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STUDIUM LÉKOVÝCH INTERAKCÍ NA ÚROVNI JATERNÍCH A RENÁLNÍCH TRANSPORTNÍCH PROTEINŮ

Dizertační práce

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Obsah

Seznam použitých zkratek

ABC	„ATP binding cassette“
BCRP	„Breast cancer resistance protein“ – transportér
BSEP	„Bile salt export pump“ – transportér
CAR	„Constitutive androstane receptor“ – nukleární receptor
CYP	izoforma cytochromu P450
FXR	„Farsenoid X receptor“ – nukleární receptor
MDR	„Multidrug resistance proteins“ – podrodina ABC transportérů
MRP	„Multidrug resistance-associated proteins“ – podrodina ABC transportérů
NTCP	„Na^+-taurocholate cotransporting polypeptide“ – polypeptid transportující organické aniony
NSAID	„Non-steroid antiinflammatory drugs“
OAT	„Organic anion transporter“ – transportéry organických aniontů
OATP	„Organic anion transporting polypeptide“ – polypeptidy transportující organické anionty
OCT	„Organic cation transporter“ – transportéry organických kationtů
P-gp	P-glykoprotein (MDR1)
PXR	„Pregnane X receptor“ – nukleární receptor
RT-PCR	„Reverse transcriptase polymerase chain reaction“
SLC	„Solute carrier“ – rodina transportních proteinů
SULT	Sulfotransferázy – enzymy II. fáze biotransformace
UGT	Uridin difosfát-glukuronosyl transferázy – enzymy II. fáze biotransformace

ÚVOD

1. Problematika farmakokinetických lékových interakcí

Jedním z hlavních předmětů farmakologického pre-/klinického výzkumu jsou klinicky významné nežádoucí lékové interakce. Popis a bližší porozumění jejich principům může vést k možnostem těmto interakcím předcházet a v konečném důsledku především přispět ke zvýšení bezpečnosti farmakoterapie. Farmakokinetické lékové interakce tvoří zásadní skupinu lékových interakcí, jejichž důsledkem je změna množství léčiva v místě jeho účinku. Současně podávaná léčiva mohou navzájem interagovat na všech úrovních kinetických dějů, tj. na úrovni absorpce (především v gastrointestinálním traktu), distribuce (kompetice o vazebná místa na plazmatické bílkoviny, ale i ovlivnění aktivních transportních procesů), metabolizmu (inhibice/indukce biotransformačních enzymů) i exkrece (opět např. modulací aktivního transportu v eliminačních orgánech). Výzkum lékových interakcí byl dosud zaměřen zejména na problematiku ovlivnění metabolizmu a následných nežádoucích změn v dispozici léčiv, především díky podrobnému hodnocení klinicky závažných interakcí působených enzymovou indukcí/inhibicí systému cytochromu P450.

Proto byla v posledních letech zavedena již do raných stádií farmaceutického výzkumu řada metod podrobně zkoumajících metabolické a farmakokinetické vlastnosti léčiv. Potenciální léčiva jsou během vývoje časně testována na vhodnou solubilitu, permeabilitu a metabolickou stabilitu. Díky těmto testům lze u řady nových léčiv pozorovat zvýšenou metabolickou stabilitu (mj. neinteragují s jaterními oxygenázami včetně cytochromu P450). Důsledkem je přesun k alternativním cestám systémové clearance, např. mimojaternímu metabolizmu a ve zvyšující se míře i eliminaci zprostředkované aktivními transportéry. Popsaný posun od relativně známých eliminačních procesů, především metabolizmu zprostředkovaného cytochromem P450, k novým, méně popsaným eliminačním procesům klade další nároky na farmakologický výzkum. Metabolicky stabilní nové molekuly léčiv (např. antihypertenzivum aliskiren, antidiabetika ze skupiny gliptinů či antimykotika charakteru echinokandinů), eliminované především biliární exkrecí nebo aktivní tubulární sekrecí, podléhají řadě aktivních transportních procesů. Výzkum potenciální indukce a inhibice těchto procesů je proto klíčový k porozumění jejich vlivu na variabilitu kinetiky transportovaných léčiv (Funk, 2008).

2. Význam aktivního transportu pro farmakokinetiku léčiv

Aktivní transport je jedním ze zásadních mechanizmů přestupu endogenních látek

i xenobiotik přes fyziologické bariéry představované buď vrstvami buněk, nebo samotnou cytoplazmatickou membránou. Vzhledem k tomu, že tyto bariéry jsou na molekulární úrovni obvykle tvořeny právě vrstvou polarizovaných buněk (hepatocytů, enterocytů apod.), má na propustnost bariér vliv také distribuce transportních molekul na apikální (luminální pól) a bazolaterální (krevní pól) části buněčné membrány. Např. unipolární lokalizace efluxních ABC (ABC – z anglického „ATP binding cassette“) transportérů je stěžejní pro směr a rozsah prostupu léčiv přes fyziologické bariéry většiny orgánů (Ito *et al.*, 2005). Transportní proteiny tak zasahují do farmakokinetiky léčiv prostřednictvím usnadnění nebo naopak zabránění přestupu léčiv mezi jednotlivými kompartmenty. Tímto způsobem výrazně modifikují a v řadě případů i určují kvalitativní i kvantitativní poměry na úrovni absorpce, distribuce i exkrece jednotlivých léčivých látek.

Počet genů kódujících proteiny membránových transportérů u člověka je odhadován na téměř 900, což představuje přibližně 3% funkčního lidského genomu (Venter *et al.*, 2001). Proteiny kódované těmito geny zastávají v buňkách důležité fyziologické úlohy: transportují živiny, odstraňují nepotřebné látky a udržují elektrochemický gradient na membránách. Podle počtu současně transportovaných látek a směru transportu se transportní děje dělí na uniport (jedna látka), symport (dvě látky stejným směrem) a antiport (dvě látky opačným směrem). Dále lze transportní procesy rozlišovat podle energetické závislosti. *Aktivní transport* využívá energii ATP (tzv. primárně aktivní transport) nebo elektrochemický gradient spřažené látky (tzv. sekundárně aktivní transport), zatímco *facilitovaná difúze* probíhá pouze ve směru elektrochemického gradientu transportované látky (Baynes, 2005). Především na základě těchto parametrů se transportéry v současnosti člení do dvou skupin: (i) **ABC transportéry** využívající energii ATP a (ii) **SLC transportéry** (z anglického „solute carrier“), které ke své činnosti vyžadují elektrochemický gradient substrátu nebo spřažené látky.

2.1. SLC transportní proteiny

Probíhající intenzivní výzkum především v posledních dvou desetiletích objevil a popsal několik rodin transportních proteinů podílejících se na vstupu léčiv do buněk. Jedná se o rozsáhlou skupinu transportních proteinů přenášejících substráty po směru koncentračního gradientu buď bez závislosti na přísunu energie, nebo využívajících energii symportu resp. antiportu dalšího substrátu (sodík, redukovaný glutathion atd.). Pro kinetiku léčiv jsou významní zástupci čtyř rodin SLC: NTCP (z anglického „Na^+-taurocholate co-

transporting polypeptide“), OATP (z anglického „Organic anion transporting polypeptide“), OAT (z anglického „Organic anion transporter“) a OCT (z anglického „Organic cation transporter“) (Zair *et al.*, 2008; Funk, 2008). Jejich stručný přehled a substrátovou specifitu nabízí Tab. 1. Značení jednotlivých transportérů vychází z mezinárodního konsenzu – označení molekuly proteinu vychází z původního názvu, do kterého je v případě existence více zástupců z dané rodiny inkorporován číselný údaj o genu; označení genu v současnosti respektuje doporučení vycházející z řešení projektu HUGO (HGNC, http://www.gene.ucl.ac.uk/nomenclature/genefamily/abc.html).

Tab. 1. Transportéry ze skupiny SLC důležité pro kinetiku léčiv
(upraveno dle Funk, 2008)

Gen (alternativně)	**Protein (starší označení)**	**Endogenní substráty**	**Exogenní substráty – léčiva (příklady)**	**Lokalizace**
SLC10A1	NTCP	žlučové kyseliny	rosuvastatin, sulindak	játra, střevo (apikálně)
SLCO1A2 (SLC21A3)	OATP1A2 (OATP-A, OATP1)	estron-3-sulfát, žlučové kyseliny, T_3, T_4 (hormony štítné žlázy)	digoxin, erythromycin, imatinib	játra (apikálně), ledviny, střevo, mozek
SLCO1B1 (SLC21A6)	OATP1B1 (OATP-C, OATP2)	žlučové kyseliny, bilirubin, estron-3-sulfát, T_3, T_4	atorvastatin, simvastatin, fluvastatin, kaspofungin, bosentan, benzylpenicilin, repaglinid, valsartan, olmesartan	játra, střevo
SLCO1B3 (SLC21A8)	OATP1B3 (OATP-8)	bilirubin, estron-3-sulfát, LTC4, T_3, T_4, žlučové kyseliny,	enalapril, rifampicin, metotrexát, fluvastatin, bosentan, valsartan, telmisartan, paklitaxel	játra
SLCO2B1 (SLC21A9)	OATP2B1 (OATP-B, OATP-RP2)	estron-3-sulfát	rosuvastatin, fluvastatin, aliskiren, benzylpenicilin	játra, střevo (apikálně)
SLCO4C1 (SLC21A20)	OATP-H	organické anionty	metotrexát, sitagliptin	ledviny
SLC22A1	OCT1	acetylcholin, progesteron, kortikosteron	aciklovir, ritonavir, lamivudin, metformin, chinidin, verapamil, ranitidin, famotidin, oxaliplatina	játra (apikálně), řada dalších orgánů

SLC22A2	OCT2	organické kationty, široké spektrum	amantadin, memantin, platina, ranitidin, vareniklin	ledviny (bazolaterálně), řada dalších orgánů
SLC22A4	OCTN1	organické kationty, široké spektrum	doxorubucin, verapamil, gabapentin	střevo, ledviny (apikálně)
SLC22A5	OCTN2	L-karnitin, organické kationty	betalaktámová ATB, verapamil	střevo, ledviny (apikálně), játra
SLC22A6	OAT1	organické anionty, široké spektrum	acyklovir, NSAID, furosemid, mykofenolát	ledviny, placenta
SLC22A7	OAT2	prostaglandiny, organické anionty	allopurinol, diklofenak, paklitaxel, salicyláty, tetracykliny	játra, ledviny, mozek
SLC22A8	OAT3	organické anionty, široké spektrum	adefovir, NSAID, rosuvastatin, sitagliptin, mykofenolát	ledviny
SLC22A11	OAT4	organické anionty, široké spektrum	NSAID, zidovudin, metotrexát	ledviny

NTCP

NTCP je klíčovým influxním přenašečem pro konjugované a s nižší afinitou též nekonjugované žlučové kyseliny a hraje tak významnou úlohu ve fyziologii jater (Alrefai a Gill, 2007). Jeho exprese byla vyjma jater detekována i v buňkách střevního epitelu. Význam NTCP pro kinetiku xenobiotik je dle aktuálního stavu poznání relativně malý, nicméně byla popsána jeho úloha v transportu NSAID sulindaku a inhibitoru HMG-CoA reduktázy rosuvastatinu (Ho *et al.*, 2006; Bolder *et al.*, 1999).

OATP

Tato skupina transportérů je označována jako zřejmě nejdůležitější, resp. doposud nejlépe prozkoumaná, rodina aktivních přenašečů zprostředkovávajících vstup léčiv do buněk. Dosud bylo identifikováno více než 50 zástupců této skupiny, z nichž je u člověka přítomno 11. Pro kinetiku léčiv je významných především pět z nich: OATP1A2, OATP1B1, OATP1B3 a OATP2B1 v játrech a OATP4C1 v ledvinách (Niemi, 2007). Aktivita těchto transportérů je nezávislá na přítomnosti kationtu sodíku a zejména první dva zmíněné mohou zprostředkovat obousměrný přenos, kde může tvořit hnací sílu

antiport redukovaného glutathionu. Spektrum substrátů těchto transportérů je široké a kromě mnoha organických aniontů zahrnuje i látky bazické povahy s větší molekulou a neutrální steroidy – viz. Tab. 1 (Hagenbuch a Gui, 2008). Mezi jednotlivými OATP transportéry je popsán podstatný překryv substrátového spektra, což na jedné straně umožňuje kompenzaci v případě porušené funkce jednoho z nich, na druhé straně značně ztěžuje individuální studium vlastností jednotlivých proteinů.

Kromě přenosu xenobiotik mají OATP transportéry přirozeně fyziologický význam i v transportu endogenních látek. OATP1B1 byl charakterizován jako hlavní (na sodíku nezávislý) bazolaterální přenašeč žlučových kyselin v lidských játrech; OATP1A2 a OATP1B3 se zdají mít spíše minoritní roli; OATP2B1 dle publikovaných informací žlučové kyseliny ve svém substrátovém spektru nemá (Kullak-Ublick *et al.*, 2001).

OAT

V této rodině transportérů bylo dosud u člověka identifikováno 5 zástupců značených OAT1 až OAT5 (Anzai *et al.*, 2006). Míra exprese a význam jednotlivých přenašečů se v exkrečních orgánech liší. V játrech je exprimován především OAT2, jiné formy zde sice byly rovněž detekovány, jejich funkce je však minoritní. Naopak v ledvinách mají hlavní význam pro transport léčiv v buňkách proximálních ledvinných tubulů OAT1 a OAT3 (van Montfoort *et al.*, 2003; Zair *et al.*, 2008). Tyto transportéry jsou schopné přenášet organické anionty oběma směry. Jejich modelovým substrátem je para-aminohippurová kyselina. Pro farmakoterapii je zejména důležitá role těchto přenašečů v jaterní i renální eliminaci např. nesteroidních antirevmatik, *β*-laktamových antibiotik, diuretik, urikosurik, metotrexátu, antivirotik, tetracyklinů, prostaglandinů a cyklických nukleosidů (Miyazaki *et al.*, 2004).

OCT

Jedná se o skupinu transportérů podobně jako výše uvedené OAT patřící do rodiny SLC22, jejíž specifitou je přenos organických kationtů, přičemž spektrum substrátů je u jednotlivých molekul značně široké. V lidských játrech byli dosud popsáni dva zástupci této skupiny transportérů OCT1 a OCT3 (Koepsell 2004). OCT1 zprostředkovává obousměrný přenos organických kationtů s menší molekulou. Mezi jeho substráty patří např. H2 antagonisté, imatinib, metformin, oxaliplatina nebo tricyklická antidepresiva. V ledvinách je nejvýznamnějším zástupcem OCT2 na bazolaterální membráně buněk proximálních tubulů.

2.2. ABC transportní proteiny

Ze 48 ABC transportérů (7 podrodin identifikovaných *ABCA* až *ABCG*) doposud popsaných u člověka jsou pro transport léčiv v eliminačních orgánech nejdůležitější MDR1/*ABCB1* resp. P-glykoprotein, BCRP/*ABCG2* (z anglického „Breast cancer resistance protein“) a MRP2/*ABCC2* (z anglického „Multidrug resistance-associated protein“) (Funk, 2008). Tyto transportéry byly původně detekovány na nádorových buňkách, kde svou širokou substrátovou specifitou a vysokou transportní kapacitou pro odstranění cytostatik z buněk působí rezistenci maligně změněných buněk na více cytostatik současně – fenomén „mnohočetné lékové rezistence“ (MDR – z anglického „multidrug resistance“), po kterém byly tyto molekuly od počátku pojmenovány.

Tab. 2. Efluxní ABC transportéry zapojené do exportu léčiv z buněk (zpracováno dle Schinkel and Jonker, 2003).

Gen	**Název proteinu (původní označení)**	**Endogenní substráty**	**Exogenní substráty – příklady léčiv**	**Lokalizace**
ABCB1	MDR1 (P-glykoprotein)	steroidní hormony	taxany, antracykliny, kalcineurinové inhibitory, HIV antiretrovirotika, ondansetron, verapamil atd.	játra, ledviny, střevo, mozek, řada dalších orgánů s bariérovou funkcí
ABCB11	BSEP	žlučové kyseliny (konjugované i nekonjugované)	pravastatin, vinblastin, sulindak	játra
ABCC1	MRP1*	oxidovaný glutathion, leukotrien C_4	antracykliny, vinka alkaloidy, etoposid	játra, plíce, testes
ABCC2	MRP2 (cMOAT)	bilirubin glukuronid, leukotrien C_4	pravastatin, indinavir, metotrexát, většina léčiv a jejich metabolitů konjugovaných s kyselinou glukuronovou nebo glutathionem	játra, ledviny, střevo, mozek
ABCC3	MRP3*	sulfatované žlučové kyseliny	metotrexát, konjugáty léčiv	střevo, plíce, ledviny, játra,

ABCC4	MRP4*	konjugované žlučové kyseliny a cyklické nukleotidy	metotrexát, topotekan, azidothymidin, nukleosidová a nukleotidová analoga	játra, ledviny, střevo, řada dalších orgánů
ABCG2	BCRP (MXR)	estron-3-sulfát a kyselina listová	rosuvastatin, imatinib, lamivudin, irinotekan, metotrexát, sulfatované metabolity, antracykliny, nitrofurantoin	játra, ledviny, placenta, střevo

* transportéry lokalizované na bazolaterální/sinusoidální membráně

MDR1 (P-glykoprotein)

MDR1/P-gp představuje základní, nejdříve popsaný a nejlépe prozkoumaný ABC transportér u člověka. Po identifikaci (Juliano a Ling, 1976) byl popsán jeho podíl na mnohočetné lékové rezistenci nádorových buněk (Bosch a Croop, 1996). Lokalizace MDR1 na apikálních membránách buněk tkání s exkreční (hepatocyty, enterocyty, buňky proximálních tubulů ledvin), resp. bariérovou funkcí (placenta, hematoencefalická a testikulární bariéra) a identifikace širokého spektra substrátů (Tab. 2) vedly k hypotézám o možném významu ve farmakokinetice léčiv (Thiebaut *et al.*, 1987). Tyto byly následně potvrzeny v četných *in vitro* i *in vivo* studiích (Fromm, 2003). MDR1 je lokalizován na apikálních membránách buněk s exkreční/bariérovou funkcí, tj. v játrech na kanalikulárních membránách hepatocytů resp. v ledvinách na kartáčovém lemu buněk proximálních tubulů či ve střevním epitelu na luminální membráně enterocytů, kde zprostředkovává jednosměrný eflux lipofilních látek charakteru kationtů s velkou molekulou – Tab. 2. MDR1 zřejmě úzce kooperuje s procesy biotransformace, konkrétně funkcí enzymu CYP3A4, o čemž svědčí překrývající se spektrum substrátů, lokalizace a společné regulační mechanizmy exprese prostřednictvím nukleárních receptorů PXR (z anglického „Pregnane X receptor“) a CAR (z anglického „Constitutive androstane receptor“) (Marzolini *et al.*, 2004).

BSEP

BSEP (z anglického „Bile salt export pump“) byl dosud detekován pouze v játrech, kde slouží jako základní transportér konjugovaných i nekonjugovaných žlučových kyselin

z hepatocytu do žluči, čímž se stává primárně zodpovědným za samotnou tvorbu žluči. Svou funkcí rovněž přímo navazuje na činnost bazolaterálně lokalizovaného jaterního NTCP transportéru (Byrne *et al.*, 2002). Snížení exprese nebo inhibice funkce tohoto přenašeče bylo popsáno jako jeden ze základních mechanizmů rozvoje intrahepatální cholestázy. Geneticky podmíněný deficit BSEP způsobuje závažnou poruchu nazývanou progresivní familiární intrahepatální cholestáza 2. typu (PFIC2). Pro jaterní eliminaci xenobiotik má BSEP spíše minoritní roli. Z dosud identifikovaných substrátů je možné uvést sulindak a vinblastin (Hirano *et al.*, 2005; Lecureur *et al.*, 2000). S ohledem na bezpečnost terapie je však důležité popsání léčiv-inhibitorů BSEP pro identifikaci možných cholestatických komplikací léčby – inhibičně působí např. perorální antidiabetikum glibenklamid (Alrefai a Gill, 2007).

MRP2

MRP2 je transportér s významnou fyziologickou funkcí spočívající v exkreci konjugovaných metabolitů endogenních látek (zejména bilirubinu) a xenobiotik do žluči a moči (Nies a Keppler, 2007). MRP2 zajišťuje v játrech jeden ze základních mechanizmů tvorby žluči, nezávislé na sekreci osmoticky aktivních solí žlučových kyselin. u člověka byl popsán raritní geneticky podmíněný deficit MRP2, který se projevuje konjugovanou hyperbilirubinémií označovanou jako Dubin-Johnsonův syndrom (Konig *et al.*, 1999). Tkáňová distribuce MRP2 koresponduje s distribucí konjugačních enzymů fáze II, UDP-glukuronyltransferázou a glutathion-S-transferázou, což potvrzuje navržený model efektivní synergie konjugačního metabolizmu a následného transportu organických aniontů, pro který svědčí i společná regulace prostřednictvím nukleárních receptorů (PXR, CAR a FXR – viz Tab. 4) (Zamek-Gliszczynski *et al.*, 2006a). Tento transportér představuje základní cestu biliární exkrece pro organické anionty, například konjugáty léčiv s kyselinou glukuronovou či glutathionem (mateřské látky i jejich metabolity), ale i nekonjugované substráty (např. metotrexát, pravastatin, azitromycin) (Nies a Keppler, 2007). Z tohoto důvodu byl MRP2 ve starší terminologii označován jako cMOAT (z anglického „canalicular Multispecific organic anion transporter“) – podrobněji k vlastnostem MRP2 viz. str. 51-59 (Fuksa *et al.*, 2006).

MRP1, MRP3, MRP4, MRP5 a MRP6

Tyto transportéry, podobně jako dosud nejlépe popsaný MRP2 rovněž z podrodiny ABCC, zprostředkovávají především v játrech eflux exo- i endogenních látek, resp. jejich

metabolitů a/nebo konjugátů z buněk zpět do krve. Jejich úloha v ledvinách je zatím objasněna minimálně, nicméně např. MRP4 byl překvapivě lokalizován (na rozdíl od hepatocytů) na apikální membráně buněk proximálních tubulů. Spektrum substrátů MRP1, MRP3 a MRP4 je do značné míry podobné MRP2 (Toyoda *et al.*, 2008; Zhou *et al.*, 2008). Aktivita těchto přenašečů v hepatocytech, zejména MRP3 a MRP4, zřejmě nabývá na důležitosti během cholestázy, resp. deficitu MRP2, kdy zvýšení jejich exprese představuje kompenzační mechanizmus pro export toxických aniontů typu žlučových kyselin a konjugovaného bilirubinu, které se při cholestatických poruchách kumulují v buňce (Zollner a Trauner, 2008). Úloha MRP5 a MRP6 v celkovém obratu léčiv není dle současného stavu poznání významná.

BCRP

BCRP je transportér, obdobně jako MDR1, původně popsaný v nádorových buňkách, kde působil jejich rezistenci k řadě cytostatik, např. mitoxantronu, topotekanu, irinotekanu a doxorubicinu (Mao a Unadkat, 2005). Ve vyšším množství byl nalezen v placentě a je rovněž typickým povrchovým indikátorem kmenových buněk (Staud *et al.*, 2006). V játrech a ledvinách je společně s MDR1 a MRP2 lokalizován na kanalikulárních/apikálních membránách buněk a působí zde jako efluxní přenašeč pro řadu steroidů, xenobiotik a jejich konjugovaných metabolitů (sulfáty a glukuronidy) (Zamek-Gliszczynski *et al.*, 2006b; Enokizono *et al.*, 2007). Lokalizací a spektrem substrátů se zdá být funkčně příbuzný s P-gp, nicméně dosud nejasným zůstává způsob regulace jeho exprese a funkce.

3. Úloha transportérů v eliminaci léčiv v jednotlivých orgánech

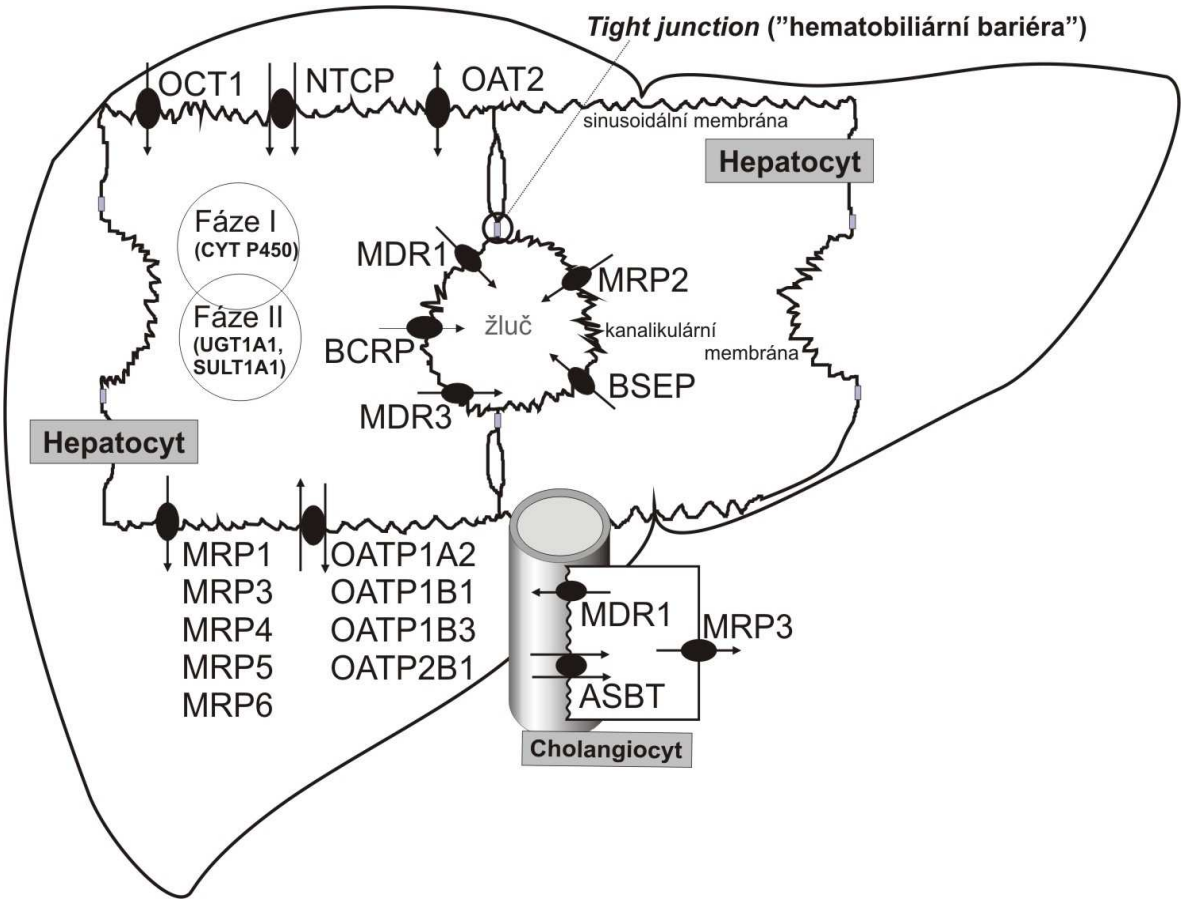
Transportní proteiny představují jeden ze základních mechanizmů eliminace léčiv zejména při zajišťování jejich biliární exkrece v hepatocytech (viz oddíl 1.3.1.), kde hydrofilní léčiva musí vždy překonat tzv. hematobiliární bariéru. Podobně klíčovou úlohu mají transportéry pro absorpci a exkreci léčiv ve střevě a rovněž v ledvinách na úrovni tubulárních buněk představují základní mechanizmus močové exkrece a reabsorpce léčiv.

3.1. Transportní procesy v játrech

Absorpce, distribuce, metabolizmus a exkrece jsou základními farmakokinetickými

ději popisujícími pohyb léčiva v organizmu. Játra představují jeden z hlavních orgánů zodpovědných za poslední dva procesy – jsou centrálním orgánem pro metabolizmus léčiv i jejich následnou exkreci do žluči. Tomu odpovídá široké spektrum biotransformačních enzymů a transportních proteinů. Tyto struktury tvoří funkční kaskádu, jejímiž kroky jsou vychytávání léčiv z krve do hepatocytů přes jejich sinusoidální (bazolaterální) membránu, metabolizmus a navazující exkrece části mateřské látky a/nebo jejích potenciálních metabolitů do žluči – viz Obr. 1 (Chandra a Brouwer, 2004). Část léčiva nebo metabolitů může být transportována zpět do krevního řečiště nebo může být vázána v hepatocytu v depotní formě. o tom, které procesy a v jakém rozsahu se podílejí na transportu konkrétního léčiva, rozhodují jeho fyzikálně-chemické vlastnosti, především struktura, velikost molekuly, rozpustnost v tucích, vazebnost a stupeň ionizace.

Absorbované léčivo se dostává do jater především prostřednictvím portální cirkulace, která představuje 60-70 % krevní saturace tohoto orgánu. Hepatocyty tedy po střevním epitelu (enterocytech) tvoří další metabolicky i exkrečně značně aktivní tkáň, se kterou přichází léčivo do kontaktu záhy po jeho perorálním podání. Ve spolupráci s intestinální bariérou tak funkčně dotváří proces presystémové eliminace („first-pass“ efekt) (Roberts *et al.*, 2002). Intenzivní přívod léčiva krví, což dosvědčuje směřování až 25 % srdečního výdeje do jater, vytváří optimální podmínky i pro eliminaci parenterálně podaných xenobiotik. Léčivo se dostává do hepatocytů přes jejich bazolaterální membránu po směru koncentračního gradientu v jaterních sinusoidech. Způsob prostupu bazolaterální membránou je závislý na molekule xenobiotika, ale i funkčním stavu aktivních molekul na membráně. u léčiv s vyšší rozpustností v tucích a menší molekulou je proces průchodu realizován zejména pasivní difúzí. Pro vychytávání léčiv s větší molekulou nebo léčiv polárních či ionizovaných je však většinou nezbytná přítomnost transportéru (van Montfoort *et al.*, 2003).



Obr. 1. Uspořádání eliminačních cest léčiv v játrech (převzato a upraveno dle Zollner and Trauner, 2008).
Léčiva jsou do hepatocytů vychytávána pomocí transportérů NTCP (z anglického „Na^+-taurocholate cotransporting polypeptide"), OAT (z anglického „Organic anion transporter") a OATP (z anglického „Organic anion transporting polypeptide") lokalizovaných na sinusoidální membráně hepatocytu. OAT2 zajišťuje vychytávání látek charakteru organických anionů a OCT1 látek charakteru organických kationů s menší molekulou. Transport do žluči je zprostředkován kanalikulárními přenašeči BSEP (z anglického „Bile salt export pump"), MRP2 (z anglického „Multidrug resistance-associated protein 2") a BCRP (z anglického „Breast cancer resistance protein") proteinem. MDR3 (z anglického „Multidrug resistance protein 3") přenáší fosfatidylcholin, který vytváří ve žluči smíšené micely společně se žlučovými kyselinami a cholesterolem. MDR1 (z anglického „Multidrug resistance protein 1") transportuje léčiva charakteru organických kationtů. MRP1, MRP3, MRP4, MRP5 a MRP6 (z anglického „Multidrug resistance-associated protein 1, 3, 4, 5 a 6") na bazolaterální membráně hepatocytu poskytují alternativní cestu exkrece žlučových kyselin a dalších látek charakteru organických aniontů do systémové cirkulace.V cholangiocytech byly popsány MDR1 a MRP3 s obdobnou úlohou jako v hepatocytech, dále ASBT (z anglického „Apical sodium-dependent bile acid transporter") transportující žlučové kyseliny.

Léčivo ve formě mateřské látky nebo případných metabolitů opouští jaterní buňku buď prostřednictvím zpětného transportu do krve, nebo je exportováno do žluči. Zatímco první děj probíhá obvykle po směru koncentračního gradientu a mohou se ho účastnit i obousměrně fungující zástupci SLC rodiny, exkrece do žluči je realizována většinou proti koncentračnímu gradientu prostřednictvím jednosměrných ABC transportérů, pro jejichž

funkci je nutná dodávka energie ve formě ATP – viz Tab. 2. Velká část ABC transportérů je za tímto účelem lokalizovaná na kanalikulární (žlučový pól) membráně hepatocytů – viz Obr. 1 (Funk, 2008). Některé proteiny (MRP1,3-6) jsou však v nezanedbatelném množství lokalizovány i na bazolaterální membráně, kde se účastní přestupu xenobiotik a jejich metabolitů z buněk zpět do krve – jejich exprese a funkce je aktivována zejména za patofyziologických situací spojených s porušenou tvorbou a odtokem žluči (cholestáza, deficit MRP2) vyžadujících ochranu hepatocytů před škodlivou kumulací endogenních látek typu bilirubinu nebo žlučových kyselin – Obr. 1 (Maher *et al.*, 2006; Zollner a Trauner, 2008). Aktivita ABC transportérů je základním mechanizmem tvorby žluči. Hlavní složku žluči představují osmoticky aktivní žlučové kyseliny, které jsou vylučovány za účasti BSEP v monovalentní formě, nebo prostřednictvím MRP2 v divalentní formě. MRP2 se navíc podílí na biliárním efluxu dalších konjugátů, včetně bilirubinu s navázaným diglukuronidem nebo glutathionem, což je funkce zabezpečující druhou složku tvorby žluči, nazývanou „na žlučových kyselinách nezávislou“ (Schinkel a Jonker, 2003). V játrech je dále lokalizován pro tento orgán specifický protein MDR3 (ABCB4). Základní aktivita tohoto kanalikulárně lokalizovaného transportéru je exkrece fosfolipidů (např. fosfatidylcholinu) do žluči, kde následně umožňují vytváření micel, čímž brání škodlivému detergentnímu působení žlučových kyselin na okolní tkáň (Lo *et al.*, 2008). Pro lipofilní léčiva navíc konstituují vazebná místa, což umožňuje jejich žlučový transport do střevního lumen a následnou enterohepatální cirkulaci nebo finální eliminaci stolicí. Význam MDR3 v eliminaci léčiv je malý, zatím byla popsána jeho schopnost s nízkou afinitou transportovat některé kationty typu digoxinu, paklitaxelu nebo vinblastinu (Smith *et al.*, 2000).

3.2. Transportní procesy v ledvinách

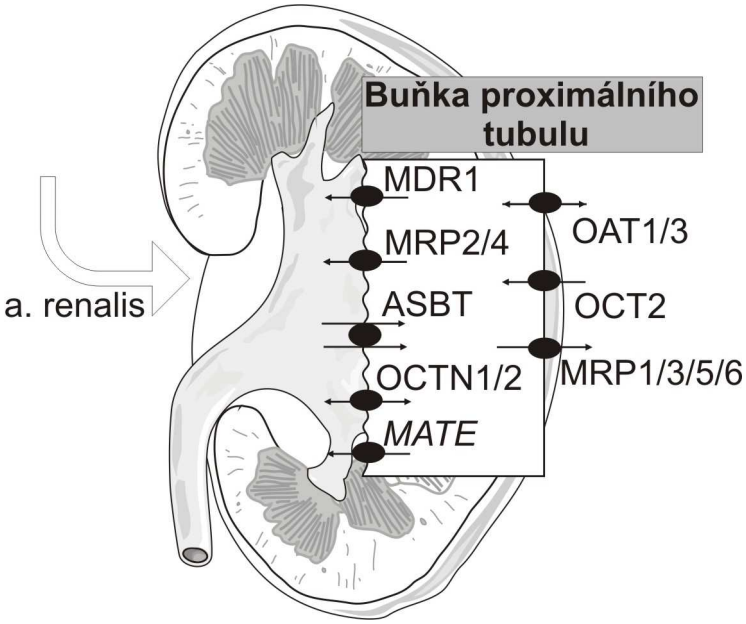
Z pohledu specifity aktivního transportu jsou v buňkách ledvin, zřejmě více než v jiných orgánech, podstatné fyzikálně-chemické vlastnosti substrátu, především stupeň a charakter ionizace. Proto jsou jednotlivé přenašeče v literatuře často rozděleny na dva systémy – dle preference pro organické anionty nebo kationty.

Systém pro transport organických aniontů v ledvinách zahrnuje dva hlavní transportéry, OAT1 a OAT3. Na bazolaterálních membránách buněk proximálních tubulů jsou dále exprimovány transportéry OAT2 a OATP4C1 (viz. Tab. 1), jejich funkční význam v kinetice léčiv však dosud není přesně znám (Kusuhara a Sugiyama, 2009). OAT1 a OAT3 byly prokázány jako hlavní aktivní transportéry pro vychytávání

a koncentrování aniontů v buňkách epitelu. Oba transportéry jsou relativně specificky inhibovány kyselinou p-aminohippurovou a benzylpenicilinem. Substrátová spektra OAT1 a OAT3 se překrývají, nicméně OAT1 se zdá více přispívat k renálnímu vychytávání nízkomolekulárních organických aniontů jako např. antivirotik (adefovir, cidofovir a tenofovir), naopak OAT3 se podílí více na influxu větších amfifilních aniontů, např. statinů, sartanů nebo benzylpenicilinu, které jsou rovněž substráty jaterních transportérů pro organické anionty (Kusuhara a Sugiyama, 2009). OAT3 dále může přenášet i některá léčiva charakteru kationtů, např. H2 antagonisty, i tzv. „zwitterionty" („obojetné" ionty), např. fexofenadin. Úloha OAT1 a OAT3 v ledvinách byla jasně popsána v *in vivo*/*in vitro* studiích za použití Oat1-/- a Oat3-/- myší, kdy u „knock-outovaných" zvířat byla pozorována významně opožděná eliminace substrátů těchto transportérů, např. diuretik. Cílové struktury farmakodynamického účinku některých diuretik jsou lokalizovány v lumen. Ve zmíněných experimentech bylo pozorováno, že nepřítomnost OAT1 a OAT3 redukuje koncentraci léčiva v lumen, čímž významně snižuje diuretický efekt furosemidu a bendroflumethiazidu (Eraly *et al.*, 2006; Vallon *et al.*, 2008). Transportéry zprostředkující luminální eflux léčiv dosud nebyly zcela identifikovány. Dle dat z *in vitro* transportních studií za použití vezikulů z membrány kartáčového lemu bylo navrženo, že apikální transport organických aniontů je tvořen dvěma funkčními systémy: (i) elektroneutrální výměnou organických aniontů a (ii) elektrochemickým gradientem usnadněným transportem. Identifikováno bylo několik transportérů, např. NPT1 (z anglického „Na^+/phosphate transporter type I") a URAT1 (z anglického „Urate transporter-1"), avšak jejich význam pro transport léčiv *in vivo* dosud nebyl objasněn. Kromě zmíněných dvou systémů byla popsána odlišná třetí funkční skupina, do které patří ABC efluxní transportéry MDR1, BCRP, MRP2 a MRP4 (Kusuhara a Sugiyama, 2009).

Hlavním transportérem organických kationtů na bazolaterální membráně proximálních tubulů je OCT2, který zprostředkovává facilitovanou difúzi různých léčiv, např. metforminu a cimetidinu (Koepsell, 2007). V experimentech zkoumajících efluxní mechanizmy bylo pozorováno, že vnější koncentrační gradient H^+ stimuluje vychytávání kationtů do vezikulů z membrány kartáčového lemu. Následně bylo vyvozeno, že systém efluxu hydrofilních organických kationtů do lumen bude využívat sekundárně aktivní transport. Nejdříve popsanými transportéry byly OCTN1 a OCTN2 (z anglického „Carnitine/organic cation transporter"). Recentně byla na apikální membráně dále identifikována pro ledviny zcela specifická skupina tzv. MATE (z anglického „Multidrug and toxin extrusion") proteinů skládající se z Mate1 (popsán v ledvinách hlodavců)

a MATE1/SLC47A1 a MATE2K/SLC47A2, které byly detekovány v lidských ledvinách (Otsuka *et al.*, 2005; Masuda *et al.*, 2006). MATE proteiny patrně transportují organické kationty vylučované močí v nemetabolizované formě, přičemž přestup látek membránou je realizován formou antiportu s protony (Kusuhara a Sugiyama, 2009).

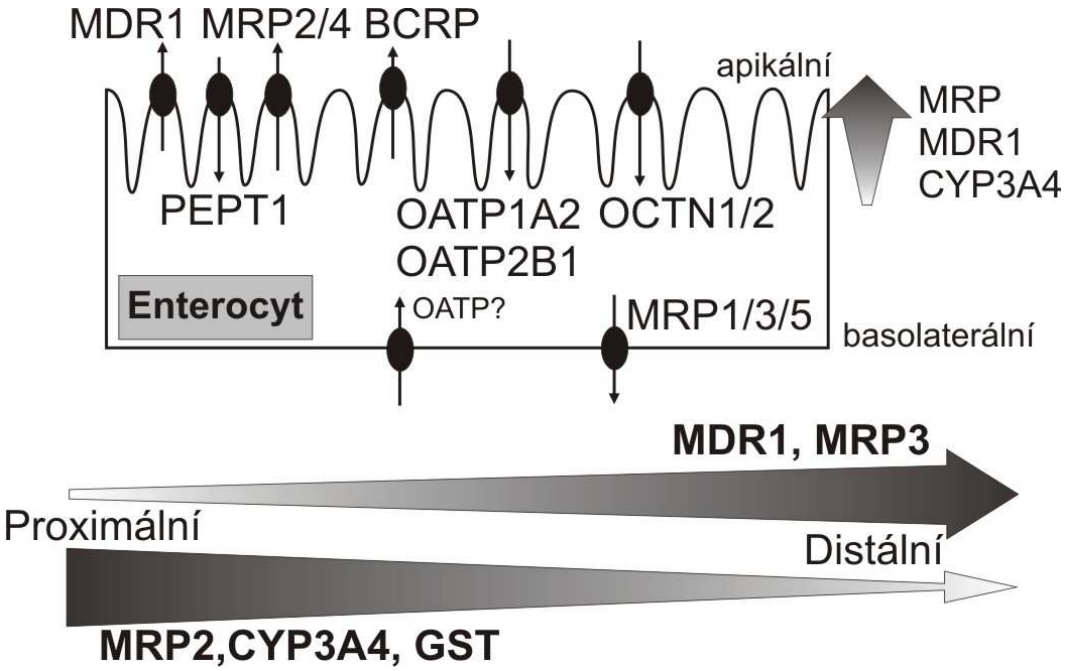


Obr. 2. Transportní proteiny v ledvinách (zpracováno dle Zollner *et al.*, 2006).
Pro vstup léčiv do buňky na bazolaterální straně je rozhodující exprese OAT1 a OAT3 (z anglického „Organic anion transporter“) pro transport aniontů a OCT2 (z anglického „Organic cation transporter“) pro transport kationtů. Na apikální membráně zajišťují exkreci látek MDR1, MRP2, MRP4 (z anglického „Multidrug resistance protein 1“ resp. „Multidrug resistance-associated protein 2, 4“), OCTN1-2 (z anglického „Carnitine/organic cation“) a MATE (z anglického „Multidrug and toxin extrusion protein) transportéry. ASBT (z anglického „Apical sodium-dependent bile acid transporter“) se podílí na reabsorpci žlučových kyselin. Za exkreci z buňky do krve na bazolaterální membráně zodpovídají MRP1, MRP3, MRP5 a MRP6 (z anglického „Multidrug resistance-associated protein 1, 3, 5, 6“).

3.3. Transportní procesy ve střevě

Střevní epitel představuje první fyziologickou překážku, kterou musí všechna léčiva a jiné látky podané per os překonat. Ačkoliv lipofilní léčiva mohou relativně snadno projít apikální plazmatickou membránou enterocytů, jejich další prostup buňkou a následně bazolaterální membránou do systému není vždy jistý. Rozsah absorpce střevním epitelem je přitom kritickým faktorem určujícím biologickou dostupnost léčiv. Funkční bariéra pro průnik xenobiotik je ve střevě tvořena synergií mezi enzymy biotransformace a efluxními transportními proteiny. Některé látky, jež nejsou substráty pro aktivní transport, pronikají do enterocytu a poté jsou konvertovány na metabolity, které již mají afinitu k transportním

efluxním proteinům. Následně tedy mohou být ze střevního epitelu aktivně exportovány zpět do lumen. Zúčastněné transportéry na apikální membráně enterocytu patří především do rodiny ABC, konkrétně je hlavní úloha připisována MDR1, MRP2 a BCRP transportérům (Chan *et al.*, 2004). Pokud jsou léčiva difundující či právě prošlá apikální membránou substráty těchto transportérů, mohou být aktivně přenášena zpět do střevního lumen, rovněž tak jejich metabolity (Walgren *et al.*, 2008). Obdobně xenobiotika již přítomná v systémové cirkulaci (bez ohledu na předchozí cestu vstupu) mohou podléhat aktivní exkreci z krve přes obě membrány enterocytu do lumen (Hunter *et al.*, 1997).



Obr. 3. Lokalizace transportérů ve střevním epitelu a na membránách enterocytu (zpracováno dle Chan *et al.*, 2004).
Pro usnadnění vstupu léčiv do buňky jsou rozhodující OATP a OCTN transportéry na apikální membráně. Aktivní eflux do střevního lumen je zajištěn ABC transportéry, konkrétně MDR1 (z anglického „Multidrug resistance protein 1"), MRP2/4 (z anglického „Multidrug resistance-associated protein 2/4")a BCRP (z anglického „Breast cancer resistance protein"). Jejich exprese se zvyšuje směrem od krypty ke špičce klku. Na basolaterální membráně byly popsány efluxní přenašeče MRP1, MRP3 a MRP5 (z anglického „Multidrug resistance-associated protein 1, 3, 5"). Intenzita exprese proteinů se mění v průběhu trávicí trubice, zvyšující se exprese distálním směrem byla pozorována u MDR1 a MRP3, naopak MRP2 (společně s enzymy biotransformace) je exprimován nejvíce v proximálních částech.

3.4. Bariérové funkce transportérů (mozek, placenta, gonády)

Hematoencefalická bariéra (HEB) přispívá k homeostáze buněk centrálního nervového systému jejich ochranou před potenciálně škodlivými endo- i exogenními látkami. HEB je fyziologické rozhraní mezi krevním řečištěm a mozkem složené ze

souvislé vrstvy endoteliálních buněk bez fenestrací těsně propojených pomocí tzv. „tight junctions“ a vrstvy podpůrných buněk (gliové buňky a výběžky astrocytů) (Begley a Brightman, 2003). HEB má dvě významné úlohy: i) umožňuje udržení stálého složení extracelulární mozkové tekutiny a ii) výrazně limituje přechod potenciálně škodlivých xenobiotik včetně terapeutik do mozkové tkáně. Právě v druhé zmíněné (ochranné) funkci HEB sehrávají významnou úlohu aktivní efluxní transportéry z ABC nadrodiny, které jsou lokalizovány na krevní (apikální) straně endoteliálních buněk, kde je jim připisován zásadní podíl na distribuci a exkreci léčiv do/z CNS. MDR1 byl prokázán jako klíčový element HEB schopný aktivního přenosu velkého množství lipofilních léčiv ven z endotelových buněk mozkových kapilár, které morfologicky tvoří vlastní bariéru. Později byly jako funkční součást HEB popsány další přenašeče z ABC nadrodiny, např. MRP1, MRP2 a BCRP. Výsledkem funkce ABC efluxních transportérů na HEB je na jedné straně zmírnění či zabránění nežádoucím účinkům, především neurotoxicitě látek, které by jinak do CNS snadno penetrovaly. Na straně druhé však tyto efluxní proteiny mohou významně omezit distribuci léčiv na místo jejich farmakodynamického účinku, tedy právě do CNS. Bylo již popsáno, že některá neurologická onemocnění mohou být asociována s nadměrnou expresí ABC transportérů, což může mít za následek rezistenci k farmakoterapii – zkoumaným neurologickým onemocněním je epilepsie. Z uvedeného důvodu se i potenciální modulace funkce ABC transportérů exprimovaných na HEB stala předmětem studia, jak zlepšit žádoucí průnik léčiv do CNS. Výzkum této oblasti může přinést například nové léčebné modality v oblasti dosud farmakorezistentních forem neurologických poruch. ABC transportéry MDR1, MRP2 nebo BCRP se nabízí jako cílové struktury pro terapeutické strategie navrhující modifikaci HEB ve smyslu zlepšení průniku žádoucích léčiv do CNS, a to díky jejich lokalizaci na luminální straně endotelových buněk mozkových kapilár, funkci efluxní pumpy a popsanému širokému spektru substrátů. Taková strategie se může jevit opodstatněná obzvláště ve světle přibývajících důkazů identifikujících nadměrnou expresi těchto transportérů jako hlavní patologický mechanizmus farmakorezistence neurologických onemocnění u některých populací pacientů (Loscher a Potschka 2005).

Placenta představuje fyziologickou bariéru, která odděluje cirkulaci matky a plodu. Většina léčiv podaných v průběhu těhotenství může, alespoň do určité míry, přestoupit do cirkulace plodu (Audus, 1999). Míra a rychlost přestupu léčiva závisí přirozeně na fyzikálně-chemických vlastnostech léčiva (molekulová hmotnost, lipofilita, stupeň

ionizace, vazebnost na plazmatické bílkoviny), anatomickém uspořádání (např. mezidruhové rozdíly (Carter a Enders, 2004), tloušťka placentární membrány) a fyziologických charakteristikách placenty (průtok krve placentou aj.).

V lidské placentě musí léčivo překonat bariéru mnohojaderného syncytiotrofoblastu, resp. bazální a apikální cytoplazmatickou membránu a dále musí prostoupit endotelem fetálních kapilár. Většina léčiv přechází přes tyto membrány prostou difúzí. Některá léčiva mohou být rozpoznána přenašeči pro endogenní substráty (především ze skupiny SLC transportérů) a placentou mohou procházet facilitovanou difúzí, která je však obecně mezi transportními ději minoritní (Ganapathy *et al.*, 2000; Syme *et al.*, 2004). Hlavní vliv na omezení prostupu farmak přes placentu maternofetálním směrem mají ABC transportéry MDR1, MRP2 a BCRP lokalizované na apikální membráně syncytiotrofoblastu (St-Pierre *et al.*, 2000; Pavek *et al.*, 2003; Staud *et al.*, 2006). Současně se tyto transportéry podílejí na odstraňování endo- a xenobiotik z krve plodu. Dosud méně objasněn byl význam transportérů MRP1 a MRP3, které byly lokalizovány v oblasti bazolaterální membrány syncytiotrofoblastu i fetálního endotelu (Atkinson *et al.*, 2003; Nagashige *et al.*, 2003).

Selektivní prostup xenobiotik skrze Sertoliho buňku varlete je determinován existencí funkční tzv. *hematotestikulární bariéry*. Zároveň spojení typu „tight junction“ u báze Sertoliho buněk varlat představují fyzikální bariéru izolující adluminální kompartment semenných tubulů od zbytku organizmu. Vyvíjející se zárodečné buňky jsou v tomto prostředí chráněny před vlivem xenobiotik i imunitního systému. Výzkum konkrétních fyziologických mechanizmů podílejících se na ochraně zrajících zárodečných buněk je dosud relativně omezený, nicméně společně s imunologickými protektivními mechanizmy je v ochraně před léčivy a toxiny připisována významná role i aktivnímu efluxu. Na membráně Sertoliho (nebo i Leydigových a myoidních) buněk byla zatím, analogicky jiným fyziologickým bariérám, popsána exprese některých zástupců z rodiny ABC transportérů, např. MRP1, BCRP a MDR1 (Bart *et al.*, 2007), u nichž se přepokládá významná úloha právě především v kontinuálním aktivním exportu menších lipofilních molekul procházejících buněčnou membránou (Maeda *et al.*, 2007).

4. Faktory ovlivňující expresi a funkci transportérů jako zdroj lékových interakcí

Variabilita aktivity transportních proteinů (fenotyp) zapojených do distribuce

a eliminace léčiv je, společně se změnami aktivity biotransformačních enzymů, jedním z hlavních zdrojů intra- a interindividuální variability v odpovědi organizmu na podání léčiva. Příčinou těchto změn je multifaktoriální regulace exprese a funkce cílových molekul. Primárním determinujícím faktorem je genetická výbava – genotyp (Kerb, 2006). Avšak přítomnost dalších modifikujících faktorů zřídka umožňuje najít přímý vztah mezi genotypem a výsledným fenotypem. Důvodem je především komplexní flexibilní systém regulace exprese konkrétních znaků, který citlivě reaguje na přítomnost látky indukující nebo suprimující expresi genu. Současně je možná přímá inhibice funkce transportéru. Aktuální výsledná aktivita kteréhokoliv transportéru (ale např. i enzymu) je tak utvářena vlivem mnoha endogenních a exogenních mechanizmů, čímž vzniká unikátní prostředí individuálního organizmu v konkrétním čase, což vede k rozdílným aktivitám transportérů mezi jednotlivci, ale i u jednotlivce v závislosti na čase.

Farmakogenetika transportu léčiv

V důsledku přítomnosti aberantní (mutované) alely se mění výsledná aktivita transportéru, což se u postiženého jedince ve většině případů projeví snížením nebo ztrátou funkce cílového proteinu. Jelikož se poškození přenáší autosomálně recesivně, následky jsou výraznější u homozygotů pro defektní gen. Rozvoj molekulárně-biologických metod a jejich automatizace umožňují provádět poměrně rozsáhlé studie, jejichž výsledkem byla např. identifikace genetické variability u některých transportérů (především MDR1, MRP2, BSEP a zástupce skupiny OATP), ale především u více prozkoumaných biotransformačních enzymů (např. CYP2D6, CYP2C9, CYP2C19, UGT1A1 a NAT). Některé příklady vlivu mutací v genech pro transportéry na farmakoterapii z klinické praxe jsou uvedeny v Tab. 3.

Tab. 3. Příklady klinicky významných polymorfismů transportérů a jejich důsledky. (zpracováno dle Kerb, 2006; Maeda a Sugiyama, 2008)

Gen	**Léčivo**	**Klinické důsledky variability**	**Prokázaný nebo předpokládaný mechanizmus**
MDR1	Fenytoin	Zvýšená absorpce léčiva	Hypofunkční alely
MDR1	HIV inhibitory proteáz	Zvýšená dostupnost do CNS	Porucha funkce P-gp na hematoencefalické bariéře
OATP	Inhibitory HMG-CoA reduktázy (statiny)	Nedostatečný efekt	Snížený vstup léčiv do jater

Změna exprese transportérů

Proces exprese genetické informace zahrnuje transkripci, translaci a postranslační modifikaci, jejichž cílem je vytvoření funkčního proteinu a jeho správná lokalizace. Ve všech dějích je zapojena řada řídících a regulačních mechanizmů a jakékoliv narušení může zapříčinit vzestup (indukci) nebo pokles (represi) ve finálním množství proteinu s korespondující změnou aktivity. V současnosti je za klíčový krok v procesu genové exprese transportérů považována regulace transkripce, a to zejména prostřednictvím nukleárních receptorů. Tato regulace je v zásadě společná pro transportéry i biotransformační enzymy. Pro produkty genů zajišťující eliminaci léčiv je důležitých několik receptorů: AhR (z anglického „Aryl hydrocarbon receptor"), PXR, CAR, FXR (z anglického „Farnesoid X receptor") a PPARα (z anglického „Peroxisome proliferator-activated receptor-α") (Urquhart *et al.*, 2007; Klaassen a Slitt, 2005; Xu *et al.*, 2005). Tyto receptory byly detekovány v jádře (některé i v cytoplazmě – AhR a CAR) jaterních buněk. Po navázání substrátu, kterým je i řada léčiv, dimerizují s RXR (z anglického „Retinoid X receptor") za vzniku komplexu, který se váže na konkrétní oblast RE (z anglického „response element") v promotorové části cílového genu. Význam nukleárních receptorů v transkripční regulaci společné pro transportní proteiny a jaterní enzymy přibližuje Tab. 4.

Tab. 4. Vliv aktivace nukleárních receptorů na funkce enzymů a transportérů. (upraveno dle Funk, 2008)

Receptor	**Ligandy**	**Regulované molekuly a jejich změny**			
		Vychytávání	**Biotransformace**		**Exkrece**
			I. fáze	II. fáze	
AhR	xenobiotika – aromatické uhlovodíky		CYP1A1 ↑ CYP1A2 ↑	UGT1A1 ↑ SULT1A1 ↑ GST-A2 ↑ aldehyd-dehydrogenáza (ALDH)↑	MDR1 ↑
PXR	léčiva – např. glukokortikoidy, rifampicin, statiny	OATP1B1 ↑ OATP1A2 ↑ OCT1 ↑	CYP2B6 ↑ CYP2C9 ↑ CYP3A4 ↑ CYP7A1 ↑	UGT1A1 ↑ GST-A2 ↑	MDR1 ↑ MRP2 ↑
CAR	léčiva – např. fenobarbital karbamazepin efavirenz fenytoin	OATP1B1 ↑	CYP2B6 ↑ CYP2C9 ↑ CYP2C19 ↑ CYP2A6 ↑ CYP3A4 ↑	UGT1A1 ↑ SULT1A1 ↑	MRP2 ↑ MRP3 ↑

FXR	žlučové kyseliny, kyselina urso-deoxycholová	OATP1B3 ↑ NTCP ↓ OATP1B1 ↓	CYP7A1 ↓	SULT2A1 ↑ UGT1A1 ↑	BSEP ↑ MRP2 ↑
PPARα	fibráty, mastné kyseliny		CYP4A1 ↑ CYP7A1 ↓		MDR3 ↑

Přímá stimulace nebo inhibice aktivního transportu

Zatímco možnost stimulace transportu prostřednictvím alosterické modulace transportního proteinu byla experimentálně popsána (indometacinem a sulfanitranem navozená stimulace efluxu estradiol 17-β-d-glukuronidu zprostředkovaného MRP2), klinický význam mají především interakce založené na inhibici funkce aktivní molekuly (Zelcer *et al.*, 2003; Lin, 2003). Právě tento typ interakcí, který může ohrozit bezpečnost terapie, vede k výzkumu enzymatických a transportních cest eliminace léčiv a kvantifikaci jejich inhibičních vlivů prostřednictvím stanovení inhibičních konstant (IC_{50} a K_i), což následně umožňuje i predikci potenciálních interakcí léčiv (Pelkonen a Turpeinen, 2007). Molekulární podstatou těchto interakcí je blokáda funkce transportéru jeho substrátem (kompetitivní – hlavní typ) nebo látkou, která působí vazbou mimo aktivní místo (nekompetitivní). Důsledkem je snížení vnitřní („intrinsic“) clearance zajišťované konkrétním přenašečem. Při současné aplikaci léčiva, pro jehož celkovou kinetiku má inhibovaná cesta klíčový význam, pak dojde k vzestupu plazmatických koncentrací tohoto léčiva. Takový nárůst může být u léčiva s úzkým terapeutickým oknem spojen s projevy toxicity (Lin, 2003). Nástup inhibiční interakce je téměř okamžitý, jelikož se jedná o přímé působení na cílovou molekulu. Rovněž časové trvání takové interakce zcela koresponduje s přítomností inhibitoru v místě lokalizace transportéru – odpovídá tedy rovněž i biologickému poločasu eliminace inhibitoru.

5. Možnosti výzkumu lékových interakcí na úrovni aktivního transportu

Základním nástrojem pro farmakologický výzkum aktivního transportu a potenciálních kinetických interakcí jsou *in vitro*, *in situ* a *in vivo* modely. V případě *in vitro* systémů zůstává stále velkou limitací na poli tohoto výzkumu nedostatek modelů zachovávajících komplexní morfologii orgánu a současně i fyziologickou funkci jednotlivých buněk. Zdánlivé výhody *in vivo* systémů či izolovaných perfundovaných orgánů (ve vyšší míře reflektující skutečný fyziologický stav orgánu) jsou vyváženy jejich značnou technickou náročností a menší efektivitou (spotřeba času a experimentálních

zvířat) a přirozeně i odlišnostmi druhu laboratorních zvířat (hlodavců), který je v experimentech používán. Dále je zřejmé, že komplexnost fungování celého orgánu (tkáně) neumožňuje uspokojivě studovat jednotlivé individuální mechanizmy pro vychytávání či eflux substrátů z exkreční tkáně. Možnosti posouzení vlivu konkrétních transportérů na celkovou dispozici jejich substrátů/léčiv jsou dále omezeny neexistencí dostatečně specifických silných inhibitorů (Funk, 2008).

5.1. Metody *in vitro*

Často používaným *in vitro* modelem pro studium jaterního transportu jsou **izolované hepatocyty**. Modely založené na hepatocytech izolovaných z lidské jaterní tkáně eliminují problémy spojené s druhovými rozdíly v hepatobiliární dispozici substrátů. Izolované hepatocyty jsou široce používány pro studium mechanizmů vychytávání léčiv, naopak jejich význam pro studium exkrečních mechanizmů je výrazně limitován redistribucí kanalikulárních membránových proteinů a ztrátou polarizace buněk (Kučera *et al.*, 2006). Od hepatocytů odvozené **buněčné linie** (WIF-B, HepG2) schopné vytvářet „exkreční domény" podobné žlučovým kanálkům jsou, např. po transfekci genu pro jeden či více přenašečů, používány pro studium intracelulárního přesunu a regulace transportérů (Sai *et al.*, 1999; Zegers *et al.*, 1998). Transfekované buněčné linie jsou rovněž modely používanými i pro průmyslový preklinický výzkum interakčního potenciálu nových molekul s transportéry (především ve smyslu inhibice jejich funkce) a stanovení základních parametrů např. IC_{50} a K_i (Zhang *et al.*, 2008). Kanalikulární exkrece substrátů je zkoumána na spárovaných hepatocytech (dvojici buněk zachované i po inkubaci s kolagenázou, kde je zachován žlučový kanálek) (Graf *et al.*, 1990). Naopak konvenční kultury hepatocytů (na rigidním substrátu) nejsou vhodné ke studiu aktivního transportu, neboť rychle „dediferencují", tj. ztrácejí fyziologickou morfologii včetně kanalikulární sítě a mizí i specifické jaterní funkce včetně aktivního transportu (LeCluyse *et al.*, 1996). Primární kultury uchované mezi dvěma vrstvami kolagenového gelu (tzv. „sendvičová kultivace") vyvíjejí intaktní kanalikulární sítě, zachovávají expresi a funkci transportérů a polarizaci buňky, a jsou tedy preferovaným a široce používaným *in vitro* modelem pro výzkum hepatobiliární dispozice látek (Liu *et al.*, 1999).

Pro výzkum tubulární sekrece v ledvinách je množství *in vitro* systémů dosud významně omezeno. Jedním ze základních používaných modelů jsou **řezy ledvin** včetně čerstvě připravených lidských tkání po nefrektomii (Nozaki *et al.*, 2003). Ke studiu transportu (substrátové specifity) byl dále používán model **vezikulů připravených**

z membrány kartáčového lemu, což je systém velmi podobný jaterním vezikulům připraveným z kanalikulární membrány hepatocytů. Tyto systémy sledují vlastnosti či modulaci aktivního transportu pomocí míry akumulace substrátů uvnitř veziklu. Limitací ledvinového modelu je skutečnost, že na rozdíl od jaterního protějšku vezikly z membrány kartáčového lemu nelze použít pro studium funkce ABC efluxních transportérů, jelikož vezikly jsou správně orientovány vně apikální membránou (Kusuhara *et al.*, 2009).

5.2. Metody *in situ/ ex vivo*

Patří sem metody využívající izolovaného perfundovaného střeva, jater či ledviny. Ve srovnání s *in vitro* modely nabízejí přesnější popis funkce transportérů v absorpci a eliminaci léčiv a její souvislost s funkcí biotransformačních enzymů. Sledovaná látka či látky jsou přidány do perfúzního média a dopraveny do sledovaného orgánu. Po ukončení perfúze jsou měřeny koncentrace látky v tkáni, perfuzátu a všech eliminačních tekutinách (např. žluči či moči). Uvedený model umožňuje sledovat úlohu a změny transportu v konkrétním orgánu jednodušeji než při *in vivo* experimentu s celým organizmem, jelikož je kontrolována koncentrace léčiva dodávaného přímo do sledované tkáně a zároveň je eliminován vliv ostatních orgánů. Značnou nevýhodou perfúzních metod je technická (chirurgická zručnost, laboratorní vybavení) i časová náročnost. Integrita orgánů a fyziologické fungování aktivních molekul rovněž s časem perfúze rychle klesají (Xia *et al.*, 2007).

5.3. Metody *in vivo*

Jednoznačné informace především o následcích lékových interakcí přinášejí klinická data demonstrující inhibiční nebo indukční potenciál léčiv u nemocných nebo zdravých dobrovolníků. Pro detailnější výzkum fyziologické úlohy transportérů i jejich role v lékových interakcích jsou však nezastupitelné *in vivo* zvířecí modely. První ze základních současných možností přístupu k testování funkce konkrétních transportérů je využití geneticky modifikovaných myší nebo přirozeně se vyskytujících mutantních živočichů v genu pro konkrétní transportér/y (tzv. „genový knock-out“) (Chen *et al.*, 2003). Geneticky „knock-outované“ myši jsou produkovány rozrušením genů pro endogenní transportér. Přírodní mutanty P-gp nebo Mrp2 byly popsány u myší, potkanů nebo psů (Xia *et al.*, 2007). Využití mutant pro popis role transportérů s sebou nese známá úskalí, především jistou nepřesnost díky popsaným (často kompenzačním) změnám

exprese a funkce dalších (nejen) transportních fyziologických mechanizmů (Schuetz *et al.*, 2000).

Další možností *in vivo* výzkumu je podávání známých specifických inhibitorů transportér/ů (tzv. „chemický knock-out“). Zatímco pro P-gp či BCRP jsou k dispozici selektivní inhibitory, např. GF120918 (oba transportéry), LX335979 (inhibitor P-gp) a Ko143 (inhibitor BCRP), pro skupinu MRP či OATP přenašečů dosud specifické inhibitory popsány nebyly (Xia *et al.*, 2007). Během podávání inhibitoru může být sledován farmakokinetický profil řady léčiv/substrátů a podrobněji popsán vliv inhibice transportéru na jejich farmakokinetiku. Obdobně jako inhibiční studie mohou být koncipovány i studie *in vivo* se známými induktory exprese transportérů. Uvedené přístupy a především možnost jejich potenciálního zobecnění čelí problematice specifity podaných inhibitorů či induktorů a výběru vhodné dávky. V prezentovaných publikacích byly použity interakční modely založené na analýze kinetických parametrů léčiva resp. modelového substrátu transportérů v ustáleném stavu po vícedenní předléčbě nebo při současném akutním podání druhého léčiva (inhibitoru resp. induktoru transportu), jehož interakční potenciál byl zkoumán.

5.3.1. Farmakokinetická analýza

Pro hodnocení změn farmakokinetiky léčiv *in vivo* se u modelů zvýšené exprese (indukce, tj. „up-regulace“) či snížené exprese („represe“, tj. „down-regulace“) transportérů používají dva non-kompartmentové přístupy. První je založený na kontinuálním podávání léčiva pro dosažení ustáleného stavu plazmatických koncentrací, druhý vychází z jednorázového podání léčiva a následného dostatečně dlouhého sledování plazmatických koncentrací pro získání jejich co nejkompletnějšího profilu (včetně eliminační fáze). Biliární a renální eliminace se počítá z koncentrací naměřených v nasbíraných vzorcích žluči a moči.

Sledování farmakokinetických parametrů léčiva **po dosažení** jeho **ustálených koncentrací** („steady-state“) **v plazmě** nabízí výhodu kratšího trvání experimentu, kdy za pomoci vhodně zvolené nárazové dávky a následné kontinuální nitrožilní infuze lze navodit tuto situaci do několika desítek minut od zahájení podávání. Pro výpočet jednotlivých parametrů se pak používají následující rovnice:

$$BE = X_{\check{Z}} \ x \ C_{\check{Z}}$$

$$RE = X_M \ x \ C_M$$

kde BE je biliární exkrece léčiva (nmol/min/kg), $X_Ž$ tok žluči (μl/min), $C_Ž$ koncentrace léčiva ve žluči (μM); RE je renální exkrece léčiva (nmol/min/kg), X_M tok moči (μl/min) a C_M koncentrace léčiva v moči (μM).

$$CL_{TOT} = R / C_{SS}$$
$$CL_B = BE / C_{SS}$$
$$CL_R = RE / C_{SS}$$

kde CL_{TOT} je celková clearance (ml/min/kg), R rychlost infúze (μmol/kg/min), C_{ss} koncentrace léčiva v ustáleném stavu, CL_B biliární clearance, BE biliární exkrece, CL_R renální clearance a RE renální exkrece. Podíl glomerulární filtrace (GF) na CL_R je pak vyjádřen pomocí stanovení clearance kreatininu (CL_{KR}):

$$CL_{KR} = \frac{X_M x C_M}{C_P}$$

kde CL_{KR} je clearance kreatininu, X_M tok moči, C_M koncentrace kreatininu v moči, C_P koncentrace kreatininu v plazmě.

Farmakokinetické parametry léčiva po jednorázovém podání vychází z komplexní analýzy časového průběhu plazmatických koncentrací léčiva. Iniciálně se z křivky určuje nejvyšší naměřená plazmatická koncentrace látky (C_{max}) a příslušná doba dosažení této koncentrace (T_{max}). Analýza terminální fáze časové křivky umožní odečíst eliminační konstantu (k_{el}), která představuje směrnici této křivky. Z ní je pak počítán biologický poločas eliminace ($t_{1/2}$) jako podíl ln2 a k_{el}. Následně je počítána plocha pod křivkou plazmatických koncentrací ($AUC_{0-\infty}$) jako součet $AUC_{0\text{-}T_{poslední}}$ určené logaritmicko-lineárním lichoběžníkovým pravidlem mezi časem 0 (podání léčiva) a časem poslední měřené koncentrace ($T_{poslední}$) a AUC extrapolované do nekonečna ($AUC_{T_{poslední}-\infty}$), která se určuje jako podíl poslední měřené koncentrace dělené k_{el}. Celková clearance (CL_{Tot}) se určuje vztahem:

$$CL_{Tot} = dávka / AUC_{0-\infty}$$

Paralelně je počítán zdánlivý distribuční objem (Vd_z):

$$Vd_z = CL_{Tot} / k_{el}$$

Distribuční objem pro ustálený stav (Vd_{ss}) je získáván vztahem:

$$Vd_{ss} = CL_{Tot} x \frac{AUMC}{AUC_{Tposlední-\infty}}$$

kde AUMC je momentová plocha pod křivkou.

Biliární (CL_B) a renální (CL_R) clearance lze při tomto způsobu hodnocení stanovit pomocí vztahu:

$$CL_B = M_B / AUC_{0 \rightarrow Tposlední}$$
$$CL_R = M_R / AUC_{0 \rightarrow Tposlední}$$

kde M_B a M_R je množství léčiva vyloučené do žluči nebo moči během sledovaného období. Výhodou tohoto přístupu je vyšší citlivost modelu na potenciální změny a komplexnější informace o kinetice (např. distribuční objem), nevýhodou je nutnost dostatečně dlouhého sledování pro získání co nejkompletnějšího profilu koncentrací v čase.

Cíle práce

Cíle dizertační práce:

Zvýšená nebo snížená exprese transportních proteinů byla opakovaně popsána *in vitro* po podání řady modelových léčiv. Pozorované změny vedly k hypotézám, že modulace funkce transportních proteinů může být u řady léčiv s nižším terapeutickým indexem (obdobně jako u jiných látek v případě změn aktivity biotransformačních enzymů) příčinou klinicky signifikantních lékových interakcí ohrožujících bezpečnost farmakoterapie.

Cílem předložené práce bylo blíže zkoumat a popsat mechanizmy lékových interakcí na transportních proteinech za využití *in vivo* modelu potkana. Konkrétně:

1. Sledovat vliv dexamethasonu jakožto modelového induktoru na expresi a funkci transportérů P-gp a Mrp2 v eliminačních orgánech.
2. Detailně popsat morfologii změn exprese Mrp2 transportéru v jaterní tkáni po opakovaném podávání dexamethasonu.
3. Popsat vliv amiodaronu a azitromycinu na kinetiku organických aniontů, nízkodávkového metotrexátu, resp. konjugovaného bilirubinu a blíže popsat mechanizmus lékové interakce.
4. Sledovat vliv chronického podávání amiodaronu na expresi a funkci Oatp2, P-gp a Mrp2 transportérů v eliminačních orgánech.

Podíl na jednotlivých publikacích

Výsledky doktorandovi vědecké experimentální i rešeršní práce a jejich diskuze jsou prezentovány souborem šesti publikací:

I. Fuksa L, Mičuda S, Cermanová J, Brčáková E and Štaud F (2006) Fyziologická funkce MRP2. Československá fyziologie 55:57-65.

II. Micuda S, Fuksa L, Mundlova L, Osterreicher J, Mokry J, Cermanova J, Brcakova E, Staud F, Pokorna P and Martinkova J (2007) Morphological and functional changes in p-glycoprotein during dexamethasone-induced hepatomegaly. Clin Exp Pharmacol Physiol 34:296-303.

III. Micuda S, Fuksa L, Brcakova E, Osterreicher J, Cermanova J, Cibicek N, Mokry J, Staud F and Martinkova J (2008) Zonation of multidrug resistance-associated protein 2 in rat liver after induction with dexamethasone. J Gastroenterol Hepatol 23:e225-e230.

IV. Fuksa L, Brcakova E, Cermanova J, Hroch M, Chladek J, Kolouchova G, Malakova J, Martinkova J, Staud F and Micuda S (2008) Amiodarone modulates pharmacokinetics of low-dose methotrexate in rats. Biopharm Drug Dispos 29:289-299.

V. Cermanova J, Fuksa L, Brcakova E, Hroch M, Kucera O, Kolouchova G, Hirsova P, Malakova J, Staud F, Martinkova J, Cervinkova Z and Micuda S (2010) Up-regulation of renal Mdr1 and Mrp2 transporters during amiodarone pretreatment in rats. Pharmacol Res 61:129-135.

VI. Fuksa L, Brcakova E, Kolouchova G, Hirsova P, Hroch M, Cermanova J, Staud F, Micuda S (2010) Dexamethasone reduces methotrexate biliary elimination and potentiates its hepatotoxicity in rats. Toxicology 267:165-171.

U kapitoly I., IV. a VI. je předkladatel dizertační práce prvním autorem, v případě kapitol II., III. a V. spoluautorem. Autor dizertace sepsal všechny tři rukopisy, u nichž je prvním autorem. U prvoautorských prací provedl doktorand rovněž kompletní zpracování dat a veškeré doplňkové práce v rámci oponentních řízení během jejich publikování.

V práci uvedené v kapitole II. se autor dizertace podílel na všech *in vivo* experimentech, odběru vzorků a analýze proteinové exprese pomocí Western blotu pod vedením Doc. MUDr. Stanislava Mičudy Ph.D. Ve spolupráci se skupinou Doc. Štauda z Farmaceutické fakulty zavedl analýzu genové exprese pomocí metodiky kvantitativní RT-PCR. Autorem imunohistochemické analýzy a hodnocení optické denzity P-gp a akumulace tuku v játrech je pplk. prof. Jan Osterreicher Ph.D. z Katedry radiobiologie, Fakulty vojenského lékařství Univerzity obrany. Analytické stanovení rhodaminu-123

pomocí metody HPLC provedla Ing. Lucie Mundlová z Ústavu farmakologie Lékařské fakulty.

V práci uvedené v kapitole III. se autor dizertace podílel na všech *in vivo* experimentech a provedl analýzu exprese proteinů pomocí Western blotu. Kvantitativní imunohistochemie byla opět provedena pplk. prof. Jan Osterreicherem Ph.D. z Katedry radiobiologie, Fakulty vojenského zdravotnictví Univerzity obrany. Stanovení konjugovaného bilirubinu ve vzorcích a sérových biochemických parametrů provedl MUDr. Norbert Cibiček Ph.D. z Ústavu klinické biochemie a diagnostiky Fakultní nemocnice HK.

V práci uvedené v kapitole IV. se doktorand podílel na *in vivo* experimentech. HPLC analytické stanovení koncentrací amiodaronu bylo provedeno PharmDr. Janou Malákovou Ph.D. z Ústavu klinické biochemie a diagnostiky Fakultní nemocnice HK, dále HPLC analytické stanovení metotrexátu a azitromycinu provedli Ing. Miloš Hroch a Bc Jitka Hájková z Ústavu farmakologie Lékařské fakulty.

V práci uvedené v kapitole V. se autor dizertace podílel na *in vivo* experimentech, provedl analýzy proteinové exprese zkoumaných transportérů a ve spolupráci s Mgr. Evou Brčákovou Ph.D. (Ústav farmakologie Lékařské fakulty) a MUDr. Ottou Kučerou Ph.D. (Ústav fyziologie Lékařské fakulty) provedl *in vitro* kumulační a efluxní experimenty s rhodaminem-123 na primárních potkaních hepatocytech. HPLC analytické stanovení koncentrací amiodaronu a měření koncentrací bilirubinu a kreatininu bylo provedeno PharmDr. Janou Malákovou Ph.D. z Ústavu klinické biochemie a diagnostiky Fakultní nemocnice HK, dále HPLC analytické stanovení rhodaminu-123 provedl Ing. Miloš Hroch z Ústavu farmakologie Lékařské fakulty.

V práci uvedené v kapitole VI. se autor dizertace podílel na všech *in vivo* experimentech a provedl analýzu exprese proteinů pomocí Western blotu a expresi mRNA pomocí kvantitativní PCR v reálném čase. Stanovení konjugovaného bilirubinu ve vzorcích a sérových biochemických parametrů bylo provedeno na Ústavu klinické biochemie a diagnostiky Fakultní nemocnice HK. HPLC analytické stanovení metotrexátu provedl Ing. Miloš Hroch z Ústavu farmakologie Lékařské fakulty.

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FYZIOLOGICKÁ FUNKCE MRP2

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Fyziologická funkce MRP2

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SOUHRN

Studium úlohy transportních proteinů ve farmakokinetice a farmakodynamice léčiv je rychle se rozvíjející oblastí biomedicínského výzkumu. MRP2 přenašeč, exprimován na apikální membráně buněk mnoha absorpčních a exkrečních orgánů, má nezanedbatelný vliv na farmakokinetické parametry řady léčiv. Jeho zvýšená exprese v nádorových buňkách také svědčí o podílu na fenoménu mnohočetné rezistence vůči cytostatikům. Ve zdravých tkáních za fyziologických podmínek naopak slouží jako prostředek detoxikace a ochrany před nežádoucími látkami. To dokládá i dobře prokázaná spojitost exprese a regulace MRP2 transportéru s dalšími mechanizmy ovlivňujícími absorpci, distribuci a eliminaci látek, kupříkladu biotransformačními enzymy I. a II. fáze a dalšími efluxními transportéry (P-glykoprotein atd.). MRP2 tak představuje významný článek v komplexním dynamickém systému ochrany organizmu před poškozením chemickými látkami. Ačkoliv současné znalosti o tomto transportéru nejsou zdaleka kompletní, poskytují dnes šanci pro lepší pochopení farmakodynamických a farmakokinetických vlastností řady xenobiotik. Poznání mechanizmů zapojených do regulace exprese a funkce MRP2 na molekulární úrovni tak přináší do budoucna možnost výrazného zefektivnění farmakoterapie pomocí modulace jeho aktivity.
Klíčová slova: MRP2, multidrug resistence, játra, biliární sekrece, transport xenobiotik

SUMMARY

The role of transport proteins in distribution, action and toxicity of drugs has recently become an increasingly popular scope of biomedicinal research. The MRP2 transporter, expressed in the apical membrane of polarized cells in organs of absorption and excretion, has a considerable impact on pharmacokinetic parameters of many substances. Increased expression of MRP2 found in tumour cells suggests its contribution to multidrug resistence phenomenon. In healthy tissues under physiological conditions, MRP2 functions as a mean of detoxification as well as protection against potentially harmful xenobiotics. Accordingly, the tissue distribution and regulation of MRP2 transporter was proven to be coordinated with other mechanisms responsible for absorption, distribution, and elimination of substances, namely with enzymes of phase I and II biotransformation, and other efflux transporters (P-glycoprotein etc.). Therefore, MRP2 plays an important role in the complex dynamic system of organism's defense against harmful chemical substances. Although present knowledge of this transporter are incomplete, it provides better understanding of farmacodynamic and farmacokinetic properties of many drugs. Furthermore, the increasing understanding of mechanisms involved in regulation of expression and function of MRP2 at the molecular level may also bring us to more effective therapeutic approaches through modulation of the transporters' function.
Key words: MRP2; multidrug resistence; liver; biliar secretion; transport of xenobiotics

ÚVOD

Aby se léčiva dostala na místo svého účinku, musí zpravidla projít přes řadu buněčných membrán. Toho mohou dosáhnout buď prostou (pasivní) difúzí nebo různými transportními procesy zprostředkovanými specifickými přenašeči. Tyto transportéry se podílejí jak na importu (influxu) látek do buněk, tak na jejich exportu (efluxu) z buněk.

Obecně lze konstatovat, že membránová permeabilita je z velké části předurčena fyzikálně-chemickými vlastnostmi látek, především jejich lipofilitou. Nicméně zvýšená lipofilita není vždy zárukou dobré absorpce a penetrace do buněk díky existenci transportních proteinů, neboť právě efluxní mechanizmy mohou velmi podstatně determinovat schopnost léčiva kumulovat se v buňkách, resp. tkáních. Klinický a toxikologický význam těchto makromolekul spočívá v jejich značném podílu na distribuci a eliminaci léčiv a xenobiotik obecně.

ABC transportní proteiny

ABC transportéry (z anglického ATP-binding cassette) jsou značně rozsáhlou nadrodinou, jejímž společným strukturním znakem je vysoce konzervativní doména pro vazbu

ATP. Hydrolýza této molekuly slouží jako zdroj energie pro přenos substrátu. V současné době je v této skupině několika desítek ABC proteinů klasifikováno 7 podrodin od ABCA po ABCG. Substráty pro tyto přenašeče tvoří fosfolipidy, peptidy, steroidy, polysacharidy, aminokyseliny, nukleotidy, organické aniony, léčiva, toxiny a jejich odpovídající metabolity, resp. konjugáty. Spektra substrátů se často mezi různými transportéry překrývají, navíc exogenní substráty mohou o transportéry soutěžit jak mezi sebou, tak i se substráty endogenními. Tím se otevírá prostor pro nejrůznější interakce, jež potom mohou významně ovlivnit distribuci příslušných přenášených látek. Klíčová role je rodině efluxních ABC transportérů přisouzena v ovlivnění absorpce, distribuce a exkrece toxických substancí. To dokladuje i jejich preferenční lokalizace v orgánech a strukturách s exkreční a bariérovou funkcí, tj. v játrech, ledvinách, střevě, plicích, placentě, mozku a gonádách (Pavek et al., 2002). Zde působí proti průniku cizorodých látek a zároveň zajišťují mechanizmus pro jejich eliminaci. Tato funkce ABC transportérů je podporována přítomností biotransformačních enzymů, které přeměňují látky na metabolity, jež jsou dále substráty pro efluxní přenašeče. V orgánech s exkreční funkcí tak vzniká funkční synergie, která chrání organizmus před nežádoucími účinky vyplývajícími z kumulace xenobiotik. Velká část současných znalostí o efluxních transportérech je založena na výzkumu P-glykoproteinu (MDR1/ABCB1) objeveného již v r. 1976 (Juliano a Ling 1976). Objev a výzkum tohoto proteinu (a později dalších) je spojen s problémem tzv. mnohočetné rezistence vůči cytostatikům (Donnenberg a Donnenberg 2005) – z anglického multidrug resistence – kdy řada nádorových buněk málo reagujících na terapii ve zvýšené míře exprimuje právě ABC transportéry. Tyto přenašeče aktivně vylučují cytostatika z buňky, brání jim tak dosažení místa účinku a činí buňku rezistentní. S narůstajícím spektrem informací se ukazuje, že kromě MDR1 je pro kinetiku endo- i xenobiotik velmi důležitý další ABC přenašeč – MRP2 (multidrug resistance-associated protein 2).

MRP2 (multidrug resistance-associated protein 2)

První informace o tomto proteinu byly získány na konci 80. let, kdy experimenty za použití organických aniontů typu konjugovaného bilirubinu a leukotrienu C4 (LTC4) prokázaly existenci jednosměrného přenašeče lokalizovaného na apikálních (kanalikulárních) membránách buněk tkání s exkreční funkcí. Původní název cMOAT (kanalikulární multispecifický transportér organických aniontů) byl změněn po identifikaci MRP2 (ABCC2) kódujícího genu (Buchler et al., 1996). Následné získání specifických protilátek, DNA primerů, izolovaných subcelulárních a celulárních systémů, umožnilo detailní studium exprese a funkce tohoto přenašeče.

Molekulární charakteristika a topologie MRP2

MRP2 je fosfoglykoprotein velikosti cca 190kDa složený z 1545 aminokyselin. Ty jsou uspořádány do pěti strukturně příbuzných částí, z nichž tři tvoří transmembránové domény skládající se z šesti (resp. pěti) α-helixů, a dvě tvoří intracelulární vazebné domény pro nukleotidy (NBD, z anglického nucleotide binding domain). Třetí transmembránová doména, charakteristická pro některé přenašeče skupiny MRP včetně MRP2, je tvořena pouze pěti α-helixy a NH2-konec je proto uložen extracelulárně (viz obr.1) (Schinkel a Jonker, 2003). Obě vazebné domény pro nukleotidy vykazují u jednotlivých proteinů vysokou identitu, obzvláště v jádrovém úseku asi 200 aminokyselin. Dvě sekvence lokalizované v každé NBD, označené „Walker A motiv“ a „Walker B motiv“ jsou klíčové pro ATP-ázovou funkci. Walker A se podílí na vazbě β-fosfátu z ATP a Walker B váže hořečnaté ionty. Přesný mechanizmus, kterým je energie uvolněná z hydrolýzy ATP využita pro transport substrátu, však není dosud znám.

Sekvenční analýza MRP2 proteinu ukázala jistou míru shody uvnitř MRP podrodiny transportérů, a to nejvyšší ve srovnání s MRP1 (48 %) a MRP3 (47 %). Naopak strukturální podobnost s P-glykoproteinem byla zjištěna poměrně malá (18 %) (Konig et al. 2003).

Buněčná lokalizace a tkáňová distribuce MRP2

MRP2 byl zpočátku identifikován na apikální membráně potkaních (Buchler et al., 1996) a lidských (Keppler et al., 1996) hepatocytů. Proto byl původně nazýván kanalikulární MRP (cMRP), resp. cMOAT (Keppler et al., 1996;Paulusma et al., 1996;Paulusma a Oude Elferink, 1997). Po naklonování MRP2 z lidských a potkaních jater byla MRP2 mRNA nalezena také v ledvinách, duodenu a periferních nervech. Pomocí imunofluorescenční mikroskopie a imunohistochemie byla přítomnost MRP2 dále potvrzena na apikálních membránách proximálních tubulů ledvin a epiteliálních buněk močového měchýře, tenkého a tlustého střeva a plic (Konig et al., 2003). V tenkém střevě bylo zjištěné množství MRP2 mRNA mezi nejvyššími ze všech ABC transportérů (Taipalensuu et al., 2001). MRP2 byl detekován i v placentě, na apikální straně membrány syncytiotrophoblastu (St-Pierre et al., 2000). Nedávno byl MRP2 objeven také na luminální (apikální) membráně endotelu mozkových kapilár, což nejspíše svědčí o jeho úloze v transportu látek z CNS do krve, a tudíž podílu na hematoencefalické bariéře (Miller et al., 2002).

Orgánová distribuce MRP2 se může měnit za různých patofyziologických podmínek. Ve zdravých játrech potkana je Mrp2 rozmístěn homogenně po celém lobulu, avšak cholestáza přesouvá jeho koncentraci směrem k centrální (perivenózní) oblasti lobulu (viz dále) (Paulusma et al., 2000). Na subcelulární úrovni byl v hepatocytech během časné cholestázy pozorován selektivní přesun proteinu z apikální membrány do intracelulárních perikanalikulárních vezikulů (Dietrich et al., 2004). V potkaním střevě je Mrp2 lokalizován na apikální membráně enterocytů s mírou exprese rostoucí od krypty k vilu, podobně jak bylo popsáno pro P-glykoprotein. Naopak oproti P-glykoproteinu (ale společně s CYP3A4 enzymem) se exprese Mrp2 snižuje

Tabulka 1. *substrátová specificita lidského a potkaního mrp2*

MRP2 (lidský rekombinantní)	**Mrp2 (potkaní rekombinantní)**	**Mrp2 (potkaní; normální/mutantní BCM)a**
LTC4	LTC4	LTC4
S-Glutathionyl 2,4-dinitrobenzen	S-Glutathionyl 2,4-dinitrobenzen	LTD4
S-Glutathionyl ethakrynová kys.	Bilirubin Monoglukuronosyl	LTE4
Bilirubin Monoglukuronosyl	Bisglukuronosyl	N-Acetyl LTE4
Bisglukuronosyl	17β-Glukuronosyl estradiol	S-Glutathionyl 2,4-dinitrobenzen
17β-Glukuronosyl estradiol	Sulfatolithocholyl taurin	S-Glutathionyl sulfobromoftalein
Sulfobromoftalein		Glutathion disulfid
p-aminohippurát		Bilirubin Monoglukuronosyl
Ochratoxin A		Bisglukuronosyl
		Glukuronosyl nafenopin
		Glukuronosyl E3040a
		Glukuronosyl grepafloxacin
		Glukuronosyl SN38 karboxylát
		Glukuronosyl SN38 lakton
		Sulfobromoftalein
		Fluo-3a
		Metotrexát
		Temokaprilát
		Pravastatin
		Sulfatolithocholyl taurin
		Sulfatochenodeoxycholyl taurin

Uvedené sloučeniny byly identifikovány jako substráty měřením jejich ATP-dependentního transportu do převrácených membránových vezikulů z buněk exprimujících rekombinantní MRP2/Mrp2 ve srovnání s vezikuly z buněk exprimujících kontrolní vektor. Dále byl měřen ATP-dependentní transport do vezikulů z kanalikulárních membrán hepatocytů z Mrp2-deficitních potkanů (GY/TR- a EHBR) ve srovnání s vezikuly z hepatocytů zdravých zvířat (převzato z König et al., 1999a).

aZkratky: BCM, bile (hepatocyte) canalicular membranes: kanalikulární membrány hepatocytů; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazol; Fluo-3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2-amino-5-methyl-phenoxy)-ethan-N,N,N_,N_-tetraoctová kyselina pentaammoniová sůl; SN38, metabolit CPT11 s hydrolyzovanou esterovou vazbou (7-ethyl-10-hydroxycamptothecin).

směrem od duodena k distálnímu ileu a tlustému střevu, kde míra exprese dosahuje pouze 30 % exprese v duodenu (Cherrington et al., 2002).

Substrátová specifita MRP2

MRP2 má velmi podobné spektrum substrátů jako dříve objevený a popsaný MRP1. Mezi substráty s vysokou afinitou patří amfifilní anionty, především konjugáty s glutathionem a kyselinou glukuronovou, např. LTC4, bilirubin glukuronid nebo 17β-glukuronosyl estradiol. MRP2 je dále transportuje nekonjugované sloučeniny, např. fluorescenční barvivo Fluo-3, modelovou látku pro studium jaterního transportu sulfobromoftalein, anionické protinádorové léčivo metotrexát, inhibitor HMG-CoA reduktázy pravastatin, ACE inhibitor temokaprilát a další. Z antimikrobních látek jsou popsanými substráty MRP2 např. inhibitory HIV proteáz nebo fluorochinolony (Suzuki a Sugiyama, 2002). Podrobný seznam spolehlivě prokázaných substrátů je uveden v tabulce 1. Kromě xenobiotik se MRP2 podílí i na transportu farmakologicky aktivních složek potravy jako např. flavonoidů (resp. jejich metabolitů) quercetin 4'-β-glukosidu a epikatechin sulfátu (Vaidyanathan a Walle, 2001;Konig et al., 2003).

Fyziologická a farmakologická funkce MRP2

Základní fyziologickou funkcí MRP2 je transmembránový transport konjugovaných metabolitů endogenních látek a xenobiotik do žluči, moči a střevního lumina. V játrech MRP2 zajišťuje jeden z mechanizmů tvorby žluče, nezávislé na sekreci osmoticky aktivních solí žlučových kyselin. Význam této funkce byl dokumentován výraznou redukcí tvorby žluče u Mrp2 deficitních potkanů (Konig et al., 2003).

Tkáňová distribuce MRP2 koresponduje s distribucí konjugačních enzymů fáze II, UDP-glukuronyltransferázou a glutathion-S-transferázou, což potvrzuje navržený model efektivní synergie metabolizmu a následně transportu organických aniontů (Gerk a Vore, 2002). Na úrovni střevní sliznice může tento transportér svým aktivním efluxem xenobiotik hrát obdobnou protektivní roli, jaká již byla navržena

pro P-glykoprotein. V závislosti na finální aktivitě MRP2 jsou potom důsledkem změny biologické dostupnosti jeho substrátů (Schinkel a Jonker, 2003, Konig et al., 2003).

Zvýšená exprese MRP2/Mrp2 v mozkových kapilárách vzbudila zájem jako potenciální příčina farmakorezistentní epilepsie. Při in vivo studiích byl u Mrp2-deficitních potkaních kmenů pozorován usnadněný průchod fenytoinu, valproátu či karbamazepinu přes hematoencefalickou bariéru. Podobné výsledky potvrdily i studie, které použily probenecid jako inhibitor Mrp2 u zdravých potkanů (Potschka et al., 2003). Obecně lze tedy konstatovat, že MRP2/Mrp2 může, stejně jako podobně lokalizovaný MDR1 (P-gp), limitovat mozkovou a fetální penetraci celé řady látek přítomných v (maternální) plazmě. Díky značné podobnosti v tkáňové distribuci obou přenašečů je pravděpodobná i jistá příbuznost ve farmakologické a toxikologické protektivní funkci, která se nicméně týká jiných (i když částečně se překrývajících) spekter substrátů.

Podíl MRP2 na multidrug resistenci naznačila zvýšená exprese tohoto transportéru v mnoha vzorcích různých karcinomů (Kool et al., 1997, Sandusky et al., 2002). Přímé důkazy přinesly transportní studie na buněčných liniích transfekovaných MRP2 cDNA, které ukázaly značnou rezistenci těchto buněk mj. k cisplatině, etoposidu, vinkristinu, mitoxantronu, doxorubicinu, epirubicinu a metotrexátu (Cui et al., 1999; Konig et al., 2003).

Polymorfismus MRP2

Gen pro MRP2, ABCC2, je lokalizován na chromozomu 10q23−q24 a jeho velikost je cca 65kbp. Samotná kódovací sekvence se skládá z 32 exonů o velikosti od 56 do 255bp. Každá vazebná doména pro nukleotidy je kódována třemi exony. Jak u lidí, tak i u potkanů byly popsány přirozeně se vyskytující mutace v MRP2 (ABCC2) genu (Konig et al., 2003). Některé z těchto mutací mají za následek nepřítomnost transportéru na kanalikulární membráně hepatocytů s následným rozvojem onemocnění označovaného u člověka jako Dubin-Johnsonův syndrom (DJS) (DUBIN a JOHNSON, 1954). Konkrétních DNA poruch vedoucích k DJS byla pozorována celá řada (zahrnující bodové mutace typu missense, nonsense i mutace ovlivňující tzv. splice site junctions). Tyto mohou vést k rychlé degradaci mRNA, ovlivnit interakce s chaperony, maturaci a stabilitu proteinu, stejně jako jeho správné umístění na membráně, přičemž přesné mechanizmy u jednotlivých fenotypů ještě nejsou dostatečně popsány.

Dubin-Johnsonův syndrom

Dubin-Johnsonův syndrom (DJS) je vzácné benigní onemocněn í se zvýšenou frekvencí výskytu (1:1300) mezi íránskými a marockými židy, u kterých bývá navíc často spojeno s deficiencí koagulačního faktoru VII. Toto autozomálně recesivní onemocnění je charakterizováno chronickou, převážně konjugovanou, nehemolytickou hyperbilirubinémií. Ta je způsobena poškozeným hepatobiliárním transportem organických aniontů vyjma solí žlučových kyselin (mají alternativní transportér), za který je právě zodpovědná nepřítomnost funkčního MRP2 na kanalikulární membráně hepatocytů. Histologie jater takto postižených pacientů nevykazuje žádné abnormality kromě charakteristické lysozomální kumulace melaninu v perikanalikulární oblasti. Pacienti navíc vykazují abnormální močovou exkreci koproporfyrinu, vedlejšího produktu syntézy hemu, který je také nejdůležitějším markerem při diagnóze tohoto syndromu (Koskelo et al., 1967). V cirkulaci se vyskytují dvě izoformy koproporfyrinu, I a III. Močová exkrece zdravých vykazuje charakteristický poměr obou izoforem výrazně ve prospěch izoformy III. U pacientů s DJS je tento poměr opačný, nicméně přesný mechanizmus tohoto jevu není zcela objasněn. Stejný fenomén byl potvrzen i u Mrp2-deficientních potkaních kmenů, kde byla také izoforma koproporfyrin I identifikována jako endogenní substrát Mrp2 (Jansen et al., 1985). Přesun od hepatobiliární exkrece této látky k renální se potom zdá být kompenzací nepřítomnosti transportéru.

Klinický význam pro kinetiku exogenních substrátů MRP2 nebyl u DJS dlouho znám. Až recentní informace naznačují významné změny spojené s kumulací substrátů a vyšší toxicitou. Příkladem je porucha eliminace metotrexátu u nemocných s DJS vedoucí při vysokodávkové terapii až k trojnásobně vyšším plazmatickým koncentracím ve srovnání s jedinci s normálním MRP2. Následkem byla významná nefrotoxicita vyžadující podání karboxypeptidázy (Hulot et al., 2005).

Modely absence Mrp2 u potkana

Konkrétní mutace byly identifikovány u dvou dobře popsaných potkaních kmenů s kongenitální hyperbilirubinemií, které jsou známy jako zvířecí modely lidského DJS. U kmene Wistar se jedná o tzv. Groningen žluté/transport-deficitní (GY/TR-) potkany, u kmene Sprague-Dawley jsou označováni jako Eisai hyperbilirubineničtí (EHBR). Objev těchto modelů měl značný význam při popisu funkce a substrátové specifity, stejně jako regulace Mrp2 transportéru. Změny vedoucí k absenci Mrp2 u obou kmenů byly identifikovány jako bodové mutace vedoucí k předčasným stop-kodonům. Zajímavé je, že stejně jako u lidí s DJS, se u těchto zvířecích modelů na kanalikulární membráně hepatocytů nepodařilo detekovat vůbec žádný (ani nefunkční) MRP2/Mrp2 protein. Obdobně i příslušná mRNA byla pod detekční hranicí Northern blottingu (Buchler et al., 1996;Paulusma et al., 1996). Nepřítomnost mRNA může vysvětlovat mechanizmus zvaný „nonsense-mediated decay“ a je pravděpodobné, že absence proteinu je následkem právě rychlé degradace mutantní RNA. Jiné popsané mutace u lidí sice vedly k syntéze defektního MRP2 proteinu, ale s velmi omezenou životností a rychlou proteolýzou. Pravděpodobné je, že v endoplazmatickém retikulu (ER) funguje striktní kontrola kvality syntetizovaných proteinů. Pouze proteiny se „správnou“ sekundární strukturou a přesnou glykosylací

jsou vypuštěny z ER do Golgiho komplexu a odtud transportovány na membránu . Naopak defektní bílkoviny jsou zadrženy v ER a následně rychle biodegradovány v proteazómech (Keitel et al., 2003).

Významným objevem v oblasti korigování absence Mrp2 na podkladě bodových mutací je úspěšné vyzkoušení možnosti inzerce funkčního genu in vivo u deficitních potkanů (Hirouchi et al., 2005). Lidský gen pro Mrp2, vnesený za pomoci adenoviru, vedl u deficitních zvířat k expresi funkčního proteinu na kanalikulárních membránách hepatocytů s následným zvýšením biliární sekrece Mrp2 substrátu, dibromosulftaleinu.

Regulace exprese a funkce MRP2

Předchozí kapitoly sumarizovaly základní informace o lokalizaci, struktuře a funkci MRP2 transportéru. Celá skupina ABC transportérů však tvoří vysoce adaptabilní systém, reagující na různorodé vlivy, mající za následek up- i down-regulaci exprese a související změnu funkce.

Exprese MRP2 může být ovlivněna celou řadou léčiv a nemocí ovlivňujících stav jater, obzvláště cholestatických onemocnění. Experimentální data prokázala, že exprese a funkce je regulována také mnoha endogenními látkami, například steroidními hormony a žlučovými kyselinami. Nedávné studie napovídají, že regulace exprese MRP2 se děje na minimálně třech různých úrovních, zahrnujících endocytózou zprostředkovaný „odsun“ z kanalikulární membrány, změny na úrovni transkripce i regulaci translace a posttranslačních úprav.

MRP2 je syntetizován v endoplazmatickém retikulu, přesunut a zpracován v Golgiho aparátu a nakonec translokován na apikální membránu polarizovaných buněk. Neporušená cytoskeletální soustava je pro intracelulární přesun nezbytná, jak dokazuje experiment s nokodazolem, mikrotubuly rozrušující látkou, který způsobil redukci apikálních vakuol, poruchu inzerce proteinů na membránu a následně redukci Mrp2 proteinu (Konig et al., 2003). Transportní funkce Mrp2 je v krátkém časovém horizontu (řádově minut) řízena dynamickou rovnováhou endocytózy a exocytózy přenašeče mezi membránou a intracelulárními vezikuly (Kipp a Arias 2002). Kupříkladu studie s dibutyryl cAMP prokázaly, že podání této látky způsobí rychlé splynutí perikanalikulárních vezikulů obsahujících Mrp2 a Bsep s membránou a inzerci proteinů (Boyer a Soroka, 1995). Stejně tak byla transportní aktivita Mrp2 zvýšena na kanalikulárních membránách získaných z jater potkanů, kterým byl před izolací podáván dibutyryl cAMP. Současné podání kolchicinu, látky s depolymerizačním účinkem na mikrotubuly, tento účinek dibutyryl cAMP blokuje. Stejný efekt „odsunu“ funkčního Mrp2 proteinu lze pozorovat po podání cholestázu navozujícího lipopolysacharidu, muchomůrkového toxinu phalloidinu (Rost et al., 1999), estradiolu 17-β-D-glukuronidu (Mottino et al., 2002) nebo po perfúzi jater hyperosmotickým pufrem. V tomto případě vedla následná změna kvality pufru na hypoosmotický k opětovné reinzerci proteinu do membrány (Konig et al., 2003).

Transkripční regulace MRP2 byla popsána jako snížení exprese během onemocnění a zvýšení exprese následkem podávání různých známých enzymových induktorů. Jaterní zánět doprovázející stavy jako sepse, alkoholová, autoimunní a virová hepatitida je spojen s cholestázou a hyperbilirubinémií. Játra jsou hlavním cílem mediátorů zánětu (např. tumor nekrotizující faktor α, interleukin-6 a interleukin-1β), které zároveň regulují změny v jaterní syntéze proteinů během odpovědi akutní fáze. Denson (Denson et al., 2002) identifikoval RXRα/RARα responzivní elementy v Mrp2 promotoru a prokázal, že transkripční suprese Mrp2 způsobená proteiny akutní fáze (především cytokiny) se děje skrze redukci nukleárních RXRα/RARα heterodimerů navozenou interleukinem-1β. Zároveň prokázal, že tato změna je specifická pro játra, kde cytokiny pravděpodobně snižují expresi Mrp2, zatímco v ledvinách zůstává množství Mrp2 nezměněno. O regulaci transportu během cholestázy viz dále.

V případě zvýšené transkripce Mrp2 se jedná o komplexní jev podobný u všech ABC transportérů, resp. biotransformačních enzymů. Modelová transkripční indukce probíhá přes jaderné (nebo cytosolické) receptory, nazývané také „sirotčí nukleární receptory“ (z anglického orphan nuclear receptors). Patří sem pregnanový X receptor (PXR), u lidí také značený SXR (steroidní X receptor), CAR (konstitutivní androstanový receptor), FXR (farnesoidní X receptor), LXR (jaterní X receptor), RXR (retinoidní X receptor) a další. Společnou vlastností těchto receptorů je hojný výskyt v orgánech exkrece a poměrně pestrá substrátová specifita. Nejvýznamnější úloha v regulaci eliminace léčiv na všech úrovních je připisována zatím nejlépe prozkoumanému PXR receptoru, jehož signální cesta probíhá dále popsaným způsobem. Po navázání ligandu dojde k translokaci PXR receptoru do jádra, kde vytvoří heterodimer vazbou na (pro všechny výše zmíněné receptory společného) RXR partnera. Ten se potom váže na příslušnou sekvenci DNA nazývanou HRE (hormon responzivní element) nebo XRE (xenobiotický responzivní element). Tyto opakující se nukleotidové sekvence se nacházejí v promotorové oblasti genů, kde po navázání receptoru (ve formě monomeru, homodimeru, ale nejčastěji heterodimeru) dojde k aktivaci transkripce (Wang a LeCluyse, 2003).

Nedávné studie ukázaly, že ligandy FXR, PXR a CAR receptorů (chenodeoxycholová kyselina, PCN (pregnenolon-16α-karbonitril), dexametazon a fenobarbital) indukovaly Mrp2 mRNA v primárních kulturách hepatocytů a podařilo se i popsat příslušné responzivní oblasti (HRE) v potkaním promotoru schopné vázat odpovídající FXR/RXR, PXR/RXR a CAR/RXR heterodimery (Kast et al., 2002). Ligandem PXR receptoru a tudíž i induktorem transkripce Mrp2 je také např. jeho substrát inhibitor HIV proteázy ritonavir (Konig et al., 2003).

V primárních kulturách potkaních hepatocytů dexametazon, 2-acetylaminofluoren, cisplatina, cykloheximid, fenobarbital, klotrimazol a PCN zvyšují množství jak mRNA, tak i proteinu během 24 hodin (Kauffmann et al., 1997;Courtois et al., 1999;Kubitz et al., 1999a; Kubitz et al., 1999b). Dexametazon a cisplatina také indukují expresi Mrp2 u potkanů v játrech i ledvinách, v prvním případě přes vazbu na jaderný PXR receptor (Demeule et al., 1999). Po perorální léčbě rifampinem, který je také PXR ligandem,

a následné biopsii byla u člověka popsána indukce MRP2 v duodenální sliznici (Konig et al., 2003).

V souvislosti s transkripčními regulačními mechanismy pozornost dále zasluhují pozorování, u nichž změny v množství mRNA ne vždy korelují s expresí proteinu a naopak. Kupříkladu podávání ethinylestradiolu značně snížilo expresi Mrp2 proteinu bez jakéhokoli vlivu na expresi Mrp2 mRNA. Podobně byla popsána o 50 % nižší exprese proteinu v játrech březích potkanů, zatímco množství mRNA se od kontrolních vůbec nelišila (Cao et al., 2001). Zdá se proto, že část MRP2 mRNA (pravděpodobně nepřekládaná oblast směrem k 5'-konci) obsahuje specifické regiony schopné snížit rychlost translace.

Zajímavé poznatky ohledně regulace funkce přinášejí interakční studie na buněčných liniích exprimujících Mrp2. Podle získaných údajů z kinetiky transportu modelových substrátů je pravděpodobné, že Mrp2 obsahuje nejen více vazebných domén pro kotransport látek (výše popsaný např. pro glutathion), ale i allosterická vazebná místa pro látky, které nemusí být substráty, ale mohou významně ovlivňovat rychlost přenosu jiných sloučenin. Například transportní studie na membránových vezikulech obsahujících MRP2 prokázaly, že přenos modelového substrátu estradiolu 17-β-D-glukuronidu byl v přítomnosti furosemidu a probenecidu zvýšen 1,5násobně a v přítomnosti indometacinu až šestinásobně (Bodo et al., 2003). Podle Zelcera a kol. (Zelcer et al., 2003) je efekt látky modulující transport přímo závislý na substrátu. Tato pozorování jsou v souladu s již dříve navrženou možností, že každá dvojice substrát-modulátor tvoří unikátní interakci na komplexu možných vazebných míst MRP2 proteinu. Podobný popis byl navržen i pro MRP1 přenašeč, jenž je svou strukturou, funkcí i spektrem substrátů značně příbuzný s MRP2. Funkční model navrhuje pro MRP2 dvě podobná vazebná místa, jedno pro transport substrátu (S místo) a druhé pro modulátor transportu (M místo). Po navázání látky do M místa dojde ke konformační změně v S místě, která vede k usnadnění/urychlení vazby substrátu na přenašeč a efektivnějšímu přenosu. Některé látky se mohou vázat pouze do M místa nebo v závislosti na koncentraci do M i S místa (samy jsou transportovány) a chovat se tak jako stimulátory a/nebo kompetitivní inhibitory přenosu příslušného substrátu (Zelcer et al., 2003).

Vliv cholestázy na MRP2

Tvorba žluče, důležité pro digesci a absorbci lipidů stejně jako pro eliminaci látek, je jedna z esenciálních funkcí jater. Žluč je produkována filtrací, která se řídí osmotickým gradientem tvořeným kontinuální aktivní sekrecí látek přes kanalikulární membránu hepatocytu. Transportní proteiny lokalizované na bazolaterální a apikální membráně hrají v tomto procesu nejdůležitější úlohu. Přenašeče zodpovídající za import látek do hepatocytu na bazolaterální straně patří do skupiny SLC (solute carrier superfamily) a OATP (organic anion transporting polypeptide). Z přítomných proteinů má pro tvorbu žluči největší význam uptake přenašeč Ntcp/NTCP (sodík/taurocholát kotransportující polypeptid), jehož substráty jsou konjugované soli žlučových kyselin. Společnou vlastností transportérů obou skupin, na rozdíl od proteinů ABC nadrodiny, je nezávislost na ATP jako zdroji energie pro přenos.

Sekrece osmoticky aktivních látek na kanalikulární straně limituje rychlost celého procesu tvorby žluče, za který jsou zodpovědny především přenašeče ze skupiny ABC transportérů. MRP2 (společně s Cl-/HCO3- pumpou) zde tvoří, díky sekreci redukovaného glutathionu (GSH), hnací sílu pro tok žluče nezávislý na solích žlučových kyselin . Hlavní složkou žluče udržující osmotický gradient nezbytný pro její tok jsou ovšem právě monovalentní soli žlučových kyselin, jejichž transportérem je Bsep/Abcb11 (z anglického Bile salt export pump). Na kanalikulární membráně jsou dále P-gp transportující především objemné amfifilní organické kationty a MDR3 (lidský analog potkaního Mdr2) zodpovědný za export fosfatidylcholinu. Pro podrobnější popis všech transportních mechanizmů na membránách hepatocytů odkazujeme na komplexní přehledový článek (Trauner a Boyer 2003).

Cholestáza je definována jako porucha odtoku žluče vedoucí ke zvýšení hladin bilirubinu a žlučových kyselin v séru. Různé formy cholestázy (1. intrahepatální – u zvířat experimentálně běžně navozena podáním lipopolysacharidu nebo ethinylestradiolu; 2.extrahepatální/obstrukční – navozena podvazem žlučovodu) vedou k podobným adaptačním změnám v expresi transportních systémů v játrech. Obecně dochází k redukci bazolaterálního importu látek do hepatocytu (snížení exprese Ntcp a Oatp přenašečů) a kanalikulární sekrece (redukce Mrp2 a Bsep) a naopak k indukci aktivity bazolaterálních efluxních přenašečů, především Mrp3 (Donner a Keppler, 2001; Donner et al., 2004). U extrahepatální cholestázy jsou tyto změny vysvětlovány jako kompenzační mechanizmy chránící hepatocyty před poškozením kumulujícími se toxiny, např. žlučovými kyselinami nebo xenobiotiky. Trvale zvýšená exprese MRP3, přenašeče s podobným spektrem substrátů jako MRP2, byla popsána u MRP2-deficitních lidí s Dubin-Johnsonovým syndromem i u GY/TR- a EHBR potkanů (Kuroda et al. 2004), což potvrzuje možnost kompenzace chybějícího MRP2/Mrp2. Během cholestázy byla také popsána zonální redukce Mrp2 pokračující směrem od periportální k pericentrální části jaterního lalůčku (Paulusma et al., 2000). V dosud studovaných experimentálních modelech je MRP2 jediným transportérem vykazujícím down-regulaci u všech forem cholestázy (Dietrich et al., 2004). Naopak v ledvinách byla opakovaně prokázána zachovaná (nebo dokonce zvýšená) exprese Mrp2 proteinu (Lee et al., 2001). Tento fenomén se zdá být kompenzačním mechanizmem pro exkreci Mrp2 substrátů ven z organizmu, avšak jeho příčiny na molekulární úrovni zatím nejsou zcela objasněny. Jaterní Kupfferovy buňky produkují zvýšené množství prozánětlivých proteinů akutní fáze včetně řady cytokinů (viz výše), které pravděpodobně snižují expresi RXRα, společného partnera pro jaterní receptory schopné modulovat transkripci celé řady transportérů včetně Mrp2. Toto vysvětlení bylo potvrzeno dalšími experimenty, kdy

intrahepatální cholestáza navozená endotoxinem byla doprovázena podáním dexametazonu nebo protilátek proti cytokinům (Kubitz et al., 1999b), což částečně zabránilo poklesu exprese Mrp2 a dalších přenašečů (Cherrington et al., 2004). Zajímavé je, že při experimentálním podávání právě těch substrátů, jejichž transport je poškozen během cholestázy, například kyselin cholové a deoxycholové, byla popsána jednoznačná indukce Mrp2. Zvýšená exprese by mohla sloužit jako ochrana před jaterní kumulací potenciálně toxických žlučových kyselin. K tomuto jevu ovšem při cholestáze nedochází, pravděpodobně díky převaze jiných (antagonistických) regulačních mechanizmů.

Souhrnem lze konstatovat, že během cholestázy dochází ke snížení exprese Mrp2, a to minimálně na krátkodobé úrovni formou endocytózy proteinu z kanalikulární membrány a eventuální proteolýzy v lyzozomech a/nebo inhibicí přepisu samotného genu snížením aktivity nezbytných transkripčních faktorů (Trauner et al. 1997; Paulusma et al., 2000). Nedostatečná sekrece organických anionů na kanalikulární membráně má pravděpodobně za následek kompenzační zvýšení Mrp3 na bazolaterální membráně, který tyto substráty exportuje zpět do sinusoidální krve (Donner a Keppler, 2001).

ZÁVĚR

Během jediného desetiletí studia MRP2 transportéru byl učiněn velký pokrok v poznání jeho fyziologické funkce a byla uspokojivě popsána jeho úloha v jaterní a částečně i ledvinné a střevní eliminaci látek. Mohla tak být navržena vysvětlení některých patofyziologických jevů, kupříkladu vznik ikteru při sepsi (down-regulace jaterního Mrp2 a následná porucha sekrece bilirubinu). Přesto stále zbývá mnoho otázek týkajících se jeho úlohy např. u fenoménu mnohočetné rezistence nebo podílu na hematoencefalické či placentární bariéře. Zavedené experimentální modely pro výzkum složitých mechanizmů regulace a vzájemných vztahů mezi transportéry, enzymy, nukleárními receptory apod. poskytují šanci nejen pro lepší pochopení mechanizmu účinku léčiv, ale do budoucna i možnost zásahů, které budou moci zefektivnit terapii ať už prevencí nežádoucích interakcí látek, zabráněním toxické kumulaci nebo naopak dosažení efektivních terapeutických hladin ovlivněním eliminace.

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MORPHOLOGICAL AND FUNCTIONAL CHANGES IN P-GLYCOPROTEIN DURING DEXAMETHASONE-INDUCED HEPATOMEGALY

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SUMMARY

1. The effect of dexamethasone on hepatic and renal P-glycoprotein (P-gp) expression, localization and activity was investigated in rats after 4 days oral administration of two dose regimens (1 or 25 mg/kg per day). Simultaneous increases in liver weight were evaluated by quantitative histological examination.

2. In the liver, dexamethasone pretreatment produced hepatomegaly as a consequence of extensive periportal fat accumulation, which was quantified by densitometry of oil red O-stained liver sections. Quantitative immunohistochemical analysis revealed preferential periportal zonation of P-gp in control animals. Dexamethasone pretreatment resulted in spatially disproportional induction of P-gp protein expression within the liver acinus characterized by preferential increase in pericentral areas, with consequent uniform panlobular distribution. Western blot analysis confirmed these results, showing increases in P-gp protein. Quantitative reverse transcription–polymerase chain reaction analysis revealed no statistically significant change in liver *mdr1b* mRNA expression after either dexamethasone treatment regimen. The expression of *mdr1a* mRNA was significantly decreased by 85–87%.

3. In the kidney, dexamethasone reduced *mdr1a* mRNA expression by 69–89%, whereas *mdr1b* mRNA expression was increased in a dose-dependent manner. However, despite tendencies, no significant increases in P-gp expression were observed at the protein level.

4. The *in vivo* function of P-gp was evaluated by measuring renal and biliary secretion of rhodamine-123 (Rho123) under a steady state plasma concentration. The biliary, renal and tubular secretory clearance of Rho123 was significantly increased only after high-dose dexamethasone.

5. In conclusion, the present study suggests that drug interactions observed during corticosteroid therapy may be mediated, at least in part, through increased biliary, and also renal, excretion of P-gp substrates. Expression of P-gp in the liver showed primary periportal zonation with differential changes during induction. Accompanying hepatomegaly may be explained by severe microvesicular steatosis selectively localized to the periportal areas.

Key words: dexamethasone, induction, P-glycoprotein, rhodamine-123, steatosis.

INTRODUCTION

P-Glycoprotein (P-gp), encoded by the *MDR1* gene in humans and *mdr1a/1b* genes in rodents, functions as an ATP-dependent efflux transporter identified on apical membranes of various tissues, including the intestine, liver, kidney, adrenal gland, brain, eye and testis.[1] In these organs, P-gp mediates the cellular efflux of many structurally and pharmacologically unrelated hydrophobic compounds, such as some anticancer agents, immunosuppressants, steroid hormones, calcium channel blockers, HIV protease inhibitors and cardiac glycosides.[2] Consequently, changes in the expression and activity of P-gp are commonly associated with substantial inter-individual variability in the pharmacokinetics of these drugs, producing unpredictable changes in patient responses to therapy. Among the most frequently reported causes of altered P-gp activity are drug–drug interactions. Numerous reports exist describing inhibition of P-gp, with consequent increases in substrate disposition and excessive drug effect or even toxicity.[3–5] Moreover, growing evidence points to the clinical importance of P-gp induction that may be associated with failure of therapy.[5,6] Therefore, considerable attention is focused on the examination and prediction of possible drug–drug interactions based on changes in P-gp activity. An important example of drugs known to impact on the pharmacokinetics of simultaneously applied P-gp substrates are corticosteroids.

Corticosteroids, commonly used immunosuppressive drugs, are potent inducers of P-gp in the liver and intestine, causing decreased disposition of P-gp substrates in humans and rodents.[5] A typical example of such a pharmacokinetic interaction is the significant decrease in blood concentrations of tacrolimus, a P-gp/CYP3A substrate, in rats and humans after coadministration with glucocorticoids.[7–9] A series of similar reports has led to the widespread use of a potent synthetic corticosteroid, namely dexamethasone (DEX), as a model inducer of P-gp and CYP3A in enterocytes and hepatocytes.[10,11] Nevertheless, little is known about the potential effect of

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DEX on renal P-gp. Demeule *et al*.[10] reported decreased expression of P-gp protein in kidneys of rats pretreated with low-dose DEX (1 mg/kg per day for 4 days), whereas others found no change in expression of *mdr1a/1b* mRNA with DEX at 1–40 mg/kg per day.[1,12] Because experiments in transgenic mice showed changes in the renal clearance of P-gp substrates in knock-out animals,[13] similar effects could be also anticipated for drug-induced changes in renal P-gp expression.

During previous P-gp induction studies, we observed concomitant increases in liver weight after DEX pretreatment.[14] Similar effects of corticosteroids have been observed repeatedly in animals and humans[15–18] and several attempts have been made to explain the pathophysiological background of this observation. Most results have indicated that hepatomegaly produced by the steroids is caused by excessive fat accumulation,[18,19] but detailed morphological information is not available.

The aim of the present study was to elucidate the contribution of hepatic, and especially renal transport, systems on DEX-mediated decreases in the disposition of P-gp substrates. Rhodamine-123 was chosen as the model drug because this compound is primarily excreted into the bile and urine in an unchanged form.[20] Changes in the expression and localization of P-gp in the rat liver and kidney were quantified using immunohistochemistry, western blot and real-time reverse transcription–polymerase chain reaction (RT-PCR). Moreover, we quantitatively evaluated changes in the localization of fat accumulation during DEX pretreatment as potentially the main cause of the hepatomegaly observed.

METHODS

Materials

Rhodamine-123 and DEX were purchased from Sigma Chemical (St Louis, MO, USA). Mouse monoclonal antibody C219, directed against *Mdr1*, was purchased from Signet Laboratories (Dedham, MA, USA). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG were obtained from GE Healthcare (Prague, Czech Republic). All other reagents were obtained from Sigma Chemical and SERVA Electrophoresis (Heidelberg, Germany) and were of the highest purity available.

Animals and treatment

Male Wistar rats, weighing 280–330 g (Velaz, Konárovice, Czech Republic), were given an olive oil solution of DEX at a dose of 1 or 25 mg/kg daily (low-dose and high-dose regimen) for 4 consecutive days by stomach intubation ($n = 6$ in each group). These doses were selected on the basis of previous experiments.[14] Untreated (control) rats ($n = 6$) received an equal volume of vehicle alone (olive oil; 2.0 mL/kg). All animals were subjected to *in vitro* and *in vivo* studies 24 h after the last dose. During pretreatment, rats were housed under controlled environmental conditions (12 h light–dark cycle; temperature 22 ± 1°C) with a commercial food diet and water freely available. Animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* (revised 1996; http://www.nap.edu/books/0309053773/html/81.html). The study protocol was approved by the animal welfare committee of Charles University in Prague, Faculty of Medicine in Hradec Kralove.

Membrane preparation

Livers and kidneys were minced in ice-cold Tris-sucrose buffer (10 mmol/L Tris-HCl, 250 mmol/L sucrose, pH 7.6), containing 0.5 mg/mL leupeptin, 0.5 mg/mL pepstatin, 2 mg/mL aprotinin, 50 mg/mL benzamidine and 40 mg/mL phenylmethylsulphonyl fluoride (PMSF), and homogenized with a motor-driven Teflon homogenizer (Braun, Melsungen, Germany) operating at 1500 r.p.m. A membrane-enriched microsomal pellet was obtained from the post-nuclear supernatant after ultracentrifugation at 100 000 *g* for 60 min at 4°C. The pellet was resuspended in Tris-HCl buffer (50 mmol/L Tris-HCl, pH 7.4, plus protease inhibitors). The protein concentration was determined according to the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA) and samples were stored at −80°C.

Immunoblot analysis

Crude membrane-containing homogenates (50 g protein) were incubated with sample buffer at room temperature for 30 min and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) on 6.25% polyacrylamide gels. Immunoblotting was performed using a tank blotting system from GE Healthcare, followed by enhanced chemiluminescence and autoradiography. Exposed Hyperfilms (GE Healthcare) were scanned with a GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and quantified using the QuantityOne imaging software (Bio-Rad Laboratories). The antibodies, dissolved in 5% low-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS), were used at the following dilutions: C219 at 1 : 500 (P-gp); and horseradish peroxidase-conjugated rabbit anti-mouse antibodies at 1 : 1000.

Light microscopy

To evaluate microscopic changes within rat livers, histological examination was performed on sections from control and DEX-pretreated animals (1 mg/kg per day). For immunohistochemical microscopy, liver samples were frozen in hexane precooled in liquid nitrogen and stored at −80°C. Cryosections (7–8 μm) were prepared with a cryotome minitome. Immunostaining was performed using C219 and horseradish peroxidase-conjugated rabbit anti-mouse antibodies diluted in phosphate-buffered saline (PBS) containing 5% fetal calf serum at 1 : 100. Visualization was achieved by incubating with 3,3′-diaminobenzidine tetrachloride (Sigma-Aldrich, St Louis, MO, USA) as the peroxidase substrate. To reveal the contribution of fat accumulation to DEX-induced hepatomegaly, frozen sections from livers were processed by oil red O stain (Sigma-Aldrich). Images of histological sections were acquired on an Olympus IMT-2 light microscope (Olympus, Prague, Czech Republic). Consequent morphometric and densitometric analyses were performed using ImagePro software (Media Cybernetics, Silver Spring, MD, USA). To evaluate P-gp expression in rat liver, 12 randomly selected viewing fields of every immunostained liver section (six from the periportal and six from the perivenous areas) were evaluated at a magnification of ×400. Consequently, the relative presence and integral optical density (OD) of positively stained elements was quantified. To measure fat accumulation within the liver, size, integral OD (vesicle area multiplied by mean density) and the proportion of the visual field area of 10 randomly selected fat drops were measured in the periportal and pericentral (perivenous) areas of the liver acinus at a magnification of ×1200.

Examination of *mdr1a/1b* expression by RT-PCR

Total hepatic and renal RNA was isolated and reverse-transcribed into complementary DNA, as described previously.[21] Real-time PCR was performed on an iCYCLER (Bio-Rad Laboratoires) as described elsewhere.[21] Primers for the target genes *mdr1a* and *mdr1b* and for β*2-microglobuline* (β*2-micro*) as a housekeeping gene (Table 1) were designed using Vector NTI Suite software (Informax, North Bethesda, MD, USA). The basic processing of real-time amplification curves was performed using iCYCLER software version 3.0 (Bio-Rad Laboratoires). Cycle thresholds (C_t) and the slopes of calibration curves were determined. The results were analysed using eqn 1, described by Mei *et al*.:[12]

$$\Delta C_t = C_{tmdr1a/b} - C_{tβ\text{-}mikro} \quad [1]$$

$$C_{tLinear} = 2^{-(\Delta CtDEX - Ctcontrol)}$$

Table 1 Sequences and specifications of primers used for real-time reverse transcription–polymerase chain reaction quantification

Gene	Accession no.	Sequence (5′→3′)	Product length (bp)	Localization
mdr1a	AF257746	ctg ctc aag tga aag ggg cta c (f) agc att tct gta tgg tat ctg caa gc (r)	329	2526–2854
mdr1b	AY082609	cgc ttc taa tgt taa agg ggc tat g (f) agc att tct gta tgg tat ctg caa gc (r)	331	2489–2819
β2-Microglobuline	Y00441	tgc cat tca gaa aac tcc cca (f) tac atg tct cgg tcc cag gtg a (r)	303	64–336

f, forward; r, reverse.

where $C_{tmdr1a/b}$ represents the C_t value of target gene, $C_{tβ\text{-}mikro}$ is the C_t value of the endogenous internal standard of *β2-microglobuline* RNA and $C_{tLinear}$ represents the fold change in mRNA expression between the control ($C_{tcontrol}$) and treated (C_{tDEX}) groups and assumes a doubling of target sequence with each PCR cycle.

Biliary and urinary excretion of Rho123 *in vivo*

Control rats and those treated for the previous 4 days with low- or high-dose DEX were anaesthetized with pentobarbital (35 mg/kg) and cannulated with polyethylene tubes introduced in the right jugular vein for drug administration and the left jugular artery for blood sampling. The urinary bladder and bile duct were also cannulated for urine and bile sampling, respectively. The body temperature of the animals was maintained at 37°C with a heat lamp. To elucidate the effect of DEX on steady state biliary excretion and renal handling of Rho123, rats received a bolus intravenous injection of Rho123 at a loading dose of 100 nmol/kg, followed by a constant-rate infusion (Perfusor Compact; Braun, Prague, Czech Republic) of a 2.5% mannitol solution delivering a dose of 130 nmol Rho123/h at a rate of 4 mL/h until the end of the study. A 60 min infusion was found to result in a steady state concentration of Rho123. The dosing schedule was based on the results of a previous study.[14] Mannitol was used to obtain a sufficient and constant urine flow rate. After 60 min infusion, bile and urine were collected in preweighed tubes at 10 min intervals for 30 min throughout the experiment. Blood samples were taken at the mid-point of the bile and urine collection period. Plasma samples were obtained by centrifugation of blood samples at 3000 *g* for 10 min. The volume of bile and urine samples was measured gravimetrically, with specific gravity assumed to be 1.0. All plasma, bile and urine samples were stored at −80°C until analysis.

Analytical procedures

The concentration of Rho123 in plasma, urine and bile was determined by modification of previously described HPLC method.[11] Briefly, the apparatus used for the HPLC was a Shimadzu LC system (Kyoto, Japan) equipped with a fluorescence detector (RF-10A; Shimadzu; excitation 480 nm; emission 520 nm), consisting of an LC-10AS liquid pump and an SIL-10A autoinjector. The conditions were as follows: column, TSKgel ODS-80TM (Tosoh, Tokyo, Japan); mobile phase, acetonitrile and 1% acetic acid (40 : 60, v/v); column temperature (LCO 100; ECOM s.r.o., Prague, Czech Republic), 50°C; flow rate, 0.7 mL/min. The calibration curve for Rho123 was prepared each time over the concentration range 0–5.0 mol/L. The correlation coefficient (linearity) was always more than 0.999 and the detection limit was approximately 1 nmol/L. The within-day coefficient of variation (CV) was below 5.6%, whereas the between-day CV was found to be below 6.8%. The concentrations of creatinine in plasma and urine were measured on a Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions.

Protein binding experiments

To estimate the difference in the protein binding of Rho123 between control and DEX-treated rats, plasma protein binding was evaluated by dialysis through Ultrafree-MC Centrifugal Filter Units (Millipore, Billerica, MA, USA). Plasma samples (400 μL) from control and pretreated animals were dialysed at 3000 *g* for 20 min. As a standard for comparison, a pH 7.4 PBS solution containing 0.1 mol/L Rho123 was dialysed according to the same conditions. The concentration of Rho123 was chosen on the basis of data obtained during *in vivo* experiments. Concentrations of Rho123 on both sides of the membrane were measured by HPLC as described above.

Pharmacokinetic analysis

Total plasma clearance (CL_{Total}) of Rho123 was estimated by dividing the constant infusion rate of Rho123 by the steady state concentration in plasma (C_{ss}). The biliary and renal clearance (CL_{Bile} and CL_R) of Rho123 during each collection period was calculated by dividing the respective excretion rate by C_{ss} determined for that collection period. Glomerular filtration rate (GFR) was evaluated as clearance of endogenous creatinine (CL_{CR}). The renal clearance ratio of Rho123 was calculated as CL_R/GFR. The renal tubular secretion clearance (CL_{RS}) for unbound drug, which represents the net tubular secretion, was calculated as CL_R/f_U minus GFR, where f_U represents the unbound fraction of the drug.

Statistical analysis

Experiments were performed in six animals per group. All data are expressed as the mean±SEM. Statistical significance was examined in groups of control and DEX-treated animals using ANOVA (Graphpad Instat 3.0 software; Graphpad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of DEX on liver weight and bodyweight, as well as hepatic histology

Over the course of the 4 day treatment, DEX administration resulted in a rapid loss of total bodyweight of 40.0 ± 1.3 and 50.3 ± 5.6 g in the low- and high-dose groups, respectively. Simultaneously, DEX exerted a stimulatory effect on liver weight, increasing it from 10.5 ± 0.5 g in control animals to 11.8 ± 0.7 g (low-dose DEX; $P < 0.05$) and 14.5 ± 1.2 g (high-dose DEX; $P < 0.01$). Consequently, the liver to bodyweight ratio was increased from 3.2 ± 0.2% in control animals to 4.3 ± 0.3% (low-dose DEX; $P < 0.05$) and 5.6 ± 0.5% (high-dose DEX; $P < 0.01$). Microscopic examination of oil red O-stained livers with morphometric and densitometric image analysis revealed no significant difference in parameters evaluated between periportal and perivenous areas of liver tissues in control animals (Fig. 1a; Table 2). In contrast, pretreatment with low-dose DEX produced severe microvesicular fat infiltration, characterized by an increase in the size of fat droplets (1.5-fold), their integral OD (3.8-fold) and

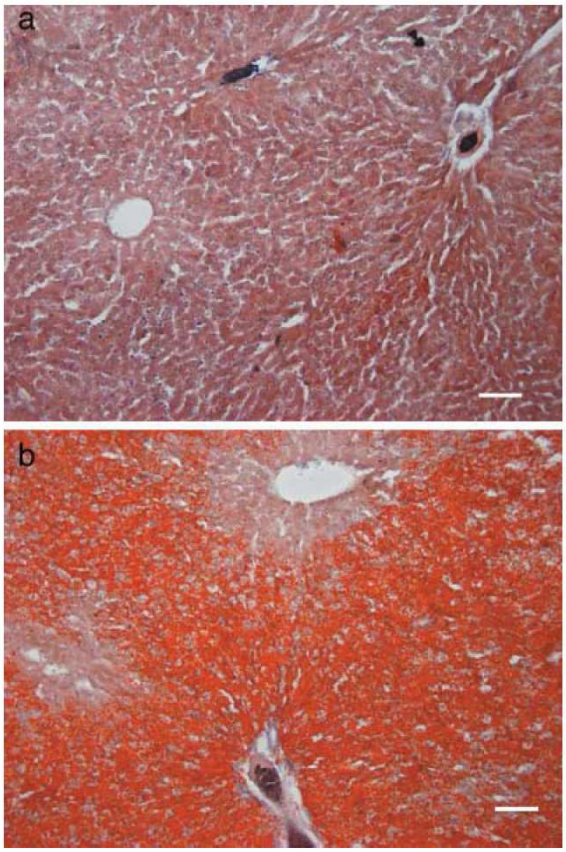

Fig. 1 Photomicrographs of oil red O-stained 7–8 μm sections of (a) control and (b) dexamethasone-pretreated rat liver, illustrating periportal lipid accumulation. Representative images are shown of six livers for each of the dexamethasone-treated and control groups. (a) Control liver. The image exemplifies pericentral (left side) and periportal (right side) zones of the liver acinus. Mild steatosis is demonstrated in adult animals as a weak staining located preferentially in the periportal areas. (b) Dexamethasone (1 mg/kg per day, p.o., for 4 days)-pretreated liver. The image exemplifies pericentral (upper part) and periportal (bottom part) zones of the liver acinus. Severe microvesicular steatosis is demonstrated as a strong staining located preferentially in periportal areas. In contrast, only a few fat droplets could be observed around the central vein. Bars, 100 μm.

proportion of visual area (3.5-fold), located exclusively in the periportal areas of the liver acinus (Fig. 1b; Table 2).

Immunohistochemistry of P-gp

Hepatocellular P-gp immunostaining performed in control and DEX-pretreated (low-dose) rat liver is shown in Fig. 2. In control rats, C219 antibody yielded a weak canalicular staining prevailing in periportal hepatocytes (Fig. 2a,b; Table 2; $P < 0.001$). Administration of DEX increased the intensity of P-gp staining, which became evenly distributed without any quantitative difference between periportal and pericentral areas (Fig. 2c,d; Table 2). Consequently, statistically significant induction of P-gp after DEX pretreatment was observed only in pericentral areas. Comparison of overall integral OD between control and DEX-treated rats yielded a 2.3-fold increase in immunostained P-gp.

Western blot analysis of P-gp expression in the liver and kidney

Representative immunoblots of hepatic and renal crude membrane proteins are shown in Fig. 3. Both groups of DEX-pretreated rats (low and high dose) showed a stronger band intensity of hepatic immunoreactive protein compared with untreated rats. Densitometric analysis of the P-gp level indicated that DEX pretreatment increased hepatic P-gp expression 1.9- and 3.4-fold in the low- and high-dose DEX groups, respectively (Fig. 3a,b), suggesting significant hepatic P-gp induction. In contrast, the expression of renal P-gp, despite tendencies, remained statistically unchanged in both DEX-treated groups (Fig. 3c,d).

Real-time RT-PCR analysis of *mdr1a/1b* expression

The effects of the two DEX dose regimens (low and high dose) on liver and renal tissue expression of *mdr1a* and *mdr1b* mRNA are summarized in Table 3. Rats treated with DEX showed dose- and tissue-dependent changes in the expression of mRNA for *mdr1a* and *mdr1b*. Expression of *mdr1a* mRNA in the liver and kidney decreased by 85–87% and 69–89%, respectively. In contrast, *mdr1b* mRNA in the kidneys increased 1.3- and 5.4-fold compared with control rats. In the liver, *mdr1b* mRNA showed no statistically significant changes after either dose of DEX.

Table 2 Quantitative evaluation of fat accumulation and P-glycoprotein expression in livers from control and dexamethasone pretreated (1 mg/kg per day, p.o., for 4 days) rats

	Control		DEX	
	Pericentral	Periportal	Pericentral	Periportal
Size of fat droplets (μm)	1.6 ± 0.2	2.0 ± 0.4	2.0 ± 0.3‡	3.5 ± 0.4**§
Mean integral OD of fat droplets per viewing field	1790 ± 760	4780 ± 1770*	8000 ± 2200§	17 080 ± 3100**§
Mean no. fat droplets per viewing field (%)	9.4 ± 4.0	12.9 ± 5.1	23.2 ± 8.0†	54.5 ± 7.9**§
Mean P-gp positivity per viewing field (%)	0.7 ± 0.3	5.3 ± 1.4**	6.1 ± 1.7§	6.7 ± 1.9
Mean P-gp integral OD per viewing field (%)	145 ± 70	1180 ± 315**	1450 ± 412§	1605 ± 469

Values are the mean±SEM ($n = 6$). *$P < 0.05$, **$P < 0.001$ compared with periportal areas; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$, $^{\S}P < 0.001$ compared with the same location in control rats.

OD, optical density; P-gp, P-glycoprotein; DEX, dexamethasone.

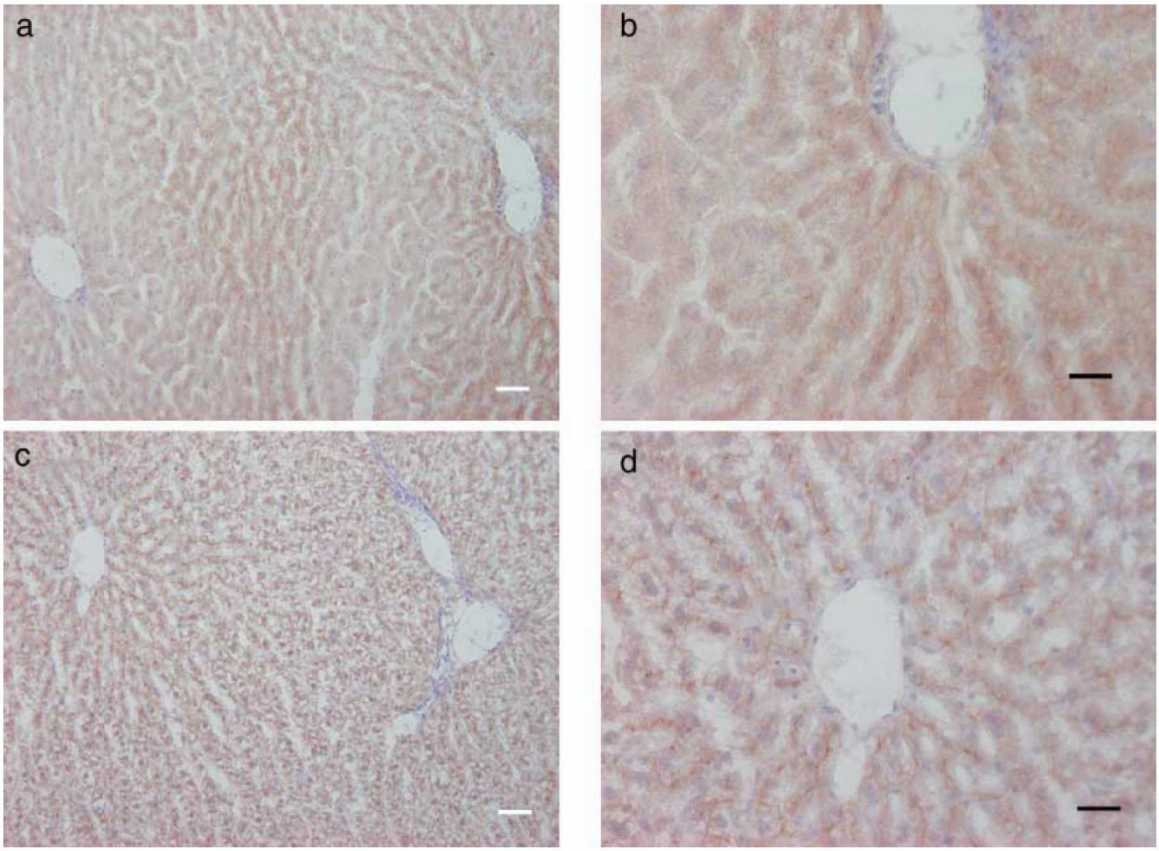

Fig. 2 Immunohistochemical microscopy for P-glycoprotein (P-gp) in (a,b) control and (c,d) dexamethasone-pretreated rat liver. Representative images are shown of six livers for each of the dexamethasone-treated and control groups. Liver cryosections (7–8 μm) were labelled with the monoclonal antibody C219 directed against rat P-gp. In normal rat liver, there was a weak P-gp immunostaining of hepatocytes in the vicinity of the periportal area (a,b). Dexamethasone increased the intensity of P-gp staining preferentially in the areas of central veins (c,d). (a) Control liver. The image exemplifies pericentral (left side) and periportal (right side) zones of the liver acinus. The intensity of P-gp immunostaining is concentrated in the periportal areas. Bar, 50 μm. (b) Control liver: periportal area in detail. The image demonstrates the preferential location of P-gp in this zone mainly as a part of canalicular membranes. Bar, 25 μm. (c) Dexamethasone (1 mg/kg per day, p.o., for 4 days)-pretreated liver. The image exemplifies panlobular P-gp immunostaining as a consequence of increased P-gp expression. Bar, 50 μm. (d) Dexamethasone pretreated liver: pericentral area in detail. The image demonstrates evenly increased expression of P-gp in areas with initially very low expression of the protein. Similar to the control group, P-gp is located mostly on canalicular membranes. Bar, 25 μm.

Effect of DEX on biliary excretion and renal handling of Rho123

Dose-dependent effects of DEX on the biliary and renal excretion of Rho123 under steady state conditions in rats are presented in Table 4. In low-dose DEX-pretreated rats, the CL_{Total} of Rho123 increased 1.4-fold. The CL_{Bile} and CL_R of Rho123 had a tendency to increase (2.2–2.5 and 0.7–2.1 mL/min, respectively), although the differences failed to reach the 5% level of significance. In contrast, significant increases in CL_{Bile} and CL_R of Rho123 were observed in rats after high-dose DEX (2.2–7.9 and 0.7–3.7 mL/min, respectively). Accordingly, CL_{Total} of Rho123 increased 1.4-fold for untreated animals. Interestingly, there was no statistically significant difference in CL_{Total} of Rho123 between low- and high-dose DEX-pretreated rats. To describe the mechanism of renal elimination of Rho123, the tubular secretion clearance (CL_{RS}) was calculated. Despite an increase in urine formation in DEX-pretreated rats, creatinine clearance remained unaffected in both groups. Consequently, CL_{RS} was increased 3.0- and 5.0-fold in accordance following 1 and 25 mg/kg DEX, respectively (Table 4).

DISCUSSION

In the present study, we used the rat *in vivo* model to evaluate the effect of the short-term administration of two dose regimens of DEX on the expression and activity of P-gp in the liver and kidney. Rhodamine-123 was chosen as the model drug for P-gp because this compound is primarily excreted into the bile and urine in an unchanged form.[20] Real-time RT-PCR, western blot and immunohistochemical microscopy analyses revealed increased expression of P-gp in the rat liver, which was dependent on the dose of DEX used. The *in vivo* biliary and renal excretion of Rho123 showed increased

transport activity, agreeing well with the increased amount of *mdr1b* mRNA and P-gp protein. In addition, we identified significant fat accumulation as the main cause of hepatomegaly produced by DEX.

A series of studies has reported that DEX is a potent inducer of intestinal and hepatic P-gp.[10,11,22] In agreement with these observations, the present data on the biliary excretion of Rho123 showed increased activity of P-gp in the rat liver caused by DEX. We found that, in case of the low-dose DEX pretreatment, the increase in biliary clearance of Rho123 was only minor and failed to reach statistical significance. However, in high-dose DEX-pretreated animals, biliary excretion and clearance of Rho123 increased significantly. Consequent analysis by western blot confirmed induction of P-gp protein expression in the respective rat livers, with the extent of the changes being in agreement with the observed increases in P-gp-mediated biliary excretion of Rho123. The present results are consistent with observations made by others, who described an increase in biliary excretion of P-gp substrates in response to treatment with P-gp inducers, with parallel changes in P-gp protein expression.[23,24] We also performed real-time RT-PCR analysis of *mdr1a/b* mRNA expression. All results showed either a decrease or no statistically

Table 3 Expression of *mdr1a/1b* mRNA following 4 day treatment with dexamethasone

	Control	Dexamethasone (mg/kg per day)			
	ΔC_t^a	1		25	
		ΔC_t	Fold change	ΔC_t	Fold change
mdr1a					
Liver	14.3 ± 0.5	17.3 ± 0.3	0.1**	17.1 ± 0.7	0.2**
Kidney	12.2 ± 0.2	15.5 ± 0.3	0.1***	13.9 ± 0.1	0.3***
mdr1b					
Liver	14.4 ± 0.6	12.6 ± 0.9	3.4	14.4 ± 1.0	1.0
Kidney	12.4 ± 0.4	12.1 ± 0.8	1.3	10.0 ± 0.5	5.4*

Results shown are the mean±SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control.

$\Delta C_t = C_{tmdr1a/b} - C_{t\beta 2\text{-}mikro}$, where where $C_{tmdr1a/b}$ represents the cycle threshold (C_t) of the target gene and $C_{t\beta\text{-}mikro}$ is the C_t value of the endogenous internal standard of *β2-microglobuline* RNA. Values were calculated as described in the Methods.

Decrease is indicated by fold change from the control value.

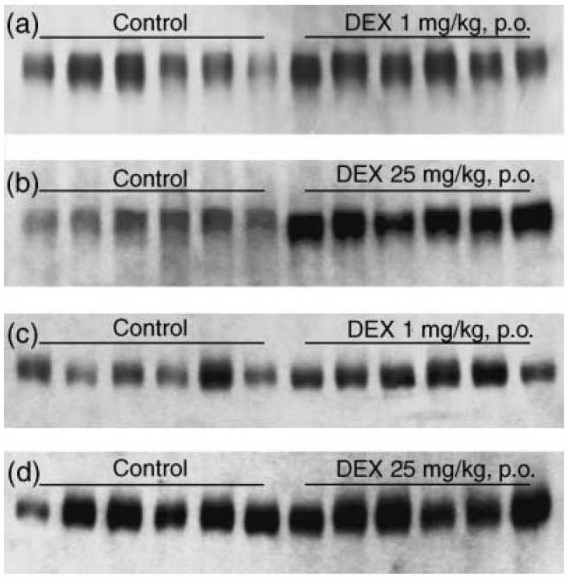

Fig. 3 Immunoblot analysis of P-glycoprotein (P-gp) in rat (a,b) liver and (c,d) kidneys from control and dexamethasone (DEX)-retreated rats. Dexamethasone was administered to rats at the doses indicated daily for 4 days.

Table 4 Pharmacokinetics of rhodamine-123 in untreated and dexamethasone-pretreated rats

	Control	DEX (mg/kg per day)			
		1		25	
			Fold change		Fold change
Urine flow rate (μL/min)	26.5 ± 3.7	79.1 ± 11.5*	3.0	95.8 ± 11.5*	3.6
Urinary excretion rate (nmol/mL per kg)	0.08 ± 0.03	0.15 ± 0.06	1.9	0.28 ± 0.05*	3.4
Bile flow rate (μL/min)	27.5 ± 2.2	18.7 ± 2.1	0.7	28.3 ± 4.2	1.0
Biliary excretion rate (nmol/mL per kg)	0.26 ± 0.04	0.18 ± 0.01	0.7	0.57 ± 0.14*	2.2
Plasma C_{ss} (μmol/L)	0.12 ± 0.01	0.07 ± 0.01*	0.6	0.08 ± 0.01*	0.6
CL_R (mL/min per kg)	0.6 ± 0.2	2.1 ± 0.8	3.4	3.7 ± 0.7*	6.1
CL_{Bile} (mL/min per kg)	2.2 ± 0.3	2.5 ± 0.2	1.1	7.9 ± 2.2*	3.5
CL_{Total} (mL/min per kg)	21.7 ± 1.0	30.0 ± 1.4*	1.4	29.3 ± 1.5*	1.3
CL_{CR} (mL/min per kg)	1.2 ± 0.1	1.4 ± 0.3	1.2	1.3 ± 0.05	1.1
f_U	0.17 ± 0.01	0.24 ± 0.05	1.4	0.28 ± 0.02*	1.6
CL_R/fu	3.6 ± 0.9	8.6 ± 3.1	2.4	13.2 ± 2.3*	3.7
CL_R/GFR	0.5 ± 0.1	1.6 ± 0.5	3.2	2.7 ± 0.4*	5.5
CL_{RS}	2.4 ± 0.9	7.2 ± 3.4	3.0	11.9 ± 2.5*	5.0

Values are the means±SEM ($n = 6$). $^*P < 0.05$ compared with control value.

DEX, dexamethasone; C_{ss}, steady state plasma concentration; CL_{Bile}, CL_{Total}, CL_R, CL_{RS}, bile, total, renal and renal secretory clearance, respectively; f_U, unbound fraction of the drug; GFR, glomerular filtration rate.

significant change in *mdr1a* and *mdr1b* mRNA in DEX-pretreated rat liver. The reason for the absence of a clear effect of DEX on hepatic *mdr1a/b* mRNA in the present study is unknown. However, no change or a decrease in hepatic *mdr1a/b* mRNA after DEX pretreatment *in vivo* in rats has also been reported by other groups.[1,12] The discrepancy in these results may be explained, at least in part, by dose-, time-, tissue-, route of administration- and probably also strain-dependent effects of DEX on P-gp expression in rats.[25] Indeed, induction of P-gp protein expression in rat liver after DEX has been repeatedly described as a function of dose, starting from 1 mg/kg daily, with the maximum being 100 mg/kg daily.[10,11,14] Considering the duration of pretreatment, treatment for 4–7 days has been reported to yield significant induction, which recovers within 2 weeks after discontinuation of DEX treatment.[7,22] The effect of rat strain on the induction of *mdr1b* mRNA was demonstrated after the prototype inducer, 2-acetaminofluorene (2-AAF) in livers from Fisher, Wistar and Sprague-Dawley rats. Treatment of primary hepatocytes with 2-AAF caused an induction of *mdr1b* expression in Fisher and Wistar hepatocytes, whereas no effect was observed in Sprague-Dawley hepatocytes.[25] Consequently, the Wistar rats pretreated with DEX in the present study under the conditions described may exhibit different responsiveness to DEX than, for example, Sprague-Dowley rats used in other studies.[12] Finally, all the mechanisms involved in the regulation of *mdr1a/b* gene expression are not yet fully understood. There is general agreement about the importance of glucocorticoid receptor (GR)-mediated stimulation of pregnane X receptor (PXR) expression with a consequent increase in *mdr1a/b* transcription.[26] Nevertheless, experiments reporting a lack of agreement between *mdr1a/b* mRNA and P-gp protein expression suggest the contribution of different mechanisms.[5] Indeed, Lee *et al*.[27] reported that induction of *mdr1b* P-gp is regulated, at least in part, post-transriptionally. Our finding of increased PXR expression (S Micuda *et al.*, unpubl. data, 2005) and discrepancies between mRNA and protein levels point to the influence of both mechanisms.

Along the liver acinus, zonal heterogeneity has been demonstrated for specific hepatocellular functions as a consequence of zonated gene expression. Secretion of bile acids and the biliary transport of several xenobiotics, including P-gp substrates, has been shown to occur predominantly in the periportal area.[28] Accordingly, *in situ* techniques revealed highest P-gp mRNA and protein expression in the periportal region of the rat hepatic acinus, with transcript levels decreasing towards the perivenous area.[29,30] Our results of positive P-gp immunostaining located almost exclusively in the periportal region of livers from control rats are consistent with these observations. Pretreatment with DEX produced induction of P-gp expression also in the normally silent cells in the perivenous areas, which resulted in a more uniform acinar expression pattern. Importantly, the extent of the overall increase in P-gp immunostaining observed in the present study corresponds well with the increase in P-gp band intensity detected by western blot (2.2-fold and 2.9-fold induction for low- and high-dose DEX, respectively). Interestingly, a similar but inverse pattern of induction has been described for cytochrome P450 enzymes, which are primarily located in the perivenous areas of the liver acinus where administration of inducer produced an increase especially in periportal hepatocytes.[31]

It has been reported that Rho123 is actively secreted into the urine by P-gp and that alterations in P-gp expression are accompanied by changes in P-gp-mediated renal tubular secretory clearance.[13,20,32,33] In the present experiments, DEX pretreatment increased the net tubular secretion clearance, the CL_R/CL_{CR} ratio and the renal clearance of Rho123, as well as the expression of *mdr1b* mRNA, in a dose-dependent manner, suggesting induction of P-gp in the brush border membrane of renal proximal tubular cells. A similar observation was made by Lilja *et al*.,[34] who described an increase in renal clerance of another P-gp substrate, celiprolol, after administration of the P-gp inducer rifampicin. Nevertheless, we failed to detect statistically significant increases in P-gp protein by western blot. The reason for our conflicting results between *mdr1b* mRNA, renal clearance of Rho123 and P-gp protein levels is unclear. One possible explanation is that another transport protein (e.g. rat organic cation transporter (rOCT)) is involved in the renal excretion of Rho123 and administration of DEX may result in upregulation of that transporter.[35]

Although glucocorticoids are known to be steatogenic agents in humans and rodents,[15,16] the exact molecular mechanisms of this effect have been explained only partly. The whole process seems to be a combination of several mechanisms, of which the most important is the direct effect of glucocorticoids on lipid metabolism. Glucocorticoids inhibit mitochondrial matrix-located long-, medium- and short-chain acyl-CoA dehydrogenases (LCAD, MCAD and SCAD, respectively) and suppress hepatic triglyceride (TG) secretion, thereby inducing hepatocellular TG accumulation. These effects are mostly acute, depending on the actual presence of steroid within the cell, although a mild decrease in the expression of MCAD and SCAD has also been described.[36] Nevertheless, steatosis induced by steroids persists several weeks or even months after discontinuation of therapy.[16,18] Therefore, long-term changes for gene expression can be anticipated. Indeed, DEX and other steroids belong to the group of peroxisome proliferating agents that produce peroxisome proliferator-activated receptor α receptor-mediated stimulation of peroxisome proliferation and β-oxidation of long-chain fatty acids therein.[37] In this situation, one logical explanation of hepatic steatosis after the administration of corticosteroids is the combination of decreased hepatic TG secretion worsened by impaired β-oxidation of medium- and short-chain fatty acids (in mitochondria) in the presence of their increased production (from long-chain fatty acids in peroxisomes). In agreement with this hypothesis, our finding of preferential periportal localization of liver steatosis corresponds well with the reported periportal zonation of β-oxidation of fatty acids in the liver.[31]

In conclusion, the present study has shown that the previously reported DEX-induced stimulation of *in vivo* clearance of Rho123 is not only attributable to changes in its intestinal exsorption, but also to significant increases in biliary and renal excretion because of increased expression of *mdr1b* mRNA and consequently mdr1 protein. Detailed quantitative immunohistochemical analysis showed primary periportal localization of P-gp within the liver acinus, with consequent preferential perivenous induction after corticosteroid administration. Finally, the present study provides, for the first time, detailed analysis of corticosteroid-induced liver steatosis, showing significant zonation of fat accumulation to periportal areas of liver acinus after corticosteroid pretreatment.

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ZONATION OF MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2 IN RAT LIVER AFTER INDUCTION WITH DEXAMETHASONE

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HEPATOLOGY

Zonation of multidrug resistance-associated protein 2 in rat liver after induction with dexamethasone

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bilirubin, dexamethasone, induction, liver, multidrug resistance-associated protein 2, zonation.

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Abstract

Background and Aim: The present study was aimed to evaluate the hepatic zonation of multidrug resistance-associated protein 2 (mrp2), an important drug transporter, and its potential changes during the induction of its expression by known inducer, dexamethasone (DEX).

Methods: The hepatic expression of mrp2 was studied by immunohistochemistry with consequent quantification by measurement of integral optical densities of mrp2 staining in the periportal and perivenous areas of the liver acinus in control and DEX-pretreated rats (1 mg/kg daily per os for 4 days). Overall changes in mrp2 expression and function produced by DEX were monitored using Western blotting and an *in vivo* clearance study of endogenous-conjugated bilirubin, a mrp2 substrate.

Results: In the control animals, a quantitative image analysis revealed the primary periportal localization of mrp2 within the liver acinus with the expression of mrp2 being 16.7-fold of that in the perivenous area. After DEX pretreatment, the expression of mrp2 increased, especially in the perivenous hepatocytes. The overall expression of mrp2 increased 3.2-fold in comparison with the control group. This observation was confirmed by Western blotting, which showed a 1.3-fold increase in the mrp2 protein after DEX pretreatment. The functional consequences of the induced mrp2 protein in the livers of the DEX-pretreated rats were demonstrated by the increased biliary excretion of conjugated bilirubin.

Conclusion: In conclusion, these results indicate the zonation of mrp2 protein expression primarily to periportal hepatocytes. The induction by DEX produced spatially disproportional changes with an increase in the mrp2 protein being most prominent in the perivenous hepatocytes.

Introduction

A spatial pattern of gene expression is commonly observed in different organs. In the mature mammalian liver, most genes appear to exhibit a zonated expression pattern, that is, in an ascending or descending gradient from the portal to the central vein within the acinus, the microcirculatory unit of the liver.[1] This heterogeneity was first described for oxidative and carbohydrate metabolism, but was later expanded to include amino acids and ammonia metabolisms, lipid metabolisms, xenobiotic reactions, cytoprotective functions, and plasma protein synthesis. In addition, zonation has been also observed for transporter-mediated bile formation, where hepatocytes in zone 1 (periportal) were shown to mediate a preferential role in the uptake and biliary excretion of bile acids and organic anions under physiological conditions, while hepatocytes in zone 3 (pericentral) may play a role only when there is a high-dose load.[2] This functional difference appears to be a consequence of the heterogeneous expression of transporting proteins, which extract organic anions from the portal circulation to the hepatocytes and excrete them into the bile canaliculus.[3–6] Concerning particular transporters, amidated bile acids are taken up by the liver by the Na^+/taurocholate cotransport polypeptide and the organic anion transporting polypeptide (oatp) family, whereas organic anions are taken up only by the oatp family. The biliary excretion of amidated bile acids is mediated by the bile salt export pump (bsep), whereas the excretion of organic anions, bile acid sulfates, and glucuronides is mediated by multidrug resistance protein 2 (mrp2).[2] Until now, clear acinar

heterogeneity of transporters' protein expression has been demonstrated for only a few transporters, for example, oatp2, bsep, and mrp3.[6,7]

Mrp2 (Abcc2) is a multispecific organic anion transporter of the hepatocellular canalicular membrane which mediates the efflux of various organic anions, including conjugated bilirubin, glucuronides, glutathione, glutathione conjugates, and sulfated and glucuronidated bile acids.[8–10] The zonal localization of mrp2 within the liver was studied by several authors who described a homogenous distribution of the mrp2 protein throughout the liver acinus.[6,11,12] However, these data are in disagreement with the previously reported preferential periportal location of bile acid uptake and excretion.[13,14] Moreover, the evaluation of mrp2 substrate biliary excretion in rats with impairment of either zone 1 or zone 3 hepatocytes had conflicting results.[2,4] Thus, further studies are needed to elucidate this problem. Interestingly, bile duct ligation, which is known to produce downregulation of mrp2 caused restriction of mrp2 expression to the central areas of the liver lobule,[11] while the administration of lipopolysaccharide endotoxin concentrated mrp2 to periportal hepatocytes.[12] In contrast, no such information is available about the morphological pattern of mrp2 induction, despite the fact that mrp2 expression could be induced by various drugs, including dexamethasone (DEX),[15–17] 2,4,5-trichlorophenoxyacetic acid,[18] acetaminophen,[19] 2-acetylaminofluorene,[20] and 1,4-bis(2-[3,5-dichloropyridyloxy])benzene.[21] In addition, the induction is of therapeutic interest in diseases where mrp2 is missing or downregulated, such as Dubin–Johnson syndrome or cholestasis.[22]

In the present investigation, we analyzed the hepatic zonation of basal mrp2 protein expression and its change following induction by known inducer DEX in the rat liver. Quantitative immunohistochemistry was used to determine mrp2 protein expression in the periportal and perivenous areas of the liver acinus. The verification of induction of mrp2 protein expression and function was performed by Western blotting and an *in vivo* clearance study with an endogenous substrate for mrp2, conjugated bilirubin.[23]

Methods

Materials

Mouse monoclonal antibodies M2-III6 and M2-III5 directed to the mrp2 were purchased from Signet Laboratories (Dedham, MA, USA). Horseradish peroxidase-conjugated goat antimouse and antirabbit immunoglobulin G (IgG) were obtained from GE Healthcare (Prague, Czech Republic). All other reagents were obtained from Sigma Chemical Co (St Louis, MO, USA) and SERVA Electrophoresis GmbH (Heidelberg, Germany), respectively, and were of the highest purity available.

Animals and treatment

Male Wistar rats ($n = 6$, in each group) weighing 270–320 g (Konárovice, Czech Republic) were given an olive oil solution of DEX at a dose of 1 mg/kg daily for four consecutive days by stomach intubations. Untreated (control) rats received an equal volume of vehicle alone (olive oil, 2.0 mL/kg). During pretreatment, the rats were housed under controlled environmental conditions (12-h light–dark cycle; temperature, 22 ± 1°C) with a commercial food diet and water freely available. The animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (http://www.nap.edu/books/0309053773/html/81.html). The study protocol was approved by the Animal Welfare Committee of Charles University in Prague, Faculty of Medicine (Hradec Kralove, Czech Republic).

Quantitative immunohistochemistry

Liver samples were frozen in hexane precooled in liquid nitrogen and stored at −80°C. Cryosections (7–8 μm thick) were prepared with a Minitome cryotome (Prague, Czech Republic). Immunostaining was carried out using M2-III6 and horseradish peroxidase-conjugated rabbit antimouse antibodies diluted in phosphate-buffered saline containing 5% fetal calf serum at 1 : 100. Visualization was achieved by incubating with 3,3′ diaminobenzidine tetrachloride as the peroxidase substrate. Images of the histological sections were acquired on an Olympus IMT-2 light microscope (Olympus, Prague, Czech Republic). Consequent analysis was performed using Image Pro software (Media Cybernetics, Bethesda, MD, USA) where relative presence and integral optical density of positive-stained elements were quantified. To evaluate the expression of mrp2 in the rat liver, 12 randomly-selected viewing fields of every immunostained liver section (six from the periportal area and six from perivenous area) were evaluated at 600-fold original magnification.

Immunoblot analysis

The immunoblast analysis was performed as described previously.[24] Briefly, a membrane-enriched fraction (50 μg protein) was separated on a 6.25% polyacrylamide gel. After being transferred to a nitrocellulose membrane (GE Healthcare, Czech Republic), the proteins were blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membrane was then incubated with the M2-III5 (1 : 500) antibody for 1 h, washed appropriately with TBST, and incubated for 1 h with a peroxidase-conjugated goat antimouse IgG antibody (1 : 1000). After washing the membrane four times with TBST buffer, chemiluminescence was developed using enhanced chemiluminescence reagents (GE Healthcare, Czech Republic). The immunoreactive bands on the autoradiography films were scanned with a GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and semiquantified using the QuantityOne imaging software (Bio-Rad, USA).

Biliary and urinary excretion of conjugated bilirubin *in vivo*

The control and DEX-pretreated rats were anesthetized with pentobarbital (50 mg/kg) and cannulated with polyethylene tubes introduced in the right jugular vein for drug administration and the left carotic artery for blood sampling. The urinary bladder and bile duct were also cannulated for urine and bile collection. The body temperature of the animals was maintained at 37°C with a heat lamp. To elucidate the effect of DEX on steady-state biliary excretion and the renal handling of endogenous-conjugated bilirubin, the rats received only constant-rate infusion (Perfusor Compact;

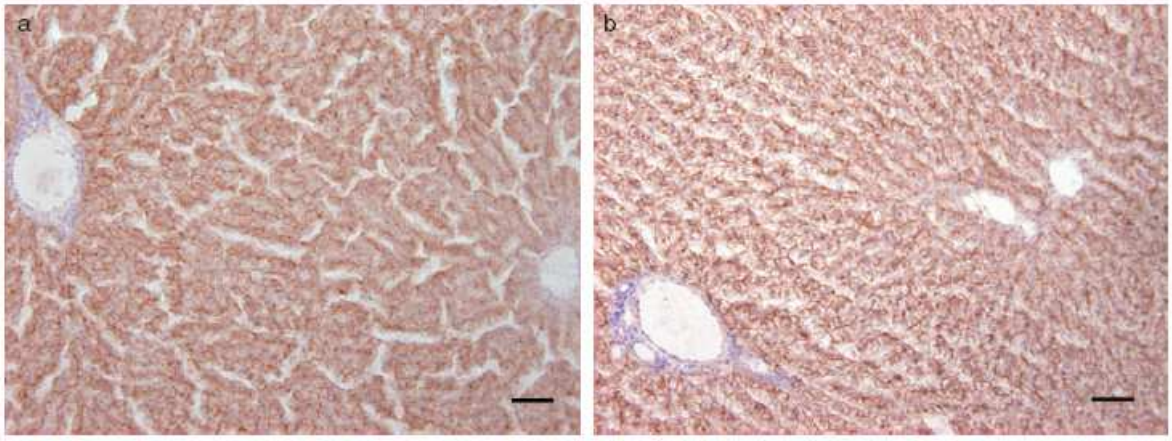

Figure 1 Immunohistochemical microscopy of multidrug resistance-associated protein 2 (mrp2) in the control and dexamethasone-pretreated (1 mg/kg daily for 4 days, per os) rat liver. Representative images of six livers per group of dexamethasone and control animals are shown. Liver cryosections (7–8 μm thick) were labeled with the monoclonal antibody M2-III6 directed against rat mrp2. In the normal rat liver, there was strong mrp2 immunostaining localized on the canalicular membrane of hepatocytes, particularly in the periportal areas (a). Dexamethasone increased the intensity of mrp2 staining in both areas with the increase being most prominent in pericentrally-localized hepatocytes (b). Scale bar, 50 μm; magnification, 160×.

Braun, Prague, Czech Republic) of a 4% mannitol solution at a rate of 2 mL/h to maintain a sufficient and constant urine flow rate. A 40-min infusion was found to result in stable urine and bile production. Thereafter, bile and urine were collected in pre-weighed tubes in 20-min intervals for another 40 min. Blood samples were taken at the midpoint of the bile and urine collection periods. Plasma samples were obtained by centrifugation of blood samples at 3000 *g* for 10 min. All plasma, bile, and urine samples were stored at −80°C until analysis. The concentrations of conjugated (direct) bilirubin and creatinine in plasma, bile, and urine were measured on the Cobas Integra 800 autoanalyzer (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Pharmacokinetic analysis

The biliary and renal clearance (CL_{Bile} and CL_R) of conjugated bilirubin during the steady state was calculated by dividing the respective excretion rate by plasma concentrations which were supposed to be in the steady state (C_{ss}). The glomerular filtration rate (GFR) was evaluated as the clearance of endogenous creatinine (CL_{CR}). The renal clearance ratio of conjugated bilirubin was calculated as CL_R/GFR.

Statistical analysis

Experiments were carried out on six animals per group. All experimental data were expressed as mean ± SEM. Statistical significance was examined in the control and DEX-treated animals using unpaired *t*-test by means of Graphpad Instat 3.0 software (Graphpad Software, San Diego, CA, USA). A difference of $P < 0.05$ was considered statistically significant.

Results

Immunohistochemistry of mrp2

Hepatocellular mrp2 immunostaining performed in the control and DEX-pretreated rat livers is shown in Figure 1. In the control rats, the M2-III6 antibody yielded a strong canalicular staining, which was highly prevalent in periportal hepatocytes (Fig. 1a, Table 1). The administration of DEX increased the intensity of mrp2 staining, which became evenly distributed throughout the liver acinus without any significant quantitative differences between the periportal and pericentral areas (Fig. 1b; Table 1). In comparison with the control group, the statistically significant induction of mrp2 after DEX pretreatment was observed in both the perivenous and pericentral areas with higher intensity in the pericentral zone. A comparison of overall integral optical densities between the control and DEX-administered rats indicated a 3.2-fold increase in the immunostained mrp2 protein.

Western blot analysis of mrp2 expression

Representative immunoblots of hepatic and kidney crude-membrane proteins are shown in Figure 2. The DEX-pretreated rats showed a stronger band intensity of hepatic immunoreactive protein when compared to the untreated rats. A densitometric analysis of the mrp2 level indicated that DEX pretreatment increased hepatic and renal mrp2 protein expression 1.3-fold ($P < 0.05$, Fig. 2) and 1.4-fold ($P < 0.01$, Fig. 2), respectively.

Effect of DEX on bilirubin excretion

The influence of DEX on the biliary and renal excretion of endogenous-conjugated bilirubin in rats is presented in Table 2.

Table 1 Quantitative evaluation of multidrug resistance-associated protein 2 expression in control and DEX-pretreated (1 mg/kg daily for 4 days, per os) rat liver

Parameter for image analysis	Control Centr	Port	DEX Centr	Port
Proportion of visual field area occupied by mrp2 staining	0.1 ± 0.05	2.1 ± 0.8‡	2.9 ± 0.9**	3.9 ± 1.1*
Integral optical densities of mrp2 in visual field	28 ± 11	467 ± 174‡	664 ± 202**	903 ± 266*

‡$P < 0.001$ periportal versus pericentral areas of the same animals; *$P < 0.05$, **$P < 0.001$ comparison of data from respective locations between control and dexamethasone (DEX)-pretreated animals.
Values are mean ± SEM of six rats. Centr, pericentral areas; Port, periportal areas.

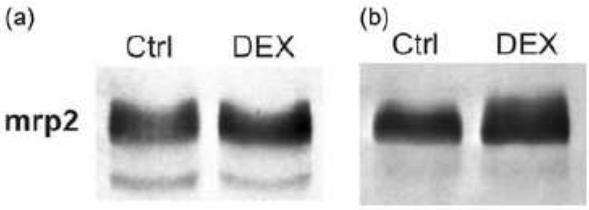

Figure 2 Immunoblot analysis of multidrug resistance-associated protein 2 (mrp2) the in rat liver (a) and kidney (b) from control (Ctrl) and dexamethasone (DEX; 1 mg/kg daily, per os for 4 days)-pretreated rats. Representative pictures of four measurements are shown.

Table 2 Renal and hepatic elimination of endogenous conjugated bilirubin in control and dexamethasone (DEX)-pretreated (1 mg/kg per day orally for 4 days) rats

	Conjugated bilirubin	
	Controls	DEX
Urine flow rate (μL/min)	10.2 ± 0.6	22.1 ± 3.2**
Bile flow rate (μL/min)	21.2 ± 1.5	21.3 ± 1.1
Urinary excretion rate (nmol/min)	0.03 ± 0.003	0.04 ± 0.003*
Biliary excretion rate (nmol/min)	0.3 ± 0.1	0.8 ± 0.2*
Plasma C_{ss} (μM)	0.3 ± 0.07	0.4 ± 0.1
CL_R (ml/min)	0.2 ± 0.05	0.2 ± 0.06
CL_{Bile} (ml/min)	2.7 ± 1.5	4.6 ± 2.3
CL_R/CL_{CR}	0.05 ± 0.01	0.05 ± 0.02
CL_{CR} (ml/min)	3.3 ± 0.3	3.1 ± 0.4
Total plasma protein (g/L)	54.2 ± 1.0	65.6 ± 0.9***
Plasma albumin (g/L)	35.4 ± 0.6	46.1 ± 0.5***
ALT (μcat/L)	0.9 ± 0.06	6.1 ± 1.7**
AST (μcat/L)	1.50 ± 0.09	10.2 ± 3.5*
GMT (μcat/L)	0.03 ± 0.01	0.06 ± 0.02

Values are mean ± SEM ($n = 6$). Significantly different from control value (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CL_{Bile}, biliary clearance; CL_{CR}, creatinine clearance; CL_R, renal clearance; C_{ss}, steady-state concentration in plasma; GMT, gamma-glutamyl transferase.

DEX produced 2.2-fold increase in the urine flow rate without changes in bile flow. The biliary excretion of conjugated bilirubin was increased 2.7-fold after DEX pretreatment. However, as plasma concentrations of conjugated bilirubin rose with wide interindividual variability, as well in response to DEX therapy, the observed 1.7-fold increase of CL_{Bile} failed to display statistical significance. Renal clearance of the compound remained unchanged.

Biochemical parameters

The changes in serum biochemical parameters after short-term DEX pretreatment are summarized in Table 2. DEX pretreatment increased the plasma total protein and albumin concentrations and induced the activity of liver enzymes, aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

Discussion

This study describes the zonation of the mrp2 protein in the rat liver and its change after administration of known inducer, DEX. We have shown that mrp2 protein expression is primarily located on the canalicular membrane of hepatocytes in periportal areas (zone 1) of the liver acinus. The short-term administration of DEX resulted in the significant induction of mrp2 protein expression in both periportal and perivenous hepatocytes, with more intensive induction observed in perivenous hepatocytes. An overall increase was observed in the liver mrp2 expression after DEX was confirmed by Western blotting and an *in vivo* pharmacokinetic study with conjugated bilirubin as an endogenous substrate of mrp2.

The expression of some hepatic genes and proteins is compartmentalized into three different zones which reflect the spatial distribution of cells near the central or portal veins. Numerous metabolism-related pathways, including xenobiotic metabolism and conjugation, amino acid utilization, cholesterol synthesis, bile formation, and nuclear hormone receptors exhibit zonal patterns of regulation and/or expression in the normal liver.[25] As for mrp2, only inconclusive knowledge is available. Studies using immunohistochemistry and immunofluorescence showed that mrp2 is equally expressed in the canalicular plasma membrane of pericentral and periportal hepatocytes.[6,11,12] In contrast, data obtained by kinetic studies with mrp2 substrates and selectively impaired zone 1 (periportal) and zone 3 (pericentral) hepatocytes demonstrated either pericentral or periportal prevalence of mrp2.[2,4,26] Our results, based on precise morphometric and densitometric quantification in multiple visual fields which have not been used for mrp2 quantification yet, suggested the periportal zonation of mrp2. Because the uptake and biliary excretion of organic anions, which are natural substrates for mrp2, is also preferably accomplished by

zone 1 hepatocytes,[4] the primary location of the mrp2 protein within the periportal area would therefore be a logical explanation of this functional heterogeneity of the liver acinus.

The induction of mrp2 expression and the subsequent increase of function have been demonstrated for a variety of compounds.[27] Nevertheless, the morphological patterns of induction within the liver acinus have not been described as yet. In the present study, we used DEX, a pregnane X receptor (PXR) receptor agonist,[28] as a clinically important drug, which has proven potency to change drug disposition based on increased activity of drug-metabolizing enzymes and transporters.[29,30] We demonstrated that pretreatment with DEX produced the induction of mrp2 expression in the normally silent cells in the perivenous areas, which resulted in a more uniform acinar expression pattern. Importantly, the extent of the overall 3.2-fold increase in mrp2 histological immunostaining observed in our study corresponds well with the 1.3-fold increase in mrp2 band intensity detected by Western blotting. Recently, we made similar observations with another important drug transporting protein, P-glycoprotein.[24] Interestingly, concerning zonation, a similar but inverse pattern of induction was described for cytochrome P450 enzymes, which are primarily located in the perivenous areas of the liver acinus, and the administration of an inducer produced an increase, especially in periportal hepatocytes.[1] Therefore, our results, together with these findings, suggest the existence of an effective cooperation/coordination mechanism (e.g. PXR receptor) between phase I/phase II drug metabolizing enzymes and drug transporters, uniting the expression of both systems within the liver acinus after the administration of inducers, such as DEX, and thus supporting the excretion of endo- and xenobiotics from the organism.

To assess the functional consequences of increased mrp2 expression, we performed an *in vivo* study with the evaluation of biliary and renal clearance of endogenous conjugated bilirubin in rats after the administration of DEX. The substrate selection was based on the knowledge that bilirubin, the main product of heme catabolism, is excreted from the hepatocytes into bile almost exclusively via mrp2, mainly as glucuronides.[23,31] The excretion across the canalicular membrane of hepatocytes is the rate-limiting step of overall hepatic transport of bilirubin,[32] and is deficient in two mutant rat strains, GY/TR$^-$[33] and Eisai hyperbilirubinemic rat[34] with the absence of mrp2.[8] Conversely, the induction of mrp2 expression contributes to an increase in conjugated bilirubin total and hepatic clearance in rodents.[35,36] Our data showed a significant increase in the biliary excretion of conjugated bilirubin, which was in agreement with the induction of mrp2. However, the observed increase in the biliary clearance of conjugated bilirubin failed to reach statistical significance due to high interindividual variability of the changes in its plasma concentrations. One possible explanation could be the fact that conjugated bilirubin is formed in the endoplasmic reticulum of hepatocytes by uridine diphosphoglucuronosyltransferase,[37] an enzyme that is also induced by PXR and constitutive androstane receptor agonists.[22] Hence, upon conversion, conjugated bilirubin is directly available for mrp2-mediated biliary excretion, and its concentration measured in plasma may not be a reliable indicator for the calculation of biliary clearance.

Interestingly, we observed a significant increase of urine production after DEX pretreatment. Nevertheless, GFR, which is represented by creatinine clearance, did not change significantly, supporting the hypothesis that increased urine production is due to changes in the renal tubular process. Indeed, increased diuresis and no or a slight increase in CL_{CR} have been repeatedly observed in humans and animals.[38] The main mechanism involved in increased diuresis after the administration of glucocorticoids seems to be marked natriuresis, accompanied by the induction of Na/K-ATPase and increased kalium excretion.[38] The observed increase of albumin concentration in plasma after DEX was also previously described and could be attributable to increased albumin synthesis by the liver.[39,40] In agreement with our results, long-term and/or high-dose treatment with oral or parenteral glucocorticoids was previously associated with the induction of liver AST and ALT activities.[41] Despite some reports suggesting an increase synthesis of enzymes rather than damage of hepatocytes, the exact mechanism of this effect remains unclear at present.[41,42]

In conclusion, we have used a precise morphometric method for the separate quantification of immunostained mrp2 within the different areas/zones of the liver acinus, which has not yet been used for mrp2 quantification. The primary periportal zonation of mrp2 was revealed in the rat liver with its significant changes during induction toward uniform panlobular distribution due to upregulation, mainly in the perivenous (zone 3) region of the liver acinus. The induction of mrp2 was confirmed by the results of Western blotting and an *in vivo* clearance study with endogenous conjugated bilirubin.

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AMIODARONE MODULATES PHARMACOKINETICS OF LOW-DOSE METHOTREXATE IN RATS

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Amiodarone Modulates Pharmacokinetics of Low-dose Methotrexate in Rats

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ABSTRACT: Clinical studies of low-dose methotrexate (LDMTX) pharmacokinetics document increased plasma concentrations of MTX after co-administration of the drug with amiodarone or macrolide antibiotics. As drug–drug interactions may increase the toxicity of LDMTX, a rat model was used to follow renal and biliary elimination of MTX during its constant-rate i.v. infusion and concomitant single bolus i.v. injections of amiodarone or azithromycin. The mean steady-state plasma concentration of 1.7 ± 0.1 μmol/l was reached and the total clearance achieved 17.7 ± 1.0 ml/min/kg. Administration of amiodarone decreased the biliary clearance of MTX to 73% of the control values ($p < 0.05$). Correspondingly, the total clearance decreased to 72% and plasma MTX concentrations were augmented to 2.5 ± 0.4 μmol/l ($p < 0.05$). Amiodarone-treated rats exhibited a 3.3-fold decrease in the renal clearance ($p < 0.05$) of conjugated bilirubin, which was associated with its increased plasma concentration. In contrast, azithromycin did not alter any of the MTX pharmacokinetic parameters. In conclusion, this is the first report describing the impairment of MTX hepatic elimination during co-administration with amiodarone. This study also provides new insight into acute amiodarone-induced hyperbilirubinaemia, where increased bilirubin production and decreased renal clearance may contribute to this effect. Importantly, azithromycin seems to be a safe co-medication during LDMTX therapy. Copyright © 2008 John Wiley & Sons, Ltd.

Key words: methotrexate; amiodarone; azithromycin; biliary excretion; interaction

Introduction

Low-dose methotrexate (LDMTX) therapy has become effective in the treatment of autoimmune and lymphoproliferative diseases [1]. In these disorders, the therapeutic outcome of MTX is related to its systemic or tissue-specific concentrations [2]. Unfortunately, the pharmacokinetics of LDMTX is individually highly variable, resulting in a different systemic exposure to the drug and unpredictable therapeutic/toxic effects in patients. Among the main causes of this variability is the inhibition of transporter-mediated MTX excretion in the liver and kidney [1] as seen in drug–drug interactions between LDMTX and, for example, nonsteroidal anti-inflammatory drugs, salicylic acid and probenecid. These interactions may result in bone marrow suppression and acute renal failure [3]. In addition, clinical data suggest that other commonly used drugs such as amiodarone and macrolides are suspected of producing these

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interactions with MTX. However, no detailed interaction study quantitatively describing changes in MTX pharmacokinetics after administration of both suspected chemical structures is available yet.

Amiodarone, a benzofuranic acid derivative, is a potent drug used in the treatment of paroxysmal supraventricular tachycardia, malignant ventricular tachyarrhythmias, atrial flutter and fibrillation [4]. As a highly lipophilic molecule, AMD is widely bound in the tissues with a huge distribution volume and a correspondingly long serum elimination half-life of 40–60 days. The main route of elimination is the hepatic metabolism to active desethylamiodarone and subsequent excretion to bile [5]. Clinical usage of AMD is hampered by a wide spectrum of drug–drug interactions based on inhibition of several important cytochrome P450 isoforms, such as CYP3A4, CYP1A2 and CYP2C9 [6,7]. In addition, amiodarone may affect the excretion of drugs that are either poorly metabolized (e.g. digoxin) [8] or the metabolism is not a rate-limiting step for their elimination (e.g. anthracyclines and vinca alkaloids) [9]. Studies with *in vitro* cellular models identified that these interactions occur via the inhibition of the hepatic P-glycoprotein (P-gp) membrane transporter [10,11]. Moreover, inhibition of another transporter, Oatp2, has been described for amiodarone [12]. Although the involvement of both transporters in MTX pharmacokinetics seems to be minor, serious interaction between AMD and MTX has been reported in patients with psoriasis [13]. Authors have suggested pharmacokinetic mechanisms but the proof is still missing.

Azithromycin, a 15-ring member macrolide antibiotic, is widely used in the therapy of community-acquired but also hospital infections [14]. In comparison to other macrolide antibiotics, AZT possesses unique pharmacokinetic characteristics with a longer half-life, greater tissue distribution and higher intracellular concentration than others known [15]. It is mainly eliminated in unchanged form in the faeces via biliary excretion and intestinal secretion, whereas urinary excretion is the minor elimination route in humans [15,16]. Recently, Sugie *et al.* [17] have demonstrated that the active excretion of azithromycin is mediated via two ATP-dependent membrane transporters, P-glycoprotein and Mrp2. At the same time, azithromycin has been shown to produce inhibition of the hepatobiliary excretion of drugs that are substrates for Mrp2, the main transporter for biliary excretion of MTX [18,19]. Regarding co-administration with MTX, the interaction between MTX and another macrolide antibiotic has already been described [20]. Moreover, an inhibitory effect of azithromycin on the renal and biliary excretion of MTX given in a high-dose regimen was demonstrated recently [21]. Therefore, the question of whether AZT could also affect LDMTX pharmacokinetics arises.

The present study aimed to investigate whether amiodarone or azithromycin influences either the hepatobiliary or renal excretion of LDMTX in rats. At the beginning, the pharmacokinetic profile of both potential inhibitors was described in rats including their renal and biliary excretion. Thereafter, an *in vivo* clearance study was performed in rats where the influence of either amiodarone or azithromycin on the pharmacokinetics of MTX was examined during steady-state MTX plasma concentrations. In addition, the kinetics of another organic anion, endogenous conjugated bilirubin, was monitored in the same animals to obtain further information on the potential inhibitory influence of both compounds on this excretory pathway.

Methods

Chemicals

Amiodarone was purchased from Sigma Chemical (St Louis, MO). Azithromycin and clarithromycin were kindly donated by Zentiva (Praha, Czech Republic). Amiodarone, methotrexate and azithromycin for injection were obtained from EBEWE Pharma (Unterach, Austria) and Pliva d.d. (Zagreb, Croatia), respectively. All other reagents are commercially available and were of analytical grade. All reagents were used without further purification.

Animals

Male Wistar rats (280–320 g) were obtained from BioTest Ltd (Konarovice, Czech Republic). The

rats were housed under controlled environmental conditions (temperature of 22 ± 1°C and humidity of 55% ± 5%) with a commercial food diet and water available *ad libitum*. All rats received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals (revised 1996; http://www.nap.edu/books/0309053773/html/81.html). The study protocol was approved by the Animal Welfare Committee of Charles University in Prague, Faculty of Medicine in Hradec Kralove.

Amiodarone and azithromycin pharmacokinetics in rats

Rats (n=3) under anaesthesia by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) were fixed in a supine position and cannulated into the right jugular vein, left carotid artery, bile duct and urinary bladder for drug administration, blood sampling, bile collection and urine collection, respectively. After surgical preparations, the rats received an intravenous injection of either azithromycin (40 mg/kg) or amiodarone (25 mg/kg). The dose was selected on the basis of experience from previous studies [17,22] to allow comparison of the pharmacokinetic data. In addition, 4% mannitol solution was infused at a rate of 2 ml/h throughout the study to maintain a constant urine flow rate. Blood samples (≈ 0.3 ml) were taken at designated time intervals (4, 10, 20, 40, 70, 90 and 120 min) after injection of the drug. Plasma was obtained from the blood samples by centrifugation at $3000 \times g$ for 5 min at 4°C. Simultaneously, bile and urine samples were collected in preweighed tubes at 20 min intervals. All specimens were stored at −80°C until analysis. The body temperature of the animals was maintained at 37°C by the placement of the animals on a heating platform.

Effect of azithromycin and amiodarone on the biliary and renal clearance of MTX in rats

Rats (n=6, in each group) under light anaesthesia with sodium pentobarbital (50 mg/kg) were fixed in a supine position and cannulated into the right jugular vein, left carotid artery, bile duct and urinary bladder for drug administration, blood sampling, bile collection and urine collection, respectively. After surgical preparations, the rats received a bolus injection of MTX in a loading dose of 4 μmol/kg, followed by a constant-rate infusion (Perfusor Compact; Braun, Prague) of a saline solution containing 4% mannitol delivering 1.8 μmol/kg of MTX per h at a rate of 2 ml/h until the end of the study. The loading and maintenance doses of MTX were determined by preliminary biliary and renal clearance experiments. Mannitol solution was used to maintain a sufficient and constant urine flow rate. After a 60 min infusion when MTX C_{ss} was attained, bile and urine samples were collected at 20 min intervals for 40 min. After a 100 min infusion, a bolus of amiodarone (25 mg/kg) or azithromycin (40 mg/kg) or isotonic saline was administered intravenously. Bile and urine samples were thereafter collected in preweighed tubes at 20 min intervals from 160 to 220 min. Blood samples were collected at the midpoints of the bile collection periods (70, 90, 170, 190 and 210 min after the start of MTX infusion). Plasma samples were obtained by immediate centrifugation of blood samples and were kept frozen (−80°C). The volume of bile and urine samples was measured gravimetrically with specific gravity assumed to be 1.0. The body temperature of the animals was maintained at 37°C by the placement of the animals on a heating platform.

Drug analysis

The concentration of amiodarone, azithromycin and methotrexate in plasma, urine and bile were determined by high-performance liquid chromatography (HPLC) methods.

The concentrations of methotrexate were measured after deproteination of samples according to a previously described method [2] with the following minor modifications. Briefly, the instrument was an Agilent 1100 series (Agilent, Palo Alto, USA) chromatograph provided with a fluorescence detector (excitation, 350 nm; emission 430 nm). Separation was achieved at 30°C using a column Gemini C18, 110A, 4.6 × 150 mm and precolumn Gemini C18, 4 × 3 mm (Phenomenex, Torrance, USA). The mobile phase flowing at the rate of 0.6 ml/min consisted of ammonium acetate and acetonitrile (87:13, v/v).

For amiodarone and desethylamiodarone analysis, plasma, bile and urine samples (0.1 ml) were diluted by using 0.4 ml of water. A solution of zinc sulfate 20 µl (10%) and acetonitrile (1 ml) was added. The samples were mixed and centrifuged for 10 min at 15000 × *g*. The injection volume of supernatant was 70 µl. Analysis was performed on a 2695 Waters Separations Module (Waters Corp., Milford, MA, USA) equipped with a 996 photodiode array detector and Peltier column-thermostat Jet-Stream (Thermotechnic Products). Empower Software (Waters Corp., Milford, MA, USA) was employed for the data acquisition and processing. The separation of amiodarone and desethylamiodarone was performed on the analytical column Symmetry C18 (Waters) 5 µm particle size (4.6 mm i.d × 150 mm). A Waters Symmetry C18 5 µm particle size Guard Column (3.9 mm i.d. × 20 mm) was used as the analytical precolumn. The isocratic flow rate of the mobile phase was set at 1.1 ml/min. The mobile phase consisted of acetonitrile (47%) and 50 mM phosphate buffer pH 3.1 (53%). UV spectra of all chromatographic peaks were recorded in the range 200–600 nm using a diode-array UV detector with a resolution at 1.2 nm.The wavelength of 242 nm was used for quantitation. The lower limit of detection was 0.10 µmol/l (amiodarone) and 0.2 µmol/l (metabolite), respectively. The inter- and intra-batch accuracies and precisions reached values of 92.6–104.7% (recovery) and 2.3–9.4% (RSD), respectively.

The HPLC method for azithromycin analysis was performed as follows. The plasma sample (150 µl) was mixed with the same volume of 0.05 M potassium carbonate, 5 µl of acetonitrile and 50 µl of internal standard (20 mg/l clarithromycin). After 5 s shaking, 1.2 ml of tert-butylmethylether was added and the mixture was vigorously vortexed for 30 s and centrifuged at 2200 × *g* for 10 min. The organic layer was transferred to an Eppendorf tube and evaporated to dryness. The remnant was dissolved in 100 µl of mobile phase and 50 µl was injected on a column. Samples of bile and urine were diluted with water and 50 µl of internal standard (20 mg/l clarithromycin) was added. The mixture was directly injected on an HPLC column. The chromatographic system consisting of HPLC pump LC-20AD, autoinjector SIL-10ADvp (Shimadzu, Japan), thermostated column compartment LCO102 (Ecom, Czech Republic) and coulochem detector with analytical cell model 5010 (ESA Inc., MA, USA) was used for all separations. Chromatographic data were captured and evaluated with Clarity Lite software (Prague, Czech Republic). Isocratic separation at a flow rate of 1.0 ml/min was carried out on a Gemini C18 reverse phase column (150 × 4.6 mm, 3 µm particle size), protected with a Gemini C18 4 × 3 mm guard column (Phenomenex, Torrance, CA, USA) at a temperature of 40°C. The mobile phase consisted of 0.05 M phosphate buffer (pH=8.0) and acetonitrile (60:40, v/v). The effluent was monitored at an electrode potential of 900 mV with a total sample run time of 20 min. The lower limit of detection was 0.156 µM. The inter- and intra-batch accuracies reached values of 2.2–19.7%.

The concentrations of creatinine and bilirubin (direct and total) were measured on Cobas Integra ® 800 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Pharmacokinetic analysis

Pharmacokinetic analyses were conducted with Kinetica (version 4.4.1). Non-compartmental analysis was used to describe the disposition of amiodarone and azithromycin during 120 min, i.e. the same period during which the drugs were present in the organism together with MTX in the subsequent steady-state interaction study. Maximum observed serum concentrations (C_{max}) of azithromycin and amiodarone were estimated for each animal directly from the serum concentration-time data. The time of the maximum concentration (T_{max}) was defined as the time of the first occurrence of C_{max} (i.e. coincident with the initial blood sample). Area under the plasma concentration-time curve (*AUC*) from time 0 to T_{last} was estimated according to the log-linear trapezoidal rule where T_{last} was the last quantified concentration. The biliary (CL_{Bile}) and renal (CL_R) clearance was calculated by Equations (1) and (2), where X_{bile} and X_{urine} were the amount of azithromycin or amiodarone excreted to bile and urine, respectively, during the evaluated

period and T_{last} was 120 min [23]:

$$CL_{Bile} = X_{Bile}/AUC_{0\text{-}Tlast} \quad (1)$$

$$CL_R = X_{Urine}/AUC_{0\text{-}Tlast} \quad (2)$$

Steady-state pharmacokinetic parameters of MTX for the interaction study were calculated for each animal as the mean of three points in 160′–220′ of experiment. The total plasma clearance (CL_{Total}) of MTX was estimated by dividing the constant infusion rate of MTX by the steady-state concentration in plasma (C_{ss}). Biliary and renal clearance (CL_{Bile} and CL_R) of MTX during each collection period was calculated by dividing the respective excretion rate by C_{ss} determined for that collection period. Kinetic parameters of endogenous conjugated bilirubin, an Mrp2 substrate, were calculated by the same approach, on the basis of the assumption that bilirubin plasma concentrations were in steady-state with the exception of CL_{Bile} as this cannot be calculated due to the fact that liver is the organ that synthesizes conjugated bilirubin synthesis. The glomerular filtration rate (GFR) was evaluated as the clearance of endogenous creatinine (CL_{CR}). The renal clearance ratio of MTX and bilirubin was calculated as CL_R/CL_{CR}.

Statistical analysis

Interaction experiments were carried out in five animals per group. All experimental data are expressed as mean ± SEM. Statistical significance was examined by unpaired *t*-test using Graphpad Prism 4.0 software (Graphpad Software, San Diego, USA). A value of $p < 0.05$ was considered statistically significant.

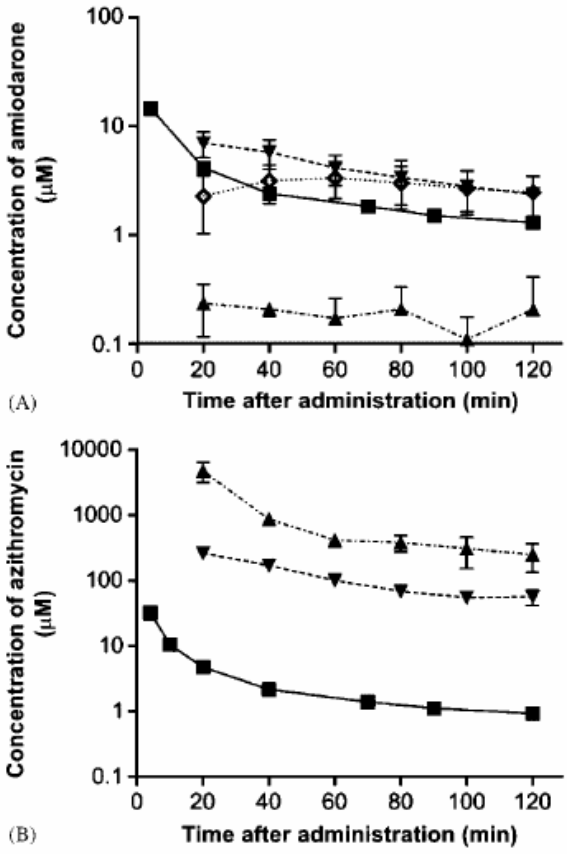

Figure 1. Semilogarithmic plots of amiodarone (A) and azithromycin (B) plasma, biliary and urinary concentration versus time curves measured in Wistar male rats after intravenous administration of a single bolus dose (25 mg/kg of amiodarone and 40 mg/kg of azithromycin). For amiodarone, the main metabolite, desethylamiodarone, was detected only in bile. Symbols: ■, drug concentrations in plasma; ▼ drug concentrations in bile; ▲, drug concentrations in urine; ◇, desethylamiodarone concentrations in bile. Values are the mean ± SEM (n=3)

Results

Plasma concentration-time curve of amiodarone and azithromycin

Semilogarithmic plots of plasma, biliary and urinary concentration-time data for amiodarone and azithromycin after a single intravenous injection are shown in Figure 1, respectively. Regarding amiodarone, the maximum concentrations of the compound in plasma (C_{max}), measured 4 min after administration, were 14.5 ± 1.9 μM. The main metabolite, desethylamiodarone, was detected only in bile. Concentrations of both amiodarone and desethylamiodarone in bile quickly exceeded amiodarone concentrations in plasma (Figure 1A). Within 120 min of administration, the rats excreted only 0.1% of the applied amiodarone dose, 97% of which appeared in bile and 3% in urine. The CL_{Bile} and CL_R of amiodarone were 5.9 ± 0.7 ml/h/kg and 0.2 ± 0.07 ml/h/kg, respectively. In the

case of azithromycin, C_{max} measured within 4 min after administration was 32.5 ± 7.8 μM. Both bile and urine concentrations were well above those measured in plasma (Figure 1B). A total of 18% of the injected azithromycin dose was excreted in urine and bile over the evaluated period (120 min), 8% of which was into the bile and 92% into urine. The CL_{Bile} and CL_R of azithromycin were 90.4 ± 17.9 ml/h/kg and 1116 ± 377 ml/h/kg, respectively.

Steady-state pharmacokinetics of methotrexate in control animals

Pharmacokinetic data of MTX during steady-state of plasma concentrations in control rats are summarized in Table 1. A steady-state concentration of MTX was attained after 60 min from the start of its constant rate infusion. Concentrations of MTX in bile were 169-fold higher than in plasma, suggesting an important contribution of the active transport mechanism. Urinary concentrations were also 98-fold higher than in plasma, but a CL_R/CL_{CR} ratio below 1 suggests that glomerular filtration together with tubular reabsorption play main roles in methotrexate renal elimination. The sum of MTX biliary and urinary excretion rates accounted for 87% of the infusion rate in the absence of inhibitors. Thus, the metabolism of methotrexate under these conditions should be minor and any potential effect of an interaction involving metabolism should be minimal.

Effect of amiodarone and azithromycin on biliary and renal clearance of MTX

The effects of amiodarone (25 mg/kg) and azithromycin (40 mg/kg) on the biliary and renal excretion of MTX were investigated under steady-state conditions obtained by the continuous intravenous infusion. The pharmacokinetic parameters of MTX are summarized in Table 1. Administration of amiodarone significantly decreased the CL_{Bile} of MTX by 27% with a corresponding reduction of CL_{Tot} by 28%. The renal clearance of MTX remained unaffected after amiodarone; however, CL_{CR} was significantly reduced by 33%. In comparison, there was no change in any of the MTX pharmacokinetic parameters after azithromycin administration (Table 1).

Effect of amiodarone and azithromycin on conjugated bilirubin (CB) excretion

To further investigate the influence of both potential inhibitors on the organic anion elimination pathways, the biliary and renal excretion of another MRP2 substrate, endogenous CB, was evaluated in the MTX-infused animals. The results of endogenous bilirubin kinetics in rats are presented in Table 2. Amiodarone produced a 5.9-fold increase in the biliary excretion of conjugated bilirubin. However, as the concentrations of CB in plasma rose 5.5-fold as well, the overall influence of amiodarone on CB biliary clearance remained insignificant. Despite the

Table 1. Effects of amiodarone and azithromycin on biliary and renal excretion of MTX in rats

	MTX		
	Control	AMD	AZT
Urine flow rate (μl/min)	11.8 ± 2.0	13.1 ± 3.1	13.8 ± 2.5
Bile flow rate (μl/min)	18.7 ± 2.1	18.7 ± 4.7	20.8 ± 0.7
Urinary excretion rate (nmol/kg/min)	7.3 ± 1.7	12.2 ± 3.3	8.1 ± 1.5
Biliary excretion rate (nmol/kg/min)	18.7 ± 1.5	18.7 ± 1.5	17.0 ± 1.9
C_{ss} (μM)	1.7 ± 0.1	2.5 ± 0.4^a	1.6 ± 0.1
CL_R (ml/kg/min)	4.3 ± 0.9	5.1 ± 1.4	5.6 ± 1.4
CL_{Bile} (ml/kg/min)	11.1 ± 1.2	7.9 ± 1.5^a	10.4 ± 0.5
CL_{Tot} (ml/kg/min)	17.7 ± 1.0	12.7 ± 1.8^a	19.7 ± 1.8
CL_{CR} (ml/kg/min)	7.7 ± 1.2	5.2 ± 0.6^a	7.6 ± 0.9
CL_R/CL_{CR}	0.6 ± 0.1	1.0 ± 0.2^a	0.7 ± 0.1

Values are mean ± SEM (n=5).
Significantly different from control values ($^a p < 0.05$).

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Table 2. Kinetics of endogenous bilirubin in rats (n=5 in each group) after administration of either amiodarone (25 mg/kg) or azithromycin (40 mg/kg)

	Control	AMD	AZT
Conjugated bilirubin			
Urinary excretion rate (nmol/kg/min)	0.12 ± 0.03	0.16 ± 0.04	0.13 ± 0.03
Biliary excretion rate (nmol/kg/min)	3.6 ± 1.2	21.4 ± 3.2[c]	3.6 ± 0.3
C_{ss} (μM)	0.2 ± 0.1	1.1 ± 0.2[b]	0.1 ± 0.002[a]
CL_R (ml/kg/min)	0.7 ± 0.2	0.2 ± 0.05[a]	1.3 ± 0.3
CL_R/CL_{CR}	0.1 ± 0.03	0.03 ± 0.008[a]	0.2 ± 0.03
Total bilirubin			
C_{ss} (μM)	4.1 ± 0.1	6.1 ± 0.4[**]	5.0 ± 0.5

Data on urine and bile flow rates are listed in Table 1—samples from the same animals were analysed. Pharmacokinetic analysis was performed on the basis of the assumption that bilirubin serum concentrations were in steady-state.
Values are mean ± SEM (n=5).
Significantly different from control values ([a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$).

administration of amiodarone not changing the CB urinary excretion rate, the renal clearance of CB was decreased. In contrast, administration of azithromycin induced a significant decrease in CB C_{ss}.

Discussion

This study initially describes in detail the biliary and urinary excretion of amiodarone and azithromycin during the first 120 min upon intravenous administration of a single bolus dose to male Wistar rats. Thereafter, the influence of both compounds on steady-state pharmacokinetics of MTX was monitored in separate groups of animals during the same period. A significant decrease of MTX biliary clearance was observed after administration of amiodarone, which was associated with increased MTX plasma concentrations and decreased total clearance of the drug. In contrast, no change in MTX pharmacokinetics was detected after administration of azithromycin. The evaluation of kinetics of another organic anion, endogenous conjugated bilirubin, showed a significant increase in its plasma concentrations and biliary excretions after administration of amiodarone, while a decrease of plasma CB concentrations was detected after application of azithromycin.

In humans, the major route of MTX elimination is renal excretion of unmetabolized drug, which accounts for approximately 60% to 90% of the total body clearance. In addition, about 3% to 4% of the applied dose is excreted in urine as 7-hydroxy-methotrexate [3]. The remaining 10–30% of methotrexate is eliminated by active biliary excretion, which is mediated mostly by MRP2 [24,25]. In rats, 62% of i.v.-administered MTX is excreted into bile, whereas 27% of the dose is excreted into urine, as shown by Masuda *et al*. [19]. Similarly to humans, biliary excretion is mediated by MRP2 as demonstrated by the almost complete abolition of methotrexate biliary recovery in Eisai hyperbilirubinaemic rats (EHBR), the strain deficient in MRP2 expression and function in the liver and intestine [26]. Consistent with previous reports, the present study demonstrated that biliary excretion represents the major route of MTX elimination in rats. Importantly, unlike these studies, a dosage regimen was used that maintained steady-state MTX concentrations equal to those measured after low-dose MTX administration [2]. The equilibrated rate of administration and the sum of urinary and biliary excretion confirmed the steady-state. An observed small discrepancy between the overall excretion and the infusion velocity could, at least partly, be explained by metabolism to 7-hydroxymethotrexate by hepatic aldehyde-oxidase, which may account for 6–10% of the administered dose [27]. Concerning renal elimination, steady-state concentrations of MTX used in our study were associated with a ratio of CL_R/CL_{CR} below 1, which suggests tubular reabsorption. A similar observation was reported previously in humans [1].

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Before the interactions study with MTX, a short study was performed to obtain information about the kinetics of amiodarone and azithromycin in rats during an appropriate time period. Complete pharmacokinetic parameters of the compounds could not be described due to the long half-life of both compounds [17,28]. Nevertheless, the study focused on actual concentrations in fluids and biliary and renal excretions during the period of planned co-administration with MTX. Regarding amiodarone, as expected [22], the principal route of the drug elimination was biliary excretion. A small proportion of the dose excreted during the evaluated periods corresponds well with the very long biological half-life of amiodarone in humans and rats [5,28]. Importantly for interactions, the measured plasma concentrations in our study comply with those observed in clinical settings where therapeutic concentrations are 1–2.5 mg/l, i.e. 1.5–3.7 μM [5]. Administration of azithromycin also yielded pharmacokinetic behaviour that complies with previously reported data [17]. The only difference was higher renal clearance of azithromycin in our study. This fact may be related to the continual infusion of fluids and the maintenance of constant urine flow throughout the study to provide the same conditions as in the case of the following interaction study. Similarly to amiodarone, plasma concentrations of azithromycin were within the range attainable in humans [29,30]. Thus, the basal condition for evaluation of MTX interactions was fulfilled for both potential inhibitors.

The study evaluated the effect of amiodarone on the steady-state biliary and renal excretion of methotrexate in rats. As reported by Reynolds *et al*. [13], administration of amiodarone to patients receiving oral LDMTX therapy induced serious skin necrosis which healed rapidly when methotrexate was discontinued. Despite no interaction study being available yet, the authors proposed that the interaction had a pharmacokinetic background. In agreement with this suggestion, our study showed, for the first time, that amiodarone significantly decreased the biliary clearance of methotrexate with a corresponding increase in its C_{ss}. Considering MTX pharmacokinetics, the interaction described in our study is clearly based on changes in activities of transport proteins in the liver. Nevertheless, we are aware that the methodology used in our study did not allow us to answer the question of which transporter is involved in the observed interaction. Studies with transfected cell lines showed the inhibitory effect of amiodarone on P-gp- and OATP2-mediated transport of digoxin and anthracycline [11,12]. No study reporting the involvement of P-gp in methotrexate biliary excretion is available. However, P-gp is a transporter of cationic compounds with large lipophilic molecules and indirect evidence of there being no influence of MTX on the biliary secretion of other P-gp substrates suggests that the contribution of P-gp to MTX biliary elimination is small [31]. In contrast, recent data demonstrated that both main members of the OATP family, human OATP1A2 and OATP1B1 and rat Oatp1a1 (Oatp1) and Oatp1a4 (Oatp2), which function similarly, may contribute to MTX transport [32–34]. Moreover, Ueda *et al*. [35] suggested that interactions of methotrexate with organic anions take place at the level of basolateral membrane, the location of OATPs in the liver. Taken together, the inhibition of Oatp2-mediated hepatic uptake of MTX seems to be the principal mechanism of increased plasma concentration of MTX during amiodarone administration. In kidneys, reduced CL_{CR} was observed in amiodarone-administered animals. This result complies with the described impairment of kidney function during acute amiodarone therapy [36]. Nevertheless, a decreased glomerular filtration rate after administration of amiodarone was not associated with reduction of MTX CL_R. The ratio of CL_{RMTX}/CL_{CR} was increased after amiodarone, suggesting a blockade of reabsorption when compared with control animals. Amiodarone's inhibitory influence on OATP1 in the kidney, where this protein is expressed on the apical membrane of the distal tubule and seems to play a role in active tubular reabsorption of MTX [33], requires further elucidation.

The idea for evaluation of potential azithromycin–MTX interaction originates from the fact that interaction of MTX with other macrolide-like antibiotics has already been described, and that azithromycin was shown as an inhibitor of the

Mrp2 transporter, an important molecule for biliary excretion of MTX [19,26]. Nevertheless, our study showed that the administration of azithromycin did not change LDMTX pharmacokinetics. This also provides important information for clinical practice where these two compounds may be co-administered in situations such as chemically induced abortion [37]. In addition, the absence of interaction supports the significance of Oatps for the pharmacokinetics of LDMTX. First, azithromycin is not an inhibitor of Oatps, thus indicating that potency to inhibit methotrexate pharmacokinetics is only through Mrp2 [38]. Second, Mrp2 is a low-affinity high-capacity MTX transporter with K_m of 300 μM, thus its importance for pharmacokinetics of LDMTX is likely less prominent than for interactions with a high-dose regimen [19,35]. This information is supported by our recent finding that azithromycin produced a decrease in biliary and renal elimination of MTX when its C_{ss} approached an anticancer regimen [21].

To further extend information on the influence of amiodarone and azithromycin on the elimination pathways for organic anions the kinetics of endogenous conjugated bilirubin was evaluated in the same (MTX-infused) animals. It is known that Mrp2-mediated biliary excretion of conjugated bilirubin serves as the main rate-limiting step in the biliary elimination of the compound and that MRP2 deficiency is associated with hyperbilirubinaemia in rats and humans [39]. Because both evaluated compounds have been demonstrated previously to produce intrahepatic cholestasis during repeated long-term administration, we expected a decrease in the biliary excretion of the compound associated with its increased plasma concentrations [40,41]. Indeed, administration of amiodarone induced a marked increase of endogenous conjugated bilirubin plasma concentration (5.9-fold); however, the biliary excretion of CB was correspondingly increased (5.5-fold), too. Because CB is the metabolite which is formed from bilirubin intrahepatically, the decreased uptake to hepatocytes is unlikely to be the cause of increased C_{ss} of the compound after amiodarone injection. Therefore, the acute increase seems to be related to increased production of CB rather than to the cholestasis reported after chronic treatment. This finding is supported by mild haemolysis observed in serum and urine of amiodarone bolus administered animals (unpublished observation). Indeed, haemolytic anaemia due to impaired erythrocyte membrane function and *in vitro* photohaemolysis was described for amiodarone [42,43]. In addition, the observed decrease in CL_{CR} which was associated with decreased renal clearance of CB may point to another mechanism of amiodarone induced hyperbilirubinaemia. These data may bring new insight into the mechanism of hyperbilirubinaemia observed after acute high-dose amiodarone administration in humans [44]. Regarding azithromycin, similar kinetic behaviour of CB, i.e. decreased CB plasma concentration, was observed in mice lacking Mrp3, the basolateral transporter of conjugated bilirubin with overlapping substrate specificity with Mrp2 [45,46]. Possibly, azithromycin also inhibits Mrp3, thus preventing backward transport of CB to blood.

In conclusion, the present study suggests that amiodarone, an inhibitor of Oatp2, increases the plasma concentration of MTX by inhibiting Oatp2-mediated hepatic uptake of MTX in rats. Although the data obtained in the present study cannot be extrapolated directly to humans, the results provide useful information about the mechanism of interaction already described in clinical practice. Therefore, the co-administration of both compounds would better be avoided in humans or, if inevitable, then careful monitoring of MTX plasma concentrations with immediate correction of its dosage is obligatory. In addition, our data show that one of the most commonly used antibiotics, azithromycin, at clinically relevant plasma concentrations, failed to demonstrate any significant effect on the *in vivo* biliary or renal excretion of low-dose methotrexate in rats and thus suggests a safe combination for therapy with low-dose methotrexate.

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UP-REGULATION OF RENAL MDR1 AND MRP2 TRANSPORTERS DURING AMIODARONE PRETREATMENT IN RATS

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Up-regulation of renal Mdr1 and Mrp2 transporters during amiodarone pretreatment in rats

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Rhodamine-123

ABSTRACT

Although amiodarone (AMD) is known to produce drug–drug interactions through inhibition of transporter-mediated excretion of drugs, its impact on these mechanisms during chronic treatment has not been described yet. Therefore, the aim of this study was to investigate the influence of AMD pretreatment on the main multidrug transporting proteins, Mdr1 and Mrp2, in the liver and kidney. The expression of the transporters and pharmacokinetics of their substrates, rhodamine-123 (Rho123) and endogenous conjugated bilirubin (CB), were evaluated in rats after either AMD oral pretreatments (4–14 days) or single intravenous bolus. AMD pretreatment of all durations up-regulated renal Mdr1 and Mrp2 protein expression to 155–190% and 152–223% of the control values, respectively. In agreement, we observed a corresponding increase in renal clearance of both substrates. Hepatic expression was increased only for Mdr1 to 234–270% of controls, which was associated with increased biliary elimination of amiodarone without change in Rho123 biliary clearance. Interestingly, hepatic expression of another Mdr transporter, Mdr2, was progressively decreased by amiodarone administration. Acute administration of AMD reduced Rho123 biliary clearance by 64%. Our results indicate that repeated administration of AMD to rats is associated with significant increase in hepatic and renal expression of Mdr1 and Mrp2 transporters, which may contribute to variability in pharmacokinetics of AMD and simultaneously applied drugs.

1. Introduction

Amiodarone is a potent drug used in the treatment of serious supraventricular and ventricular tachyarrhythmias. However, its wide clinical use is precluded by extensive adverse effects and drug–drug interactions. In order to limit these complications, careful monitoring of the therapy is recommended. In addition, prospective identification of the mechanisms involved in such therapeutic complications may allow their better prediction and even prevention.

The most frequently reported drug–drug interactions of amiodarone are those based on the inhibition of biotransformation [1]. As a highly lipophilic molecule, amiodarone is widely bound in the tissues with huge distribution volume and a correspondingly long serum elimination half-life of 40–60 days [2]. The main route of its elimination is the hepatic biotransformation to desethylamiodarone (DEA) by CYP3A4 and CYP2C8, with further metabolism by CYP3A4 followed by excretion into the bile [3]. Amiodarone was demonstrated as a potent inhibitor of CYP3A4, CYP1A2, CYP2C9, CYP2C19 and CYP2D6 [4,5]. Based on this mechanism, serious amiodarone drug–drug interactions were described with agents such as warfarin, simvastatin, and cyclosporine A [5]. In addition, amiodarone may affect excretion of drugs that either are poorly metabolized, e.g. digoxin [6], or those whose metabolism is not the rate-limiting step in their elimination, e.g. anthracyclines and vinca alcaloids [7]. Studies with cellular models identified that these interactions may occur in the liver via the inhibition of either canalicular multidrug resistance protein 1 (Mdr1) [8,9] or basolateral organic anion transporting polypeptide 2 (Oatp2) [10,11]. As a consequence, information about the principal underlying mechanism of these interactions has become less conclusive. In addition, it is well known that a long term treatment by Mdr1 inhibitors (e.g. cyclosporine A or ritonavir) may result in the transporter induction [12,13]. No such information is currently available for amiodarone.

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The present study aims to investigate amiodarone influence on the expression and function of two main drug transporting multidrug resistance proteins, Mdr1 and Mrp2, in the liver and kidneys. The function of the transporters was evaluated by elimination kinetics of two substrates, rhodamine-123 (Rho123 for Mdr1) and endogenous conjugated bilirubin (CB for Mrp2) in rats after either chronic (4–14 days) or acute (i.v. bolus) AMD administration. Detailed analysis of hepatic transport was evaluated using cultured primary hepatocytes. Changes in Mdr1, Mrp2, and Oatp2 protein expression were evaluated by Western blot.

2. Materials and methods

2.1. Materials

Rho123 and amiodarone were purchased from Sigma Chemical Co. (St. Louis, MO). Cyclosporine A (CsA) was a commercially available original formulation of Sandimmun (Novartis Ltd.). Mouse monoclonal antibodies C219, directed to the Mdr1 (150–170 kDa) and M2III-5, directed to Mrp2 (170–190 kDa) were purchased from Signet Laboratories, Inc. (Dedham, MA, USA). Rabbit anti-Oatp2 (75 kDa) polyclonal antibody was obtained from Millipore Corporate Headquarters (Billerica, MA, USA). Rabbit anti-Mdr2 (170 kDa) and anti-Cyp3a2 (55 kDa) polyclonal antibodies were obtained from Abcam (Cambridge, UK) and Daiichi Pure Chemicals Co. (BD Gentest, Woburn, Massachusetts, USA), respectively. As a loading control for Western blot, rabbit polyclonal β-actin antibody (42–45 kDa) was purchased from Sigma (St. Louis, MO). Horseradish peroxidase-linked sheep anti-mouse and donkey anti-rabbit immunoglobulin G were purchased from GE Healthcare (Prague, Czech Republic). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and Bio-Rad laboratories (Hercules, CA, USA), respectively, and were of the highest purity available.

2.2. Animals and treatment

Adult male Wistar rats (Konárovice, Czech Republic) with initial weight of 270–280 g were used throughout the study. Animals were housed under controlled environmental conditions (12-h light–dark cycle; temperature, 22 ± 1 °C) with a commercial food diet and water freely available. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication, 1996) and under the supervision of the Ethical Committee of the Faculty of Medicine in Hradec Kralove.

Rats were divided into six groups ($n = 6$, in each group). Amiodarone pretreated animals (25 mg/kg) received the drug once daily by stomach intubation for 4, 7, and 14 consecutive days. Corresponding three control groups were treated with an equal volume of vehicle alone (PBS, 2.0 ml/kg). Pharmacokinetic study with Rho123 was performed 24 h after the last dose administration. During this experiment, rats under light anesthesia with sodium pentobarbital (50 mg/kg) were fixed in supine position and cannulated in jugular vein, carotid artery, ductus choledochus and bladder for drug administration, blood, bile and urine sampling, respectively. The body temperature of the animals was maintained at 37 °C by a heating platform. Thereafter, a bolus dose of Rho123 (80 μg/kg) was applied intravenously, followed by a constant-rate infusion (Perfusor Compact; Braun, Prague) of a saline solution containing 4% mannitol delivering 400 μg (i.e. 1050 μmol)/kg of Rho123 per hour at a rate of 2 ml/h for 2 h. The loading and maintenance doses of Rho123 were determined by previous experiments [14]. Mannitol solution was used to maintain sufficient and constant urine flow rate. Bile and urine samples were collected in preweighed tubes at 20-min intervals from 60 to 120 min. Blood samples were collected at the midpoint of each collection period (70, 90 and 110 min after the infusion was started). Plasma samples were obtained from the whole blood by centrifugation at 1200 × *g* for 5 min at 4 °C. Organs for consequent evaluation of protein expression were immediately frozen in liquid nitrogen and together with plasma, bile and urine samples stored at −80 °C until analysis.

Acute effect of amiodarone, CsA (a known inhibitor of multidrug resistance transporters) or vehicle on Rho123 kinetics was tested similarly as described for pretreatments. After surgical preparations, the same loading and maintenance doses of Rho were introduced. Since the 60th min of Rho123 infusion, bile and urine samples were collected at 20-min interval for 40 min. At 100 min of Rho123 infusion, amiodarone (25 mg/kg), CsA (25 mg/kg) or isotonic saline was administered intravenously. Bile and urine samples were thereafter collected in preweighed tubes at 20-min intervals from 160 to 220 min. Blood samples were collected at the midpoint of each collection period (70, 90, 170, 190, and 210 min after the infusion was started). Samples were processed as described above.

2.3. Rho123 accumulation and efflux in primary rat hepatocytes

Hepatocytes were isolated as described previously [15], seeded in a density of 2×10^6 cells per Petri dish (60 mm diameter), and cultured for one day. *In Rho123 accumulation experiments*, adherent cells were incubated in Williams E medium containing 1 μM Rho123 in the absence or presence of amiodarone (0.1 μM, 1 μM, and 5 μM) or CsA (1 μM and 10 μM), respectively, at 37 °C for 60 and 120 min. *In dye efflux experiments*, cells were preloaded with Rho123 by exposure to 1 μM Rho123 in the medium for 2 h and then incubated with Williams E medium without Rho123 in the absence (control efflux) or presence of amiodarone or CsA for up to 120 min. Subsequently, hepatocytes were washed three times with 4 °C cold PBS and intracellular dye was extracted by incubation with 0.5% Triton X (1.5 ml per dish) for 10 min at room temperature. The accumulated or excreted amount of Rho123 was normalized for the protein content per dish.

2.4. Drug analyses

The concentrations of amiodarone, and Rho123 were determined by high-performance liquid chromatography (HPLC) methods as described previously [14,16]. The concentrations of bilirubin and creatinine in plasma, bile, and urine were measured on Cobas Integra® 800 (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instructions.

2.5. Pharmacokinetic analysis

Total plasma clearance (CL_{TOT}) of Rho123 was estimated by dividing the constant infusion rate of Rho123 by the steady-state concentration in plasma (C_{ss}). Biliary and renal clearance (CL_{Bile} and CL_R) of Rho123 during each collection period was calculated by dividing the respective excretion rate (BE, biliary; UE, urinary) by C_{ss} determined for that collection period. Biliary excretion and clearance of AMD was calculated using the same method from measurements performed in bile and plasma samples obtained during the final collection periods (100–120′ in AMD orally pretreated rats, i.e. 24 h after last AMD administration; or 100–120′ after AMD intravenous bolus). Clearance of endogenous creatinine (CL_{CR}) was determined as an indicator of glomerular filtration rate.

2.6. Immunoblot analysis

This method was performed as described previously [17]. Briefly, a membrane-enriched fraction (50 μg protein) was

separated on a 6.25% polyacrylamide gel. After being transferred to a nitrocellulose membrane, the proteins were blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST). The membrane was then incubated with C219, M2-III5, anti-Mdr2, anti-Oatp2 (1:500) or anti-Cyp3a2 (1:5000) antibody for 1 h, washed four times with TBST, and incubated for 1 h with a peroxidase-conjugated secondary antibody (1:1000). After washing the membrane four times with TBST buffer, chemiluminescence was developed using an ECL reagents (GE Healthcare). The immunoreactive bands on the autoradiography films were scanned with calibrated densitometer ScanMaker i900 (UMAX, Prague, CZ) and quantified using the QuantityOne imaging software (Bio–Rad Laboratories, Hercules, CA). Equal loading of proteins onto the gel was confirmed by immunodetection of β-actin.

2.7. Statistical analysis

All quantitative data are presented as means and SDs. The results of the three control groups for AMD pretreatment are presented together because no statistically significant differences have been found among these groups. Differences between experimental and control values were assessed by one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 5.0 software (San Diego, California).

3. Results

3.1. Protein expression

To determine the potential influence of amiodarone therapy on the protein expression of two transporters important for hepatic and renal drug excretion, Mdr1 and Mrp2, Western blot analysis was performed in total hepatic and renal membrane fractions obtained from rats treated for 4, 7 or 14 days either with amiodarone (25 mg/kg/day orally) or with corresponding volume of vehiculum (PBS) alone. As demonstrated in Fig. 1A/B, amiodarone pretreatment significantly induced Mdr1 expression in both liver and kidneys. The increase was most pronounced after 4 days (223% and 190% of controls in the liver and kidneys, respectively). To exclude a possibility of false results due to slight cross-reactivity of C219 antibody with Mdr2 phospholipid transporter, we analyzed also expression of this protein, which was progressively reduced throughout the AMD pretreatment (Fig. 1A). The expression of the main drug metabolizing enzyme co-operating with Mdr1, Cyp3a2 (rat orthologue of human CYP3A4), was unchanged in the liver, but increased in the kidney after 4 and 7 days of AMD administration (Fig. 1B). The expression of Mrp2 followed different patterns in the liver and kidneys. While in the latter (Fig. 1B) it was increased during the whole 14-day period of treatment by 234–270% compared to controls, the liver expression of the protein remained unchanged. Similarly, the expression of Oatp2, one of the main drug transporters localized at the basolateral membrane of hepatocytes, was also not influenced by amiodarone pretreatment.

3.2. Effect of amiodarone oral pretreatment on the kinetics of Rho123, AMD, and CB

Pharmacokinetic parameters of Rho123 are summarized in Table 1. Repeated administration of amiodarone produced progressive increase in urinary excretion rate of Rho123, which was associated with increased renal clearance of the dye. The biliary excretion of Rho123 demonstrated tendency to increase, nevertheless the rise was not followed in biliary clearance parameter. Measurement of plasma and biliary concentrations of AMD showed that the drug was present in low concentrations in plasma of pretreated animals while bile concentrations and thus also excretions were comparable to those detected after single intravenous administration (Table 2). Importantly, there was statistically significant rise in biliary clearance of amiodarone, which reflected patterns of induced expression of Mdr1 in the liver (Fig. 1A, Table 2). The influence of oral amiodarone pretreatment (4, 7 and 14 days) on the renal and biliary clearances of conjugated bilirubin is shown in

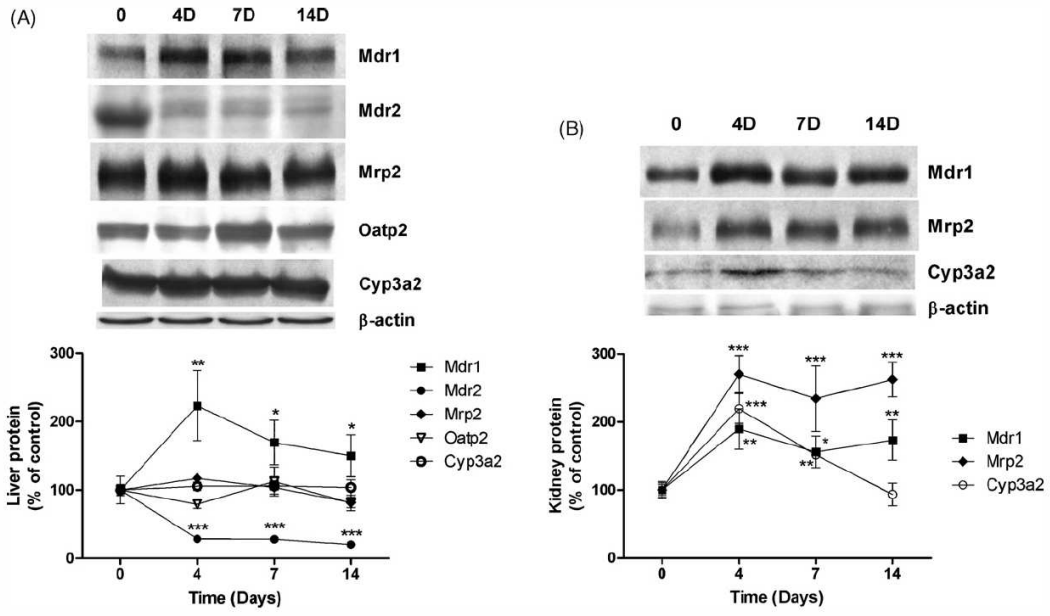

Fig. 1. Protein expression of Mdr1, Mrp2, Cyp3a2 in the liver (A) and kidneys (B) and Mdr2, Oatp2 in the liver (A) after 4-, 7-, and 14-day amiodarone pretreatment determined by Western blot. Expression (mean ± SD; $n = 6$) is presented as a relative optical density compared to the control animals ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

Table 1
Steady-state pharmacokinetics of Rho123 in amiodarone (4, 7, and 14 days) pretreated rats. Control groups (4, 7, and 14 days of oral PBS, $n = 6$ for each group) are presented together, because no significant difference was noted among them in any of evaluated parameter.

	Pretreatment control	Amiodarone		
		4 Days	7 Days	14 Days
Urine flow rate (μl/min)	9.4 ± 2.2	13.3 ± 2.2*	14.7 ± 2.9**	13.2 ± 1.4*
Urinary excretion rate (nmol/min/kg)	2.4 ± 0.6	3.7 ± 0.2	4.0 ± 1.6*	4.7 ± 0.6**
Bile flow rate (μl/min)	18.8 ± 2.4	19.6 ± 4.2	17.5 ± 3.2	20.6 ± 4.2
Biliary excretion rate (nmol/min/kg)	1.1 ± 0.2	1.8 ± 1.0	1.2 ± 0.3	1.9 ± 0.3*
Plasma (μM)	0.5 ± 0.05	0.6 ± 0.1	0.7 ± 0.2	0.7 ± 0.2
CL_R (ml/min/kg)	4.7 ± 1.1	6.1 ± 0.9	7.3 ± 1.6*	6.9 ± 1.9
CL_{Bile} (ml/min/kg)	2.0 ± 0.3	2.9 ± 1.3	1.8 ± 0.6	2.6 ± 0.4
CL_{TOT} (ml/min/kg)	34.2 ± 2.4	28.7 ± 4.6	28.2 ± 7.7	29.1 ± 8.9
CL_{CR} (ml/min/kg)	7.7 ± 2.1	8.9 ± 2.1	9.2 ± 1.2	9.7 ± 2.3

Values are shown as means ± SD ($n = 6$).
* $p < 0.05$.
** $p < 0.01$—significantly different from control groups.

Table 2
Plasma concentrations, biliary excretions and biliary clearances of amiodarone after its either single intravenous dose or repeat oral (4, 7, and 14 days) dose administration (25 mg/kg/day).

	Amiodarone i.v.	Amiodarone		
		4 Days	7 Days	14 Days
Plasma (μM)	1.3 ± 0.2	0.14 ± 0.09	0.17 ± 0.15	0.14 ± 0.08
Biliary excretion rate (pmol/min/kg)	92 ± 35	132 ± 97	109 ± 41	58 ± 17
CL_{Bile} (ml/min/kg)	0.07 ± 0.02	0.9 ± 0.3***	0.8 ± 0.5**	0.5 ± 0.3

Values are shown as means ± SD ($n = 6$).
** $p < 0.01$.
*** $p < 0.001$—significantly different from control groups.

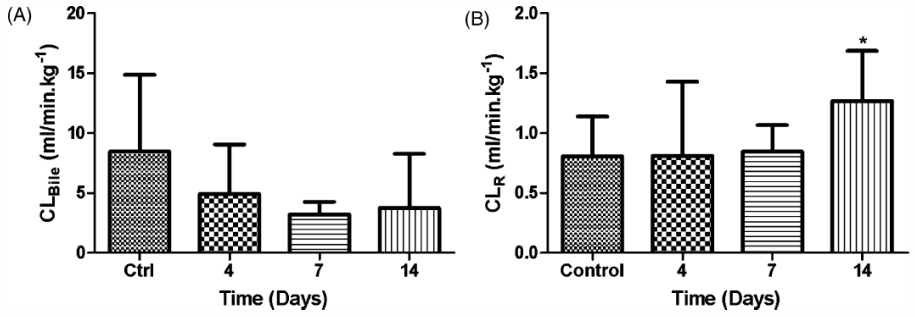

Fig. 2. Biliary (CL_{Bile}; A) and renal (CL_R; B) clearance of conjugated bilirubin in amiodarone (4, 7, and 14 days) pretreated rats. Bar chart presents means ± SD ($n = 6$); *$p < 0.05$—significantly different from control groups. Control groups (4, 7, and 14 days of oral PBS, $n = 6$ for each group) are presented together, because no significant difference was noted among them in any of evaluated parameter.

Fig. 2. Repeated administration of amiodarone produced progressive rise in CB renal clearance reaching the highest value on day 14. The biliary clearance of CB followed an inverse pattern, decreasing along the AMD treatment.

3.3. *Effects of amiodarone intravenous administration on kinetics of Rho123*

We investigated the effects of amiodarone (25 mg/kg) on the biliary and renal excretion of Rho123 under steady-state conditions during a continuous infusion, and the parameters are summarized in Table 3. Intravenous AMD reduced both biliary excretion and biliary clearance of Rho123 to 43% and 36% of control values, respectively, while renal elimination of the dye remained unaffected. The total clearance of Rho123 remained unchanged, however. In line with expectations, in the rats administered a bolus of known Mdr1 inhibitor, CsA, both biliary excretion and biliary clearance of Rho123 were significantly decreased compared to both control and amiodarone-administered groups (Table 3).

Table 3
Steady-state pharmacokinetics of Rho123 in amiodarone and cyclosporine bolus-administered rats.

	Control	Amiodarone	Cyclosporine
Urine flow rate (μl/min)	14.7 ± 4.2	16.9 ± 5.0	12.9 ± 5.7
Urinary excretion rate (nmol/min/kg)	5.7 ± 2.7	6.8 ± 1.6	3.8 ± 1.6
Bile flow rate (μl/min)	19.6 ± 2.3	17.4 ± 2.9	18.0 ± 3.4
Biliary excretion rate (nmol/min/kg)	2.1 ± 0.6	0.9 ± 0.3***	0.3 ± 0.07***
Plasma (μM)	0.7 ± 0.1	0.7 ± 0.05	0.7 ± 0.2
CL_R (ml/min/kg)	9.8 ± 3.8	10.2 ± 2.9	5.4 ± 2.2
CL_{Bile} (ml/min/kg)	3.6 ± 1.2	1.3 ± 0.4***	0.5 ± 0.2***
CL_{TOT} (ml/min/kg)	29.6 ± 5.7	26.2 ± 2.2	25.2 ± 4.8
CL_{CR} (ml/min/kg)	8.2 ± 0.8	11.5 ± 2.4*	7.3 ± 2.6

Values are shown as means ± SD ($n = 6$).
* $p < 0.05$.
*** $p < 0.001$—significantly different from control group.

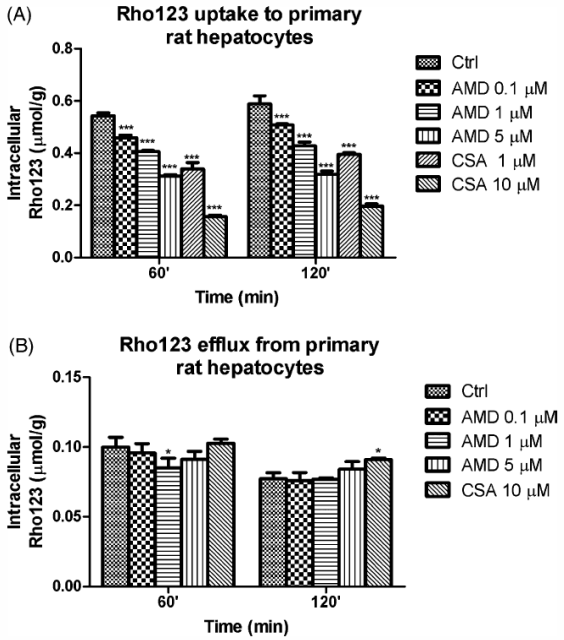

Fig. 3. Influence of amiodarone (0.1, 1, and 5 μM) and cyclosporine A (1 and 10 μM) on intracellular accumulation and efflux of Rho123 in primary rat hepatocytes after 60 and 120 min of incubation. Bar chart presents means ± SD ($n = 3$). Significantly different from control group ($^{*}p < 0.05$ and $^{***}p < 0.001$).

3.4. Rho123 transport in primary rat hepatocytes

To determine localization of AMD– and CsA–Rho123 interaction in the liver, accumulation and efflux study was performed using primary rat hepatocytes (Fig. 3). After 60 min of incubation, amiodarone showed a significant concentration-dependent decrease in Rho123 accumulation, and 5 μM amiodarone inhibited accumulation of the dye down to 58% of the control, which was comparable to the effects of CsA in 1 μM concentration. The incubation for 120 min resulted in similar but more pronounced effects. No clear effects of amiodarone were observed in the efflux study, while CsA significantly inhibited also the efflux of Rho123 from hepatocytes.

4. Discussion

The principal findings of this study relate to the induction effects of amiodarone (AMD), a widely used antiarrhythmic agent, on the expression and function of the main efflux drug transporters in the kidney, Mdr1 and Mrp2. The data indicate that 4–14 days AMD administration to rats is associated with constantly increased Mdr1 and Mrp2 protein expression with corresponding increased renal clearance of their model substrates, Rho123 and conjugated bilirubin. AMD pretreatment induced also Mdr1 expression in the liver but without significant influence on Rho123 biliary clearance. In contrast, acute AMD administration markedly reduced biliary clearance of Rho123 by blockade of its uptake to hepatocytes.

Induction of renal transporting protein expression, namely Mdr1, by its inhibitor has already been documented for CsA. This widely used immunosuppressive agent is a potent inhibitor of cytochrome P450 enzymes and Mdr1 transporter [18] as confirmed also by results of our study. However, repeated administration of CsA to patients with transplanted kidney results in increased expression of Mdr1 in the organ. Importantly, Mdr1 is not increased in patients with CsA nephrotoxicity. This indicates that CsA induces its own detoxification by Mdr1 and that inadequate up-regulation of the protein may contribute to the drug's nephrotoxicity [19,20]. These data have been confirmed by preclinical studies in rats [21,22]. Regarding AMD, expression changes of any transporting protein during AMD treatment has never yet been described. Therefore we can only scale our results to a drug with very similar pharmacokinetic profile such as CsA. We detected similar increase in Mdr1 expression, which was further associated with higher expression of another important efflux transporter, Mrp2. Although the presence of AMD blocks Mdr1 with the lowest reported IC_{50} being 5.48 μM, the plasma concentrations measured in our study were deeply below this value. This may explain the observed increase in renal excretion of Mdr1 and Mrp2 substrates, Rho123 and CB, respectively. In addition, similarly to inducing effects formerly reported for two Mdr1 inhibitors, CsA and ritonavir [12,13], we also demonstrated an increase in hepatic Mdr1 expression after AMD pretreatment. The up-regulation was associated with increased biliary elimination of the drug, which indicates that AMD, apart from inhibiting Mdr1 function by direct binding to the transporter, may augment its own elimination. To improve the accuracy of Mdr1 detection, we analyzed also the expression of hepatic Mdr2 canalicular transporter, which exclusively mediates biliary excretion of phospholipids [23]. Unique information about amiodarone induced down-regulation of this transporter and its role in the pathophysiology of phospholipidosis [24] induced in clinical practice by the antiarrhythmic needs further evaluation. Moreover, Mdr1 as one of the main xenobiotic transporting proteins tightly cooperates with the main xenobiotic-metabolizing cytochrome P450 isoform, Cyp3a2 (rat orthologue of human CYP3A4), and these two molecules share similar spectrum of substrates and regulatory mechanisms [25]. The liver expression of the enzyme was however not influenced by amiodarone pretreatment, so strong inhibitory potential of the drug against this isoform could be expected [4]. Amiodarone-induced transient increase in the kidney expression of Cyp3a2 must be interpreted in the view of its very low renal expression in comparison with hepatic tissue [26]. Thus the impact of this effect on overall metabolism of xenobiotics would be minimal.

Interesting information was a raised urine production in AMD-pretreated animals. Nevertheless, the systemic concentrations of amiodarone (Table 2) were well bellow the therapeutic limit (1–2.5 mg/L, i.e. 1.5–3.7 μM). Thus, only minimal systemic effect of amiodarone on heart rate and blood pressure could be expected. This complies with unchanged creatinine clearance, the parameter which is a reliable marker of glomerular filtration/kidney perfusion and rapidly drops during, e.g. hypotension. In agreement, recent reports demonstrated that significant reduction of blood pressure and heart rate with a subsequent reduction of glomerular filtration rate is achievable in rats only with amiodarone dose of 50 mg/kg given intravenously or intraperitoneally [27,28]. This may also explain why we have not observed the reduction of creatinine clearance after intravenous AMD administration. In contrast, increased urine production after oral pretreatment with AMD with unchanged creatinine clearance suggests that this increase may be associated with induction of tubular transporters. No such data are available for amiodarone yet, but we observed similar phenomenon when another Mdr1 inducing agent (dexamethasone) was applied to rats [14].

High potential of AMD to produce drug–drug interactions based on inhibition of cytochrome P450 enzymes has been demonstrated in numerous studies [5]. Moreover, its capability to increase plasma concentrations of digoxin, a poorly metabolized agent, has been known for a long time [29]. When digoxin was recognized as a

substrate of Mdr1 [30], and the ability of AMD to decrease multidrug resistance in tumors and to potentiate the intracellular accumulation of cytostatics [31,32] was demonstrated, inhibition of Mdr1 was suggested as the mechanism of this interaction. Consequent experiments on Mdr1-transfected cells confirmed the inhibitory influence of AMD on Mdr1 with IC_{50} within the range of 5.48–45.6 μM. Further studies however suggested that the main mechanism of AMD–digoxin interaction is a blockade of digoxin uptake into the cells at the level of basolateral transporter Oatp2. The model employed was transfected *Xenopus leavis* oocytes, where AMD inhibited transport of digoxin with K_i of 1.8 μM [11]. AMD affinity to Oatp2 in therapeutic concentrations was thus shown to be much higher than to Mdr1. Subsequent *in vivo* study showed an increase in digoxin plasma concentration after an intravenous bolus of AMD, as a result of impaired biliary and intestinal excretion of digoxin [10]. The determination of digoxin tissue concentrations indicated that the impairment in biliary excretion was a result of its decreased accumulation in hepatocytes, i.e. at the level of basolateral uptake rather than canalicular efflux.

Our results support the data that AMD-mediated interaction takes place rather at the basolateral membrane and not at the canalicular Mdr1 [10,11]. The addition of AMD to primary hepatocytes in our study resulted in a decrease in cellular uptake of the dye without significant influence on the efflux velocity. Moreover, *in vivo* study showed that Rho123 biliary excretion was not influenced by AMD pretreatment despite the fact that biliary (and thus intracellular) concentrations of AMD were similar to those observed during i.v. bolus administration experiments. Because AMD plasma concentrations were very low in pretreated animals (24 h after the last amiodarone dose), while therapeutic concentrations were attained in the bolus dose animals, we could anticipate that the main site of AMD inhibition of drug transport in the liver is basolateral membrane of hepatocytes. Indeed, using the same dose of amiodarone, and reaching identical plasma concentrations, we have recently demonstrated similar mechanism of alteration of methotrexate biliary elimination [16]. Thus, formerly reported inhibition of basolateral Oatp2 by amiodarone is suspected of this interaction. Nevertheless, the affinity of Rho123 to Oatp2 has not been evaluated yet, and further studies are needed for this Mdr1 model substrate.

In conclusion, the present findings indicate AMD capability to modify elimination routes of Rho123, showing an increase in renal excretion of the dye along the pretreatment with this potent antiarrhythmic agent. The expression of the main drug efflux transporters in the kidney, Mdr1 and Mrp2, suggested that this increase may be related to the up-regulation of the transporters. Induction of Mdr1 in liver may speed the elimination of AMD from the body. In contrast, the drug's plasma presence within the range of therapeutic concentrations resulted in marked decrease of Rho123 hepatic transport. As demonstrated during *in vitro* study, this effect originates from an inhibition of basolater uptake of the dye into hepatocytes. Therefore a combination of the described mechanisms may be considered as the cause of AMD-mediated drug–drug and drug–endogenous compound interactions.

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DEXAMETHASONE REDUCES METHOTREXATE BILIARY ELIMINATION AND POTENTIATES ITS HEPATOTOXICITY IN RATS

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Dexamethasone reduces methotrexate biliary elimination and potentiates its hepatotoxicity in rats

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ABSTRACT

Increased hepatotoxicity of methotrexate has been reported during dexamethasone therapy in humans. Despite the observed inducing effect of dexamethasone on some methotrexate transporting proteins in the liver, the kinetic aspects of this interaction have not been studied yet. Thus, the aim of the present study was to evaluate the influence of dexamethasone on the hepatic and overall pharmacokinetics of methotrexate. Pharmacokinetics of methotrexate was evaluated in rats during an *in vivo* steady-state clearance study after either single intravenous dose of dexamethasone or its four-day oral administration in a dose optimized for transport proteins induction. Dexamethasone oral pretreatment reduced biliary clearance of methotrexate by 53%. Although liver tissue concentration of methotrexate increased only slightly in these animals, a significant increase in liver weights produced by dexamethasone pretreatment revealed a marked increase in liver content of the drug. An evaluation of plasma liver enzyme activities measured before and after methotrexate administration demonstrated a potentiation of corticosteroid hepatotoxicity by the cytostatic. Analysis of methotrexate transporter expression in the liver showed up-regulation of Mrp2, Oatp1a4, and Oat2, and down-regulation of Mrp3. These observations comply with increased biliary excretion and reduced plasma concentrations of their endogenous substrate, conjugated bilirubin. In contrast, single intravenous bolus of dexamethasone did not influence any pharmacokinetic parameter of methotrexate. In conclusion, these results indicate that hepatocellular impairment associated with reduced biliary elimination of methotrexate, and its raised liver content may contribute to increased hepatotoxicity of the drug when co-administered with dexamethasone. Moreover, an influence of dexamethasone on protein expression of anionic drugs transporters in the liver and kidney was demonstrated.

1. Introduction

Methotrexate (MTX), a folate antimetabolite, is an anionic cytostatic drug commonly used in the therapy of many malignancies such as acute lymphoblastic leukemia, osteosarcoma, and head and neck tumors (Sterba et al., 2009; van Dalen and de Camargo, 2009). In these indications, the drug is applied in high-dose regimen, which may be associated with severe toxic reactions, particularly in those patients who attain higher plasma concentrations for prolonged period (Hansen et al., 1971; Jolivet et al., 1983). Administered via a long-term intravenous infusion, high-dose MTX steady-state plasma concentrations are primary dependent on the function of excretory pathways. In humans, the main route of MTX elimination is urinary excretion which takes 70–90% of the applied dose. The remaining part is mostly excreted into bile with some minor metabolism to 7-hydroxy MTX. The driving processes of MTX elimination are glomerular filtration in the kidneys and several active transport processes in hepatocytes and renal tubular cells (Grim et al., 2003).

The most important transporters for methotrexate kidney/liver elimination are the organic anion transporters (Oat1–3), organic anion transporting polypeptide 2 (Oatp2, Oatp1a4), members of the multidrug resistance-associated protein subfamily (Mrp2–4; Abcc2–4) and breast cancer resistance protein (Bcrp; Abcg2) (Badagnani et al., 2006; Borst et al., 2007; Masuda et al., 1997; Takeda et al., 2002; Takeuchi et al., 2001; Vlaming et al., 2009). The function of these transporters can be altered by various drugs, and serious drug–drug interactions may result from impairment of MTX elimination. Bone marrow suppression and acute renal failure have been described as a consequence of raised MTX plasma

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concentration during co-administration with inhibitors of renal and/or liver organic anion transporting proteins such as nonsteroidal anti-inflammatory drugs or probenecid (McLeod, 1998). Logically, induction of the transport pathways may enhance elimination of the drug and thus reduce the incidence of adverse events. However, co-administration of MTX with a potent inducer of some of these active transport pathways in the liver and kidneys, dexamethasone (Demeule et al., 1999; Luttringer et al., 2002; Micuda et al., 2008), did not alleviate the adverse events, but even increased the hepatotoxicity of the cytostatic (English et al., 1987; van Outryve et al., 2002; Wolff et al., 1998). The mechanism of this interaction is currently unknown.

The aim of the present study was to evaluate the effects of a single- or repeated-dose dexamethasone administration on methotrexate systemic, hepatic and renal kinetics in rats. As a reference inhibitor of organic anion transporters, probenecid was used to confirm the sensitivity of the *in vivo* model also to inhibition. Changes in the expression of the main methotrexate transporters (Mrp2–4, Bcrp, Oatp1a4, and Oat1–3) in the liver and kidney after dexamethasone pretreatment were evaluated using Western blot and qRT-PCR. In addition, kinetic parameters of a typical endogenous substrate of organic anion transporters, endogenous conjugated bilirubin, were evaluated in both control and dexamethasone-pretreated animals.

2. Materials and methods

2.1. Materials

Dexamethasone was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Lyophilized methotrexate for infusion was purchased from Ebewe Pharma, and the pure substance was a generous gift from Pliva-Lachema (Brno, Czech Republic). Mouse monoclonal antibody M_2III-5, directed to Mrp2 (170–190 kDa), and BXP-21 directed to Bcrp (70 kDa), were obtained from Signet Laboratories, Inc. (Dedham, MA, USA). Rabbit anti-Oatp1a4 (75 kDa) polyclonal antibody was obtained from Millipore Corporate Headquarters (Billerica, MA, USA). Mouse monoclonal antibody M_3II-21 directed to Mrp3 (180–190 kDa) was obtained from Alexis Corporation (Lausen, Switzerland). Anti-Mrp4 antibody was purchased from Abcam (Cambridge, UK). Oat1 and Oat2 monoclonal antibodies were purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA). As a loading control for Western blot, rabbit polyclonal β-actin antibody (42–45 kDa) was purchased from Sigma (Prague, Czech Republic). Horseradish peroxidase-linked sheep anti-mouse and donkey anti-rabbit immunoglobulin G were purchased from GE Healthcare (Prague, Czech Republic). All other reagents and supplies were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Bio-Rad (Hercules, CA, USA), respectively, and were of the highest purity available.

2.2. Animals and treatment

Male Wistar rats (Konarovice, Czech Republic) weighing 280–330 g were divided into four groups ($n = 6$ in each). One group received dexamethasone pretreatment (25 mg/kg daily) while the three remaining were applied with vehicle alone. Both regimens were applied for four days by stomach intubation. Dexamethasone dose used for pretreatments was selected as previously established optimum dose for hepatic transport protein induction and was within the range of dexamethasone doses used for this purpose (Chandra et al., 2005; Cherrington et al., 2002; Maher et al., 2005; Micuda et al., 2005). All animals were subjected to *in vivo* clearance studies 24 h after the last oral drug/vehicle application. Rats were housed under controlled environmental conditions (12-h light–dark cycle; temperature, 22 ± 1 °C) with a commercial food diet and freely available water. The study protocol was approved by the animal welfare committee of the Charles University in Prague, Faculty of Medicine in Hradec Kralove.

2.3. In vivo clearance study of methotrexate and bilirubin

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and cannulated with polyethylene tubes in the right jugular vein for drug administration, and the left carotid artery for blood sampling. The urinary bladder and bile duct were also cannulated for urine and bile collections. Body temperature of animals was maintained at 37 °C with a heat lamp. To evaluate the steady-state pharmacokinetic of methotrexate, all rats received a bolus intravenous injection of methotrexate at a loading dose of 8 μmol/kg followed by a constant-rate infusion (Perfusor Compact; Braun, Prague) of a 4% mannitol solution delivering dose of 10 μmol/kg of methotrexate/h at a rate of 2 ml/h until the end of the study. A 60-min infusion was found to result in a steady-state plasma concentration of methotrexate. The previously tested dosage was selected to obtain a methotrexate plasma concentration below reported K_m of basolateral (23 μM) and canalicular (300 μM) transport (Ueda et al., 2001). One untreated group of animals received a single intravenous bolus of dexamethasone (1 mg/kg – maximum daily dose in humans) 10 min before initiation of methotrexate application. Another group of untreated animals received, together with methotrexate, also an intravenous infusion of probenecid (200 μmol/kg/h) starting with a loading dose of 70 μmol/kg. Mannitol was always used as a constituent of the vehicle to obtain a constant and sufficient urine flow rate. After a 60-min infusion (attained steady-state), bile and urine were collected in preweighed tubes at 10-min intervals for 30 min. Blood samples were taken at the midpoint of the bile and urine collection periods. Plasma samples were obtained by centrifugation at 3000 × *g* for 10 min. The volume of bile and urine was measured gravimetrically, with specific gravity assumed to be 1.0. All plasma, bile, and urine samples were stored at −80 °C until analysis.

The protocol of pretreatment and *in vivo* study of bilirubin clearance was identical to that mentioned above, with the only exception that animals received an infusion of 4% mannitol only.

2.4. Immunoblot analysis

Crude membrane-containing homogenates were prepared from freshly harvested livers and kidneys as described previously (Micuda et al., 2007). Homogenates (50 μg) were incubated with sample buffer at room temperature for 30 min and separated on a 7.5% polyacrylamide gel. After the proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA), the membrane was blocked for 1 h at room temperature with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TTBS). The membrane was then incubated with primary antibodies (1:500) for 1 h, washed, and incubated for 1 h with a peroxidase-conjugated secondary antibody (1:1000). After washing five times with TTBS, the membranes were developed using enhanced chemiluminescent reagent (GE Healthcare, Prague, CZ) and subjected to autoluminography for 1–5 min. The immunoreactive bands on the exposed films were scanned with a densitometer ScanMaker i900 (UMAX, Prague, CZ) and semiquantified using the QuantityOne imaging software (Bio-Rad).

2.5. Examination of transporter gene expression by qRT-PCR

RNA was isolated from liver and kidney tissue samples using TRIzol reagent (Invitrogen, USA) and converted into cDNA via an iScript reverse transcription kit (Bio-Rad Laboratories, Hercules, USA). Ten nanograms of cDNA were loaded into one reaction, performed in triplicate. The amplification was completed using the TaqMan® Fast Universal PCR Master Mix and pre-designed TaqMan® Gene Expression Assay kit for the following genes: Mrp2 (Abcc2, Rn00563231_m1), Mrp3 (Abcc3, Rn00589786_m1), Mrp4 (Abcc4, Rn01465702_m1), Bcrp (Abcg2, Rn00710585_m1), Oatp1a4 (Slco1a4, Rn00756233_m1), Oat1 (Slc22a6 Rn00568143_m1), Oat2 (Slc22a7, Rn00585513_m1), Oat3 (Slc22a8, Rn00580082_m1). Rat GAPDH (P/N 4308313) and Eukaryotic 18S rRNA (P/N4319413E) endogenous control kits were used as housekeeping genes. All pre-made assays were provided by Applied Biosystems (Foster City, USA). The time–temperature profile used in the "fast" mode was: 95 °C for 20 s; 40 times: 95 °C for 3 s, 60 °C for 30 s, and run on 7500HT Fast Real-Time PCR System (Applied Biosystems, Foster City, USA). The relative expression ratio (R) was then calculated according to Pfaffl (2001):

$$R = \frac{E_{\text{target}}^{\Delta Ct(\text{control}-\text{sample})/t_{\text{target}}}}{E_{\text{housekeeping}}^{\Delta Ct(\text{control}-\text{sample})/t_{\text{housekeeping}}}}$$

where E_{target} and $E_{\text{housekeeping}}$ are the effectivity values determined from the calibration curve slopes for each gene, $\Delta Ct_{\text{target}}$ and $\Delta Ct_{\text{housekeeping}}$ are the differences in threshold values (Ct) between control and pretreated (dexamethasone) sample for each of the genes, i.e. target and housekeeping.

2.6. Analytical procedures

The concentrations of methotrexate in the liver homogenate, plasma, urine and bile were determined by HPLC after deproteination of samples according to a previously described method (Fuksa et al., 2008). Briefly, the instrument was an Agilent 1100 series (Agilent, Palo Alto, USA) chromatograph provided with a fluorescence detector (excitation, 350 nm; emission 430 nm). Separation was achieved at 30 °C using a Gemini C18, 110A, 4.6 × 150 mm column and Gemini C18, 4 mm × 3 mm pre-column (Phenomenex, Torrance, USA). The mobile phase, flowing at a rate of 0.6 ml/min, consisted of ammonium acetate and acetonitrile (87:13, v/v). The concentrations of bilirubin and creatinine in plasma and urine were measured on a Cobas Integra® 800 (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. This instrument was also used for determination of liver enzyme activities (ALT, AST), cholesterol and triglyceride concentration in plasma.

2.7. Protein binding experiments

The plasma protein binding of methotrexate was evaluated by ultrafiltration through Ultrafree-MC Centrifugal Filter Units (Millipore, Billerica, MA, USA). Four

Table 1
Changes in steady-state pharmacokinetic parameters of methotrexate after either single intravenous dose (1 mg/kg) or repeated four-day oral (25 mg/kg/day) administration of dexamethasone. Probenecid, a reference inhibitor of organic anion transporters, was applied intravenously to a separate group of rats.

MTX parameters	Control	Dexamethasone oral	Dexamethasone i.v.	Probenecid i.v.
Systemic				
C_{ss} (μM)	9.3 ± 0.3	13 ± 1.6*	10 ± 0.3	19 ± 1.4***
CL_{TOT} (ml/min/kg)	22 ± 0.8	17 ± 1.6*	19 ± 0.6	11 ± 0.8***
Biliary elimination				
Bile flow rate (μl/min)	24 ± 1.0	23 ± 1.0	23 ± 0.8	28 ± 5.0
Biliary excretion rate (nmol/min/kg)	107 ± 3.1	89 ± 6.2*	118 ± 3.2	89 ± 15
CL_{Bile} (ml/min/kg)	12 ± 0.4	7.4 ± 1.0*	11 ± 0.5	5.1 ± 0.9***
Biliary excretion rate (nmol/min/g of liver)	2.5 ± 0.2	1.3 ± 0.1*	2.9 ± 0.1	2.2 ± 0.5
$CL_{Bile/Ctissue}$(ml/min/g of liver tissue)	0.17 ± 0.02	0.08 ± 0.01*	0.18 ± 0.01	0.14 ± 0.04
Liver weight (g)	15 ± 0.4	19 ± 0.8***	14 ± 0.5	13 ± 0.3
Liver concentration (μM)	15 ± 1.4	17 ± 1.3	17 ± 1.2	19 ± 3.5
Liver content of MTX (nmol)	224 ± 19	324 ± 32*	238 ± 13	256 ± 43
Renal elimination				
Urine flow rate (μl/min)	11 ± 1.0	40 ± 9.0**	14 ± 1.4	10 ± 0.5
Urinary excretion rate (nmol/min/kg)	62 ± 3.6	86 ± 7.4*	71 ± 5.0	79 ± 6.4
GFR (ml/min/kg)	5.9 ± 0.7	3.7 ± 0.4	4.7 ± 0.5	4.1 ± 0.6
CL_R (ml/min/kg)	6.7 ± 0.5	7.4 ± 1.0	6.8 ± 0.5	4.4 ± 0.6*
CL_R/GFR	1.2 ± 0.1	2.0 ± 0.3*	1.7 ± 0.4	1.2 ± 0.2
f_U	0.9 ± 0.01	0.8 ± 0.01	0.8 ± 0.01	0.9 ± 0.01
CL_{RS}	1.9 ± 0.4	5.0 ± 1.2*	3.9 ± 1.1	0.9 ± 0.7

Values are presented as means ± S.E.M. in groups of 6 animals.
* Significantly different from control values $P < 0.05$.
** Significantly different from control values $P < 0.01$.
*** Significantly different from control values $P < 0.001$.

hundred microliters of plasma samples from control and pretreated animals were dialyzed at 3000 × *g* for 20 min. As a standard for recovery comparison, a pH 7.4 phosphate-buffered saline solution containing 10 μM of methotrexate was dialyzed under the same conditions. This methotrexate concentration was chosen based on the data obtained during *in vivo* experiments. Concentrations on both sides of the membrane were measured by HPLC as described above. Tissue concentrations of methotrexate were measured in liver homogenates diluted serially with PBS (40%, 20%, 10%, and 5%), which were upon measurement extrapolated to 100%. Each homogenate (50 μl) was deproteinized with 200 μl of acetonitrile and centrifuged at 6000 × *g* for 10 min. The resulting supernatant (200 μl) was evaporated to dryness under a nitrogen gas stream at 45 °C. The residue was reconstituted with 200 μl of the mobile phase and injected into the HPLC system. This assay was shown to be linear for the concentrations tested, with correlation coefficients of >0.99. No interference with the peak of methotrexate was observed in any sample.

2.8. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated using MS Excel software (Microsoft Corporation). Total plasma clearance (CL_{TOT}) of methotrexate was estimated by dividing the constant infusion rate by the steady-state concentration in plasma (C_{ss}). Biliary and renal clearance (CL_{Bile} and CL_R) of methotrexate during each collection period was calculated by dividing the respective excretion rate by the C_{ss} determined for that collection period. In addition, tissue biliary clearance ($CL_{Bile/Ctissue}$) was also calculated using biliary excretion rate normalized to liver weight and liver concentrations of MTX (C_{tissue}) instead of C_{ss}. Glomerular filtration rate (GFR) was measured by clearance of endogenous creatinine. The renal clearance ratio of methotrexate was calculated as CL_R/GFR. The renal tubular secretion clearance (CL_{RS}) for unbound drug was calculated as CL_R/f_U minus GFR, where f_U represents the unbound fraction of the drug. Each parameter was calculated using the mean value of three points during 30 min of steady state.

2.9. Statistical analysis

Experiments were carried out in 6 animals per group. All experimental data are expressed as mean ± S.E.M. Statistical significance was examined by unpaired *t*-test or ANOVA followed by Tukey's post-hoc test using Graphpad Prism 5.0 software (Graphpad Software, San Diego, USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Clearance study with methotrexate

Detailed analysis of methotrexate pharmacokinetics is presented in Table 1. Dexamethasone four-day oral pretreatment produced a 33% reduction of MTX total clearance, which was associated with corresponding increase in its plasma concentrations. Detailed analysis of MTX excretory parameters demonstrated a reduction of its biliary clearance to 62% of control values. This reduction was more marked (to 47%), when the biliary clearance was calculated using tissue concentrations of the drug and the biliary excretion rates related to weight of the liver, which was markedly increased by dexamethasone (Table 1). Importantly, the intrahepatic concentrations of methotrexate slightly increased, and the following correction to dexamethasone-induced hepatomegaly pointed to a significant rise in the liver content of the drug. Regarding renal clearance of methotrexate, it predictably accounted for 30% of the total clearance in control rats. Despite the fact that this parameter remained unaffected by dexamethasone pretreatment, we detected a slight decrease in glomerular filtration rate, which was compensated by a significant increase in tubular secretion of the drug. In contrast to the pretreatment, acute bolus of dexamethasone had no effect on any of the evaluated parameters of methotrexate kinetics. To further verify the sensitivity of the *in vivo* model to co-administration of a potentially interacting drug, we applied also probenecid, a proven inhibitor of MTX elimination. This antiuratic drug significantly reduced both biliary and renal clearance of methotrexate to 43% and 66% of control values, respectively.

3.2. Biochemical parameters

To assess the potential hepatotoxicity of dexamethasone and methotrexate co-administration we measured activities of ALT and AST in plasma samples taken before and 90 min after initiation of MTX administration (Fig. 1A and B). Dexamethasone pretreatment alone was associated with increased ALT and AST activities to 2140% and 1240% of control values, respectively. High-dose MTX administration for 90 min produced a further increase of both liver enzyme activities only in dexamethasone-pretreated groups. Furthermore, dexamethasone pretreatment was associated with increased cholesterol and TAG levels, which tended to decrease upon MTX administration (Fig. 1C and D).

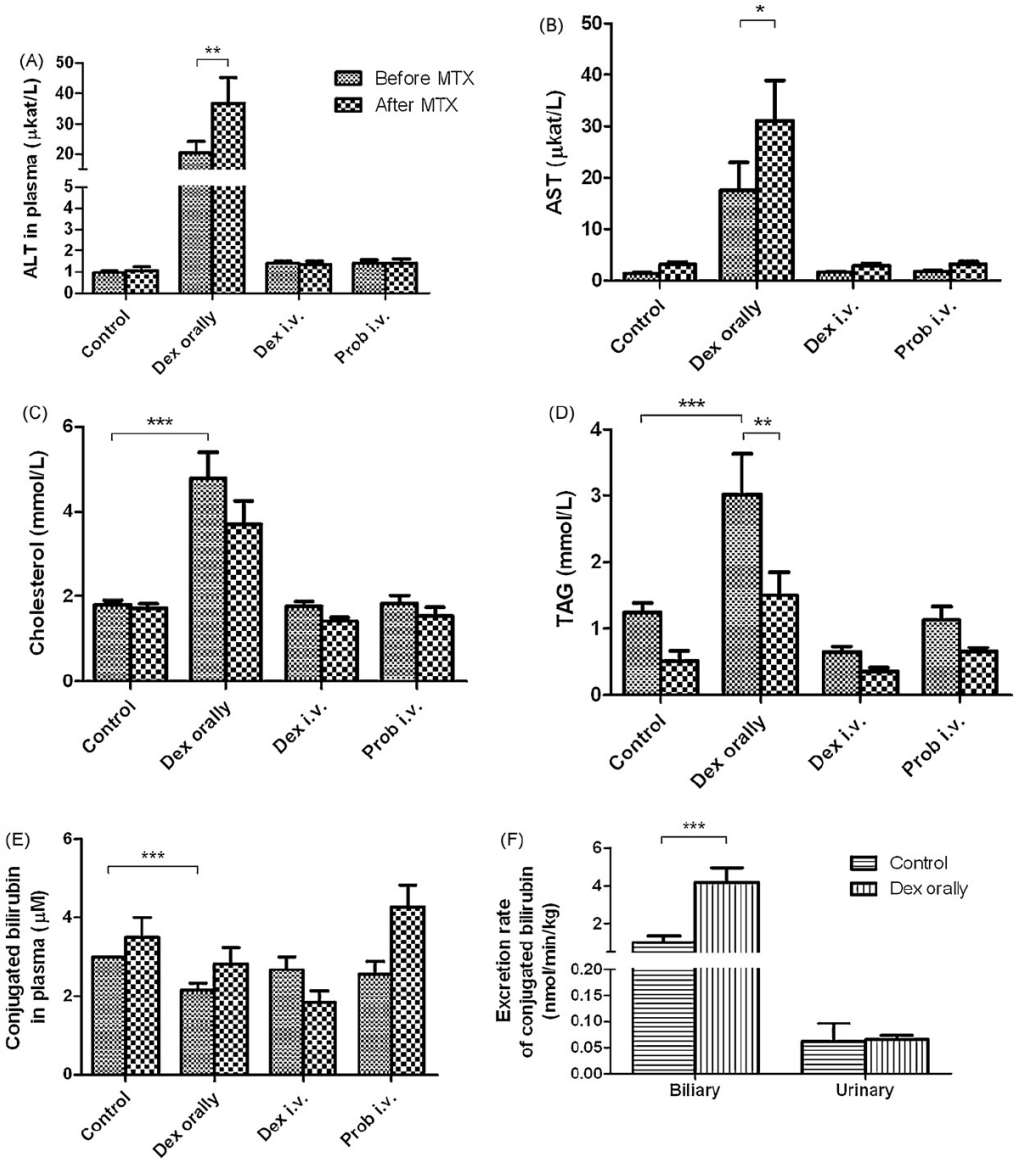

Fig. 1. Selected serum biochemical parameters in control, and dexamethasone (oral: 25 mg/kg/day or i.v.: 1 mg/kg) or probenecid (i.v.) applied rats. Changes in biliary and urinary excretion rates of conjugated bilirubin after dexamethasone oral pretreatment are depicted in part F. Values are means ± SEM ($n = 6$, in each group). Statistical significance of the difference within the same group before and 90 min after the start of MTX infusion or between the experimental and control group is marked as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3.3. Conjugated bilirubin

Renal and biliary excretion of conjugated bilirubin, a prototype endogenous substrate for transporters of anionic compounds, was evaluated to further describe the influence of potent corticosteroids on these pathways. We observed a rise in the biliary excretion rate in dexamethasone-pretreated animals to 422% of control values which was associated with decreased plasma concentrations of the substance (Fig. 1E and F).

3.4. Expression of MTX transporters

Protein expression, evaluated by Western blot assay, is presented in Table 2 and Fig. 2. In the liver, the amount of Mrp2 increased to 220%, and in the kidneys to 179%. Mrp3 expression decreased to 39% of the control values in the liver and was unchanged in the kidneys. Mrp4 protein remained stable in the liver, while in the kidney it decreased to 31%. Oatp1a4 expression (detected only in the liver) significantly increased to 856% of the

Table 2
Relative expression of principal hepatic and renal MTX transporters after dexamethasone pretreatment (25 mg/kg/day orally for four days).

Gene symbol	Protein synonym	% of control values							
		Liver				Kidney			
		mRNA		Protein		mRNA		Protein	
Abcc2	Mrp2	↓	54 ± 6.6***	↑	220 ± 13**	↓	36 ± 8.2*	↑	179 ± 8.9**
Abcc3	Mrp3	↓	21 ± 10**	↓	39 ± 2.9**	↓	32 ± 8.3**		102 ± 18
Abcc4	Mrp4	↓	55 ± 11**		98 ± 11		64 ± 16	↓	31 ± 5.4*
Abcg2	Bcrp	↓	30 ± 7.3**		89 ± 14		48 ± 19	↓	79 ± 6.4*
Slco1a4	Oatp1a4	↑	340 ± 55**	↑	856 ± 61***		NP		NP
Slc22a6	Oat1		NP		NP		76 ± 13		74 ± 0.7
Slc22a7	Oat2		83 ± 5.9	↑	128 ± 3.1*	↓	13 ± 3.7*		119 ± 27
Slc22a8	Oat3	↓	49 ± 9.1*		ND		90 ± 39		ND

Values are means ± S.E.M ($n = 6$, in each group). NP: not present, ND: not defined (functional antibody was not available yet).
Arrows highlight whether the significant change in expression compared to control values represents an increase (↑) or decrease (↓) in the amount of mRNA or protein.
* Significantly different from control values $P < 0.05$.
** Significantly different from control values $P < 0.01$.
*** Significantly different from control values $P < 0.001$.

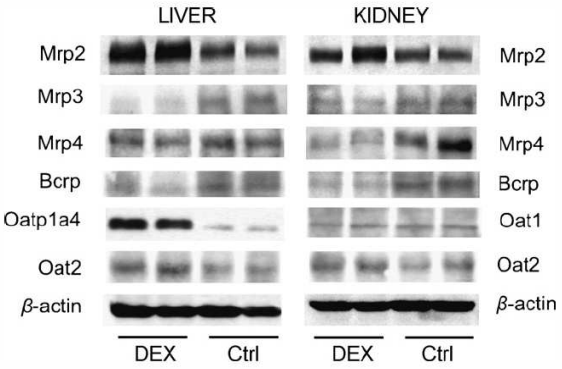

Fig. 2. Protein expression of methotrexate transporters in the liver and kidneys of rats pretreated for four days orally by either dexamethasone (25 mg/kg/day) or vehicle alone—representative Western blots of all evaluated transporters are shown.

control level, while Bcrp expression was unchanged in the liver and decreased to 79% in the kidneys. Of the organic anion transporters (Oats), only Oat2 in the liver was found to be slightly higher (128%) than control values.

Relative gene expression, evaluated by qRT-PCR, is presented in Table 2. In the liver, a significant decrease was found in canalicular transporters at the mRNA level; Mrp2 decreased to 54% and Bcrp to 30% compared to the control group. The expression of basolaterally located ABC transporters Mrp3 and Mrp4 was also decreased to 21% and 55%, respectively. In contrast, uptake transporter Oatp1a4 mRNA was up-regulated to 340% of the control values. In the kidneys, all examined transporter genes were down-regulated, Mrp2, Mrp3 and Oat2 significantly to 36%, 32% and 13%, respectively.

4. Discussion

In the present study, we investigated the *in vivo* effects of either single- or repeated-dose administration of dexamethasone on the steady-state pharmacokinetics of methotrexate in rats. Daily oral administration of dexamethasone for four days produced a significant decrease in methotrexate total clearance due to the reduction of its biliary elimination. Enlarged liver with slightly increased intracellular concentrations of methotrexate resulted in increased liver content of the cytostatic in the dexamethasone-pretreated animals. This effect was associated with increased plasma activities of liver enzymes after methotrexate administration in dexamethasone-pretreated rats. The expression of most methotrexate transporters in both the liver and kidneys was impaired by repeated dexamethasone doses. In contrast, no effect on the methotrexate pharmacokinetics was seen immediately after a single dexamethasone administration.

The elimination of intravenously administered methotrexate in rats is preferentially accomplished via the biliary route and only about one third of the applied dose is excreted into urine (Chen et al., 2003; Ueda et al., 2001). In humans, the situation is opposite, and up to 70–90% of the applied methotrexate is eliminated in urine (Grim et al., 2003). Nevertheless, evaluation of the biliary elimination revealed a marked enterohepatic circulation of the drug in patients, so the actual biliary excretion rate may be even equal to urinary excretion rate (Hendel and Brodthagen, 1984). Therefore, an impairment of either pathway may gain a high clinical significance due to accumulation of methotrexate in the organism and implying raised toxicity. Such alterations typically originate from a blockade of methotrexate transporting proteins by a simultaneously applied anionic drug, e.g. NSAID or probenecid (Aherne et al., 1978; Brouwers and de Smet, 1994). However, a potentiation of methotrexate toxicity has been reported also by an inducer of such transporting pathways, dexamethasone (English et al., 1987; Wolff et al., 1998). Because the pharmacokinetic consequences of this interaction have been unknown, we pretreated the experimental animals using the dosage of dexamethasone previously optimized for hepatic transporter induction (Micuda et al., 2005), and performed a detailed kinetic study with methotrexate. The pretreatment was indeed associated with an impairment in the biliary excretion of the cytostatic. Pharmacokinetic analysis showed that the biliary excretion of the drug is reduced while its tissue concentrations are slightly raised, which may suggest a problem at the level of the intracellular drug distribution and/or canalicular biliary export. The observed up-regulation of the main canalicular methotrexate protein, Mrp2 (Masuda et al., 1997), and steady levels of the second one, Bcrp (Vlaming et al., 2009), pointed to an alteration of intracellular distribution of methotrexate during corticosteroid therapy. A direct blockade of participating canalicular transporters by dexamethasone is ruled out by the observation that when given as an intravenous bolus, dexamethasone did not change the pharmacokinetic parameters of methotrexate. At the same time, dexamethasone-produced increase in Mrp2 protein expression was associated with an increase in the biliary excretion of its prototype substrate, conjugated bilirubin (Kamisako et al., 1999), and consequently, with a reduction in its plasma concentration, which proved that the functional capacity of Mrp2 during induction was raised. Other important methotrexate transporters were changed differently. The protein expression of liver basolateral efflux transporter Mrp3 was reduced, while the bidirectional

basolateral transporters Oatp1a4 and Oat2 were up-regulated. This may suggest an increased exchange of their substrates between plasma and intracellular space.

Corticosteroids act typically as gene transcription regulators (Czock et al., 2005). Our comparison of transporter protein expression with gene expression data comply with this mechanism. Dexamethasone interacts with glucocorticoid receptor (GR) and pregnane X receptor (PXR) which control transcription of xenobiotic metabolizing enzyme and transporter genes in excretory organs (Pascussi et al., 2001; Pavek and Dvorak, 2008). In contrast, some discrepancies were observed in our study between mRNA and protein level of the key transporter Mrp2. This may be a consequence of its complex regulation with a significant contribution of post-transcriptional mechanisms as suggested previously (Jones et al., 2005; Kubitz et al., 1999).

Hepatotoxicity is a common adverse effect of the therapy with methotrexate. Liver damage may develop immediately after methotrexate administration, especially if given in a high-dose regimen, and is characterized by a transient elevation of liver enzyme levels or hyperbilirubinaemia (Perez et al., 1979). Chronic therapy with the drug may also induce microvesicular steatosis, portal tract inflammation, focal liver cell necrosis, and fibrosis which resembles non-alcoholic steatohepatitis (Langman et al., 2001; van Outryve et al., 2002). Our data indicate acute elevation of liver enzymes immediately after methotrexate high-dose administration to dexamethasone-pretreated animals. Simple acute co-administration of both compounds did not produce such changes indicating necessity of primary steroid-induced cellular damage for potentiation of the hepatotoxicity of methotrexate. Indeed, dexamethasone pretreatment alone was apparently toxic to the hepatic parenchyme. As suggested by the increase in the liver weight and by other previous reports (Iancu et al., 1986; Micuda et al., 2007; Thatcher and Caldwell, 1994), the corticosteroid is capable to induce a severe hepatic steatosis in periportal areas of the liver acinus as a consequence of an impaired peroxisomal and mitochondrial β-oxidation of fatty acids. Recently, Lee et al. (2008) demonstrated this action also for methotrexate, which indicates a common pathway for the liver damage incurred by both drugs. In agreement, non-alcoholic fatty liver disease is one of the main predisposing factors for methotrexate hepatotoxicity and vice-versa (Lee et al., 2008; van Outryve et al., 2002). In this situation, the observed increased liver content of methotrexate during corticosteroid therapy may also contribute to the tissue damage.

In conclusion, this work provides a detailed analysis of methotrexate pharmacokinetics in rats after both single- and repeated-dose administration of dexamethasone. Whereas the acute administration had no effect on methotrexate pharmacokinetics, the repeated administration of the corticosteroid produced a decrease in biliary excretion of the cytostatic, which was associated with its increased plasma concentrations. These data were contradictory to the observed up-regulation of the principal basolateral and canalicular methotrexate transporters. This indicates an alteration in the liver intracellular sequestration of methotrexate, which results in increased liver content of the drug. This effect, together with dexamethasone-induced liver damage, may predispose to the observed acute hepatotoxicity of methotrexate.

Conflict of interest statement

None declared.

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SOUHRN

Aktivní transmembránový transport je významným faktorem zasahujícím do farmakokinetiky léčiv, a to buď usnadněním nebo naopak zabráněním v přestupu léčiv přes fyziologické bariéry nebo mezi různými kompartmenty. Membránové transportéry tak mají vedle enzymů podstatný vliv na osud léčiva v organizmu. Spektrum a míra exprese transportérů a jejich lokalizace na cytoplazmatických membránách buněk pak ovlivňuje průběh základních farmakokinetických dějů absorpce, distribuce, metabolizmu a exkrece léčiva. Nicméně aktivita transportních proteinů může být modifikována řadou vlivů, mj. současně podávanými léčivy. Kombinace léčiv, kde jedno moduluje transport dalšího, tak může podmínit změny farmakokinetiky a následně i farmakodynamiky, dochází k lékové interakci.

Studie popsané v předložené dizertační práci se věnovaly bližšímu popisu léčivy navozených změn exprese a funkce hlavních transportních proteinů v játrech a ledvinách a jejich vlivu na farmakokinetiku modelových substrátů. Pozornost byla soustředěna především na ABC efluxní transportéry (konkrétně P-gp, Mrp2) lokalizované na apikálních membránách buněk exkrečních orgánů, a Oatp2 transportér důležitý pro vstup léčiv do buněk přes bazolaterální membránu. Použitými léčivy pro bližší popis změn v aktivním transportu byly dexamethason a amiodaron. Dexamethason je velmi silný syntetický kortikosteroid, který *in vitro* i *in vivo* prokázal významný indukční vliv na eliminační procesy, tj. biotransformační enzymy i transportéry. Naopak amiodaron, život zachraňující antiarytmikum, je známým inhibitorem metabolizmu léčiv a recentně byl popsán jeho přímý inhibiční vliv i na aktivní transportéry.

Bylo pozorováno, že podávání dexamethasonu stimuluje celkovou clearance rhodaminu-123, modelového substrátu P-gp, zvýšením biliární i močové exkrece. Na molekulární úrovni byla potvrzena zvýšená genová exprese i zvýšené množství proteinu na membráně. Detailní kvantitativní imunohistochemická analýza vzorků jaterní tkáně ukázala, že P-gp je primárně exprimován především v periportálně lokalizovaných hepatocytech jaterního acinu, přičemž relativní nárůst exprese během podávání dexamethasonu je významně vyšší v perivenózní zóně. Současně jsme popsali jaterní steatózu navozenou podáváním dexamethasonu – histologicky prokázanou především v periportálních buňkách.

Obdobně jako u P-gp, i u Mrp2 transportéru jsme za použití stejné metody kvantitativně popsali fyziologickou lokalizaci především v periportální oblasti a významný relativní nárůst směrem k uniformní expresi v celém acinu při podávání steroidu. Zvýšení exprese Mrp2 po podání dexamethasonu bylo prokázáno i analýzou exprese proteinu

a zvýšením biliární exkrece konjugovaného bilirubinu – endogenního substrátu tohoto transportéru.

V další studii jsme pozorovali výraznou modifikaci kinetiky metotrexátu, široce používaného antirevmatika a antineoplastika, za současného podání amiodaronu. Po aplikaci amiodaronu v dávce navozující plazmatické koncentrace identické s terapeutickými u člověka bylo pozorováno podstatné snížení biliární (a korespondující pokles celkové) clearance za současného významného nárůstu plazmatické koncentrace metotrexátu. Uvedená pozorování, byť sotva přímo přenositelná na situaci podávání u člověka, poukazují na možnou klinickou interakci a zvýšené riziko projevů toxicity metotrexátu. Důsledek interakce, především hematologická toxicita, byl již ve formě kazuistik popsán v klinické praxi u nemocných s psoriázou. Dále jsme na obdobném modelu zkoumali vliv makrolidového antibiotika azitromycinu na kinetiku metotrexátu, avšak nepozorovali jsme žádné významné změny. Obě léčiva jsou substráty Mrp2 proteinu, nicméně jejich současné podání nevedlo k modifikaci kinetických parametrů žádného z nich oproti samostatnému podání.

Antiarytmikum amiodaron bylo použito i v další studii, kde jsme blíže zkoumali jeho vliv na eliminaci rhodaminu-123. Již dříve bylo popsáno, že amiodaron je inhibitorem funkce P gp, avšak význam interakce v klinické praxi není zcela objasněn. Provedený *in vivo* experiment potvrdil teoretický předpoklad, že akutní intravenózní aplikace amiodaronu významně sníží biliární exkreci rhodaminu-123. Současně provedená *in vitro* transportní studie s primárními hepatocyty blíže osvětlila mechanizmus *in vivo* pozorovaného snížení jaterní eliminace, kdy konkrétním místem blokády transportu není eflux, tj. kanalikulární membrána s ABC transportéry (P-gp), ale naopak vychytávání léčiva a jeho přestup bazolaterální membránou. Opakované podání antiarytmika vedlo ke zvýšení renální eliminace substrátu v závislosti na době podávání. Z výsledků analýzy exprese transportních proteinů vyplynulo, že se současně zvýšilo množství efluxních P-gp a Mrp2 v ledvinách. Rovněž bylo pozorováno, že opakované podávání inhibitoru vedlo k indukci exprese P-gp i v játrech.

Cílem poslední studie bylo zhodnotit vliv dexamethasonu na jaterní eliminaci a celkovou kinetiku metotrexátu. Farmakokinetické parametry metotrexátu byly hodnoceny u potkanů v průběhu *in vivo* studie za ustálených koncentrací po aplikaci dexamethasonu v různých dávkovacích schématech. Předléčba vedla k významné redukci biliární clearance metotrexátu. Současně bylo pozorováno zvětšení jater spojené s výrazně zvýšenou kumulací metotrexátu v játrech. Analýza plazmatické aktivity jaterních enzymů

provedená před a po podání metotrexátu ukázala potenciaci jaterní toxicity navozené předléčbou samotným kortikosteroidem. Výsledky této práce poukazují na hepatocelulární změny navozené narušením biliární eliminace metotrexátu. Zvýšení jaterní kumulace tohoto cytostatika může přispívat ke zvýšení jeho hepatotoxicity za současného podávání dexamethasonu. Dále byly *in vivo* popsány významné změny v expresi transportních proteinů pro organické anionty v játrech i ledvinách navozené podáváním dexamethasonu.

Shrnuté výsledky uvedených prací popisují aspekty farmakokinetických interakcí, kdy konkrétním mechanizmem je modulace (ve smyslu zvýšení či snížení) aktivního transportu léčiva způsobená podáním léčiva jiného. Naše výsledky také zdůrazňují význam *in vivo* studií při hodnocení vlivu exogenních látek na aktivitu transportérů v eliminačních orgánech.

SUMMARY

Active transport is a substantial factor affecting drug pharmacokinetics by either facilitating or hampering the penetration of the drug through physiological barriers or between different compartments. Thus the membrane transporters exert, along with enzymes of biotransformation, significant influence on the disposition of the drug in the organism. The spectrum and extent of transporter expression and their localization at the cytoplazmic membranes consequently affect the principal pharmacokinetic processes, i.e., absorption, distribution, metabolism, and excretion. However, the activity of the transport proteins can be modified by many factors including concomitantly administered drugs. Combination of drugs in which one modulates the transport of the other can bring about a drug-drug interaction resulting in profound changes in pharmacokinetics and subsequently pharmacodynamic effects.

The studies included and discussed in the present thesis focused on closer research of drug-induced changes in the expression and function of the main hepatic and renal transporters and their effects on the pharmacokinetics of the model substrates. The subject of our particular interest were ABC efflux transporters (namely P-gp and Mrp2) localized to the apical membranes of polarized epithelium cells in the excretory organs, and also Oatp2 transporter playing important role in the basolateral uptake of drugs. Dexamethasone and amiodarone were employed to bring about changes in the active transport. Dexamethasone is a potent corticosteroid that showed capability to increase elimination processes, i.e. to induce enzymes and transporters both *in vitro* and *in vivo*. Amiodarone, a life-saving antiarrhythmic, is a well-known inhibitor of drug metabolism. Its direct inhibitory effects on the active transport have recently been reported.

Our data showed that dexamethasone treatment stimulates the total clearance of rhodamine-123, a model substrate of P-gp, by increasing its biliary and urinary excretion. Investigating the changes at the molecular level, we observed increased gene expression, i.e. higher amount of both protein and mRNA. a detailed quantitative immunohistochemical sample analysis subsequently showed that P-gp is primarily expressed in hepatocytes localized periportally within the liver acinus. The relative rise in its expression during dexamethasone treatment was, however, significantly higher in the perivenous zone. Dexamethasone treatment also produced liver steatosis – histologically proven especially in the periportal hepatocytes.

Similarly to P-gp, we described the physiological localization of Mrp2 preferentially in periportal areas using identical methodology. Dexamethasone treatment produced (again as in P-gp induction) a relative rise of the protein amount towards

a uniform expression throughout the acinus. Significant induction of Mrp2 activity was confirmed also by protein expression analysis using Western blotting and by an *in vivo* study, where we detected an increase in the biliary excretion of conjugated bilirubin – an endogenous substrate of the transporter.

Our next study demonstrated marked amiodarone-induced modification in the kinetics of methotrexate, a widely used antirheumatic and antineoplastic agent. After amiodarone administration at a dose producing plasma concentrations identical to those observed in clinical practice we observed a substantial drop in biliary (and correspondingly in total) clearance accompanied by significant rise in steady-state plasma concentration. These data, although not directly transferrable to clinical situation in human, point to a possibility of a clinically important interaction leading to a higher incidence of methotrexate toxicity. Such adverse events, especially myelosuppression, have already been reported in case reports in psoriatic patients. Using the same model we further investigated the influence of azithromycin, a macrolide antibiotic, on methotrexate kinetics. Both drugs are Mrp2 substrates. Concomitant application of azithromycin and methotrexate did not change any of the kinetic parameters of either drug when compared to their separate administrations.

Amiodarone was used also in the next study, in which we investigated its influence on the elimination of rhodamine-123. Amiodarone has previously been described as a P-gp inhibitor, yet the clinical significance of this issue has not been elucidated to date. Our *in vivo* experiments confirmed the hypothesis that acute intravenous amiodarone administration can significantly decrease biliary excretion of rhodamine-123. Subsequent *in vitro* transport study using rat primary hepatocytes further explained the mechanism of the *in vivo* observed elimination blockade, where the specific site of the transport inhibition is not efflux, i.e. canalicular membrane with ABC transporters (incl. P-gp), but conversely the uptake through the basolateral membrane. Repeated administration of amiodarone produced also a progressive time-dependent increase in the renal elimination of rhodamine-123. The expression analyses of the transporters demonstrated an induction in renal P-gp and Mrp2 proteins. At the same time we observed an induction of P-gp also in the liver after long-term amiodarone treatment.

The aim of the last study was to evaluate the effects of dexamethasone treatment on the biliary elimination and overall kinetics of methotrexate. The dexamethasone pretreatment led to a marked reduction in methotrexate biliary clerance. a significant increase was observed also in the liver weights along with cumulation of methotrexate in

the liver tissue. According to the plasma enzyme activities determined before and after methotrexate administration, the dexamethasone-mediated hepatotoxicity was apparently further potentiated. Our results thus suggest a hepatocellular injury connected with the impaired biliary elimination of methotrexate. With the concomitant dexamethasone treatment the increase in hepatic accumulation of methotrexate may contribute to an increase in the hepatotoxicity of the antifolate.

The summarized results of the publications describe various aspects of the pharmacokinetic drug-drug interactions, where the underlying mechanism of the interactions is a modulation (either induction/activation or inhibition) of the active transport. Such modulation is produced by a simultaneous or previous administration of another drug known to affect the expression and/or function of transporting proteins. Our results also accent the importance of the usage of *in vivo* models in studies evaluating the effects of xenobiotics on transport activities in the organs of elimination.

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