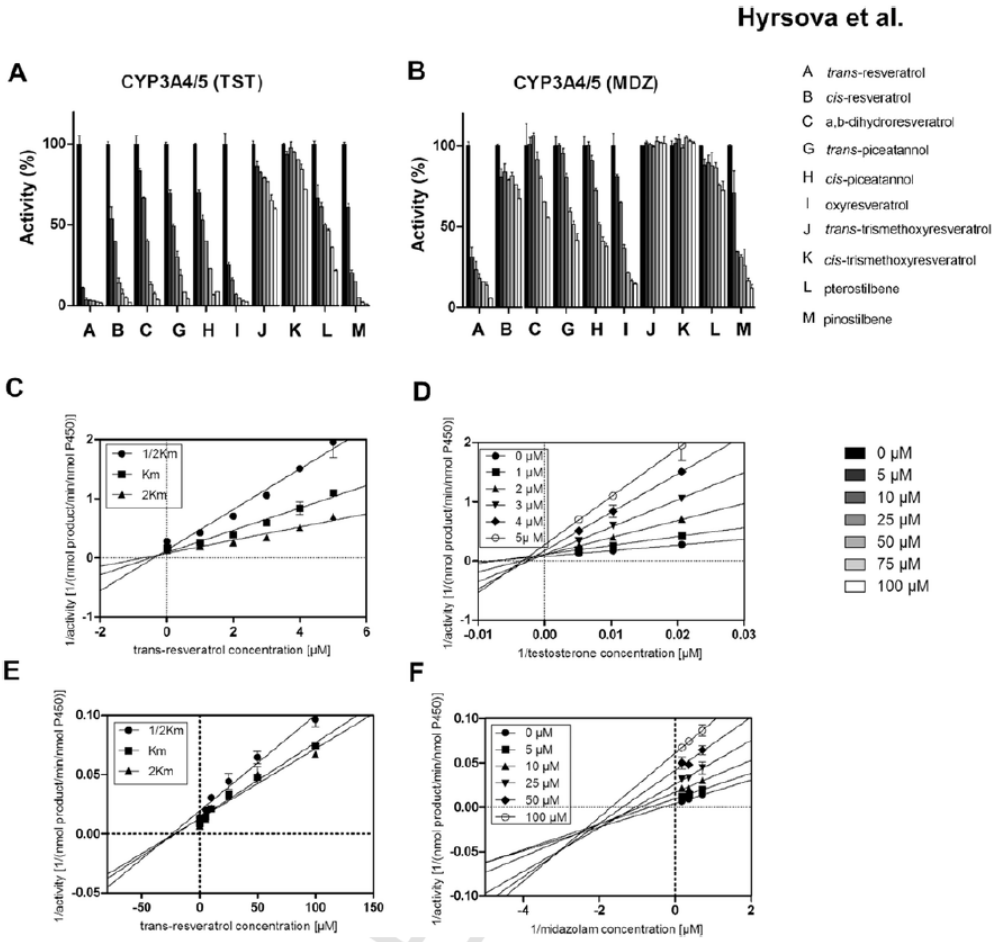

Fig. 4. Inhibitory effects of stilbenes on CYP3A4/5 activity in human liver microsomes examined with testosterone (TST) (panel A) and midazolam (MDZ) (panel B) CYP3A4/5 substrates. Dixon (panels C and E) and Lineweaver-Burk (panels D and F) plots for CYP3A4/5 activity in the presence of *trans*-resveratrol are shown. CYP3A4/5 substrate testosterone (panels C and D) and midazolam (panels E and F) were used.*trans*- and *cis*-resveratrol (A and B), a,b-dihydroresveratrol (C), *trans*-3,4,5,4′-tetramethoxystilbene (D), *trans*-2,4,3′,5′-tetramethoxystilbene (E), *trans*-4-methoxystilbene (F), *trans*-piceatannol (G), *cis*-piceatannol (H), oxyresveratrol (I), *trans*- and *cis*-trismethoxyresveratrol (J and K), pterostilbene (L) and pinostilbene (M).

Table 1
Inhibitory effects of stilbenes expressed as IC_{50} values on CYPs enzymatic activities in human liver microsomes.

Cytochrome P450	1A2	2A6	2B6	2C8	2C9	2C19	2D6	2E1	3A4/5 TST	3A4/5 MDZ
Studied compound	IC_{50} (μM)									
trans-resveratrol (A)	7.8	–	–	17.2	–	22.4	–	–	2.6	1.6
cis-resveratrol (B)	–	–	–	–	–	41.1	–	–	4.5	–
dihydroresveratrol (C)	–	–	–	35.6	–	–	–	–	20.6	–
cis-piceatannol (G)	–	–	–	53.9	–	57.9	–	–	8.9	57.4
trans-piceatannol (H)	–	–	–	80.6	–	82.7	–	–	8.4	–
oxyresveratrol (I)	5.9	–	–	19.4	–	28.9	–	–	0.35	14.5
trans-trismethoxyresveratrol (J)	–	–	–	–	–	–	–	–	–	–
cis-trismethoxyresveratrol (K)	–	–	–	–	–	–	–	–	–	–
pterostilbene (L)	–	–	–	–	–	13.2	–	–	7.9	–
pinostilbene (M)	–	–	–	96.3	–	12.0	–	–	5.8	4.6

(–) Indicates an IC_{50} value that is >100 and could not be determined for the experimental set-up; TST, testosterone; MDZ, midazolam.
All data are presented as the mean from three independent experiments ($n = 3$).

free control was observed only for pinostilben (M) and *cis*- and *trans*piceatannol (H and G) (Fig. 5).

Oxyresveratrol (I) and *trans*resveratrol (A) were found to be weak inhibitors of CYP2D6 activity (Fig. 5); other tested compounds caused only marginal effects.

Enzyme activity CYP2C9 was affected by *trans*-resveratrol (A) (it was decreased by approximately 40%). A weak inhibitory effect was observed with pinostilbene (M) and *cis*- and *trans*piceatannol (H and G). CYP2A6 and CYP2E1 enzymatic activities were not changed in the presence of the compounds studied, and only *cis*-piceatannol (H) re-

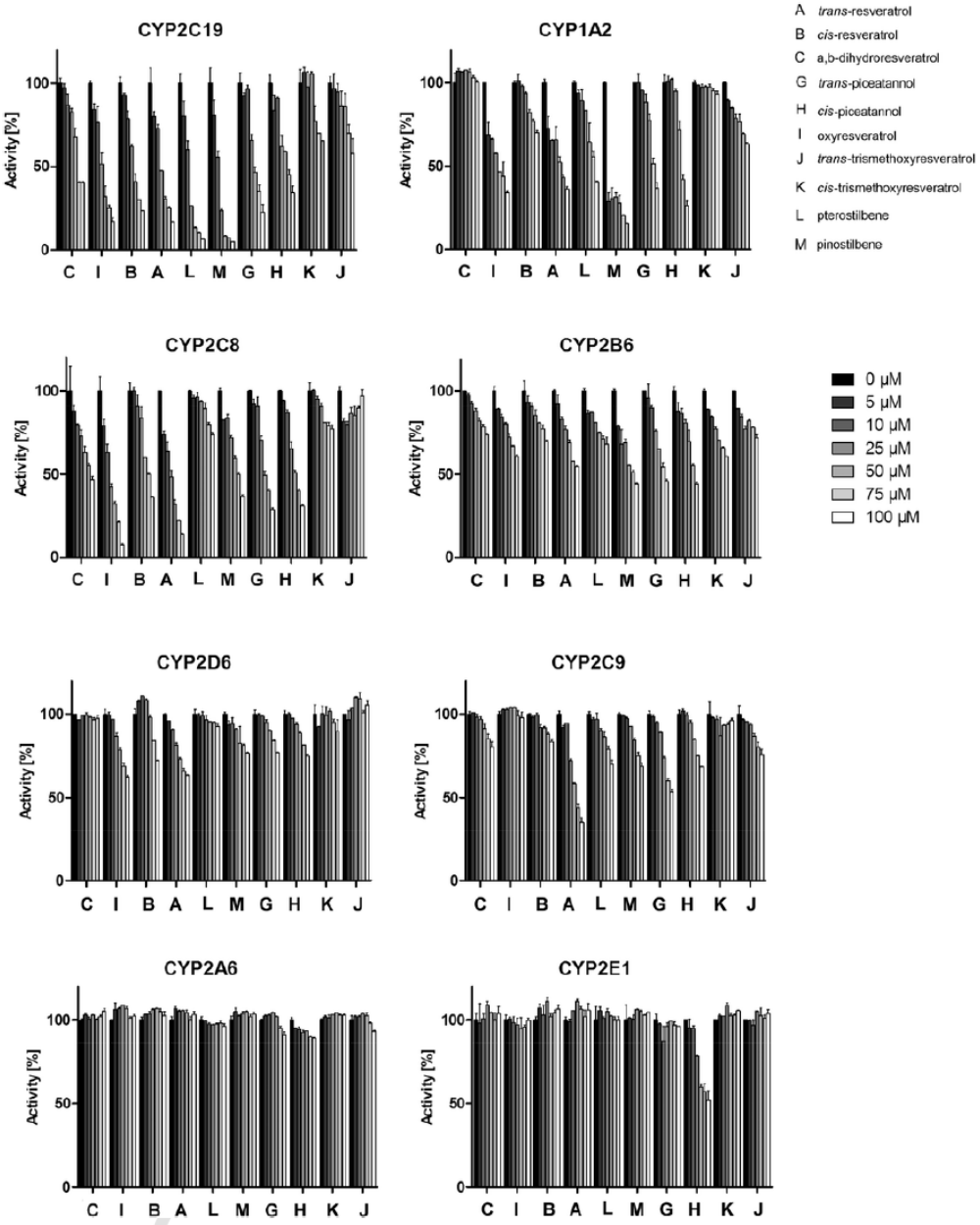

Fig. 5. Effects of tested stilbenes on enzymatic activities of CYP2C19, CYP1A2, CYP2C8, CYP2B6, CYP2D6, CYP2C9, CYP2A6 and CYP2E1 in human microsomes. Inhibitory effects of *trans*- and *cis*-resveratrol (A and B), a,b-dihydroresveratrol (C), *trans*-3,4,5,4′-tetramethoxystilbene (D), *trans*-2,4,3′,5′-tetramethoxystilbene (E), *trans*-4-methoxystilbene (F), *trans*-piceatannol (G), *cis*-piceatannol (H), oxyresveratrol (I), *trans*- and *cis*-trismethoxyresveratrol (J and K), pterostilbene (L) and pinostilbene (M) on CYPs activities in human liver microsomes were examined with specific probes indicated in the *Materials and Methods* section. All data are presented as the mean ± standard deviations (SDs) from at least three independent experiments ($n = 3$).

duced CYP2E1 activity to 52% of control value (Fig. 5). IC_{50} and K_i parameters, as well as the inhibition type by the tested stilbenes, are summarized in Table 1 and Table 2.

4. Discussion

We report herein that polymethoxylated stilbenes are ligands of PXR, but natural stilbenes abundant in food such as *trans*- and *cis*-resveratrol, *cis*-piceatannol, oxyresveratrol and pterostilbene do not significantly induce CYP3A4 or CYP2B6 expression in primary human hepatocytes. In contrast, all tested natural stilbenoids suppressed the enzymatic activities of at least one CYP and most of the tested stilbenes inhibit the CYPs in a non-competitive manner. *Trans*-resveratrol, *cis*-resveratrol, pterostilbene and pinostilbene were found to inhibit CYP3A4 activity with K_i lower than 10 µM. However, only *trans*-RSV significantly inhibits both testosterone 6β-hydroxylation and midazolam 1′hydroxylation CYP3A4/5 activity in micromolar concentrations by a non-competitive mechanism, suggesting a potential risk of food-

Table 2
Inhibition constant K_i and types of inhibition for each stilbene were determined from Dixon plots with three substrate concentrations used.

Tested compound	Cytochrome P450	Type of inhibition	K_i (μM)
trans-resveratrol (A)	CYP3A4/5 MDZ	non-competitive	5.5
	CYP3A4/5 TST	non-competitive	1.5
	CYP2C8	mixed	26.0
	CYP1A2	mixed	38.8
cis-resveratrol (B)	CYP3A4/5 TST	non-competitive	6.2
trans-piceatannol (G)	CYP3A4/5 TST	non-competitive	10.7
cis-piceatannol (H)	CYP3A4/5 TST	non-competitive	14.0
oxyresveratrol (I)	CYP1A2	non-competitive	35.7
	CYP3A4/5 TST	mixed	12.3
	CYP3A4/5 MDZ	non-competitive	19.9
	CYP2C8	competitive	33.3
pterostilbene (L)	CYP3A4/5 TST	competitive	8.7
	CYP2C19	mixed	26.3
pinostilbene (M)	CYP3A4/5 TST	non-competitive	6.7
	CYP3A4/5 MDZ	non-competitive	53.7
	CYP2C19	mixed	72.7

TST, testosterone 6β-hydroxylation; MDZ, midazolam 1′-hydroxylation.
All data are presented as the mean from three independent experiments ($n = 3$).

drug interactions with CYP3A4/5 substrates. In the study, we for the first time tested stilbenes in several different primary human hepatocyte preparations on CYP3A4 and CYP2B6 mRNA induction, we used two CYP3A4 substrates, testosterone and midazolam, in enzymatic assays and we for the first time used non-cellular TR-FRET ligand binding assays for PXR and CAR receptors.

RSV, the most abundant naturally occurring stilbene, is a common constituent of the average everyday diet. In red wine, the average concentration of *trans*-resveratrol and *cis*-resveratrol is approximately 1.9 mg/L (8.3 μM) and 1.0 mg/L, respectively (Detampel et al., 2012); RSV plasma concentration does not exceed 6 ng/mL (0.03 μM/L) after an intake of 600 mL of wine (Vitaglione et al., 2005). However, numerous high-dosage food supplements or medications with RSV are commercially available to the public. A dose of RSV received from such formulations may reach up to 2–5 g/day, and RSV plasma concentrations after a gram dosage of RSV achieve micromolar concentrations (Detampel et al., 2012; Howells et al., 2011). RSV dosage in dietary supplements often highly exceeds the amounts of RSV taken in from natural sources. For instance, naturally occurring amounts of RSV in grape skin are in the range of 5–7 mg/kg, and approximately 1 mg/kg of RSV are found in grape seeds (Detampel et al., 2012). The question thus arises as to whether we can assume an RSV-drug interaction at a standard dosage of RSV in dietary supplements or natural sources. Nevertheless, we can propose portal vein blood concentrations >10 μM after a number of food supplements or after the ingestion of some natural sources. This speculation is based on the observation that the portal vein concentration or local gut concentration often significantly exceeds plasma levels by at least an order of magnitude (Detampel et al., 2012; Kadono et al., 2014). The portal vein or intestinal concentration then corresponds to the concentration to which the hepatocytes or enterocytes are exposed.

The inhibition constant (K_i) is the valuable prediction for assessment of potential drug interactions. If plasma concentrations are greater than K_i, an interaction is likely. Conversely, if the plasma concentrations are less than K_i, an interaction is unlikely (Kakkar et al., 1999). Since we have confirmed that K_i for CYP3A4 of RSV isomers, pterostilbene and pinostilbene are lower than 10 μM (Table 2), the potential food-drug interaction for stilbenes at high doses should be considered both at the level of intestinal as well as hepatic first-pass metabolism.

The inhibitory activity of RSV towards CYP3A4 enzymes discovered in vitro has stimulated several clinical studies with volunteer subjects by other researchers. In these studies, RSV was found to inhibit the phenotypic activities of CYP3A4, CYP2D6, and CYP2C9 (Chow et al., 2010), CYP2E1 (Bedada and Neerati, 2016) and to induce the phenotypic index of CYP1A2 (Chow et al., 2010). RSV-drug pharmacokinetic interactions have been described for buspirone and carbamazepine as well as with caffeine, losartan and dextromethorphan. Based on these clinical studies, authors have concluded that therapeutic doses of RSV can modulate drug metabolism, leading to increased drug toxicity, adverse drug reactions and/or altered drug efficacy (Chow et al., 2010). In contrast, the inhibition of CYP2E1 by RSV has been proposed to elevate the hepatotoxicity of the CYP2E1 substrate ethanol (Bedada and Neerati, 2016). Thus, these pivotal studies with RSV confirm the possibility of food-drug interactions based on molecular interactions with CYPs. On the other hand, only limited data have so far been reported on the interactions of other stilbenes with nuclear receptors or cytochrome P450 enzymes.

RSV is known to be an in vitro inhibitor of CYP3A4 activity in human liver (IC_{50} = 4 μM) and rat liver microsomes (IC_{50} = 20 μM) (Piver et al., 2001). In the present work, two CYP3A4 substrates, testosterone and midazolam, were used for the first time (Table 1). It was found that RSV is able to inhibit the activity of CYP3A4/5, with IC_{50} = 2.6 μM for testosterone and IC_{50} = 1.6 μM for midazolam (Fig. 4A and B); K_i = 1.5 μM for testosterone hydroxylation was calculated using both Dixon and Lineweaver-Burk plots (Fig. 4C–F). In contrast, Piver et al. reported K_i = 25 μM, i.e., one order of magnitude higher than in our study. Interestingly, in the case of other CYP3A enzyme prototypic activity (midazolam 1′hydroxylation), the inhibition of CYP3A4/5 was not as prominent as it was with testosterone. We can speculate about a different mode of binding of midazolam in the active site of CYP3A4 (Krasulova et al., 2016).

As to the other CYP forms, CYP1A2 was inhibited by RSV, with K_i values of 38.8 μM (Table 1 and 2), which is again in agreement with published data (25 μM) (Piver et al., 2003). No inhibition or a very low inhibition of CYP2A6 and CYP2B6 was observed by RSV, which agrees with previously published data (K_i = 150 μM for CYP2A6) (Piver et al., 2003). In contrast to the latter study, no inhibition of CYP2E1 was observed in our current study (Table 1 and 2, Fig. 5) (Piver et al., 2003). This difference may be explained in terms of the different properties of the experimental material (e.g., the use of an in-house bank of human liver microsomes) (Piver et al., 2003) versus the commercial and characterized microsomes used in the present study.

Interestingly, we found that most of the tested stilbenes inhibit the tested CYPs in a non-competitive manner, a finding which should encourage further structural examinations (Table 2). Apart from RSV, *cis*-RSV, (6.2 μM), pinostilbene (6.7 μM), pterostilbene (8.7 μM), *trans*-piceatannol (10.7 μM) and oxyresveratrol (12.3 μM) have their K_i parameters for testosterone 6β-hydroxylation close to 10 μM (Table 2), indicating a theoretical risk of FDI. Therefore, further clinical studies should be conducted to confirm potential of these compounds for DDIs or drug interactions with food supplements.

In addition to inhibition of CYP, the up-regulation of PXR target genes *CYP3A4* and *CYP2B6* mRNA by tested stilbenoids has been investigated in several preparations of human hepatocytes in the research work. We observed the induction of these genes and interactions of methylated stilbenes such as of *trans*-3,4,5,4′-tetramethoxystilbene (D), *trans*-2,4,3′,5′-tetramethoxystilbene (E), *trans*-4-methoxystilbene (F), *trans*-trismethoxyresveratrol (J), *cis*-trismethoxyresveratrol (K) and pterostilbene (L) with PXR-LBD and CAR-LBD, respectively (Fig. 3A and B). Interestingly, a much higher effect of these stilbenes on *CYP2B6* mRNA up-regulation than on the *CYP3A4* gene was observed, even though CYP3A4 is more sensitive to PXR-mediated transactivation than CYP2B6 in primary human hepatocytes. Therefore, we also looked at the interactions of the tested stilbenes with CAR and its low constitutively active variant CAR3, a key transcription factor in *CYP2B6* gene transactivation in human hepatocytes (Auerbach et al., 2005). Only a

weak activation of CAR or its variant CAR3 with *cis*-trismethoxyresveratrol (compound K) was observed (Fig. 3B and D), indicating no effect of CAR activation by the tested stilbenes in primary human hepatocytes (Fig. 2A and B). This phenomenon can also be explained considering activation of the nuclear factor, erythroid 2 like 2 (NRF2) by these compounds in the *CYP2B6* gene promoter activation (Piton et al., 2010). RSV has been shown to activate NRF2 through direct conjugation with the reactive cysteine of KEAP1 (Forman et al., 2014); however, data for other stilbenes are not available. Further research thus should focus on whether methoxylated stilbenes are activators of the NRF2 transcription factor in human CYP enzyme induction. Therefore, clinical studies should confirm potential of methylated stilbenes developed as anticancer drugs such as of *trans*-3,4,5,4′-tetramethoxystilbene (D) and *trans*-2,4,3′,5′-tetramethoxystilbene (E) to cause DDIs.

5. Conclusions

In summary, this is the first detailed study of thirteen stilbene derivatives, including naturally occurring stilbenes in the human diet and their induction of *CYP3A4* and *CYP2B6* mRNA in primary human hepatocytes. The interactions of tested stilbenes with CYP2C8/9/19, CYP2D6, CYP2A6, CYP2E1, CYP1A2 and CYP2B6 enzymes are also reported here. We discuss the potential of RSV and other stilbenes to influence drug metabolism, assuming that *in vivo* CYP3A4-mediated biotransformation may be affected by high doses of these stilbenes present in the diet. Nevertheless, further clinical studies should be conducted to confirm this hypothesis.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

This work was funded by GACR 303/12G163 (to P.P.), EFSA-CDN (No. CZ.02.1.01/0.0/0.0/16_019/0000841) co-funded by ERDF, (GAUK 338315 to L.H.), a student grant from the Palacky University (PrF-2017-004 to Z.D.) and by SVV project 260 414. We thank Ms. Barbora Pastorkova for performing the qRT-PCR analyses.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxlet.2018.10.028.

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8.4 Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor.

Carazo A, **Hyrsova L**, Dusek J, Chodounska H, Horvatova A, Berka K, Bazgier V, Gan-Schreier H, Chamulitrat W, Kudova E and Pavek P (2017) Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor. *Toxicology Letters* 265:86-96.

(IF 2016: **3,858**)

V naší další práci jsme se zabývali možným vlivem acetylace nebo oxidace žlučových kyselin (např. prostřednictvím střevní mikroflóry) na jejich schopnost interagovat s jadernými receptory PXR a VDR, o nichž je známo, že se podílejí na biosyntéze a metabolizmu žlučových kyselin.

V průběhu experimentů jsme zkoumali vliv na reportérové konstrukty v genových reportérových experimentech provedených na HepG2 buněčné linii. Stanovovali jsme také interakce derivátů žlučových kyselin s LBD v nebuněčných experimentech a vliv na expresi cílových genů PXR v diferencované buněčné linii HepaRG. Jako velmi silní agonisté PXR se v reportérových experimentech projevili dvě acetylované žlučové kyseliny, konkrétně 3,7,12-triacetát kyseliny cholové a 3,12-diacetát DCA. 3,12-diacetát DCA navíc i významně zvýšil expresi MDR1, CYP3A4 a CYP2B6.

Výsledky práce lze shrnout tak, že 3,7,12-triacetát kyseliny cholové a 3,12-diacetát DCA by mohly být endogenními ligandy PXR.

Toxicology Letters 265 (2017) 86–96

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Full Length Article

Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of pregnane X (PXR) receptor

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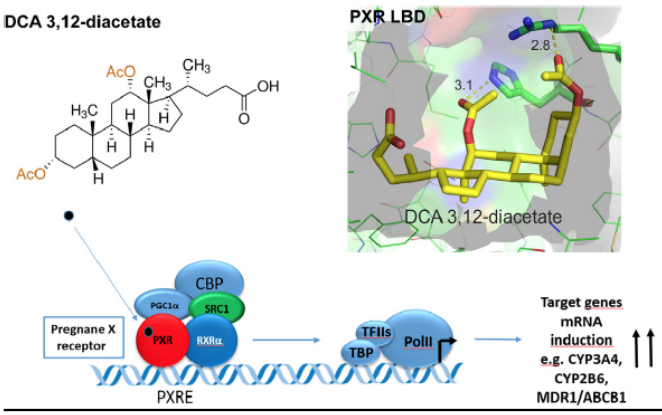
HIGHLIGHTS

- Acetylated deoxycholic (DCA) and cholic (CA) acids are potent ligands of PXR.
- Acetylated DCA and CA enhance PXR target genes expression.
- Dehydrogenation or acetylation of DCA, CA, lithocholic (LCA) or chenodeoxycholic (CDCA) do not lead to increased affinity to FXR or VDR.
- Acetylated DCA and CA were not found in bile.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 12 September 2016
Received in revised form 10 November 2016
Accepted 15 November 2016
Available online 18 November 2016

Keywords:
PXR
Metabolism
Bile acids

ABSTRACT

The Pregnane X (PXR), Vitamin D (VDR) and Farnesoid X (FXR) nuclear receptors have been shown to be receptors of bile acids controlling their detoxification or synthesis. Chenodeoxycholic (CDCA) and lithocholic (LCA) acids are ligands of FXR and VDR, respectively, whereas 3-keto and acetylated derivates of LCA have been described as ligands for all three receptors.

In this study, we hypothesized that oxidation or acetylation at position 3, 7 and 12 of bile acids DCA (deoxycholic acid), LCA, CA (cholic acid), and CDCA by detoxification enzymes or microbiome may have an effect on the interactions with bile acid nuclear receptors. We employed reporter gene assays in HepG2 cells, the TR-FRET assay with recombinant PXR and RT-PCR to study the effects of acetylated and

Abbreviations: BA, bile acid; CA, cholic acid; CDCA, chenodeoxycholic acid; CYP, cytochrome P450; DCA, deoxycholic acid; 6-ECDCA, 6α-ethyl-chenodeoxycholic acid, obeticholic acid; FXR, farnesoid X receptor; LBD, ligand binding domain; LCA, lithocholic acid; PXR, pregnane X receptor; TR-FRET, time-resolved fluorescence energy transfer; VDR, vitamin D receptor.

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http://dx.doi.org/10.1016/j.toxlet.2016.11.013
0378-4274/

Nuclear receptors
FXR

keto bile acids on the nuclear receptors activation and their target gene expression in differentiated hepatic HepaRG cells.

We demonstrate that the DCA 3,12-diacetate and CA 3,7,12-triacetate derivatives are ligands of PXR and DCA 3,12-diacetate induces PXR target genes such as CYP3A4, CYP2B6 and ABCB1/MDR1.

In conclusion, we found that acetylated DCA and CA are potent ligands of PXR. Whether the acetylated bile acid derivatives are novel endogenous ligands of PXR with detoxification or physiological functions should be further studied in ongoing experiments.

1. Introduction

Three nuclear receptors of the nuclear receptor superfamily – the Pregnane X receptor (PXR, NR1I2), Farnesoid X (FXR, NR1H4) and Vitamin D receptor (VDR, NR1I1) – have been recently established as bile acid receptors in the liver and in the intestine and their role in bile acid (BA) synthesis regulation or detoxification has been clearly documented (Ishizawa et al., 2008; Makishima et al., 2002, 1999; Ridlon and Bajaj, 2015; Staudinger et al., 2001b; Wang et al., 1999; Xie et al., 2001). 3-Keto LCA has been found as a potent ligand for the VDR, FXR and PXR (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b), and the LCA acetate and LCA acetate methyl ester as highly potent VDR ligands (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b). It was also proposed that gut microbiome may produce endocrine molecules from steroids to activate nuclear receptors and several oxidized bile acid derivatives such as 7-oxo CA, 7-oxo DCA and 7-oxo CDCA have been identified as products of prokaryotic hydroxysteroid dehydrogenases (Ridlon and Bajaj, 2015).

We hypothesized that dehydrogenation (oxidation) or acetylation of bile acids may increase affinity to these nuclear receptors and that liver biotransformation enzymes or intestinal microflora may modify bile acids at position 3α, 7α and 12α of DCA, LCA, CA, and CDCA to derivatives more avidly interacting with the bile acid receptors (Ridlon and Bajaj, 2015). Therefore, to study structure-activity relationships (SAR) of dehydrogenated and acetylated bile acids with PXR, VDR and FXR receptors, we synthetized a series of dehydrogenated (keto) and acetylated derivatives of DCA, LCA, CA, and CDCA (Fig. 1). Some of these compounds are known products of gut microbiome bile salt 3α-, 7α, 12α-hydroxysteroid dehydrogenases or deconjugation enzymes as well as potential products of cytochrome P450-mediated biotransformation (compounds underlined in Fig. 1) (Deo and Bandiera, 2009; Ridlon and Bajaj, 2015; Ridlon et al., 2006).

The PXR has been identified as a "master" xenobiotic sensor regulating the expression of a wide variety of genes involved in the transport, metabolism and elimination of xenobiotics along with a number of endogenous substances. In addition, PXR has a function in regulating several cellular signaling pathways related to physiological processes (Banerjee et al., 2015). In the case of PXR, mainly lithocholic acid and its 3-keto derivative have been found to activate both human and mouse PXR (Krasowski et al., 2005; Staudinger et al., 2001b; Xie et al., 2001). 3-Keto LCA was found to be an even more potent ligand of PXR than LCA; whereas CDCA, DCA and CA only mildly activate PXR (Krasowski et al., 2005; Staudinger et al., 2001b). Therefore, PXR has been established as the receptor of LCA responsible for the detoxification of the highly hepatotoxic and a potentially enteric carcinogenic bile acid via induction of its metabolism (Staudinger et al., 2001b; Xie et al., 2001).

The FXR is localized mainly in the liver, intestine (ileum) and kidneys. FXR regulates the enterohepatic circulation and metabolism of bile acids, and it also modulates liver regeneration, inflammation and growth (Ali et al., 2015). Chenodeoxycholic acid (CDCA), and to a lesser extent lithocholic (LCA) and deoxycholic acid (DCA), are natural ligands of human FXR and able to transactivate the receptor, whereas cholic acid (CA) has weak effect on FXR activation (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). Among keto-bile acids, 7-keto and 3,7-keto LCA are known to activate FXR, although with lower potency (Wang et al., 1999). Conjugates of CDCA, LCA, and DCA with taurine and glycine and to a lesser extent CA conjugates can also activate FXR when transported into cells by a conjugate transporter such as ABST (IBAT, SLC10A2) (Makishima et al., 1999; Parks et al., 1999). Ligands of FXR are considered therapeutically useful for the treatment of liver disorders including various forms of cholestasis and fatty liver (steatosis) disease (Ali et al., 2015).

The VDR mediates the effect of the vitamin D active form 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2$vitD_3). VDR is primarily associated with calcium and phosphate homeostasis, but it is also an important regulator of cell growth and differentiation, cell death and immunity (Dusso et al., 2005). It has been shown that VDR also functions as a receptor for the secondary bile acid lithocholic acid (LCA). VDR is the most sensitive receptor to activation by LCA and its metabolites in comparison with other nuclear receptors. Activation of VDR by LCA or by vitamin D induces expression of CYP3A4, a cytochrome P450 enzyme that detoxifies LCA in the liver and intestine (Makishima et al., 2002). Interestingly, derivatives of LCA such as 3-keto LCA (Makishima et al., 2002), LCA propionate and methylester LCA acetate display significantly higher affinity and potency to activate VDR (Adachi et al., 2005; Ishizawa et al., 2008).

In the current work, we attempted to determine if dehydrogenation or acetylation at position 3, 7 and 12 of unconjugated DCA, LCA, CA and CDCA has effects on interactions with the bile acid receptors PXR, FXR and VDR. We investigated interactions of synthetized acetylated, diacetylated, triacetylated and dehydrogenated bile acids CDCA, LCA, DCA and CA at positions 3 (α, β), 7α and 12α (Fig. 1) with PXR, FXR, and VDR nuclear receptors in a cellular assay as well as with recombinant nuclear receptor proteins in coactivator TR-FRET assays. Employing RT-PCR and *in silico* docking, we confirmed interactions of acetylated DCA and CA bile acids with PXR ligand binding domain (LBD) and PXR target genes regulation in HepaRG cells by DCA 3,12-diacetate. We further analyzed lipid extracts of mouse liver and human bile samples using HPLC/MS-MS.

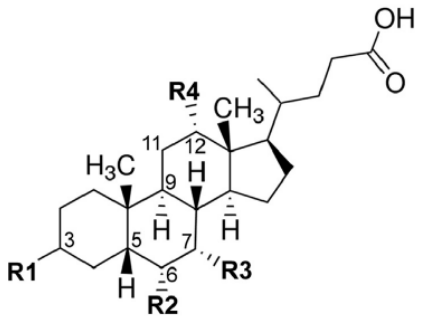
2. Material and methods

2.1. Chemicals

The bile acid derivatives (Fig. 1) were synthesized at the Institute of Organic Chemistry and Biochemistry, Czech Academy of Sciences. Synthesis procedures and NMR spectra are available upon request and will be published elsewhere.

2.2. Plasmids

The FXR response elements (FXRE)-driven luciferase reporter plasmid (pFXRE-luc2P) was constructed by inserting



	Position			
	3 (R1)	6 (R2)	7 (R3)	12 (R4)
LCA	OH		-	-
Iso LCA	OH		-	-
Iso LCA 5(6)-en 3-acetate	OAc		-	-
6-hydroxy LCA	OH	OH	-	-
CA	OH		OH	OH
7-oxo CA	OH		=O	OH
3,7,12-trioxo CA	=O		=O	=O
CA 3,7,12-triacetate	OAc		OAc	OAc
CA 3,7-diacetate	OAc		OAc	OH
DCA	OH		-	OH
Iso DCA	OH		-	OH
DCA 3-acetate	OAc		-	OH
DCA 3,12-diacetate	OAc		-	OAc
3,12-dioxo DCA	=O		-	=O
12-oxo DCA	OH		-	=O
12-oxo 9(11)-en- DCA	OH		-	=O
12-oxo DCA 3-acetate	OAc		-	=O
CDCA	OH		OH	-
CDCA 3,7-diacetate	OAc		OAc	-
CDCA 3-acetate	OAc		OH	-
7-oxo CDCA	OH		=O	-

Systematic Names (Lipid Maps™ database):

LCA, 3α-Hydroxy-5β-cholan-24-oic Acid, Lithocholic acid; Iso LCA, 3β-Hydroxy-5β-cholan-24-oic Acid, Isolithocholic Acid; Iso LCA 5(6)en 3-acetate, 3β-Hydroxy-5β-cholan-5-en-24-oic Acid 3-acetate; 6-hydroxy LCA, 3α,6α-Dihydroxy-5β-cholan-24-oic Acid, Hyodeoxycholic Acid; CA, 3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid, Cholic Acid; 7-oxo CA, 3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oic Acid; 3,7,12-trioxo CA, 3,7,12-Trioxo-5β-cholan-24-oic Acid; CA 3,7,12-Triacetate, 3α,7α,12α-Trihydroxy-5β-cholan-24-oic Acid 3,7,12-triacetate; CA 3,7-diacetate, 3α,7α,12α-Trihydroxy-5β-cholan-24-oic Acid 3,7-diacetate; DCA, 3α,12α-Dihydroxy-5β-cholan-24-oic Acid, Deoxycholic Acid; Iso DCA, 3β,12α-Dihydroxy-5β-cholan-24-oic Acid, Isodeoxycholic Acid; DCA 3-acetate, 3α,12α-Dihydroxy-5β-cholan-24-oic Acid 3-acetate; DCA 3,12-diacetate, 3α,12α-Dihydroxy-5β-cholan-24-oic Acid 3,12-diacetate; 3,12-dioxo DCA, 3,12-Dioxo-5β-cholan-24-oic Acid; 12-oxo DCA, 3α-Hydroxy-12-oxo-5β-cholan-24-oic Acid; 12-oxo 9(11)en- DCA, 3α-Hydroxy-12-oxo-5β-chol-9(11)-en-24-oic Acid; 12-oxo DCA 3-acetate, 3α-Hydroxy-12-oxo-5β-cholan-24-oic Acid 3-acetate; CDCA, 3α,7α-Dihydroxy-5β-cholan-24-oic Acid, Chenodeoxycholic Acid; CDCA 3,7-diacetate, 3α,7α-Dihydroxy-5β-cholan-24-oic Acid 3,7-diacetate; CDCA 3-acetate, 3α,7α-Dihydroxy-5β-cholan-24-oic Acid 3-acetate; 7-oxo CDCA, 3α-Hydroxy-7-oxo-5β-cholan-24-oic Acid.

Fig. 1. Common names of tested bile acids.
Underlined bile acids are formed by gut microbiota (Ridlon and Bajaj, 2015; Ridlon et al., 2006, 2014).

complementary oligonucleotides containing two copies of FXRE, an inverted repeat in which consensus receptor-binding hexamers are separated by one nucleotide (IR-1) from the phospholipid transfer protein (PLTP) promoter (5′-aaactgaGGGTCAgTGACC-Caagtgaa-3′) and one FXRE (IR-1) of the SHP gene promoter (−291GAGTTAaTGACCT−279) into *KpnI-XhoI* cloning sites of pGL4.27 (Promega, Hecules, CA, USA) upstream of the minimal promoter. The experiments with pCMX-GAL-hFXR, PXR-responsive p3A4-luc and pM-GAL4-PXR LBDmut (S247W/C284W) constructs were performed as we have previously described (Hirsova et al., 2013; Krausova et al., 2011; Rulcova et al., 2010). pDR_3-luc plasmid containing four repeats of consensus VDR response element $(AGGTCANNNNN)_5$ was used (DR3 *cis*-Reporting System, Stratagene). The pGL5-luc, pGL4.23 and pRL-TK constructs were purchased from Promega (Madison, WI, USA). pSG5-hFXR and pSG-hPXR expression constructs were kindly provided by Dr. S.

Kliewer (University of Texas, Dallas, TX, USA). The expression plasmids pSG5-hRXRα and pSG5-hVDR were a generous gift from Dr. C. Carlberg (University of Kuopio, Kuopio, Finland).

2.3. Reporter gene assay and mammalian two-hybrid assays

All transient transfection reporter gene assays were performed with Lipofectamine® 2000 transfection reagent (Life Technologies, Carlsbad, CA, USA, now part of Thermo Fisher) in the HepG2 cells (between passages 15 to 25). Cells were seeded at the density of 40,000 cells/cm^2 onto 48-well Plates 24 h before transfection. HepG2 cells were transfected with luciferase reporter gene constructs (150 ng/well) together with NRs expression vectors (100 ng/well) and pRL-TK *Renilla* construct (30 ng/well) 24 h after seeding, according to the manufacturers protocol. For pilot screening of bile acids interactions with PXR, VDR and FXR receptors, 30 μM concentration has been used. In the concentration, tested bile acids have no effect on HepG2 viability (*data not shown*).

Mammalian two-hybrid assays were carried out with the pM-GAL4-PXRwt fusion expression plasmid for wild-type PXR and the double mutant pM-GAL4-PXRmut (S247W/C284W) expression plasmid. The double PXR mutant (S247W/C284W) has replaced the serine at position 247 with the larger tryptophan, which effectively fills the ligand binding pocket of PXR. This replacement blocks the ligand binding pocket-dependent activity of PXR, rendering the construct constitutively active independently of a ligand. HepG2 cells were transiently transfected with the pGL5-luc reporter plasmid (150 ng per well in 48-well plates) together with either the pM-GAL4-PXR LBDwt or pM-GAL4-PXR LBDmut (S247W, C248W) (100 ng per well) fusion expression plasmid, the VP16-SRC1 fusion expression plasmid or the VP16 empty vector (100 ng per well), as well as the pRL-TK control plasmid (30 ng per well) for transfection normalization. After 24 h of stabilization, the cells were treated with the DCA 3,12-diacetate and CA 3,7,12-triacetate (30 μM), rifampicin (RIF, 10 μM) or vehicle (DMSO; 0.1%, v/v) for an additional 24 h. The VP16-SRC1-RID fusion expression construct for SRC1 coactivator 1 has been described previously (Krausova et al., 2011). During the length of the experiments, cells were kept in an incubator at temperature 37 °C and 5% CO_2. After treatment, the cells were lysed and assayed for both firefly and *Renilla* luciferase activities with the use of the Dual-Luciferase Reporter Assay kit (Promega, Hercules, CA, USA). All test values were normalized to the mean value of the experimental control group (empty expression constructs with vehicle) and the data are presented as fold of the control group's mean value ($n = 3$ or more). More detailed protocols can be found in our previous papers (Krausova et al., 2011; Smutny et al., 2014).

2.4. PXR ligand binding assay

The LanthaScreen® TR-FRET PXR Competitive Binding Assay (Invitrogen/Life Technologies, Carlsbad, CA) was performed according to the protocol described in our previous paper (Smutny et al., 2014).

2.5. HepaRG cell cultivation

Cryopreserved HepaRG™ (GIBCO®) cells and media were obtained from Life Technologies (Carlsbad, CA, USA). The HepaRG™ cell line is an immortalized and terminally differentiated hepatic cell line that retains many liver-specific characteristics of primary human hepatocytes including high endogenous activities of nuclear receptors and biotransformation enzymes. The HepaRG cells were initially isolated from a liver tumor of a female patient suffering from hepatocarcinoma (Gripon et al., 2002). The HepaRG cell line was cultivated and differentiated for qRT-PCR expression experiment as previously described (Gripon et al., 2002; Hyrsova et al., 2016). On the contrary, HepG2 cells have been preferred to HepaRG cells in transient transfection experiments, since majority of transfection reagents display low transfection efficiency or toxicity in HepaRG cells.

2.6. qRT-PCR assay

Total RNA isolation, cDNAs synthesis and RT-PCR experiments were performed as described previously (Smutny et al., 2014).

2.7. CYP3A4 enzymatic activity assay

Human recombinant CYP3A4 (P450-Glo™ CYP3A4 Screening System with Luciferin-PPXE) was used to evaluate the interaction of the DCA 3,12-diacetate with human CYP3A4 (Smutny et al., 2014).

2.8. HPLC/MS-MS analyses of the DCA 3,12-acetate and CA 3,7, 12-triacetate

Three mouse liver samples and human bile samples (collected according to the Declaration of Helsinki of 1964 and approved by the local ethics committee) have been used for the analyzed. Patients with a diagnosis of biliary complications were admitted at the Endoscopy unit, Gastroenterology department of the University of Heidelberg Hospital. Samples have been prepared as described in *Supplementary data* protocol.

For HPLC–MS/MS analyses, the separation of bile acids was achieved using a Luna C18 column (Phenomenex, CA; 100 mm × 2.0 mm, 3 μm particle size) fitted on a separation module (Waters 2695, Milford, MA). Binary solvents used for the analysis were 80% H_2O/methanol with 8 mM ammonium acetate, pH = 8.0 (solvent A) and 95% methanol/H_2O with 8 mM ammonium acetate, pH = 8.0 (solvent B). The flow rate was maintained at 0.2 mL/min, and the gradient started with 100% solvent A for 2.5 min, changed to 100% solvent B in 1 min, held for 16.5 min, and finally switched back to the initial condition within 3 min. The HPLC column was maintained at 40 °C and coupled with an electrospray ionization source of the tandem mass spectrometer (Quattro micro API, Micromass Waters, Manchester, UK). The mass spectrometer was operated with the source, with desolvation temperatures set at 130 °C and 300 °C, respectively. The bile acids in free acid form and their taurine- glycine −conjugates were detected in a negative mode. The capillary, cone, extractor, and RF voltages were used at 4100, 70, 10, and 0.6 V, respectively. The source and desolvation gases (nitrogen) were set at a flow rate of 800 and 90 L/h, respectively. Collision energies were 60, 48, and 30 eV for glyco-, tauro-, and unconjugated BAs, respectively. The DCA 3,7-diacetate and CA 3,7,12-triacetate were detected by the multiple reaction monitoring (MRM) transitions at 475.1 > 433.3 and 533.2 > 491.2, respectively. Peak assignment was achieved by direct comparison with the retention times at 9.19 min and 8.16 min of authentic standards of the DCA 3,7-diacetate and CA 3,7,12-triacetate, respectively. The concentrations of bile acids were determined by the peak area ratio between the bile acid and the internal standard.

2.9. Statistical and bioinformatic analyses

All data are presented as the mean ± standard deviations (SDs). A one-way analysis of variance (ANOVA) with a Dunnett's *post hoc* test was applied. EC_{50} (BAs concentration required to achieve half-maximum promoter activation), IC_{50} (BAs concentration required to achieve 50% inhibition in TR-FRET assay or CYP3A4 enzymatic

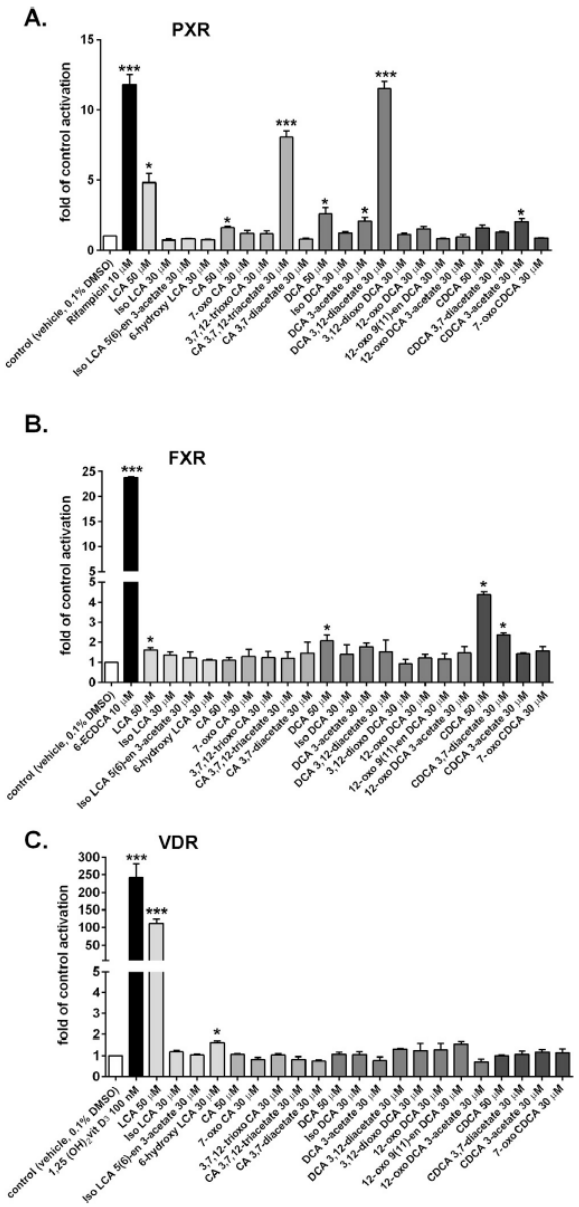


Fig. 2. Interaction of the bile acid derivates with PXR, FXR and VDR in luciferase reporter gene assays. Transient transfection reporter gene experiments with NRs responsive luciferase constructs (p3A4-luc, pFXRE-luc2P and pDR3-luc) were performed in HepG2 cells cotransfected with appropriate NRs expression constructs to establish the interactions of the tested compounds with PXR, FXR and VDR. The data are presented as the means ± SD from three independent experiments ($n = 3$) and are expressed as the fold-change of normalized luciferase activities relative to the vehicle-treated sample (control) group mean value (set to 1).
$^{*}p < 0.05$, $^{***}p < 0.001$—statistically significant difference compared to vehicle-treated cells (ANOVA with Dunnett's post-hoc test).

assay) and I_{max} (representing the overall maximal calculated induction produced by the tested compound; maximal responding capacity; maximal efficacy) values were determined according to Hill's equation by nonlinear regression analysis. All of the statistical analyses were performed using GraphPad Prism 6 Software (GraphPad Software, Inc., San Diego, CA) based on at least three independent experiments ($n = 3$). A p value of <0.05 was considered to be statistically significant.

2.9.1. Molecular modeling

3D structures of the compounds were prepared and all hydrogens were added with the Marvin 14.9.8 program (ChemAxon, http://www.chemaxon.com). The crystal structure of PXR with ethinyl estradiol and *trans*-nonachlor (PDB ID: 4 × 1G) was used as final docking template with a docking grid of 14 Å around the center of the ligand in the crystal structure (Delfosse et al., 2015), which was deleted prior to docking. Polar hydrogens were added to the receptor and all ligands with the AutoDock Tools 1.5.4 program (Morris et al., 2009) prior to docking with the AutoDock Vina program (Trott and Olson, 2010), whereas side-chains of the flexible residues identified within available PXR crystal structures (amino acids H407 and R410) were set flexible.

3. Results

3.1. Interaction of the tested bile acid derivatives with PXR, FXR and VDR in reporter gene assays

In the first set of experiments, we tested a series of interactions between bile acids derivatives with PXR, FXR and VDR by using reporter gene assays in HepG2 cells. Validated prototype ligands of the receptors such as rifampicin, 6-ECDCA and $1,25(OH)_2vitD_3$, respectively, have been used as comparators (black columns) (Fig. 2A–C). We found that the DCA 3,12-diacetate and CA 3,7,12-triacetate derivatives significantly ($p < 0.001$) activated human PXR at a concentration of 30 μM (Fig. 2A). We also observed a mild but significant ($p < 0.05$) PXR activation by the DCA 3-acetate and CDCA 3-acetate, and with LCA, DCA and CA, which are known to interact with PXR.

In the experiments, known FXR ligands 6-ECDCA, CDCA, LCA, and DCA significantly activated FXR-responsive luciferase construct (Fig. 2B). We also observed a weak but statistically significant ($p < 0.05$) activation of FXR by the CDCA 3,7-diacetate (Fig. 2B).

Finally, in the experiments with the VDR-responsive luciferase reporter gene construct, we did not observe any significant activation of VDR by any of the tested compounds except for the known ligands LCA and 6-hydroxy LCA (Fig. 2C). Furthermore, the 3β epimers Iso LCA and Iso LCA 5(6)en 3-acetate did not have any significant effects on any of the nuclear receptors examined (Fig. 2).

Thus our results showed that the acetylation of CA and DCA was able to increase the activation of PXR, and the acetylation of CDCA may increase the activation of FXR.

3.2. The DCA 3,12-diacetate interacts with the PXR ligand binding domain (LBD) in a dose-dependent manner

Next we performed dose-response studies using the gene reporter assays in HepG2 cells with the p3A4-luc construct to measure the affinity of the DCA 3,12-diacetate to PXR LBD. We found that the DCA 3,12-diacetate had lower affinity, but higher efficacy to activate PXR (Fig. 3A). EC_{50} of the DCA 3,12-diacetate and rifampicin was 32.1 ± 1.13 and 9.1 ± 1.65 μM, respectively.

In the next experiments, we aimed to determine the affinity of the DCA 3,12-diacetate to PXR-LBD in an *in vitro* TR-FRET PXR coactivation assay using recombinant PXR-LBD protein. In this assay, the affinity of the tested compounds to PXR was evaluated based on competition with a fluorescent PXR ligand. We found that the DCA 3,12-diacetate had similarly low IC_{50} in comparison with prototype non-fluorescent PXR ligand SR12813 (IC_{50} 3.68 ± 1.80

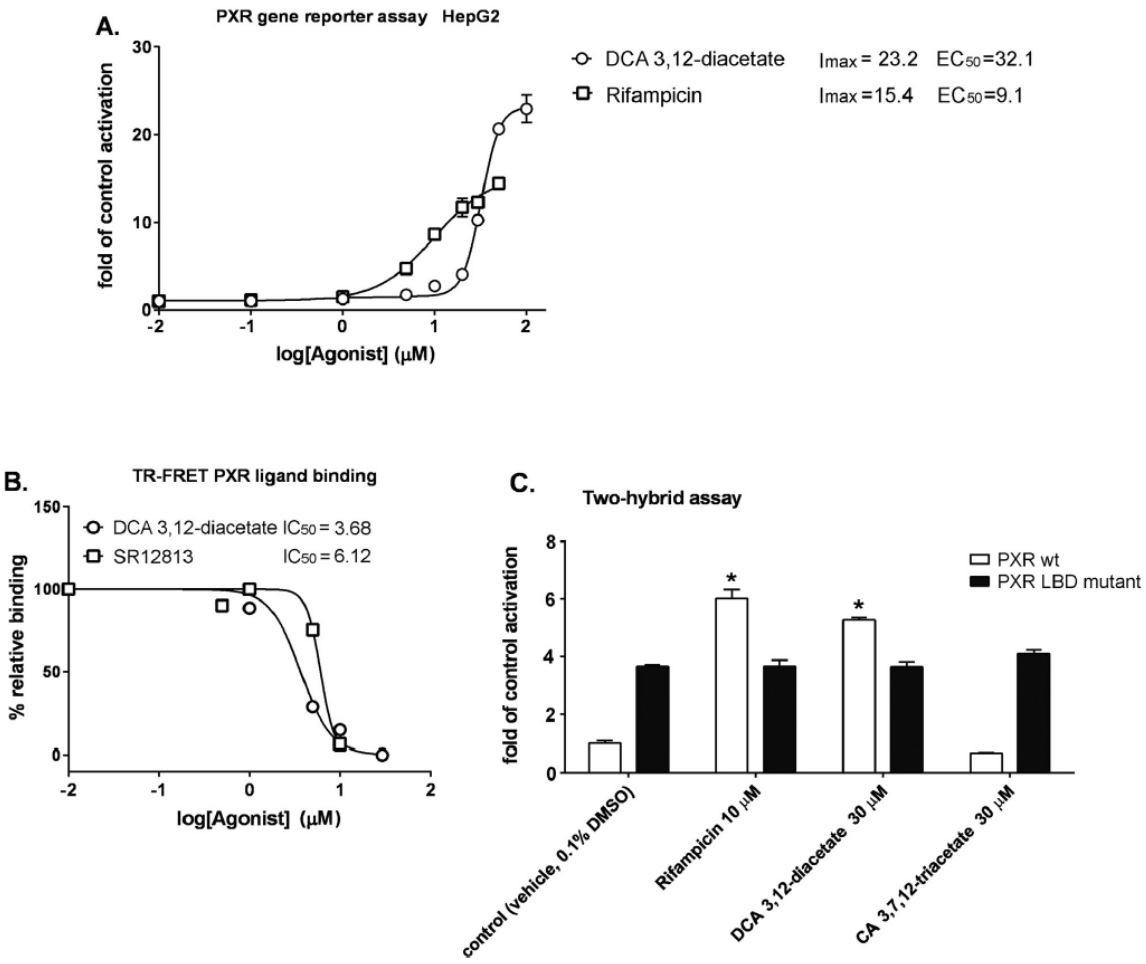


Fig. 3. Interaction of the DCA 3,12-diacetate and CA 3,7,12-triacetate with the PXR ligand binding domain.
(A) Dose-response activation of PXR by the DCA 3,12-diacetate in reporter gene assays. Reporter gene experiments were performed with a p3A4-luc luciferase construct in HepG2 cells treated with the indicated range of concentrations of PXR ligands for 24 h. The data are presented as the means ± SD from three independent experiments ($n = 3$) and are expressed as the fold-change in activation relative to the vehicle-treated samples (control) means. EC_{50} is the concentration required to achieve half-maximum promoter activation; I_{max} represents the overall maximal calculated induction (in μM).
(B) Interaction of the DCA 3,12-diacetate with PXR in a LanthaScreen® TR-FRET Pregnane X Receptor competitive binding assay. The TR-FRET assay was performed to determine the affinity of the DCA 3,12-diacetate to recombinant PXR. SR12813, a model non-fluorescent PXR agonist, was used as a positive control. Data are presented as the relative binding of fluorescent PXR substrate to PXR LBD versus the DCA 3,12-diacetate or SR12813 concentrations. The maximum value was set as 100% in the absence of a competitor. IC_{50} (a tested compound concentration required to achieve 50% inhibition in PXR-SRC-1 interaction measured by TR-FRET fluorescence) was calculated. The data are presented as the mean ± SD from three independent experiments ($n = 3$) performed in triplicate measurements.
(C) The mammalian two-hybrid assay in HepG2 cells transiently transfected with the pGL5-luc reporter plasmid together with either the pM-GAL4-PXR LBD wt or pM-GAL4-PXR LBD mut (S247W, C248W) fusion expression plasmid, the VP16-SRC1 fusion expression plasmid or the VP16 empty vector, as well as the pRL-TK control plasmid. After 24 h of stabilization, the cells were treated with the DCA 3,12-diacetate and CA 3,7,12-triacetate (30 μM), rifampicin (RIF, 10 μM) or vehicle (DMSO; 0.1%, v/v) for an additional 24 h. The data are presented as the means ± SD from three independent experiments ($n = 3$).
*$p < 0.05$—statistically significant difference compared to vehicle-treated cells transfected with wild-type PXR LBD (ANOVA with Dunnett's post-hoc test).

and 6.12 ± 1.05 μM, respectively) (Fig. 3B). These data indicated a high affinity of the DCA 3,12-diacetate to PXR.

Finally, we analyzed whether the DCA 3,12-diacetylate could affect the recruitment of the SRC1 coactivator in mammalian two hybrid assay to either the wild-type (wt) PXR LBD or the constitutively active mutant of PXR (S247W/C284W) with an obstructed ligand-binding pocket. We observed that the DCA 3,12-diacetylate at 30 μM significantly ($p < 0.05$) augmented the interaction between the wt PXR LBD and SRC1, as detected by an increase of pGL5-luc reporter vector activity (Fig. 3C). On the other hand, the CA 3,7,12-triacetate did not stimulate an interaction between the wild-type PXR LBD and SRC1 coactivator. Mutated PXR was not stimulated by any of the compounds tested. These results indeed demonstrated that the DCA 3,12-diacetylate was a direct agonist in the LBD of PXR.

3.3. The DCA 3,12-diacetate induces PXR-target genes mRNAs in differentiated HepaRG cells

The HepaRG cells were cultivated under differentiated conditions and treated with rifampicin (10 μM) and DCA 3,12-diacetate (30 μM). As shown in Fig. 4A, the expression of CYP3A4

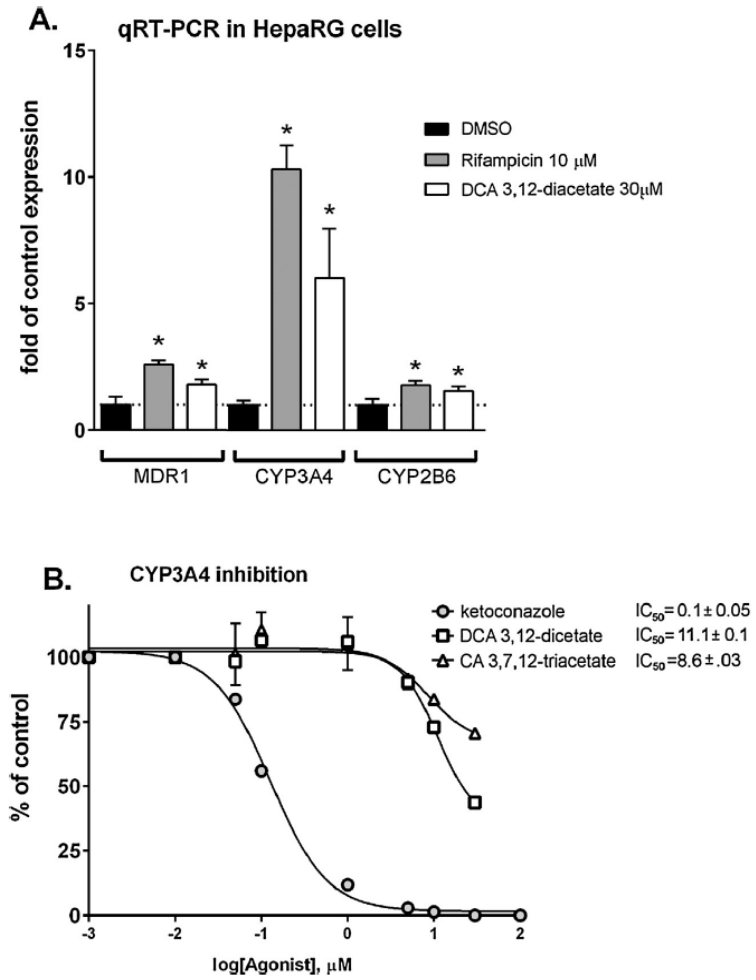


Fig. 4. The DCA 3,12-diacetate significantly induces PXR target genes mRNA expression and inhibits CYP3A4 catalytic activity.
(A) RT-PCR experiments were performed in differentiated HepaRG cells with TaqMan primers/probes after a 24 h treatment with rifampicin 10 μM (Rif) and the DCA 3,12-diacetate (30 μM), which significantly induced CYP3A4, CYP2B6, MDR1 mRNA expression in differentiated HepaRG cells. The data are presented as the means ± SD from three experiments ($n = 3$) and are expressed as the fold-change in induction relative to vehicle-treated controls (normalized to 1). *$p < 0.05$ represents a statistically significant difference compared to control (vehicle-treated) cells (ANOVA with a Dunnett's post hoc test).
(B) The P450-Glo™ CYP3A4 Screening System with Luciferin-PPXE was used to evaluate the interaction of the DCA 3,12-acetate with human CYP3A4 in CYP3A4-expressed microsomes. Ketoconazole was used as a prototype CYP3A4 inhibitor. CYP3A4 reactions were performed according to manufacturer's protocol in three independent experiments ($n = 3$) in triplicates. Luminescence was recorded using a plate-reader, with values displayed as relative light units related to control vehicle-treated samples. Data are presented as the means ± SD of CYP3A4 inhibition related to vehicle-treated membranes (control mean was set to be 100%). IC_{50} is the tested compound concentration required to achieve 50% inhibition of CYP3A4 activity (in μM).

mRNA, a major PXR target gene, was induced by the DCA 3,12-diacetate to ~7 folds in comparison with vehicle (DMSO)-treated controls while the activation by rifampicin was ~10 folds (Fig. 5). In addition, the DCA 3,12-diacetate significantly induced other PXR target genes including CYP2B6 and ABCB1/MDR1.

3.4. The DCA 3,12-diacetate and CA 3,7,12-triacetate are weak inhibitors of CYP3A4 enzymatic activity

Next, we examined whether the DCA 3,12-diacetylate could inhibit the catalytic activity of CYP3A4, which is an important cytochrome P450 enzyme involved in BA metabolism and is a major target gene induced by PXR activation. We observed an inhibition with IC_{50} ~10 μM for both the DCA 3,7-diacetate and CA 3,7,12-triacetate (Fig. 4B). These data indicate that the DCA 3,12-diacetate up-regulates CYP3A4 expression and at the same time inhibits CYP3A4 enzymatic activity.

3.4.1. Docking of DCA derivatives to PXR structure

Recently, a PXR structure with the steroid ligand inside – ethinylestradiol – (PDB ID: 4X1G) has been released (Delfosse et al., 2015) and this was used as a docking template (Fig. 5A). Furthermore, as some side-chain conformations have been shown to differ among individual published PXR-LBD crystal structures, we set two polar residues as flexible—H407 and R410. With this approach, the molecular docking of DCA derivatives showed a common binding pose for all molecules (Fig. 5).

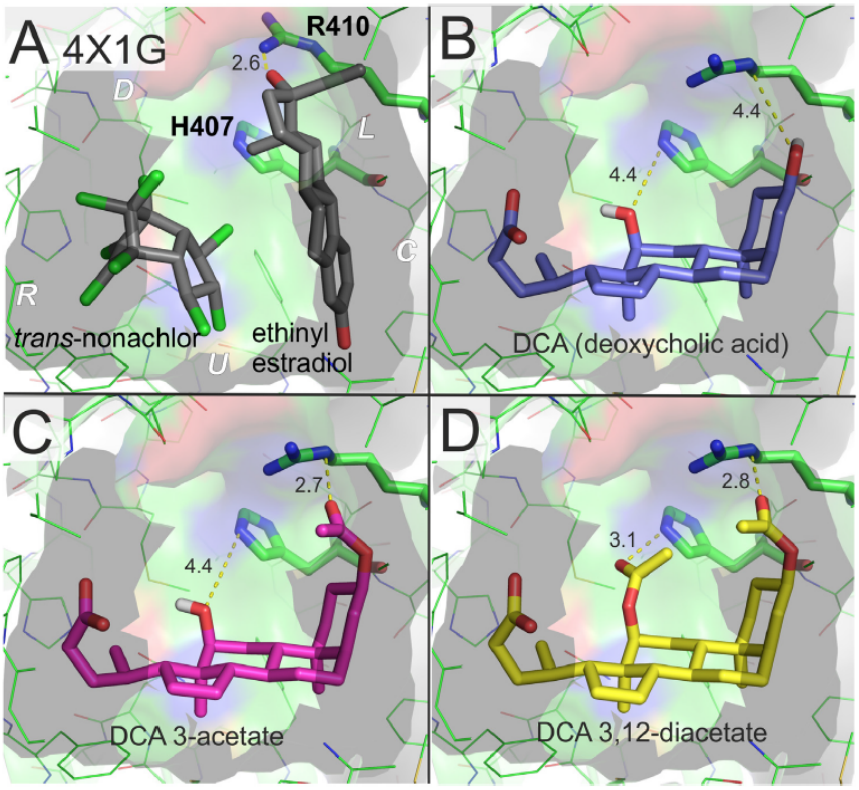


Fig. 5. Binding poses within PXR structure.
Best binding poses of derivatives of deoxycholic acid in comparison with crystal structure with bound ethinylestradiol and *trans*-nonachlor (PDB ID: 4 × 1G[1]) with annotated positions of hotspots (see main text for definition). Derivatives of DCA share the same binding pose, whereas acetylation affects hydrogen bonds from residues H407 and R410, which are known for their role in PXR activation.

DCA occupies several of hotspots, the regions of protein surface that are major contributors to the binding energy (Ngan et al., 2009). The hydrophobic part of the steroid tail occupies hotspot R, whereas carboxylic group is exposed towards water inside of the cavity center. The hydrophobic groove in hotspot U is filled with methyls between the A/B and C/D rings and the steroid edge interacts with hotspot C as well through hydrophobic interactions. Nevertheless, nonpolar interactions with hotspot D were too far, as interactions with hotspot L are important for its influence on the activation function helix (Fig. 5B). On the other hand, this hotspot was fulfilled with the DCA 3-acetate, which had one hydrogen bond acceptor interacting with R410, with the DCA 3,12-diacetate, which had two hydrogen bonds with both flexible residues R410, and more importantly with H407 (Fig. 5C and D). As a result of this docking, the DCA 3,12-diacetate as a PXR ligand exhibited the strongest interaction energy.

3.5. Analysis of the DCA 3,12-acetate and CA 3,7,12-triacetate in human bile and mouse liver samples

We further determined the presence of the DCA 3,12-acetate and CA 3,7,12-triacetate in biological samples to provide evidence as to whether these bile acids could be endogenous ligands *in vivo*. We had previously set-up bile acid profiling by HPLC/MS-MS (Jiao et al., 2015), which was implemented for detection of the DCA 3,12-acetate and CA 3,7,12-triacetate. By using pure standards, the mass for a precursor scan (Fig. 6A and A′) and corresponding daughter scan (Supplemental material Fig. S1) was detected for the DCA 3,12-acetate and CA 3,7,12-triacetate at retention time 9.19 min and 8.16 min, respectively.

We further analyzed lipid extracts of mouse liver and several human bile samples. However, none of these two acetyl bile acids was detected in these samples (Fig. 6B–D and 6B′–6D′). We therefore concluded that these acetylated bile acids may not be endogenous bile acid PXR ligands in bile or in the liver or that their concentrations may be lower than of the detection limit of HPLC/MS-MS.

4. Discussion

Three nuclear receptors of the nuclear receptor superfamily have been recently established as bile acid receptors, and their role in bile acid synthesis regulation or detoxification has been clearly documented (Ishizawa et al., 2008; Makishima et al., 2002, 1999; Staudinger et al., 2001b; Wang et al., 1999; Xie et al., 2001). Bile acids have thus been established as endogenous regulatory ligands of nuclear receptors in the regulation of numerous target genes. Recently, 3-keto LCA has been found as a potent ligand for the VDR, FXR and PXR (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b), and the LCA acetate methyl ester as a highly potent VDR ligand (Adachi et al., 2005; Makishima et al., 1999; Staudinger et al., 2001b). Therefore, we hypothesized that oxidized or acetylated bile acids DCA, LCA, CA, and CDCA at position 3, 7 and 12 may have an effect on the interactions with bile

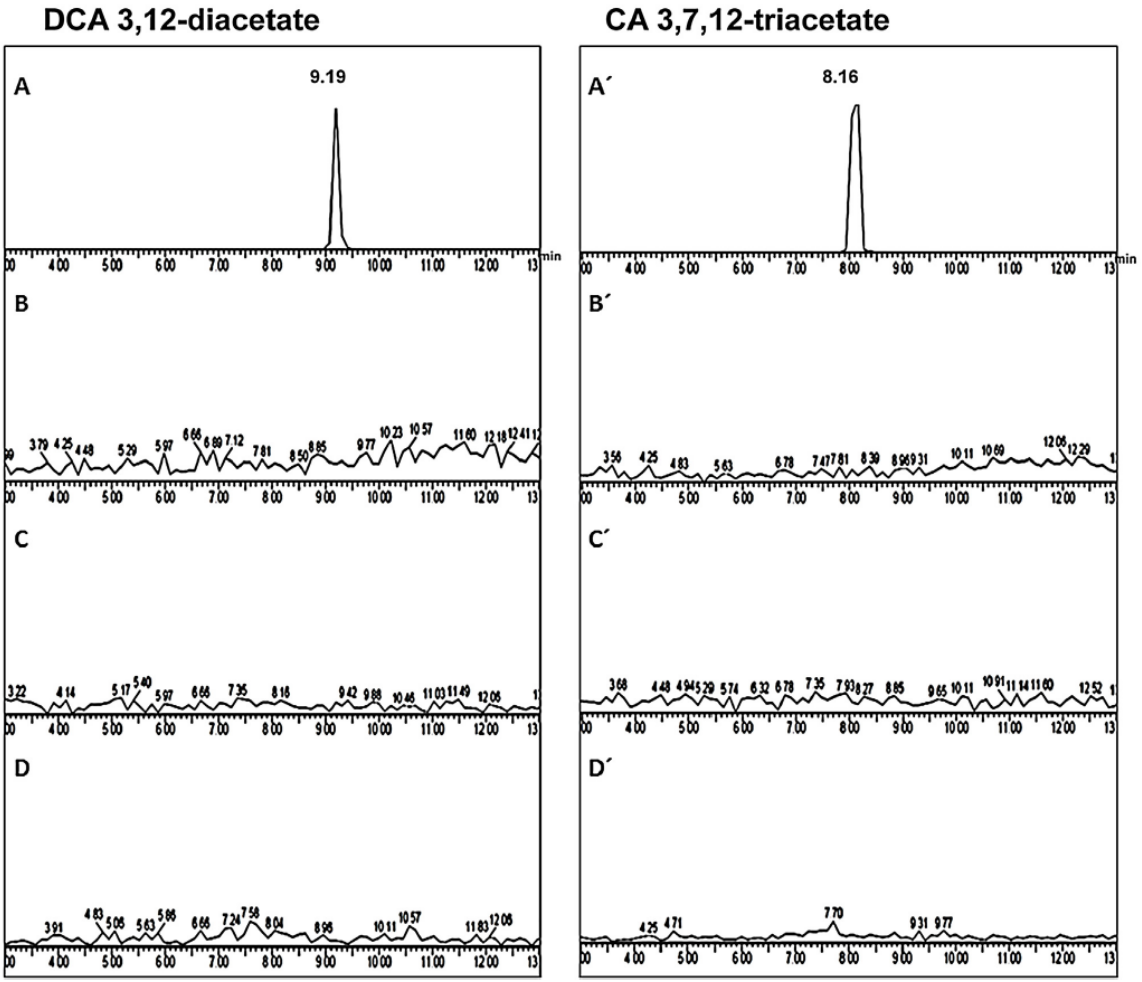


Fig. 6. HPLC/MS-MS analyses of acetylated bile acids.
In the left panel, HPLC–MSMS analysis of the DCA 3,7-diacetate by MRM of 475.1 > 433.2 in an authentic standard detected at 9.19 min (A) as well as in a representative lipid extract from mouse liver homogenate (B), and human bile samples collected in 2010 (C) and in 1995 (D) from a representative donor. In the right panel, HPLC–MSMS analysis of CA 3,7,12-triacetate by MRM of 533.2 > 491.2 in an authentic standard detected at 8.16 min (A′) as well as in a representative lipid extract from mouse liver homogenate (B′), and human bile samples collected in 2010 (C′) and in 1995 (D′) from a representative donor.

acid nuclear receptors. For this purpose, we synthetized a series of dehydrogenated (keto) and acetylated derivatives of DCA, LCA, CA, and CDCA, and, to our knowledge for the first time, we tested their interactions with human PXR, VDR, and FXR in cellular and *in vitro* assays.

We demonstrated that the DCA 3,12-diacetate was an efficient and high-affinity PXR ligand with greater potency than the parent secondary bile acid DCA (EC_{50} = 32.1 μM versus 150.2 μM, respectively) (Krasowski et al., 2005). We showed consistently that the DCA 3,12-diacetate was able to upregulate the mRNA expression of PXR target genes CYP3A4, CYP2B6, and MDR1 in a differentiated HepaRG cells, a superior human hepatocyte model with endogenous expression of functional PXR. Dehydrogenation or acetylation of the 3, 7 and 12 hydroxyl groups of CDCA, LCA, DCA and CA did not result in any significant increase of VDR or FXR activities.

We could only observe, respectively, a weak activation of FXR with the CDCA 3,7-diacetate and of PXR with the DCA 3-acetate and CDCA 3-acetate. Oxidation of 3, 7 and 12 hydroxyl groups did not result in any augmented activity of the tested compounds to all the nuclear receptors studied. Although Iso LCA has been reported as a weak activator of VDR (Adachi et al., 2005), our data did not confirm the results. Cotransfection of ASBT bile acid transporter into HepG2 cells in a parallel experiment did not significantly improve interaction of the tested bile acids with any of the nuclear receptors examined (*A.C., P.P. unpublished observations*).

Based on docking experiments, we proposed that the acetylation of DCA may improve interactions with PXR ligand-binding domain residues, even though we should also take into account the easier entry of the acetylated bile acid into cells due to their high lipophilicity.

PXR was found to be efficiently activated by lithocholic acid, a hydrophobic bile acid formed by 7α-dehydroxylation of CDCA by intestinal anaerobic bacteria. CDCA, DCA and CA have only a mild effect to activate PXR in high micromolar concentrations (Krasowski et al., 2005; Staudinger et al., 2001b).

Activation of PXR induces genes encoding enzymes involved in the metabolism and detoxification of secondary bile acids (mainly CYP3A4 cytochrome P450 enzyme) and down-regulates CYP7A1

(cholesterol 7alpha-hydroxylase), a rate limiting enzyme of BA synthesis (Banerjee et al., 2015; Staudinger et al., 2001a; Xie et al., 2001). DCA is metabolized by CYP3A4, a main target enzyme of PXR and VDR in the liver and in the intestine, into 1β-hydroxy-DCA and 3-dehydro-DCA (3-oxo DCA) (Chen et al., 2014). By this mechanism, PXR has been documented as a key factor conferring resistance to LCA toxicity in various transgenic or knockout animal models (Staudinger et al., 2001b; Xie et al., 2001). In addition, activation of PXR has been proposed beneficial in the elevation and potential treatment of inflammatory liver and bowel diseases (Cheng et al., 2012; Wallace et al., 2010). Recently, it was shown that symbiotic bacteria regulate gastrointestinal barrier function and inflammation via the xenobiotic sensor PXR and Toll-like receptor 4 by production of indole 3-propionic acid (IPA) (Venkatesh et al., 2014). Therefore, discovery of a potent endogenous bile acid ligand of PXR receptor, produced by either gut microflora or liver metabolism, may help us to uncover additional regulatory functions of microflora or other regulatory pathways of bile acid synthesis and detoxification In addition, the potent endogenous PXR ligands could be considered as a safe therapy in inflammatory and cholesteric liver diseases (Ridlon and Bajaj, 2015).

In our study, we presented the novel finding that the acetylation of DCA and CA led to a significant increase in binding to PXR, and the DCA 3,7-diacetate was identified as a potent PXR ligand which was able to induce the PXR target genes CYP3A4 and CYP2B6 as well as the P-glycoprotein/MDR1 transporter in a HepaRG hepatocyte model. Since there have been no reports of the existence of acetylated BAs in biological samples, we analyzed the presence of these BAs in mouse liver and human bile samples. However, we observed neither the DCA 3,12-diacetate nor CA 3,7,12-triacetace in these samples. It is possible that these acetylated BAs do not exist in free form but in conjugated forms with glycine, taurine, sulfates, and glucuronide. At this time, it is unlikely that the DCA 3,12-diacetate can be considered as an important endogenous PXR ligand. Whether the conjugates of the DCA 3,12-diacetate exist *in vivo* and whether they are capable of activating PXR awaits further investigation in our laboratories. Another possibility is that intestinal microflora may produce the DCA 3,12-diacetate, which may in turn activate intestinal PXR. Further studies are needed to examine the presence of the DCA 3,12-diacetate and its conjugates within enterohepatic circulation employing HPLC/MS-MS.

5. Conclusions

Taken together, the majority of the newly-synthesized acetylated derivatives of LCA, CA, DCA, and CDCA did not show any improvement in their activity towards the nuclear receptors tested, the two exceptions being the DCA 3,12-diacetate and CA 3,7,12-triacetate, which were found to be powerful agonists of PXR. Dehydrogenation of position 3, 7 and 12 did not stimulate interactions with the PXR, FXR and VDR. Since we did not observe the DCA 3,12-diacetate or CA 3,7,12-triacetate in mouse liver and human bile samples, these bile acids may not be natural ligands for PXR. Our next experiments are focused on analysis of acetylated bile acid derivatives or their conjugates in the intestine and on their putative physiological function in the intestinal barrier.

Acknowledgements

This work was funded by GACR303/12/G163 (to P.P.), DFGCH288/6-3 (to W.C.), project LO1305 (to K.B.), IGA PrF2016028 (to V.B), the Charles University grant SVV 260293 (to A.C, A.H., L.H.), TACRTE01020028 and RVO61388963 (to E.K. and H.C.). We thank Dr. A. Gauss of University Heidelberg Hospital in providing us with human bile samples. A. Carazo gratefully acknowledges Zentiva for funding of his work in Albert Einstein College of Medicine in New York.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2016.11.013.

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9 Diskuse

Základním principem při eliminaci léčiv je koordinace biotransformace a transportu exogenních látek, resp. jejich metabolitů, prostřednictvím současné exprese biotransformačních enzymů a transportérů. Hladiny obou těchto skupin proteinů jsou na transkripční úrovni silně kontrolovány nukleárními receptory. Pregnanový X receptor (PXR) je v lidském organismu jedním ze stěžejních jaderných receptorů podílející se na regulaci metabolismu (Mani et al., 2013).

PXR hraje klíčovou roli při regulaci metabolismu jak endogenních látek, tak i látek cizorodého původu. Svou aktivitou se podílí na koordinaci exprese biotransformačních enzymů I. a II. fáze a transportních proteinů umožňujících přenos léčiv a dalších xenobiotik přes buněčné membrány. Vliv PXR je zásadní při indukci exprese některých enzymů cytochromu P450. Týká se to především CYP3A4, který se podílí na metabolismu více než 50 % používaných léčiv. Z transportérů je významný vliv na indukci P-glykoproteinu (P-gp, MDR1), který umožňuje přenos široké škály léčiv z buněk, například hepatocytů nebo enterocytů, čímž ovlivňuje jejich celkovou farmakokinetiku (Klaassen and Aleksunes, 2010; Wang and LeCluyse, 2003; Xie and Evans, 2001). Ovlivněním těchto systémů tak může docházet k četným lékovým interakcím, které se mohou v důsledku vystupňovaného metabolismu projevit selháním léčby aplikované podle jinak běžného dávkovacího schématu. Z tohoto důvodu jsme se v některých našich pracích zaměřili na identifikaci dalších potenciálních ligandů PXR (agonistů i antagonistů).

V průběhu našeho výzkumu jsme se zaměřili na základní vlastnost PXR – regulování hlavních biotransformačních enzymů a transportérů. Vzhledem k absenci dat o transportéru pro organické kationty 1 (OCT1, SLC22A1) a vzhledem k jeho dominantní jaterní expresi jsme zjišťovali možnosti regulace OCT1 jaderným receptorem PXR.

Transport látek do hepatocytů je nezbytným předstupněm jaterního metabolismu a exkrece většiny léčiv. Membránové transportéry umožňují přenos látek přes bazolaterální membránu hepatocytů a mohou tak hrát i klíčovou roli při odpovědi organismu na léčivo

v případě, že jaterní buňky jsou cílovým místem účinku léčiv. OCT1 transportér patří mezi jeden z nejdůležitějších transportérů zprostředkovávající transport kladně nabitých látek, mezi nimiž lze nalézt poměrně široké spektrum běžně používaných léčiv (Nies et al., 2009). Proto ovlivňování množství OCT1 v hepatocytech, stejně jako jeho funkce, může mít závažné klinické důsledky. Např. v roce 2008 byla publikována klinická studie, v níž se autoři zabývali vlivem genového polymorfizmu OCT1 transportéru na farmakokinetiku metforminu. U pacientů, u nichž se vyskytovala alela s redukovanou funkcí, došlo k významnému ovlivnění řady farmakokinetických parametrů včetně snížení farmakodynamické odpovědi na terapii metforminem (Shu et al., 2008).

Na základě údajů jedné z dříve uveřejněných studií se předpokládalo, že rifampicin zvyšuje množství OCT1 transportéru a tím zvyšuje transport metforminu do hepatocytů (Cho et al., 2011). Oproti tomu v dalších dvou studiích bylo naznačeno, že aktivace PXR rifampicinem vede ke snížení hladiny OCT1 mRNA v primárních lidských hepatocytech (Badolo et al., 2015; Jigorel et al., 2006). Tato pozorování jsou v souladu i s našimi výsledky, kdy jsme ve skupině zahrnující hepatocyty od 15 lidských dárců pozorovali významné snížení hladiny SLC22A1 ve 13 případech. Stejný fenomén jsme zaznamenali i při použití diferencovaných HepaRG buněk. I v případě genových reportérových experimentů jsme pozorovali sníženou aktivaci OCT1 reportérového plazmidu po jeho ovlivnění rifampicinem (Hyrsova et al., 2016a).

Hepatocytární nukleární faktor 4 α (HNF4α) patří mezi jaderné receptory a hraje zásadní roli při regulaci široké řady genů s převážně jaterní lokalizací (Watt et al., 2003). Exprese OCT1 je tímto faktorem podstatně ovlivňována. V promotoru OCT1 byly identifikovány dvě responzivní oblasti pro HNF4α, tzv. DR-2 oblasti a oblast zvaná E-box, která interaguje s USF1 a USF2 a zesiluje aktivační působení HNF4α (Kajiwara et al., 2008; Nies et al., 2009; Saborowski et al., 2006). Absencí HNF4α lze vysvětlit opačný fenomén, který pozorovali Austin a její spolupracovníci u krevních buněk. V této studii provedené na krevní buněčné linii KCL a u pacientů s chronickou myeloidní leukémií bylo množství SLC22A1 v přítomnosti rifampicinu naopak zvýšené (Austin et al., 2015).

V rámci našich experimentů jsme zkoušeli také schopnost různých mutantů PXR inhibovat OCT1 genový reportérový konstrukt. Pouze nefunkční mutant T422D s mutovanou AF-2 oblastí, který není schopný vázat koaktivátory, nevykázal žádnou

inhibiční aktivitu vůči reportérovému konstruktu (Hyrsova et al., 2016a). Již dříve bylo popsáno, že jak PXR tak HNF4α používají stejné koaktivátory, konkrétně SRC-1 a peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) (Moreau et al., 2008; Wang et al., 1998). Na tomto podkladě jsme v další řadě experimentů zkoumali vliv obou těchto koaktivátorů na inhibiční efekt PXR na OCT1 reportérový konstrukt. Buněčná linie HepG2 byla postupně transfekována zvyšujícím se množstvím koaktivátorů, což vedlo v případě SRC-1 ke snižujícímu se inhibičnímu efektu. Tento výsledek naznačil, že jako možný inhibiční mechanismus se zde uplatňuje kompetice o tento koaktivátor. Pomocí chromatinové imunoprecipitace jsme zaznamenali sníženou vazbu SRC-1 do DR-2 oblastí (responzivní oblasti pro HNF4α) i do oblasti E-boxu (místo vazby USF1 a 2 faktorů) a tím tento kompetiční mechanismus potvrdili (Hyrsova et al., 2016a).

Nepřímá regulace OCT1 transportéru byla popsána již v minulosti u dalších jaderných receptorů, konkrétně u farnesoidního X receptoru (FXR) a glukokortikoidního receptoru (GR). V těchto případech nedochází ke kompetitivnímu odebírání koaktivátorů, ale k přímé stimulaci exprese korepresoru SHP a tím zastavení transkripce OCT1 respektive ke zvýšení hladiny HNF4α aktivací GR a následnému zvýšení hladiny i OCT1 (Rulcova et al., 2013; Saborowski et al., 2006). Také kompetice o stejné koaktivátory mezi různými jadernými receptory není novinkou, před několika lety byla popsána kompetice mezi PXR a CAR o SRC-1 koaktivátor (Saini et al., 2005) nebo třeba mezi HNF4α a CAR o koaktivátory PCG1α a GRIP1 (Miao et al., 2006).

Prohloubení znalostí o látkách, které mohou být ligandy PXR a ovlivňovat jeho funkci, je další oblastí, kde zatím chybí dostatek informací. Proto jsme se v některých našich studiích zaměřili na identifikaci dalších potenciálních agonistů či antagonistů PXR. V naší práci jsme se zabývali skupinou 13 látek stilbenoidní struktury, z nichž 8 látek se přirozeně vyskytuje (*trans*- a *cis*-resveratrol, *trans*-4-methoxystilben, *trans*- a *cis*-piceatanol, oxyresveratrol, pterostilben a pinostilben), jedna látka je metabolitem (a,b-dihydroresveratrol) a 4 látky jsou syntetického původu a v přírodních zdrojích nebyly popsány (*trans*-3,4,5,4'-tetramethoxystilben, *trans*-2,4,3',5'-tetramethoxystilben a *trans*- a *cis*-trismethoxyresveratrol) (Hyrsova et al., 2018). Resveratrol patří mezi jednoznačně nejzkoumanější látku celé této skupiny, k říjnu roku 2018 lze v databázi PubMed nalézt více než 11 000 vědeckých publikací týkajících se resveratrolu. Jedná se o přirozeně se vyskytující látku stilbenoidního charakteru, která se běžně vyskytuje

v každodenní stravě. Mimo jiné byl její výskyt popsán v červeném víně, hroznových džusech, pistáciích nebo arašídech. Resveratrol je široce studovanou látkou kvůli svým pleiotropním účinkům na živé organismy včetně protinádorového, antioxidačního a protizánětlivého působení (Carrizzo et al., 2013).

Několik vědeckých prací se již v minulosti zabývalo interakcí mezi PXR a resveratrolem. Některé z těchto studií identifikovaly resveratrol jako agonistu PXR (Dolezelova et al., 2017; Dring et al., 2010; Jacobs et al., 2005; Kluth et al., 2007; Smutny and Pavek, 2014; Yu et al., 2011), v případě práce publikované čínskou pracovní skupinou byl dokonce *trans*-resveratrol popsán jako silnější agonista PXR než jeho *cis*-izomer (Yu et al., 2011). Avšak poměrně nedávno byl popsán i antagonistický efekt resveratrolu na PXR (Deng et al., 2014). Navíc bylo také prokázáno, že i další stilbenoidní sloučenina – pterostilben je schopná aktivovat PXR, což naznačuje možný skupinový efekt stilbenů (Dring et al., 2010).

V průběhu experimentů jsme nejprve stanovili schopnost vybraných stilbenoidních látek vázat se do rekombinantní ligand vázající domény PXR pomocí nebuněčné metody TR-FRET. Zjistili významnou interakci zkoumaných stilbenoidních látek s ligand vázající doménou (LBD) PXR a identifikovali jsme jako slabé agonisty PXR látky *trans*-2,4,3',5'-tetramethoxystilben, *trans*-4-methoxystilben, *trans*- a *cis*-trismethoxyresveratrol a pterostilben. Avšak indukce cílového genu CYP3A4 nebyla u žádné ze zmiňovaných látek významná (Hyrsova et al., 2018).

Následně jsme testovali jejich vliv na p3A4-luc reportérový konstrukt v genových reportérových experimentech. Vzhledem k tomu, že resveratrol a ostatní stilbenoidy zasahují do různých signálních kaskád včetně inhibice světluškové i *Renilla* luciferázy, došlo k detekci falešně negativních výsledků. Z tohoto důvodu jsme nejprve zkoušeli vyhodnocení těchto experimentů pomocí detekce mRNA luciferázy RT-PCR, což byl přístup poměrně komplikovaný. Poté jsme se rozhodli pro vhodnější vyhodnocení poměrem mezi buňkami s kotransfekovaným PXR a s buňkami bez PXR, abychom odstranili nežádoucí inhibiční působení stilbenoidů na luciferázu. Látky *trans*-2,4,3',5'-tetramethoxystilben a *cis*-trismethoxyresveratrol signifikantně zvýšily aktivitu reportérového konstruktu a potvrdili tak slabě agonistické působení derivátů resveratrolu (Hyrsova et al., 2018).

Červené víno obsahuje zhruba 1,9 mg/l (8,3 μM) *trans*-resveratrolu a přibližně 1,0 mg/l *cis*-resveratrolu (Detampel et al., 2012). Hladina resveratrolu v plazmě po konzumaci 600 ml červeného vína byla stanovena na přibližně 6 ng/ml, což odpovídá 0,03 μM (Vitaglione et al., 2005). Ačkoliv tato hladina je pro vznik interakcí příliš nízká, existuje řada doplňků stravy, které obsahují obrovské množství resveratrolu a člověk tak denně zkonzumuje i 2-5 g látky. Při takových dávkách může hladina v plazmě dosáhnout i mikromolárních koncentrací a resveratrol se tak může stát zdrojem interakcí mezi současně podávanou léčbou a takovými doplňky stravy (Detampel et al., 2012; Howells et al., 2011).

Mezi ligandy PXR se neřadí jenom léčiva a jiná xenobiotika, mohou to být i některé endogenní látky, včetně žlučových kyselin. Konkrétně byl popsán agonistický efekt kyseliny litocholové (LCA) a jejího metabolitu kyseliny 3-ketolitocholové. PXR se účastní udržování homeostázy žlučových kyselin působením na jejich syntézu a transport (Staudinger et al., 2001a; Staudinger et al., 2001b). V naší práci jsme se zaměřili na zkoumání efektu acetylovaných žlučových kyselin – kyseliny cholové (CA) a deoxycholové (DCA), na PXR (Carazo et al., 2017). Předpokládali jsme, že tyto deriváty žlučových kyselin by mohli vznikat v játrech nebo ve střevě v důsledku metabolismu, eventuálně ve střevě v důsledku metabolismu žlučových kyselin enzymy střevní mikroflóry. Acetylované kyseliny byly vybrány také na základě schopnosti acetylované LCA aktivovat vitaminový D receptor, který je členem stejné podrodiny jaderných receptorů NR1I jako PXR, ještě silněji než samotná LCA (Adachi et al., 2005). Efekt samotných CA, DCA a kyseliny chenodeoxycholové (CDCA) byl již dříve popsán, tyto kyseliny mají pouze slabý aktivační účinek na PXR, a to navíc ve vysokých koncentracích (Staudinger et al., 2001b). Námi testovaný 3,12-diacetát DCA se projevil jako silný agonista PXR schopný jej aktivovat při nižších koncentracích než samotná DCA. Navíc jsme také prokázali schopnost 3,12-diacetátu DCA signifikantně zvýšit hladinu cílových genů PXR, konkrétně CYP3A4 a transportéru MDR1 v hepatocytární progenitorové buněčné linii HepaRG. Ačkoliv se nám nepodařilo zmiňované acetylované žlučové kyseliny prokázat ve vzorcích lidské žluče, existuje možnost jejich přítomnosti ve formě konjugátu např. s kyselinou glukuronovou nebo glycinem (Carazo et al., 2017).

Závěrem lze shrnout, že PXR je jedním z široce studovaných nukleárních receptorů, zejména kvůli své schopnosti podílet se na řízení metabolismu jak endogenních látek, tak i xenobiotik. Je znám především pro své indukční působení na biotransformační enzymy

I. a II. fáze metabolismu. Naopak suprese (down-regulace) cílových genů PXR byla doposud jen velmi omezeně studována. OCT1 je tak prvním lékovým transportérem, u kterého byla prokázána down-regulace a podrobně popsán její mechanismus. Antagonistů PXR bylo prozatím identifikováno pouze omezené množství oproti široké škále agonistů. Ostatně, oblast studia a identifikace potenciálních endogenních ligandů PXR receptoru, včetně produktů střevní mikroflóry je málo probádána a proto intenzivně studována.

10 Ústní prezentace na konferencích

22. Mezioborová toxikologická konference TOXCON 2017

Plzeň, 21. - 23. 6. 2017

The pregnane X receptor down-regulates organic cation transporter 1 (SLC22A1) in human hepatocytes by competing for ("squelching") SRC-1 coactivator

6. Postgraduální a 4. Postdoktorandská vědecká konference

Farmaceutická fakulta Univerzity Karlovy, 9. - 10. 2. 2016

Regulation of OCT1 transporter by nuclear receptor PXR in different hepatic models

5. Postgraduální a 3. postdoktorandská vědecká konference

Farmaceutická fakulta Univerzity Karlovy, 3. - 4. 2. 2015

Organic cation transporter 1 is down-regulated by pregnan X receptor in HepaRG cell line and primary human hepatocytes

4. Postgraduální a 2. postdoktorandské vědecké konference

Farmaceutická fakulta Univerzity Karlovy, 28. a 29. 1. 2014

Organic cation transporter 1 is downregulated by pregnan X receptor

11 Plakátová sdělení na konferencích

22. Mezinárodní Symposium Microsomes and Drug Oxidations (MDO2018)

Kanazawa, Japonsko, 1. - 5. 10. 2018

Trans-resveratrol, but not other natural stilbenes occurring in food, carries the risk of drug-food interaction via inhibition of CYP450 enzymes or with xenosensor receptors

21. Mezinárodní Symposium Microsomes and Drug Oxidations (MDO2016)

Davis, Kalifornie, USA, 2. - 6. 10. 2016

The pregnane X receptor down-regulates organic cation transporter 1 (SLC22A1) in human hepatocytes by competing for ("squelching") SRC-1 coactivator

Biomedical transporters 2013

Svatý Mořic, Švýcarsko, 11. – 15. 8. 2013

Glucocorticoid receptor regulates organic cation transporter 1 (*OCT1*, *SLC22A1*) expression via HNF4α up-regulation in primary human hepatocytes

63. Farmakologické dny

Olomouc, 11. – 13. 9. 2013

Regulation of organic cation transporter 1 (*OCT1*, *SLC22A1*) expression via major nuclear receptors in primary human hepatocytes

12 Seznam zkratek

ABC	nadrodina transportérů ATP-binding cassette
ABCG5	člen G5 transportérové nadrodiny ATP-binding cassette
AF-1, 2	aktivační funkce 1, 2
AhR	arylhydrokarbonový receptor
ALDH1	aldehyddehydrogenáza 1
ASP^+	4-(4-(dimethylamino)-styryl)-N-methylpyridinium
ATP7B	ATP-ase copper-transporting β polypeptide
BCRP	breast cancer resistence protein
BSEP	transportér pro žlučové kyseliny (bile salt export pump)
C/EBPα, β	CCAAT/enhancer-binding protein α, β
CA	kyselina cholová (cholic acid)
CAR	konstitutivní androstanový receptor (constitutive androstane receptor)
CBP	CREB vázající protein (CREB [CRE (cAMP response element)-binding protein] binding protein)
CDCA	chenodeoxycholová kyselina (chenodeoxycholic acid)
CES	karboxyesteráza
CITCO	6(4chlorfenyl)imidazo[2,1-b]thiazol-5-karbaldehyd O(3,4dichlorbenzyl)oxim
CNT2	concentrative nucleoside transporter 2

CYP3A4	cytochrom P450 3A4
DBD	DNA vázající doména (DNA-binding domain)
DCA	deoxycholová kyselina (deoxycholic acid)
DR	direct repeat
ER	everted repeat
ENT1	equilibrative nucleoside transporter 1
FXR	farnesoidní X receptor
FXRE	FXR odpovídající oblast (FXR responsive element)
G6Páza	glukóza-6-fosfatáza
GR	glukokortikoidní receptor
GRE	GR odpovídající oblast (GR responsive element)
GSTA1	glutathion-S-transferáza A1
HNF4α	hepatocytární nukleární faktor 4α
IBABP	střevní protein vázající žlučové kyseliny (intestinal bile acid-binding protein)
LBD	ligand vázající doména (ligand-biding domain)
LEFTs	transkripční faktory s bohatým výskytem v játrech (liver enriched transcription factors)
MATE1	multidrug and toxin extrusion protein 1
MDR1	multidrug resistance 1
MPP^+	1-methyl-4-fenylpyridinium (1-methyl-4-phenylpyridinium)
mRNA	informační RNA (messenger RNA)
MRP	multidrug resistence protein
NCoR	korepresor jaderných receptorů (nuclear receptor corepressor)

NR	nukleární (jaderný) receptor
NR1I2	nukleární receptor člen 1I2
NTCP1	polypeptid kontransportující sodík a taurocholát 1 (natrium/taurocholatecotransporting polypeptide 1)
OAT2	transportér pro organické antionty 2
OATP	polypeptid transportující organické anionty
OCT1	transportér pro organické kationty 1
PEPCK1	fosfoenolpyruvátkarboxykináza 1
PEPT	peptidový transportér
PGC1α	PPARγ koaktivátor 1α (PPAR (peroxisome proliferator-activated receptor) gamma coactivator 1α)
P-gp	P-glykoprotein
PolII	RNA polymeráza II
PXR	pregnanový X receptor
PXRE	PXR odpovídající oblast (PXR responsive element)
RAR	receptor kyseliny retinové (retinoid acid receptor)
RT-PCR	reverse transcription polymerase chain reaction
RXRα	retinoidní X receptor α
SHP	malý heterodimerní partner (small heterodimer partner)
siRNA	malá interferující RNA (small interfering RNA)
SLC	nadrodina transportérů solute carrier
SLC22A1	člen 22A1 transportérové nadrodiny solute carrier
SMRT	tlumící mediátor retinoidních a tyroidních receptorů (silencing mediator of retinoid and thyroid receptors)

SRC1	koaktivátor steroidních receptorů 1 (streoid receptor coactivator 1)
SULT1A1	sulfotransferáza 1A1
TBP	TATA box vázající protein (TATA box-binding protein)
TEA	tetraethylamonium
TFII	transkripční faktory II
TR	receptor tyroidních hormonů
TR-FRET	time resolved fluorescence resonance energy transfer
UGT1A1	UDP-glukuronosyltransferáza 1A1
USF1, 2	nadřazený stimulační faktor 1, 2 (upstream stimulating factor 1, 2)
VDR	vitaminový D receptor