UNIVERZITA KARLOVA

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Interakce membránových transportérů s léčivy v placentě a duktálním adenokarcinomu pankreatu

DISERTAČNÍ PRÁCE

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V Hradci Králové, 2020

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

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Mgr. Lucie Jirásková

Poděkování

Na tomto místě bych ráda poděkovala prof. PharmDr. Františku Štaudovi, Ph.D. za vložení důvěry do „nefarmakologa z Olomouce“ a že mi umožnil se stát členem jeho výzkumného týmu. Avšak největší a srdečné díky patří mému školiteli doc. PharmDr. Lukáši Červenému, Ph.D. V prvé řadě za jeho neuvěřitelnou trpělivost, kterou sama nemám, a proto ji nejvíce oceňuji. Jeho neustálé bytí online a rychlost poskytnutí rad či korekcí textů je obdivuhodné. Ochota poradit při provádění experimentů i při sepisování odborných vědeckých publikací a vstřícný přístup vždy s úsměvem je pro něho samozřejmostí.

Dále děkuji všem mým spolupracovníkům z Katedry farmakologie a toxikologie, se kterými jsem měla tu čest pracovat. Za vytvoření přátelského prostředí, bez kterého by veškerá práce byla mnohem náročnější a ochotu s čímkoliv poradit jmenovitě děkuji svým blízkým přátelům Mgr. Sáře Karbanové, PharmDr. Aleši Šorfovi, Ph.D. a Mgr. Lence Ťupové.

Speciální poděkování patří také doc. MUDr. Filipu Čečkovi, Ph.D., prof. MUDr. Aleši Ryškovi, Ph.D., MUDr. Heleně Hornychové, Ph.D. a doc. Dipl.-Math. Jurjenovi Duintjerovi Tebbensovi, Ph.D. za spolupráci při sepisování druhého prvoautorského článku.

Velké díky patří mým rodičům, ti mi dali do života tu nejdůležitější školu a bez nich bych nebyla tam, kde jsem. V neposlední řadě děkuji svým prarodičům, příteli a „mimofakultním“ přátelům, že mi byli silnou oporou po celou dobu mého doktorského studia a mnohdy ve mě věřili víc, než já v sebe samu.

Závěrem bych chtěla rovněž poděkovat za finanční podporu poskytnutou Grantovou agenturou Univerzity Karlovy (GAUK 812216), Grantovou agenturou České republiky (GAČR17−16169S) a fondem Specifického vysokoškolského výzkumu SVV 260-414.

Abstrakt

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Kandidátka:	Mgr. Lucie Jirásková
Školitel:	doc. PharmDr. Lukáš Červený, Ph.D.
Název disertační práce:	Interakce membránových transportérů s léčivy v placentě a duktálním adenokarcinomu pankreatu

Membránové transportéry jsou přítomny napříč celým organismem a zastávají řadu vitálně důležitých funkcí. Významnými zástupci membránových transportérů jsou p-glykoprotein (ABCB1), breast cancer resistance protein (ABCG2) a multidrug resistance-associated protein 2 (ABCC2) patřící do rodiny ATP-biding cassette (ABC) transportérů. Dalšími důležitými zástupci jsou nukleosidové transportéry, které patří do rodiny Solute Carriers (SLC) transportérů. V poslední době je sledována jejich důležitost ve farmakokinetice léčiv a vzniku rezistence k protinádorové terapii. V rámci této disertační práce jsme se uvedeným transportérům (zejména nukleosidovým) věnovali komplexně. Podařilo se nám popsat expresní profil nukleosidových transportérů v placentě v různých fázích gestace. Dále jsme sledovali, zda se exprese nukleosidových transportérů mění v závislosti na míře diferenciace trofoblastu či může být ovlivněna epigeneticky a popsali důležitost cAMP/proteinkináza A signalizační dráhy v jejich regulaci. Z pohledu farmakokinetického jsme zjistili, že z placentárních nukleosidových transportérů ekvilibrativní nukleosidový transportér 1 signifikantně usnadňuje materno-fetální přestup nukleosidových analogů anti-HIV abakaviru a anti-HCV ribavirinu, nicméně neovlivňují placentární kinetiku anti-HIV léčiv emtricitabinu a zidovudinu. Podobně ABC transportéry zajišťující v placentě aktivní ochranu plodu nesnižovaly materno-fetalní transfer ribavirinu. Zabývali jsme se i rolí ekvilibrativního nukleosidového transportéru 1 ve farmakorezistenci duktálního adenokarcinomu pankreatu vůči adjuvantnímu podávání gemcitabinu. V rámci naší kohorty pacientů se nám nepodařilo potvrdit korelaci mezi expresí ekvilibrativního nukleosidového transportéru 1 a přežíváním pacientů. Obdobný výsledek jsme pozorovali i při analýze dalších potenciálních prognostických markerů homologní protein 3 pro neurogenní lokus (NOTCH3) a mikroRNA 21 (miR-21). Naše data a publikované práce přinesly řadu poznatků

o regulaci nukleosidových transportérů a jejich zapojení do farmakokinetiky léčiv a rozšířily také znalost o zapojení ABC transportérů v placentární kinetice nukleosidových analogů.

Abstract

Charles University, Faculty of Pharmacy in Hradec Králové

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Candidate:	Mgr. Lucie Jirásková
Supervisor:	doc. PharmDr. Lukáš Červený, Ph.D.
Title of doctoral thesis:	Interactions of membrane transporters with drugs in the placenta and pancreatic ductal adenocarcinoma

Membrane transporters are found throughout the body, where they are responsible for many vital functions. Important representatives of membrane transporters are P-glycoprotein (ABCB1), Breast cancer resistance protein (ABCG2) and multidrug resistance-associated protein 2 (ABCC2) belonging to the ATP-biding Cassette (ABC) family. Nucleoside transporters belonging to Solute Carriers family (SLC) transporters represent another important group. It has been well evidenced that these transporters also affect drug disposition and contribute to tumor resistance to anticancer therapy. Over working on this dissertation thesis, we investigated the mentioned transporters (with special focus on nucleoside transporters) in complex fashion. We described the expression profile of nucleoside transporters in the placenta at different stages of gestation. We also examined whether the expression of nucleoside transporters changes depending on the degree of differentiation or can be affected epigenetically, and we demonstrated the importance of the cAMP / protein kinase A signaling pathway in their regulation. Regarding drug disposition, we found that placental equilibrative nucleoside transporter 1 significantly facilitates mather-to-fetus transfer of nucleoside-derive drugs, anti-HIV abacavir and anti-HCV ribavirin, but do not affect placental kinetics of anti-HIV drugs, emtricitabine and zidovudine. Similarly, ABC transporters providing fetal protection did not reduce maternal-fetal transfer of ribavirin. We also addressed the role of equilibrative nucleoside transporter 1 in the chemoresistance of pancreatic ductal adenocarcinoma to adjuvant gemcitabine therapy. Within our cohort of patients, we were unable to confirm the correlation between equilibrative nucleoside transporter 1 expression and patients' survival. We achieved a similar result in the analysis of other potential prognostic markers neurogenic locus notch homolog protein 3 (NOTCH3) and microRNA 21 (miR-21). Our data and published studies

have broadened knowledge on the regulation of nucleoside transporters and their involvement in drug pharmacokinetics, as well as they evidenced lacking involvement of ABC transporters in the placental kinetics of nucleoside analogs.

OBSAH

1 SEZNAM ZKRATEK

ABC	ATP-vázající transportéry (z angl. ATP-binding Cassette)
ABCB1	P-glykoprotein (P-gp), MDR1
ABCC2	z angl. Multidrug Resistance-associated Protein 2, MRP2
ABCG2	z angl. Breast Cancer Resistance Protein, BCRP
ATP	adenosintrifosfát
ATRA	all-trans retinoic acid (tretinoin)
ART	kombinovaná antiretrovirová terapie (z angl. combination antiretroviral therapy)
CCR5	inhibitory C-C chemokinového receptoru 5
CNTs	koncentrační nukleosidové transportéry (z angl. Concetrative Nucleoside Transporters)
dCK	deoxycitidinkináza
DNMT	DNA metyltransferáza
dNTP	deoxyribonukleotid
DSS	nádorově specifické přežití (z angl. Disease-Specific Survival)
ENTs	ekvilibrativní nukleosidové transportéry (z angl. Equilibrative Nucleoside Transporters)
GEM	gemcitabin
HDAC	histondeacetyláza
HDACi	inhibitory histondeacetyláz
HIV	virus lidské imunitní nedostatečnosti (z angl. Human Immunodeficiency Virus)
HUGO	projekt mapování lidského genomu (z angl. Human Genome Organisation)
INSTIs	inhibitory integrázy (z angl. Integrase Strand Transfer Inhibitors)

MATE	z angl. Multidrug and Toxin Extrusion Proteins
miR-21	mikroRNA 21
MVM	vezikuly z izolované mikrovilózní membrány (z angl. Microvillous Plasma Membrane)
NNRTIs	nenukleosidové inhibitory reverzní transkriptázy (z angl. Non-nucleoside Reverse Trancriptase Inhibitor)
NOTCH3	homologní protein 3 pro neurogenní lokus (z angl. Neurogenic Locus Notch Homolog Protein 3)
NRTIs	nukleosidové inhibitory reverzní transkriptázy (z angl. Nucleoside/Nucleotide Reverse Transcriptase Inhibitor)
NTs	nukleosidové transportéry
OAT	transportéry pro přenos organických aniontů (angl. Organic Anion Transporter)
OATP	transportní polypeptidy pro přenos organických aniontů (z angl. Organic Anion-Tranporting Polypetides)
OCT	transportéry pro přenos organických kationtů (z angl. Organic Cation Transporter)
PDAC	duktální adenokarcinom pankreatu (z angl. Pancreatic Ductal Adenocarcinoma)
PKA	proteinkináza A
PIs	inhibitor proteázy (z angl. Protease Inhibitors)
RAR	z angl. Retinoic Acid Receptor
RRM1	podjednotka M1 ribonukleotid-reduktázy
RRM2	podjednotka M2 ribonukleotid-reduktázy
RXR	z angl. Retinoid X Receptor
SLC	Solute Carrier rodina transportérů
TDF	tenofovir disoproxil fumarát

TFV	tenofovir
WHO	Mezinárodní zdravotnická organizace (z angl. World Health Organization)

2 ÚVOD

Buněčné membrány ohraničují jednotlivé buňky a organely, zajišťují komunikaci s okolím a jsou zapojeny do metabolických a transportních procesů. Aby toto bylo možné, jsou membrány vybaveny transportními systémy, zahrnující membránové transportéry. Membránových transportérů je velké množství typů a jsou různým stupněm specializovány pro transport specifických molekul a zajišťování různých fyziologických procesů včetně absorpce a distribuce živin. Některé transportéry naopak zajišťují protekci celého organismu/citlivých tkání tím, že zpomalují absorpci, omezují distribuci a urychlují exkreci potenciálně nebezpečných látek včetně xenobiotik.

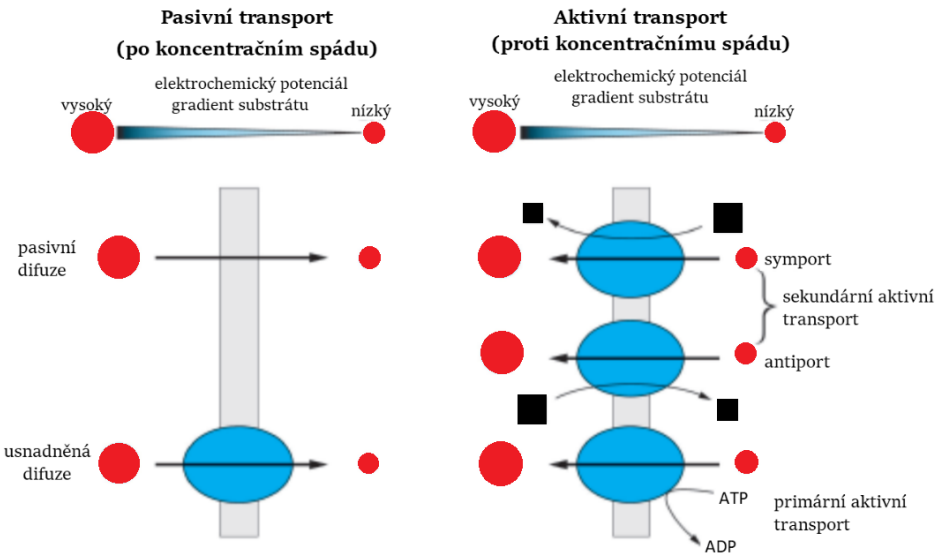
Historie výzkumu membránového transportu sahá do 30. let 20. století (po objevení lipidové dvojvrstvy vědci Gorterem a Grendelem v roce 1925), kdy byl autory Osterhount et al. popsán iontový transport spřažený s membránovým proteinem. V druhé půlce 20. století se pak dále prohlubovaly poznatky o mechanismech membránového transportu [1] a membránové transportéry se staly tématem základního i aplikovaného výzkumu; 1.4.2020 bylo nalezeno v databázi PubMed téměř čtvrt milionu vědeckých publikací (klíčová slova: „MEMBRANE TRANSPORTERS and HUMANS"). V roce 1975 byl pak v laboratoři Viktora Linga objeven první transportér důležitý pro transport léčiv, P-glykoprotein [2].

3 TEORETICKÁ ČÁST

3.1 Membránové transportéry

Membránový transport je soubor mechanismů umožňující iontům, vitálně důležitým látkám či léčivům překonat buněčnou membránu. Transport přes biologické membrány a bariéry je jedním z nejdůležitějších faktorů ovlivňující absorpci, distribuci a exkreci látek. Řada látek přestupuje přes membránu pasivní difúzí bez spotřeby energie po směru koncentračního spádu, avšak u mnoho látek je jejich přenos zprostředkován usnadněnou difúzi či aktivní transportem. Pro usnadněnou difúzi je charakteristická účast obousměrného membránového transportéru a přenos látky ve směru koncentračního gradientu, tedy bez spotřeby energie. Aktivní transport je jednosměrný, probíhá proti koncentračnímu gradientu a je závislý na dodávce energie ve formě ATP. Aktivní transport dále dělíme podle hnací síly na primární a sekundární. Primární aktivní transport využívá energii přímo z hydrolýzy ATP. Sekundární aktivní transport neboli kotransport využívá jako hnací sílu elektrochemický gradient vytvořený transportem jiné částice, může tak docházet k symportu či antiportu částic [3]. Mechanismy membránového transportu jsou znázorněny na obrázku 1.

Membránové transportéry jsou integrální membránové proteiny, které se dělí podle směru transportu substrátů na jednosměrné influxní (transport do buňky), efluxní (transport ven z buňky) a obousměrné. Buňce mohou sloužit k přijímání důležitých látek, jako jsou např. minerály, vitamíny, aminokyseliny, cukry a nukleosidy nebo naopak k vylučování toxických látek a produktů metabolismu. V lidském genomu bylo dle HUGO GeneNomenclature Committee identifikováno více než 400 membránových transportérů, které jsou v současné době rozděleny do dvou významných rodin, a to na ATP-binding cassette (ABC) a Solute-carrier (SLC) [4]. V následujících kapitolách budou, s ohledem na zaměření této práce, detailně popsány efluxní lékové ABC transportéry a nukleosidové transportéry patřící do rodiny SLC.



Obr. 1: Transport látek přes biologické membrány. Modré ovály znázorňují membránové transportéry. Červené kruhy a černé čtverce znázorňují substráty, jejich velikost odpovídá koncentraci. Šipky znázorňují směr transportu (po či proti koncentračnímu spádu). Převzato a upraveno dle [3].

3.1.1 ABC transportéry

ABC transportéry, představují širokou skupinu membránových transportérů využívající hydrolýzy ATP (adenosintrifosfát) jako zdroj energie k efluxu substrátů z buňky nebo transportu látek z/do buněčných organel proti koncentračnímu gradientu [5]. V lidském těle je exprimováno 49 ABC transportérů a dle fylogeneze je lze rozdělit do 7 podrodin A-G [2, 3]. Z hlediska farmakokinetiky, patří mezi nejvýznamnější, a tedy nejvíce studované ABC transportéry, P-glykoprotein (P-gp, ABCB1, MDR1), breast cancer resistance protein (BCRP, ABCG2) a multidrug resistance-associated proteiny (MRPs, ABCCs). Tyto transportéry se nacházejí v membránách enterocytů, hepatálních a renálních buňkách, kde modulují absorpci, distribuci a eliminaci substrátů, dále pak v biologických bariérách (hematoencefalická, hematotestikulární a placentární), kde hlavně chrání mozek a vyvíjející se plod před nežádoucími účinky xenobiotik [6, 7].

3.1.1.1 P-glykoprotein (P-gp, ABCB1, MDR1)

ABCB1 je prvním objeveným, a díky své schopnosti způsobovat mnohočetnou farmakorezistenci v nádorových buňkách, nejvíce probádaným ABC transportérem. ABCB1 se skládá ze 2 homologních aminokyselinových řetězců, z nichž každý obsahuje 6 hydrofobních transmembránových domén a intracelulární ATP-vázající doménu [5, 8, 9]. Substráty ABCB1 jsou

strukturně i funkčně velmi různorodé. Společným znakem mnoha z nich je přítomnost aromatické skupiny a amfifilní povaha [5]. Mezi substráty ABCB1 patří řada cytostatik, antiretrovirotik, antibiotik, antiarytmik či analgetik [5, 8]. ABCB1 se v lidském organismu hojně nachází v apikální membráně buněk tvořících zejména biologické bariéry. Hraje klíčovou roli ve farmakokinetice léčiv a je místem vzniku lékových interakcí [10-12]. Je často ve zvýšené míře exprimován v nádorových buňkách, kde může snižovat intracelulární koncentraci cytostatik, a tak se podílet na zvýšené rezistenci nádorové buňky k chemoterapii. Proto je dlouhodobě zkoumaný s cílem nalézt způsob, jak nádorovou lékovou rezistenci překonat [13, 14]. Exprese ABCB1 je prokázána i v lidské placentě [15]. Je lokalizován na apikální straně syncytiotrofoblastu, odkud pumpuje látky zpět do intervilózního prostoru obsahující krev matky a tím snižuje materno-fetální transfer některých látek [16].

3.1.1.2 Breast cancer resistance protein (BCRP, ABCG2)

Jak již název tohoto transportéru napovídá, poprvé byl ABCG2 izolován z karcinomu prsu [5]. Odlišuje se svou strukturou. Na rozdíl od ABCB1 obsahuje ve své struktuře pouze 6 transmembránových jednotek a jednu intracelulární ATP-vázající doménu, a proto pro správnou funkci musí tvořit homo–dimery spojené disulfidickými můstky [5, 8, 17]. Stejně jako ABCB1 je ABCG2 lokalizován v apikální membráně buněk tvořících biologické bariéry [5, 17, 18]. Nejvíce je exprimovaný v placentárním syncytiotrofoblastu, kde se podílí na ochraně plodu tím, že stejně jako ABCB1 omezuje materno-fetální přestup svých substrátů. ABCG2 ovlivňuje i samotný vývoj placentárního syncytiotrofoblastu [19, 20]. Jeho výskyt byl potvrzen také na povrchu krevních a solidních nádorových buněk, kde se v součinnosti nebo nezávisle na ABCB1 podílí na vzniku mnohočetné lékové rezistence [17, 21]. ABCG2 vykazuje podobnou substrátovou specifitu jako ABCB1. Mezi jeho substráty patří např. cytostatika, antivirotika, antiretrovirotika, antibiotika, antidiabetika, flavonoidy, hormony a další látky [8, 22].

3.1.1.3 Multidrug resistance-associated proteins (MRPs, ABCCs)

Označení celé této skupiny transportérů je odvozeno ze schopnosti způsobovat mnohočetnou lékovou rezistenci nádorových buněk prvním objeveným transportérem této skupiny, a to ABCC1 [23, 24]. Strukturou jsou velmi podobné ABCB1, obsahují 12 transmembránových domén a 2 intracelulární ATP-vázající domény, to platí pro ABCC4, 5, 8 a 9. ABCC1, 2, 3, 6 a 7 obsahují navíc ještě pět transmembránových segmentů s volným NH2 koncem [25]. ABCC transportéry mohou být, v závislosti na typu buněk, exprimovány v apikální i v bazolaterální membráně [23]. Typickým, bazolaterálně lokalizovaným transportérem z této

podskupiny je ABCC1, apikálně lokalizovaným (včetně placentární bariéry) je pak ABCC2 [24]. Kromě jejich role v mnohočetné lékové rezistenci, tyto transportéry zprostředkovávají transport mnohých endogenních i exogenních látek, a to především konjugátů s navázaným glutathionovým, glukuronidovým nebo sulfátovým zbytkem, za což je zodpovědný především ABCC2 [5, 23]. Ovlivňují farmakokinetiku některých léčiv, např. cytostatik, antivirotik, hypolipidemik či antiarytmik [23, 26, 27].

3.1.2 SLC transportéry

Jedná se o velice rozmanitou skupinu transportérů, která čítá více než 300 zástupců, lokalizovaných v celé řadě tkání a orgánů, např. játra, ledviny, střeva, srdce, plíce, placenta a další [28]. Na rozdíl od ABC transportérů, které mají ve své struktuře hydrolytické místo pro ATP a mohou tak uvolněnou energii využít přímo, většina SLC transportérů patří mezi sekundárně aktivní transportéry. ATP dependentní přenašeč vytváří koncentrační gradient iontů, který je hnací silou pro transport jiné látky. Mezi nejvýznamnější podskupiny SLC transportérů patří transportéry pro přenos organických aniontů, OAT (organic anion transporters) a OATP (organic anion-transporting polypeptides), organických kationtů, OCT (organic cation transporters), MATE (multidrug and toxin extrusion proteins) a nukleosidů, nukleosidové transportéry (NTs). S ohledem na mé experimentální zaměření a publikační výstupy této práce jsou v následujících podkapitolách blíže popsány zástupci NTs a jejich význam ve studovaných oblastech.

3.1.2.1 Nukleosidové transportéry

Purinové i pyrimidinové nukleosidy jsou hydrofilní látky, které mohou jen velmi omezeně přestupovat přes buněčnou membránu pasivní difúzí, proto jsou nezbytné pro jejich vychytávání buňkou specializované membránové NTs. V poslední době se NTs věnuje čím dál větší pozornost, díky zjištěním, že významně ovlivňují extracelulární a intracelulární koncentrace nukleosidů. Nukleosidy mají nezastupitelnou roli v mnoha buněčných funkcích, a jsou proto považovány za regulátory buněčné homeostázy. Přirozeně se vyskytující nukleosidy dělíme na purinové, mezi které patří adenosin, guanosin a inosin, a pyrimidinové jako je uridin, cytidin a thymidin. Tyto nukleosidy slouží jako prekurzory pro syntézu nukleotidů a nukleových kyselin, které jsou nezbytné pro kontrolu růstu a metabolismu ve všech živých systémech, dále pak koenzymů (NADP a FAD) a intracelulárních signálních molekul (cAMP a cGMP). Adenosin je neuromodulátor, který reguluje fyziologické procesy, jako je neurotransmise a kardiovaskulární aktivita [29, 30], ale podílí se i na řadě patofyziologických dějích (např. pre-eklampsie či gestační diabetes) [31-33]. Díky nezastupitelné roli nukleosidů v syntéze nukleových kyselin jsou jejich

analogy klinicky využívány v léčbě solidních nádorů (např. 5-fluorouracil, gemcitabin, merkaptopurin, azacytidin a kladribin), virových infekcí jako je AIDS (např. lamivudin, abakavir, zidovudin, emtricitabin a tenofovir), hepatitida B (entekavir, adefovir a telbivudin) a hepatitida C (ribavirin) [34-39].

Dle dřívějších funkčních a kinetických studií byly ustanoveny dvě genové rodiny nukleosidových transportérů - *SLC28A* a *SLC29A*, kódující aktivní koncentrační nukleosidové transportéry (CNT; *SLC28*) a pasivní ekvilibrativní nukleosidové transportéry (ENT; *SLC29*) [40].

3.1.2.1.1 CNTs

CNT transportéry zprostředkovávají aktivní jednosměrný Na^+-dependentní transport nukleosidů a jejich derivátů do buňky. Jsou exprimovány nejčastěji na apikální straně buněčných membrán orgánů. Rodina těchto transportérů má tři zástupce, CNT1 (*SLC28A1*), CNT2 (*SLC28A2*) a CNT3 (*SLC28A3*), lišící se substrátovou specifitou [41]. CNT1 přenáší hlavně pyrimidinové nukleosidy, CNT2 purinové a CNT3 transportuje oba strukturní typy nukleosidů [42]. Genová exprese *SLC28A1* byla detekována hlavně v epiteliálních buňkách jater, ledvin a střeva. V případě *SLC28A2*, exprese byla popsána v placentě, játrech, ledvinách, střevě, srdci, mozku, pankreatu či prostatě. *SLC28A3* mRNA se nachází například v terminální placentě, plicích, kostní dřeni, prostatě a játrech [43-45]. Na úrovni proteinu byly CNT1 i CNT2 detekovány v buňkách jater, ledvin, střeva a pankreatu [46]. CNTs jsou zapojené do buněčných signalizačních kaskád. CNT1 je dokonce považovaný za transceptor (kombinuje vlastnosti transportéru a receptoru). Jeho role byla popsána v buněčné proliferaci, ovšem ta je prokazatelně nezávislá na jeho transportní funkci. V případě CNT2 a CNT3 jako transceptorech se dosud pouze spekuluje. CNT2 je důležitým mechanismem regulace extracelulárních koncentrací fyziologicky nejvýznamnějšího nukleosidu, adenosinu; který je jeho modelovým substrátem s vysokou afinitou [41, 47, 48]. Ve studiích Dufflot et al. (2004) a Pastor-Anglada et Pérez-Torras (2018), byla popsána aktivace CNT2 po předchozí aktivaci adenosinových receptorů A1 v hepatocytech [41, 49]. Tento mechanismus spočívá v aktivaci a otevření K^+ kanálů pomocí ATP, což způsobí zvýšení absorpce nukleosidu přes CNT2. CNT2 se účastní i dalších dějů spojených s tvorbou energie, konkrétně s procesem glykolýzy a glukoneogeneze [50]. Snížené vychytávání adenosinu vede k vyšším extracelulárním koncentracím adenosinu, což vede k aktivaci AMP-dependentních kinázy (AMPK). Tento děj také přispívá k regulaci energetického metabolismu buňky [41].

Klinický význam CNTs spočívá v transportu protinádorových či antivirových léčiv a ovlivnění jejich farmakokinetiky. Příkladem je gemcitabin (GEM; 2´,2´- difluorodeoxycytidin), indikovaný k léčbě karcinomu pankreatu, který je transportovaný pomocí CNT1 a CNT3, a dále např. fludarabin, který je lékem volby u chronické lymfocytární leukémie a je substrátem CNT3

[51, 52]. Známým částečným inhibitorem CNTs je phloridzin a specifickými inhibitory CNT2 jsou KGO-2142 a KGO-2173 [53, 54], nespecificky se jejich aktivita inhibuje deplecí sodných kationtů [42].

3.1.2.1.2 ENTs

Tato podskupina nukleosidových transportérů má čtyři zástupce ENT1 (*SLC29A1*), ENT2 (*SLC29A2*), ENT3 (*SLC29A3*) a ENT4 (*SLC29A4*) [42]. ENTs, kromě ENT4, jsou na Na^+ nezávislé a zprostředkovávají facilitovanou difúzi. ENT1-3 kontrolují transport purinových i pyrimidinových nukleosidů [42]. ENT4 je pH-dependentní a podílí se na transportu adenosinu a organických kationtů [55]. ENT transportéry mají širokou substrátovou specifitu. V řadě případů jsou lokalizované v bazolaterální membráně polarizovaných buněk a tkání. Na rozdíl od CNTs mají menší afinitu k substrátům, ale větší kapacitu přenosu [56, 57]. Za specifický inhibitor ENTs je považován nitrobenzylthionosin (NBMPR), který je po desetiletí používám k funkční charakterizaci těchto transportérů v buněčných kulturách i tkáních [58]. ENTs mají rozdílnou sensitivitu k NBMPR. ENT1 je NBMPR sensitivní transportér a je inhibován v nanomolárních koncentracích NBMPR, zatímco ENT2 je k působení NBMPR méně citlivý a je inhibován v mikromolárních koncentracích NBMPR. Funkce ENT3 není NBMPR ovlivněna [42]. Mezi další inhibitory ENTs patří vasodilatační léčiva jako je dipyridamol, dilazep a draflazin [42], nicméně o nich je známo, že inhibují i jiné transportéry [59].

3.2 Molekulární mechanismy regulace nukleosidových transportérů

Z minulosti je známo, že dysregulace nukleosidových transportérů může být způsobena patologickými změnami [60-66]. Dále bylo zjištěno, že exprese CNT2 je snížena v zanícené střevní tkáni u pacientů s Crohnovou chorobou [67]. Jejich inhibice se experimentálně využívá v léčbě akutního poškození plic [68] a snížená exprese ENT1 nebo CNT3 může způsobovat nádorovou farmakorezistenci [38]. Mezi jeden z nejběžnějších mechanismů způsobující variabilní expresi genů a následně funkci proteinu patří genetický polymorfismus. Vliv polymorfismů na funkční expresi nukleosidových transportérů není nijak významný [59]. Naproti tomu, je exprese nukleosidových transportérů ovlivňována hypoxií, fosforylačními ději, regulačním faktorem RS1, glukózou či transaktivací nukleárních receptorů [69-73].

Množství nukleosidových transportérů v buňkách a tkáních částečně závisí na řadě mechanismů spojených se stimulací proliferace a diferenciace (např. aktivace proteinkinázy nebo kyseliny retinové [74-76], může být také ovlivněno řadou různých endogenních a

exogenních faktorů [28], např. jejich hladina je regulovaná složením a množstvím výživy [70, 77-79]. Proto se jejich hladina liší mezi jednotlivci a exprese se může výrazně měnit v průběhu těhotenství [45, 80-82].

Exprese nukleosidových transportérů v placentě v průběhu gestace, inter-individuální rozdíly a mechanismy molekulární regulace nukleosidových transportérů v placentě nebyly dosud uspokojivě popsány. Z epigenetických mechanismů v placentě, metylace cytosinových zbytků na CpG ostrůvcích obvykle vede k potlačení transkripce genů a acetylace histonů způsobují naopak genovou indukci [83]. V rakovinných buňkách je popsáno, že míru exprese nukleosidových transportérů lze ovlivnit indukcí diferenciace [84].

3.3 Nukleosidové transportéry v placentě a PDAC

Jak již bylo zmíněno výše, nukleosidové transportéry jsou exprimovány v řadě důležitých orgánů a tkání. Tato práce se zaměří na roli nukleosidových transportérů v lidské placentě a duktálním adenokarcinomu pankreatu (PDAC), kde ENT1 určuje míru cytotoxicity GEM tím, že je zásadní cestou, pro jeho transport do nádorové tkáně (viz kapitola 3.4).

3.3.1 Role transportérů v placentárním přestupu

Placenta je důležitý dočasný orgán, zajišťující vývin plodu. Po celou dobu gestace odděluje maternální a fetální cirkulaci a zajišťuje látkovou výměnu (živiny, kyslík a produkty metabolismu plodu) mezi matkou a plodem. Zároveň produkuje důležité hormony kontrolující správný vývoj placenty a plodu [85]. Vedle těchto funkcí má placenta aktivní mechanismy snižující přechod lipofilních (potenciálně toxických) látek z oběhu krve matky do plodu [27, 28]. Nejdůležitější částí placentární bariéry je polarizovaná mnohobuněčná vrstva tzv. syncytiotrofoblast. Ten se nachází na povrchu choriových klků, které jsou omývány mateřskou krví, a tak dochází k výměně látek mezi matkou a plodem (obr. 2A, B). Syncytiotrofoblast exprimuje celou řadu ABC i SLC transportérů zajišťujících výměnu živin, signálních molekul, růstových faktorů a xenobiotik (obr. 2C) [27]. V poslední době přibývá publikací o roli těchto transportérů v placentární farmakokinetice léčiv [36, 80, 86, 87].

Placentární ENT1 je lokalizován na apikální (maternální) straně membrány [46, 88], zatímco ENT2 se vyskytuje na obou stranách, apikální i bazolaterální (fetální) membráně cytotrofoblastu [89]. Oproti ENTs je proteinová exprese CNTs v lidské placentě mnohem méně prozkoumána a v řadě publikací se výsledky rozchází. Dle studie Errasti-Murugarren et al. (2011), bylo na BeWo buněčné linii zjištěno, že se v placentě nachází geny *SLC28A1, SLC28A2, SLC28A3, SLC29A1 i SLC29A2.* Na proteinové úrovni byl CNT1 prokázán na obou pólech

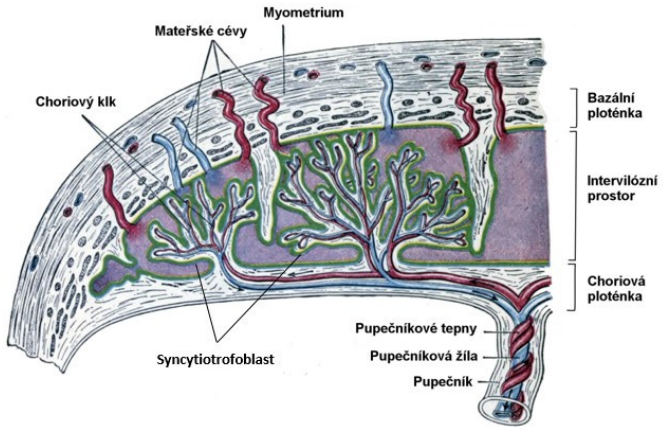
syncytiotrofoblastu, zatímco CNT2 ani CNT3 detekovány vůbec nebyly [89]**.** Dále Govindarajan et al. (2007), prokázali expresi *SLC28A2*, *SLC29A1* a *SLC29A2*, ale na úrovni proteinu detekovali pouze ENT1 a ENT2 [46]. Vzhledem k těmto nejasnostem jsme se expresi a lokalizaci nukleosidových transportérů v placentě blíže věnovali a výsledky jsme zveřejnili v publikacích I a V [36, 45].

Nukleosidové transportéry zajišťují vychytávání nukleosidů placentou a přispívají k jejímu správnému vývoji [69]. Nukleosidové transportéry také umožňují placentární vychytávání a přenos léčiv odvozených od struktury nukleosidů [37, 87]. Mezi léčiva odvozená od nukleosidů patří řada antivirotik a antiretrovirotik, která jsou běžně podávána při léčbě těhotných žen s HIV, hepatitidou B či C, nebo jako prevence přenosu viru z matky na dítě během těhotenství [27, 90-96].

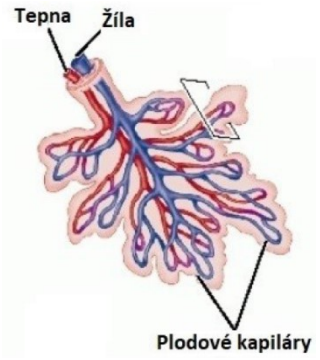
Z ABC transportérů jsou ABCB1 a ABCG2 považovány za nejvýznamnější a nejvíce prostudované ve farmakokinetice léčiv [97]. Jsou lokalizovány na apikální membráně syncytiotrofoblastu a exprese ABCG2 je v celém organismu nejvyšší právě v placentě [15, 19, 20, 98]. Zde chrání plod tím, že pumpují své substráty do krevního oběhu matky [99-103]. Další rodinou ABC transportérů nacházející se v placentě jsou ABCCs. Exprese některých zástupců je potvrzena na apikální i bazolaterální straně syncytiotrofoblastu. Například ABCC1 se nachází na bazolaterální straně, zatímco ABCC2 na apikální straně syncytiotrofoblastu [104-106]. O funkci ABCCs není doposud příliš známo, nicméně bylo zjištěno, že inhibicí ABCC2 došlo ve směru materno-fetálním ke zvýšení přestupu talinololu [107].

Exprese placentárních ABC transportérů se v průběhu gestace mění. Exprese ABCG2 mRNA v lidské placentě je od počátku gestace stabilní směrem ke konci těhotenství, zatímco proteinová exprese mírně roste a nevykazuje známky významné inter-individuální variability [108]. U potkana exprese ABCG2 v placentě v průběhu gestace klesá. ABCB1 je v lidské placentě exprimován od časné fáze gestace do konce těhotenství. U člověka míra exprese tohoto proteinu v placentě v průběhu gestace klesá, zatímco u potkana je tomu naopak [28].

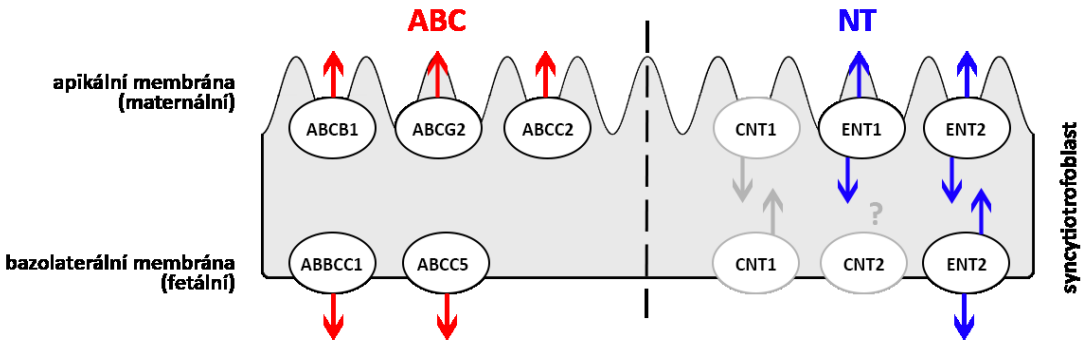
A



B



C



Obr. 2: Schéma anatomie lidské placenty (A). Lidská placenta je tvořena bazální ploténkou (maternální část) a choriovou ploténkou (fetální část), mezi nimiž se nachází intervilózní prostor, kde cirkuluje krev matky, která omývá trofoblast na povrchu klků (znázorněny zeleně). Detail klku s fetálními cévami, kde dochází k výměně látek mezi matkou a plodem (B). Schématické znázornění vybraných ABC transportérů (znázorněny červeně) a nukleosidových transportérů (znázorněny modře) v syncytiotrofoblastu a směr transportu látek, který zprostředkovávají (C). Evidence o expresi a distribuci CNTs v placentě se v různých publikacích rozchází (znázorněno šedě). Obrázky A a B byly převzaty a upraveny dle [109].

3.3.2 HIV infekce v těhotenství

Dle Mezinárodní zdravotnické organizace (WHO) bylo v roce 2014 přibližně 17,4 milionů žen v reprodukčním věku nakažených virem HIV. Z těchto žen asi 1,5 milionů každý rok otěhotní. Vertikální přenos viru HIV z matky na dítě, ke kterému může dojít v průběhu celého těhotenství, při porodu či kojení je nejčastější příčinou nákazy u dětí [110-112]. Pokud se matka neléčí vhodnou farmakoterapií v podobě kombinované antiretrovirové terapie (cART), riziko přenosu

HIV infekce na dítě je 45 %, přičemž progrese tohoto onemocnění u dětí, které se nakazily transmisí z matky, je rychlejší a polovina z nich umírá do dvou let života. Užívání cART je součástí terapeutických postupů, které snižuje pravděpodobnost přenosu HIV z matky na dítě pod 2 %.

Farmakoterapie HIV ve formě cART je vždy složené z minimálně tří léčiv z alespoň dvou různých farmakodynamických skupin tak, aby bylo zasaženo více fází životního cyklu HIV: nukleosidové inhibitory reverzní transkriptázy - NRTI (př. zidovudin, lamivudin, emtricitabin, tenofovir a abakavir) [113-116], nenukleosidové inhibitory reverzní transkriptázy - NNRTIs (př. etravirin a rilpivirin) [117, 118], inhibitory retrovirové aspartylproteázy - PIs (př. ritonavir a atazanavir) [119, 120], inhibitory integrázy - INSTIs (př. raltenavir, elvitegravir a dolutegravir) [121], inhibitory C-C chemokinového receptoru 5 - CCR5 (př. maravirok) [122] a inhibitory fúze HIV s buněčnou membránou (př. enfuvirtid) [123]. Součástí cART u těhotných žen by mělo vždy být léčivo s vysokým placentárním přestupem [124, 125]. Lékem první volby u těhotných žen je tenofovir disoproxil fumarát (TDF) [126], dalšími standardně používanými léčivy ze skupiny NRTIs jsou emtricitabin, abakavir a zidovudin.

Řada klinicky významných antiretrovirotik jsou substráty a/nebo inhibitory některého ABC transportéru [127]. Lze tedy předpokládat, že u těhotných žen transportéry v placentě ovlivňují placentární přestup těchto léčiv z maternální do fetální krve. PIs v terminální fázi těhotenství přecházejí přes placentu omezeně [128], což může poukazovat na roli efluxních transportérů [127]. Naproti tomu NRTIs jsou léčiva s výrazným placentárním přestupem [110, 114]. Nejvýznamnějšími a nejvíce prostudovanými placentárními transportéry jsou ABCB1 a ABCG2, které zajišťují eflux některých klinicky významných antiretrovirotik (např. TDF) z trofoblastu zpět do krve matky [129]. Dále bylo zjištěno, že AZT je substrátem ABCB1 a ABCG2, nicméně přestup AZT přes placentu je limitován do určité míry pouze ABCB1 [130] a taktéž abakavir interaguje s ABCB1 a ABCG2, avšak jeho transplacentární přestup je ovlivňován pouze v ekvilibriu maternální a fetální koncentrace [131]. Role placentárních nukleosidových transportérů v materno-fetálním přestupu léčiv doposud nebyla příliš studována [37, 87, 132]. Znalost interakcí antiretrovirotik s transportéry a lékových interakcí na membránových transportérech je velice důležitá pro výběr efektivní a bezpečné léčby. Proto jsme se v publikacích č. III a V zaměřili na studium role nukleosidových transportérů v placentární kinetice některých NRTI (zidovudin, emtricitabin a abakavir) [36, 133].

3.3.3 HCV infekce v těhotenství

Hepatitida C je onemocnění postihující zhruba 3 % světové populace [134, 135]. U přibližně 20 % těhotných žen dochází ke ko-infekci HCV a HIV [136, 137]. Přenos infekce z matky na dítě během těhotenství je nejběžnější příčinou nákazy u dětí (5 – 10 %) [138] a ko-infekce HIV

tuto pravděpodobnost ještě zvyšuje. U dětí nakažených vertikálním přenosem od matky častěji dochází k rozvoji chronické infekce a fibrózy se zánětem jater [138]. Bylo zjištěno, že riziko přenosu HCV infekce souvisí s množstvím viru, proto se předpokládá, že snížením viremie u těhotných žen vhodnou léčbou, by mohlo vést ke snížení pravděpodobnosti vertikální nákazy [139]. Je tedy potřeba nalézt účinné a bezpečné farmakoterapeutické režimy pro těhotné ženy, které však nepoškodí vývoj plodu.

Jedním z kandidátů pro prevenci vertikální infekce HCV je ribavirin. Ribavirin je purinový analog se širokou antivirovou aktivitou [140, 141]. Je nezbytný pro léčbu život ohrožujících virových infekcí jako je respirační syncytiální virus nebo virus chřipky a je kombinován s přímo působícími antivirotiky k léčbě HCV [142].

Doposud byla jeho teratogenita zaznamenána pouze u zvířat [143], nikoli u lidí [144]. Přesto je užívání ribavirinu zatím během těhotenství přísně kontraindikováno. Existují však případy, kdy byl ribavirin pacientkám podáván a nezpůsobil vývojové vady plodu [145]. Aby bylo možné začít ribavirin prakticky využívat, je nutné shromáždit další data o jeho bezpečnosti a také o mechanismech jeho placentárního přestupu. Tímto tématem jsme se blíže zabývali v publikaci č. IV [146].

3.4 Role transportérů v nádorové farmakorezistenci

Nádorová onemocnění představují jednu z nejčastějších příčin úmrtí mezi chorobami a jejich incidence každým rokem vzrůstá [147]. I přes zavedení nových postupů ve farmakoterapii je léčitelnost některých typů nádorů komplikována, mimo jiné rezistencí na léčbu. Jedním z hojně studovaných mechanismů rezistence nádorových buněk je aktivita ABC transportérů, mezi jejichž substráty patří celá řada cytostatik [148, 149]. Nadměrná exprese ABCB1, ABCG2 a ABCC1 transportérů v nádorové tkáni je spojována s horší prognózou pacientů se solidní či hematologickou malignitou [150]. V průběhu let bylo ve snaze takto navozenou lékovou rezistenci překonat vyvinutím specifických inhibitorů, nicméně klinického úspěchu nebylo dosaženo. Možnými příčinami může být design studie, který nezohlednil interindividuální variabilitu v expresi transportérů a selektivní cílení na jeden přenašeč, přičemž na farmakorezistenci se podílí více ABC transportérů zároveň [149]. V současné době se výzkum více soustředí na popis synergického interakčního potenciálu látek, které jsou samy úspěšně využívány jako protinádorová léčiva [13, 14, 151, 152].

V porovnání s ABC transportéry, role nukleosidových transportérů v nádorové terapii byla dosud studována méně [44, 153, 154]. Na rozdíl od ABC transportérů je u nukleosidových

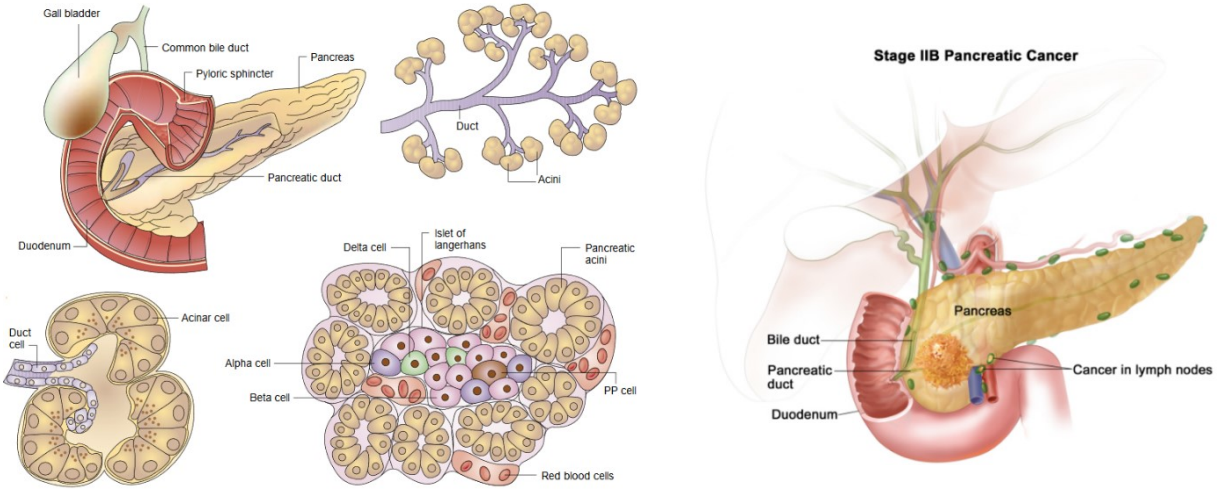
transportérů pozornost zaměřena především na snížení exprese nukleosidových transportérů (ENT1, CNT1 a CNT3) v nádorových buňkách [155], které koreluje se sníženým vychytáváním cytostatik odvozených od nukleosidů do buněk nádoru, a je tedy zřejmě příčinou jejich snížené účinnosti [44, 154]. Tento mechanismus rezistence nádorových buněk k cytostatikům se uplatňuje např. u akutní lymfoblastické leukémie [154], avšak zásadním příkladem je duktální adenokarcinom pankreatu (PDAC), u kterého jsou značně omezené možnosti léčby a GEM zůstává už více jak dekádu lékem volby. GEM je hydrofilní a je tedy striktně závislý na přítomnosti transportérů, které tak mohou významně ovlivnit jeho protinádorový účinek. Podrobně se tomuto tématu věnujeme v kapitole 3.5.2 a v publikaci II.

3.5 Duktální adenokarcinom pankreatu

Karcinom pankreatu je maligní tumor vycházející z exokrinní části pankreatu s incidencí přibližně 170 tisíc onemocnění za rok (Česká republika je druhá v incidenci v Evropě) [117, 153, 156, 157]. Většina typů pankreatických nádorů se řadí mezí PDAC (obr. 3A, B, C). PDAC se klinicky dělí na tři skupiny: resekovatelný (≈ 10 – 20 %; přežití 23 měsíců), lokálně pokročilý neresekovatelný (≈ 30 – 40 %; přežití 6-12 měsíců) a metastazující (≈ 50 %; přežití 6 měsíců) [153, 158].

Obecně se jedná o nádor se slabou odpovědí na farmakoterapii a velice špatnou prognózou. Počet úmrtí na toto onemocnění za rok je přitom prakticky identický s incidencí a činí kolem 196 tisíc ročně [159]; to je 2,8 % všech zemřelých na nádorová onemocnění. Vysoká mortalita je dána především biologickou agresivitou tumoru, pozdní diagnózou a rezistencí na většinu chemoterapeutik. Pětileté přežití všech pacientů se pohybuje pod hranicí 5 %.

A B C



Obr. 3: Schéma anatomie pankreatu (A). Pankreas je žláza s vnitřní i vnější sekrecí. Anatomicky se dělí na hlavu, tělo a ocas, který sahá až ke slezině. Lalůčky vnější sekretorické části pankreatu produkují pankreatickou šťávu (B). Duktální adenokarcinom pankreatu (C). Převzato a upraveno dle [160, 161].

3.5.1 Léčba PDAC

V současné době je jediným potenciálně kurabilním terapeutickým postupem chirurgická resekce. K té je však možné přistoupit u méně než 20 % pacientů [162]. Karcinom pankreatu je onemocnění, které se vyznačuje značnou chemorezistencí. Dosud základní chemoterapeutické postupy jsou založeny na podávání nukleosidového derivátu GEM, a to jak v adjuvantním, tak i paliativním režimu [117, 153, 156, 157]. GEM je užíván v monoterapii u starších pacientů v horší kondici a v některých případech i v kombinaci s jiným cytostatikem (např. cisplatina, oxaliplatina, a irinotekan) [163-167] u pacientů v relativně dobré kondici, kteří lépe snášející toxicitu léčby [153, 168]. Neoptolemos et al. (2017) publikoval klinická data porovnávající účinnost GEM v monoterapii a GEM v kombinaci s kapecitabinem. Doba přežití u pacientů s PDAC po resekci byla 28 měsíců v kohortě s kombinovaným režimem a 25,5 měsíců u skupiny pacientů v GEM monoterapii. U GEM s kapecitabinem byl zaznamenán vyšší výskyt závažných nežádoucích účinků stupně 3 a 4 [169]. Dalším adjuvantním chemoterapeutickým postupem podávaným pacientům s PDAC, které úspěšně prošlo klinickým testováním je modifikovaný FOLFIRINOX (mFOLFIRINOX) obsahující oxaliplatinu, leukovorin, irinotekan a 5-fluoroucil. V porovnání s GEM monoterapií je tento chemoterapeutický postup významně

účinnější z pohledu celkového přežití. Jedná se však o léčbu vysoce toxickou s velkým množstvím nežádoucích účinků a ekonomicky velmi nákladnou [170].

3.5.2 Role prognostického markeru ENT1 v adjuvantní léčbě GEM v PDAC

GEM v adjuvantní terapii je zatím z pohledu poměru nákladů a účinnosti nejlepší volbou. Zároveň je relativně dobře pacienty snášen, avšak řada pacientů díky významné chemorezistenci nádoru z této léčby neprofituje. GEM patří mezi nukleosidové analogy a stejně jako fyziologické nukleosidy je vysoce hydrofilní. Aby bylo dosaženo cytotoxického účinku (mechanismem blokády *de novo* syntézy DNA) [171], a jeho transfer přes buněčnou membránu musí být facilitován. Hlavní úlohu v transportu GEM přes plasmatickou membránu, a tedy i jeho akumulaci v buňce, zprostředkovává ENT1. Dosud publikované *in vitro* studie poukazují na korelaci mezi množstvím ENT1 transportéru v cytoplasmatické membráně a cytotoxicitou GEM. Kolektiv Spratlin et al. (2004), využitím imunohistochemické analýzy zjistil, že pacienti s nižší hladinou ENT1 v rakovinné tkáni mají medián přežití signifikantně kratší, než pacienti s vyšší hladinou ENT1 [84]. Nižší exprese je tedy spojována s horší odpovědí na GEM, a tedy i s horší prognózou [172-174]. Ke stejnému závěru, pomocí metody kvantitativní RT-PCR, došli i Fujita et al. (2010) a Giovannetti et al. (2006) [175, 176]. Ale protože výsledky těchto studií byly v menší či větší míře kontaminovány nehomogenní testovanou skupinou, (užitím radioterapie, různých postupů hodnocení výsledků, užitím GEM v neodajuvantních/adjuvantních/paliativních režimech), zaměřili jsme se na testování vztahu GEM terapie a celkového přežití v kohortě pacientů striktně definované monoterapií GEM u resekovaných pacientů s PDAC. V rámci řešení dizertační práce jsme se ovšem nesoustředili pouze na vztah mezi množstvím ENT1 a celkovým přežitím pacientů léčených monoterapií GEM, ale rozšířili jsme náš zájem i o další molekuly ovlivňující míry cytotoxicity GEM.

3.5.3 Další potenciální prognostické markery v PDAC

Role řady dalších pankreatických proteinů a faktorů byla studována pro individualizaci adjuvantní léčby GEM u pacientů s PDAC. Jedná se však zatím o jednotlivé publikace nebo byly tyto faktory studované na malé kohortě či nebyly sledovány v homogenní skupině pacientů. CNT3 je vedle ENT1 dalším transportérem, který zprostředkovává přenos GEM do buňky. Maréchal et al. (2009) zjistili korelaci mezi vyšší hladinou CNT3 a delším přežitím u pacientů po resekci s adjuvantní léčbou GEM [174]. Dalším zajímavým kandidátem je deoxycytidin kinasa (dCK), která fosforyluje GEM po vstupu do buňky na monofosfát. Ten je pak dále fosforylován až na trifosfátový aktivní metabolit. V některých studiích je spojována vyšší exprese dCK s delším

přežitím [173, 177], avšak v jiné publikaci uvádí opačné výsledky [178]. Jedním ze studovaných a možných prediktivních faktorů GEM rezistence v PDAC je i ribonukleotid-reduktáza M1 (RRM1). Tato jednotka tvoří dimer s ribonukleotid-reduktázou M2 (RRM2) a účastní se syntézy deoxyribonukleotidů dNTP, které jsou základem pro syntézu DNA. Vazbou GEM na katalytické místo RRM1 se inhibuje aktivita ribonukleotid-reduktázy. V tomto případě jsou výsledky studií také rozporuplné. Některé studie uvádí, že vysoká exprese RRM1 způsobuje rezistenci ke GEM [179, 180]. Maréchal et al. (2012) ve studii s větší kohortou pacientů (n=103) neshledal korelaci mezi mírou exprese RRM1 a odpovědí nádoru na léčbu GEM [173]. Avšak dvě studie zjistily korelaci mezi nízkou expresí RRM1 a současně vyšší expresí ENT1 s delším přežití pacientů s PDAC.

V našem výzkumu (publikaci II.) jsme se zaměřili na molekuly homologní protein 3 pro neurogenní lokus (NOTCH3) a mikroRNA 21 (miR-21).

NOTCH3 se podílí na řadě buněčných extracelulárních interakcích a ovlivňuje přežití pankreatických buněk [181]. Jeho množství je signifikantně zvýšeno v buňkách rakovinné tkáně, kde se podílí na inhibici apoptózy a podporuje proliferaci buněk [182]. Bylo popsáno, že snížená exprese NOTCH3 vede ke zvýšení citlivosti pankreatických buněčných linií ke GEM. Pacienti se sníženou expresí mRNA NOTCH3 mají v případě léčby GEM lepší prognózu a zvyšuje se u nich doba celkového přežití [178, 183].

MikroRNA jsou krátké nekódující jednovláknové řetězce, které jsou zapojeny v posttrankripčních modifikacích mnoha genů. Jedna z těchto malých molekul, miR-21, hraje důležitou roli v hematologických a solidních malignitách. Ukazuje se, že její rozdílná exprese u pacientů s PDAC koreluje s dobou přežití, přičemž s vysokou expresí miR-21 signifikantně klesá medián přežití nezávisle na GEM [184-186].

K dnešnímu dni jsou dostupné studie týkající se těchto nádorových markerů provedené řádově pouze na stovkách pacientů [84, 173-176, 178, 185-187]. Z tohoto důvodu další poznatky o expresi uvedených genů v resekované nádorové tkáni mohou být užitečné pro přípravu protokolů vedoucích k personalizované léčbě pacientů s PDAC.

4 CÍLE PRÁCE

Obsahem předkládané disertační práce je studium nukleosidových transportérů a ABC transportérů v placentě a v případě nukleosidových transportérů i v PDAC a jejich roli v materno-fetálním přestupu léčiv odvozených od nukleosidů a rezistenci PDAC vůči terapii GEM.

Jedná se konkrétně o splnění následujících cílů:

I. Popsat expresi a funkci nukleosidových transportérů v placentě a buněčné linii BeWo, testování vlivu diferenciaci nebo epigenetiku ovlivňujících agens na expresi nukleosidových transportérů v BeWo buněčné linii

II. Studovat roli placentárních nukleosidů v materno-fetálním přestupu anti-HIV léčiv ze skupiny NRTI (abakavir, zidovudin a emtricitabin) a anti-HCV ribavirinu; vliv ABC a nukleosidových transportérů na placentární kinetiku.

III. Studovat korelaci mezi mírou exprese ENT1, NOTCH3 a miR-21 a DSS pacientů s resekovaným PDAC léčených adjuvantně GEM.

5 SEZNAM PRACÍ A PODÍL KANDIDÁTKY NA JEDNOTLIVÝCH PUBLIKACÍCH

Tato disertační práce je předkládána jako komentovaný soubor šesti prací publikovaných v zahraničních časopisech s IF.

Kandidátka je první autorkou dvou prací (I a II) a spoluautorkou čtyř prací (III, IV, V a VI). Její podíl na jednotlivých publikacích je následující:

I. Jiraskova L, Cerveny L, Karbanova S, Ptackova Z, Staud F. Expression of Concentrative Nucleoside Transporters (SLC28A) in the Human Placenta: Effects of Gestation Age and Prototype Differentiation-Affecting Agents. Mol Pharm. 2018 Jul 2;15(7):2732-2741. doi: 10.1021/acs.molpharmaceut.8b00238. Epub 2018 May 25.

- praktické provedení buněčných experimentů, analýza dat a příprava manuskriptu

II. Jiraskova L, Ryska A, Duintjer Tebbens EJ, Hornychova H, Cecka F, Staud F, Cerveny L. Are *ENT1*/ENT1, *NOTCH3,* and miR-21 Reliable Prognostic Biomarkers in Patients with Resected Pancreatic Adenocarcinoma Treated with Adjuvant Gemcitabine Monotherapy? Cancers (Basel). 2019 Oct 23;11(11). pii: E1621. doi: 10.3390/cancers11111621.

- praktické provedení většiny experimentů (stanovení genové exprese pomocí RT-PCR), příprava dat pro statistickou analýzu, příprava manuskriptu

III. Karbanova S, Cerveny L, Ceckova M, Ptackova Z, Jiraskova L, Greenwood S, Staud F. Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine. Placenta. 2017 Dec;60:86-92. doi: 10.1016/j.placenta.2017.10.011. Epub 2017 Nov 10.

- podíl na experimentech, konzultace experimentálně získaných dat, revize manuskriptu

IV. Karbanova S, Cerveny L, Jiraskova L, Karahoda R, Ceckova M, Ptackova Z, Staud F. Transport of ribavirin across the rat and human placental barrier: Roles of nucleoside and ATP-binding cassette drug efflux transporters. Biochem Pharmacol. 2019 May;163:60-70. doi: 10.1016/j.bcp.2019.01.024. Epub 2019 Feb 2.

- podíl na experimentech, konzultace experimentálně získaných dat, revize manuskriptu

V. Cerveny L, Ptackova Z, Ceckova M, Karahoda R, Karbanova S, Jiraskova L, Greenwood SL, Glazier JD, Staud F. Equilibrative Nucleoside Transporter 1 (ENT1, *SLC29A1*) Facilitates Transfer of the Antiretroviral Drug Abacavir across the Placenta. Drug Metab Dispos. 2018 Nov;46(11):1817-1826. doi: 10.1124/dmd.118.083329. Epub 2018 Aug 10.

- podíl na provedení akumulačních buněčných experimentů a revizi manuskriptu

VI. Karbanova S, Sorf A, Jiraskova L, Lalinska A, Ptackova Z, Staud F, Cerveny L. S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) is not a selective inhibitor of equilibrative nukleoside transporters but also blocks efflux activity of breast cancer resistence protein. Pharml Res. 2020 Feb 21;37(3):58. doi: 10.1007/s11095-020-2782-5. Epub 2019 Nov 25.

- podíl na konzultaci experimentálně získaných dat, akumulační experimenty provedené na buněčné linii BeWo, revize manuskriptu

6 SEZNAM POUŽITÉ LITERATURY

1. Lombard, J., *Once upon a time the cell membranes: 175 years of cell boundary research.* Biol Direct, 2014. **9**: p. 32.
2. Ling, V., *Drug resistance and membrane alteration in mutants of mammalian cells.* Can J Genet Cytol, 1975. **17**(4): p. 503-15.
3. Giacomini, K.M., Sugiyama, Y, *Goodman and Gilman's Pharmacological basis of therapeutics*. 12th ed. 2011, New York: McGraw Hill Companies.
4. Hillgren, K.M., et al., *Emerging transporters of clinical importance: an update from the International Transporter Consortium.* Clin Pharmacol Ther, 2013. **94**(1): p. 52-63.
5. Schinkel, A.H. and J.W. Jonker, *Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview.* Adv Drug Deliv Rev, 2003. **55**(1): p. 3-29.
6. Staud, F., et al., *Expression and function of p-glycoprotein in normal tissues: effect on pharmacokinetics.* Methods Mol Biol, 2010. **596**: p. 199-222.
7. Staud, F. and P. Pavek, *Breast cancer resistance protein (BCRP/ABCG2).* Int J Biochem Cell Biol, 2005. **37**(4): p. 720-5.
8. Choudhuri, S. and C.D. Klaassen, *Structure, function, expression, genomic organization, and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2 (BCRP) efflux transporters.* Int J Toxicol, 2006. **25**(4): p. 231-59.
9. Li, Y., et al., *The structure and functions of P-glycoprotein.* Curr Med Chem, 2010. **17**(8): p. 786-800.
10. *Digoxin: serious drug interactions.* Prescrire Int, 2010. **19**(106): p. 68-70.
11. de Lannoy, I.A. and M. Silverman, *The MDR1 gene product, P-glycoprotein, mediates the transport of the cardiac glycoside, digoxin.* Biochem Biophys Res Commun, 1992. **189**(1): p. 551-7.
12. Schinkel, A.H., et al., *Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A.* J Clin Invest, 1995. **96**(4): p. 1698-705.
13. Hofman, J., et al., *Interactions of Alectinib with Human ATP-Binding Cassette Drug Efflux Transporters and Cytochrome P450 Biotransformation Enzymes: Effect on Pharmacokinetic Multidrug Resistance.* Drug Metab Dispos, 2019. **47**(7): p. 699-709.
14. Sorf, A., et al., *Cyclin-dependent kinase inhibitors AZD5438 and R547 show potential for enhancing efficacy of daunorubicin-based anticancer therapy: Interaction with carbonyl-reducing enzymes and ABC transporters.* Biochem Pharmacol, 2019. **163**: p. 290-298.
15. Ceckova-Novotna, M., P. Pavek, and F. Staud, *P-glycoprotein in the placenta: expression, localization, regulation and function.* Reprod Toxicol, 2006. **22**(3): p. 400-10.
16. Ushigome, F., et al., *Human placental transport of vinblastine, vincristine, digoxin and progesterone: contribution of P-glycoprotein.* Eur J Pharmacol, 2000. **408**(1): p. 1-10.
17. Doyle, L. and D.D. Ross, *Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2).* Oncogene, 2003. **22**(47): p. 7340-58.
18. Borst, P. and R.O. Elferink, *Mammalian ABC transporters in health and disease.* Annu Rev Biochem, 2002. **71**: p. 537-92.
19. Ceckova, M., et al., *Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo.* Clin Exp Pharmacol Physiol, 2006. **33**(1-2): p. 58-65.
20. Maliepaard, M., et al., *Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues.* Cancer Res, 2001. **61**(8): p. 3458-64.
21. Lee, J.S., et al., *Reduced drug accumulation and multidrug resistance in human breast cancer cells without associated P-glycoprotein or MRP overexpression.* J Cell Biochem, 1997. **65**(4): p. 513-26.

22. Szakacs, G., et al., *The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox).* Drug Discov Today, 2008. **13**(9-10): p. 379-93.
23. Keppler, D., *Multidrug resistance proteins (MRPs, ABCCs): importance for pathophysiology and drug therapy.* Handb Exp Pharmacol, 2011(201): p. 299-323.
24. Deeley, R.G., C. Westlake, and S.P. Cole, *Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins.* Physiol Rev, 2006. **86**(3): p. 849-99.
25. Borst, P., et al., *A family of drug transporters: the multidrug resistance-associated proteins.* J Natl Cancer Inst, 2000. **92**(16): p. 1295-302.
26. Kruh, G.D. and M.G. Belinsky, *The MRP family of drug efflux pumps.* Oncogene, 2003. **22**(47): p. 7537-52.
27. Staud, F., L. Cerveny, and M. Ceckova, *Pharmacotherapy in pregnancy; effect of ABC and SLC transporters on drug transport across the placenta and fetal drug exposure.* J Drug Target, 2012. **20**(9): p. 736-63.
28. Staud, F. and M. Ceckova, *Regulation of drug transporter expression and function in the placenta.* Expert Opin Drug Metab Toxicol, 2015. **11**(4): p. 533-55.
29. Jennings, L.L., et al., *Distinct regional distribution of human equilibrative nucleoside transporter proteins 1 and 2 (hENT1 and hENT2) in the central nervous system.* Neuropharmacology, 2001. **40**(5): p. 722-31.
30. Loffler, M., et al., *Physiological roles of vascular nucleoside transporters.* Arterioscler Thromb Vasc Biol, 2007. **27**(5): p. 1004-13.
31. Pardo, F., et al., *Role of equilibrative adenosine transporters and adenosine receptors as modulators of the human placental endothelium in gestational diabetes mellitus.* Placenta, 2013. **34**(12): p. 1121-7.
32. Pastor-Anglada, M. and S. Perez-Torras, *Who Is Who in Adenosine Transport.* Front Pharmacol, 2018. **9**: p. 627.
33. Salsoso, R., et al., *Adenosine and preeclampsia.* Mol Aspects Med, 2017. **55**: p. 126-139.
34. Jordheim, L.P., et al., *Advances in the development of nucleoside and nucleotide analogues for cancer and viral diseases.* Nat Rev Drug Discov, 2013. **12**(6): p. 447-64.
35. Minuesa, G., et al., *Drug uptake transporters in antiretroviral therapy.* Pharmacol Ther, 2011. **132**(3): p. 268-79.
36. Cerveny, L., et al., *Equilibrative Nucleoside Transporter 1 (ENT1, SLC29A1) Facilitates Transfer of the Antiretroviral Drug Abacavir across the Placenta.* Drug Metab Dispos, 2018. **46**(11): p. 1817-1826.
37. Ma, Z., et al., *Multiple SLC and ABC Transporters Contribute to the Placental Transfer of Entecavir.* Drug Metab Dispos, 2017. **45**(3): p. 269-278.
38. Pastor-Anglada, M. and S. Perez-Torras, *Nucleoside transporter proteins as biomarkers of drug responsiveness and drug targets.* Front Pharmacol, 2015. **6**: p. 13.
39. Pastor-Anglada, M., N. Urtasun, and S. Perez-Torras, *Intestinal Nucleoside Transporters: Function, Expression, and Regulation.* Compr Physiol, 2018. **8**(3): p. 1003-1017.
40. Kong, W., K. Engel, and J. Wang, *Mammalian nucleoside transporters.* Curr Drug Metab, 2004. **5**(1): p. 63-84.
41. Pastor-Anglada, M. and S. Perez-Torras, *Emerging Roles of Nucleoside Transporters.* Front Pharmacol, 2018. **9**: p. 606.
42. Molina-Arcas, M., F.J. Casado, and M. Pastor-Anglada, *Nucleoside transporter proteins.* Curr Vasc Pharmacol, 2009. **7**(4): p. 426-34.
43. Pennycooke, M., et al., *Differential expression of human nucleoside transporters in normal and tumor tissue.* Biochem Biophys Res Commun, 2001. **280**(3): p. 951-9.
44. Gray, J.H., R.P. Owen, and K.M. Giacomini, *The concentrative nucleoside transporter family, SLC28.* Pflugers Arch, 2004. **447**(5): p. 728-34.

45. Jiraskova, L., et al., *Expression of Concentrative Nucleoside Transporters (SLC28A) in the Human Placenta: Effects of Gestation Age and Prototype Differentiation-Affecting Agents.* Mol Pharm, 2018. **15**(7): p. 2732-2741.
46. Govindarajan, R., et al., *In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta.* Am J Physiol Regul Integr Comp Physiol, 2007. **293**(5): p. R1809-22.
47. Iriyama, T., et al., *Elevated placental adenosine signaling contributes to the pathogenesis of preeclampsia.* Circulation, 2015. **131**(8): p. 730-41.
48. Nishimura, T., et al., *Mechanism of nucleoside uptake in rat placenta and induction of placental CNT2 in experimental diabetes.* Drug Metab Pharmacokinet, 2012. **27**(4): p. 439-46.
49. Duflot, S., et al., *ATP-sensitive K(+) channels regulate the concentrative adenosine transporter CNT2 following activation by A(1) adenosine receptors.* Mol Cell Biol, 2004. **24**(7): p. 2710-9.
50. Huber-Ruano, I., et al., *Link between high-affinity adenosine concentrative nucleoside transporter-2 (CNT2) and energy metabolism in intestinal and liver parenchymal cells.* J Cell Physiol, 2010. **225**(2): p. 620-30.
51. Errasti-Murugarren, E., M. Pastor-Anglada, and F.J. Casado, *Role of CNT3 in the transepithelial flux of nucleosides and nucleoside-derived drugs.* J Physiol, 2007. **582**(Pt 3): p. 1249-60.
52. Kermani, I.A., M. Dehdilani, and R. Dolatkhah, *Chronic lymphocytic leukemia in the recent 10 years and treatment effects of Fludarabin.* Asian Pac J Cancer Prev, 2007. **8**(3): p. 367-71.
53. Hiratochi, M., et al., *Hypouricemic effects of novel concentrative nucleoside transporter 2 inhibitors through suppressing intestinal absorption of purine nucleosides.* Eur J Pharmacol, 2012. **690**(1-3): p. 183-91.
54. Gupte, A. and J.K. Buolamwini, *Synthesis and biological evaluation of phloridzin analogs as human concentrative nucleoside transporter 3 (hCNT3) inhibitors.* Bioorg Med Chem Lett, 2009. **19**(3): p. 917-21.
55. Barnes, K., et al., *Distribution and functional characterization of equilibrative nucleoside transporter-4, a novel cardiac adenosine transporter activated at acidic pH.* Circ Res, 2006. **99**(5): p. 510-9.
56. Baldwin, S.A., et al., *The equilibrative nucleoside transporter family, SLC29.* Pflugers Arch, 2004. **447**(5): p. 735-43.
57. Young, J.D., et al., *Human equilibrative nucleoside transporter (ENT) family of nucleoside and nucleobase transporter proteins.* Xenobiotica, 2008. **38**(7-8): p. 995-1021.
58. Karbanova, S., et al., *S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) is Not a Selective Inhibitor of Equilibrative Nucleoside Transporters but Also Blocks Efflux Activity of Breast Cancer Resistance Protein.* Pharm Res, 2020. **37**(3): p. 58.
59. Biotechnology, S., *The Transporters Book*. 2nd ed. 2015, Hungary.
60. Escudero, C., P. Casanello, and L. Sobrevia, *Human equilibrative nucleoside transporters 1 and 2 may be differentially modulated by A2B adenosine receptors in placenta microvascular endothelial cells from pre-eclampsia.* Placenta, 2008. **29**(9): p. 816-25.
61. Espinoza, J., A.F. Espinoza, and G.G. Power, *High fetal plasma adenosine concentration: a role for the fetus in preeclampsia?* Am J Obstet Gynecol, 2011. **205**(5): p. 485 e24-7.
62. Fernandez-Calotti, P. and M. Pastor-Anglada, *All-trans-retinoic acid promotes trafficking of human concentrative nucleoside transporter-3 (hCNT3) to the plasma membrane by a TGF-beta1-mediated mechanism.* J Biol Chem, 2010. **285**(18): p. 13589-98.
63. Flanagan, S.A. and K.A. Meckling, *Nucleoside transporter expression and activity is regulated during granulocytic differentiation of NB4 cells in response to all-trans-retinoic acid.* Leuk Res, 2007. **31**(7): p. 955-68.

64. Guzman-Gutierrez, E., et al., *Differential expression of functional nucleoside transporters in non-differentiated and differentiated human endothelial progenitor cells.* Placenta, 2010. **31**(10): p. 928-36.
65. Jones, K.W., R.J. Rylett, and J.R. Hammond, *Effect of cellular differentiation on nucleoside transport in human neuroblastoma cells.* Brain Res, 1994. **660**(1): p. 104-12.
66. Perez-Torras, S., et al., *Concentrative nucleoside transporter 1 (hCNT1) promotes phenotypic changes relevant to tumor biology in a translocation-independent manner.* Cell Death Dis, 2013. **4**: p. e648.
67. Ye, J.H. and V.M. Rajendran, *Adenosine: an immune modulator of inflammatory bowel diseases.* World J Gastroenterol, 2009. **15**(36): p. 4491-8.
68. Eckle, T., et al., *Crosstalk between the equilibrative nucleoside transporter ENT2 and alveolar Adora2b adenosine receptors dampens acute lung injury.* FASEB J, 2013. **27**(8): p. 3078-89.
69. Errasti-Murugarren, E., et al., *Role of the transporter regulator protein (RS1) in the modulation of concentrative nucleoside transporters (CNTs) in epithelia.* Mol Pharmacol, 2012. **82**(1): p. 59-67.
70. Grden, M., et al., *High glucose suppresses expression of equilibrative nucleoside transporter 1 (ENT1) in rat cardiac fibroblasts through a mechanism dependent on PKC-zeta and MAP kinases.* J Cell Physiol, 2008. **215**(1): p. 151-60.
71. Cheng, X. and C.D. Klaassen, *Regulation of mRNA expression of xenobiotic transporters by the pregnane x receptor in mouse liver, kidney, and intestine.* Drug Metab Dispos, 2006. **34**(11): p. 1863-7.
72. Morote-Garcia, J.C., et al., *Hypoxia-inducible factor-dependent repression of equilibrative nucleoside transporter 2 attenuates mucosal inflammation during intestinal hypoxia.* Gastroenterology, 2009. **136**(2): p. 607-18.
73. Reyes, G., et al., *The Equilibrative Nucleoside Transporter (ENT1) can be phosphorylated at multiple sites by PKC and PKA.* Mol Membr Biol, 2011. **28**(6): p. 412-26.
74. Damaraju, V.L., et al., *Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy.* Oncogene, 2003. **22**(47): p. 7524-36.
75. Seamon, K.B., W. Padgett, and J.W. Daly, *Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells.* Proc Natl Acad Sci U S A, 1981. **78**(6): p. 3363-7.
76. Tomi, M., et al., *Role of protein kinase A in regulating steroid sulfate uptake for estrogen production in human placental choriocarcinoma cells.* Placenta, 2014. **35**(8): p. 658-60.
77. Grden, M., et al., *Diabetes-induced alterations of adenosine receptors expression level in rat liver.* Exp Mol Pathol, 2007. **83**(3): p. 392-8.
78. Leung, G.P., R.Y. Man, and C.M. Tse, *D-Glucose upregulates adenosine transport in cultured human aortic smooth muscle cells.* Am J Physiol Heart Circ Physiol, 2005. **288**(6): p. H2756-62.
79. Valdes, R., et al., *Nutritional regulation of nucleoside transporter expression in rat small intestine.* Gastroenterology, 2000. **119**(6): p. 1623-30.
80. Ahmadimoghaddam, D. and F. Staud, *Transfer of metformin across the rat placenta is mediated by organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) protein.* Reprod Toxicol, 2013. **39**: p. 17-22.
81. Gil, S., et al., *P-glycoprotein expression of the human placenta during pregnancy.* Placenta, 2005. **26**(2-3): p. 268-70.
82. Meyer zu Schwabedissen, H.E., et al., *Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP).* Drug Metab Dispos, 2006. **34**(4): p. 524-33.
83. Kohan-Ghadr, H.R., et al., *Potential role of epigenetic mechanisms in regulation of trophoblast differentiation, migration, and invasion in the human placenta.* Cell Adh Migr, 2016. **10**(1-2): p. 126-35.

84. Spratlin, J., et al., *The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma.* Clin Cancer Res, 2004. **10**(20): p. 6956-61.
85. Napso, T., et al., *The Role of Placental Hormones in Mediating Maternal Adaptations to Support Pregnancy and Lactation.* Front Physiol, 2018. **9**: p. 1091.
86. Tomi, M., T. Nishimura, and E. Nakashima, *Mother-to-fetus transfer of antiviral drugs and the involvement of transporters at the placental barrier.* J Pharm Sci, 2011. **100**(9): p. 3708-18.
87. Yamamoto, T., et al., *Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters.* Eur J Pharmacol, 2007. **557**(1): p. 1-8.
88. Barros, L.F., et al., *Immunolocalisation of nucleoside transporters in human placental trophoblast and endothelial cells: evidence for multiple transporter isoforms.* Pflugers Arch, 1995. **429**(3): p. 394-9.
89. Errasti-Murugarren, E., et al., *Expression and distribution of nucleoside transporter proteins in the human syncytiotrophoblast.* Mol Pharmacol, 2011. **80**(5): p. 809-17.
90. Arshad, M., S.S. El-Kamary, and R. Jhaveri, *Hepatitis C virus infection during pregnancy and the newborn period--are they opportunities for treatment?* J Viral Hepat, 2011. **18**(4): p. 229-36.
91. Azim, H.A., Jr., N. Pavlidis, and F.A. Peccatori, *Treatment of the pregnant mother with cancer: a systematic review on the use of cytotoxic, endocrine, targeted agents and immunotherapy during pregnancy. Part II: Hematological tumors.* Cancer Treat Rev, 2010. **36**(2): p. 110-21.
92. Azim, H.A., Jr., F.A. Peccatori, and N. Pavlidis, *Treatment of the pregnant mother with cancer: a systematic review on the use of cytotoxic, endocrine, targeted agents and immunotherapy during pregnancy. Part I: Solid tumors.* Cancer Treat Rev, 2010. **36**(2): p. 101-9.
93. Brown, R.S., Jr., et al., *Antiviral therapy in chronic hepatitis B viral infection during pregnancy: A systematic review and meta-analysis.* Hepatology, 2016. **63**(1): p. 319-33.
94. Gingelmaier, A., et al., *Mitochondrial toxicity in HIV type-1-exposed pregnancies in the era of highly active antiretroviral therapy.* Antivir Ther, 2009. **14**(3): p. 331-8.
95. Koren, G., et al., *Cancer chemotherapy and pregnancy.* J Obstet Gynaecol Can, 2013. **35**(3): p. 263-278.
96. Wiesweg, M., et al., *Administration of Gemcitabine for Metastatic Adenocarcinoma during Pregnancy: A Case Report and Review of the Literature.* AJP Rep, 2014. **4**(1): p. 17-22.
97. Ni, Z. and Q. Mao, *ATP-binding cassette efflux transporters in human placenta.* Curr Pharm Biotechnol, 2011. **12**(4): p. 674-85.
98. Mathias, A.A., J. Hitti, and J.D. Unadkat, *P-glycoprotein and breast cancer resistance protein expression in human placentae of various gestational ages.* Am J Physiol Regul Integr Comp Physiol, 2005. **289**(4): p. R963-9.
99. Molsa, M., et al., *Functional role of P-glycoprotein in the human blood-placental barrier.* Clin Pharmacol Ther, 2005. **78**(2): p. 123-31.
100. Nanovskaya, T., et al., *Role of P-glycoprotein in transplacental transfer of methadone.* Biochem Pharmacol, 2005. **69**(12): p. 1869-78.
101. Nekhayeva, I.A., et al., *Role of human placental efflux transporter P-glycoprotein in the transfer of buprenorphine, levo-alpha-acetylmethadol, and paclitaxel.* Am J Perinatol, 2006. **23**(7): p. 423-30.
102. Pavek, P., et al., *Influence of P-glycoprotein on the transplacental passage of cyclosporine.* J Pharm Sci, 2001. **90**(10): p. 1583-92.

103. Staud, F., et al., *Expression and transport activity of breast cancer resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line.* J Pharmacol Exp Ther, 2006. **319**(1): p. 53-62.
104. Meyer zu Schwabedissen, H.E., et al., *Variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation.* Drug Metab Dispos, 2005. **33**(7): p. 896-904.
105. Nagashige, M., et al., *Basal membrane localization of MRP1 in human placental trophoblast.* Placenta, 2003. **24**(10): p. 951-8.
106. St-Pierre, M.V., et al., *Expression of members of the multidrug resistance protein family in human term placenta.* Am J Physiol Regul Integr Comp Physiol, 2000. **279**(4): p. R1495-503.
107. May, K., et al., *Role of the multidrug transporter proteins ABCB1 and ABCC2 in the diaplacental transport of talinolol in the term human placenta.* Drug Metab Dispos, 2008. **36**(4): p. 740-4.
108. Yeboah, D., et al., *Expression of breast cancer resistance protein (BCRP/ABCG2) in human placenta throughout gestation and at term before and after labor.* Can J Physiol Pharmacol, 2006. **84**(12): p. 1251-8.
109. Gray, H., *Anatomy of the Human Body* 2000.
110. Backe, E., et al., *Demonstration of HIV-1 infected cells in human placenta by in situ hybridisation and immunostaining.* J Clin Pathol, 1992. **45**(10): p. 871-4.
111. Fondoh, V.N. and N.A. Mom, *Mother-to-child transmission of HIV and its predictors among HIV-exposed infants at Bamenda Regional Hospital, Cameroon.* Afr J Lab Med, 2017. **6**(1): p. 589.
112. Sprecher, S., et al., *Vertical transmission of HIV in 15-week fetus.* Lancet, 1986. **2**(8501): p. 288-9.
113. Best, B.M., et al., *Pharmacokinetics of tenofovir during pregnancy and postpartum.* HIV Med, 2015. **16**(8): p. 502-11.
114. Best, B.M., et al., *Impact of pregnancy on abacavir pharmacokinetics.* AIDS, 2006. **20**(4): p. 553-60.
115. Moodley, J., et al., *Pharmacokinetics and antiretroviral activity of lamivudine alone or when coadministered with zidovudine in human immunodeficiency virus type 1-infected pregnant women and their offspring.* J Infect Dis, 1998. **178**(5): p. 1327-33.
116. Stek, A.M., et al., *Effect of pregnancy on emtricitabine pharmacokinetics.* HIV Med, 2012. **13**(4): p. 226-35.
117. Regine, W.F., et al., *Fluorouracil vs gemcitabine chemotherapy before and after fluorouracil-based chemoradiation following resection of pancreatic adenocarcinoma: a randomized controlled trial.* JAMA, 2008. **299**(9): p. 1019-26.
118. Reznicek, J., et al., *MDR1 and BCRP Transporter-Mediated Drug-Drug Interaction between Rilpivirine and Abacavir and Effect on Intestinal Absorption.* Antimicrob Agents Chemother, 2017. **61**(9).
119. Le, M.P., et al., *Pharmacokinetics, safety and efficacy of ritonavir-boosted atazanavir (300/100 mg once daily) in HIV-1-infected pregnant women.* Antivir Ther, 2015. **20**(5): p. 507-13.
120. Ripamonti, D., et al., *Atazanavir plus low-dose ritonavir in pregnancy: pharmacokinetics and placental transfer.* AIDS, 2007. **21**(18): p. 2409-15.
121. Mesplede, T. and M.A. Wainberg, *Integrase Strand Transfer Inhibitors in HIV Therapy.* Infect Dis Ther, 2013. **2**(2): p. 83-93.
122. Tupova, L., et al., *Interactions between Maraviroc and the ABCB1, ABCG2, and ABCC2 Transporters: An Important Role in Transplacental Pharmacokinetics.* Drug Metab Dispos, 2019. **47**(9): p. 954-960.
123. Kitchen, C.M., et al., *Enfuvirtide antiretroviral therapy in HIV-1 infection.* Ther Clin Risk Manag, 2008. **4**(2): p. 433-9.

124. Hirt, D., et al., *Population pharmacokinetics of tenofovir in HIV-1-infected pregnant women and their neonates (ANRS 12109).* Clin Pharmacol Ther, 2009. **85**(2): p. 182-9.
125. *Recommendations for the Use of Antiretroviral Drugs in Pregnant Women with HIV Infection and Interventions to Reduce Perinatal HIV Transmission in the United States. Department of Health and Human Services.* . Dostupné z https://aidsinfo.nih.gov/guidelines/html/3/perinatal/0. (15.3.2020).
126. WHO, *Antiretroviral drugs for treating pregnant women and preventing HIV infection in infants. Recommendations for a public health approach (2010 version).* Dostupné z http://whqlibdoc.who.int/publications/2010/9789241599818_eng.pdf. (26.2.2020).
127. Kis, O., et al., *The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters.* Trends Pharmacol Sci, 2010. **31**(1): p. 22-35.
128. Marzolini, C., et al., *Transplacental passage of protease inhibitors at delivery.* AIDS, 2002. **16**(6): p. 889-93.
129. Neumanova, Z., et al., *Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta.* AIDS, 2014. **28**(1): p. 9-17.
130. Neumanova, Z., et al., *Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine.* Biopharm Drug Dispos, 2016. **37**(1): p. 28-38.
131. Neumanova, Z., et al., *Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir.* Reprod Toxicol, 2015. **57**: p. 176-82.
132. Nishimura, T., et al., *Enhancement of zidovudine uptake by dehydroepiandrosterone sulfate in rat syncytiotrophoblast cell line TR-TBT 18d-1.* Drug Metab Dispos, 2008. **36**(10): p. 2080-5.
133. Karbanova, S., et al., *Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine.* Placenta, 2017. **60**: p. 86-92.
134. Alter, M.J., *Epidemiology of hepatitis C virus infection.* World J Gastroenterol, 2007. **13**(17): p. 2436-41.
135. Mohd Hanafiah, K., et al., *Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence.* Hepatology, 2013. **57**(4): p. 1333-42.
136. Marine-Barjoan, E., et al., *HCV/HIV co-infection, HCV viral load and mode of delivery: risk factors for mother-to-child transmission of hepatitis C virus?* AIDS, 2007. **21**(13): p. 1811-5.
137. Polis, C.B., et al., *Impact of maternal HIV coinfection on the vertical transmission of hepatitis C virus: a meta-analysis.* Clin Infect Dis, 2007. **44**(8): p. 1123-31.
138. Reid, M., et al., *Contribution of Liver Fibrosis and Microbial Translocation to Immune Activation in Persons Infected With HIV and/or Hepatitis C Virus.* J Infect Dis, 2018. **217**(8): p. 1289-1297.
139. Benova, L., et al., *Vertical transmission of hepatitis C virus: systematic review and meta-analysis.* Clin Infect Dis, 2014. **59**(6): p. 765-73.
140. Novakova, L., et al., *Current antiviral drugs and their analysis in biological materials-Part I: Antivirals against respiratory and herpes viruses.* J Pharm Biomed Anal, 2018. **147**: p. 400-416.
141. Sidwell, R.W., et al., *Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide.* Science, 1972. **177**(4050): p. 705-6.
142. Novakova, L., et al., *Current antiviral drugs and their analysis in biological materials - Part II: Antivirals against hepatitis and HIV viruses.* J Pharm Biomed Anal, 2018. **147**: p. 378-399.
143. Naik, G.S. and M.G. Tyagi, *A pharmacological profile of ribavirin and monitoring of its plasma concentration in chronic hepatitis C infection.* J Clin Exp Hepatol, 2012. **2**(1): p. 42-54.

144. Sinclair, S.M., et al., *The Ribavirin Pregnancy Registry: An Interim Analysis of Potential Teratogenicity at the Mid-Point of Enrollment.* Drug Saf, 2017. **40**(12): p. 1205-1218.
145. Roberts, S.S., et al., *The Ribavirin Pregnancy Registry: Findings after 5 years of enrollment, 2003-2009.* Birth Defects Res A Clin Mol Teratol, 2010. **88**(7): p. 551-9.
146. Karbanova, S., et al., *Transport of ribavirin across the rat and human placental barrier: Roles of nucleoside and ATP-binding cassette drug efflux transporters.* Biochem Pharmacol, 2019. **163**: p. 60-70.
147. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.
148. Gillet, J.P. and M.M. Gottesman, *Mechanisms of multidrug resistance in cancer.* Methods Mol Biol, 2010. **596**: p. 47-76.
149. Robey, R.W., et al., *Revisiting the role of ABC transporters in multidrug-resistant cancer.* Nat Rev Cancer, 2018. **18**(7): p. 452-464.
150. Fletcher, J.I., et al., *ABC transporters as mediators of drug resistance and contributors to cancer cell biology.* Drug Resist Updat, 2016. **26**: p. 1-9.
151. Hofman, J., et al., *Brivanib Exhibits Potential for Pharmacokinetic Drug-Drug Interactions and the Modulation of Multidrug Resistance through the Inhibition of Human ABCG2 Drug Efflux Transporter and CYP450 Biotransformation Enzymes.* Mol Pharm, 2019. **16**(11): p. 4436-4450.
152. Sorf, A., et al., *Ribociclib shows potential for pharmacokinetic drug-drug interactions being a substrate of ABCB1 and potent inhibitor of ABCB1, ABCG2 and CYP450 isoforms in vitro.* Biochem Pharmacol, 2018. **154**: p. 10-17.
153. Burris, H.A., 3rd, et al., *Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.* J Clin Oncol, 1997. **15**(6): p. 2403-13.
154. Cai, J., et al., *Two distinct molecular mechanisms underlying cytarabine resistance in human leukemic cells.* Cancer Res, 2008. **68**(7): p. 2349-57.
155. Li, Q. and Y. Shu, *Role of solute carriers in response to anticancer drugs.* Mol Cell Ther, 2014. **2**: p. 15.
156. Neoptolemos, J.P., et al., *Adjuvant chemotherapy with fluorouracil plus folinic acid vs gemcitabine following pancreatic cancer resection: a randomized controlled trial.* JAMA, 2010. **304**(10): p. 1073-81.
157. Oettle, H., et al., *Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial.* JAMA, 2007. **297**(3): p. 267-77.
158. Neoptolemos, J.P., et al., *Adjuvant 5-fluorouracil and folinic acid vs observation for pancreatic cancer: composite data from the ESPAC-1 and -3(v1) trials.* Br J Cancer, 2009. **100**(2): p. 246-50.
159. Kleeff, J., et al., *Pancreatic cancer.* Nat Rev Dis Primers, 2016. **2**: p. 16022.
160. (US), N.C.I., *PDQ Cancer Information Summaries*. 2018.
161. Bardeesy, N. and R.A. DePinho, *Pancreatic cancer biology and genetics.* Nat Rev Cancer, 2002. **2**(12): p. 897-909.
162. Kommalapati, A., et al., *Contemporary Management of Localized Resectable Pancreatic Cancer.* Cancers (Basel), 2018. **10**(1).
163. Moore, M.J., et al., *Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group.* J Clin Oncol, 2007. **25**(15): p. 1960-6.
164. Louvet, C., et al., *Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial.* J Clin Oncol, 2005. **23**(15): p. 3509-16.

165. Poplin, E., et al., *Phase III, randomized study of gemcitabine and oxaliplatin versus gemcitabine (fixed-dose rate infusion) compared with gemcitabine (30-minute infusion) in patients with pancreatic carcinoma E6201: a trial of the Eastern Cooperative Oncology Group.* J Clin Oncol, 2009. **27**(23): p. 3778-85.
166. Postlewait, L.M., et al., *Combination gemcitabine/cisplatin therapy and ERCC1 expression for resected pancreatic adenocarcinoma: Results of a Phase II prospective trial.* J Surg Oncol, 2016. **114**(3): p. 336-41.
167. Rocha Lima, C.M., et al., *Irinotecan/gemcitabine combination chemotherapy in pancreatic cancer.* Oncology (Williston Park), 2001. **15**(3 Suppl 5): p. 46-51.
168. Gong, J.F., et al., *[Efficacy of gemcitabine-based chemotherapy on advanced pancreatic cancer].* Ai Zheng, 2007. **26**(8): p. 890-4.
169. Neoptolemos, J.P., et al., *Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial.* Lancet, 2017. **389**(10073): p. 1011-1024.
170. Conroy, T., et al., *FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer.* N Engl J Med, 2018. **379**(25): p. 2395-2406.
171. Plunkett, W., et al., *Gemcitabine: preclinical pharmacology and mechanisms of action.* Semin Oncol, 1996. **23**(5 Suppl 10): p. 3-15.
172. Hagmann, W., et al., *Membrane drug transporters and chemoresistance in human pancreatic carcinoma.* Cancers (Basel), 2010. **3**(1): p. 106-25.
173. Marechal, R., et al., *Levels of gemcitabine transport and metabolism proteins predict survival times of patients treated with gemcitabine for pancreatic adenocarcinoma.* Gastroenterology, 2012. **143**(3): p. 664-674 e6.
174. Marechal, R., et al., *Human equilibrative nucleoside transporter 1 and human concentrative nucleoside transporter 3 predict survival after adjuvant gemcitabine therapy in resected pancreatic adenocarcinoma.* Clin Cancer Res, 2009. **15**(8): p. 2913-9.
175. Fujita, H., et al., *Gene expression levels as predictive markers of outcome in pancreatic cancer after gemcitabine-based adjuvant chemotherapy.* Neoplasia, 2010. **12**(10): p. 807-17.
176. Giovannetti, E., et al., *Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine.* Cancer Res, 2006. **66**(7): p. 3928-35.
177. Ashida, R., et al., *m.* J Exp Clin Cancer Res, 2009. **28**: p. 83.
178. Eto, K., et al., *Human equilibrative nucleoside transporter 1 and Notch3 can predict gemcitabine effects in patients with unresectable pancreatic cancer.* Br J Cancer, 2013. **108**(7): p. 1488-94.
179. Bergman, A.M., et al., *In vivo induction of resistance to gemcitabine results in increased expression of ribonucleotide reductase subunit M1 as the major determinant.* Cancer Res, 2005. **65**(20): p. 9510-6.
180. Nakahira, S., et al., *Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer.* Int J Cancer, 2007. **120**(6): p. 1355-63.
181. Dang, L., et al., *Notch3 signaling initiates choroid plexus tumor formation.* Oncogene, 2006. **25**(3): p. 487-91.
182. Mann, C.D., et al., *Notch3 and HEY-1 as prognostic biomarkers in pancreatic adenocarcinoma.* PLoS One, 2012. **7**(12): p. e51119.
183. Drouillard, A., et al., *DLL4 expression is a prognostic marker and may predict gemcitabine benefit in resected pancreatic cancer.* Br J Cancer, 2016. **115**(10): p. 1245-1252.

184. Bauer, A.S., et al., *Diagnosis of pancreatic ductal adenocarcinoma and chronic pancreatitis by measurement of microRNA abundance in blood and tissue.* PLoS One, 2012. **7**(4): p. e34151.
185. Dillhoff, M., et al., *MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival.* J Gastrointest Surg, 2008. **12**(12): p. 2171-6.
186. Giovannetti, E., et al., *MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity.* Cancer Res, 2010. **70**(11): p. 4528-38.
187. Raffenne, J., et al., *hENT1 Testing in Pancreatic Ductal Adenocarcinoma: Are We Ready? A Multimodal Evaluation of hENT1 Status.* Cancers (Basel), 2019. **11**(11).
188. Jiraskova, L., et al., *Are ENT1/ENT1, NOTCH3, and miR-21 Reliable Prognostic Biomarkers in Patients with Resected Pancreatic Adenocarcinoma Treated with Adjuvant Gemcitabine Monotherapy?* Cancers (Basel), 2019. **11**(11).
189. Cygalova, L.H., et al., *Transplacental pharmacokinetics of glyburide, rhodamine 123, and BODIPY FL prazosin: effect of drug efflux transporters and lipid solubility.* J Pharmacol Exp Ther, 2009. **331**(3): p. 1118-25.
190. Gerbaud, P., K. Tasken, and G. Pidoux, *Spatiotemporal regulation of cAMP signaling controls the human trophoblast fusion.* Front Pharmacol, 2015. **6**: p. 202.
191. Janneh, O. and S.H. Khoo, *Interactions of tenofovir, Lamivudine, abacavir and Didanosine in primary human cells.* Pharmaceutics, 2011. **3**(2): p. 326-37.
192. Neoptolemos, J.P., et al., *Effect of adjuvant chemotherapy with fluorouracil plus folinic acid or gemcitabine vs observation on survival in patients with resected periampullary adenocarcinoma: the ESPAC-3 periampullary cancer randomized trial.* JAMA, 2012. **308**(2): p. 147-56.
193. Huang, J., et al., *Cost-effectiveness analysis of adjuvant treatment for resected pancreatic cancer in China based on the ESPAC-4 trial.* Cancer Manag Res, 2018. **10**: p. 4065-4072.
194. Andersson, R., et al., *Gemcitabine chemoresistance in pancreatic cancer: molecular mechanisms and potential solutions.* Scand J Gastroenterol, 2009. **44**(7): p. 782-6.
195. Blackford, A., et al., *SMAD4 gene mutations are associated with poor prognosis in pancreatic cancer.* Clin Cancer Res, 2009. **15**(14): p. 4674-9.
196. Donahue, T.R., et al., *Integrative survival-based molecular profiling of human pancreatic cancer.* Clin Cancer Res, 2012. **18**(5): p. 1352-63.

7 JEDNOTLIVÉ PRÁCE A JEJICH KOMENTÁŘE

Plné znění článků se nachází v příloze.

7.1 Expression of concentrative nucleoside transporters (SLC28A) in the human placenta: effects of gestation age and prototype differentiation-affecting agents

Jirásková Lucie, Červený Lukáš, Karbanová Sára, Ptáčková Zuzana, Štaud František

Molecular Pharmaceutics, 2018; 15(7): p. 2732-2741

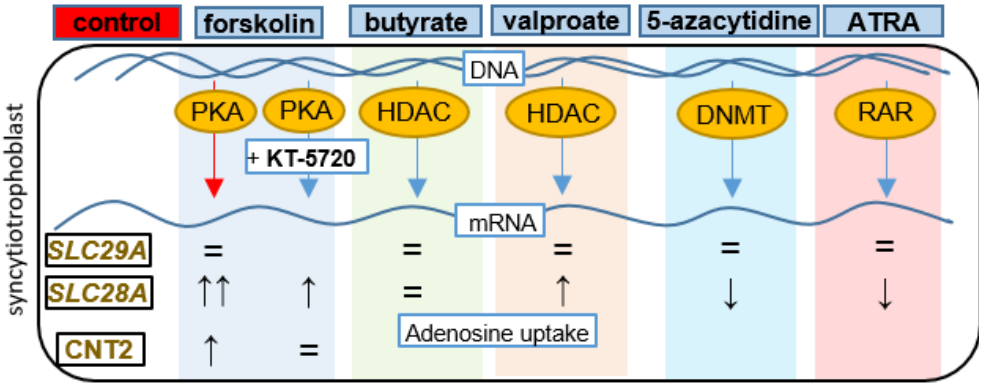
($IF_{[2018]}$ – 4.396, Q1)

Tato práce vychází ze dvou základních hypotéz naznačených již v hlavním textu disertační práce. Protože placenta je rychle se vyvíjející orgán, který vyžaduje dodávku dostatečného množství stavebních látek (včetně nukleosidů) pro správný růst, předpokládali jsme změny exprese nukleosidových transportérů (zejména vysokoafinitních CNTs) v závislosti na délce gestace. Dále byla snaha zodpovědět otázku, zda případná změna exprese je dána mírou diferenciace a/nebo epigenetickou regulací.

Proto cílem tohoto projektu bylo nejprve stanovit a porovnat pomocí kvantitativní reverzní transkriptázové PCR (qRT-PCR) analýzy hladinu exprese CNTs v prvotrimestrální a terminální lidské placentě. Zjistili jsme, že dominantním typem CNT transportéru je v placentě CNT2, přičemž jeho exprese se stejně jako u ostatních zástupců CNT během gestace zvyšuje. Nicméně, mRNA exprese *SLC28A1* byla na hranici detekce v obou gestačních fázích. Obdobný expresní profil mRNA pro geny *SLC28A* jsme pozorovali i v buněčné linii BeWo.

Dále jsme sledovali efekt diferenciačních (forskolin, all-trans-retinoic acid) a epigenom ovlivňujících látek (butyrát sodný, valproát sodný a 5-azacytidin) na expresi *SLC29A* a *SLC28A* v buněčné linii BeWo. Největší efekt způsobil forskolin, který indukcí cAMP/PAK signalizační dráhy upreguloval *SLC28A2*, což se promítlo do zvýšené míry vychytávání adenosinu v buněčné linii BeWo. Exprese ENT transportérů nebyla ovlivněna žádnou z testovaných látek, což naznačuje jejich možnou konstitutivní expresi (Obr. 4). Výsledky naší studie přináší nové poznatky o regulaci NTs, a to především CNT2. Lze tedy spekulovat, že substráty CNT2 budou vychytávány ve větší míře do terminální placenty a/nebo placenty se zvýšenou aktivitou

adenylylcyklázy. Upregulace CNT2 způsobená forskolinem byla pozorována i buněčné linii JEG-3, která nevytváří mnohojaderný syncytiotrofoblast. Proto předpokládáme, že zvýšené hladiny SLC28A2/CNT2 nesouvisí s diferenciací cytotrofoblastu v syncytiotrofoblast.



Obr. 4: Schématické znázornění efektu diferenciaci nebo epigenetiku ovlivňujících látek na mRNA expresi nukleosidových transportérů v BeWo buněčné linii. Z testovaných látek měl nejvyšší efekt forskolin, který signifikantně indukoval expresi CNT2. Tato indukce byla současně inhibována inhibitorem PKA, KT-5720. Zvýšení CNT2 bylo potvrzeno v BeWo buňkách i na úrovni funkce.

Grafický abstrakt publikace I [45].

PKA = proteinkináza A; HDAC = histondeacetyláza; DNMT = DNA metyltransferáza; RAR = retinoic acid receptor

7.2 Are *ENT1*/ENT1, *NOTCH3,* and miR-21 reliable prognostic biomarkers in patients with resected pancreatic adenocarcinoma treated with adjuvant gemcitabine monotherapy?

Jirásková Lucie, Ryška Aleš, Duintjer Tebbens Erik Jurjen, Hornychová Helena, Čečka Filip, Štaud František, Červený Lukáš

Cancers, 2019; 11(11): pi. E1621

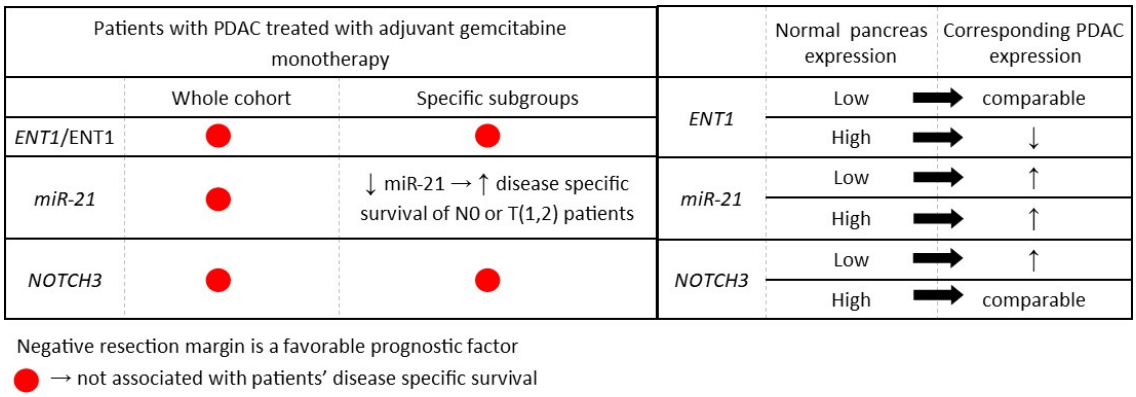
($IF_{[2018/2019]}$ – 6.162, Q1)

Tato práce vychází z hypotézy, že vyšší množství membránově vázaného ENT1 a nižší exprese molekul *NOTCH3* a miR-21 může být asociována s delším DSS u pacientů s PDAC léčených adjuvantně GEM.

Cílem tohoto projektu bylo využití tenkých parafínových řezů PDAC a zdravé tkáně pankreatu získaných z uniformní skupiny pacientů definované striktně resekcí PDAC a adjuvantní léčbou GEM, pro kvantifikaci potenciálních rakovinných biomarkerů *SLC29A1*/ENT1, *NOTCH3* a miR-21 a asociaci těchto markerů s odpovědí pacienta na léčbu GEM.

V rámci výzkumu jsme pomocí qRT-PCR analýzy zjistili, že nižší exprese *miR-21* zvyšuje DSS (disease-specific survival) u pacientů s negativním nálezem metastází v lymfatických uzlinách a stádiem tumoru 1 a 2. Dále jsme zjistili, že velmi důležitým prognostickým faktorem je parametr R0 („negative resection margins“). Množství *SLC29A1* a *NOTCH3* v našem souboru pacientů nekorelovalo s DSS pacientů. Tento negativní výsledek byl v případě *SLC29A1* potvrzen i pomocí imunohistochemické analýzy s anti-ENT1 protilátkou 10D7G2 (Obr 5). Tato publikace přinesla i několik zajímavých zjištění týkajících se expresního profilu *SLC29A1*, *NOTCH3* a miR-21 v nádorové a nenádorové tkáni. V naší kohortě vzorku nádorová tkáň měla v porovnání s nenádorovou tkání signifikantně nižší mRNA expresi *SLC29A1* a vyšší v případě *NOTCH3* a miR-21. Další data této studie naznačují zvýšené riziko down-regulace *SLC29A1* u pacientů s vyšší expresí *SLC29A1* v nenádorové tkáni pankreatu. Exprese *NOTCH3* má tendenci se zvyšovat pouze u pacientů s nízkou expresí v nenádorové tkáni, zatímco ke zvýšení miR-21 pravděpodobně dochází nezávisle na hladinách v normálním tkáni pankreatu (Obr. 5).

Naše data nevylučují potenciální budoucí použití *SLC29A1* a miR-21 jako prognostických biomarkerů pro personalizaci léčby GEM u pacientů s PDAC po resekci. Naznačují však, že tento postup ještě není připraven k implementaci do klinického prostředí.



Obr. 5: Schématické znázornění kvantifikace potenciálních prognostických biomarkerů *SLC29A1*/ENT1, *NOTCH3* a miR-21 a asociaci těchto markerů s DSS pacientů s PDAC adjuvantně léčenými GEM.

Grafický abstrakt publikace II [188].

N0 = negativní nález metastází v lymfatických uzlinách; T(1,2) = stádium tumoru 1 a 2

7.3 Role of nucleoside transporters in transplacental pharmacokinetics of nukleoside reverse transcriptase inhibitors zidovudine and emtricitabine

Karbanová Sára, Červený Lukáš, Čečková Martina, Ptáčková Zuzana, Jirásková Lucie, Greenwood S, Štaud František

Placenta, 2017; 60: p. 86-92

($IF_{[2017]}$ – 2.434)

Zidovudin a emtricitabin jsou efektivní antiretrovirotika s dobrým bezpečnostním profilem, která jsou součástí doporučovaných farmakoterapeutických postupů používaných k léčbě a prevenci perinatálního přenosu HIV z matky na dítě. Jedná se o léčiva s popsaným vysokým placentárním přestupem, avšak přesný mechanismus přenosu těchto léčiv přes placentu není zatím zcela znám.

AZT i FTC jsou léčiva odvozená od nukleosidů, proto jsme předpokládali, že se na jejich placentárním přestupu mohou podílet ekvilibrativní nukleosidové transportéry, ENT1 a ENT2.

Pomocí akumulačních studií provedených na buněčné linii BeWo a čerstvých vilózních fragmentech izolovaných z lidské placenty jsme sledovali, zda ENT1 a ENT2 usnadňují vychytávání zidovudinu a amtricitabinu. Experimenty *in situ* provedené pomocí duální perfuze potkaní placenty byly posléze využity ke sledování, zda ENT1 a/nebo ENT2 ovlivňují materno-fetální a/nebo feto-maternální přestup zidovudinu a emtricitabinu. Placentární akumulace zidovudinu i emtricitabunu nebyla ovlivněna přítomností NBMPR při koncentracích 0,1 µM a 0,1 mM. Na základě těchto výsledků jsme vyvodili závěr, že ani ENT1 ani ENT2 se nepodílejí na transplacentárním přenosu zidovudinu a emtricitabinu.

7.4 Transport of ribavirin across the rat and human placental barrier: roles of nucleoside and ATP-binding cassette drug efflux transporters

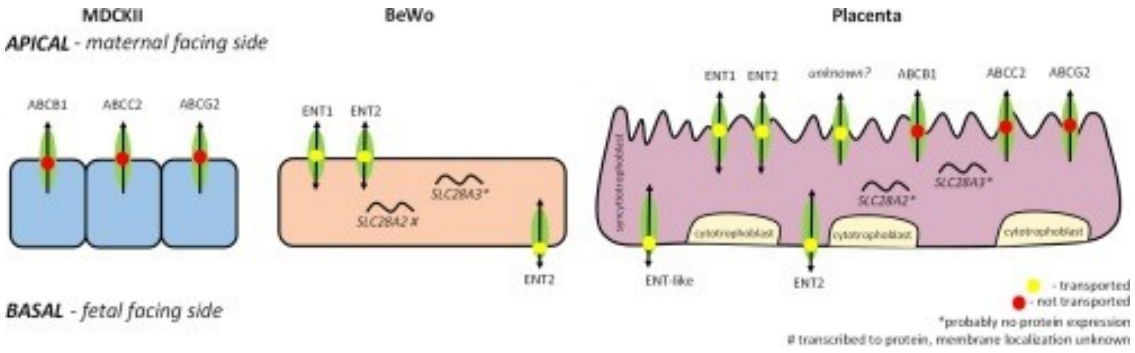
Karbanová Sára, Červený Lukáš, Jirásková Lucie, Karahoda Rona, Čečková Martina, Ptáčková Zuzana, Štaud František

Biochemical Pharmacology, 2019; 163: p. 60-70.

($IF_{[2018/2019]}$ – 4.825, Q1)

Ribavirin je nukleosidový derivát se širokou antivirální aktivitou užívaný v kombinované farmakoterapii HCV. Studie na zvířecích modelech prokázaly teratogenitu ribavirinu, nicméně dosud získaná data o jeho užití těhotných žen, zatím teratogenitu u člověka nepotvrdily. Je tak jedním z kandidátů pro léčbu chronické hepatitidy C u těhotných žen a prevenci přenosu viru z matky na plod. Aby bylo možné ribavirin v těhotenství užívat je kromě vlivu na plod nutné popsat mechanismy přispívající k materno-fetálnímu prostupu ribavirinu. V této studii jsme se tedy zaměřili na roli nukleosidových transportérů a ABC transportérů v placentární kinetice ribavirinu.

Naše výsledky poukázaly na to, že ENT1 signifikantně přispívá k vychytávání ribavirinu v BeWo buněčné linii, placentárních vilózních fragmentech a veziklech z izolované mikrovilózní membrány (MVM), zatímco CNTs, pravděpodobně CNT2, se podílí na transportu ribavirinu pouze v BeWo buněčné linii. Metodou *in situ* duálně perfundované potkaní placenty (otevřené cirkulace) jsme inhibicí ENTs prokázali snížení materno-fetální i feto-maternální clearance. Zapojení ABC transportérů (ABCB1, ABCG2 a ABCC2) v transplacentárním přenosu ribavirinu nebylo pomocí experimentů s MDCKII buněčnou linií a uzavřené duální perfundované potkaní placenty prokázáno (Obr. 6). Tato data významně přispívají k pochopení mechanismů podílejících se na placentárním přestupu ribavirinu a dávají podnět k dalšímu studiu, např. korelace exprese placentárního ENT1 s koncentrací ribavirinu ve fetální cirkulaci, případně dopad lékových interakcí na ENT1 a na hladiny ribavirinu v krvi plodu.



Obr. 6: Schématické znázornění exprese a transportní funkce jednotlivých transportérů v přenosu ribavirinu v různých experimentálních modelech.

Grafický abstrakt publikace IV [146].

7.5 Equilibrative nucleoside transporter 1 (ENT1, *SLC29A1*) facilitates transfer of the antiretroviral drug abacavir across the placenta.

Červený Lukáš, Ptáčková Zuzana, Čečková Martina, Karahoda Rona, Karbanová Sára, Jirásková Lucie, Greenwood Susan, Glazier Jocelyn, Štaud František.

Drug Metabolism and Disposition, 2018; 46(11): p. 1817-1826

($IF_{[2018]}$ – 3.354, Q2)

Abakavir je antiretrovirotikum odvozené od nukleosidu, které je součástí doporučovaných kombinovaných režimů pro prevenci vertikálního přenosu viru HIV z matky na dítě v průběhu těhotenství. Je považován za léčivo s vysokým transplacentárním přestupem, přičemž jeho přítomnost ve fetální cirkulaci zajišťuje profylaxi plodu či případně eliminuje virus, který přestoupí placentu. V této publikaci jsme se tak zaměřili na nukleosidové transportéry a jejich roli v materno-fetálním přestupu abakaviru.

Pro potvrzení této hypotézy, jsme provedli akumulační experimenty na i) buněčné linii BeWo, odvozené od lidského placentárního choriokarcinomu, ii) v lidských vilózních fragmentech a iii) v mikrovilózních (MVM) veziklech. Nejprve bylo nutné ověřit aktivitu nukleosidových transportérů v těchto experimentálních modelech. Použitím [^{3}H]-adenosinu (modelový substrát ENTs, CNT2 a CNT3) a [^{3}H] thymidinu (modelový substrát pro ENTs, CNT1 a CNT3) jsme prokázali signifikantní aktivitu ENT1 a CNT2 v BeWo buněčné linii, přičemž experimenty na vilózních fragmentech a MVM veziklech poukázaly pouze na ENT1 aktivitu. Placentární vychytávání [^{3}H]-abakaviru bylo inhibováno NBMPR (0,1 µM), což demonstruje, že ENT1 usnadňuje vychytávání abakaviru placentou. Role CNTs byla pozorována pouze v BeWo buňkách. Abychom ověřili, že ENT1 ovlivňuje i přenos přes placentu, provedli jsme duální perfuzi potkaní placenty. Tyto experimenty ukázaly, že Ent1 významně přispívá k celkovému transportu [^{3}H]-abakaviru přes placentu. Na závěr jsme kvantifikovali expresi ENT1 a ENT2 ve vzorcích prvotrimestrálních a terminálních placent. Prokázali jsme, že jejich exprese se v průběhu gestace nemění, nicméně je značně variabilní, což by mohlo být předmětem dalších studií zabývajících se vztahem mezi expresí ENT1 v placentě a koncentracemi abakaviru ve fetální krvi.

7.6 S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) is not a selective inhibitor of equilibrative nukleoside transporters but also blocks efflux activity of breast cancer resistence protein

Karbanová Sára, Šorf Aleš, Jirásková Lucie, Lalinská Anežka, Ptáčková Zuzana, Štaud František, Červený Lukáš.

Pharmaceutical Research, 2020; 37(3): p. 58.

($IF_{[2018/2019]}$ – 3.896, Q1)

S-(4-Nitrobenzyl)-6-thioinosin (NBMPR) je od roku 1972 rutinně používám jako specifický inhibitor ekvilibrativních transportérů ENT1 a ENT2. ENT1 je NBMPR sensitivní transportér a je inhibován v mikromolárních koncentracích NBMPR. ENT2 je méně sensitivní a je inhibován v milimolárních koncentracích NBMPR. Při studiu role transportérů v placentární kinetice zidovudinu a TDF jsme zjistili, že NBMPR (0,1 mM) inhibuje aktivní feto-maternální přestup těchto látek. Protože zidovudin a TDF jsou popsanými substráty ABCB1 a ABCG2, rozhodli jsme se testovat hypotézu, že NBMPR tyto transportéry inhibuje.

Při studiu jsme pomocí akumulačních studií provedených na *in vitro* modelu placentární bariéry (buněčná linie BeWo) nejdříve ověřili, že TDF, stejně jako zidovudin [133] není substrátem nukleosidových transportérů. Dále jsme provedli na buněčné linii MDCKII (parentní linii a linii exprimující příslušný lidský transportér) i) akumulační studie, kde jsme využili fluorescenční modelový substrát ABCB1/ABCG2, Hoechst 33342, a specifické inhibitory ABCB1 a ABCG2 a ii) obousměrný transport přes buněčnou monovrstvu s radioaktivně značeným [^{3}H]-glyburidem, který je považován za preferenční substrát ABCG2 [189]. Na závěr jsme provedli *in situ* duální perfuzi potkaní placenty s [^{3}H]-glyburidem.

Pomocí těchto metod jsme zjistili, že NBMPR v koncentraci 0,1 mM zvyšuje akumulaci Hoechst 33342 v MDCKII-ABCG2 buňkách. V transportním experimentu s [^{3}H]-glyburidem přes MDCKII-ABCG2 jsme pozorovali vyšší transport ve směru bazolaterálně-apikálním než apikálně-bazolaterálním, přičemž tento transport byl inhibován NBMPR (0,1 mM). NBMPR (0,1 mM) také inhiboval aktivní feto-maternílní transport [^{3}H]-glyburidu Tato publikace tedy jako první přináší důkazy, že NBMPR v koncentraci 0,1 mM, která je běžně používán k inhibici ENT1 a ENT2, inhibuje transport nukleosidů a nukleosidových derivátů zprostředkovaný, nejen ENT1/ENT2, ale inhibuje také aktivitu ABCG2.

8 ZÁVĚR

Membránové transportéry jsou již po řadu desetiletí intenzivně studovaným tématem v mnoha laboratořích na celém světě. Tuto pozornost si získaly mimo jiné díky jejich nezastupitelné roli v procesech spojených s lékovým transportem přes buněčnou membránu. Dlouhodobě je diskutováno jejich zapojení v rozvoji lékové rezistence snižující efektivitu protinádorové terapie, nicméně zásadní zůstává i jejich podíl na absorpci, distribuci a exkreci léčiv. Pokud jde o distribuci, bylo popsáno, že ABCB1, ABCG2 a ABCC2 svou efluxní aktivitou snižují materno-fetální přestup některých látek, a představují tak aktivní složku placentární ochrany vyvíjejícího se plodu [129, 130]. Na druhé straně stojí nukleosidové transportéry, jejichž role je zřejmě především nutriční (příjem nukleosidů placentou) [41], nicméně se také podílejí na placentárním přestupu látek odvozených od nukleosidů [42]. V porovnání s ABC transportéry je skupina placentárních nukleosidových transportérů mnohem méně studována. V rámci řešení této disertační práce jsme popsali expresi nukleosidových transportérů na úrovní mRNA v prvotrimestrální a terminální lidské placentě (publikace I) a přinesli jsme důkazy, že exprese nízkoafinitních, ale vysokokapacitních ENTs je v průběhu gestace konstitutivní, zatímco exprese vysokoafinitních, ale nízkokapacitních CNTs v průběhu gestace pravděpodobně roste. Naše domněnka, že exprese ENTs je v placentě konstitutivní byla dále podpořena daty z buněčné linie BeWo, kde jsme nepozorovali žádný efekt látek ovlivňujících diferenciaci nebo epigenom, zatímco CNTs (zejména CNT2) byly mnohem náchylnější ke změně exprese, což je plně v souladu s literaturou [39, 41]. Jako zásadní regulační faktor se ukázala být aktivace signalizační dráhy adenylylcykláza/cAMP/PKA. Tato dráha je důležitým faktorem při přeměně cytotrofoblastu na syncytiotrofoblast [190]. Naše výsledky ale ukázaly, že exprese a aktivita CNT2 se zvyšuje při aktivaci adenylylcyklázy bez ohledu na tento diferenciační proces (publikace I), přičemž předpokládáme, že nárůst CNT2 může souviset se zvětšujícími se nároky placenty na dodávky nukleosidů v průběhu gestace [27, 28].

Dále jsme prokázali, že ENT1 usnadňuje materno-fetální transfer antiretrovirotika abakaviru (publikace V). Kromě relativně vysoké lipofility [191] bude interakce s placentárním ENT1 důvodem popsaného vysokého placentární přestupu této látky. Na druhou stranu naše práce demonstrovala, že ENT1 je zásadním přenašečem určujícím rychlost placentárního přestupu ribavirinu (publikace IV). V této práci jsme navíc přinesli důkazy, že placentární kinetika ribavirinu není ovlivňována aktivitou ABCB1, ABCG2 a ABCC2 (publikace IV). Další testované látky ze skupiny NRTIs, emtricitabin a zidovudin, s placentárními nukleosidovými transportéry neinteragovaly (publikace III). Při řešení interakcí placentárních nukleosidových transportérů se zidovudinem a další látkou ze skupiny NRTIs, TDF (oba popsané substráty ABCB1 a ABCG2

transportéru [129, 130]) jsme zaznamenali inhibiční efekt NBMPR (0,1 mM) na aktivní feto-maternální transport obou látek. Testovali jsme tedy hypotézu, že NBMPR při této koncentraci inhibuje ABCB1 a/nebo ABCG2. Práce (publikace VI), pak poprvé popsala NBMPR v koncentraci 0,1 mM jako neselektivní inhibitor ENTs a ABCG2. Toto zjištění bude tedy nutné zohledňovat při navrhování experimentů využívajících NBMPR v koncentraci 0,1 mM a interpretaci výsledků získaných se substráty, u nichž je předpoklad, že jejich membránový přenos je ovlivňován ENTs a zároveň ABCG2. Týká se to zejména tkání s velkým množstvím těchto transportérů, mezi které patří (syncytiotrofoblast, hepatocyty a střevní tkáň) [5, 18, 28].

Role ENT1 byla sledována také v retrospektivní klinické studii zabývající se korelací exprese vybraných potenciálních prognostických markerů a délkou DSS pacientů s PDAC adjuvantně léčených GEM. GEM je stále lékem volby u pacientů s PDAC [117, 153, 157, 169, 192]. Důvodem je nejlepší poměr mezi finančními náklady na terapii, efektivitou a dále relativně akceptovatelnou toxicitou [193]. Efektivita léčby může být významně zlepšena personalizovaným podáním GEM, a to ve správné dávce a pouze pacientům, kteří mohou z léčby profitovat [173, 175]. Proto je snaha hledat prognostické/prediktivní molekuly, které by umožnily s ohledem na terapii GEM klinikům identifikovat ty pacienty, kteří budou z léčby profitovat ve smyslu prodloužení života či prodloužení doby bez progrese onemocnění. Protože se jedná o hydrofilní léčivo, jeho přestup přes buněčnou membránu závisí na přítomnosti transportérů, a to zejména ENT1 [84, 194]. Korelace nízké exprese ENT1 s prognózou pacientů s PDAC je tedy celkem zevrubně studována [84, 172-175], nicméně i přes téměř dvě dekády trvající výzkum není dostatek dat, aby bylo možné vytvořit klinické doporučení pro stratifikaci pacientů využívající ENT1 jako prognostického či prediktivního markeru. Jedním z důvodů je neexistence jednotných protokolů pro kvantifikaci ENT1 v PDAC a studie provedené na heterogenních skupinách pacientů. V této práci jsme provedli paralelní analýzu exprese ENT1 pomocí dvou odlišných metodických přístupů - kvantifikace počtu transkriptů pomocí real-time PCR a imunohistochemické analýzy v tenkých parafínových řezech tkáně pankreatu u jednotně definované skupiny pacientů s PDAC po resekci s adjuvantní léčbou pouze GEM.

Naše výsledky nepotvrdily korelaci mezi expresí ENT1 a prognózou pacientů. Zjistili jsme však, že existuje pravděpodobně zvýšené riziko down-regulace ENT1, u pacientů, kteří mají vyšší expresi ENT1 ve zdravé tkáni pankreatu.

Dalšími studovanými potenciálními markery v této práci byly NOTCH3 a miR-21, jejichž prognostická role v PDAC byla doposud studována jen velmi málo [178, 182, 184-186]. Jako první jsme se pokusili zjistit prognostickou hodnotu transkriptů markeru NOTCH3 u pacientů s PDAC adjuvantně léčenými GEM ve smyslu délky DSS pacientů, avšak nepozorovali jsme žádnou souvislost mezi hladinami NOTCH3 v PDAC a delším DSS. Nízká exprese miR-21 byla významná

pouze u pacientů s negativním nálezem metastází v lymfatických uzlinách (N0) a stádiem tumoru 1 a 2. Exprese NOTCH3 je zvýšena u pacientů s PDAC s nízkou expresí v normálním pankreatu, zatímco zvýšení exprese miR-21 u pacientů s PDAC není závislé na hladinách v normálním pankreatu.

Nepotvrdili jsme dříve publikovanou korelaci mezi množstvím ENT1 nebo miR-21 a DSS pacientů s PDAC léčených adjuvantně GEM. Tyto výsledky však mohly být ovlivněny řadou faktorů, např. vysokým zastoupením pacientů s R1, N1 a T(3,4) v kohortě, či vysokým zastoupení pacientů s významným množstvím markerů negativně ovlivňujících progresi PDAC [195, 196]. Řada pacientů v naší kohortě také ukončila GEM terapii předčasně. Kromě tohoto nelze vyloučit, že naše kohorta měla celkově nízkou expresi ENT1 a/nebo vysokou v případě miR-21. Naše data nevylučují potenciál využití ENT1 a miR-21 jako prognostických biomarkerů u resekovaných pacientů s PDAC. Naznačují však, že tento postup ještě není připraven k implementaci do klinických procesů.

Naše výsledky přispívají do mozaiky informací o placentárním přestup a mohou přispět k upřesnění terapie těhotných žen s HIV či HCV případně k rozvoji „on table“ modelů či PBPK computing modelů. Dále naše výsledky ukázaly, že v případě personalizované léčby pacientů s PDAC léčených adjuvantně je nutné dále upřesnit protokol evaluace prognostických markerů a zaměřit se na více specifikované pacienty, jelikož ENT1 není jedinou biomolekulou ovlivňující odpověď pacienta na léčbu.

9 SEZNAM DOPOSUD PUBLIKOVANÝCH PRACÍ KANDIDÁTKY

9.1 Recenzované publikace v odborných časopisech s IF týkající se tématu práce

Jiraskova, L., Cerveny, L., Karbanova, S., Ptackova, Z., Staud, F., ***Expression of Concentrative Nucleoside Transporters (SLC28A) in the Human Placenta: Effects of Gestation Age and Prototype Differentiation-Affecting Agents***. Molecular Pharmaceutics, 2018. 15(7): p. 2732-2741.

($IF_{[2018]}$ – 4.396, Q1)

Jiraskova, L., Ryska, A., Duintjer Tebbens, EJ., Hornychova, H., Cecka, F., Staud, F., Cerveny, L., ***Are ENT1/ENT1, NOTCH3, and miR-21 Reliable Prognostic Biomarkers in Patients with Resected Pancreatic Adenocarcinoma Treated with Adjuvant Gemcitabine Monotherapy?*** Cancers (Basel), 2019. 11(11): p. E1621.

($IF_{[2019]}$ – 6.162, Q1)

Karbanova, S., Cerveny, L., Ceckova, M., Ptackova, Z., Jiraskova, L., Greenwood, S., Staud, F., ***Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine***. Placenta, 2017. 60: p. 86-92.

($IF_{[2017]}$ – 2.434, Q1)

Karbanova, S., Cerveny, L., Jiraskova, L., Karahoda, R., Ceckova, M., Ptackova, Z., Staud, F., ***Transport of ribavirin across the rat and human placental barrier: Roles of nucleoside and ATP-binding cassette drug efflux transporters.*** Biochemical Pharmacology. 2019. 163: p. 60-70.

($IF_{[2018/2019]}$ – 4.825, Q1)

Cerveny, L., Ptackova, Z., Ceckova, M., Karahoda, R., Karbanova, S., Jiraskova, L., Greenwood, SL., Glazier, JD., Staud, F., ***Equilibrative Nucleoside Transporter 1 (ENT1, SLC29A1) Facilitates Transfer of the Antiretroviral Drug Abacavir across the Placenta.*** Drug Metabolism and Disposition, 2018. 46(11): p. 1817-1826.

($IF_{[2018]}$ – 3.354, Q2)

Karbanova, S., Sorf, A., Jiraskova, L., Lalinska, A., Ptackova, Z., Staud, F., Cerveny, L., ***S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) is not a selective inhibitor of equilibrative nucleoside transporters but also blocks efflux activity of breast cancer resistance protein.*** Pharmaceutical Research, 2020. 37(3): p. 58.

($IF_{[2018/2019]}$ – 3.896, Q1)

9.2 Přednášky na konferencích

Jiraskova L., Cerveny L., Staud F. **Differentiation and epigenome affecting drugs change expression of nucleoside transporters in placental BeWo cell line.**

6. Postgraduální a 4. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 9. – 10. února 2016, Hradec Králové, Česká republika

Jiraskova L., Cerveny L., Ryska A., Hornychova H., Cecka F., Staud F. **Human equilibrative nucleoside transporter 1, NOTCH3 and micro RNA-21 can predict gemcitabine effects in patients with pancreactic cancer.**

7. Postgraduální a 5. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 7. – 8. února 2017, Hradec Králové, Česká republika

Jiraskova L., Cerveny L., Karbanova S., Ryska A., Hornychova H., Cecka F., Tebbens E.J.D., Ceckova M., Ptackova Z., Karahoda R., Staud F. **Nucleoside transporters; role in pharmacokinetics and mechanisms of regulation.**

8. Postgraduální a 6. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 24. – 25. ledna 2018, Hradec Králové, Česká republika

9.3 Postery prezentované na konferencích

Jiraskova L., Cerveny L., Staud F. **Epigenome and differentiation affecting drugs change expression of nucleoside transporters in placental BeWo and JEG-3 cell lines.**

65. Farmakologické dny, 16. – 18. září 2015, Praha, Česká republika

Jiraskova L., Cerveny L., Staud F. **Expression of nucleoside transporters in BeWo cells is changed by differentiation and epigenome affecting drugs.**

SOLVO Biotechnology, Meet the Experts Transporter Conference, 10. - 14.5. 2016, Budapešť 2016

Jiraskova L., Cerveny L., Staud F. **Changes of expression of nucleoside transporters in human choriocarcinoma derived BeWo cell line are mediated by differentiation and epigenome affecting drugs.**

Slovak Toxicology Society, 21. 6. - 24. 6. 2016, Stará Lesná, Slovensko

Jiraskova L., Cerveny L., Staud F. **Expression of nucleoside transporters in human choriocarcinoma derived BeWo cells is changed by differentiation and epigenome affecting drugs.**

66. Farmakologické dny, 13. – 15. září 2016, Brno, Česká republika

Jiraskova L., Cerveny L., Ryska A., Hornychova H., Cecka F., Staud F. **Human equilibrative nucleoside transporter 1, Notch3 and micro RNA-21 can predict survival after gemcitabine therapy in resected pancreactic adenocarcinoma.**

10th international BioMedical Transporters Conferences, 6. – 10. srpna 2017, Lausanne, Švýcarsko

Jiraskova L., Cerveny L., Ryska A., Hornychova H., Tebbens EJD., Cecka F., Staud F. **Predictive role of human ENT1, NOTCH3 and miRNA-21 for patient survival after gemcitabine therapy of resected pancreatic adenocarcinoma.**

68. Farmakologické dny, 5. – 7. září 2018, Hradec Králové, Česká republika

Jiraskova L., Cerveny L., Karbanova S., Ptackova Z., Staud F. **Expression of concentrative nucleoside transporters (*SLC28A*) in the human placenta; effects of gestation age and prototype differentiation-affecting agents**

22nd North American ISSX Meeting, 15. – 19. července, 2018, Montreal, Kanada

10 ODBORNÁ STÁŽ

University of Graz, 17. - 22. července 2016, Rakousko

V rámci zahraniční stáže jsem si osvojila metodu na izolaci placentárních trofoblastů. Tuto metodu jsme zavedli na našem pracovišti.

11 PŘÍLOHY

Publikace I: Expression of Concentrative Nucleoside Transporters (SLC28A) in the Human Placenta: Effects of Gestation Age and Prototype Differentiation-Affecting Agents.

molecular pharmaceutics

Article

Cite This: *Mol. Pharmaceutics* 2018, 15, 2732−2741

Expression of Concentrative Nucleoside Transporters (*SLC28A*) in the Human Placenta: Effects of Gestation Age and Prototype Differentiation-Affecting Agents

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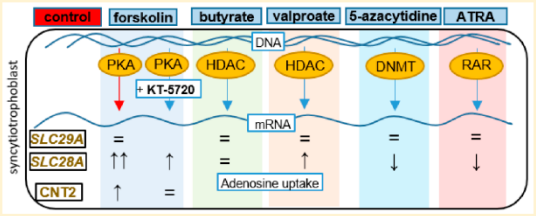
ABSTRACT: Equilibrative (*SLC29A*) and concentrative (*SLC28A*) nucleoside transporters contribute to proper placental development and mediate uptake of nucleosides/nucleoside-derived drugs. We analyzed placental expression of *SLC28A* mRNA during gestation. Moreover, we studied in choriocarcinoma-derived BeWo cells whether *SLC29A* and *SLC28A* mRNA levels can be modulated by activity of adenylyl cyclase, retinoic acid receptor activation, CpG islands methylation, or histone acetylation, using forskolin, all-*trans*-retinoic acid, 5-azacytidine, and sodium butyrate/sodium valproate, respectively. We found that expression of *SLC28A1*, *SLC28A2*, and *SLC28A3* increases during gestation and reveals considerable interindividual variability. *SLC28A2* was shown to be a dominant subtype in the first-trimester and term human placenta, while *SLC28A1* exhibited negligible expression in the term placenta only. In BeWo cells, we detected mRNA of *SLC28A2* and *SLC28A3*. Levels of the latter were affected by 5-azacytidine and all-*trans*-retinoic acid, while the former was modulated by sodium valproate (but not sodium butyrate), all-*trans*-retinoic acid, 5-azacytidine, and forskolin that caused 25-fold increase in *SLC28A2* mRNA; we documented by analysis of *syncytin-1* that the observed changes in *SLC28A* expression do not correlate with the morphological differentiation state of BeWo cells. Upregulated *SLC28A2* mRNA was reflected in elevated uptake of [^{3}H]-adenosine, high-affinity substrate of concentrative nucleoside transporter 2. Using KT-5720 and inhibitors of phosphodiesterases, we subsequently confirmed importance of cAMP/protein kinase A pathway in *SLC28A2* regulation. On the other hand, *SLC29A* genes exhibited constitutive expression and none of the tested compounds increased *SLC28A1* expression to detectable levels. In conclusion, we provide the first evidence that methylation status and activation of retinoic acid receptor affect placental *SLC28A2* and *SLC28A3* transcription and substrates of concentrative nucleoside transporter 2 might be taken up in higher extent in placentas with overactivated cAMP/protein kinase A pathway and likely in the term placenta.



KEYWORDS: *concentrative nucleoside transporters, human placenta, cAMP/protein kinase A signaling pathway, gene regulation, epigenetics, differentiation*

1. INTRODUCTION

The placenta is an important temporary organ that connects the developing fetus to the mother's uterine while maintaining maternal and fetal circulations separated throughout the whole gestation.[1,2] The maternal−fetal interface is formed by polarized multinucleated syncytiotrophoblast that creates a barrier protecting the developing fetus from potentially harmful xenobiotics.[1] Syncytiotrophoblast layer expresses various transporters and ensures reciprocal exchange of nutrients, signal molecules, cytokines, growth factors, and xenobiotics as well as those limiting the placental transfer of lipophilic compounds.[1]

Nucleoside transporters (NTs) are ubiquitously occurring proteins predominantly needed for maintaining nucleoside homeostasis.[3] On the basis of genetic/functional similarities, NTs are categorized into two subfamilies: concentrative nucleoside transporters (CNTs; *SLC28A*) consisting of three members CNT1−3 that mediate unidirectional sodium-dependent influx and equilibrative nucleoside transporters (ENTs; *SLC29A*) that have four representatives ENT1−4 conferring sodium-independent facilitated diffusion.[3] Dysregulation of NTs might be associated with, for example, impaired cell differentiation or pre-eclampsia,[4−10] and their inhibition might be beneficial in treatment of acute lung injury.[11] Beyond the salvage of endogenous nucleosides, NTs are of importance for pharmacokinetics and drug−drug interactions of most nucleoside-derived anticancer and antiviral drugs;[3] decreased expression of ENT1 or CNT3 might be the cause of tumor pharmacoresistance.[12] Hypoxia, transporter phosphorylation, transporter regulator RS1, exposure to glucose, or transactivation of nuclear receptors have been suggested to regulate NT expression in nonplacental tissue.[7,13−23]

In the human placenta, NTs mediate placental uptake of nucleosides and likely contribute to proper placental develop-

Received: March 6, 2018
Revised: April 17, 2018
Accepted: May 21, 2018
Published: May 21, 2018

DOI: 10.1021/acs.molpharmaceut.8b00238
Mol. Pharmaceutics 2018, 15, 2732−2741

ment.[21] They also enable placental uptake and transfer of nucleoside-derived drugs.[24,25] This type of pharmacotherapy is frequently administered to treat pregnant mothers or to prevent mother-to-child HIV, hepatitis B, or hepatitis C viral transmission,[1,26−32] although these compounds may be harmful to the placenta/developing fetus.[33,34] Placental ENT1 is localized in the apical (mother-facing) membrane,[35,36] while ENT2 is on both apical and basal (fetus-facing) sites of the trophoblast layer.[18] ENT3 resides intracellularly and seems to be particularly abundant in the placenta; however, its role in the placenta has not been specified to date.[37] ENT4 is considered to be only low-affinity H^+ coupled adenosine transporter that is expressed ubiquitously (mainly in cardiovascular system) and transports mostly organic cations.[38] All CNTs were detected at mRNA level in the term human placenta, and CNT1 was suggested to be functionally expressed.[18,24,36]

The expression of drug transporters can be affected by various endogenous and exogenous factors[39] and, therefore, is not uniform in the course of gestation and may significantly differ among individuals.[40−42] Differentiation-associated mechanisms (e.g., protein kinases or retinoic acid activation)[5,43,44] or epigenetics have been suggested to play a role in regulation of transporter expression. Of epigenetics mechanisms, methylation of cytosine residues in CpG islands usually leading to suppression of gene transcription and histone acetylation causing the opposite effect have been reported in the placenta.[45] Knowledge on NT expression during gestation is lacking, and mechanisms involved in regulation of placental NTs have not been studied.

In the present study, we sought to investigate expression of *SLC28A1*, *SLC28A2*, and *SLC28A3* genes in samples of the first-trimester and term placentas. As positive correlation between cell differentiation status and expression of NTs has been proposed,[8−10] we further studied in BeWo cells effects of the following prototype differentiation agents; forskolin (FSK), an activator of adenylyl cyclase, all-*trans*-retinoic acid (ATRA), agonist of nuclear retinoic acid receptor inducing differentiation in various tumor cell lines, 5-azacytidine, DNA methyltransferase inhibitor, sodium butyrate, and sodium valproate, histone deacetylases inhibitors (HDACi).

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. The compounds for treatment of BeWo cells, sodium butyrate, sodium valproate, 5-azacytidine, ATRA, FSK, KT-5720 (a model PKA inhibitor), nonspecific phosphodiesterase (PDE) inhibitors theophylline and 3-isobutyl-1-methylxanthine (IBMX), and further specific PDE7 inhibitor 5-Nitro-2,N,N-trimethylbenzenesulfonamide (BRL 50481), were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Specific inhibitor of PDE4 (rolipram) was provided by Scintila, s.r.o. (Uvoz, Jihlava, Czech Republic). The radiolabeled [^{3}H]-adenosine was obtained from Moravek Biochemicals (Brea, California, USA). Solvent dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The bicinchoninic acid assay kit (BCA assay) was obtained from ThermoFisher Scientific (Rockford, USA).

2.2. Cell Culture. The human choriocarcinoma-derived cell line BeWo was obtained from the European Cell Culture Collection (ECACC; Salisbury, Wiltshire, UK). Cells were cultured in Ham's F-12 (Sigma-Aldrich, St. Louis, Missouri, USA) medium supplemented with 10% fetal bovine serum maintained in a humidified atmosphere of 5% CO_2 at 37 °C.[46]

2.3. Collection of Human Placenta Samples. In this study, randomly selected 12 term- and 4 first-trimester placentas were used, previously collected for the study by Ahmadimoghaddam et al.[40] All term placentas were obtained following elective cesarean section at term (38−41 week of gestation), and first-trimester placentas were acquired from interruption of the pregnancy between 9 and 13 week of gestation from healthy women. The procedure was approved by the University Hospital Research Ethics Committee (201006S15P), Hradec Kralove. All participants provided written informed consent. The samples were frozen in liquid nitrogen immediately after surgery and then stored at −80 °C until analysis.

2.4. Treatment of BeWo Cells with Differentiation-Inducing Agents. BeWo cells were seeded (3.5×10^5) on 24-well plates (passage 5−25). Cultivation medium was replaced after 24 h with fresh cultivation medium (control cells) or the medium containing tested compound: sodium butyrate (1 mM), sodium valproate (2 mM), 5-azacytidine (2.5 μM), ATRA (1 μM), theophylline (5 mM), IBMX (200 μM), BRL (20 μL), or rolipram (400 μM). Furthermore, we tested effect of FSK (50 μM) in either presence or absence of KT-5720 (5 μM). The cells were exposed to a tested compound for 48 or 72 h with everyday change of the respective medium. Volume/volume concentration of DMSO was in all experiments 0.1%. The reported concentrations of individual compounds were used, which will be described in detail in the Discussion.

2.5. Isolation of RNA from BeWo Cells and Placental Samples and Reverse Transcription. Total RNA was isolated from (i) control and treated BeWo cells; (ii) weighted samples of precisely cut villous part of the term placenta; (iii) weighted samples of dissected first-trimester placental tissue. Tri Reagent solution purchased from Molecular Research Centre (Cincinnati, Ohio, USA) was used according to the manufacturer's instructions. The purity of the isolated RNA was checked by the $A_{260/280}$ ratio and RNA integrity was confirmed by electrophoresis on a 1% agarose gel. The concentration of RNA was calculated by A_{260} measurement. RNA (1 μg) was converted into cDNA using the gb Reverse Transcription Kit from Generi Biotech s.r.o. (Hradec Kralove, Czech Republic) on Bio-Rad T100 Thermal Cycler (Hercules, California, USA) according to the manufacturer's protocol.

2.6. Qualitative End-Point PCR Analysis. End-point PCR analysis of *SLC28A* genes expression was carried out with cDNA (25 ng) in 20 μL volume reaction with OneTaq Red DNA Polymerase (Bioline, Taunton, Massachusetts, USA) according to the manufacturer's instructions using Bio-Rad T100 Thermal Cycler (Hercules, California, USA). For amplification of human *SLC28A1*, *SLC28A2*, and *SLC28A3*, we employed primers previously designed by Yamamoto et al.[24] The PCR cycling conditions were 95 °C for 3 min followed by 40 cycles at 95 °C for 30 s, 56 °C for 30 s and 72 °C 45 s, followed by 72 °C for 10 min. Amplicons were analyzed on a 1.5% agarose gel labeled by GelRed Nucleic Acid Stain (Biotium, Hayward, California, USA) using the HyperLadder 100bp length marker (Bioline, Taunton, Massachusetts, USA).

2.7. Quantitative PCR Analysis. Quantitative PCR (qPCR) analysis of NTs expression in control and treated BeWo cells and first-trimester and term human placentas was performed using QuantStudio 6 (Thermo Fisher Scientific, Waltham, USA). cDNA (25 ng) was amplified in 10 μL reaction in a 96-well plate using TaqMan Universal Master Mix II, no UNG (Thermo Fisher Scientific, Waltham, USA), and

DOI: 10.1021/acs.molpharmaceut.8b00238
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predesigned TaqMan Real Time Expression PCR assays for *SLC28A1* (*CNT1*, Hs00984403_m1), *SLC28A2* (*CNT2*, Hs00188407_m1), *SLC28A3* (*CNT3*, Hs00910439_m1), *SLC29A1* (*ENT1*, Hs01085704_g1), *SLC29A2* (*ENT2*, Hs00155426_m1), and *syncytin-1* (Hs02341206_g1) coding, a marker of cytotrophoblast differentiation.[47] The amplification of each sample was performed in triplicate using the following PCR cycling profile: 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. Data are presented as fold of expression values for BeWo cells or are reported in arbitrary units (a.u.) for placental tissue samples. Gene expressions were normalized against the geometric mean of expression of the predesigned reference genes *B2M* (Hs00984230_m1) and *GAPDH* (Hs02758991_g1) (Thermo Fisher Scientific, Waltham, USA)[40,48,49] that had been tested for stable expression in all samples. The data were processed by the comparative $\Delta\Delta C_T$ method.

2.8. *In Vitro* Uptake of [^{3}H]-Adenosine into BeWo Cells. For uptake experiment, BeWo cells were seeded at a density 3.5×10^5 on 24-well culture plates (TPP, Trasadingen, Switzerland). FSK (50 μM) with or without 5 μM KT-5720 was added after 24 h and subsequently cultivated for following 48 h with everyday medium replacement. Experiments were performed in uptake buffer containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM Tris as previously suggested.[24] At the beginning of experiment, cells were washed with 0.5 mL of the uptake buffer. Then the cells were incubated in 0.25 mL of the uptake buffer containing model substrate [^{3}H]-adenosine at final activity 0.4 μCi/mL that corresponded to 17.4 nM. The activity of the radioisotope used in experiments was the lowest possible given the specific activity required for the analysis. Accumulation was stopped after 5 min by quick aspiration of radioactivity containing uptake buffer and washed twice with the ice-cold buffer (1 mL) and then the cells were lysed in 0.25 mL of SDS (0.02%).[24,46] The concentration of accumulated [^{3}H]-adenosine was determined by liquid scintillation counting (Tricarb 2900 TR; PerkinElmer, Waltham, MA, USA) and normalized to protein content (pmol/mg protein; Pierce BCA Protein Assay Kit, thermos Fisher Scientific, Waltham, MA, USA). Data are presented as fold of increase to control.

2.9. Statistical Analyses. Statistical significance in *in vitro* studies was performed by unpaired Student's *t* test or one-way ANOVA followed by Dunnett's *post hoc* test. For statistical analysis of NTs mRNA expression in the first-trimester and term human placenta, paired nonparametric Friedman followed by Dunn's multiple comparison and Mann−Whitney tests were applied. All data were processed using GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, California, USA).

3. RESULTS

3.1. Qualitative RT-PCR Analysis of *SLC28A* mRNA Expression in BeWo Cells and Human Term Placenta. We first confirmed mRNA expression of *SLC28A* in BeWo cells (Figure 1A) and one randomly selected human placenta from our sample collection (Figure 1B). We detected amplicons specific for the primers used for *SLC28A2 and SLC28A3*, but not for *SLC28A1*.

3.2. Quantitative RT-PCR Analysis of *SLC28A* mRNA Expression in First-Trimester and Term Human Placenta. We carried out qRT-PCR to evaluate mRNA expression profile of *SLC28A* genes in the first-trimester and term human placentas. Further, we compared the expression in both developmental placental stages and quantified interindividual differences of *SLC28A2* genes. *SLC28A2* exhibited the highest expression in both the first-trimester and term placental samples. In the first-trimester placental samples, expression of *SLC28A1* and *SLC28A3* was under detection limit, while in the term placenta median expression of *SLC28A1* was marginal (only four term placentas revealed low levels of *SLC28A1* expression) and *SLC28A2* showed ∼2.6-fold higher expression than that observed for *SLC28A3*. To confirm a functional system for detection of *SLC28A1* expression, liver and kidney tissues were used as positive controls (data not shown). *SLC28A2* exhibited ∼5-fold higher median expression in the term placenta than in the first-trimester placenta. The interindividual differences were ∼1 (*SLC28A1*, *SLC28A3*) and ∼2 (*SLC28A2*) orders of magnitude in the term placenta and ∼1 (*SLC28A2*) in the first-trimester placentas (Figure 2).

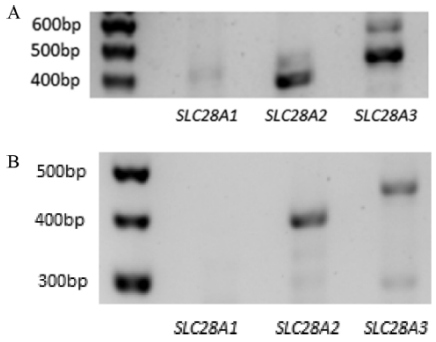

Figure 1. Qualitative RT-PCR analysis of NTs in (A) BeWo cells and (B) human placenta. Representative agarose gels show that BeWo cell line and randomly selected term human placenta express *SLC28A2* and *SLC28A3*, but not *SLC28A1*. The expected sizes of the PCR products for the primers used were 610bp for *SLC28A1*, 390bp for *SLC28A2*, and 440bp for *SLC28A3*.

3.3. Effect of Differentiation-Affecting Drugs on Expression of NTs in BeWo Cells. While testing the effect of selected compounds on NTs mRNA expression, we observed that FSK (50 μM) increased expression of *SLC28A2* by ∼25-fold (Figure 3A). Sodium valproate also elevated expression of *SLC28A2* and the effect was time dependent observing ∼2-fold and ∼2.5-fold increase in 48 and 72 h-time points, respectively (Figure 3B). Interestingly, another HDACi, sodium butyrate (1 mM), did not exhibit any effect on NTs expression in tested time points (Figure 3C). 5-Azacytidine (2.5 μM) and ATRA (1 μM) decreased expression of *SLC28A2* and *SLC28A3* by ∼2-fold; however, 5-azacytidine revealed this effect only after 48 h (Figure 3D, E). None of the tested compounds increased *SLC28A1* expression; its expression remained below the detection limit of our method (data not shown).

3.4. Effect of PKA Inhibitor, KT-5720, on FSK-Induced *SLC28A2* mRNA Upregulation in BeWo Cells. As the effect of FSK in BeWo cells is attributed mostly to activation of cyclic adenosine monophosphate-protein kinase A (cAMP-PKA) signaling pathway,[43] we tested whether the PKA specific inhibitor, KT-5720, may reverse observed induction of *SLC28A2*. Exposure to KT-5720 (5 μM) alone did not show any effect and significantly decreased (by ∼2-fold) FSK-induced *SLC28A2* upregulation following 48-h incubation

DOI: 10.1021/acs.molpharmaceut.8b00238
Mol. Pharmaceutics 2018, 15, 2732−2741

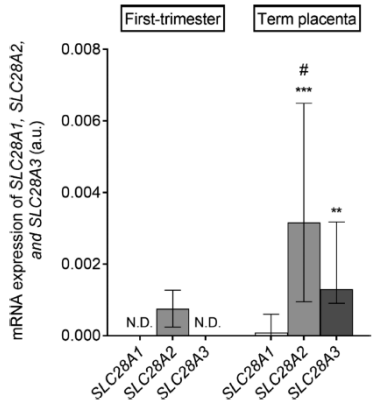

Figure 2. Quantitative PCR analysis of *SLC28A* mRNA expression in first- and term human placentas. First-trimester placenta expresses only *SLC28A2* mRNA, while term placenta reveals expression of all *SLC28A* genes, although median expression of *SLC29A1* is marginal; only four term placentas revealed low levels of *SLC28A1* expression. Gene expressions were appropriately normalized against the geometric mean of expression of two housekeeping genes, *GAPDH* and *B2M*.[40,48,49] Data are presented as median of arbitrary units (a.u.) with interquartile range. Statistical analysis was examined using nonparametric Friedman paired test followed by Dunn's multiple comparisons test to compare median expression within one developmental placental stage, and Mann−Whitney was applied to evaluate differences between the first-trimester and term placenta; significance obtained by Friedman test was denoted as follows ** $p \leq 0.01$; *** $p \leq 0.001$. Mann−Whitney evaluation was used to compare *SLC28A2* expression in the first-trimester and term placenta and the significance was denoted # $p \leq 0.05$; $n = 4$ (first-trimester placentas), $n = 12$ (term placentas); N.D., not detected.

(Figure 4), though it did not reach the level of the control. It indicates only partial inhibitory PKA effect of KT-5720 (5 μM) or contribution of mechanisms affecting *SLC28A2* expression different from PKA regulation.

3.5. Effect of Phosphodiesterases Inhibitors on *SLC28A2* Expression. Having shown that *SLC28A2* might be induced by increased activity of cAMP/PKA pathway, we further tested effects of phosphodiesterases inhibitors that might prolong effect of cAMP and, therefore, mimic the effect of FSK on expression of *SLC28A2* in BeWo cells. Only IBMX (200 μM), but not theophylline (5 mM), rolipram (400 μM), or BRL (20 μM), affected *SLC28A2* expression (Figure 5).

3.6. Effect of FSK Treatment on Intracellular Accumulation of [^{3}H]-Adenosine in BeWo Cells. We further investigated whether FSK (50 μM)-mediated induction of *SLC28A2* may be reflected in elevated CNT2 function. Using uptake study with [^{3}H]-adenosine (17.4 nM), a model substrate of NTs revealing the highest affinity to CNT2, we did observe significant increase in cells treated with FSK (50 μM) for 48 h. It confirms that elevated *SLC28A2* expression in FSK-treated cells is reflected at functional level. Interestingly, KT-5720 (5 μM) or FSK (50 μM)/KT-5720 (5 μM) significantly decreased [^{3}H]-adenosine intracellular accumulation in BeWo cells (Figure 6) suggesting that blockade of PKA might result in decreased function of membrane-embedded CNT2.

3.7. Effect of Tested Compounds on BeWo Cells Differentiation: Monitoring of *Syncytin-1* Gene Levels. To evaluate whether observed effects of tested compounds on expression of NTs is related to morphological differentiation of BeWo cells, we monitored mRNA expression of *syncytin-1*, a marker of syncytiotrophoblast formation in BeWo cells.[50] Only FSK (50 μM) increased *syncytin-1* expression by ~7.5 fold (Figure 7).

4. DISCUSSION

In the present study, we are bringing the first evidence about quantitative mRNA expressions of *SLC28A* genes and their inter/intraindividual variability in the first-trimester and term human placenta. Using prototype differentiation-affecting agents, we also investigated whether levels of NTs expression might be given by the activity of adenylyl cyclase, retinoic acid receptor activation or by CpG islands methylation/histone acetylation status.

We showed that of all *SLC28A* genes, *SLC28A2* and *SLC28A3*, are expressed in BeWo cells and in one randomly selected human placenta (Figure 1). These data do not correlate with previously published reports demonstrating that BeWo cell line and human placenta express *SLC28A3*[24,25] and all *SLC28A* genes,[18,24] respectively. The observed difference in CNTs expression pattern in BeWo cells might be related to clonal diversity or different cultivation conditions as NTs are regulated by the level of nutrition supply.[22,23,51,52] Lack of *SLC28A1* transcript in the term placenta can be explained by low and variable mRNA expression as subsequently shown in samples of human placenta from 12 donors (Figure 2). *SLC28A* genes increase expression toward term of gestation (Figure 2); *SLC28A1* and *SLC28A3* are transcribed only in the term placenta, while the most abundant *SLC28A2* is expressed in both tested developmental stages (Figure 2). This expression profile contrasts to that found for protective efflux transporters, P-glycoprotein and breast cancer resistance protein, exhibiting decreasing trend,[41,42] and also for *SLC29A1* and *SLC29A2*, showing constitutive expression.[53] This phenomenon is likely related to increased placental needs for nucleoside supply,[54] energy metabolism,[55] or for purinergic control as reported for liver cells.[56]

Subsequently, we tested an effect of prototype differentiation-affecting agents on mRNA expression of NTs in BeWo cells. FSK, in the concentration of 50 μM, known to induce syncytiotrophoblast formation in BeWo cells,[57,58] significantly increased *SLC28A2* expression (Figure 3A), whereas it revealed no effect on *SLC29A* genes, suggesting that *SLC28A2* reflects state of differentiation while *SLC29A* are constitutively expressed as previously described in hepatoma cell line FAO.[59] We observed comparable effect of FSK in another choriocarcinoma-derived JEG-3 cell line either (data not shown), therefore proposing that this phenomenon is not BeWo cells specific. We were not the first who analyzed effect of FSK on NTs expression in BeWo cells; Ma et al. showed that FSK restored *SLC28A2* expression in *SLC28A2*-lacking BeWo cells and in contrast to our findings they also observed effect on *SLC29A2* and *SLC28A3*.[25] This discrepancy can be partially related to the different cultivation conditions used (difference in fetal bovine serum concentration and D-glucose) or their different PCR detection technique based on SYBR Green nonspecifically intercalating the double-stranded DNA; primer specificity (melting curve analysis) is not declared in the report.[25] We also tested concentrations 10 μM and 100 μM of FSK as used in others studies,[50,60] but we observed similar effect on *SLC28A2* (data not shown).

DOI: 10.1021/acs.molpharmaceut.8b00238
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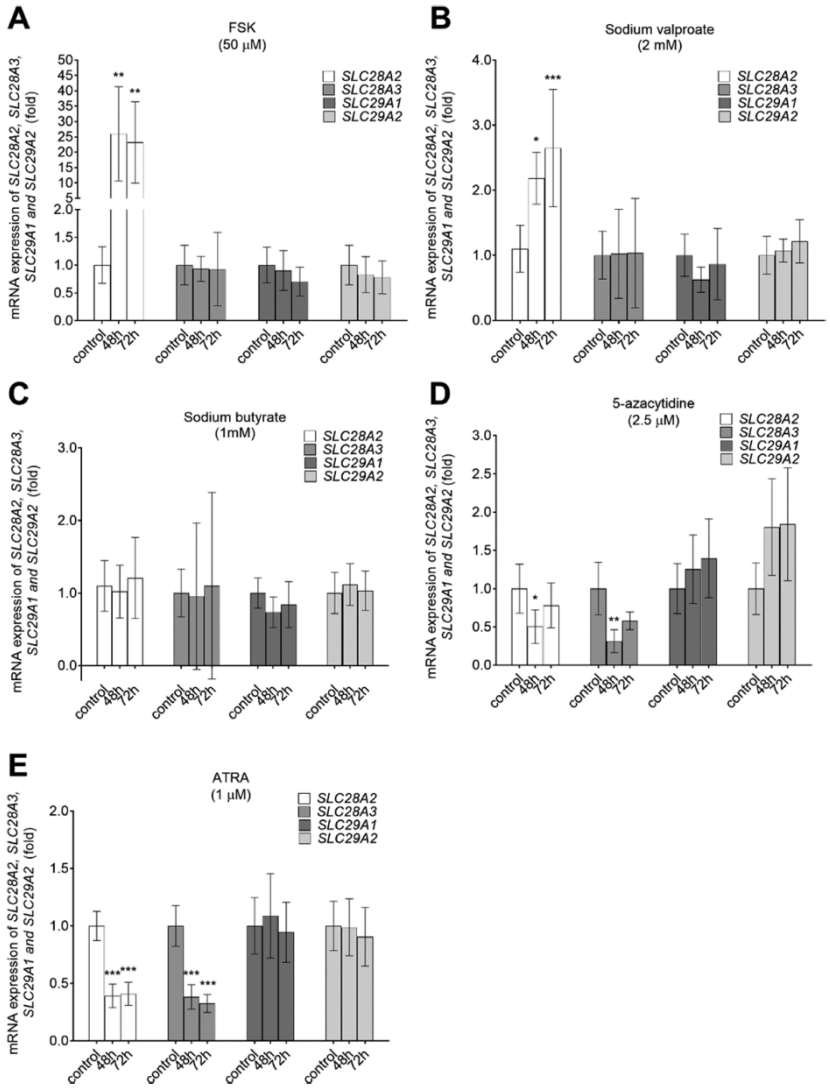

Figure 3. Effect of differentiation affecting drugs on *SLC28A2*, *SLC28A3*, *SLC29A1*, and *SLC29A2* mRNA expression levels in BeWo cells. Cells were exposed to (A) FSK (50 μM), (B) sodium valproate (2 mM), (C) sodium butyrate (1 mM), (D) 5-azacytidine (2.5 μM), and (E) ATRA (1 μM) for 48 and 72 h. Data were analyzed using the comparative $\Delta\Delta C_T$ method and presented as fold-change of expression; geometric mean of expression of two housekeeping genes *B2M* and *GAPDH* was applied for data normalization. Each column represents the mean ± SD ($n \geq 3$). Statistical analysis was performed by one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test; $*$ $p < 0.05$, $**$ $p < 0.01$, $***$ $p < 0.001$, significantly different from nontreated cell.

As BeWo cells have been described to express retinoic acid receptor,[61] we tested its agonist ATRA (1 μM) that has been recently shown by Le Vee et al. to downregulate SLC transporters in HepaRG cells and primary human hepatocytes.[62] ATRA (1 μM) was also employed by Huebner et al. to investigate epigenetic and transcriptional regulation of retinoic acid receptor responder 1 in BeWo cells.[61] *SLC28A2* and *SLC28A3* levels were decreased following exposure to ATRA (Figure 3E). Retinoic acid receptor response element has not been identified in regulatory sequence of *SLC28A2* so far, and Ensembl.org database predicts only binding site of transcriptional repressor CTCF. Therefore, we speculate about coordinated activity of retinoic acid receptor and CTCF as observed for estrogen receptor.[63]

To test the role of histone deacetylases inhibition in regulation of NTs, we used sodium butyrate and sodium valproate in concentrations 1 mM and 2 mM.[64−66] Sodium butyrate increases expression and activity of vitamin D receptor,[66] while sodium valproate affects proliferation and hormone secretion, both in BeWo cells.[65] Sodium butyrate did not modify expression of NTs (Figure 3C), whereas sodium valproate caused time-dependent increase in expression of

DOI: 10.1021/acs.molpharmaceut.8b00238
Mol. Pharmaceutics 2018, 15, 2732−2741

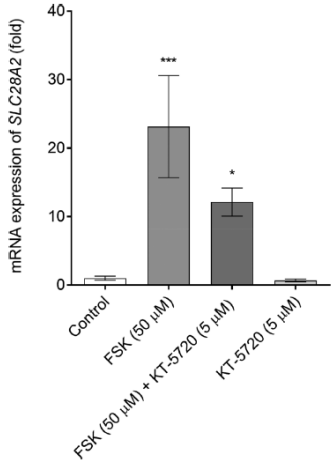

Figure 4. Effect of PKA inhibitor KT-5720 on FSK-induced *SLC28A2* mRNA upregulation in BeWo cells. KT-5720 (5 μM), a PKA inhibitor, significantly reversed the FSK-induced (50 μM) increase in *SLC28A2* mRNA expression level after 48-h incubation. Data were analyzed using the comparative $\Delta\Delta C_T$ method and are presented as fold-change of expression; geometric mean of expression of two housekeeping genes *B2M* and *GAPDH* was applied for data normalization. Each column represents the mean ± SD ($n \geq 3$). Statistical analysis was performed by one way ANOVA followed by *post hoc* Dunnett's multiple comparison test; $*$ $p < 0.05$, $***$ $p < 0.001$, compared to nontreated cells.

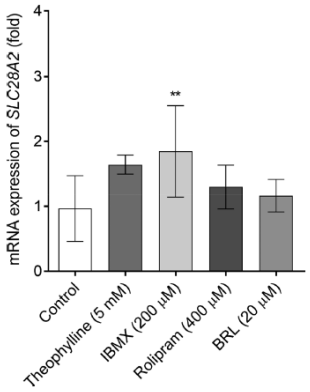

Figure 5. Effect of phosphodiesterases inhibitors on *SLC29A2* mRNA expression in BeWo cells. Cells were exposed to theophylline (5 mM), IBMX (200 μM), rolipram (400 μM), or BRL (20 μM) for 48 h. Data were analyzed using the comparative $\Delta\Delta C_T$ method are presented as fold-change of expression; geometric mean of expression of two housekeeping genes *B2M* and *GAPDH* was applied for data normalization. Each column represents the mean ± SD ($n \geq 3$). Statistical analysis was performed by one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test; $**p < 0.01$, compared to control cells.

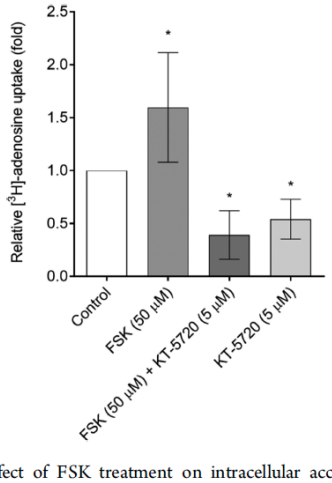

Figure 6. Effect of FSK treatment on intracellular accumulation of [^{3}H]-adenosine in BeWo cells. BeWo cells were treated with FSK (50 μM), combination FSK (50 μM) with KT-5720 (5 μM), or KT-5720 (5 μM) for 48 h and then 5 minute accumulation of [^{3}H]-adenosine (17.4 nM, 0.4 μCi/ml) was analyzed. Each column represents the mean of ratio of concentrations determined in treated cells and respective control ± SD ($n = 5$). One-way ANOVA was used to evaluate statistical significance followed by *post hoc* Dunnett's multiple comparison test.

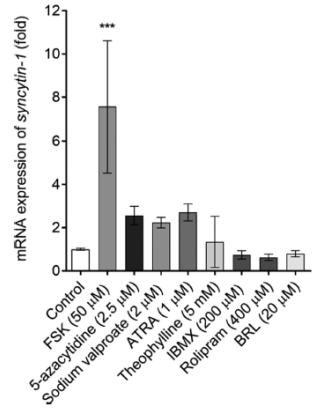

Figure 7. Effect of tested compounds on *syncytin-1* mRNA expression levels in BeWo cells. Cells were exposed to FSK (50 μM), 5-azacytidne (2.5 μM), sodium valproate (2 μM), ATRA (1 μM), theophylline (5 mM), IBMX (200 μM), rolipram (400 μM), or BRL (20 μM) for 48 h. Data were analyzed applying the comparative $\Delta\Delta C_T$ method and presented as fold-change of expression; geometric mean of expression of two housekeeping genes *B2M* and *GAPDH* was applied for normalization of *syncytin-1* expression. Each column represents the mean ± SD ($n \geq 3$). Statistical analysis was performed by one-way ANOVA followed by *post hoc* Dunnett's multiple comparison test; $***p < 0.001$, showing statistical difference from control cells.

SLC28A2 (Figure 3B). We hypothesize that *SLC28A2* is not regulated by histone acetylation and the observed effect of sodium valproate may be explained by its complex mechanism of action.[67,68]

DNA methyltransferase inhibitor 5-azacytidine (1 mM), previously tested to induce vitamine D receptor or retinoic acid receptor responder 1,[55,66] decreased *SLC28A2* and *SLC28A3* expression in time point of 48 h, but not after 72 h (Figure 3C). 5-azacytidine-mediated downregulation of gene expression is less frequent and can be related to demethylation of gene expression suppressors.[69] As 5-azacytidine is a suggested substrate of both CNT2 and CNT3,[70,71] we suggest that

DOI: 10.1021/acs.molpharmaceut.8b00238
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decreased *SLC28A2*/*SLC28A3* functional expression observed after 48 h may limit 5-azacytidine uptake.

Because of extensive effect of FSK in BeWo (and also in JEG-3 cells), we tested hypothesis that *SLC28A2* expression may be controlled by cAMP-PKA signaling pathway.[72] Application of KT-5720, an established inhibitor of PKA,[44] partly reversed the FSK-induced expression of *SLC28A2* (Figure 4). KT-5720 (5 μM) was reported to cause approximately half-reduction of FSK-mediated increase in expression of *cytochrome P450 19A1*, *organic anion transporter 4*, and *organic anion-transporting polypeptide*,[44] which correlate with our observation for the *SLC28A2*. Despite testing higher concentration of KT-5720 (15 μM), full abolishment of FSK-mediated induction of *SLC28A2* was not observed (data not shown). Therefore, we suggest that KT-5720 is low affinity inhibitor or additional cAMP-activated mechanism such as Rap1/MEK/$ERK_{1/2}$ signaling pathway participates in *SLC28A2* gene regulation. To investigate the cAMP-PKA regulatory pathway of *SLC28A2* in more detail, we inhibited activity of PDE that are responsible for cAMP biodegradation. Only nonselective PDE inhibitor IBMX (200 μM), previously reported to decrease activity of placental PDE to highest extent,[73] significantly increased *SLC28A2* expression (Figure 5). It provided additional evidence that *SLC28A2* expression could be regulated by cAMP/PKA pathway.

There are no specific CNT2 substrates. For the uptake study, we selected adenosine that reveals high affinity to CNT2 and CNT3 and is much weaker substrate of ENT1 and ENT2[3]. The increased adenosine accumulation in BeWo cells (Figure 6) should be a consequence of up-regulated *SLC28A2* coding CNT2 as only this gene was affected by exposure to FSK (Figure 3). It confirms that cAMP/PKA activation results in higher functional expression in plasma membrane. Interestingly, KT-5720 alone and combination FSK/KT-5720 decreased adenosine uptake (Figure 6). We hypothesize that KT-5720 might cause insufficient trafficking of CNT2 from subcellular structures into plasma membrane.[74]

Subsequently, we analyzed *syncytin-1* encoding human endogenous retrovirus glycoprotein, which mediates cell−cell fusion of cytotrophoblast into syncytiotrophoblast,[58] to evaluate association between level of *SLC28A2* expression and syncytiotrophoblast formation. Interestingly, we showed that of the prototype differentiation agents tested only FSK significantly changed the *syncitin-1* expression (Figure 7). This might indicate that sodium valproate, 5-azacytidine, and ATRA reveal their effect on *SLC28A* gene expression independently of state of BeWo cells differentiation. On the other hand, there was correlation between *SLC28A2* and *syncytin-1* expression in FSK-treated cells. However, we also observed increase in *SLC28A2* expression in FSK-treated JEG-3 cells that do not undergo syncytiotrophoblast formation.[50,58] Therefore, we conclude that functional *SLC28A2* expression is dependent on cAMP/PKA signaling pathway activity but does not reflect state of morphological differentiation.

We here provide the first evidence that expression of *SLC28A* genes increases during gestation, reveals considerable interindividual variation, and *SLC28A2* is the dominant placental *SLC28A* subtype in both first-trimester and term placenta. We showed that *SLC28A* transcription is affected by sodium valproate, 5-azacytidine, ATRA, and to highest extent by FSK, while *SLC29A* genes exhibited constitutive expression. The observed changes in *SLC28A* are not associated with the morphological differentiation state of BeWo cells and cAMP/PKA signaling pathway plays an important role in regulation of *SLC28A2* functional expression. In conclusion, we suggest that methylation status and activation of retinoic acid receptor affect placental *SLC28A2* and *SLC28A3* transcription and substrates of concentrative nucleoside transporter 2 might be taken up in higher extent in placentas with overactivated cAMP-PKA pathway and likely in the term placenta. Additional studies should be performed to specify molecules involved in cAMP-mediated regulation of *SLC28A2* expression in BeWo cells and to show whether this regulation is also of general importance for regulation of *SLC28A2* gene expression in nonplacental cells.

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Funding

This work was supported by the Czech Science Foundation (GACR 17−16169S), by Grant Agency of Charles University (GAUK 812216/C/2016; GAUK 324215/C/2015), and SVV 2017/260-414.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We would like to thank Dr. Marian Kacerovsky (Department of Obstetrics and Gynecology, University Hospital in Hradec Kralove) for providing us with human placentas and Martina Hudeckova for the help with the human placenta collection and sampling.

■ ABBREVIATIONS

ATRA, all-*trans*-retinoic acid; BCA, bicinchoninic acid assay; BRL, 5-nitro-2,N,N-trimethylbenzenesulfonamide; B2M, beta−2-microglobulin; cAMP, cyclic adenosine monophosphate; CNT1−3, concentrative nucleoside transporters; DNMT, DNA methyltransferase; ENT1−4, equilibrative nucleoside transporters; FSK, forskolin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDACi, histone deacetylases inhibitor; IBMX, 3-isobutyl-1-methylxanthine; KT-5720, inhibitor of protein kinase A; NTs, nucleoside transporters; PDE, phosphodiesterase; PKA, protein kinase A; RAR, retinoic acid receptor; SLC, solute carrier transporters

■ REFERENCES

(1) Staud, F.; Cerveny, L.; Ceckova, M. Pharmacotherapy in pregnancy; effect of ABC and SLC transporters on drug transport across the placenta and fetal drug exposure. *J. Drug Target* **2012**, *20*, 736−63.

(2) Carter, A. M.; Enders, A. C. Comparative aspects of trophoblast development and placentation. *Reprod. Biol. Endocrinol.* **2004**, *2*, 46.

(3) Molina-Arcas, M.; Casado, F. J.; Pastor-Anglada, M. Nucleoside transporter proteins. *Curr. Vasc. Pharmacol.* **2009**, *7*, 426−34.

(4) Fernandez-Calotti, P.; Pastor-Anglada, M. All-trans-retinoic acid promotes trafficking of human concentrative nucleoside transporter-3 (hCNT3) to the plasma membrane by a TGF-beta1-mediated mechanism. *J. Biol. Chem.* **2010**, *285*, 13589−98.

(5) Espinoza, J.; Espinoza, A. F.; Power, G. G. High fetal plasma adenosine concentration: a role for the fetus in preeclampsia? *Am. J. Obstet. Gynecol.* **2011**, *205*, 485.e24−7.

(6) Escudero, C.; Casanello, P.; Sobrevia, L. Human equilibrative nucleoside transporters 1 and 2 may be differentially modulated by

DOI: 10.1021/acs.molpharmaceut.8b00238
Mol. Pharmaceutics 2018, 15, 2732−2741

A2B adenosine receptors in placenta microvascular endothelial cells from pre-eclampsia. *Placenta* **2008**, *29*, 816−25.
(7) Perez-Torras, S.; Vidal-Pla, A.; Cano-Soldado, P.; Huber-Ruano, I.; Mazo, A.; Pastor-Anglada, M. Concentrative nucleoside transporter 1 (hCNT1) promotes phenotypic changes relevant to tumor biology in a translocation-independent manner. *Cell Death Dis.* **2013**, *4*, e648.
(8) Flanagan, S. A.; Meckling, K. A. Nucleoside transporter expression and activity is regulated during granulocytic differentiation of NB4 cells in response to all-trans-retinoic acid. *Leuk. Res.* **2007**, *31*, 955−68.
(9) Guzman-Gutierrez, E.; Sandoval, C.; Nova, E.; Castillo, J. L.; Vera, J. C.; Lamperti, L.; Krause, B.; Salomon, C.; Sepulveda, C.; Aguayo, C.; Sobrevia, L. Differential expression of functional nucleoside transporters in non-differentiated and differentiated human endothelial progenitor cells. *Placenta* **2010**, *31*, 928−36.
(10) Jones, K. W.; Rylett, R. J.; Hammond, J. R. Effect of cellular differentiation on nucleoside transport in human neuroblastoma cells. *Brain Res.* **1994**, *660*, 104−12.
(11) Eckle, T.; Hughes, K.; Ehrentraut, H.; Brodsky, K. S.; Rosenberger, P.; Choi, D. S.; Ravid, K.; Weng, T.; Xia, Y.; Blackburn, M. R.; Eltzschig, H. K. Crosstalk between the equilibrative nucleoside transporter ENT2 and alveolar Adora2b adenosine receptors dampens acute lung injury. *FASEB J.* **2013**, *27*, 3078−89.
(12) Pastor-Anglada, M.; Perez-Torras, S. Nucleoside transporter proteins as biomarkers of drug responsiveness and drug targets. *Front. Pharmacol.* **2015**, *6*, 13.
(13) Morote-Garcia, J. C.; Rosenberger, P.; Nivillac, N. M.; Coe, I. R.; Eltzschig, H. K. Hypoxia-inducible factor-dependent repression of equilibrative nucleoside transporter 2 attenuates mucosal inflammation during intestinal hypoxia. *Gastroenterology* **2009**, *136*, 607−18.
(14) Reyes, G.; Nivillac, N. M.; Karim, M. Z.; Desouza, L.; Siu, K. W.; Coe, I. R. The Equilibrative Nucleoside Transporter (ENT1) can be phosphorylated at multiple sites by PKC and PKA. *Mol. Membr. Biol.* **2011**, *28*, 412−26.
(15) Fernandez-Calotti, P. X.; Lopez-Guerra, M.; Colomer, D.; Pastor-Anglada, M. Enhancement of fludarabine sensitivity by all-trans-retinoic acid in chronic lymphocytic leukemia cells. *Haematologica* **2012**, *97*, 943−51.
(16) Cheng, X.; Klaassen, C. D. Regulation of mRNA expression of xenobiotic transporters by the pregnane x receptor in mouse liver, kidney, and intestine. *Drug Metab. Dispos.* **2006**, *34*, 1863−7.
(17) Aymerich, I.; Pastor-Anglada, M.; Casado, F. J. Long term endocrine regulation of nucleoside transporters in rat intestinal epithelial cells. *J. Gen. Physiol.* **2004**, *124*, 505−12.
(18) Errasti-Murugarren, E.; Diaz, P.; Godoy, V.; Riquelme, G.; Pastor-Anglada, M. Expression and distribution of nucleoside transporter proteins in the human syncytiotrophoblast. *Mol. Pharmacol.* **2011**, *80*, 809−17.
(19) Bicket, A.; Mehrabi, P.; Naydenova, Z.; Wong, V.; Donaldson, L.; Stagljar, I.; Coe, I. R. Novel regulation of equlibrative nucleoside transporter 1 (ENT1) by receptor-stimulated Ca2+-dependent calmodulin binding. *Am. J. Physiol Cell Physiol* **2016**, *310*, C808−20.
(20) Coe, I.; Zhang, Y.; McKenzie, T.; Naydenova, Z. PKC regulation of the human equilibrative nucleoside transporter, hENT1. *FEBS Lett.* **2002**, *517*, 201−5.
(21) Errasti-Murugarren, E.; Fernandez-Calotti, P.; Veyhl-Wichmann, M.; Diepold, M.; Pinilla-Macua, I.; Perez-Torras, S.; Kipp, H.; Koepsell, H.; Pastor-Anglada, M. Role of the transporter regulator protein (RS1) in the modulation of concentrative nucleoside transporters (CNTs) in epithelia. *Mol. Pharmacol.* **2012**, *82*, 59−67.
(22) Grden, M.; Podgorska, M.; Kocbuch, K.; Rzepko, R.; Szutowicz, A.; Pawelczyk, T. High glucose suppresses expression of equilibrative nucleoside transporter 1 (ENT1) in rat cardiac fibroblasts through a mechanism dependent on PKC-zeta and MAP kinases. *J. Cell. Physiol.* **2008**, *215*, 151−60.
(23) Leung, G. P.; Man, R. Y.; Tse, C. M. D-Glucose upregulates adenosine transport in cultured human aortic smooth muscle cells. *Am. J. Physiol Heart Circ Physiol* **2005**, *288*, H2756−62.
(24) Yamamoto, T.; Kuniki, K.; Takekuma, Y.; Hirano, T.; Iseki, K.; Sugawara, M. Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters. *Eur. J. Pharmacol.* **2007**, *557*, 1−8.
(25) Ma, Z.; Yang, X.; Jiang, T.; Bai, M.; Zheng, C.; Zeng, S.; Sun, D.; Jiang, H. Multiple SLC and ABC Transporters Contribute to the Placental Transfer of Entecavir. *Drug Metab. Dispos.* **2017**, *45*, 269−278.
(26) Azim, H. A., Jr.; Peccatori, F. A.; Pavlidis, N. Treatment of the pregnant mother with cancer: a systematic review on the use of cytotoxic, endocrine, targeted agents and immunotherapy during pregnancy. Part I: Solid tumors. *Cancer Treat. Rev.* **2010**, *36*, 101−9.
(27) Azim, H. A., Jr.; Pavlidis, N.; Peccatori, F. A. Treatment of the pregnant mother with cancer: a systematic review on the use of cytotoxic, endocrine, targeted agents and immunotherapy during pregnancy. Part II: Hematological tumors. *Cancer Treat. Rev.* **2010**, *36*, 110−21.
(28) Wiesweg, M.; Aydin, S.; Koeninger, A.; Stein, A.; Schara, U.; van Roye, C.; Hense, J.; Welt, A.; Schuler, M. Administration of Gemcitabine for Metastatic Adenocarcinoma during Pregnancy: A Case Report and Review of the Literature. *AJP Rep* **2014**, *4*, 17−22.
(29) Koren, G.; Carey, N.; Gagnon, R.; Maxwell, C.; Nulman, I.; Senikas, V. Society of, O., and Gynaecologists of, C. Cancer chemotherapy and pregnancy. *J. Obstet Gynaecol Can.* **2013**, *35*, 263−80.
(30) Gingelmaier, A.; Grubert, T. A.; Kost, B. P.; Setzer, B.; Lebrecht, D.; Mylonas, I.; Mueller-Hoecker, J.; Jeschke, U.; Hiedl, S.; Friese, K.; Walker, U. A. Mitochondrial toxicity in HIV type-1-exposed pregnancies in the era of highly active antiretroviral therapy. *Antivir Ther* **2009**, *14*, 331−8.
(31) Arshad, M.; El-Kamary, S. S.; Jhaveri, R. Hepatitis C virus infection during pregnancy and the newborn period−are they opportunities for treatment? *J. Viral Hepat* **2011**, *18*, 229−36.
(32) Brown, R. S., Jr.; McMahon, B. J.; Lok, A. S.; Wong, J. B.; Ahmed, A. T.; Mouchli, M. A.; Wang, Z.; Prokop, L. J.; Murad, M. H.; Mohammed, K. Antiviral therapy in chronic hepatitis B viral infection during pregnancy: A systematic review and meta-analysis. *Hepatology* **2016**, *63*, 319−33.
(33) Eudaly, J. A.; Tizzano, J. P.; Higdon, G. L.; Todd, G. C. Developmental toxicity of gemcitabine, an antimetabolite oncolytic, administered during gestation to CD-1 mice. *Teratology* **1993**, *48*, 365−81.
(34) Serman, L.; Vlahovic, M.; Sijan, M.; Bulic-Jakus, F.; Serman, A.; Sincic, N.; Matijevic, R.; Juric-Lekic, G.; Katusic, A. The impact of 5-azacytidine on placental weight, glycoprotein pattern and proliferating cell nuclear antigen expression in rat placenta. *Placenta* **2007**, *28*, 803−11.
(35) Barros, L. F.; Yudilevich, D. L.; Jarvis, S. M.; Beaumont, N.; Young, J. D.; Baldwin, S. A. Immunolocalisation of nucleoside transporters in human placental trophoblast and endothelial cells: evidence for multiple transporter isoforms. *Pfluegers Arch.* **1995**, *429*, 394−9.
(36) Govindarajan, R.; Bakken, A. H.; Hudkins, K. L.; Lai, Y.; Casado, F. J.; Pastor-Anglada, M.; Tse, C. M.; Hayashi, J.; Unadkat, J. D. In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. *Am. J. Physiol Regul Integr Comp Physiol* **2007**, *293*, R1809−22.
(37) Beal, P. R.; Yao, S. Y.; King, A. E.; Cass, C. E.; Young, J. D.; Baldwin, S. A. The equilibrative nucleoside transporter family, SLC29. *Pfluegers Arch.* **2004**, *447*, 735−43.
(38) Barnes, K.; Dobrzynski, H.; Foppolo, S.; Beal, P. R.; Ismat, F.; Scullion, E. R.; Sun, L.; Tellez, J.; Ritzel, M. W.; Claycomb, W. C.; Cass, C. E.; Young, J. D.; Billeter-Clark, R.; Boyett, M. R.; Baldwin, S. A. Distribution and functional characterization of equilibrative nucleoside transporter-4, a novel cardiac adenosine transporter activated at acidic pH. *Circ. Res.* **2006**, *99*, 510−9.

DOI: 10.1021/acs.molpharmaceut.8b00238
Mol. Pharmaceutics 2018, 15, 2732−2741

(39) Staud, F.; Ceckova, M. Regulation of drug transporter expression and function in the placenta. *Expert Opin. Drug Metab. Toxicol.* **2015**, *11*, 533−55.
(40) Ahmadimoghaddam, D.; Staud, F. Transfer of metformin across the rat placenta is mediated by organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) protein. *Reprod. Toxicol.* **2013**, *39*, 17−22.
(41) Gil, S.; Saura, R.; Forestier, F.; Farinotti, R. P-glycoprotein expression of the human placenta during pregnancy. *Placenta* **2005**, *26*, 268−70.
(42) Meyer zu Schwabedissen, H. E.; Grube, M.; Dreisbach, A.; Jedlitschky, G.; Meissner, K.; Linnemann, K.; Fusch, C.; Ritter, C. A.; Volker, U.; Kroemer, H. K. Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP). *Drug Metab. Dispos.* **2006**, *34*, 524−33.
(43) Seamon, K. B.; Padgett, W.; Daly, J. W. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78*, 3363−7.
(44) Tomi, M.; Miyata, Y.; Noguchi, S.; Nishimura, S.; Nishimura, T.; Nakashima, E. Role of protein kinase A in regulating steroid sulfate uptake for estrogen production in human placental choriocarcinoma cells. *Placenta* **2014**, *35*, 658−60.
(45) Kohan-Ghadr, H. R.; Kadam, L.; Jain, C.; Armant, D. R.; Drewlo, S. Potential role of epigenetic mechanisms in regulation of trophoblast differentiation, migration, and invasion in the human placenta. *Cell Adh Migr* **2016**, *10*, 126−35.
(46) Karbanova, S.; Cerveny, L.; Ceckova, M.; Ptackova, Z.; Jiraskova, L.; Greenwood, S.; Staud, F. Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine. *Placenta* **2017**, *60*, 86−92.
(47) Vargas, A.; Toufaily, C.; LeBellego, F.; Rassart, E.; Lafond, J.; Barbeau, B. Reduced expression of both syncytin 1 and syncytin 2 correlates with severity of preeclampsia. *Reprod. Sci.* **2011**, *18*, 1085−91.
(48) Radilova, H.; Libra, A.; Holasova, S.; Safarova, M.; Viskova, A.; Kunc, F.; Buncek, M. COX-1 is coupled with mPGES-1 and ABCC4 in human cervix cancer cells. *Mol. Cell. Biochem.* **2009**, *330*, 131−40.
(49) Vandesompele, J.; De Preter, K.; Pattyn, F.; Poppe, B.; Van Roy, N.; De Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **2002**, *3*, No. RESEARCH0034.
(50) Al-Nasiry, S.; Spitz, B.; Hanssens, M.; Luyten, C.; Pijnenborg, R. Differential effects of inducers of syncytialization and apoptosis on BeWo and JEG-3 choriocarcinoma cells. *Hum. Reprod.* **2006**, *21*, 193−201.
(51) Grden, M.; Podgorska, M.; Szutowicz, A.; Pawelczyk, T. Diabetes-induced alterations of adenosine receptors expression level in rat liver. *Exp. Mol. Pathol.* **2007**, *83*, 392−8.
(52) Valdes, R.; Ortega, M. A.; Casado, F. J.; Felipe, A.; Gil, A.; Sanchez-Pozo, A.; Pastor-Anglada, M. Nutritional regulation of nucleoside transporter expression in rat small intestine. *Gastroenterology* **2000**, *119*, 1623−30.
(53) Cerveny, L.; Ptackova, Z.; Ceckova, M.; Jiraskova, L.; Karbanova, S.; Karahoda, R.; Greenwood, S.; Glazier, J. D.; Staud, F. Equilibrative nucleoside transporter 1 (ENT1) facilitates transfer of the antiretroviral drug abacavir across the placenta, submitted.
(54) Raivio, K. O.; Vettenranta, K. Changes in trophoblastic purine metabolism with aging of the placenta. *Adv. Exp. Med. Biol.* **1989**, *253A*, 399−406.
(55) Huber-Ruano, I.; Pinilla-Macua, I.; Torres, G.; Casado, F. J.; Pastor-Anglada, M. Link between high-affinity adenosine concentrative nucleoside transporter-2 (CNT2) and energy metabolism in intestinal and liver parenchymal cells. *J. Cell. Physiol.* **2010**, *225*, 620−30.
(56) Duflot, S.; Riera, B.; Fernandez-Veledo, S.; Casado, V.; Norman, R. I.; Casado, F. J.; Lluis, C.; Franco, R.; Pastor-Anglada, M. ATP-sensitive K(+) channels regulate the concentrative adenosine transporter CNT2 following activation by A(1) adenosine receptors. *Mol. Cell. Biol.* **2004**, *24*, 2710−9.
(57) Wice, B.; Menton, D.; Geuze, H.; Schwartz, A. L. Modulators of Cyclic-Amp Metabolism Induce Syncytiotrophoblast Formation Invitro. *Exp. Cell Res.* **1990**, *186*, 306−316.
(58) Borges, M.; Bose, P.; Frank, H. G.; Kaufmann, P.; Potgens, A. J. A two-colour fluorescence assay for the measurement of syncytial fusion between trophoblast-derived cell lines. *Placenta* **2003**, *24*, 959−64.
(59) del Santo, B.; Valdes, R.; Mata, J.; Felipe, A.; Casado, F. J.; Pastor-Anglada, M. Differential expression and regulation of nucleoside transport systems in rat liver parenchymal and hepatoma cells. *Hepatology* **1998**, *28*, 1504−11.
(60) Neelima, P. S.; Rao, A. J. Gene expression profiling during Forskolin induced differentiation of BeWo cells by differential display RT-PCR. *Mol. Cell. Endocrinol.* **2008**, *281*, 37−46.
(61) Huebner, H.; Strick, R.; Wachter, D. L.; Kehl, S.; Strissel, P. L.; Schneider-Stock, R.; Hartner, A.; Rascher, W.; Horn, L. C.; Beckmann, M. W.; Ruebner, M.; Fahlbusch, F. B. Hypermethylation and loss of retinoic acid receptor responder 1 expression in human choriocarcinoma. *J. Exp. Clin. Cancer Res.* **2017**, *36*, 165.
(62) Le Vee, M.; Jouan, E.; Stieger, B.; Fardel, O. Differential regulation of drug transporter expression by all-trans retinoic acid in hepatoma HepaRG cells and human hepatocytes. *Eur. J. Pharm. Sci.* **2013**, *48*, 767−74.
(63) Ross-Innes, C. S.; Brown, G. D.; Carroll, J. S. A co-ordinated interaction between CTCF and ER in breast cancer cells. *BMC Genomics* **2011**, *12*, 593.
(64) Gurvich, N.; Tsygankova, O. M.; Meinkoth, J. L.; Klein, P. S. Histone deacetylase is a target of valproic acid-mediated cellular differentiation. *Cancer Res.* **2004**, *64*, 1079−86.
(65) Kwiecinska, P.; Wisniewska, J.; Gregoraszczuk, E. L. Effects of valproic acid (VPA) and levetiracetam (LEV) on proliferation, apoptosis and hormone secretion of the human choriocarcinoma BeWo cell line. *Pharmacol. Rep.* **2011**, *63*, 1195−202.
(66) Pospechova, K.; Rozehnal, V.; Stejskalova, L.; Vrzal, R.; Pospisilova, N.; Jamborova, G.; May, K.; Siegmund, W.; Dvorak, Z.; Nachtigal, P.; Semecky, V.; Pavek, P. Expression and activity of vitamin D receptor in the human placenta and in choriocarcinoma BeWo and JEG-3 cell lines. *Mol. Cell. Endocrinol.* **2009**, *299*, 178−87.
(67) Kostrouchova, M.; Kostrouch, Z.; Kostrouchova, M. Valproic acid, a molecular lead to multiple regulatory pathways. *Folia Biol. (Praha)* **2007**, *53*, 37−49.
(68) Cerveny, L.; Svecova, L.; Anzenbacherova, E.; Vrzal, R.; Staud, F.; Dvorak, Z.; Ulrichova, J.; Anzenbacher, P.; Pavek, P. Valproic acid induces CYP3A4 and MDR1 gene expression by activation of constitutive androstane receptor and pregnane X receptor pathways. *Drug Metab. Dispos.* **2007**, *35*, 1032−41.
(69) Shin, J. S.; Jeong, H. S.; Kim, M. K.; Yun, H. Y.; Baek, K. J.; Kwon, N. S.; Kim, D. S. The DNA methylation inhibitor 5-azacytidine decreases melanin synthesis by inhibiting CREB phosphorylation. *Pharmazie* **2015**, *70*, 646−9.
(70) Damaraju, V. L.; Mowles, D.; Yao, S.; Ng, A.; Young, J. D.; Cass, C. E.; Tong, Z. Role of human nucleoside transporters in the uptake and cytotoxicity of azacitidine and decitabine. *Nucleosides, Nucleotides Nucleic Acids* **2012**, *31*, 236−55.
(71) Rius, M.; Keller, D.; Brom, M.; Hummel-Eisenbeiss, J.; Lyko, F.; Keppler, D. Vectorial transport of nucleoside analogs from the apical to the basolateral membrane in double-transfected cells expressing the human concentrative nucleoside transporter hCNT3 and the export pump ABCC4. *Drug Metab. Dispos.* **2010**, *38*, 1054−63.
(72) Hakeda, Y.; Ikeda, E.; Kurihara, N.; Nakatani, Y.; Maeda, N.; Kumegawa, M. Induction of osteoblastic cell differentiation by forskolin. Stimulation of cyclic AMP production and alkaline phosphatase activity. *Biochim. Biophys. Acta, Gen. Subj.* **1985**, *838*, 49−53.
(73) Gerbaud, P.; Tasken, K.; Pidoux, G. Spatiotemporal regulation of cAMP signaling controls the human trophoblast fusion. *Front. Pharmacol.* **2015**, *6*, 202.
(74) Fernandez-Veledo, S.; Huber-Ruano, I.; Aymerich, I.; Duflot, S.; Casado, F. J.; Pastor-Anglada, M. Bile acids alter the subcellular

DOI: 10.1021/acs.molpharmaceut.8b00238
Mol. Pharmaceutics 2018, 15, 2732−2741

localization of CNT2 (concentrative nucleoside cotransporter) and increase CNT2-related transport activity in liver parenchymal cells. *Biochem. J.* **2006**, *395*, 337−44.

DOI: 10.1021/acs.molpharmaceut.8b00238
Mol. Pharmaceutics 2018, 15, 2732−2741

Publikace II: Are *ENT1*/ENT1, *NOTCH3,* and miR-21 Reliable Prognostic Biomarkers in Patients with Resected Pancreatic Adenocarcinoma Treated with Adjuvant Gemcitabine Monotherapy?

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Are *ENT1*/ENT1, *NOTCH3*, and miR-21 Reliable Prognostic Biomarkers in Patients with Resected Pancreatic Adenocarcinoma Treated with Adjuvant Gemcitabine Monotherapy?

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Received: 13 September 2019; Accepted: 18 October 2019; Published: 23 October 2019

Abstract: Evidence on equilibrative nucleoside transporter 1 (ENT1) and microRNA-21 (miR-21) is not yet sufficiently convincing to consider them as prognostic biomarkers for patients with pancreatic ductal adenocarcinoma (PDAC). Here, we investigated the prognostic value of *ENT1*/ENT1, miR-21, and neurogenic locus homolog protein 3 gene (*NOTCH3*) in a well-defined cohort of resected patients treated with adjuvant gemcitabine chemotherapy ($n = 69$). Using a combination of gene expression quantification in microdissected tissue, immunohistochemistry, and univariate/multivariate statistical analyses we did not confirm association of *ENT1*/ENT1 and *NOTCH3* with improved disease-specific survival (DSS). Low miR-21 was associated with longer DSS in patients with negative regional lymph nodes or primary tumor at stage 1 and 2. In addition, downregulation of *ENT1* was observed in PDAC of patients with high *ENT1* expression in normal pancreas, whereas *NOTCH3* was upregulated in PDAC of patients with low *NOTCH3* levels in normal pancreas. Tumor miR-21 was upregulated irrespective of its expression in normal pancreas. Our data confirmed that patient stratification based on expression of *ENT1*/ENT1 or miR-21 is not ready to be implemented into clinical decision-making processes. We also conclude that occurrence of *ENT1* and *NOTCH3* deregulation in PDAC is dependent on their expression in normal pancreas.

Keywords: adjuvant gemcitabine monotherapy; equilibrative nucleoside transporter 1; neurogenic locus homolog protein 3; miR-21; resected pancreatic ductal adenocarcinoma; prognostic biomarker

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid malignancies, representing the fourth leading cause of cancer-related mortality in the modern world [1]. Nearly as many people die of the disease as are diagnosed each year [2], and by 2030, PDAC is expected to be the second leading cause of cancer-related death [3]. The main treatment for potentially curative therapy is still surgical removal of the tumor with tumor-free resection margins (R0). Resection is

achievable in less than 20% of patients [4] and is associated with 10% and 7.7% five- and ten-year survival, respectively [5–7].

Adjuvant gemcitabine monotherapy (GEM) doubles the five-year overall survival to up to 21% in patients with R0 resection [5–8]. Recently, Neoptolemos et al. (2017) published results of a multicenter, open-label, randomized phase clinical trial (ESPAC-4) which demonstrated superiority of GEM plus capecitabine combination over monotherapy with GEM: the overall survival was 28 months in the GEM with capecitabine group vs. 25.5 months in the GEM monotherapy group. However, a higher frequency of grade 3–4 adverse events has been reported for GEM plus capecitabine combination [9]. Another multicenter, open-label, randomized phase III clinical trial (PRODIGE 24/CCTG PA.6 (NCT01526135) showed that modified-dose FOLFIRINOX (mFOLFIRINOX), consisting of oxaliplatin at 85 mg/m^2, leucovorin at 400 mg/m^2, irinotecan at 150 mg/m^2, and 5-fluorouicil at 2.4 g/m^2 increases three-year survival compared with GEM monotherapy (63.4% vs. 48.6%). Nevertheless, the safety profile of the mFOLFIRINOX regimen was suggested as less favorable than that based on gemcitabine monotherapy [10].

Besides showing more acceptable toxicity, it has been documented that GEM monotherapy is a cost-effective option when compared with GEM plus capecitabine in an adjuvant regimen [11]. However, to improve the cost-effectiveness of adjuvant GEM monotherapy, it is important to identify patients that could significantly profit from the treatment [12–15].

Over the last decade, there has been a hunt for valuable prognostic/predictive biomarkers and reliable methods for their analysis that could be helpful in the estimation of PDAC patients' responsiveness to GEM. Of the biomarkers investigated so far, human equilibrative nucleoside transporter 1 (ENT1), microRNA-21 (miR-21), and neurogenic locus homolog protein 3 (NOTCH3) are considered promising.

ENT1 is the most important transporter for GEM influx into pancreatic cells [16,17], and hence has been extensively investigated. Currently, some evidence from immunohistochemistry and mRNA analyses performed in formalin-fixed paraffin-embedded (FFPE) samples supports a hypothesis that low expression of ENT1 might be an indicator of chemoresistance to GEM in resected patients [12,13,18,19], but contradictory findings have also been published [20,21].

miR-21 is a short (22 nt), very stable, noncoding RNA targeting *Bcl-2* [22] that likely plays an important role in preventing apoptosis, thus functioning as a proto-oncogene [23]. High miR-21 expression has been associated with significantly shorter overall survival in resected patients [24,25].

To date, most studies investigating the prognostic value of ENT1 and miR-21 have been conducted in a small cohort [19], on patients treated with a combination of adjuvant GEM and radiation [12,19,26], a cohort mixing patients with adjuvant and palliative settings [25] and/or for which chemotherapy is not reported [27]. Only a limited number of studies have been performed in a well-defined homogenous cohort of resected patients with adjuvant GEM monotherapy [13,28]. The overall evidence on ENT1/*ENT1* and miR-21 is thus encouraging, but not yet sufficiently convincing to implement this procedure in the clinical environment.

NOTCH3 is linked to the GEM-resistant PDAC phenotype. NOTCH3 confers cell extracellular interactions, such as invasion, migration, motility, and modification of survival of pancreatic cells [29]. NOTCH3 is related to GEM-induced caspase-mediated apoptosis [30]. Using multivariate analysis, high *NOTCH3* mRNA levels have been associated with shorter survival of GEM-treated patients with advanced PDAC [31]. However, to date, this biomarker has not been evaluated in resected patients with GEM monotherapy.

Several studies have indicated that low miR-21, low NOTCH3, and high *ENT1* may be used as GEM-independent favorable prognostic factors of the effect of GEM therapy [26,32–34]. Considerable inter-individual expression of NOTCH3 and miR-21 in tumor tissue, ranging from negative to strongly positive, has been reported [32,35], and increased expression of these molecules in PDAC has been suggested [25,27,32,33,35–37]. However, upregulation does not appear in all patients and it remains to be elucidated whether elevated levels of these molecules correspond with either low or high expression

in normal pancreas or are independent. Moreover, data about expression of *ENT1* in PDAC compared with normal pancreas are completely lacking.

In this study, we aimed to use quantitative real-time polymerase chain reaction (qRT-PCR) analysis of *ENT1*, miR-21, and *NOTCH3* expression in FFPE samples collected from a homogenous group of patients with resected PDAC, treated with adjuvant GEM therapy (n = 69) to evaluate the prognostic value of the associated transcripts for the estimation of disease-specific survival (DSS). Moreover, we analyzed expression profiles of *ENT1*, miR-21, and *NOTCH3* in PDAC tissue of different patient subgroups, defined by the median of expression in normal pancreas.

2. Results

2.1. Clinical–Pathological Characteristics of Patients

Clinical characteristics, including age, gender, surgery type, resection margin status, stage of primary tumor, regional lymph nodes, distant metastasis, DSS, and American Society of Anesthesiologists (ASA) score, of the patients (n = 69) are summarized in Table 1. Thirty five (50.7%) patients finished all the cycles of chemotherapy, whereas 34 patients (49.3%) prematurely terminated treatment because of disease progression (14; 20.2%), toxicity (17; 24.6%), heart failure (1; 1.5%), respiratory failure (1; 1.5%), or sudden death (1; 1.5%). In the monitored cohort, one patient had small metastases in the peritoneum in close proximity to the pancreas; the metastases were surgically removed. Fourteen patients were alive at the end of follow-up (31st December 2018).

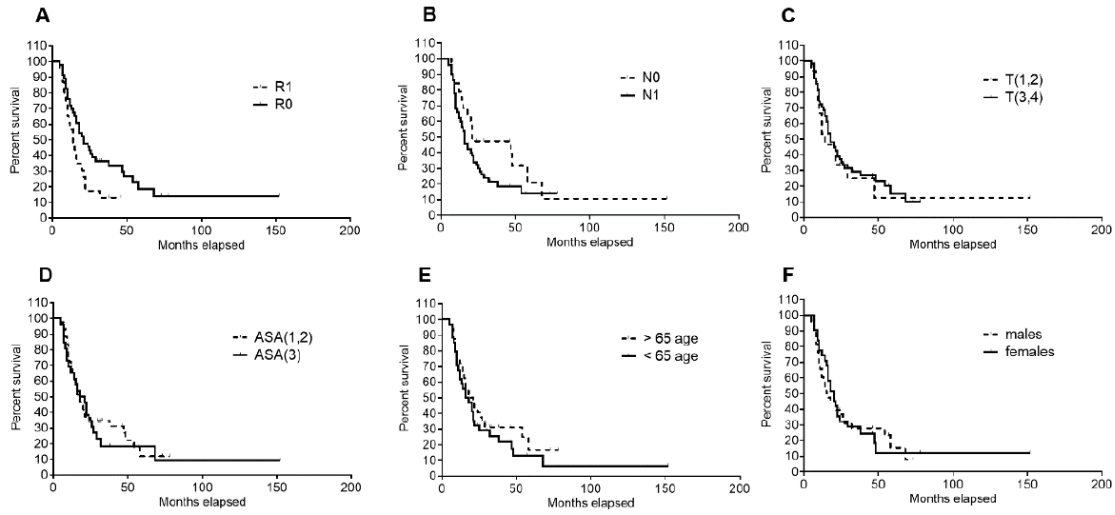
Table 1. Clinical–pathological characteristics of patients.

Number of Patients	69
Gender (females/males)	31/38
Age (years)	
Median	65
Range	39–80
Surgery (type of resection)	
PD	55
DP	14
Resection margin status	
R0	46
R1	23
T: stage of primary tumor	
T1	3
T2	12
T3	53
T4	1
N: regional lymph nodes	
N0	19
N1	50
M: distant metastasis	
M0	68
M1	1
DSS from surgery (months)	
Median	21
Range	5–152
ASA (I-III)	
I	1
II	42
III	26

ASA, American Society of Anesthesiologists score; DSS, disease-specific survival; GEM, gemcitabine; DP, distal pancreatectomy; PD, pancreaticoduodenectomy.

2.2. Clinical–Pathological Factors and Chemotherapy Response as Survival Markers

To evaluate the effects of tumor and patients' characteristics and type of resection on DSS, we performed a statistical analysis using Kaplan–Meier curves and the log rank test. We dichotomized the tested cohort based on resection margin status (R0/R1), presence of metastatic involvement of regional lymph nodes (N0/N1), primary tumor stage T(1,2)–T(3,4), ASA score ASA(1,2)/ASA(3,4), patients' age (>65 age/<65 age), gender, and type of resection (PD/DP). R0 was found to be associated with significantly longer DSS over R1 (21 months vs. 14 months, $p = 0.0314$, hazard ratio = 0.5663, and 95% confidence interval = 0.3062–1.047) (Figure 1A), whereas other analyzed parameters did not show any association with DSS (Figure 1).



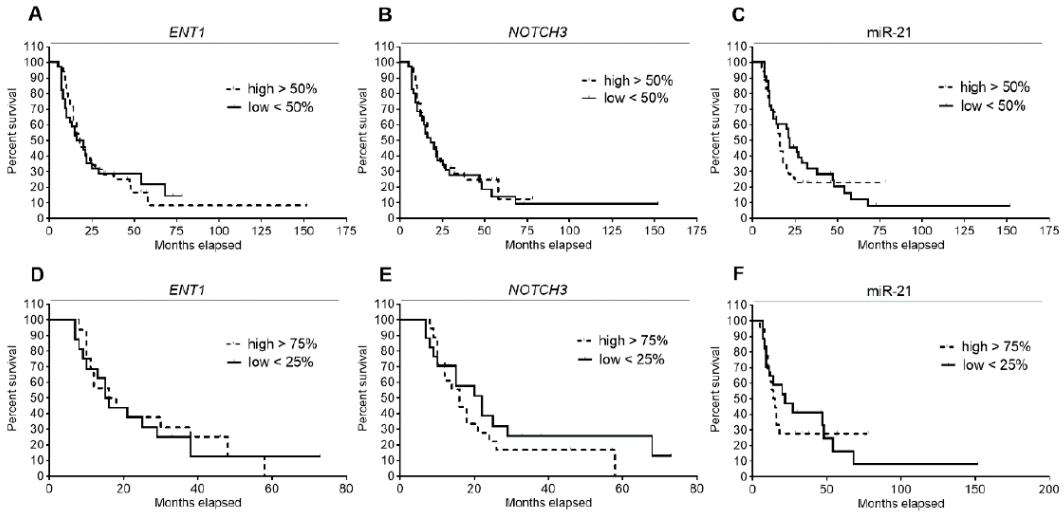
Characteristics	*n*	DSS (months)	*P* value	Hazard ratio	95 % CI
R0	46	21	0.0314*	0.5663	0.3062 - 1.047
R1	23	14		1.766	0.9548 - 3.266
N0	19	21	0.1520	0.6533	0.3744 - 1.140
N1	50	16		1.531	0.8774 - 2.671
T(1,2)	15	14	0.6812	1.140	0.5864 - 2.217
T(3,4)	54	18		0.8771	0.4511 - 1.705
ASA(1,2)	43	18	0.6620	0.8891	0.5146 - 1.536
ASA(3,4)	26	19.5		1.125	0.6511 - 1.943
Age > 65	32	19.5	0.3308	0.7696	0.4490 - 1.319
Age < 65	34	17		1.299	0.7581 - 2.227
Male	38	15.5	0.8591	1.048	0.6170 - 1.780
Female	31	20		0.9542	0.5617 - 1.621
PD	55	18	0.7067	0.8875	0.4575 - 1.722
DP	14	21		1.127	0.5809 - 2.186

Figure 1. Analysis of patients' DSS association with resection margin status, regional lymph node involvement, primary tumor stage, ASA score, age, gender, and type of resection using Kaplan–Meier curves. Patients ($n = 69$) were dichotomized based on (**A**) tumor border (R0/R1), (**B**) negative/positive regional lymph nodes (N0/N1), (**C**) primary tumor stage T(1,2)/T(3,4), (**D**) ASA score ASA(1,2)/ASA(3,4), (**E**) patients' age (> 65 age/< 65 age), (**F**) gender, and (**G**) type of resection (PD/DP). The data were analyzed using the log rank test. Statistical significance is denoted by *, $p < 0.05$.

2.3. Analysis of ENT1, NOTCH3 mRNA, and miR-21 Levels in PDAC Tissue and Their Association with DSS

To investigate possible association of *ENT1*, *NOTCH3*, and miR-21 levels with patients' DSS, we quantified gene expression of the transcripts in tumor tissue microdissected from FFPE samples ($n = 69$). For the purpose of Kaplan–Meier survival analysis, patients were dichotomized by the median of expression of the respective molecules into two groups (low <50 and high >50%). However, we did not observe any association between *ENT1*, *NOTCH3*, or miR-21 expression and patients' DSS (Figure 2). Only patients with low miR-21 showed a trend for longer DSS (22 vs. 16 months, p value 0.4649, Figure 2C). Subsequently, patients were divided based on the expression of individual markers into

quartile subgroups. We selected only subgroups of patients with the lowest (<25%, first quartile) and highest (>75%, fourth quartile) expression of each marker for subsequent analyses. However, despite using these more polarized subsets of patients, no significant differences were observed (Figure 2D–F).



ENT1	**low (< 50%)**	**high (> 50%)**	**low (< 25 %)**	**high (> 75 %)**
n	33	35	16	17
DSS (months)	18	18	15.5	17
P value	0.939		0.7968	
Hazard ratio	1.02	0.981	1.1	0.9088
95 % CI	0.5933 – 1.758	0.5687 - 1.685	0.5161 - 2.346	0.4262 - 1.938
NOTCH3	**low (< 50%)**	**high (> 50%)**	**low (< 25 %)**	**high (> 75 %)**
n	34	34	16	18
DSS (months)	18	18	22	16
P value	0.7019		0.3325	
Hazard ratio	1.112	0.8997	0.7091	1.41
95 % CI	0.6469 - 1.91	0.5236 - 1.546	0.3418 - 1.471	0.6799 - 2.925
miR-21	**low (< 50%)**	**high (> 50%)**	**low (< 25 %)**	**high (> 75 %)**
n	35	33	17	18
DSS (months)	22	16	22	14.5
P value	0.4649		0.8045	
Hazard ratio	0.8272	1.209	1.097	0.9114
95 % CI	0.4859 - 1.408	0.7102 - 2.058	0.5147 - 2.339	0.4275 - 1.943

Figure 2. Analysis of patients' DSS association with *ENT1*, *NOTCH3* mRNA, and miR-21 levels in PDAC tissue using Kaplan–Meier curves. Patients (n = 69) were divided into two groups according to the median (low <50%, high >50%) (**A**–**C**) or, alternatively, only subgroups of patients belonging to the first and fourth quartiles (low <25%, high >75%) (**D**–**F**) of expression of *ENT1* (**A**,**D**), *NOTCH3* (**B**,**E**), or miR-21 (**C**,**F**) were selected. No significant differences between the tested subgroups of patients were observed. Statistical significance was evaluated by applying log-rank test analysis.

In multivariate DSS analysis adjusted to resection margin status (R0/R1), gender (female/male), ASA score (I–III), primary tumor stage (T1–T4), regional lymph node (N0/N1), and type of resection (PD/DP), we did not observe any significant association between patients' DSS and mRNA expression of *ENT1* (Table 2), *NOTCH3* (Table 3), and miR-21 (Table 4). As shown in Table 4, only high levels of miR-21 tended to statistical significance (p = 0.089, hazard ratio = 0.475), and T3 was shown as a favorable factor (p = 0.036, hazard ratio = 0.085).

Table 2. Results of multivariate analysis (Cox's proportional hazards model) of the effects of clinical and pathological characteristics and *ENT1* mRNA expression on patients' DSS.

Patient Stratification	*p* Value	Hazard Ratio	95.0% CI	
Gender (male)	0.918	0.970	0.543	1.731
ENT1 (above median)	0.821	0.934	0.514	1.695
Resection margin status (R1)	0.135	0.624	0.336	1.159
ASA score				
ASA III (reference value)	0.601			
ASA I	0.421	2.395	0.286	20.063
ASA II	0.639	0.869	0.484	1.561
Primary tumor stage				
T4 (reference value)	0.272			
T1	0.434	0.354	0.026	4.786
T2	0.182	0.212	0.022	2.068
T3	0.106	0.169	0.019	1.463
Regional lymph nodes (N1)	0.149	0.591	0.289	1.207
Resection type (DP)	0.675	1.159	0.582	2.309

ASA, American Society of Anesthesiologists score; CI, confidence interval; DP, distal pancreatectomy. Reference levels are displayed between brackets.

Table 3. Results of multivariate analysis (Cox's proportional hazards model) of the effects of clinical and pathological characteristics and *NOTCH3* mRNA expression on patients' DSS.

Patient Stratification	*p* Value	Hazard Ratio	95.0% CI	
Gender (male)	0.956	0.984	0.553	1.751
NOTCH3 (above median)	0.383	1.286	0.731	2.263
Resection margin status (R1)	0.126	0.614	0.328	1.147
ASA score				
ASA III (reference value)	0.725			
ASA I	0.484	2.120	0.259	17.379
ASA II	0.773	0.915	0.501	1.671
Primary tumor stage				
T4 (reference value)	0.299			
T1	0.507	0.424	0.034	5.358
T2	0.267	0.275	0.028	2.686
T3	0.144	0.200	0.023	1.728
Regional lymph nodes (N1)	0.152	0.598	0.296	1.209
Resection type (DP)	0.661	1.163	0.593	2.283

ASA, American Society of Anesthesiologists score; CI, confidence interval; DP, distal pancreatectomy. Reference levels are displayed between brackets.

Table 4. Results of multivariate analysis (Cox's proportional hazards model) of the effects of clinical and pathological characteristics and miR-21 expression on patients' DSS.

Patient Stratification	*p* Value	Hazard Ratio	95.0% CI	
Gender (male)	0.817	0.935	0.530	1.649
miR-21 (above median)	0.089	0.475	0.201	1.120
Resection margin status (R1)	0.113	0.604	0.324	1.126
ASA score				
ASA III (reference value)	0.587			
ASA I	0.477	2.138	0.263	17.375
ASA II	0.530	0.828	0.460	1.491
Primary tumor stage				
T4 (reference value)	0.091			
T1	0.280	0.237	0.017	3.234
T2	0.088	0.131	0.013	1.355
T3	0.036	0.085	0.009	0.846
Regional lymph nodes (N1)	0.175	0.619	0.310	1.238
Resection type (DP)	0.658	1.169	0.586	2.335

ASA, American Society of Anesthesiologists score; CI, confidence interval; DP, distal pancreatectomy. Reference levels are displayed between brackets.

2.4. Immunostaining of ENT1 in FFPE Samples of PDAC

Immunohistochemical evaluation is the most frequently used procedure for evaluating ENT1 as a prognostic/predictive biomarker for patients with PDAC. Therefore, it is considered as a "standard" method. ENT1 staining showed predominantly membranous positivity in the cells of Langerhans islets and lymphocytes. Thus, the presence of this type of staining in normal Langerhans islets served as an internal positive control of the method (Figure 3). Quantitative scoring using light microscopy was conducted by a single experienced pathologist (AR). Of the 63 tissue samples, 54 had detectable ENT1 immunostaining (intensity score from 1 to 3), some of which revealed heterogeneous expression with regions lacking ENT1. Nine samples of PDAC were without any detectable ENT1 expression (intensity score 0). The percentage of adenocarcinoma cell staining at each intensity level was recorded for each specimen. Patients with a high histoscore (values 6–9) for ENT1 (n = 24) revealed a median DSS of 23 months, whereas patients with a low (values 1–5) histoscore (n = 30) and negative (n = 9) ENT1 showed a median DSS of 18 months (Figure 4).

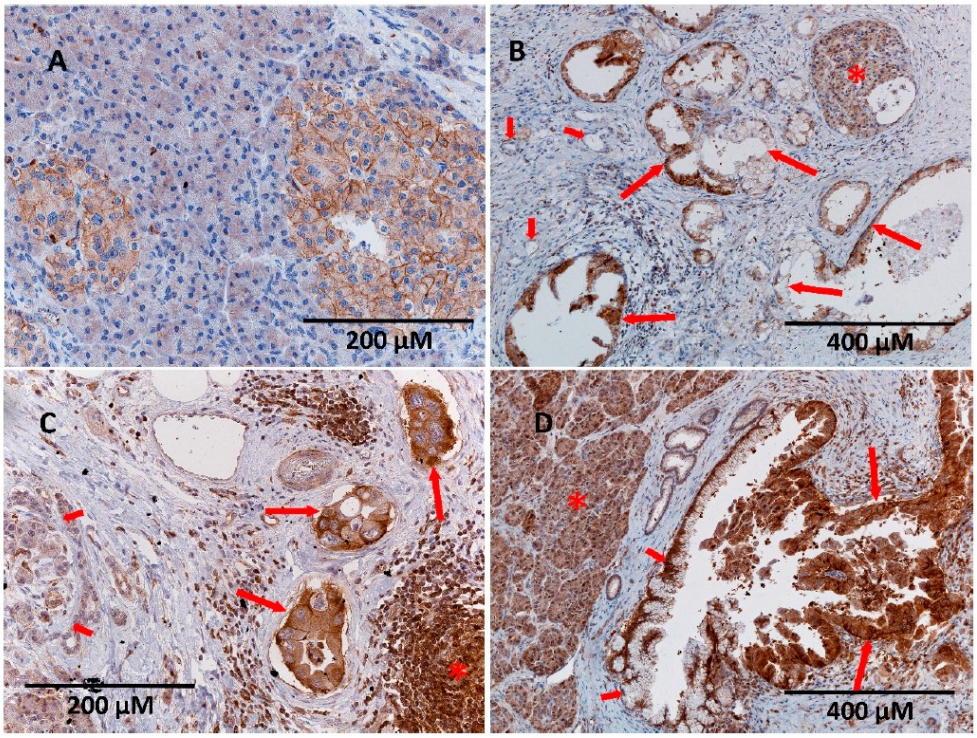
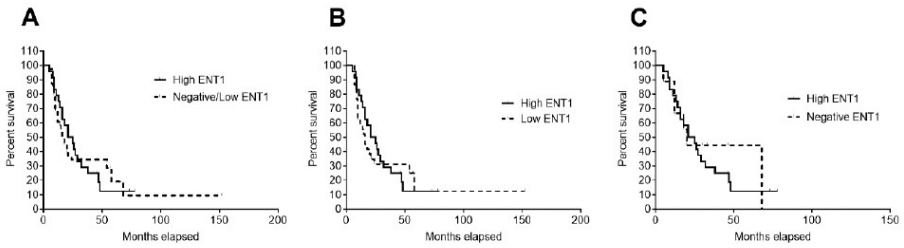


Figure 3. Variability of expression of ENT1 transporter in normal pancreas and pancreatic ductal adenocarcinoma (PDAC). (**A**) Membranous expression is seen in cells of normal Langerhans islets, whereas pancreatic acini are completely negative. (**B**) In some cases, heterogeneous positivity of neoplastic cells of PDAC could be observed (long arrows). Note that the non-neoplastic ducts are negative (short arrows). Membranous positivity in the cells of normal Langerhans islets served as an internal positive control (asterisk). (**C**) PDAC cells with strong membranous and cytoplasmic staining for ENT1 (long arrows). Lymphoid elements show the same level of positivity (asterisk), whereas non-neoplastic exocrine pancreas is virtually negative (short arrows). (**D**) Strong cytoplasmic staining in both the acini of normal pancreas (asterisk) and neoplastic cells lining the dilated duct (long arrows), whereas non-neoplastic ductal cells show only weak membranous, predominantly basal, positivity (short arrows). Original magnification was 200× (**A**,**C**) and 100× (**B**,**D**).



ENT1 staining	negative + low/high	low/high	negative/high
P value	0.9012	0.7435	0.6876
Hazard ratio	1.036	1.103	0.8345
95 % CI	0.5847 – 1.863	0.6026 – 2.020	0.3480 – 1.986

Figure 4. Kaplan–Meier curves showing differences in DSS based on immunohistochemical expression of ENT1. Patients (n = 63) were divided according to histoscore (see Methods section) into three groups with negative (n = 9), low (n = 30), and high (n = 24) protein expression of ENT1. Statistical significance was evaluated by applying log-rank test analysis between subgroups with (**A**) high and negative/low ENT1 expression, (**B**) subgroups with high and low ENT1 expression, and (**C**) subgroups with high and negative ENT1 expression. No significant association of ENT1 expression with patients' DSS was observed.

In the multivariate model adjusted to resection margin status (R0/R1), gender (female/male), ASA score (I–III), primary tumor stage (T1–T4), regional lymph node (N1/N0, i.e., positivity/negativity), type of resection (PD/DP), and ENT1 protein expression analyzed by immunohistochemistry, we observed significant positive association between patients' DSS and N0 ($p = 0.049$, hazard ratio = 0.424; Table 5). The R0 resection margin status also revealed a trend for positive association with patients' DSS ($p = 0.051$, hazard ratio = 0.505; Table 5).

Table 5. Results of multivariate analysis (Cox's proportional hazards model) of the effect of clinical and pathological characteristics and ENT1 protein levels analyzed by immunohistochemistry in FFPE samples on patients' DSS.

Patient Stratification	*p* Value	Hazard Ratio	95.0% CI	
Gender (male)	0.836	0.936	0.500	1.752
ENT1				
High (reference value)	0.277			
Negative	0.471	1.469	0.517	4.174
Low	0.109	1.800	0.877	3.695
Resection margin status (R1)	0.051	0.505	0.254	1.003
ASA score				
ASA III (reference value)	0.592			
ASA I	0.768	1.385	0.159	12.091
ASA II	0.367	0.747	0.396	1.408
Primary tumor stage				
T4 (reference value)	0.179			
T1	0.637	0.513	0.032	8.162
T2	0.175	0.211	0.022	2.001
T3	0.075	0.138	0.016	1.222
Regional lymph nodes (N1)	0.049	0.424	0.180	0.996
Resection type (DP)	0.865	1.070	0.492	2.325

ASA, American Society of Anesthesiologists score; DP, CI, confidence interval; distal pancreatectomy. Reference levels are displayed between brackets.

2.5. Analysis of ENT1 Transcripts in Subgroups with Negative, Low, and High ENT1 Protein Expression Analyzed by Immunohistochemistry

Using absolute qRT-PCR analysis, we compared the number of transcripts in samples of PDAC, stratified as tumors with no, low, or high protein expression. Despite an increasing trend from negative to high subpopulations, we did not observe any significant differences in mRNA expression among these groups of samples (Figure 5).

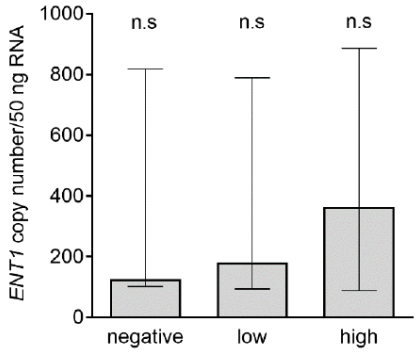


Figure 5. Analysis of *ENT1* transcripts in subgroups with negative, low, and high ENT1 protein expression. Patients were divided based on histoscore of ENT1 expression (and denoted negative ($n = 9$), low ($n = 30$) or high ($n = 24$)); see Methods section. Data are presented as median with interquartile range. Differences in the number of transcripts among subpopulations were analyzed using the nonparametric Kruskal–Wallis test, followed by Dunn's multiple comparison; n.s., not significant.

2.6. Analysis of Patients' DSS Association with ENT1, NOTCH3 mRNA, and miR-21 Levels in Patients' Subgroups, Defined by Clinical–Pathological Characteristics

As we hypothesized that our data might be affected by a high proportion of patients with positive resection margin status (R1), shown to be associated with a significantly shorter DSS (Figure 1A), N1, and/or more advanced primary tumor (T3,4), we analyzed the effect of expression of selected molecules separately in subgroups categorized as R0, N0, or T(1,2) and subsequently, subgroups R1, N1, or T(3,4). In the N0 subgroup, we observed a significantly improved DSS in patients expressing low levels of miR-21 (<50%) over those expressing high levels (>50%) of miR-21 (48 months vs. 15 months; $p = 0.0308$; hazard ratio = 0.3706; 95% confidence interval = 0.1139 to 1.205) (Figure 6A). Comparable to the T(1,2) subgroup, patients with low miR-21 expression (<50%) demonstrated longer DSS than those with high miR-21 expression (>50%) (29 months vs. 10 months; $p = 0.0438$; hazard ratio = 0.3341; 95% confidence interval = 0.09289 to 1.255) (Figure 6B). In other cases, we did not find any correlation between the expression of any tested molecule and patients' DSS.

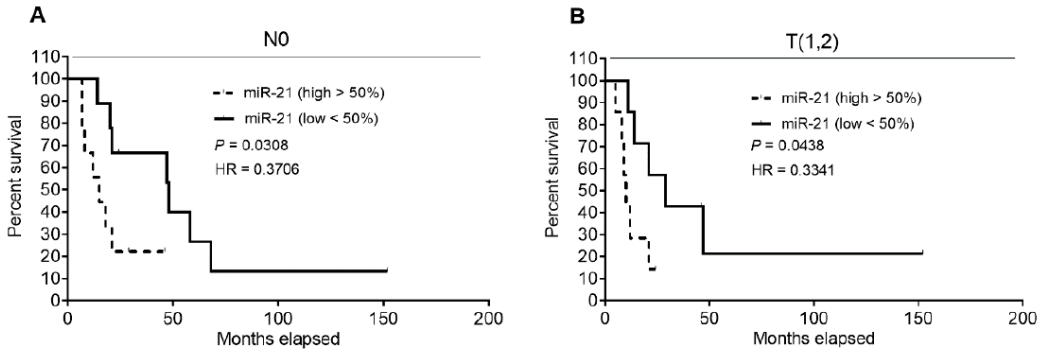


Figure 6. Analysis of patients' DSS association with miR-21 levels in subgroups with N0 and T(1,2) tumors using Kaplan–Meier curves. Patients in the N0 (**A**) and T(1,2) (**B**) subgroups were divided according to the median (low <50%, high >50%) of expression of miR-21. Low expression of miR-21 was significantly associated with improved DSS in both tested cohorts. Statistical significance was evaluated by applying log-rank test analysis, reaching $p = 0.0308$ for N0 patients and $p = 0.0438$ for patients with T(1,2) tumors.

2.7. Quantitative RT-PCR Analysis of ENT1, NOTCH3 mRNA, and miR-21 Expression in FFPE Samples of PDAC

Gene expression of selected markers *ENT1*, *NOTCH3*, and miR-21 was determined in both tumor and normal tissue. This experiment was performed in samples collected from 65 patients, for whom we had FFPE blocks containing sufficient amounts of both tumor and normal tissue. Considerable expression variability was observed for all the analyzed transcripts in both tumor and normal pancreas: *ENT1* (2.4 and 2.6 logs for tumor and normal pancreas, respectively), *NOTCH3* (2.8 logs for both types of tissues) and miR-21 (1.7 and 2.1 logs for tumor and normal pancreas, respectively). Using nonparametric unpaired Mann–Whitney test we found significantly reduced overall *ENT1* mRNA expression in tumor tissue compared with normal pancreas (Figure 7A): decreased *ENT1* expression was detected in tumor tissue of 67.7% (44/65) patients. When analyzing medians of *NOTCH3* and miR-21 expression, both were significantly increased in tumor tissue: upregulation was observed in 72.3% (47/65) and 95.4% (62/65) of patients, respectively (Figure 7B,C). Because we hypothesized that the extent of *ENT1*, *NOTCH3*, and miR-21 deregulation in PDAC might be dependent on their levels in corresponding normal pancreas, we divided patients based on the median of expression of each marker in healthy pancreas (high >50%; low <50%) and compared the expression of each marker in tumor and normal pancreas in these subgroups in paired fashion. Wilcoxon matched-pair signed-rank test showed significantly decreased *ENT1* in PDAC of the high (>50%) subgroup. *NOTCH3* showed

significant upregulation in tumor tissue of the low (<50%) subgroup specimens only, and the amount of miR-21 was increased in both tested subgroups. Subsequently, Mann–Whitney test demonstrated that overall *ENT1* expression in PDAC was still significantly higher than that found in normal pancreas of the low (<50%) subgroup (Figure 7D). *NOTCH3* expression in tumor tissue of the low (<50%) subgroup was lower compared with normal pancreas in the high (>50%) subgroup (Figure 7E). Levels of miR-21 in PDAC of the low (<50%) subgroup were comparable with those in normal pancreas in the high (>50%) subgroup (Figure 7F).

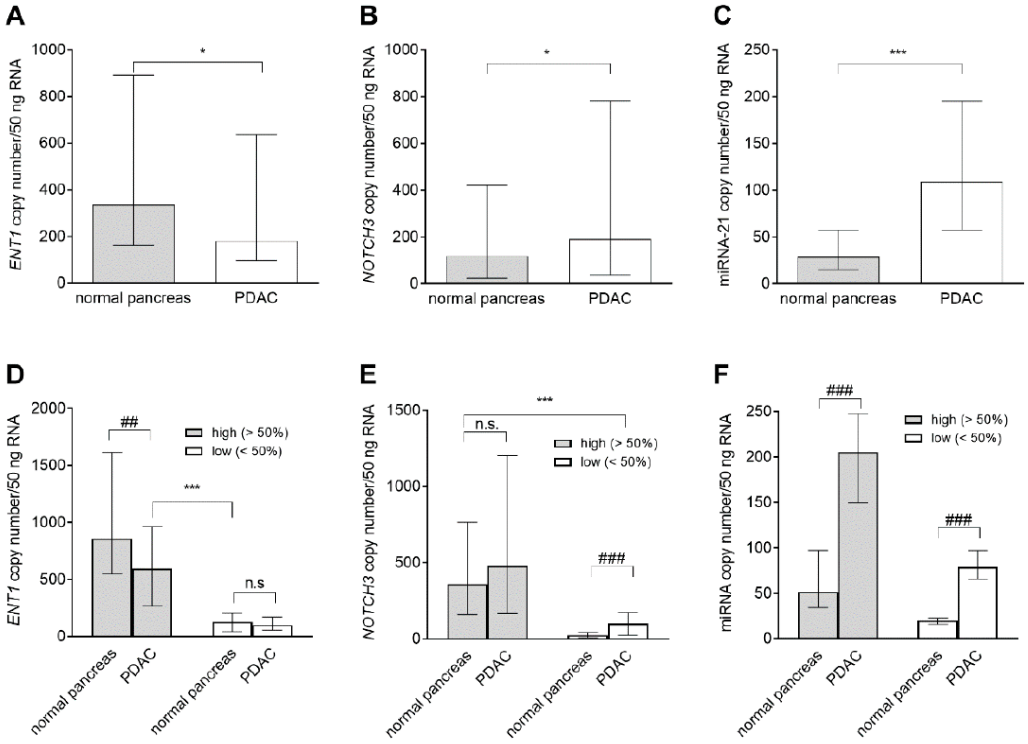


Figure 7. Comparison of mRNA expression of *ENT1*, *NOTCH3* mRNA, and miR-21 in PDAC and normal pancreas. We compared overall expression between normal pancreas and tumor tissues in the whole sample cohort (**A**–**C**) and subsequently, in subpopulations (high >50% and low <50%) sorted according to the median of the respective molecule expression in healthy tissue (**D**–**F**). The data are presented as median of copy number/50 ng of total RNA with the interquartile range. The nonparametric unpaired Mann–Whitney test was used to evaluate differences in overall expression between the PDAC and normal pancreas (**A**–**C**), $n = 65$, and to compare overall expression in respective subgroups (**D**–**F**); significance was denoted * $p < 0.05$ and *** $p < 0.001$. Statistical significance between expression in PDAC and normal pancreas in subgroups defined by medians (**D**–**F**) was evaluated using the Wilcoxon matched-pair signed-rank test: $n = 37$ (high >50%) and $n = 27$ (low <50%) for *ENT1* (**D**); $n = 40$ (high >50%) and $n = 34$ (low <50%) for *NOTCH3* (**E**), and $n = 31$ (high >50%) and $n = 33$ (low <50%) for miR-21 (**F**); ## $p < 0.01$ and ### $p < 0.001$.

3. Discussion

When considering the economic aspects of healthcare interventions [38], adjuvant GEM monotherapy of PDAC represents a cost-effective option [11]. However, the cost-effectiveness of this regimen can potentially be further improved by identification of biomarkers for personalized GEM administration [39]. Considerable attention has been devoted to ENT1 [12–15,18], whereas, to the best of our knowledge, there have only been two studies on miR-21 [24,25] and none on *NOTCH3*.

Immunohistochemistry analysis is an essential tool in everyday clinical practice. However, the performance of this method depends on the availability of a high-quality antibody [40,41] and experienced pathologist [42–45]. Further, the heterogeneity of outcomes obtained with different types of anti-ENT1 antibody [15,21,46] and lack of established standardized scoring procedure for evaluation of ENT1 expression [47] represent critical obstacles preventing full adoption of ENT1 analysis into clinical practice [20]. When staining with antibody 10D7G2, a correlation between high ENT1 expression and improved survival of PDAC patients with adjuvant GEM treatment was shown [18,19,46,48], whereas staining with SP120 antibody failed to reveal any such correlation [20,21]. Recently, Kalloger et al. (2017) attempted to explain the differences between these antibodies. Using a unique statistical approach, they concluded that both antibodies are suitable for stratification of patients but, surprisingly, SP120 is the more useful [28], making the issue of ENT1 evaluation in PDAC even more complicated. In contrast, qRT-PCR analysis might offer a more straightforward method for decision-making. Moreover, qRT-PCR allows quantification of multiple molecules in parallel. However, the applicability qRT-PCR analysis outcomes may be hampered as levels of transcripts may not be proportionally reflected by protein amounts and/or tumor and normal tissue differ only in the subcellular distribution of protein but not the total amount. Moreover, consensus about how to stratify patients based on *ENT1* gene expression and miR-21 levels has not been established: groupings based on (i) tertile of expression [19], median of expression [25], or (iii) results of recursive descent partition analysis have been suggested [12,26]. Despite these methodological drawbacks, high levels of *ENT1* mRNA detected by qRT-PCR in FFPE samples have been demonstrated to be a favorable prognostic marker in PDAC patients with adjuvant GEM therapy [12,19].

Contrary to published data [12,19,24,25], we did not demonstrate in our cohort, strictly defined by administration of the recommended adjuvant GEM regimen, that high *ENT1* and low miR-21 were favorable prognostic factors. We also did not observe an association of low *NOTCH3* with improved DSS (Figure 2). However, this latter finding corresponds with results obtained using univariate analysis performed in patients with advanced disease [31]. Subsequent multivariate analyses confirmed our observation that *ENT1*, *NOTCH3*, and miR-21 are not prognostic biomarkers of patients' responsiveness to GEM (Tables 2–4). However, when we divided the patients into two subgroups based on clinical–pathological characteristics, univariate analysis revealed that low miR-21 is a favorable prognostic factor in N0 patients, as previously demonstrated in another cohort with unspecified post-surgery treatment [32] and also in T(1,2) patients (Figure 6).

As our data on *ENT1* mRNA are in conflict with previous reports [12,19], we also performed an immunohistochemical analysis. DSS in patients with a high histoscore was 23 months, compared with 18 months in patients with low/negative staining, which is comparable to reported data [13]. However, in accordance with the analysis of *ENT1* mRNA, high levels of ENT1 protein were not shown to be a favorable prognostic factor (Figure 3, Table 5). This is in contrast with previous studies obtained using 10D7G2 antibody [13,14,18,28]. Only N0 was shown as an independent factor ($p = 0.049$), and R0 almost reached statistical significance ($p = 0.051$) in multivariate analysis of ENT1 protein expression, analyzed by immunohistochemistry and covariates (Table 5).

Clinical–pathological factors (especially N0/N1, R0/R1) have been widely discussed in terms of patients' prognosis [49–51]. Similarly to Fujita et al. [12], we observed an association of R0 with improved DSS (Figure 1). However, other factors, including primary tumor stage, ASA score, age, gender, and type of resection, did not exhibit an association with DSS (Figure 1).

Like in [12], all patients included in our study received more than three cycles of full-dose chemotherapy. However, the number of patients who refused to continue with or were advised to terminate chemotherapy by an oncologist was 49.3%, which is a higher proportion when compared with data reported from clinical trials [9,10,52]. Clinical–pathologic characteristics of our cohort, patients' chemotherapy intolerability (24.6%), and/or disease progression (20.2%) might explain the observed overall short DSS median (21 months), whereas overall survival longer than 23 months has been reported for patients on GEM therapy, irrespective of ENT1 expression [9,12,24]. Moreover, in

our study, patients with expression of *ENT1* above the median or above 75% demonstrated DSS of 18 and 17 months, respectively, whereas in other studies, 23 [12] and 25.7 [19] months were reported.

Regarding expression of analyzed molecules in PDAC, there is no strictly defined cut-off value to distinguish patients with high and low expression [26]. Only relative values are available, but they differ in the procedure of calculation [12,19]. In Fujita et al. (2010), mRNA levels of a target gene were normalized by expression of the *B2M* housekeeping gene [12], whereas in the study by Giovannetti et al. (2006), values of gene expression were calculated by the *GAPDH* housekeeping/target gene ratio [19]. Importantly, in both studies, information about the stability of these housekeeping genes across the sample cohort was lacking, which complicates interpretation of the data [53]. In our experimental setting, we used the absolute PCR quantification with linear vector with cloned DNA sequence, which is adopted in preferred procedures [54–56]. Therefore, it was not possible to compare our values of expression with previously published ones.

Considering our rigorous approach of analysis, the number of observed subjects in the cohort, and the fact that surgery was performed in the high-volume center by specialist surgeons [57–59], we hypothesize that the prognostic value of *ENT1*/ENT1 and miR-21 was not confirmed because the cohort contained (i) a relatively high proportion of R1, N1, and T(3,4) patients, (ii) a high number of patients who prematurely terminated GEM therapy, (iii) a high proportion of patients expressing markers or bearing a gene expression signature linked to pancreatic cancer disease progression [60–63], factors ignored in all previous studies, and/or (iv) a high proportion of patients with low *ENT1* and high miR-21.

Regarding the last point, we did not demonstrate a significant difference in medians of *ENT1* gene expression among patients with negative, low, and high protein expression, as stratified using the histoscore (Figure 5). A median higher than 100 transcripts of *ENT1*/50 ng RNA in samples collected from negative ENT1 PDAC and approximately 300 transcripts of *ENT1*/50 ng RNA in high ENT1 PDAC indicated that *ENT1* gene expression was not fully proportional to levels of cytoplasmic membrane-embedded ENT1 protein. Posttranscriptional and/or posttranslational regulation [64] and/or altered subcellular localization may play a role in this phenomenon [65–67].

Miyamoto et al. (2003) reported upregulated expression of *NOTCH3* in resected PDAC samples [68]. This finding was subsequently confirmed by Vo et al. (2011) and very recently by Song et al. (2018) [35,69]. Several studies have described upregulation of miR-21 in PDAC [25,27,32,36,37]. However, to date, it has not been demonstrated whether *ENT1*/ENT1 is generally downregulated in PDAC. Our results confirmed an overall increase in expression of *NOTCH3* and miR-21 in tumor tissue when compared with normal pancreas (Figure 7B,C). Deregulation of *miRNAs* has been associated with cell growth, promotion of metastatic phenotype, and/or chemoresistance in PDAC. Upregulation of miR-21 is particularly linked to promotion of cell proliferation, invasion, chemoresistance, and escape from apoptosis [22,70]. Although evidence acquired using hepatocytes and placental cells has suggested constitutive expression of *ENT1* [71–73], we found decreased *ENT1* expression in PDAC (Figure 7A). When dividing the cohort based on medians of expression in normal pancreas we observed upregulation of *NOTCH3* only in the subgroup, with low expression of *NOTCH3* below median (<50%) in normal pancreas (Figure 7E), while *ENT1* was downregulated in PDAC of patients with *ENT1* expression above the median (>50%) in normal pancreas (Figure 7D). Upregulation of miR-21 was independent of levels in normal pancreas (Figure 7F). Considering both outcomes of previous reports on *ENT1* expression in PDAC [12,19] and our data (Figure 7D), we hypothesize that length of survival depends on an individual patient's physiological expression of *ENT1* that seems to determine its expression in tumor. Further investigation is, however, needed, because there is the possibility that the expression of *ENT1* and/or *NOTCH3* in normal pancreas was influenced by factors produced by the tumor.

Considerable expression variability was observed for all the analyzed transcripts in both tumor and normal pancreas. Inter-individual differences in expression of analyzed molecules and, in case of *ENT1*, use of the expression assay recognizing almost all the transcript variants of *ENT1* (Hs01085704_g1) may explain this phenomenon [70,74].

4. Materials and Methods

4.1. Patients and Pancreatic Cancer Staging

Analysis of *ENT1*, *NOTCH3*, and miR-21 was performed in FFPE samples collected from 69 patients with PDAC who underwent surgical resection between 2006 and 2016 at the Department of Surgery, University Hospital, Hradec Kralove [57–59] and showed no substantial postoperative complications. Pancreatic cancer primary tumor/regional lymph nodes/distant metastasis (TNM) staging was performed using the American Joint Committee on Cancer (AJCC) 7th edition system [75]. The resection margins were classified as R0 (tumor-free resection) or R1 (microscopic margin involvement) [76]. Patients were given three or more cycles of adjuvant GEM monotherapy at a dose of 1000 mg/m^2 on days 1, 8, and 15 in six 28 day cycles and were monitored until 31st December 2018. This research was approved by the Ethics Committee of University Hospital Hradec Kralove (reference number 201607 SO2P).

4.2. Preparation of Formalin-Fixed Paraffin-Embedded Samples of Pancreas

All resection specimens were routinely histologically processed, that is, fixed in 10% neutral buffered formalin for 24–72 h, grossly described, cut-up, and sampled in a standardized fashion to evaluate relationship of the tumor to individual resection margins [76,77]. Multiple tissue samples were taken from both PDAC and non-neoplastic surrounding tissue for standard histological examination. The material was first embedded in paraffin, then 3 µm tissue sections were cut and stained with hematoxylin and eosin. For immunohistochemical analysis, in each case, one FFPE block from the tumor periphery containing both normal pancreatic parenchyma and neoplastic tissue was selected. In cases where there was available tissue block with normal pancreatic tissue without any tumor structures (usually tissue samples from resection margin), these blocks were selected and used to validate the method. For qRT-PCR analyses, two individual FFPE blocks (one with tumor tissue and the other containing solely normal pancreatic tissue) were selected for each patient. In the case of tumor tissue blocks, a microdissection method was used to remove parts of the tissue containing only stroma without neoplastic cells. Microdissection was performed by the principal pathologist prior to mRNA extraction, as a low percentage of neoplastic cells in the sample could have affected the outcomes of the analysis [19].

4.3. Extraction of mRNA from FFPE Samples and Reverse Transcription

For mRNA isolation from FFPE samples, a well-established method for extraction of total nucleic acids from FFPE tissues was used based on the Recover All™ total nucleic acid isolation kit (ThermoFisher Scientific, Waltman, MA, USA) [78]. The purity of the isolated RNA was checked by the $A_{260/280}$ ratio. RNA (1 µg) was converted into cDNA in 20 µL reaction using the gb reverse transcription kit from Generi Biotech s.r.o. (Hradec Kralove, Czech Republic) for *ENT1* and *NOTCH3* and a special assay for miR-21 (ThermoFisher Scientific, Waltman, MA, USA) in a Bio-Rad T100™ thermal cycler (Hercules, CA, USA), according to the manufacturer's protocol.

4.4. Quantitative Analysis of ENT1, NOTCH3, and miR-21 Expression

qRT-PCR analysis of *ENT1*, *NOTCH3*, and miR-21 expression in FFPE samples of normal and cancer tissues was performed using QuantStudio™ 6 Flex (Thermo Fisher Scientific, Waltham, MA, USA). cDNA (25 ng) was amplified in 5 µL reaction volumes in a 384-well plate using a TaqMan® Universal Master Mix II, no UNG (Thermo Fisher Scientific, Waltham, MA, USA) and predesigned TaqMan® real-time expression assays for *SLC29A1* (*ENT1*, Hs01085704_g1) [71], *NOTCH3* (Hs01128537_m1), and miR-21 (Hs04231424_s1) [25]. The PCR product sizes of each primer pair were 52, 67, and 64, respectively, enabling accurate and sensitive PCR analysis of gene expression in FFPE [79–81]. The amplification of each sample was performed in triplicate, applying the following PCR cycling profile: 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s. We used absolute

quantification for genes *NOTCH3* and *ENT1*. To determine the number of *SLC29A1* and *NOTCH3* transcripts, calibration was undertaken with a linear vector containing recombinant cDNA [54–56] prepared with primers Hs01085704_g1 and Hs01128537_m1 by company Generi Biotech (Hradec Kralove, Czech Republic). For miR-21, we used arbitrary units calculated as ΔC_T, that is, expression of miR-21 normalized by expression of the reference *RNU43* (assay ID 000397 and 001095, respectively, Thermo Fisher Scientific, Waltham, USA) [25,82].

4.5. Immunohistochemical Analysis of ENT1 Expression

Levels of ENT1 protein expression were studied using immunohistochemistry in 63 patients with sufficient remaining material for analysis after microdissection for qRT-PCR quantification. Anti-ENT1 antibody 10D7G2 was obtained from Prof. John Mackey (Cross Cancer Institute, University of Alberta, Canada), and detection was performed as recommended in the original protocol [13,18,20,46]. FFPE sections (2 µm thick) were deparaffinized, followed by pretreatment in DAKO pH 9 buffer (Glostrup, Denmark) for 10 min in a microwave (900 W). The antibody was diluted 1:10 and incubated overnight at 4 °C. A DAKO Envision+ kit (Glostrup, Denmark) was used for detection according to the manufacturer's instructions. Slides were counterstained with hematoxylin.

Immunohistochemical staining was evaluated semiquantitatively by a histoscore, which included evaluation of the proportion of cells/tissue expressing ENT1 and the intensity of staining, similarly to described previously for endometrial cancer [83]. The percentage of the cancer area stained in high-power fields was examined. The staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong), whereas the percentage of positive cells examined was scored as 0 (negative), 1 (<10%), 2 (11–50%), or 3 (>50%). The two values were multiplied, and the histoscore (values from 0 to 9) was determined: 0 (negative), values 1–5 (low), values 6–9 (high) [13]. The person evaluating the immunohistochemical slides was blinded of the results of other tests as well as of patients' outcomes.

4.6. Statistical Analyses

Disease-specific survival (DSS) from the date of surgery was assessed by employing the Kaplan–Meier method, and respective subgroups were compared by the log-rank test [84]. A Cox's proportional hazards multivariate model was used to corroborate any association of clinical and pathological factors and expression of *ENT1*/ENT1, *NOTCH3*, and/or miR-21 with patients' DSS [19,25,48,84,85]. Differences in the number of transcripts in subpopulations defined by the calculated histoscore were analyzed by the nonparametric Kruskal–Wallis test. Differences in medians of *ENT1*, *NOTCH3*, and/or miR-21 of expressions in normal pancreas and PDAC were determined by the nonparametric unpaired Mann–Whitney test, and the nonparametric Wilcoxon matched-pair signed-rank test was used to evaluate differences in expression of *ENT1*, *NOTCH3*, and miR-21 among subgroups given by the median of expression in normal pancreas (Figure 7D–F). The data were analyzed using SPSS 18.0 and GraphPad Prism 8.0.2. Statistical significance was set at $p < 0.05$.

5. Conclusions

In this retrospective study performed on a well-defined cohort of patients with resected PDAC treated with adjuvant GEM monotherapy, we did not confirm high *ENT1*/ENT1 and low miR-21 as prognostic biomarkers of improved DSS. Low miR-21 demonstrated prognostic value in N0 and T(1,2) patients only. For the first time, we attempted to assess the prognostic value of *NOTCH3* in such a cohort of patients. However, low *NOTCH3* did not show any association with improved DSS. Additionally, we confirmed that N0 patients had longer DSS. Our data do not preclude the potential application of *ENT1*/ENT1 and miR-21 as prognostic biomarkers for resected patients to improve the cost-effectiveness of chemotherapy in the future. However, they indicate that this procedure is not yet ready to be implemented into clinical decision-making processes. Standard procedures of immunohistochemistry staining scoring and qRT-PCR analyses must be established, and patients' characteristics other than *ENT1*/ENT1 and miR-21 expression that likely affect patients' survival should

be considered. Additionally, the data of this study suggested that there is a limited proportional dependence between *ENT1* gene expression evaluated by qRT-PCR in FFPE samples and protein levels assessed by immunohistochemistry and that there is likely an increased risk of *ENT1* downregulation in PDAC of patients with higher *ENT1* expression in normal pancreas, *NOTCH3* tends to be increased in PDAC of patients with low expression in normal pancreas, whereas the increase in miR-21 is independent of levels in normal pancreas. Even when *ENT1* and *NOTCH3* are deregulated in tumors of patients with high and low expression, respectively, they do not reach levels in the normal pancreas of patients with low expression of *ENT1* and high expression of *NOTCH3*. These findings may serve as a cornerstone of future experimental efforts focusing on inter-individual differences in regulation of *ENT1*, *NOTCH3* and miR-21 in PDAC as well as normal pancreas.

Author Contributions: Writing original draft, L.C., L.J., A.R., E.J.D.T., F.S.; investigation, L.C., L.J., A.R., H.H.; funding acquisition, F.S., A.R., L.C., L.J.; formal analysis, L.C., E.J.D.T., L.J.; resources, A.J., F.C.; conceptualization and supervision, L.C. All authors have read and approved the final version of the manuscript.

Funding: This work was supported by the Grant Agency of Charles University (GAUK 812216/C/2016), European Regional Development Funds BBMRICZ EF16 013/0001674, EFSA-CDN (No. CZ.02.1.01/0.0/0.0/16_019/0000841) co-funded by ERDF, Charles University (SVV 2017/260-414, program PROGRES Q40/11) and project of Czech Ministry of Education, Youth and Sports (BBMRI-CZ LM2015089).

Acknowledgments: We would like to thank John Mackey (Cross Cancer Institute, University of Alberta, Canada) for providing anti-ENT1 antibody 10D7G2.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Garrido-Laguna, I.; Hidalgo, M. Pancreatic cancer: From state-of-the-art treatments to promising novel therapies. *Nat. Rev. Clin. Oncol.* **2015**, *12*, 319–334. [CrossRef]
2. Kleeff, J.; Korc, M.; Apte, M.; la Vecchia, C.; Johnson, C.D.; Biankin, A.V.; Neale, R.E.; Tempero, M.; Tuveson, D.A.; Hruban, R.H.; et al. Pancreatic cancer. *Nat. Rev. Dis. Primers* **2016**, *2*, 16022. [CrossRef]
3. Rahib, L.; Smith, B.D.; Aizenberg, R.; Rosenzweig, A.B.; Fleshman, J.M.; Matrisian, L.M. Projecting cancer incidence and deaths to 2030: The unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res.* **2014**, *74*, 2913–2921. [CrossRef]
4. Kommalapati, A.; Tella, S.H.; Goyal, G.; Ma, W.W.; Mahipal, A. Contemporary management of localized resectable pancreatic cancer. *Cancers* **2018**, *10*, 24. [CrossRef]
5. Neoptolemos, J.P.; Dunn, J.A.; Stocken, D.D.; Almond, J.; Link, K.; Beger, H.; Bassi, C.; Falconi, M.; Pederzoli, P.; Dervenis, C.; et al. Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: A randomised controlled trial. *Lancet* **2001**, *358*, 1576–1585. [CrossRef]
6. Neoptolemos, J.P.; Stocken, D.D.; Friess, H.; Bassi, C.; Dunn, J.A.; Hickey, H.; Beger, H.; Fernandez-Cruz, L.; Dervenis, C.; Lacaine, F.; et al. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N. Engl. J. Med.* **2004**, *350*, 1200–1210. [CrossRef]
7. Oettle, H.; Neuhaus, P.; Hochhaus, A.; Hartmann, J.T.; Gellert, K.; Ridwelski, K.; Niedergethmann, M.; Zulke, C.; Fahlke, J.; Arning, M.B.; et al. Adjuvant chemotherapy with gemcitabine and long-term outcomes among patients with resected pancreatic cancer: The CONKO-001 randomized trial. *JAMA* **2013**, *310*, 1473–1481. [CrossRef]
8. Neoptolemos, J.P.; Stocken, D.D.; Smith, C.T.; Bassi, C.; Ghaneh, P.; Owen, E.; Moore, M.; Padbury, R.; Doi, R.; Smith, D.; et al. Adjuvant 5-fluorouracil and folinic acid vs observation for pancreatic cancer: Composite data from the ESPAC-1 and-3(v1) trials. *Br. J. Cancer* **2009**, *100*, 246–250. [CrossRef]
9. Neoptolemos, J.P.; Palmer, D.H.; Ghaneh, P.; Psarelli, E.E.; Valle, J.W.; Halloran, C.M.; Faluyi, O.; O'Reilly, D.A.; Cunningham, D.; Wadsley, J.; et al. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): A multicentre, open-label, randomised, phase 3 trial. *Lancet* **2017**, *389*, 1011–1024. [CrossRef]
10. Conroy, T.; Hammel, P.; Hebbar, M.; Abdelghani, M.B.; Wei, A.C.; Raoul, J.L.; Chone, L.; Francois, E.; Artru, P.; Biagi, J.J.; et al. FOLFIRINOX or gemcitabine as adjuvant therapy for pancreatic cancer. *N. Engl. J. Med.* **2018**, *379*, 2395–2406. [CrossRef]

11. Huang, J.; Liao, W.; Zhou, J.; Zhang, P.; Wen, F.; Wang, X.; Zhang, M.; Zhou, K.; Wu, Q.; Li, Q. Cost-effectiveness analysis of adjuvant treatment for resected pancreatic cancer in China based on the ESPAC-4 trial. *Cancer Manag. Res.* **2018**, *10*, 4065–4072. [CrossRef]
12. Fujita, H.; Ohuchida, K.; Mizumoto, K.; Itaba, S.; Ito, T.; Nakata, K.; Yu, J.; Kayashima, T.; Souzaki, R.; Tajiri, T.; et al. Gene expression levels as predictive markers of outcome in pancreatic cancer after gemcitabine-based adjuvant chemotherapy. *Neoplasia* **2010**, *12*, 807–817. [CrossRef]
13. Greenhalf, W.; Ghaneh, P.; Neoptolemos, J.P.; Palmer, D.H.; Cox, T.F.; Lamb, R.F.; Garner, E.; Campbell, F.; Mackey, J.R.; Costello, E.; et al. Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. *J. Natl. Cancer Inst.* **2014**, *106*, djt347. [CrossRef]
14. Marechal, R.; Bachet, J.B.; Mackey, J.R.; Dalban, C.; Demetter, P.; Graham, K.; Couvelard, A.; Svrcek, M.; Bardier-Dupas, A.; Hammel, P.; et al. Levels of gemcitabine transport and metabolism proteins predict survival times of patients treated with gemcitabine for pancreatic adenocarcinoma. *Gastroenterology* **2012**, *143*, 664–674. [CrossRef]
15. Morinaga, S.; Nakamura, Y.; Watanabe, T.; Mikayama, H.; Tamagawa, H.; Yamamoto, N.; Shiozawa, M.; Akaike, M.; Ohkawa, S.; Kameda, Y.; et al. Immunohistochemical analysis of human equilibrative nucleoside transporter-1 (hENT1) predicts survival in resected pancreatic cancer patients treated with adjuvant gemcitabine monotherapy. *Ann. Surg. Oncol.* **2012**, *19* (Suppl. 3), S558–S564. [CrossRef]
16. Andersson, R.; Aho, U.; Nilsson, B.I.; Peters, G.J.; Pastor-Anglada, M.; Rasch, W.; Sandvold, M.L. Gemcitabine chemoresistance in pancreatic cancer: Molecular mechanisms and potential solutions. *Scand. J. Gastroenterol.* **2009**, *44*, 782–786. [CrossRef]
17. Garcia-Manteiga, J.; Molina-Arcas, M.; Casado, F.J.; Mazo, A.; Pastor-Anglada, M. Nucleoside transporter profiles in human pancreatic cancer cells: Role of hCNT1 in 2′,2′-difluorodeoxycytidine-induced cytotoxicity. *Clin. Cancer Res.* **2003**, *9*, 5000–5008.
18. Farrell, J.J.; Elsaleh, H.; Garcia, M.; Lai, R.; Ammar, A.; Regine, W.F.; Abrams, R.; Benson, A.B.; Macdonald, J.; Cass, C.E.; et al. Human equilibrative nucleoside transporter 1 levels predict response to gemcitabine in patients with pancreatic cancer. *Gastroenterology* **2009**, *136*, 187–195. [CrossRef]
19. Giovannetti, E.; del Tacca, M.; Mey, V.; Funel, N.; Nannizzi, S.; Ricci, S.; Orlandini, C.; Boggi, U.; Campani, D.; del Chiaro, M.; et al. Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res.* **2006**, *66*, 3928–3935. [CrossRef]
20. Svrcek, M.; Cros, J.; Marechal, R.; Bachet, J.B.; Flejou, J.F.; Demetter, P. Human equilibrative nucleoside transporter 1 testing in pancreatic ductal adenocarcinoma: A comparison between murine and rabbit antibodies. *Histopathology* **2015**, *66*, 457–462. [CrossRef]
21. Sinn, M.; Riess, H.; Sinn, B.V.; Stieler, J.M.; Pelzer, U.; Striefler, J.K.; Oettle, H.; Bahra, M.; Denkert, C.; Blaker, H.; et al. Human equilibrative nucleoside transporter 1 expression analysed by the clone SP 120 rabbit antibody is not predictive in patients with pancreatic cancer treated with adjuvant gemcitabine—Results from the CONKO-001 trial. *Eur. J. Cancer* **2015**, *51*, 1546–1554. [CrossRef]
22. Brunetti, O.; Russo, A.; Scarpa, A.; Santini, D.; Reni, M.; Bittoni, A.; Azzariti, A.; Aprile, G.; Delcuratolo, S.; Signorile, M.; et al. MicroRNA in pancreatic adenocarcinoma: Predictive/prognostic biomarkers or therapeutic targets? *Oncotarget* **2015**, *6*, 23323–23341. [CrossRef]
23. Chan, J.A.; Krichevsky, A.M.; Kosik, K.S. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. *Cancer Res.* **2005**, *65*, 6029–6033. [CrossRef]
24. Dhayat, S.A.; Abdeen, B.; Kohler, G.; Senninger, N.; Haier, J.; Mardin, W.A. MicroRNA-100 and microRNA-21 as markers of survival and chemotherapy response in pancreatic ductal adenocarcinoma UICC stage II. *Clin. Epigenet.* **2015**, *7*, 132. [CrossRef]
25. Giovannetti, E.; Funel, N.; Peters, G.J.; del Chiaro, M.; Erozenci, L.A.; Vasile, E.; Leon, L.G.; Pollina, L.E.; Groen, A.; Falcone, A.; et al. MicroRNA-21 in pancreatic cancer: Correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res.* **2010**, *70*, 4528–4538. [CrossRef]
26. Kim, R.; Tan, A.; Lai, K.K.; Jiang, J.; Wang, Y.; Rybicki, L.A.; Liu, X. Prognostic roles of human equilibrative transporter 1 (hENT-1) and ribonucleoside reductase subunit M1 (RRM1) in resected pancreatic cancer. *Cancer* **2011**, *117*, 3126–3134. [CrossRef]

27. Vychytilova-Faltejskova, P.; Kiss, I.; Klusova, S.; Hlavsa, J.; Prochazka, V.; Kala, Z.; Mazanec, J.; Hausnerova, J.; Kren, L.; Hermanova, M.; et al. MiR-21, miR-34a, miR-198 and miR-217 as diagnostic and prognostic biomarkers for chronic pancreatitis and pancreatic ductal adenocarcinoma. *Diagn. Pathol.* **2015**, *10*, 38. [CrossRef]
28. Kalloger, S.E.; Riazy, M.; Tessier-Cloutier, B.; Karasinska, J.M.; Gao, D.; Peixoto, R.D.; Samimi, S.; Chow, C.; Wong, H.L.; Mackey, J.R.; et al. A predictive analysis of the SP120 and 10D7G2 antibodies for human equilibrative nucleoside transporter 1 (hENT1) in pancreatic ductal adenocarcinoma treated with adjuvant gemcitabine. *J. Pathol. Clin. Res.* **2017**, *3*, 179–190. [CrossRef]
29. Dang, L.; Fan, X.; Chaudhry, A.; Wang, M.; Gaiano, N.; Eberhart, C.G. Notch3 signaling initiates choroid plexus tumor formation. *Oncogene* **2006**, *25*, 487–491. [CrossRef]
30. Ashida, R.; Nakata, B.; Shigekawa, M.; Mizuno, N.; Sawaki, A.; Hirakawa, K.; Arakawa, T.; Yamao, K. Gemcitabine sensitivity-related mRNA expression in endoscopic ultrasound-guided fine-needle aspiration biopsy of unresectable pancreatic cancer. *J. Exp. Clin. Cancer Res.* **2009**, *28*, 83. [CrossRef]
31. Eto, K.; Kawakami, H.; Kuwatani, M.; Kudo, T.; Abe, Y.; Kawahata, S.; Takasawa, A.; Fukuoka, M.; Matsuno, Y.; Asaka, M.; et al. Human equilibrative nucleoside transporter 1 and Notch3 can predict gemcitabine effects in patients with unresectable pancreatic cancer. *Br. J. Cancer* **2013**, *108*, 1488–1494. [CrossRef] [PubMed]
32. Dillhoff, M.; Liu, J.; Frankel, W.; Croce, C.; Bloomston, M. MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J. Gastrointest. Surg.* **2008**, *12*, 2171–2176. [CrossRef] [PubMed]
33. Mann, C.D.; Bastianpillai, C.; Neal, C.P.; Masood, M.M.; Jones, D.J.; Teichert, F.; Singh, R.; Karpova, E.; Berry, D.P.; Manson, M.M. Notch3 and HEY-1 as prognostic biomarkers in pancreatic adenocarcinoma. *PLoS ONE* **2012**, *7*, e51119. [CrossRef] [PubMed]
34. Zhou, J.X.; Zhou, L.; Li, Q.J.; Feng, W.; Wang, P.M.; Li, E.F.; Gong, W.J.; Kou, M.W.; Gou, W.T.; Yang, Y.L. Association between high levels of Notch3 expression and high invasion and poor overall survival rates in pancreatic ductal adenocarcinoma. *Oncol. Rep.* **2016**, *36*, 2893–2901. [CrossRef] [PubMed]
35. Song, H.Y.; Wang, Y.; Lan, H.; Zhang, Y.X. Expression of Notch receptors and their ligands in pancreatic ductal adenocarcinoma. *Exp. Ther. Med.* **2018**, *16*, 53–60. [CrossRef] [PubMed]
36. Volinia, S.; Calin, G.A.; Liu, C.G.; Ambs, S.; Cimmino, A.; Petrocca, F.; Visone, R.; Iorio, M.; Roldo, C.; Ferracin, M.; et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 2257–2261. [CrossRef] [PubMed]
37. Bloomston, M.; Frankel, W.L.; Petrocca, F.; Volinia, S.; Alder, H.; Hagan, J.P.; Liu, C.G.; Bhatt, D.; Taccioli, C.; Croce, C.M. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA* **2007**, *297*, 1901–1908. [CrossRef]
38. Gerard, C.; Fagnoni, P.; Vienot, A.; Borg, C.; Limat, S.; Daval, F.; Calais, F.; Vardanega, J.; Jary, M.; Nerich, V. A systematic review of economic evaluation in pancreatic ductal adenocarcinoma. *Eur. J. Cancer* **2017**, *86*, 207–216. [CrossRef]
39. Ansari, D.; Tingstedt, B.; Andersson, R. Pancreatic cancer—Cost for overtreatment with gemcitabine. *Acta Oncol.* **2013**, *52*, 1146–1151. [CrossRef]
40. Bordeaux, J.; Welsh, A.; Agarwal, S.; Killiam, E.; Baquero, M.; Hanna, J.; Anagnostou, V.; Rimm, D. Antibody validation. *Biotechniques* **2010**, *48*, 197–209. [CrossRef]
41. Voskuil, J.L. The challenges with the validation of research antibodies. *F1000Research* **2017**, *6*, 161. [CrossRef] [PubMed]
42. Uson, P.L.S.J.; Macarenco, R.; Oliveira, F.N.; Smaletz, O. Impact of pathology review for decision therapy in localized prostate cancer. *Clin. Med. Insights Pathol.* **2017**, *10*. [CrossRef] [PubMed]
43. Middleton, L.P.; Feeley, T.W.; Albright, H.W.; Walters, R.; Hamilton, S.H. Second-opinion pathologic review is a patient safety mechanism that helps reduce error and decrease waste. *J. Oncol. Pract.* **2014**, *10*, 275–280. [CrossRef]
44. Hidalgo, M.; Cascinu, S.; Kleeff, J.; Labianca, R.; Lohr, J.M.; Neoptolemos, J.; Real, F.X.; van Laethem, J.L.; Heinemann, V. Addressing the challenges of pancreatic cancer: Future directions for improving outcomes. *Pancreatology* **2015**, *15*, 8–18. [CrossRef] [PubMed]
45. Ansari, D.; Tingstedt, B.; Andersson, B.; Holmquist, F.; Sturesson, C.; Williamsson, C.; Sasor, A.; Borg, D.; Bauden, M.; Andersson, R. Pancreatic cancer: Yesterday, today and tomorrow. *Future Oncol.* **2016**, *12*, 1929–1946. [CrossRef] [PubMed]

46. Spratlin, J.; Sangha, R.; Glubrecht, D.; Dabbagh, L.; Young, J.D.; Dumontet, C.; Cass, C.; Lai, R.; Mackey, J.R. The absence of human equilibrative nucleoside transporter 1 is associated with reduced survival in patients with gemcitabine-treated pancreas adenocarcinoma. *Clin. Cancer Res.* **2004**, *10*, 6956–6961. [CrossRef]
47. Nordh, S.; Ansari, D.; Andersson, R. hENT1 expression is predictive of gemcitabine outcome in pancreatic cancer: A systematic review. *World J. Gastroenterol.* **2014**, *20*, 8482–8490. [CrossRef]
48. Marechal, R.; Mackey, J.R.; Lai, R.; Demetter, P.; Peeters, M.; Polus, M.; Cass, C.E.; Young, J.; Salmon, I.; Deviere, J.; et al. Human equilibrative nucleoside transporter 1 and human concentrative nucleoside transporter 3 predict survival after adjuvant gemcitabine therapy in resected pancreatic adenocarcinoma. *Clin. Cancer Res.* **2009**, *15*, 2913–2919. [CrossRef]
49. Baldwin, S.; Kukar, M.; Gabriel, E.; Attwood, K.; Wilkinson, N.; Hochwald, S.N.; Kuvshinoff, B. Pancreatic cancer metastatic to a limited number of lymph nodes has no impact on outcome. *Hpb* **2016**, *18*, 523–528. [CrossRef]
50. Demir, I.E.; Jager, C.; Schlitter, A.M.; Konukiewitz, B.; Stecher, L.; Schorn, S.; Tieftrunk, E.; Scheufele, F.; Calavrezos, L.; Schirren, R.; et al. R0 versus R1 resection matters after pancreaticoduodenectomy, and less after distal or total pancreatectomy for pancreatic cancer. *Ann. Surg.* **2018**, *268*, 1058–1068. [CrossRef]
51. Valsangkar, N.P.; Bush, D.M.; Michaelson, J.S.; Ferrone, C.R.; Wargo, J.A.; Lillemoe, K.D.; Fernandez-del Castillo, C.; Warshaw, A.L.; Thayer, S.P. N0/N1, PNL, or LNR? The effect of lymph node number on accurate survival prediction in pancreatic ductal adenocarcinoma. *J. Gastrointest. Surg.* **2013**, *17*, 257–266. [CrossRef] [PubMed]
52. Lim, J.Y.; Cho, J.H.; Lee, S.J.; Lee, D.K.; Yoon, D.S.; Cho, J.Y. Gemcitabine combined with capecitabine compared to gemcitabine with or without erlotinib as first-line chemotherapy in patients with advanced pancreatic cancer. *Cancer Res. Treat.* **2015**, *47*, 266–273. [CrossRef] [PubMed]
53. Vandesompele, J.; de Preter, K.; Pattyn, F.; Poppe, B.; van Roy, N.; de Paepe, A.; Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* **2002**, *3*, research0034-1. [CrossRef] [PubMed]
54. Hou, Y.; Zhang, H.; Miranda, L.; Lin, S. Serious overestimation in quantitative PCR by circular (supercoiled) plasmid standard: Microalgal pcna as the model gene. *PLoS ONE* **2010**, *5*, e9545. [CrossRef]
55. Pfaffl, M.W.; Hageleit, M. Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. *Biotechnol. Lett.* **2001**, *23*, 275–282. [CrossRef]
56. Lin, C.H.; Chen, Y.C.; Pan, T.M. Quantification bias caused by plasmid DNA conformation in quantitative real-time PCR assay. *PLoS ONE* **2011**, *6*, e29101. [CrossRef]
57. Cecka, F.; Jon, B.; Subrt, Z.; Ferko, A. Solid pseudopapillary tumour of the pancreas: Diagnosis, treatment, and prognosis. *Acta Chir. Belg.* **2014**, *114*, 58–62. [CrossRef]
58. Hlavsa, J.; Cecka, F.; Zaruba, P.; Zajak, J.; Gurlich, R.; Strnad, R.; Pavlik, T.; Kala, Z.; Lovecek, M. Tumor grade as significant prognostic factor in pancreatic cancer: Validation of a novel TNMG staging system. *Neoplasma* **2018**, *65*, 637–643. [CrossRef]
59. Lidsky, M.E.; Sun, Z.; Nussbaum, D.P.; Adam, M.A.; Speicher, P.J.; Blazer, D.G., 3rd. Going the extra mile: Improved survival for pancreatic cancer patients traveling to high-volume centers. *Ann. Surg.* **2017**, *266*, 333–338. [CrossRef]
60. Blackford, A.; Serrano, O.K.; Wolfgang, C.L.; Parmigiani, G.; Jones, S.; Zhang, X.; Parsons, D.W.; Lin, J.C.; Leary, R.J.; Eshleman, J.R.; et al. SMAD4 gene mutations are associated with poor prognosis in pancreatic cancer. *Clin. Cancer Res.* **2009**, *15*, 4674–4679. [CrossRef]
61. Donahue, T.R.; Tran, L.M.; Hill, R.; Li, Y.; Kovochich, A.; Calvopina, J.H.; Patel, S.G.; Wu, N.; Hindoyan, A.; Farrell, J.J.; et al. Integrative survival-based molecular profiling of human pancreatic cancer. *Clin. Cancer Res.* **2012**, *18*, 1352–1363. [CrossRef] [PubMed]
62. Newhook, T.E.; Blais, E.M.; Lindberg, J.M.; Adair, S.J.; Xin, W.; Lee, J.K.; Papin, J.A.; Parsons, J.T.; Bauer, T.W. A thirteen-gene expression signature predicts survival of patients with pancreatic cancer and identifies new genes of interest. *PLoS ONE* **2014**, *9*, e105631. [CrossRef] [PubMed]
63. Stratford, J.K.; Bentrem, D.J.; Anderson, J.M.; Fan, C.; Volmar, K.A.; Marron, J.S.; Routh, E.D.; Caskey, L.S.; Samuel, J.C.; Der, C.J.; et al. A six-gene signature predicts survival of patients with localized pancreatic ductal adenocarcinoma. *PLoS Med.* **2010**, *7*, e1000307. [CrossRef] [PubMed]
64. Liu, Y.; Beyer, A.; Aebersold, R. On the dependency of cellular protein levels on mRNA abundance. *Cell* **2016**, *165*, 535–550. [CrossRef]

65. Aust, D.E.; Terdiman, J.P.; Willenbucher, R.F.; Chew, K.; Ferrell, L.; Florendo, C.; Molinaro-Clark, A.; Baretton, G.B.; Lohrs, U.; Waldman, F.M. Altered distribution of β-catenin, and its binding proteins E-cadherin and APC, in ulcerative colitis-related colorectal cancers. *Mod. Pathol.* **2001**, *14*, 29–39. [CrossRef]
66. Sajic, T.; Ciuffa, R.; Lemos, V.; Xu, P.; Leone, V.; Li, C.; Williams, E.G.; Makris, G.; Banaei-Esfahani, A.; Heikenwalder, M.; et al. A new class of protein biomarkers based on subcellular distribution: Application to a mouse liver cancer model. *Sci. Rep.* **2019**, *9*, 6913. [CrossRef] [PubMed]
67. Emanuelli, A.; Ayyathan, D.M.; Koganti, P.; Shah, P.A.; Apel-Sarid, L.; Paolini, B.; Detroja, R.; Frenkel-Morgenstern, M.; Blank, M. Altered expression and localization of tumor suppressive E3 ubiquitin ligase SMURF2 in human prostate and breast cancer. *Cancers* **2019**, *11*, 556. [CrossRef]
68. Miyamoto, Y.; Maitra, A.; Ghosh, B.; Zechner, U.; Argani, P.; Iacobuzio-Donahue, C.A.; Sriuranpong, V.; Iso, T.; Meszoely, I.M.; Wolfe, M.S.; et al. Notch mediates TGFα-induced changes in epithelial differentiation during pancreatic tumorigenesis. *Cancer Cell* **2003**, *3*, 565–576. [CrossRef]
69. Vo, K.; Amarasinghe, B.; Washington, K.; Gonzalez, A.; Berlin, J.; Dang, T.P. Targeting notch pathway enhances rapamycin antitumor activity in pancreas cancers through PTEN phosphorylation. *Mol. Cancer* **2011**, *10*, 138. [CrossRef]
70. Gnoni, A.; Licchetta, A.; Scarpa, A.; Azzariti, A.; Brunetti, A.E.; Simone, G.; Nardulli, P.; Santini, D.; Aieta, M.; Delcuratolo, S.; et al. Carcinogenesis of pancreatic adenocarcinoma: Precursor lesions. *Int. J. Mol. Sci.* **2013**, *14*, 19731–19762. [CrossRef]
71. Cerveny, L.; Ptackova, Z.; Ceckova, M.; Karahoda, R.; Karbanova, S.; Jiraskova, L.; Greenwood, S.L.; Glazier, J.D.; Staud, F. Equilibrative nucleoside transporter 1 (ENT1, SLC29A1) facilitates transfer of the antiretroviral drug abacavir across the placenta. *Drug Metab. Dispos.* **2018**, *46*, 1817–1826. [CrossRef] [PubMed]
72. del Santo, B.; Valdes, R.; Mata, J.; Felipe, A.; Casado, F.J.; Pastor-Anglada, M. Differential expression and regulation of nucleoside transport systems in rat liver parenchymal and hepatoma cells. *Hepatology* **1998**, *28*, 1504–1511. [CrossRef] [PubMed]
73. Jiraskova, L.; Cerveny, L.; Karbanova, S.; Ptackova, Z.; Staud, F. Expression of concentrative nucleoside transporters (SLC28A) in the human placenta: effects of gestation age and prototype differentiation-affecting agents. *Mol. Pharm.* **2018**, *15*, 2732–2741. [CrossRef]
74. Hulse, A.M.; Cai, J.J. Genetic variants contribute to gene expression variability in humans. *Genetics* **2013**, *193*, 95–108. [CrossRef]
75. Edge, S.B.; Compton, C.C. The American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann. Surg. Oncol.* **2010**, *17*, 1471–1474. [CrossRef] [PubMed]
76. Verbeke, C.S. Resection margins and R1 rates in pancreatic cancer—Are we there yet? *Histopathology* **2008**, *52*, 787–796. [CrossRef] [PubMed]
77. Menon, K.V.; Gomez, D.; Smith, A.M.; Anthoney, A.; Verbeke, C.S. Impact of margin status on survival following pancreatoduodenectomy for cancer: The Leeds Pathology Protocol (LEEPP). *Hpb* **2009**, *11*, 18–24. [CrossRef] [PubMed]
78. Kotorashvili, A.; Ramnauth, A.; Liu, C.; Lin, J.; Ye, K.; Kim, R.; Hazan, R.; Rohan, T.; Fineberg, S.; Loudig, O. Effective DNA/RNA co-extraction for analysis of microRNAs, mRNAs, and genomic DNA from formalin-fixed paraffin-embedded specimens. *PLoS ONE* **2012**, *7*, e34683. [CrossRef]
79. Abrahamsen, H.N.; Steiniche, T.; Nexo, E.; Hamilton-Dutoit, S.J.; Sorensen, B.S. Towards quantitative mRNA analysis in paraffin-embedded tissues using real-time reverse transcriptase-polymerase chain reaction: A methodological study on lymph nodes from melanoma patients. *J. Mol. Diagn.* **2003**, *5*, 34–41. [CrossRef]
80. Antonov, J.; Goldstein, D.R.; Oberli, A.; Baltzer, A.; Pirotta, M.; Fleischmann, A.; Altermatt, H.J.; Jaggi, R. Reliable gene expression measurements from degraded RNA by quantitative real-time PCR depend on short amplicons and a proper normalization. *Lab. Investig.* **2005**, *85*, 1040–1050. [CrossRef]
81. Szafranska, A.E.; Davison, T.S.; Shingara, J.; Doleshal, M.; Riggenbach, J.A.; Morrison, C.D.; Jewell, S.; Labourier, E. Accurate molecular characterization of formalin-fixed, paraffin-embedded tissues by microRNA expression profiling. *J. Mol. Diagn.* **2008**, *10*, 415–423. [CrossRef] [PubMed]
82. Hashimoto, K.; Ueno, H.; Ikeda, M.; Kojima, Y.; Hagihara, A.; Kondo, S.; Morizane, C.; Okusaka, T. Do recurrent and metastatic pancreatic cancer patients have the same outcomes with gemcitabine treatment? *Oncology* **2009**, *77*, 217–223. [CrossRef]

83. Metindir, J.; Dilek, G.B.; Pak, I. Staining characterization by immunohistochemistry of tumor cancer antigen in patients with endometrial cancer. *Eur. J. Gynaecol. Oncol.* **2008**, *29*, 489–492.
84. Clark, T.G.; Bradburn, M.J.; Love, S.B.; Altman, D.G. Survival analysis part I: Basic concepts and first analyses. *Br. J. Cancer* **2003**, *89*, 232–238. [CrossRef] [PubMed]
85. Bradburn, M.J.; Clark, T.G.; Love, S.B.; Altman, D.G. Survival analysis part II: Multivariate data analysis—An introduction to concepts and methods. *Br. J. Cancer* **2003**, *89*, 431–436. [CrossRef] [PubMed]

Publikace III: Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine

Placenta 60 (2017) 86–92

Contents lists available at ScienceDirect

Placenta

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Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine

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ARTICLE INFO

Article history:
Received 15 May 2017
Received in revised form
29 October 2017
Accepted 30 October 2017

Keywords:
Zidovudine
Emtricitabine
Placenta
Pregnancy
Nucleoside transporters
ENTs

ABSTRACT

Introduction: Zidovudine (AZT) and emtricitabine (FTC) are effective and well tolerated antiretroviral drugs, routinely used in the prevention of perinatal HIV transmission. However, precise mechanism(s) involved in their transfer from mother to fetus are not fully elucidated. Since both drugs are nucleoside analogues, we hypothesized that the mechanisms of their transplacental passage might include equilibrative nucleoside transporters, ENT1 and/or ENT2.
Methods: To address this issue, we performed *in vitro* accumulation assays in the BeWo placental trophoblast cell line, *ex vivo* uptake studies in fresh villous fragments isolated from human placenta and *in situ* dually perfused rat term placenta experiments.
Results: Applying this complex array of methods, we did not prove that ENTs play a significant role in transfer of AZT or FTC across the placenta.
Discussion: We conclude that the transplacental passage of AZT and FTC is independent of ENTs. Disposition of either compound into the fetal circulation should thus not be affected by ENT-mediated drug-drug interactions or placental expression of the transporters.

1. Introduction

Mother-to-child transmission (MTCT) is the most common route of HIV infection in children [1]. To minimize the risk of perinatal MTCT all pregnant women should receive combination antiretroviral therapy (cART) that contains three or more antiretrovirals, regardless of plasma HIV RNA copy number or CD4 T-lymphocyte count [2]. It has been recently emphasized that cART during pregnancy should contain nucleoside reverse transcriptase inhibitors (NRTIs) with high placental transfer that provides pre-exposure prophylaxis that further reduces possibility of fetal infection [1].

As the number of women of childbearing age or pregnant women with HIV receiving antiretroviral pharmacotherapy is steadily increasing [1], new personalized therapeutic options have been continuously pursued. Currently, clinicaltrials.gov registers 39 open clinical trials (key words "ANTIRETROVIRALS AND PREGNANCY" accessed 24th November 2016) including 12 clinical trials aimed to provide clinicians with further data about efficacy, safety or pharmacokinetics of NRTIs zidovudine (AZT) and/or emtricitabine (FTC), drugs frequently used in current cART regimens for prevention of MTCT of HIV [1].

AZT is a lipophilic molecule (logP = 0.05; pK_a = 9.68) that permeates cell membranes by passive diffusion, but its permeability can also be affected by the activity of certain efflux (ABC) or influx (SLC) transporters such as resistance-associated protein 4 (MRP4), organic anion transporters 1–4 (OAT1-4), P-glycoprotein (P-gp), or breast cancer resistance protein (BCRP) [3–7]. FTC is a weak base (logP = -0.43; pK_a = 2.65) that seems to cross cell membranes by passive diffusion despite its hydrophilicity [8]. Its transmembrane permeation may be affected by the efflux activity of MRP1 and multidrug and toxin extrusion protein 1 (MATE1) [9]. Both drugs are largely unionized (>99.8%) at physiological pH of the bloodstream [10–12].

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https://doi.org/10.1016/j.placenta.2017.10.011
0143-4004/

Over the last two decades it has been suggested that membrane transport of AZT and FTC might be facilitated by nucleoside transporters of two subfamilies [10,13–15], equilibrative nucleoside transporters (ENTs, *SLC29A*) and concentrative nucleoside transporters (CNTs, *SLC28A*), which are responsible for cellular uptake of naturally occurring nucleosides or nucleoside-derived drugs [16]. Syncytiotrophoblast, a thin layer in the placental barrier separating maternal and fetal circulations, expresses two members of ENTs (ENT1 and ENT2). ENT1 has been localized on the apical membrane whereas ENT2 is expressed in both apical and basal poles of syncytiotrophoblast mediating facilitative diffusion of their substrates [17–21]. ENT1 and ENT2 can be distinguished by their sensitivity to a specific inhibitor, S-(4-Nitrobenzyl)-6-thioinosine (NBMPR). While ENT1 is inhibited by nanomolar concentrations of NBMPR, ENT2 is blocked by higher than micromolar concentrations of the inhibitor [22]. In addition, all placental NTs can be at least partially inhibited by uridine [16].

Detailed knowledge on maternal-to-fetal transfer of AZT and FTC and a possible role of ENTs therein might contribute to further optimization of antiretroviral therapy to avoid (or take advantage of) drug-drug interactions that may limit transplacental transfer [23–25]. Investigation of AZT interactions with placental ENTs have been attempted but without conclusive results [14,26], and to the best of our knowledge there is no information on the interaction of FTC with ENTs. Therefore, the aim of the present study was to employ a portfolio of various experimental approaches, including *in vitro* (accumulation studies in BeWo cells), *ex vivo* (accumulation studies in fresh fragments of villous human placental tissue) and *in situ* (dually perfused rat term placenta) methods to elucidate whether ENT1 or ENT2 play any role in transplacental pharmacokinetics of AZT and/or FTC.

2. Materials and methods

2.1. Reagents and chemicals

Radiolabeled zidovudine ([^{3}H]-AZT) 12.9 Ci/mM, clinically used (-) enantiomer of emtricitabine ([^{3}H]-FTC) 2 Ci/mM, and model ENTs substrate thymidine [27,28] ([^{3}H]-thymidine) 74 Ci/mM were purchased from Moravek Biochemicals (Brea, California, USA). Inhibitors of ENT1 and ENT2, S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) and uridine, and solvent DMSO were obtained from Sigma-Aldrich (St. Louis, Missouri, USA); the final volume/volume concentration of DMSO used was 0.1% in all experiments. Pentobarbital natrium was acquired from INDIS Nv (Aartselaar, Belgium). All other chemicals were of analytical grade. The bicinchoninic acid assay kit was purchased from ThermoFisher Scientific (Rockford, USA).

2.2. Cells

Human choriocarcinoma cell line BeWo was obtained from the European Cell Culture Collection (ECACC; Salisbury, Wiltshire, UK). Cells were cultured in HAM's F-12 (Sigma Aldrich, St. Louis, Missouri, USA) medium containing D-glucose at a concentration of 10 mM, and supplemented with 10% fetal bovine serum maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Animals

Pregnant Wistar rats were obtained from Meditox s.r.o. (Konarovice, Czech Republic) and maintained under standard conditions (12-h/12-h day/night, water and pellets *ad libitum*). Experiments were performed on 21st day of gestation. Fasted rats were anesthetized with i.v. administration of 40 mg/kg pentobarbital into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University, Czech Republic) and were carried out in agreement with the Guide for the Care and Use of Laboratory Animals (1996) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

2.4. In vitro uptake experiments

For uptake experiments, 3.5×10^5 BeWo cells per well were seeded on 24-well culture plates. Modifications include the pre-incubating 10 min period in the buffer (0.25 ml) with or without inhibitor, NBMPR (0.1 μM or 100 μM) or uridine (5 mM). The cells were then incubated in 0.25 ml buffer containing the established ENT1/ENT2 model substrate [^{3}H]-thymidine (0.005 μM), [^{3}H]-AZT (0.032 μM) or [^{3}H]-FTC (0.184 μM) with/without an inhibitor. Final activity of all tested compounds was 0.4 μCi/ml. Accumulation was stopped after 5 min by quick aspiration of radioactivity containing buffer and by quick washing with 0.5 ml ice-cold buffer containing inhibitor and then the cells were lysed in 0.02% SDS. The concentration of accumulated isotopes was normalized to protein content and presented in pmol/mg protein.

2.5. Uptake study in fresh villous fragments from human placenta

This method was performed as previously described by Greenwood and Sibley [29]. Placentas were collected at term after uncomplicated pregnancies from the St. Mary's hospital in Manchester or from the Faculty Hospital, Hradec Kralove following written informed consent as approved by the local research Ethics Committee (REC 12/NW/0574 and 201006S15P, respectively). Villous fragments were pre-incubated for 10 min in Tyrode's buffer with subsequent incubation in Tyrode's buffer containing 0.5 μCi/ml of tested radioisotopes in presence/absence of ENTs inhibitors. The activities of radioisotopes used corresponded to the following concentrations: [^{3}H]-AZT 0.038 μM, [^{3}H]-FTC 0.242 μM or [^{3}H]-thymidine 0.006 μM. Accumulation was terminated by aspiration of radioisotope solutions and by washing twice with 6 ml of Tyrode's buffer. Subsequently, villous fragments were placed into distilled water for 18 h and then concentration of the released radioisotope was determined. Finally, fragments were removed from the water, dissolved in 0.3 M NaOH solution for 10 h at 37 °C and the total protein quantified using a BCA assay. Levels of [^{3}H]-AZT, [^{3}H]-FTC or [^{3}H]-thymidine uptakes were normalized to fragment protein content and expressed in fmol/mg.

2.6. In situ dual perfusion of the rat placenta in open circuit setup

The method of dually perfused rat term placenta was used as described previously [30]. [^{3}H]-AZT or [^{3}H]-FTC 0.06 μCi/ml (corresponding to concentrations of 0.005 μM and 0.03 μM, respectively) with/without inhibitor (0.1 μM NBMPR, 100 μM NBMPR or 5 mM uridine) were added to either maternal (M→F studies) or fetal (F→M studies) reservoir immediately after successful surgery. After 5 min of stabilization the sample collection started (time 0) and fetal effluent was sampled into pre-weighted vials at 5 min intervals. Concentrations of [^{3}H]-AZT or [^{3}H]-FTC were measured and transplacental clearance was calculated from all measured intervals. At the end of the experiment, the placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue, weighed, and dissolved in tissue solubilizer (Solvable; PerkinElmer), its radioactivity was measured to detect tissue-bound [^{3}H]-AZT or [^{3}H]-FTC.

Pharmacokinetic analysis of placental transport was performed

using standard equations for clearance as described previously [30] and total transplacental clearance was expressed in ml/min/g placenta.

2.7. Radioisotope analysis

The activities used in all experiments were dictated by the lowest specific activity of radioisotopes required for the analysis and concentrations of radioisotopes were determined by liquid scintillation counting employing Tri-Carb 2900 (TR Perkin Elmer, Waltham, MA, USA) as described elsewhere [5,9].

2.8. Statistical analyses

For *in vitro* and *in situ* studies, statistical significance was examined by one-way ANOVA followed by Dunnet's test. For statistical analysis of *ex vivo* experiments, we used non-parametric two-tailed unpaired Mann-Whitney test (for time course of accumulation) and Kruskal-Wallis test with Dunn's multiple comparison test (comparison of effect of particular inhibitors on [^{3}H]-thymidine uptake). All data were processed using GraphPad Prism 6.0 software (GraphPad Software, Inc. San Diego, California, USA).

3. Results

3.1. In vitro effects of ENTs inhibitors on AZT or FTC uptake by BeWo cells

Using [^{3}H]-thymidine (0.005 μM) as a positive control/model substrate we first evaluated function of ENT1 and ENT2 in BeWo cells. We observed that NBMPR (0.1 μM) decreased uptake of [^{3}H]-thymidine by ≈ 70% while NBMPR (100 μM) caused even more profound decrease by ≈ 85% (Fig. 1A) indicating contribution of both ENT1 and ENT2 in [^{3}H]-thymidine uptake into BeWo cells. Compared with both concentrations of NBMPR, uridine (5 mM) resulted in the weakest inhibition (by ≈ 55%) (Fig. 1A). When analyzing [^{3}H]-AZT (0.032 μM) or [^{3}H]-FTC (0.184 μM) uptake by BeWo cells, we did not observe any effect of the tested inhibitors (Fig. 1B and C) suggesting negligible role of ENTs.

3.2. Effect of ENTs inhibitor on AZT or FTC uptake by villous fragments

Using *ex vivo* accumulation studies in fresh villous fragments derived from human term placenta, we observed a time-dependent increase in accumulation of a model substrate, [^{3}H]-thymidine. Time-dependent accumulation of [^{3}H]-thymidine was significantly reduced in all the time points measured in the presence of the inhibitors. When comparing effects of 100 μM NBMPR, 0.1 μM NBMPR and 5 mM uridine after 5 min accumulation, we observed that 100 μM NBMPR revealed the most pronounced inhibition (Fig. 2A). Regarding [^{3}H]-AZT and [^{3}H]-FTC, a time-dependent increase in intracellular uptake was also observed, but there was no significant effect of NBMPR (100 μM). Because lack of NBMPR (100 μM) effect was observed, neither lower concentration of NBMPR (0.1 μM) nor uridine (5 mM) were further tested in 5 min accumulation (Fig. 2B and C).

3.3. Effect of ENTs inhibitors on transplacental clearance of AZT or FTC using in situ open circuit dual perfusion of rat placenta

Using the model of dually perfused rat term placenta, we did not observe any significant effect of the inhibitors tested (100 μM NBMPR and 5 mM uridine) on total M→F and F→M clearances of [^{3}H]-AZT (0.005 μM) (Fig. 3A–C) and [^{3}H]-FTC (0.03 μM) (Fig. 3C–D). Less than 1% of administered [^{3}H]-AZT or [^{3}H]-FTC was detected in the placenta after perfusion experiments, indicating limited tissue binding and its negligible effect on clearance calculation. Clearances reached values ≈ 0.2 ml/min/g for [^{3}H]-AZT and ≈0.07 ml/min/g placenta for [^{3}H]-FTC.

4. Discussion

The number of pregnant women with HIV entering antiviral therapy is steadily increasing [1,31], so is the evidence of adverse outcomes associated with exposure to antiretrovirals and their drug-drug interactions [31,32]. AZT and FTC reach plasma concentrations in the range of 8.1–26.3 μM and 4.5–10.1 μM, respectively [11,12,33] and belong to antiretrovirals with high transplacental transfer that are currently recommended for prevention of MTCT of HIV [34]. Although AZT or FTC interact with various drug transporters including P-glycoprotein, BCRP, OAT4, and MATE1 [3–6,9] that might decrease their fetal concentrations, the umbilical cord-to-maternal blood concentrations ratios of both antiretrovirals are 0.7–1.1 and 1.0–1.5, respectively. This implies that AZT and FTC likely cross placenta predominantly passively in a concentration gradient-dependent manner. Considering the nucleoside origin of these drugs, we hypothesized possible contribution of nucleoside transporters in their placental transfer. To address this issue, we tested interactions of AZT and FTC with ENT1/ENT2 using accumulation studies in BeWo cells, fresh villous fragments from the human placenta and *in situ* dual perfusion of rat placenta. To avoid transporter saturation we tested both drugs at low concentrations [24] (by at least one order of magnitude lower than those found in plasma).

BeWo cell line is a well-established *in vitro* model of the placental barrier that has previously been used to evaluate drug interactions with placental BCRP, P-gp, OAT4 and ENTs/CNTs transporters [28,35–38]. Gene/protein expression of ENT1 and ENT2 in BeWo cells has been documented in several studies [27,28,36]. ENT1-mediated transport in BeWo cells was suggested by Boumah et al. [27], who reported inhibition of thymidine uptake with NBMPR in a concentration range up to 10 μM. Subsequently, a binding assay with NBMPR was used by Mani et al. [28] showing protein function of both, ENT1 and ENT2, and proposing ENT1 as the more prevalent type in this cell line. Contrary to previous reports, our accumulation study with [^{3}H]-thymidine was performed for a longer time (5 min) employing two concentrations of NBMPR (0.1 μM and 100 μM). Using our setup, we confirmed ENT1 function in BeWo cells; in addition we observed a significant contribution of ENT2 (Fig. 1A). When analyzing accumulation of AZT or FTC in BeWo cells, neither NBMPR nor uridine showed any effect (Fig. 1B and C) suggesting lack of interactions with ENTs. Our observation is in conflict with that of Yao et al. [15] who revealed ENT2-mediated uptake into *Xenopus oocytes*. This discrepancy can be explained by very low expression of ENT2 in BeWo cells [28] and low affinity of AZT to ENT2. We presume no presence and therefore no effect of OAT4 that would compensate the blocked ENT2 [39]. On the other hand our findings are in agreement with more recent studies performed in the rat syncytiotrophoblast cell line TR-TBT 18d-1 [26] concluding that the uptake of AZT into syncytiotrophoblast is transporter-mediated but not via ENTs [14,26]. Interestingly, Sai et al. [14] reported effect of thymidine on AZT accumulation in TR-TBT 18d-1, however, that might be caused by interactions on concentrative nucleoside transporters (CNTs). *In vitro* FTC interactions with nucleoside transporters have been investigated only sparsely. Paff et al. [13] reported that (+)-FTC enantiomer enters HepG2 cells via a NBMPR sensitive transport system, while (-)-FTC was less NBMPR sensitive. The lack of inhibitors effect on FTC cell uptake observed in our study might, therefore, be a

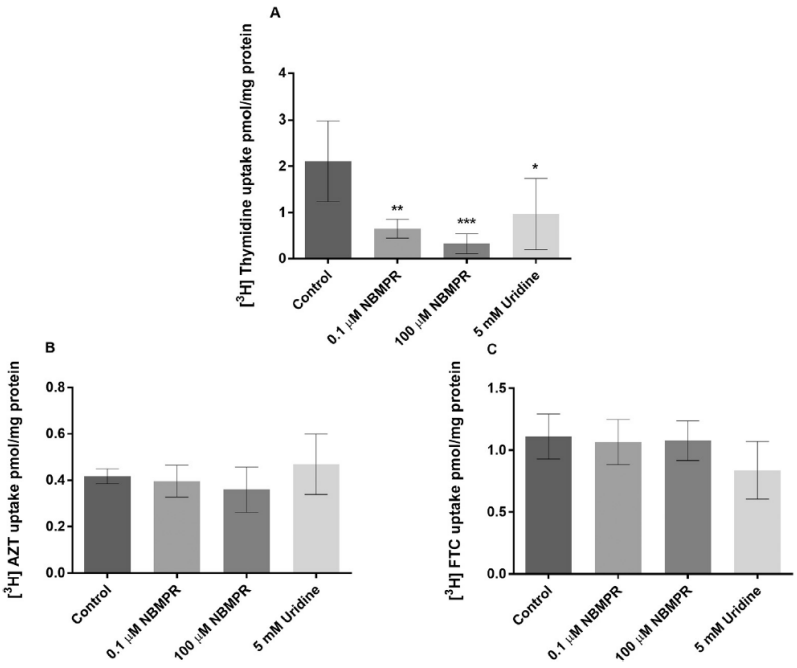

Fig. 1. Accumulation of a model ENTs substrate [^{3}H]-thymidine (0.005 μM) (A), [^{3}H]-AZT (0.032 μM) (B), and [^{3}H]-FTC (0.184 μM) (C) in BeWo cells in the presence/absence of ENTs inhibitors. [^{3}H]-thymidine accumulation showed significant sensitivity to 100 nM NBMPR and 5 mM uridine. Application of 100 μM NBMPR caused more pronounced effect when compared with 0.1 μM NBMPR indicating functional state of both ENT1 and ENT2 in BeWo cells. No effect of tested inhibitors on [^{3}H]-AZT and [^{3}H]-FTC uptake was observed, suggesting AZT and FTC cellular uptake by BeWo cells is not dependent on ENTs. Data are presented as means ± SD ($n \geq 4$), n represents number of biological replicates. An ordinary one-way ANOVA test with following Dunnett's test was used to evaluate statistical significance compared to control samples; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

consequence of (-)-FTC testing, which is, indeed, the clinically used enantiomer as it exhibits higher activity against HIV-1, HIV-2 and HBV than (+)-FTC [12,13,40]. Higher concentrations of D-glucose (25 mM) were previously documented to induce changes of ENTs expression in human artery smooth muscle cells and human umbilical vein endothelial cells from full-term normal pregnancies compared to controls (5 mM of D-glucose) [41,42]. We, therefore, cannot exclude possible effect of 10 mM D-glucose present in F-12 medium that we used in accumulation studies in BeWo cells.

To further test interactions of AZT and FTC with placental ENTs we performed accumulation studies in villous fragments derived from the human placenta. This model maintains placental villous architecture, cell-to-cell interactions, and intracellular signaling pathways [29]. It has been utilized to study amino acid transporter systems [43,44] and to elucidate interactions of some drugs with placental ABC transporters [43–45]. Using [^{3}H]-thymidine we confirmed activity of ENT1 and ENT2 herein (Fig. 2A and B). However, although 100 μM NBMPR resulted in decreased uptake of AZT and FTC in villous fragments, it did not reach statistical significance (Fig. 2C and D).

However, we cannot rule out that ENTs' inhibition in BeWo and villous fragments is effectively substituted by other mechanisms that remain to be elucidated. Moreover, the effect of the tested inhibitors may also appear during the initial phase (in seconds), when the highest uptake velocity of studied molecules is anticipated. Intervals shorter than 5 min have not been performed for [^{3}H]-AZT and [^{3}H]-FTC due to critical technical obstacles associated with their low cell/tissue accumulation. We tested three activities (0.1 μCi/ml, 0.4 μCi/ml, 0.5 μCi/ml) for *in vitro* and *ex vivo* methods detecting sufficient reproducible signal only when using 0.4 μCi/ml for uptake BeWo cells model and 0.5 μCi/ml for villous fragments after 5 min accumulation and longer (data not shown). This time setting also corresponded to previously published data [36]. The low accumulation of radioisotopes observed should not be significantly related to placental AZT and FTC biodegradation. Only up to 2% of AZT is metabolized to glucuronide form in the placenta, representing major biotransformation pathway. Data on FTC placental metabolism are not available, but should be negligible as most FTC (~90%) is excreted unchanged.

To investigate whether ENTs affect overall transplacental clearances of AZT and FTC in M→F or F→M directions, we employed the method of *in situ* dually perfused rat term placenta in open circuit setup. This is a well-justified method to evaluate transplacental pharmacokinetics and interactions of variety of drugs with placental ABC as well as SLC transporters [5,35,44,46–51]. On the other hand, interspecies differences in placental anatomy and/or levels of transporter expression and substrate affinity must be considered when extrapolating to human conditions [23,52,53]. Because of thymidine metabolism in placental tissue [18] we did not perform control experiments with [^{3}H]-thymidine as we did in other experimental approaches. However, functional expression of ENTs in the rat term placenta has been previously described by Sato et al. using 2′,3′-dideoxyinosine and uridine as substrates [54]. In our experimental setup, total [^{3}H]-AZT and [^{3}H]-FTC clearances in either direction were not significantly affected by NBMPR (100 μM) suggesting ENTs do not participate in transplacental permeation of

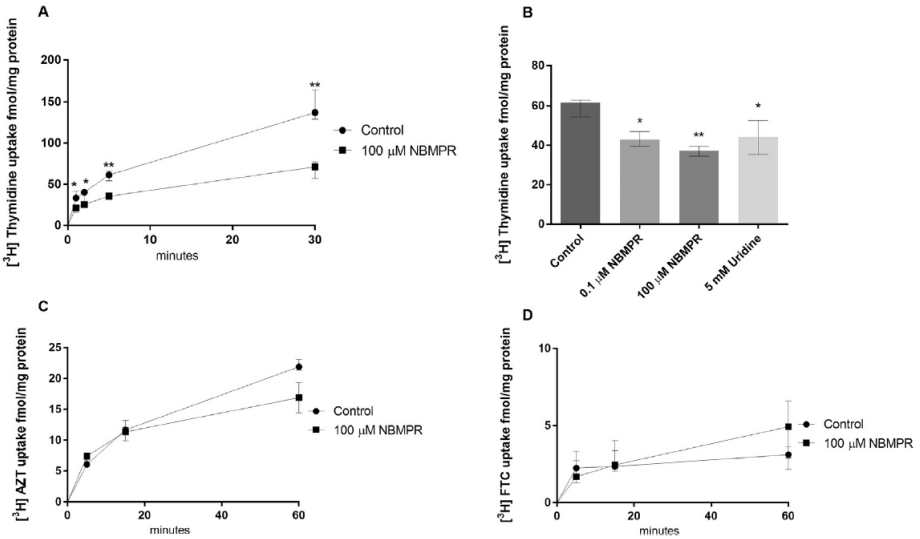

Fig. 2. Time dependent accumulation of a model ENTs substrate [^{3}H]-thymidine (0.006 μM) (A) and its uptake in presence/absence of chosen inhibitors (B), and time dependent accumulation of tested drugs [^{3}H]-AZT (0.038 μM) (C), and [^{3}H]-FTC (0.242 μM) (D) in fresh human villous fragments in the presence/absence of ENTs inhibitors. [^{3}H]-thymidine accumulation showed the significant sensitivity to 0.1 μM NBMPR and 5 mM uridine. Application of 100 μM NBMPR caused more pronounced effect when compared with 0.1 μM NBMPR indicating functional state of both ENT1 and ENT2 (B). No effect of tested inhibitors on [^{3}H]-AZT and [^{3}H]-FTC uptake was observed, suggesting AZT and FTC cellular uptake by villous fragments is not mediated by ENTs. Data are presented as median with interquartile range ($n \geq 3$), n represents number of donors. Non-parametrical Mann-Whitney (for time course of accumulation) or Kruskal-Wallis (comparison of effect of particular inhibitors on [^{3}H]-thymidine uptake) tests were used to evaluate statistical significance comparing accumulation in villous fragment with and without inhibitor. *$p < 0.05$, **$p < 0.01$.

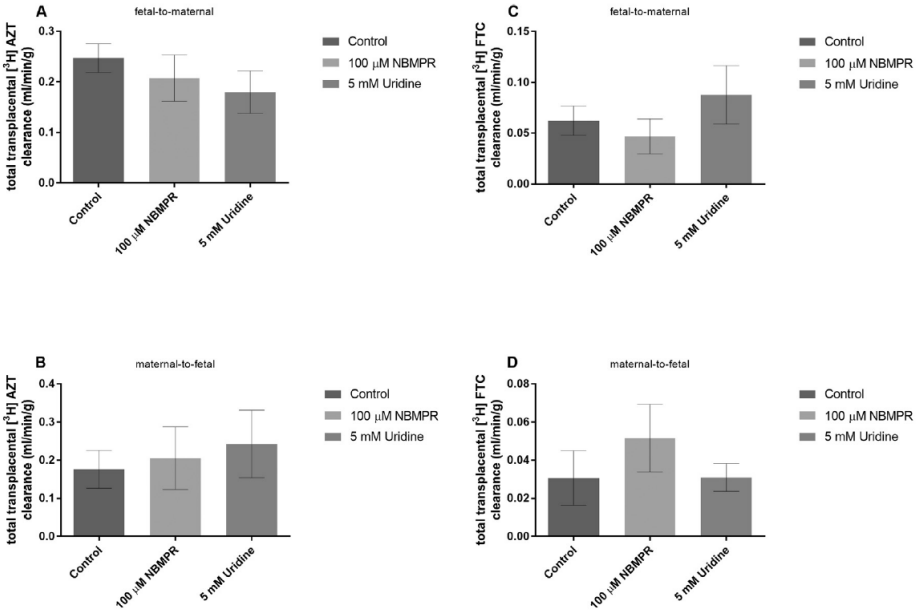

Fig. 3. Analysis of total transplacental clearances of [^{3}H]-AZT (0.005 μM) (A, B) and [^{3}H]-FTC and (0.03 μM) (C, D) across rat term placenta in the presence/absence of ENTs inhibitors. No effect of tested inhibitors on [^{3}H]-AZT and [^{3}H]-FTC clearance was observed, suggesting ENTs play no significant role in AZT and FTC transport across rat term placenta. Data are presented as means ± SD ($n \geq 4$), n represent number of experiments. One-way ANOVA followed by Dunnet's test was used to evaluate statistical significance.

tested drugs. We believe that neither placental metabolism of AZT/FTC [55–58] nor tissue binding (less than 1%) plays an important role in our findings. As we did not observe significant asymmetry in fetal-to-maternal vs. maternal-to-fetal clearances in the presence of NBMPR, we do not suppose any role of placental transporters that transfer AZT in fetal-to-maternal direction (P-gp, Bcrp or Oat4) [4,59]. Regarding FTC, our data correlate well with those of Nakatani-Freshwater et al. [10], who did not observe significant interactions of FTC with nucleoside transporters in the rat kidney.

Using various experimental models, we did not prove that ENT transporters play a significant role in transfer of AZT or FTC across the placenta. Therefore, it can be hypothesized that maternofetal disposition of either drug should not be extensively affected by ENT-mediated drug-drug interactions or by possible inter- or intra-individual variability in ENT expression. However, additional experimental setups, such as *ex vivo* perfusion of the human placenta, should be performed to draw more detailed conclusions on ENTs' role in placental transfer of AZT an FTC.

Conflict of interest

No conflict of interest.

Acknowledgement

This research was financially supported by the Czech Science Foundation (GACR 17-16169S) and Grant Agency of Charles University (GAUK 324215/C/2015; 2017/260-414). We would like to thank Dr. Marian Kacerovsky (Department of Obstetrics and Gynecology, University Hospital in Hradec Kralove) for providing us with human placentas and Martina Hudeckova for her help with the human placenta collection and sampling. We also thank Dana Souckova and Renata Exnarova for skillful assistance with the perfusion experiments.

References

[1] AidsInfo, Panel on Treatment of HIV-infected Pregnant Women and Prevention of Perinatal Transmission. Recommendations for Use of Antiretroviral Drugs in Pregnant HIV-1-infected Women for Maternal Health and Interventions to Reduce Perinatal HIV Transmission in the United States, 2016, pp. 1–13.
[2] WHO, Consolidated Guidelines on: the Use of Antiretroviral Drugs for Treating and Preventing HIV Infection, WHO Library Cataloguing-in-Publication Data, 2016.
[3] D.B. Kell, P.D. Dobson, S.G. Oliver, Pharmaceutical drug transport: the issues and the implications that it is essentially carrier-mediated only, Drug Discov. Today 16 (15–16) (2011) 704–714.
[4] O. Kis, K. Robillard, G.N. Chan, R. Bendayan, The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters, Trends Pharmacol. Sci. 31 (1) (2010) 22–35.
[5] Z. Neumanova, L. Cerveny, M. Ceckova, F. Staud, Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine, Biopharm. drug Dispos. 28 (2016) 135–143.
[6] M.W.L. Ritzel, A.M.L. Ng, S.Y.M. Yao, K. Graham, S.K. Loewen, K.M. Smith, R.J. Hyde, E. Karpinski, C.E. Cass, S.A. Baldwin, J.D. Young, Recent molecular advances in studies of the concentrative Na+-dependent nucleoside transporter (CNT) family: identification and characterization of novel human and mouse proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (s, Mol. Membr. Biol. 18 (2001) 65–72.
[7] C.D. Klaassen, L.M. Aleksunes, Xenobiotic, bile acid, and cholesterol transporters: function and regulation, Pharmacol. Rev. 62 (1) (2010) 1–96.
[8] A.M. Stek, B.M. Best, W. Luo, E. Capparelli, S. Burchett, C. Hu, H. Li, J.S. Read, A. Jennings, E. Barr, E. Smith, S.S. Rossi, M. Mirochnick, Effect of pregnancy on emtricitabine pharmacokinetics, HIV Med. 13 (2012) 226–235.
[9] J. Reznicek, M. Ceckova, L. Cerveny, F. Müller, F. Staud, Emtricitabine is a substrate of MATE1 but not of OCT1, OCT2, P-gp, BCRP or MRP2 transporters, Xenobiotica 8254 (2016) 1–9.
[10] T. Nakatani-Freshwater, D.R. Taft, Renal excretion of emtricitabine I: effects of organic anion, organic cation, and nucleoside transport inhibitors on emtricitabine excretion, J. Pharm. Sci. 97 (2008) 5401–5410.
[11] G. Sciences, RETROVIR® Package Insert, 2008, pp. 1–23.
[12] G. Sciences, EMTRIVA® Package Insert, 2012, pp. 1–30.
[13] M.T. Paff, D.R. Averett, K.L. Prus, W.H. Miller, D.J. Nelson, Intracellular metabolism of (-)- and (+)-cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine in HepG2 derivative 2.2.15 (subclone P5A) cells, Antimicrob. Agents Chemother. 38 (6) (1994) 1230–1238.
[14] Y. Sai, T. Nishimura, S. Shimpo, T. Chishu, K. Sato, N. Kose, T. Terasaki, C. Mukai, S. Kitagaki, N. Miyakoshi, Y.S. Kang, E. Nakashima, Characterization of the mechanism of zidovudine uptake by rat conditionally immortalized syncytiotrophoblast cell line TR-TBT, Pharm. Res. 25 (2008) 1647–1653.
[15] S.Y.M. Yao, A.M.L. Ng, M. Sundaram, C.E. Cass, S.a. Baldwin, J.D. Young, Transport of antiviral 3′-deoxy-nucleoside drugs by recombinant human and rat equilibrative, nitrobenzylthioinosine (NBMPR)-insensitive (ENT2) nucleoside transporter proteins produced in Xenopus oocytes, Mol. Membr. Biol. 18 (2001) 161–167.
[16] M. Molina-Arcas, F.J. Casado, M. Pastor-Anglada, Nucleoside transporter proteins, Curr. Vasc. Pharmacol. 7 (4) (2009) 426–434.
[17] S.a. Baldwin, S.Y.M. Yao, R.J. Hyde, A.M.L. Ng, S. Foppolo, K. Barnes, M.W.L. Ritzel, C.E. Cass, J.D. Young, Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes, J. Biol. Chem. 280 (2005) 15880–15887.
[18] J. Dancis, J. Lee, S. Mendoza, L. Liebes, Nucleoside transport by perfused human placenta, Placenta 14 (1993) 547–554.
[19] E. Errasti-Murugarren, P. Diaz, V. Godoy, G. Riquelme, M. Pastor-Anglada, Expression and distribution of nucleoside transporter proteins in the human syncytiotrophoblast, Mol. Pharmacol. 80 (5) (2011) 809–817.
[20] R. Govindarajan, A.H. Bakken, K.L. Hudkins, Y. Lai, F.J. Casado, M. Pastor-Anglada, C.-M. Tse, J. Hayashi, J.D. Unadkat, In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta., American journal of physiology, Regul. Integr. Comp. physiology 293 (2007) R1809–R1822.
[21] L.F. Barros, J.C. Bustamante, D.L. Yudilevich, S.M. Jarvis, Adenosine transport and nitrobenzylthioinosine binding in human placental membrane vesicles from brush-border and basal sides of the trophoblast, J. Membr. Biol. 119 (2) (1991) 151–161.
[22] J.L. Ward, A. Sherali, Z.P. Mo, C.M. Tse, Kinetic and pharmacological properties of cloned human equilibrative nucleoside transporters, ENT1 and ENT2, stably expressed in nucleoside transporter-deficient PK15 cells. Ent2 exhibits a low affinity for guanosine and cytidine but a high affinity for inosine, J. Biol. Chem. 275 (12) (2000) 8375–8381.
[23] F. Staud, M. Ceckova, Regulation of drug transporter expression and function in the placenta, Expert Opin. drug metabolism Toxicol. 11 (2015) 533–555.
[24] F. Staud, L. Cerveny, M. Ceckova, Pharmacotherapy in pregnancy; effect of ABC and SLC transporters on drug transport across the placenta and fetal drug exposure, J. drug Target. 20 (2012) 736–763.
[25] P. Brownbill, I. Chernyavsky, B. Bottalico, G. Desoye, S. Hansson, G. Kenna, L.E. Knudsen, U.R. Markert, N. Powles-Glover, H. Schneider, L. Leach, An international network (PlaNet) to evaluate a human placental testing platform for chemicals safety testing in pregnancy, Reprod. Toxicol. 64 (2016) 191–202.
[26] T. Nishimura, Y. Seki, K. Sato, T. Chishu, N. Kose, T. Terasaki, Y.S. Kang, Y. Sai, E. Nakashima, Enhancement of zidovudine uptake by dehydroepiandrosterone sulfate in rat syncytiotrophoblast cell line TR-TBT 18d-1, Drug Metabolism Dispos. 36 (2008) 2080–2085.
[27] C.E. Boumah, D.L. Hogue, C.E. Cass, Expression of high levels of nitrobenzylthioinosine-sensitive nucleoside transport in cultured human choriocarcinoma (BeWo) cells, Biochem. J. 288 (Pt 3) (1992) 987–996.
[28] R.S. Mani, J.R. Hammond, J.M. Marjan, K.A. Graham, J.D. Young, S.A. Baldwin, C.E. Cass, Demonstration of equilibrative nucleoside transporters (hENT1 and hENT2) in nuclear envelopes of cultured human choriocarcinoma (BeWo) cells by functional reconstitution in proteoliposomes, J. Biol. Chem. 273 (46) (1998) 30818–30825.
[29] S.L. Greenwood, C.P. Sibley, Placenta and Trophoblast - Methods and Protocols, vol. II, 2005, p. 122.
[30] F. Staud, Z. Vackova, K. Pospechova, P. Pavek, M. Ceckova, A. Libra, L. Cygalova, P. Nachtigal, Z. Fendrich, Expression and transport activity of breast cancer resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line, J. Pharmacol. Exp. Ther. 319 (2006) 53–62.
[31] C.H.Z. Siederdissen, B. Maasoumy, F. Marra, K. Deterding, K. Port, M.P. Manns, M. Cornberg, D. Back, H. Wedemeyer, Drug-drug interactions with novel all oral interferon-free antiviral agents in a large real-world cohort, Clin. Infect. Dis. 62 (5) (2016) 561–567.
[32] P.L. Williams, R. Hazra, R.B. Van Dyke, C. Yildirim, M.J. Crain, G.R. Seage 3rd, L. Civitello, A. Ellis, L. Butler, K. Rich, H.I.V.A.C.S. Pediatric, Antiretroviral exposure during pregnancy and adverse outcomes in HIV-exposed uninfected infants and children using a trigger-based design, AIDS 30 (1) (2016) 133–144.
[33] D. Gibb, M. Barry, S. Ormesher, L. Nokes, M. Seefried, C. Giaquinto, D. Back, Pharmacokinetics of zidovudine and dideoxyinosine alone and in combination in children with HIV infection, Br. J. Clin. Pharmacol. 39 (5) (1995) 527–530.
[34] S.A. McCormack, B.M. Best, Protecting the fetus against HIV infection: a systematic review of placental transfer of antiretrovirals, Clin. Pharmacokinet. 53 (11) (2014) 989–1004.
[35] M. Ceckova-Novotna, P. Pavek, F. Staud, P-glycoprotein in the placenta: expression, localization, regulation and function, Reprod. Toxicol. 22 (2006) 400–410.

[36] T. Yamamoto, K. Kuniki, Y. Takekuma, T. Hirano, K. Iseki, M. Sugawara, Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters, Eur. J. Pharmacol. 557 (2007) 1–8.
[37] N. Utoguchi, G.A. Chandorkar, M. Avery, K.L. Audus, Functional expression of P-glycoprotein in primary cultures of human cytotrophoblasts and BeWo cells, Reprod. Toxicol. 14 (3) (2000) 217–224.
[38] J.D. Unadkat, A. Dahlin, S. Vijay, Placental drug transporters, Curr Drug Metab 5 (1) (2004) 125–131.
[39] T. Nabekura, T. Kawasaki, Y. Kamiya, Y. Uwai, Effects of antiviral drugs on organic anion transport in human placental BeWo cells, Antimicrob. Agents Chemother. 59 (12) (2015) 7666–7670.
[40] R.F. Schinazi, A. McMillan, D. Cannon, R. Mathis, R.M. Lloyd, A. Peck, J.P. Sommadossi, M. St Clair, J. Wilson, P.A. Furman, et al., Selective inhibition of human immunodeficiency viruses by racemates and enantiomers of cis-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine, Antimicrob. Agents Chemother. 36 (11) (1992) 2423–2431.
[41] C. Aguayo, J. Casado, M. Gonzalez, J.D. Pearson, R.S. Martin, P. Casanello, M. Pastor-Anglada, L. Sobrevia, Equilibrative nucleoside transporter 2 is expressed in human umbilical vein endothelium, but is not involved in the inhibition of adenosine transport induced by hyperglycaemia, Placenta 26 (8–9) (2005) 641–653.
[42] G.P. Leung, R.Y. Man, C.M. Tse, D-Glucose upregulates adenosine transport in cultured human aortic smooth muscle cells, Am. J. Physiol. Heart Circ. Physiol. 288 (6) (2005) H2756–H2762.
[43] S.L. Greenwood, C.P. Sibley, In vitro methods for studying human placental amino acid transport placental villous fragments, Methods Mol. Med. 122 (2006) 253–264.
[44] Z. Neumanova, L. Cerveny, S.L. Greenwood, M. Ceckova, F. Staud, Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir, Reprod. Toxicol. 57 (2015) 176–182.
[45] D.E. Atkinson, C.P. Sibley, L.J. Fairbairn, S.L. Greenwood, MDR1 P-gp expression and activity in intact human placental tissue; Upregulation by retroviral transduction, Placenta 27 (6–7) (2006) 707–714.
[46] D. Ahmadimoghaddam, J. Hofman, L. Zemankova, P. Nachtigal, E. Dolezelova, L. Cerveny, M. Ceckova, S. Micuda, F. Staud, Synchronized activity of organic cation transporter 3 (Oct3/Slc22a3) and multidrug and toxin extrusion 1 (Mate1/Slc47a1) transporter in transplacental passage of MPP+ in rat., Toxicological sciences, official J. Soc. Toxicol. 128 (2012) 471–481.
[47] D. Ahmadimoghaddam, F. Staud, Transfer of metformin across the rat placenta is mediated by organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) protein (Elmsford, N.Y.), Reprod. Toxicol. 39 (2013) 17–22.
[48] L.H. Cygalova, J. Hofman, M. Ceckova, F. Staud, Transplacental pharmacokinetics of glyburide, rhodamine 123, and BODIPY FL prazosin: effect of drug efflux transporters and lipid solubility, J. Pharmacol. Exp. Ther. 331 (2009) 1118–1125.
[49] Z. Neumanova, L. Cerveny, M. Ceckova, F. Staud, Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta, AIDS Lond. Engl. 28 (2014) 9–17.
[50] M. Novotna, A. Libra, M. Kopecky, P-glycoprotein expression and distribution in the rat placenta during pregnancy, Reprod. Toxicol. 18 (2004) 785–792.
[51] T.M. Leazer, C.D. Klaassen, The presence of xenobiotic transporters in rat placenta, Drug Metab. Dispos. 31 (2) (2003) 153–167.
[52] E.P. de Rijk, E. van Esch, G. Flik, Pregnancy dating in the rat: placental morphology and maternal blood parameters, Toxicol. Pathol. 30 (2) (2002) 271–282.
[53] L. Hahnova-Cygalova, M. Ceckova, F. Staud, Fetoprotective activity of breast cancer resistance protein (BCRP, ABCG2): expression and function throughout pregnancy, Drug metab. Rev. 43 (2011) 53–68.
[54] K. Sato, Y. Sai, T. Nishimura, T. Chishu, S. Shimpo, N. Kose, E. Nakashima, Influx mechanism of 2',3'-dideoxyinosine and uridine at the blood-placenta barrier, Placenta 30 (3) (2009) 263–269.
[55] A.C. Collier, J.A. Keelan, P.E. Van Zijl, J.W. Paxton, M.D. Mitchell, M.D. Tingle, Human placental glucuronidation and transport of 3'azido-3'-deoxythymidine and uridine diphosphate glucuronic acid, Drug Metab. Dispos. 32 (8) (2004) 813–820.
[56] Drugbank, Emtricitabine.
[57] Drugbank, Zidovudine.
[58] M.R. Syme, J.W. Paxton, J.A. Keelan, Drug transfer and metabolism by the human placenta, Clin. Pharmacokinet. 43 (8) (2004) 487–514.
[59] M. Tomi, T. Nishimura, E. Nakashima, Mother-to-fetus transfer of antiviral drugs and the involvement of transporters at the placental barrier, J. Pharm. Sci. 100 (9) (2011) 3708–3718.

Publikace IV: Transport of ribavirin across the rat and human placental barrier: Roles of nucleoside and ATP-binding cassette drug efflux transporters.

Biochemical Pharmacology 163 (2019) 60–70

Contents lists available at ScienceDirect

Biochemical Pharmacology

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

Transport of ribavirin across the rat and human placental barrier: Roles of nucleoside and ATP-binding cassette drug efflux transporters

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ARTICLE INFO

Keywords:
Ribavirin
Placenta
Pregnancy
ABC transporters
Nucleoside transporters

ABSTRACT

Ribavirin is a broad-spectrum nucleoside-derived antiviral drug used in combination pharmacotherapy treatment of hepatitis C virus infection. Current evidence indicates that ribavirin-associated teratogenicity is not significant in humans, but more information about the developmental toxicity and mechanisms involved in ribavirin placental kinetics is required to assure its safe use in pregnancy. Thus, we have investigated potential roles of equilibrative nucleoside transporters (ENTs, *SLC29A*), Na^+-dependent influx-mediating concentrative nucleoside transporters (CNTs, *SLC28A*), and ATP-binding cassette (ABC) efflux pumps, in ribavirin placental pharmacokinetics. Our data indicate that ENT1 participates in uptake of ribavirin by BeWo cells, fresh human placental villous fragments and microvillous plasma membrane (MVM) vesicles while activity of CNTs (probably CNT2) was only observed in BeWo cells. *In situ* dual perfusion experiments with rat term placenta in an open circuit setup showed that ENT inhibition significantly decreases total ribavirin maternal-to-foetal and foetal-to-maternal clearances. In contrast, no contribution of ABC transporters, p-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2), or multidrug resistance-associated protein (ABCC2) was detected in assays with MDCKII cells overexpressing them, or in closed circuit dual perfusion experiments with rat term placenta. In summary, our data show that ribavirin placental pharmacokinetics are largely controlled by ENT1 activity and independent of ABCB1, ABCG2, and ABCC2 efflux pumps.

1. Introduction

Hepatitis C virus (HCV) infection is a global problem affecting about 3% of the world's population [1,2]. Mother-to-child transmission of HCV is the major route of the infection in children occurring in 5–10% [3,4] and up to 20% in HIV co-infected pregnant women [5,6]. High proportions (73–92%) of vertically infected children suffer from chronic disease [3]; liver biopsies typically show liver inflammation and fibrosis [3]. Multiple studies have suggested an association between risk of transmission and HCV viral loads [7]. Therefore, treatments that decrease HCV viremia in pregnant women are expected to reduce rates of vertical HCV transmission [8].

Ribavirin, a purine analogue with broad-spectrum anti-viral activity [9,10], is a WHO essential medicine for adults and children [11,12] that is used (*inter alia*) as the backbone of various HCV therapeutic regimens [10,13]. Due to its mechanism of action (interference with biosynthesis of guanine nucleotides) and teratogenicity observed in animal studies [14], ribavirin has been assigned to FDA Pregnancy Category X [15]. However, in humans, single and series case reports have documented normal pregnancies with no birth defects [14,16–21] unless concomitant teratogen was administered [22]. Importantly, preliminary findings obtained from an interim analysis of potential teratogenicity at the mid-point of enrolment (ClinicalTrials.gov identifier: NCT00114712) "do not suggest a clear signal of human teratogenicity for ribavirin" [15]. However, knowledge of ribavirin's placental transfer mechanisms is also important for robust evaluation of the safety of its use in pregnancy [23,24].

Ribavirin is a highly hydrophilic drug (log P −1.85), reaching

Abbreviations: AB, apical-to-basolateral; ABC, ATP-binding cassette; ABCB1, p-glycoprotein; ABCC2, multidrug resistance-associated protein 2; ABCG2, breast cancer resistance protein; BA, basolateral-to-apical; CNT, concentrative nucleoside transporter; DMEM, Dulbecco's modified Eagle's medium; ENT, equilibrative nucleoside transporter; F → M, foetus-to-mother; FDA, US Food and Drug Administration; HCV, hepatitis C virus; HIV, human immunodeficiency virus; M → F, mother-to-foetus; MDCKII, Madin-Darby canine kidney; MVM, microvillous plasma membrane; NBMPR, S-(4-nitrobenzyl)-6-thioinosine; NT, nucleoside transporter; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; r_{net}, net efflux ratio; Vd, distribution volume; WHO, World Health Organization

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https://doi.org/10.1016/j.bcp.2019.01.024
Received 20 December 2018; Accepted 31 January 2019
Available online 02 February 2019
0006-2952/

maximal plasmatic concentrations (C_{max}) in healthy volunteers of 2.6 μM after a 400 mg single oral dose. It accumulates strongly in the plasma, reaching 4-fold C_{max} at steady-state [25], and is metabolised via two pathways with no participation of cytochrome P450 enzymes: reversible phosphorylation or deribosylation and amide hydrolysis. Following a single dose, it has a relatively short plasma half-life (2 h), but its active metabolite ribavirin triphosphate is eliminated slowly, with a plasma half-life of 120–170 h [14,25]. Ribavirin does not bind plasma proteins and is extensively distributed (Vd = 4500–6000 L), especially to cells of skeletal muscles, liver, and erythrocytes, indicating involvement of specific membrane transporters [25]. *In vitro* and *in vivo* experiments have indicated that ribavirin may cross the placenta, and/or distribute into milk in humans [26,27], but these possibilities have not been unequivocally demonstrated [28].

Pharmacokinetics of nucleoside-derived antiviral drugs are frequently modulated by activities of nucleoside transporters (NTs) and ATP-binding cassette (ABC) efflux pumps [27,29–32]. There are two subfamilies of NTs: equilibrative (ENTs, mediating facilitated diffusion) and concentrative (CNTs, mediating Na^+-dependent active transport) [27,33–36]. ENTs are sensitive to S-(4-nitrobenzyl)-6-thioinosine (NBMPR). NBMPR is considered a specific ENTs inhibitor [37]: at a concentration of 0.1 μM NBMPR inhibits ENT1/Ent1 selectively, while at 100 μM NBMPR blocks activities of both ENT1/Ent1 and ENT2/Ent2 [38,39]. Up to date, no selective inhibitor of CNTs has been identified. As cellular uptake of nucleosides is believed to be mediated predominantly by nucleoside transporters [30,40], inhibitory effect of Na^+ depletion on nucleosides cellular uptake has been established as sufficient evidence of involvement of CNTs in nucleosides membrane transport [27,30,32,33,35–37,40–45]. Both ENTs and CNTs reportedly control maternal-to-foetal transfer of nucleosides and nucleoside-derived drugs [27,31–34]. ABC transporters are a vast superfamily of proteins that participate in diverse processes. Potentially important ABC transporters in the context of this study include p-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2), and multidrug resistance-associated protein 2 (ABCC2), all localized in apical membrane of human placental syncytiotrophoblast. These efflux pumps protect the developing foetus against potentially harmful xenobiotics by limiting transfer of their substrates from maternal to foetal circulation [46,47]. It has been suggested that ribavirin may be a substrate of human ENT1 [27,44,48,49], CNT2 [50] and CNT3 [27,44,51], and mouse Ent1 [52]. *In vitro* studies have indicated that ENT1 and CNT3 are important for placental uptake of ribavirin [27] and subsequent analysis showed that Ent1 is required for transfer of ribavirin into foetal circulation in mice [26]. However, possible roles of NTs in ribavirin pharmacokinetics in the human placenta have not been directly studied. Moreover, the possibility that ABCB1, ABCG2, or ABCC2 may interact with ribavirin and reduce its placental maternal-to-foetal transfer rates has not been rigorously tested in previous investigations.

Here we report the first attempts to assess roles of NTs and ABC transporters in transfer of ribavirin across the placental barrier using human placental villous fragments and microvillous plasma membrane (MVM) vesicles, *in situ* dually perfused rat term placenta, and cell-based models.

2. Materials and methods

2.1. Reagents and chemicals

Radiolabelled ribavirin (1-[(2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl) oxolan-2-yl]-1H-1,2,4-triazole-3-carboxamide; [^{3}H]-ribavirin; 17.5 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA, USA). NBMPR was purchased from Sigma Aldrich (St. Louis, MO, USA) and was used at concentrations 0.1 μM and 100 μM to inhibit ENT1/Ent1 and ENT2/Ent2, respectively [35,43,45,53,54]. Non-radiolabelled ribavirin, uridine, a competitive inhibitor of NTs [30,36], and the solvent dimethyl sulfoxide (DMSO) were also obtained from Sigma-Aldrich (St. Louis, MO, USA). The concentration of DMSO in media used in all experiments was 0.1%. Pentobarbital natrium was acquired from INDIS Nv (Aartselaar, Belgium). All other chemicals were of analytical grade. A bicinchoninic acid (BCA) assay kit was purchased from ThermoFisher Scientific (Rockford, IL, USA).

2.2. Cells

Cells of the human choriocarcinoma line BeWo were obtained from the European Cell Culture Collection (ECACC; Salisbury, UK), and cultured in HAM's F-12 medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS). MDCKII (Madine-Darby Canine Kidney) parental cells and MDCKII cells overexpressing human ABCB1 (MDCKII-ABCB1), ABCG2 (MDCKII-ABCG2), and ABCC2 (MDCKII-ABCC2) were provided by the Netherlands Cancer Institute (Dr A. Schinkel) and cultured in DMEM complete high glucose medium with L-glutamine (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. All cell lines were cultured at 37 °C under an atmosphere containing 5% CO_2.

2.3. Animals

Pregnant Wistar rats were obtained from Meditox s.r.o. (Konarovice, Czech Republic) and Velaz (Prague, Czech Republic). They were maintained under standard conditions (12 h/12 h day/night cycles, water and pellets *ad libitum*). Experiments were performed on the 21st day of gestation. Fasted rats were anesthetized with i.v. administration of 40 mg/kg pentobarbital into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (approval no. MSMT-4312/2015-8; Charles University, Czech Republic) and applied procedures were consistent with recommendations in both the Guide for the Care and Use of Laboratory Animals (1996) [55] and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [56].

2.4. In vitro uptake experiments

For uptake experiments, 24-well culture plates supplied by TPP (Trasadingen, Switzerland) were seeded with 2.5×10^5 BeWo cells per well, and cultured for 3 days until confluence with daily medium replacement. Ribavirin uptake experiments were then performed as previously described [27], with modifications, using a Na^+-containing medium (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose and 25 mM Tris, pH adjusted to 7.4 by HEPES) and a Na^+-free counterpart (with Na^+ replaced by N-methyl-D-glucamine). Briefly, sets of cells were pre-incubated for 10 min in the Na^+-containing buffer at 4 °C and/or 37 °C, with and without an inhibitor (0.1 μM or 100 μM NBMPR, or 5 mM uridine) [32,35]. Each set was then incubated in 0.25 ml of buffer containing [^{3}H]-ribavirin (0.0222 μM; final activity of radioisotopes 0.4 μCi/ml), under otherwise identical conditions. Uptake was stopped after 2, 15, 30, and 60 min (to study time-dependency of ribavirin accumulation) and at 5-min intervals (for comparison of the inhibitors' effects and analysis of the concentration dependency of [^{3}H]-ribavirin accumulation). This was done by rapidly aspiring the radioactivity-containing buffer, washing twice with 0.75 ml portions of the pre-incubation buffer (with or without inhibitor), then lysing the cells in 0.02% SDS. In saturation experiments we tested effects of adding 1, 10, and 50 μM of non-radiolabelled ribavirin, plus [^{3}H]-ribavirin (0.0222 μM, 0.4 μCi/ml) as a tracer for quantification, to the Na^+-containing buffer with/without inhibitor in incubations at 37 °C or at 4 °C.

2.5. In vitro bidirectional transport assays

Transport assays were performed using microporous polycarbonate

membrane filters (3.0 μm pore size, 24 mm diameter; Transwell 3414; Costar, Corning, NY, USA) together with MDCKII parental and ABC transporter-overexpressing MDCKII cells, as previously described [57]. MDCKII-ABCB1, MDCKII-ABCG2, MDCKII-ABCC2 or MDCKII-parental cells were seeded at a density of 1.5×10^6 per insert and cultured for 3–4 days in standard cultivation medium until confluence with daily replacement of medium. Before starting each transport experiment, cells were washed with pre-warmed phosphate buffered saline (PBS). Then the experiment was initiated by adding Opti-MEM (ThermoFisher Scientific, Rockford, IL, USA) with 0.04 mCi/ml [^{3}H]-ribavirin (corresponding to 0.0022 μM) to the donor compartment (time = 0 min). In parallel, we performed bi-directional transport experiments using the same setup, but with addition of 1 mM uridine to block canine NTs that might potentially mask ABC transporter-mediated transport of [^{3}H]-ribavirin [30]. Samples (50 μl) were collected after 30, 60 and 120 min from the acceptor compartment. Medium was immediately removed at the end of the incubation period and cells were washed twice with ice-cold PBS. The inserts were excised, and cells were lysed in 0.02% SDS to determine the percentage of intracellular radioactivity. Leakage of FITC-dextran (0.5 mg/ml) (Sigma Aldrich, St. Louis, MO, USA) was analysed at the end of the transport experiments and deemed acceptable if ≤1% per hour [57–59]. Efflux ratios of basal-to-apical to apical-to-basal translocation of [^{3}H]-ribavirin after 2 h incubation were calculated as previously described [57,60], then net efflux ratios (r_{net}) were assessed by normalization of the efflux ratios for the ABCB1/ABCG2/ABCC2-transfected or parental MDCK cell line with respect to the efflux ratio obtained for the parental cells [61]. A net efflux ratio > 2.0 was the criterion for regarding ribavirin as a substrate of a tested transporter [62].

Before each of these experiments, the transepithelial electrical resistance (TEER) across cell monolayers was measured using a Millicell-ERS instrument (Millipore Corporation, Bedford, MA) [63]. TEER values ranged from 800 to 1000 Ω cm^2 on 24 mm diameter transwells (area 4.7 cm^2), which are consistent with previously published data [64,65]. Monolayer integrity and transporter activity were further validated by tests with the model substrates [^{3}H]-digoxin, [^{3}H]-abacavir and [^{3}H]-saquinavir (for MDCKII-ABCB1, MDCKII-ABCG2 and MDCKII-ABCC2 cells, respectively), in which comparable to previously published efflux ratios were obtained [59,65–67].

2.6. Ex vivo uptake assays with fresh villous fragments of human placenta

These assays were performed as previously described [32,35,59,68,69]. Placentas were collected at term after uncomplicated pregnancies from the Faculty Hospital, Hradec Kralove, following written informed consent as approved by the local research Ethics Committee (approval no. 201006S15P). Samples of placental villous tissue (1 cm^3 cubes) were dissected from the fresh term placenta and rinsed in a 1:1 mixture of Dulbecco modified Eagle's medium (DMEM)/Tyrode's buffer mixture at room temperature within 30 min of delivery. Fresh control (Na^+-containing) Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 5.6 mM glucose, pH 7.4) and Na^+-free buffer (containing choline chloride instead of NaCl) were prepared on the day of the experiment. Samples were washed in the DMEM/Tyrode's buffer once again to remove blood. From each sample, 6–8 evenly sized (ca. 4 mm^3) villous fragments were dissected and fixed with thread on hooks then immersed in vials containing DMEM/Tyrode's solution mix and stabilized for 30 min at 37 °C. Then they were transferred and pre-incubated for 10 min in Tyrode's buffer with/without inhibitors followed by incubation in Tyrode's buffer containing 0.5 μCi/ml of [^{3}H]-ribavirin (corresponding to 0.0285 μM) and an inhibitor where desired. Uptake was stopped after 5, 30 or 60 min by removing radioactive solutions then all fragments were washed twice with 6 ml of Tyrode's buffer. Finally, villous fragments were lysed for 18 h in distilled water to release the accumulated radioactivity, removed from water and lysed in 0.3 M NaOH solution for 10 h at 37 °C. The radioactivity released was quantified by liquid scintillation counting (see below) and normalized to total protein levels, determined using the BCA assay.

2.7. Preparation of microvillous plasma membrane vesicles and uptake assays

Microvillous plasma membrane (MVM) vesicles were used to directly analyse the role of NTs in [^{3}H]-ribavirin uptake in the apical membrane of the human syncytiotrophoblast layer. Human placentas were obtained from uncomplicated pregnancies at term (38–40 weeks of gestation) delivered by Caesarean section in the University Hospital in Hradec Kralove, with the approval of the hospital's Research Ethics Committee (approval no. 201006 S15P). MVM vesicles were isolated by Mg^{2+} precipitation and differential centrifugation as previously described [58,70]. Each resulting MVM pellet was resuspended in intravesicular buffer (IVB; 290 mM sucrose, 5 mM HEPES and 5 mM Tris, pH 7.4) and vesiculated by 15 passages through a 25-gauge needle. The protein concentration and purity of the vesicles were determined using the BCA assay and enrichment of MVM alkaline phosphatase activity relative to placental homogenate, respectively [70]. The alkaline phosphatase enrichment factor was 25.4 ± 4.3 (mean ± SD, n = 12) and the percentage of physiologically orientated vesicles 86.3 ± 7.94%, showing that potential contamination of the MVM vesicles with basal plasma membrane and/or intracellular membranes was negligible [71–73]. Right-side-out orientation of vesicles was evaluated by comparing specific alkaline phosphatase activity upon vesicular disruption by detergent (exceeded 80%), as described previously [70]. Uptake of 0.1905 μM [^{3}H]-ribavirin (corresponding to 3.3 μCi/ml activity) into MVM vesicles was measured at room temperature by rapid vacuum filtration followed by liquid scintillation counting [74]. MVM vesicles (20–30 mg/ml) were equilibrated to room temperature (21–25 °C) prior to uptake experiments. Samples of MVM suspensions (10 μl) were pre-incubated for 10 min with or without inhibitor (0.1 μM and 100 μM NBMPR or 1 mM uridine, in extravesicular buffer) [32]. Uptake of [^{3}H]-ribavirin was initiated by adding the substrate diluted in extravesicular buffer (EVB; 145 mM KCl, 5 mM HEPES and 5 mM Tris, pH 7.4, with KCl instead of NaCl for Na^+-free buffer) to the pre-incubated MVM vesicles. Uptake was halted after predefined time points, by adding 2 ml ice-cold stopping buffer (130 mM NaCl, 10 mM Na_2HPO_4, 4.2 mM KCl, 1.2 mM $MgSO_4$, 0.75 mM $CaCl_2$, 100 μM NBMPR, pH 7.4) and filtering the resulting mixture through a 0.45 μM mixed cellulose ester filter (HAWP02500 membrane filter, MF-Millipore, Darmstadt, Germany) under vacuum. Filters were washed with 10 ml stopping buffer containing 100 μM NBMPR where appropriate, and the filter-associated radioactivity was determined. Protein-free controls (with IVB instead of MVM vesicle extract) were analysed in parallel to determine the amount of tracer that bound to the filter, which was subtracted from total vesicle counts. Non-specific binding of [^{3}H]-ribavirin to the plasma membrane was excluded by measuring time zero uptakes, which were comparable to values obtained for protein-free controls.

2.8. In situ dual perfusion of rat term placenta

Placental transfer of [^{3}H]-ribavirin was evaluated using dually perfused rat term placenta in open and closed setups, as previously described [32,35,57–59,75]. In the course of perfusion experiment, we monitored the physical condition of the rat (maternal and foetal perfusion pressures, breath, pulse, and limbs swelling) and selected placenta (swelling, gelling, and colouring). If bulk fluid leakage was detected or deviation from normal physical condition was monitored the experiment was terminated and obtained data were omitted from analysis.

2.8.1. Open circuit setup

Immediately after successful Wistar rat surgery, [^{3}H]-ribavirin (0.06 μCi/ml, 0.0035 μM) was added, with and without inhibitor (0.1 μM NBMPR, 100 μM NBMPR, or 5 mM uridine), to either maternal or foetal reservoirs, for maternal-to-foetal (M → F) and foetal-to-maternal (F → M) transfer analysis, respectively. After five minutes of stabilization, sample collection was started (time 0) and foetal effluent samples were collected in pre-weighed vials at 5-min intervals. [^{3}H]-ribavirin concentrations were measured and transplacental clearance was calculated from data obtained for all intervals. At the end of the experiment, the placenta was perfused with radioactivity-free buffer for 10 min, excised from the uterine tissue, weighed, and lysed in Solvable tissue solubilizer (PerkinElmer Life and Analytical Sciences, Boston, MA, USA) to detect tissue-bound [^{3}H]-ribavirin. Pharmacokinetic analysis of placental transport was performed as previously described [76].

2.8.2. Closed circuit (recirculation) setup

A closed circuit perfusion system was used to identify placental transporter(s) responsible for active transport of ribavirin from the foetal to maternal circulation. Both maternal and foetal sides of the Wistar rat placenta were infused with a non-saturating concentration (0.0035 μM) of [^{3}H]-ribavirin (0.06 μCi/ml) and after short stabilization period (5 min), the foetal perfusate (10 ml) was recirculated for 60 min. Samples (100 μl) were collected at 10-min intervals from the maternal and foetal reservoirs, and their concentrations of [^{3}H]-ribavirin were measured. This experimental setup maintains a steady concentration on the maternal side of the placenta and enables detection of changes in the foetal/maternal ratio. Any net transfer of the substrate implies transport against a concentration gradient and thus provides evidence of active transport. To block NTs that could potentially mask effects of efflux transporters on placental passage of ribavirin, uridine (5 mM) a competitive inhibitor of all NTs [30,36], was added to both maternal and foetal reservoirs and the foetal/maternal concentration ratio at equilibrium was calculated

2.9. Placental tissue sampling, isolation of RNA and qRT-PCR analysis

Rat term placentas were collected on the 21st gestation day. Immediately after sectioning, the tissue was moved to a −80 °C freezer. Total RNA was isolated using Tri Reagent solution (Molecular Research Centre, Cincinnati, OH, USA) from weighed samples (n = 5; one placenta from each of five animals) according to the manufacturer's instructions. The purity of the isolated RNA was verified by measuring its A_{260}/A_{280} ratio, and its integrity by electrophoresis in a 1% agarose gel followed by GelRed (Biotium, Fremont, CA, USA) staining. The concentration of RNA was calculated from its A_{260} value. RNA (1 μg) was converted into cDNA using a gb Reverse Transcription Kit (Generi Biotech s.r.o., Hradec Kralove, Czech Republic) and a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). Expression of the rat *Slc28a1*, *Slc28a2*, *Slc28a3*, *Slc29a1* and *Slc29a2* mRNA was analysed by quantitative RT-PCR (qRT-PCR) with a QuantStudio™ 6 Flex Real-Time PCR System (ThermoFisher Scientific, Rockford, IL, USA). Triplicate portions (25 ng) of cDNA from each sample were amplified using Universal PCR Master Mix and TaqMan PCR Gene Expression Assay sets (ThermoFisher Scientific, Rockford, IL, USA) for *Slc28a1* (Rn01477882_m1), *Slc28a2* (Rn00581463_m1), *Slc28a3* (Rn00590238_m1), *Slc29a1* (Rn01648953_m1), *Slc29a2* (Rn01479421_m1). To improve precision, the data obtained were normalized to expression of two housekeeping genes, *Ywhaz* (Rn00755072_m1) and *Gapdh* (Rn01775763_g1), analysed in each sample [77]. The PCR temperature program consisted of 95 °C for 3 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 25 s. The abundance of the amplified transcripts in the samples was calculated using the $2^{-\Delta\Delta ct}$ method.

2.10. Radioisotope analyses

We quantified radioisotopes from [^{3}H]-ribavirin in experimental samples by liquid scintillation counting, using a Tri-Carb 2910 TR instrument (PerkinElmer, Waltham, MA, USA) as previously reported [26,27]. The concentrations of [^{3}H]-ribavirin used in the tests depended on the experimental system, because the lowest concentration providing sufficient measurable activity was used to minimise risks of transporter saturation and maximize the methods' sensitivity.

2.11. Statistical analyses

The statistical significance of results of both *in vitro* and *in situ* experiments was assessed by one-way ANOVA followed by Dunnett's test, or (for experiments with MVM vesicles) Wilcoxon's matched-pairs signed rank test. For statistical analysis of time courses of [^{3}H]-ribavirin accumulation in the *ex vivo* experiments, we used non-parametric two-tailed unpaired Mann-Whitney test. Quantitative RT-PCR data were analysed using unpaired Student's *t* test. All data were processed using GraphPad Prism 7.04 software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. In vitro effects of NT inhibitors on [^{3}H]-ribavirin uptake by BeWo cells

We first analysed whether inhibition of NTs affects accumulation of [^{3}H]-ribavirin (at 0.0222 μM) by BeWo cells. In 5-min incubations at 37 °C, both Na^+ depletion and uridine (5 mM) caused significant reductions in uptake of [^{3}H]-ribavirin (2- and 2.5-fold, respectively), while NBMPR at either 0.1 μM and 100 μM increased its uptake up to 2-fold (Fig. 1a). Therefore, we suggest that Na^+-dependent transport participates in [^{3}H]-ribavirin uptake, hypothesizing that CNTs concentrates [^{3}H]-ribavirin in cells when ENTs are inhibited, as previously found in *Xenopus laevis* oocytes expressing human CNT [78]. To assess the contribution of ENTs to [^{3}H]-ribavirin uptake by BeWo cells, we monitored accumulation at a temperature (4 °C) that inhibits ATP-dependent cellular processes, including CNT activity. Under these conditions, NBMPR (0.1 μM and 100 μM) caused a ca. 2-fold decrease in uptake of [^{3}H]-ribavirin into BeWo cells (Fig. 1b). In addition, a significant effect of ENT inhibition was detected at all tested time points (2, 15, 30, and 60 min), suggesting that 100 μM NBMPR affects not only the initial velocity of the uptake (within 2 min) but also the plateau level (Fig. 1c). Our data indicate that both ENTs and CNTs (probably CNT2) participate in ribavirin uptake into BeWo cells.

3.2. Effects of ribavirin concentration, presence/absence of 100 μM NBMPR at 37 °C and low temperature (4 °C) on uptake by BeWo cells

Uptake of ribavirin by BeWo cells was concentration-dependent in all experimental systems. As shown in Fig. 2, accumulation of ribavirin (at 0.0222 μM) was significantly higher at 37 °C without NBMPR (100 μM) than at either 37 °C or 4 °C with no inhibitor of ENTs or CNTs (Fig. 2). The higher accumulation of ribavirin at 37 °C in the presence of 100 μM NBMPR may be due to ribavirin's previously reported high affinity for CNTs [78]. This phenomenon was abolished at concentrations of ribavirin > 1 μM, suggesting saturation of CNTs, probably followed by saturation of ENTs, resulting in a plateau phase.

3.3. Bidirectional transport of [^{3}H]-ribavirin across MDCKII-parental, MDCKII-ABCB1, MDCKII-ABCG2, and MDCKII-ABCC2 cells

To evaluate [^{3}H]-ribavirin interaction with placental ABCB1, ABCG2, and ABCC2, we determined the transepithelial transport of [^{3}H]-ribavirin (0.0022 μM) across monolayers of parental, ABCB1-, ABCG2-, or ABCC2-overexpressing cells. Uridine (1 mM) was used to

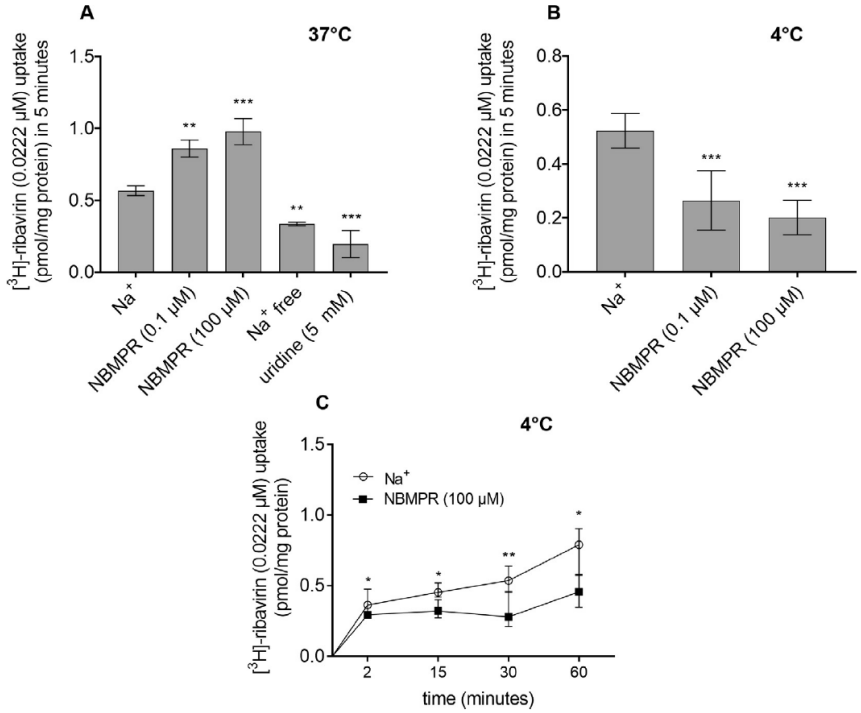

Fig. 1. Accumulation of [^{3}H]-ribavirin (0.0222 µM) by BeWo cells. At 37 °C, NBMPR (0.1 µM and 100 µM) increased while uridine (5 mM) and Na^+ depletion decreased 5-min accumulation of [^{3}H]-ribavirin (A). At 4 °C the 5-min uptake was significantly reduced in presence of NBMPR (0.1 µM and 100 µM) (B). NBMPR (100 µM) affected accumulation of [^{3}H]-ribavirin at all test time points at 4 °C (C). Data presented are means ± SD (n ≥ 5). One-way ANOVA followed by Dunnett's test was used to evaluate the significance of differences between treated and control samples after 5-min incubation and Student's *t*-test to evaluate the significance of differences in time-courses: $^*p < 0.1$, $^{**}p < 0.01$, $^{***}p < 0.001$.

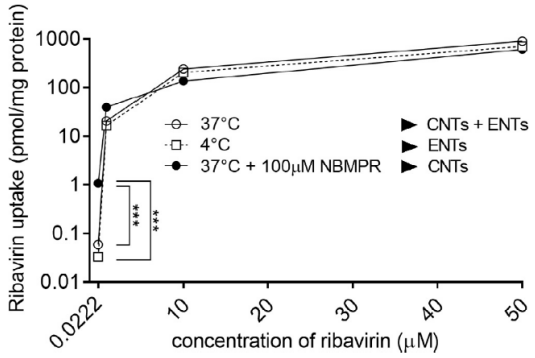

Fig. 2. Concentration-dependent accumulation of ribavirin by BeWo cells in the presence and absence of NBMPR (100 µM) at 37 °C and 4 °C, with [^{3}H]-ribavirin (0.0222 µM, 0.4 µCi/ml) as a tracer for quantification. At the lowest ribavirin concentration (0.0222 µM) and 37 °C, accumulation was significantly higher with 100 µM NBMPR than with no inhibitor at 37 °C or in experiments at 4 °C. Saturation of NTs resulting in a plateau phase was observed at concentrations of ribavirin > 1 µM. Data presented are means ± SD (n = 3). One-way ANOVA followed by Dunnett's test was used to evaluate statistical significance; $^{***}p < 0.001$.

Table 1
Net efflux ratios (r_{net}) of [^{3}H]-ribavirin (0.0022 µM) after 120 min incubation, means ± SD, n = 3.

Cells	No inhibitor	Uridine (1 mM)
MDCKII-parental	1 ± 0.007	1 ± 0.002
MDCKII-ABCB1	0.870 ± 0.008	1.12 ± 0.036
MDCKII-ABCG2	0.995 ± 0.010	0.972 ± 0.004
MDCKII-ABCC2	0.553 ± 0.034	0.526 ± 0.008

r_{net} – efflux ratios obtained for the ABCB1/ABCG2/ABCC2-transfected or parental MDCK cell line normalized to the ratio obtained for the parental cells [61].

block potential canine NT activity that could influence obtained efflux ratios. We detected no significant contribution of the tested ABC transporters at any of the sampling times (30, 60, and 120 min). Calculated values of r_{net} after 120 min incubation ranged from 0.5528 ± 0.034 (MDCKII-ABCC2) to 0.9945 ± 0.010 (MDCKII-ABCG2) with no inhibitor; and in the presence of the competitive NT inhibitor uridine (1 mM) from 0.5263 ± 0.008 (MDCKII-ABCC2) to 1.1281 ± 0.036 (MDCKII-ABCB1). Thus the results indicate that no ABCB1-, ABCG2-, or ABCC2-mediated transport was involved (Table 1). Values obtained for r_{net} after 30 and 60 min incubation (not shown) are consistent with this conclusion.

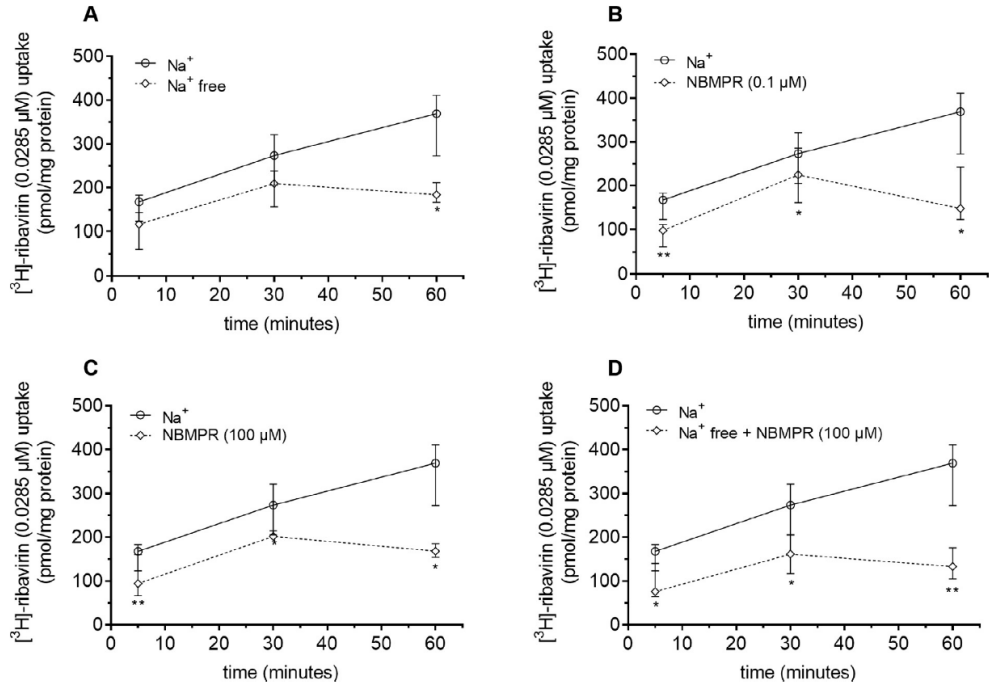

Fig. 3. *Ex vivo* uptake of [^{3}H]-ribavirin by fresh human villous placental fragments. Time-dependent uptake of [^{3}H]-ribavirin (0.0285 µM) was not affected by Na^+ depletion (A), but significantly decreased by 0.1 µM NBMPR (B), 100 µM NBMPR (C), and a combination of Na^+ depletion and 100 µM NBMPR (D). Data presented are medians with interquartile ranges, n ≥ 4 placentas. The significance of inhibitory effects of tested conditions at selected time points was assessed using the Mann-Whitney unpaired non-parametric two-tailed test ($^*p < 0.1$; $^{**}p < 0.01$).

3.4. Effects of NT inhibitors on [^{3}H]-ribavirin uptake by ex vivo human placental villous fragments

In *ex vivo* experiments with fresh villous fragments isolated from human term placenta, we observed time-dependent increases in accumulation of [^{3}H]-ribavirin (0.0285 µM) in Na^+-containing (control) buffer. In tests of effects of NT inhibitors, NBMPR at both concentrations (0.1 µM and 100 µM) and 100 µM NBMPR in combination with Na^+ depletion significantly affected the time course of [^{3}H]-ribavirin accumulation, but Na^+ depletion alone had no significant effect. These findings indicate that ENTs, but not CNTs, contribute to ribavirin uptake into placental trophoblast.

3.5. Effects of NT inhibitors on [^{3}H]-ribavirin accumulation by human placental MVM vesicles

In further assessments of NTs' contribution to [^{3}H]-ribavirin placental pharmacokinetics, we found that 100 µM NBMPR, but not Na^+ depletion, significantly reduced time-dependent uptake (with sampling after 1, 5, and 10 min) of [^{3}H]-ribavirin (0.1905 µM) by MVM vesicles isolated from human placental tissue (Fig. 4a). In subsequent comparisons, at 1- and 10-min time-points, we found that 0.1 µM NBMPR, 100 µM NBMPR and 1 mM uridine had similar effects, and Na^+ deletion had no apparent effect, relative to inhibitor-free, Na^+-containing control systems (Fig. 4b, c). The results indicate involvement of ENTs, but not CNTs, in vesicular uptake of [^{3}H]-ribavirin.

3.6. Effects of NT inhibitors on transplacental clearance of [^{3}H]-ribavirin determined by in situ dual perfusion of rat placenta

Using the dually perfused rat term placenta model, we observed low M → F and F → M total clearances, reaching values of 0.06 and 0.15 ml/min/g, respectively. Application of NBMPR (0.1 µM or 100 µM) or uridine (5 mM) significantly decreased total F → M clearances of [^{3}H]-ribavirin (0.0035 µM) relative to clearances in control (inhibitor-free) media. The only difference observed in analyses of clearance in the opposite (M → F) direction was that 0.1 µM NBMPR had no significant effect (Fig. 5a). Less than 1% of administered ribavirin was detected in the placenta after perfusion experiments, indicating that tissue binding had negligible effect on calculated clearances (data not shown). F → M clearances reached 2.4-fold higher values than M → F clearances, suggesting transporter-mediated transfer of [^{3}H]-ribavirin back to maternal circulation. In perfusion assays with a closed circuit setup, designed to check involvement of active transporters mediating F → M transfer, no significant differences in of [^{3}H]-ribavirin concentration between maternal and foetal reservoirs were detected, even when the potential masking effect of NTs was abolished by adding 5 mM uridine (Fig. 5b).

3.7. qRT-PCR analysis of Slc28a1, Slc28a2 and Slc28a3 in rat term placenta

As dual perfusion studies could not be performed under Na^+ depletion and 5 mM uridine (a high affinity substrate of CNT1, CNT2, and CNT3) profoundly affected M → F and F → M clearances of [^{3}H]-ribavirin, we quantified expression of CNTs in our Wistar rat placental tissue. Results show that *Slc28a2* was expressed 12.7-fold more strongly than *Slc28a3*, and no *Slc28a1* transcript was detected (Fig. 6).

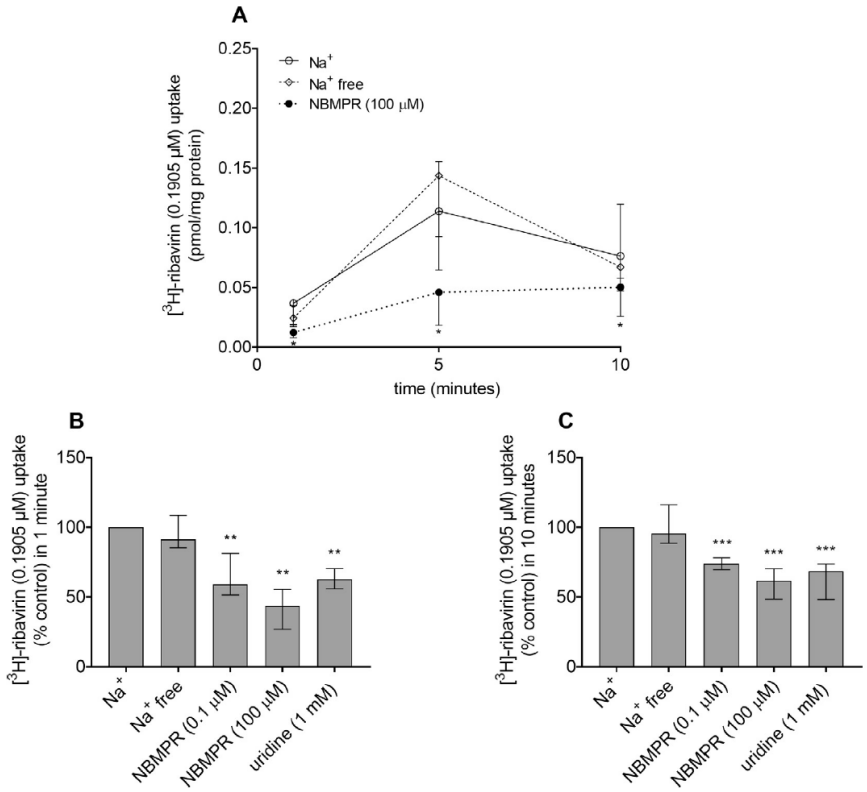

Fig. 4. Uptake of [^{3}H]-ribavirin by MVM vesicles isolated from human term placenta. Time courses of [^{3}H]-ribavirin (0.1905 μM) uptake indicate involvement of ENTs, but not Na^+-dependent mechanisms (A). Additions of NBMPR (0.1 and 100 μM) and uridine (1 mM) resulted in similar reductions in uptake after 1 min (B) and 10 min (C). Presented data are medians with interquartile ranges ($n \geq 4$). The significance of differences, relative to controls, was evaluated by the Wilcoxon matched-pairs signed rank test; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

4. Discussion

As anti-HCV treatments may be potentially used to reduce rates of vertical HCV transmission [7,8], it is important to identify safe and effective pharmacotherapy regimens for both pregnant woman and developing foetus. For more than a decade ribavirin has been the backbone of various HCV drug combinations [4,13,79]. Nowadays, it is frequently combined with directly acting antivirals against HCV [4] and is essential for treating life-threatening viral infections such as respiratory syncytial virus or influenza virus [10]. Clear signals of ribavirin teratogenicity in animals have been detected [25], but not in humans [15]. Therefore, ribavirin remains a candidate for prevention of HCV vertical infection [14,21,22].

Knowledge of ribavirin transplacental kinetics mechanisms is required to assure safe use of ribavirin in prevention of vertical transmission of HCV [23]. Thus, the role of NTs in placental ribavirin pharmacokinetics has been previously investigated using *in vitro* (BeWo cells) and *in vivo* (murine model) experimental approaches [26,27]. However, this study addresses a gap through use of experimental systems derived from human placenta and *in situ* dually perfused rat term placenta (in addition to *in vitro* models) to assess roles of NTs and ABC in transfer of ribavirin across the placental barrier.

In an initial experimental approach, we applied the well-established and routinely used *in vitro* model of accumulation into BeWo cells [27,32,35,80,81]. We observed elevated accumulation of [^{3}H]-ribavirin in the presence of NBMPR (Fig. 1a) at 37 °C, suggesting that CNTs may concentrate [^{3}H]-ribavirin in the cells when ENTs are inhibited. This phenomenon has been previously shown in *Xenopus leavis* oocytes suggesting that CNTs mediate high affinity uptake concentrating ribavirin in cells. It generates concentration gradient for ENTs that mediate opposite action, i.e. outward transfer decreasing cellular ribavirin concentration. Therefore, when ENTs are inhibited, elevated ribavirin uptake can be observed [78]. Moreover, Na^+ depletion and uridine (a high affinity substrate of all CNTs) significantly decreased [^{3}H]-ribavirin uptake relative to controls (Fig. 1a), confirming involvement of CNTs [27]. In a previous study we observed Na^+-sensitive uptake of adenosine (a CNT2 and CNT3 substrate), but not thymidine (a CNT1 and CNT3 substrate) concluding that the only CNT sub-group functionally expressed in BeWo cells is CNT2 [32]. Moreover, based on mRNA quantification, our clone of BeWo expresses *SLC28A2* more strongly than *SLC28A3* (respectively encoding CNT2 and CNT3), while no *SLC28A1* transcript (encoding CNT1) was detected [81]. Therefore, we suggest that ribavirin is a substrate of CNT2, which mediates its uptake into BeWo cells. To avoid masking effects of CNT2 on [^{3}H]-ribavirin uptake in NBMPR-treated cells we performed experiments at 4 °C to inhibit ATP-dependent cellular processes (Fig. 1b). Low temperature (4 °C) is a condition commonly used to indirectly assess involvement of active membrane transport in drug kinetics as shown in Caco-2 cell line [82,83]. Moreover, Archer et al. have described that temperature below 15 °C abolishes the activity of CNTs in rat

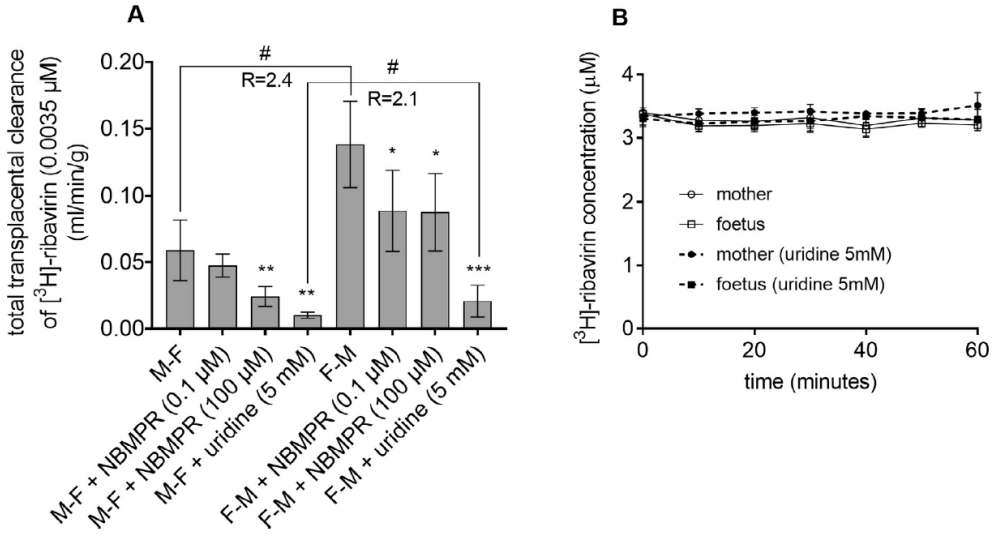

Fig. 5. [^{3}H]-Ribavirin transport across rat term placenta in open (A) and closed setups (B). NBMPR (100 µM) and uridine (5 mM) significantly decreased total transplacental clearances of [^{3}H]-ribavirin (0.0035 µM) in both M → F and F → M directions, while NBMPR (0.1 µM) only affected clearance in the F → M direction (A). No active F → M transport of [^{3}H]-ribavirin (0.0035 µM) was observed in the absence or in the presence of uridine (5 mM) used to inhibit NTs that might mask effects of ABC efflux pumps on [^{3}H]-ribavirin placental kinetics (B). Data obtained with the open-circuit setup are means ± SD (n ≥ 3), and the significance of differences was assessed using one-way ANOVA followed by Dunnett's test. The significance of differences in results obtained with the closed-circuit setup was assessed by Student's *t*-test. In both cases: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

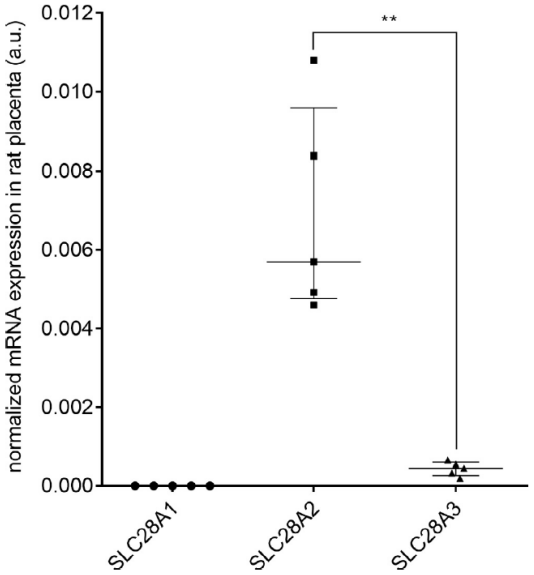

Fig. 6. Results of quantitative RT-PCR analysis of *Slc28a1*, *Slc28a2* and *Slc28a3* mRNA expression in rat term placenta. *Slc28a2* was expressed much more strongly than *Slc28a3*, and *Slc28a1* expression was below the analytical detection limit. Gene expression levels were normalized against the geometric mean expression of two housekeeping genes: *Gapdh* and *Ywhaz*. Data are reported in arbitrary units (a.u.) as mean ± SD (n = 5). The significance of differences was assessed using the unpaired parametric two-tailed Student's *t* test: $^{**}p < 0.01$.

microvascular endothelial cells while ENTs preserve the sensitivity to NBMPR and dipyridamole [84]. Jarvis et al. have shown in guinea pig erythrocytes that CNTs-controlled transport was 70-fold higher at 37 °C than at 10 °C while ENTs-mediated transport was less temperature-dependent [85]. Moreover, using vesicles prepared from human erythrocytes it has been recently evidenced that uridine K_m is comparable at ice-cold temperature and 23 °C while V_{max} was decreased by only 23% suggesting functional state of ENTs at very low temperatures [86]. In line with this finding, ENT1-mediated ribavirin uptake at ice-cold temperature was confirmed in this experimental system [86]. Under these conditions, we found that NBMPR had similar effects at both concentrations, confirming that ENTs, particularly ENT1 probably as it is most strongly expressed in the placenta [32], also contribute to [^{3}H]-ribavirin uptake into BeWo cells. The suggested ENT1- and CNT2-mediated transport of [^{3}H]-ribavirin is consistent with results of previous experiments with BeWo cells [27] and non-placental models, e.g. OR6 cells, HHL-5 cells, human hepatocytes, and mice [26,32,44,48–51,87]. Analysis of the concentration-dependence of ribavirin accumulation in BeWo cells (Fig. 2), indicated that CNTs were largely responsible for its accumulation in the presence of 100 µM NBMPR at ribavirin concentrations < 1 µM as previously found in experiments with *Xenopus* oocytes expressing human NTs [78]. However, higher concentrations resulted in saturation and thus abolishment of NTs' effects (Fig. 2).

Next, we investigated the accumulation of [^{3}H]-ribavirin in fresh villous fragments of human placenta, which provide a physiologically relevant, well-described and validated model for assessing drug interactions with ENTs [32,35,69,70]. The uptake of [^{3}H]-ribavirin was time-dependent and significantly sensitive to treatment with 0.1 µM and 100 µM NBMPR, but not Na^+ depletion alone (Fig. 3), suggesting that only ENTs are involved in its placental uptake. We propose that the dominantly expressed ENT1 [32] is responsible for this uptake. Moreover, the apparent lack of CNTs' participation in placental uptake of ribavirin is consistent with our findings that functional CNTs are not expressed in placenta [32].

In analyses of [^{3}H]-ribavirin accumulation in MVM vesicles we also detected contributions of ENT1 but not CNTs (Fig. 4), and inhibitory effects of uridine, presumably due solely to inhibition of ENTs (Fig. 4b, c). These results are consistent with findings from our experiments with villous fragments and previously reported studies with human and rat MVM vesicles [32,33,45].

In the next step, we employed an *in situ* model based on dually perfused rat placenta in open- and closed-circuit setups to evaluate the overall contribution of NTs and potentially ABC proteins to placental [^{3}H]-ribavirin transfer at the organ level. Dual perfusion of rat term placenta is an established and well-justified method that has been used to investigate interactions of placental ABC and SLC transporters with various drugs, including antivirals [57–59,88–90]. ENTs in the apical membrane of trophoblast layer of Wistar rats have been functionally characterized by analysing adenosine uptake from the maternal blood circulation into the foeto-placental unit and MVM vesicles prepared from rat placenta [45]. Analysis of placental M → F and F → M clearances of radiolabelled [^{3}H]-adenosine is impossible, with current limitations, due to extensive adenosine placental metabolism [34], so we could not directly validate this experimental system. With the open-circuit setup, we obtained the first evidence that transport of [^{3}H]-ribavirin across rat placenta from mother to foetus and *vice versa* is low overall (Fig. 5a), especially relative to antipyrine clearance across the placenta driven by passive diffusion [91]. Both M → F and F → M clearances of [^{3}H]-ribavirin were significantly reduced by presence of NBMPR (at 0.1 μM and 100 μM) or 5 mM uridine, suggesting a profound contribution of ENTs, most likely ENT1, not only to placental cell uptake but also to transport of ribavirin across rat syncytiotrophoblast. Effects of Na^+ depletion could not be investigated using this model as it deleteriously affects rat term placenta, causing oedema and high pressure, during experiments.

Lower M → F clearance than F → M clearance indicated that ribavirin is predominantly transferred in the F → M direction, therefore we performed dual perfusion experiments with a closed-circuit setup (Fig. 5b). We observed no changes in ribavirin concentrations on maternal or foetal sides, even after adding uridine to block potential masking activity of NTs, indicating no active efflux transport from foetus to mother (Fig. 5). These findings are consistent with our suggestions, based on results of *in vitro* experiments, that active efflux placental pumps do not recognize ribavirin as a substrate (Table 1).

To further assess potential involvement of CNTs in ribavirin placental kinetics we quantified mRNA expression of *slc28A1*, *slc28A2*, and *slc28A3* in our experimental animal model. We found that *slc28A2* is by far the most strongly expressed CNT gene, *slc28A1* expression is not detectable, in term placenta of our Wistar rat (Fig. 6), in accordance with previous reports [45,81]. Nevertheless, as lack of CNT activity was observed in MVM vesicles derived from Wistar rat placenta [45], these transporters do not seem to be functionally expressed in the trophoblast apical membrane [45]. Therefore, we speculate that only CNT2/CNT3 embedded in the basal membrane may potentially contribute to the higher F → M clearances observed in rat placenta.

In conclusion, using animal and (for the first time) human tissue-derived models we have shown that passage of ribavirin across the placenta from mother to foetus could be gated mainly by ENT1. On the other hand, CNT2 seems to take up ribavirin into BeWo cells, but play no role in its uptake in the human placenta. We also provide the first evidence that ABCB1, ABCG2, and ABCC2 do not recognize ribavirin as a substrate. We hypothesize that levels of ribavirin placental transport may be affected by inter-individual variabilities and interactive drug-drug effects on placental ENT1. Thus, our data broaden knowledge of placental pharmacokinetics of ribavirin, but further studies with clinical setups are needed to fully assess the pharmacokinetics and risks associated with ribavirin's use in pregnancy.

Acknowledgements

This research was financially supported by the Czech Science Foundation (grant no. GACR 17-16169S), Grant Agency of Charles University (grant nos. GAUK 324215/C/2015; SVV/2016/260-293) and EFSA-CDN (No. CZ.02.1.01/0.0/0.0/16_019/0000841). We thank Dr Marian Kacerovsky (Department of Obstetrics and Gynecology, University Hospital in Hradec Kralove) for providing us with human placentas and Martina Hudeckova for her help with the human placenta collection and sampling. We also thank Dana Souckova for skilful assistance with the perfusion experiments.

Declaration of interests

The authors declare no conflicts of interest.

References

[1] M.J. Alter, Epidemiology of hepatitis C virus infection, World J. Gastroenterol. 13 (17) (2007) 2436–2441.
[2] K.M. Hanafiah, J. Groeger, A.D. Flaxman, S.T. Wiersma, Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence, Hepatology 57 (4) (2013) 1333–1342.
[3] M. Reid, Y. Ma, R. Scherzer, J.C. Price, A.L. French, G.D. Huhn, M.W. Plankey, M. Peters, C. Grunfeld, P.C. Tien, Contribution of liver fibrosis and microbial translocation to immune activation in HIV and HCV, J. Infect. Dis. (2018).
[4] European Association for the Study of the Liver, EASL recommendations on treatment of hepatitis C 2018, J. Hepatol. (2018), https://doi.org/10.1016/j.jhep.2018.03.026.
[5] E. Marine-Barjoan, A. Berrebi, V. Giordanengo, S.F. Favre, H. Haas, M. Moreigne, J. Izopet, J. Tricoire, A. Tran, C. Pradier, A. Bongain, HCV/HIV co-infection, HCV viral load and mode of delivery: risk factors for mother-to-child transmission of hepatitis C virus? AIDS 21 (13) (2007) 1811–1815.
[6] C.B. Polis, S.N. Shah, K.E. Johnson, A. Gupta, Impact of maternal HIV coinfection on the vertical transmission of hepatitis C virus: a meta-analysis, Clin. Infect. Dis. 44 (8) (2007) 1123–1131.
[7] L. Benova, Y.A. Mohamoud, C. Calvert, L.J. Abu-Raddad, Vertical transmission of hepatitis C virus: systematic review and meta-analysis, Clin. Infect. Dis. 59 (6) (2014) 765–773.
[8] C.Y. Yeung, H.C. Lee, W.T. Chan, C.B. Jiang, S.W. Chang, C.K. Chuang, Vertical transmission of hepatitis C virus: current knowledge and perspectives, World J. Hepatol. 6 (9) (2014) 643–651.
[9] R.W. Sidwell, J.H. Huffman, G.P. Khare, L.B. Allen, J.T. Witkowski, R.K. Robins, Broad-spectrum antiviral activity of Virazole: 1-beta-D-ribofuranosyl-1,2,4-triazole-3-carboxamide, Science 177 (4050) (1972) 705–706.
[10] L. Novakova, J. Pavlik, L. Chrenkova, O. Martinec, L. Cerveny, Current antiviral drugs and their analysis in biological materials-Part I: Antivirals against respiratory and herpes viruses, J. Pharm. Biomed. Anal. 147 (2018) 400–416.
[11] WHO, WHO Model List of Essential Medicines for Children. < http://www.who.int/medicines/publications/essentialmedicines/en/ > , 2018 (accessed 14-02-2018.2018).
[12] WHO, WHO Model List of Essential Medicines. < http://www.who.int/medicines/publications/essentialmedicines/en/ > , 2018 (accessed 14-02-2018.2018).
[13] FDA, Ribavirin Pregnancy Registry. < http://www.ribavirinpregnancyregistry.com/ > , 2005 (accessed 06.12.2016.).
[14] M. Rezvani, G. Koren, Pregnancy outcome after exposure to injectable ribavirin during embryogenesis, Reprod. Toxicol. 21 (1) (2006) 113–115.
[15] S.M. Sinclair, J.K. Jones, R.K. Miller, M.F. Greene, P.Y. Kwo, W.C. Maddrey, The ribavirin pregnancy registry: an interim analysis of potential teratogenicity at the mid-point of enrollment, Drug Saf. 40 (12) (2017) 1205–1218.
[16] R.L. Atmar, J.A. Englund, H. Hammill, Complications of measles during pregnancy, Clin. Infect. Dis. 14 (1) (1992) 217–226.
[17] D. Mishkin, M. Deschenes, Conception soon after discontinuing interferon/ribavirin therapy: a successful outcome, Am. J. Gastroenterol. 96 (7) (2001) 2285–2286.
[18] K. Hegenbarth, U. Maurer, P.M. Kroisel, P. Fickert, M. Trauner, R.E. Stauber, No evidence for mutagenic effects of ribavirin: report of two normal pregnancies, Am. J. Gastroenterol. 96 (7) (2001) 2286–2287.
[19] W.C. Maddrey, Safety of combination interferon alfa-2b/ribavirin therapy in chronic hepatitis C-relapsed and treatment-naive patients, Semin. Liver Dis. 19 (Suppl 1) (1999) 67–75.
[20] M. De Santis, B. Carducci, A.F. Cavaliere, L. De Santis, A. Lucchese, G. Straface, A. Caruso, Paternal exposure to ribavirin: pregnancy and neonatal outcome, Antivir. Ther. 8 (1) (2003) 73–75.
[21] P. Labarga, J. Pinilla, I. Cachorro, Y.R. del Prado, Infant of 22 months of age with no anomalies born from a HCV- and HIV-infected mother under treatment with pegylated interferon, ribavirin and antiretroviral therapy during the first 16 weeks of pregnancy, Reprod. Toxicol. 24 (3–4) (2007) 414–416.
[22] M. Valentin, G. Ducarme, C. Yver, E. Vuillard, N. Belarbi, D. Renier, D. Luton, Trigonocephaly and valproate: a case report and review of literature, Prenat. Diagn. 28 (3) (2008) 259–261.

[23] P. Brownbill, I. Chernyavsky, B. Bottalico, G. Desoye, S. Hansson, G. Kenna, L.E. Knudsen, U.R. Markert, N. Powles-Glover, H. Schneider, L. Leach, An international network (PlaNet) to evaluate a human placental testing platform for chemicals safety testing in pregnancy, Reprod. Toxicol. (2016).
[24] S.H. Thomas, L.M. Yates, Prescribing without evidence – pregnancy, Br. J. Clin. Pharmacol. 74 (4) (2012) 691–697.
[25] G.S. Naik, M.G. Tyagi, A pharmacological profile of ribavirin and monitoring of its plasma concentration in chronic hepatitis C infection, J. Clin. Exp. Hepatol. 2 (1) (2012) 42–54.
[26] C.J. Endres, A.M. Moss, K. Ishida, R. Govindarajan, J.D. Unadkat, The role of the equilibrative nucleoside transporter 1 on tissue and fetal distribution of ribavirin in the mouse, Biopharm. Drug Dispos. 37 (6) (2016) 336–344.
[27] T. Yamamoto, K. Kuniki, Y. Takekuma, T. Hirano, K. Iseki, M. Sugawara, Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters, Eur. J. Pharmacol. 557 (1) (2007) 1–8.
[28] J. Morello, S. Rodriguez-Novoa, I. Jimenez-Nacher, V. Soriano, Usefulness of monitoring ribavirin plasma concentrations to improve treatment response in patients with chronic hepatitis C, J. Antimicrob. Chemother. 62 (6) (2008) 1174–1180.
[29] O. Kis, K. Robillard, G.N. Chan, R. Bendayan, The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters, Trends Pharmacol. Sci. 31 (1) (2010) 22–35.
[30] M. Molina-Arcas, F.J. Casado, M. Pastor-Anglada, Nucleoside transporter proteins, Curr. Vasc. Pharmacol. 7 (4) (2009) 426–434.
[31] Z. Ma, X. Yang, T. Jiang, M. Bai, C. Zheng, S. Zeng, D. Sun, H. Jiang, Multiple SLC and ABC transporters contribute to the placental transfer of entecavir, Drug Metab. Dispos. (2017).
[32] L. Cerveny, Z. Ptackova, M. Ceckova, R. Karahoda, S. Karbanova, L. Jiraskova, S.L. Greenwood, J.D. Glazier, F. Staud, Equilibrative nucleoside transporter 1 (ENT1, SLC29A1) facilitates transfer of the antiretroviral drug abacavir across the placenta, Drug Metab. Dispos. 46 (11) (2018) 1817–1826.
[33] L.F. Barros, J.C. Bustamante, D.L. Yudilevich, S.M. Jarvis, Adenosine transport and nitrobenzylthioinosine binding in human placental membrane vesicles from brush-border and basal sides of the trophoblast, J. Membr. Biol. 119 (2) (1991) 151–161.
[34] J. Dancis, J. Lee, S. Mendoza, L. Liebes, Nucleoside transport by perfused human placenta, Placenta 14 (5) (1993) 547–554.
[35] S. Karbanova, L. Cerveny, M. Ceckova, Z. Ptackova, L. Jiraskova, S. Greenwood, F. Staud, Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine, Placenta 60 (2017) 86–92.
[36] E. Errasti-Murugarren, P. Diaz, V. Godoy, G. Riquelme, M. Pastor-Anglada, Expression and distribution of nucleoside transporter proteins in the human syncytiotrophoblast, Mol. Pharmacol. 80 (5) (2011) 809–817.
[37] M. Pastor-Anglada, S. Perez-Torras, Nucleoside transporter proteins as biomarkers of drug responsiveness and drug targets, Front. Pharmacol. 6 (2015) 13.
[38] M. Sundaram, S.Y. Yao, J.C. Ingram, Z.A. Berry, F. Abidi, C.E. Cass, S.A. Baldwin, J.D. Young, Topology of a human equilibrative, nitrobenzylthioinosine (NBMPR)-sensitive nucleoside transporter (hENT1) implicated in the cellular uptake of adenosine and anti-cancer drugs, J. Biol. Chem. 276 (48) (2001) 45270–45275.
[39] S.Y. Yao, A.M. Ng, W.R. Muzyka, M. Griffiths, C.E. Cass, S.A. Baldwin, J.D. Young, Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues, J. Biol. Chem. 272 (45) (1997) 28423–28430.
[40] M. Pastor-Anglada, S. Perez-Torras, Emerging roles of nucleoside transporters, Front. Pharmacol. 9 (2018) 606.
[41] M. Pastor-Anglada, S. Perez-Torras, Who is who in adenosine transport, Front. Pharmacol. 9 (2018) 627.
[42] M. Pastor-Anglada, N. Urtasun, S. Perez-Torras, Intestinal nucleoside transporters: function, expression, and regulation, Compr. Physiol. 8 (3) (2018) 1003–1017.
[43] T. Chishu, Y. Sai, T. Nishimura, K. Sato, N. Kose, E. Nakashima, Potential of various drugs to inhibit nucleoside uptake in rat syncytiotrophoblast cell line, TR-TBT 18d–1, Placenta 29 (5) (2008) 461–467.
[44] M.K. Choi, M.H. Kim, H.J. Maeng, I.S. Song, Contribution of CNT1 and ENT1 to ribavirin uptake in human hepatocytes, Arch. Pharm. Res. 38 (5) (2015) 904–913.
[45] T. Nishimura, T. Chishu, M. Tomi, R. Nakamura, K. Sato, N. Kose, Y. Sai, E. Nakashima, Mechanism of nucleoside uptake in rat placenta and induction of placental CNT2 in experimental diabetes, Drug Metab. Pharmacokinet. 27 (4) (2012) 439–446.
[46] F. Staud, M. Ceckova, Regulation of drug transporter expression and function in the placenta, Expert Opin. Drug Metab. Toxicol. 11 (4) (2015) 533–555.
[47] F. Staud, L. Cerveny, M. Ceckova, Pharmacotherapy in pregnancy; effect of ABC and SLC transporters on drug transport across the placenta and fetal drug exposure, J. Drug Target. 20 (9) (2012) 736–763.
[48] M. Iikura, T. Furihata, M. Mizuguchi, M. Nagai, M. Ikeda, N. Kato, A. Tsubota, K. Chiba, ENT1, a ribavirin transporter, plays a pivotal role in antiviral efficacy of ribavirin in a hepatitis C virus replication cell system, Antimicrob. Agents Chemother. 56 (3) (2012) 1407–1413.
[49] Y. Fukuchi, T. Furihata, M. Hashizume, M. Iikura, K. Chiba, Characterization of ribavirin uptake systems in human hepatocytes, J. Hepatol. 52 (4) (2010) 486–492.
[50] I. Pinilla-Macua, P. Fernandez-Calotti, S. Perez-Del-Pulgar, M. Pastor-Anglada, Ribavirin uptake into human hepatocyte HHL5 cells is enhanced by interferon-alpha via up-regulation of the human concentrative nucleoside transporter (hCNT2), Mol. Pharmacol. 11 (9) (2014) 3223–3230.
[51] A.M. Moss, C.J. Endres, A. Ruiz-Garcia, D.S. Choi, J.D. Unadkat, Role of the equilibrative and concentrative nucleoside transporters in the intestinal absorption of the nucleoside drug, ribavirin, in wild-type and Ent1(-/-) mice, Mol. Pharmacol. 9 (9) (2012) 2442–2449.
[52] C.J. Endres, A.M. Moss, B. Ke, R. Govindarajan, D.S. Choi, R.O. Messing, J.D. Unadkat, The role of the equilibrative nucleoside transporter 1 (ENT1) in transport and metabolism of ribavirin by human and wild-type or Ent1-/- mouse erythrocytes, J. Pharmacol. Exp. Ther. 329 (1) (2009) 387–398.
[53] T. Nishimura, J. Tanaka, M. Tomi, Y. Seki, N. Kose, Y. Sai, E. Nakashima, Enhancement of zidovudine transfer to molt-4 cells, a human t-cell model, by dehydroepiandrosterone sulfate, J. Pharm. Sci. 100 (9) (2011) 3959–3967.
[54] Y. Sai, T. Nishimura, S. Shimpo, T. Chishu, K. Sato, N. Kose, T. Terasaki, C. Mukai, S. Kitagaki, N. Miyakoshi, Y.S. Kang, E. Nakashima, Characterization of the mechanism of zidovudine uptake by rat conditionally immortalized syncytiotrophoblast cell line TR-TBT, Pharm. Res. 25 (7) (2008) 1647–1653.
[55] Guide for the Care and Use of Laboratory Animals, in: N. A. Press (Ed.) National Research Council, Washington, DC, 1996.
[56] European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, in: E. t. s. n. 123 (Ed.) Council of Europe, Strasbourg, France, 1986.
[57] Z. Neumanova, L. Cerveny, M. Ceckova, F. Staud, Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta, AIDS 28 (1) (2014) 9–17.
[58] Z. Neumanova, L. Cerveny, M. Ceckova, F. Staud, Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine, Biopharm. Drug Dispos. 37 (1) (2016) 28–38.
[59] Z. Neumanova, L. Cerveny, S.L. Greenwood, M. Ceckova, F. Staud, Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir, Reprod. Toxicol. 57 (2015) 176–182.
[60] L. Tong, T.K. Phan, K.L. Robinson, D. Babusis, R. Strab, S. Bhoopathy, I.J. Hidalgo, G.R. Rhodes, A.S. Ray, Effects of human immunodeficiency virus protease inhibitors on the intestinal absorption of tenofovir disoproxil fumarate in vitro, Antimicrob. Agents Chemother. 51 (10) (2007) 3498–3504.
[61] B. Feng, J.B. Mills, R.E. Davidson, R.J. Mireles, J.S. Janiszewski, M.D. Troutman, S.M. de Morais, In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system, Drug Metab. Dispos. 36 (2) (2008) 268–275.
[62] J.W. Polli, S.A. Wring, J.E. Humphreys, L. Huang, J.B. Morgan, L.O. Webster, C.S. Serabjit-Singh, Rational use of in vitro P-glycoprotein assays in drug discovery, J. Pharmacol. Exp. Ther. 299 (2) (2001) 620–628.
[63] I. Hubatsch, E.G. Ragnarsson, P. Artursson, Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers, Nat. Protoc. 2 (9) (2007) 2111–2119.
[64] J. de Souza, L.Z. Benet, Y. Huang, S. Storpirtis, Comparison of bidirectional lamivudine and zidovudine transport using MDCK, MDCK-MDR1, and Caco-2 cell monolayers, J. Pharm. Sci. 98 (11) (2009) 4413–4419.
[65] E. Hellinger, S. Veszelka, A.E. Toth, F. Walter, A. Kittel, M.L. Bakk, K. Tihanyi, V. Hada, S. Nakagawa, T.D. Duy, M. Niwa, M.A. Deli, M. Vastag, Comparison of brain capillary endothelial cell-based and epithelial (MDCK-MDR1, Caco-2, and VB-Caco-2) cell-based surrogate blood-brain barrier penetration models, Eur. J. Pharm. Biopharm. 82 (2) (2012) 340–351.
[66] M.T. Huisman, J.W. Smit, K.M. Crommentuyn, N. Zelcer, H.R. Wiltshire, J.H. Beijnen, A.H. Schinkel, Multidrug resistance protein 2 (MRP2) transports HIV protease inhibitors, and transport can be enhanced by other drugs, AIDS 16 (17) (2002) 2295–2301.
[67] G. Pan, N. Giri, W.F. Elmquist, Abcg2/Bcrp1 mediates the polarized transport of antiretroviral nucleosides abacavir and zidovudine, Drug Metab. Dispos. 35 (7) (2007) 1165–1173.
[68] D.E. Atkinson, C.P. Sibley, L.J. Fairbairn, S.L. Greenwood, MDR1 P-gp expression and activity in intact human placental tissue; upregulation by retroviral transduction, Placenta 27 (6–7) (2006) 707–714.
[69] S.L. Greenwood, C.P. Sibley, In vitro methods for studying human placental amino acid transport placental villous fragments, Methods Mol. Med. 122 (2006) 253–264.
[70] J.D. Glazier, C.J. Jones, C.P. Sibley, Purification and Na+ uptake by human placental microvillus membrane vesicles prepared by three different methods, Biochim. Biophys. Acta 945 (2) (1988) 127–134.
[71] K.M. Godfrey, N. Matthews, J. Glazier, A. Jackson, C. Wilman, C.P. Sibley, Neutral amino acid uptake by the microvillous plasma membrane of the human placenta is inversely related to fetal size at birth in normal pregnancy, J. Clin. Endocrinol. Metab. 83 (9) (1998) 3320–3326.
[72] B. Harrington, J. Glazier, S. D'Souza, C. Sibley, System A amino acid transporter activity in human placental microvillous membrane vesicles in relation to various anthropometric measurements in appropriate and small for gestational age babies, Pediatr. Res. 45 (6) (1999) 810–814.
[73] D. Mahendran, S. Byrne, P. Donnai, S.W. D'Souza, J.D. Glazier, C.J. Jones, C.P. Sibley, Na+ transport, H+ concentration gradient dissipation, and system A amino acid transporter activity in purified microvillous plasma membrane isolated from first-trimester human placenta: comparison with the term microvillous membrane, Am. J. Obstet. Gynecol. 171 (6) (1994) 1534–1540.
[74] J.D. Glazier, C.P. Sibley, In vitro methods for studying human placental amino acid transport: placental plasma membrane vesicles, Methods Mol. Med. 122 (2006) 241–252.
[75] L. Cerveny, Z. Ptackova, M. Durisova, F. Staud, Interactions of protease inhibitors atazanavir and ritonavir with ABCB1, ABCG2, and ABCC2 transporters: effect on transplacental disposition in rats, Reprod. Toxicol. 79 (2018) 57–65.
[76] F. Staud, Z. Vackova, K. Pospechova, P. Pavek, M. Ceckova, A. Libra, L. Cygalova, P. Nachtigal, Z. Fendrich, Expression and transport activity of breast cancer

resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line, J. Pharmacol. Exp. Ther. 319 (1) (2006) 53–62.
[77] L. Cerveny, Z. Neumanova, S. Karbanova, I. Havlova, F. Staud, Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on Abcb1a, Abcb1b and Abcg2 expression in the placenta and in maternal and fetal organs, J. Pharm. Pharmacol. 68 (1) (2016) 84–92.
[78] T. Yamamoto, M. Sugawara, T. Kikukawa, S. Miyauchi, M. Yamaguchi, A. Tero, S. Takagi, T. Nakagaki, Kinetic study of anti-viral ribavirin uptake mediated by hCNT3 and hENT1 in Xenopus laevis oocytes, Biophys. Chem. 147 (1–2) (2010) 59–65.
[79] L. Novakova, J. Pavlik, L. Chrenkova, O. Martinec, L. Cerveny, Current antiviral drugs and their analysis in biological materials – Part II: Antivirals against hepatitis and HIV viruses, J. Pharm. Biomed. Anal. 147 (2018) 378–399.
[80] N. Utoguchi, G.A. Chandorkar, M. Avery, K.L. Audus, Functional expression of P-glycoprotein in primary cultures of human cytotrophoblasts and BeWo cells, Reprod. Toxicol. 14 (3) (2000) 217–224.
[81] L. Jiraskova, L. Cerveny, S. Karbanova, Z. Ptackova, F. Staud, Expression of concentrative nucleoside transporters (SLC28A) in the human placenta: effects of gestation age and prototype differentiation-affecting agents, Mol. Pharm. 15 (7) (2018) 2732–2741.
[82] E. Liang, J. Proudfoot, M. Yazdanian, Mechanisms of transport and structure-permeability relationship of sulfasalazine and its analogs in Caco-2 cell monolayers, Pharm. Res. 17 (10) (2000) 1168–1174.
[83] A.K. Lalloo, F.R. Luo, A. Guo, P.V. Paranjpe, S.H. Lee, V. Vyas, E. Rubin, P.J. Sinko, Membrane transport of camptothecin: facilitation by human P-glycoprotein (ABCB1) and multidrug resistance protein 2 (ABCC2), BMC Med. 2 (2004) 16.
[84] R.G. Archer, V. Pitelka, J.R. Hammond, Nucleoside transporter subtype expression and function in rat skeletal muscle microvascular endothelial cells, Br. J. Pharmacol. 143 (1) (2004) 202–214.
[85] S.M. Jarvis, B.W. Martin, Effects of temperature on the transport of nucleosides in guinea pig erythrocytes, Can. J. Physiol. Pharmacol. 64 (2) (1986) 193–198.
[86] M. Takano, E. Kimura, S. Suzuki, J. Nagai, R. Yumoto, Human erythrocyte nucleoside transporter ENT1 functions at ice-cold temperatures, Drug Metab. Pharmacokinet. 25 (4) (2010) 351–360.
[87] S.M. Jarvis, J.A. Thorn, P. Glue, Ribavirin uptake by human erythrocytes and the involvement of nitrobenzylthioinosine-sensitive (es)-nucleoside transporters, Br. J. Pharmacol. 123 (8) (1998) 1587–1592.
[88] D. Ahmadimoghaddam, J. Hofman, L. Zemankova, P. Nachtigal, E. Dolezelova, L. Cerveny, M. Ceckova, S. Micuda, F. Staud, Synchronized activity of organic cation transporter 3 (Oct3/Slc22a3) and multidrug and toxin extrusion 1 (Mate1/Slc47a1) transporter in transplacental passage of MPP+ in rat, Toxicol. Sci. 128 (2) (2012) 471–481.
[89] D. Ahmadimoghaddam, F. Staud, Transfer of metformin across the rat placenta is mediated by organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) protein, Reprod. Toxicol. 39 (2013) 17–22.
[90] M. Ceckova, J. Reznicek, Z. Ptackova, L. Cerveny, F. Muller, M. Kacerovsky, M.F. Fromm, J.D. Glazier, F. Staud, Role of ABC and solute carrier transporters in the placental transport of lamivudine, Antimicrob. Agents Chemother. 60 (9) (2016) 5563–5572.
[91] L.H. Cygalova, J. Hofman, M. Ceckova, F. Staud, Transplacental pharmacokinetics of glyburide, rhodamine 123, and BODIPY FL prazosin: effect of drug efflux transporters and lipid solubility, J. Pharmacol. Exp. Ther. 331 (3) (2009) 1118–1125.

Publikace V: Equilibrative Nucleoside Transporter 1 (ENT1, *SLC29A1*) Facilitates Transfer of the Antiretroviral Drug Abacavir across the Placenta.

1521-009X/46/11/1817–1826$35.00
Drug Metabolism and Disposition

https://doi.org/10.1124/dmd.118.083329
Drug Metab Dispos 46:1817–1826, November 2018

Equilibrative Nucleoside Transporter 1 (ENT1, *SLC29A1*) Facilitates Transfer of the Antiretroviral Drug Abacavir across the Placenta

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Received June 24, 2018; accepted August 8, 2018

ABSTRACT

Abacavir is a preferred antiretroviral drug for preventing mother-to-child human immunodeficiency virus transmission; however, mechanisms of its placental transfer have not been satisfactorily described to date. Because abacavir is a nucleoside-derived drug, we hypothesized that the nucleoside transporters, equilibrative nucleoside transporters (ENTs, *SLC29A*) and/or Na^+-dependent concentrative nucleoside transporters (CNTs, *SLC28A*), may play a role in its passage across the placenta. To test this hypothesis, we performed uptake experiments using the choriocarcinoma-derived BeWo cell line, human fresh villous fragments, and microvillous plasma membrane (MVM) vesicles. Using endogenous substrates of nucleoside transporters, [^{3}H]-adenosine (ENTs, CNT2, and CNT3) and [^{3}H]-thymidine (ENTs, CNT1, and CNT3), we showed significant activity of ENT1 and CNT2 in BeWo cells, whereas experiments in the villous fragments and MVM vesicles, representing a model of the apical membrane of a syncytiotrophoblast, revealed only ENT1 activity. When testing [^{3}H]-abacavir uptakes, we showed that of the nucleoside transporters, ENT1 plays the dominant role in abacavir uptake into placental tissues, whereas contribution of Na^+-dependent transport, most likely mediated by CNTs, was observed only in BeWo cells. Subsequent experiments with dually perfused rat term placentas showed that Ent1 contributes significantly to overall [^{3}H]-abacavir placental transport. Finally, we quantified the expression of *SLC29A* in first- and third-trimester placentas, revealing that *SLC29A1* is the dominant isoform. Neither *SLC29A1* nor *SLC29A2* expression changed over the course of placental development, but there was considerable interindividual variability in their expression. Therefore, drug-drug interactions and the effect of interindividual variability in placental ENT1 expression on abacavir disposition into fetal circulation should be further investigated to guarantee safe and effective abacavir-based combination therapies in pregnancy.

Introduction

Mother-to-child transmission is the most common route of HIV infection in children. The risk of vertical HIV transmission can be minimized by perinatal administration of a combination antiretroviral therapy that suppresses viral replication in maternal blood and genital secretions. It has recently been emphasized that combination antiretroviral therapy should include an antiretroviral drug with high placental transfer for pre-exposure prophylaxis of the fetus (http://aidsinfo.nih.gov/contentfiles/lvguidelines/PerinatalGL.pdf).

The functional part of the placental barrier is the polarized multinucleated syncytiotrophoblast, which expresses various carriers, including equilibrative nucleoside transporters (ENTs) (Staud et al., 2012). ENTs are ubiquitously occurring proteins belonging to the solute carrier (SLC) transporter superfamily that mediate bidirectional facilitated diffusion of nucleosides in tissues to maintain nucleoside homeostasis (Molina-Arcas et al., 2009). Beyond their physiologic role, ENTs, especially the two important isoforms ENT1 and ENT2 (Griffiths et al., 1997), affect the pharmacokinetics of a broad array of nucleoside-derived drugs, including nucleoside reverse transcriptase inhibitors (didanosine and zalcitabine), antihepatitis therapeutics (ribavirin and entecavir), or antineoplastic drugs (gemcitabine and cladribine) (Yamamoto et al., 2007; Molina-Arcas et al., 2009; Ma et al., 2017). Both ENT1 (encoded by *SLC29A1*) and ENT2 (encoded by *SLC29A2*) are transcribed in the human syncytiotrophoblast (Govindarajan et al., 2007; Yamamoto et al., 2007; Errasti-Murugarren et al., 2011). ENT1 has been detected in the maternal-facing (apical) microvillous plasma membrane of the syncytiotrophoblast, whereas ENT2 is localized on both the apical and fetal-facing (basal) plasma membrane (Barros et al., 1995; Govindarajan et al., 2007; Errasti-Murugarren et al., 2011). ENT1-like activity has also been observed on the syncytiotrophoblast basal plasma membrane despite undetectable ENT1 expression at this locus (Errasti-Murugarren et al., 2011).

Other than ENTs, placental expression of concentrative nucleoside transporters (CNTs, *SLC28A*) that mediate unidirectional Na^+-dependent

This project was financially supported by the Czech Science Foundation [Grant GACR 17-16169S] and Charles University in Prague [Grants GAUK 324215/C/2015, 812216/C/2016, and SVV/2017/260 414].

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https://doi.org/10.1124/dmd.118.083329.

ABBREVIATIONS: ABCB1, P-glycoprotein; ABCG2, breast cancer resistance protein; BCA, bicinchoninic acid; BeWo, choriocarcinoma-derived cell line; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; F→M, fetal-to-maternal clearance; h, human; M→F, maternal-to-fetal clearance; MVM, microvillous plasma membrane; NBMPR, S^6-(4-nitrobenzyl)mercaptopurine riboside; PCR, polymerase chain reaction; r, rat; SLC, solute carrier.

influx of nucleosides and nucleoside analogs (Molina-Arcas et al., 2009) has also been suggested, although with conflicting results. Whereas Errasti-Murugarren et al. (2011) observed protein expression of CNT1 despite very low mRNA expression, Govindarajan et al. (2007) did not detect protein expression of CNTs in the term placenta. In addition, Barros et al. (1991) demonstrated that there is no effect of Na^+ depletion on nucleoside uptake into microvillous plasma membrane (MVM) vesicles.

The expression of drug transporters in the placenta frequently varies during gestation and differs among individuals (Gil et al., 2005; Mao, 2008; Ahmadimoghaddam et al., 2013). This holds true also for *SLC28A*, exhibiting significant interindividual variability and higher expression in the term placenta compared with the first trimester (Jiraskova et al., 2018); however, such data are lacking for *SLC29A*.

Abacavir is a nucleoside analog belonging to the family of nucleoside reverse-transcriptase inhibitors. It is currently a preferred anti–HIV compound for mother-to-child transmission prevention (http://aidsinfo.nih.gov/contentfiles/lvguidelines/PerinatalGL.pdf). Transplacental transfer of abacavir in humans has been investigated only sparsely, and its cord-to-maternal-blood concentration ratio is variable, ranging from 62% to 163% at term (Chappuy et al., 2004; Best et al., 2006; Fauchet et al., 2014). The pharmacokinetics of antiviral drugs are frequently affected by the activity of drug transporters (Kis et al., 2010; Neumanova et al., 2014, 2015, 2016; Ceckova et al., 2016). Abacavir is a known substrate of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) (Shaik et al., 2007; Giri et al., 2008; Neumanova et al., 2015), and we recently demonstrated that these transporters may limit abacavir maternal-to-fetal transfer (Neumanova et al., 2015). Importantly, abacavir was reported to reduce nucleoside uptake in vitro, raising the possibility that abacavir is also a substrate for ENTs (Hong et al., 2000; Li et al., 2015).

Because a detailed understanding of drug interactions with placental transporters is required to guarantee safe and effective therapy during pregnancy (Staud et al., 2012; Thomas and Yates, 2012; Staud and Ceckova, 2015), we sought to determine whether ENTs play a role in the transplacental transfer of abacavir. We analyzed abacavir uptake into the placental choriocarcinoma-derived cell line BeWo and fresh villous placental fragments and MVM vesicles, both derived from the human term placenta. We then performed in situ dual-perfusion studies in the rat term placenta to quantify the role of ENTs in total transplacental abacavir clearance. In all experimental models, we also considered the potential contribution of CNTs to abacavir placental kinetics. Finally, using a quantitative polymerase chain reaction (PCR) method, we investigated the expression of *SLC29A1* and *SLC29A2* and their inter- and intraindividual variability in the first- and third-trimester human placenta.

Material and Methods

Chemicals and Reagents. The radiolabeled compounds [^{3}H]-abacavir (0.05 or 0.1 Ci/mM), [^{3}H]-adenosine (23 Ci/mM), and [^{3}H]-thymidine (74 Ci/mM) were purchased from Moravek Biochemicals (Brea, CA); adenosine represents a model substrate of ENT1, ENT2, CNT2, and CNT3, whereas thymidine is transported by ENT1, ENT2, CNT1, and CNT3 (Molina-Arcas et al., 2009). The specific ENT inhibitor S^6-(4-nitrobenzyl)mercaptopurine riboside (NBMPR) and the competitive inhibitors of ENTs and CNTs, uridine and adenosine (Molina-Arcas et al., 2009; Errasti-Murugarren et al., 2011), were purchased from Sigma-Aldrich (St. Louis, MO); NBMPR (0.1 μM) selectively inhibits human and rat ENT1/Ent1, whereas a concentration of 100 μM abolishes the activity of both human and rat ENT1/Ent1 and ENT2/Ent2 (Chishu et al., 2008; Sai et al., 2008; Molina-Arcas et al., 2009; Nishimura et al., 2011, 2012; Karbanova et al., 2017). Pentobarbital (Nembutal) was purchased from Abbott Laboratories (Abbott Park, IL). Solvent dimethylsulfoxide was obtained from Sigma-Aldrich, and its volume/volume concentration was 0.1% in all experiments. All other chemicals were of analytical grade. Bicinchoninic acid (BCA) assay reagents were purchased from Thermo Scientific (Rockford, IL), and Bradford Reagents were purchased from Sigma-Aldrich.

Cell Lines. The human choriocarcinoma-derived BeWo cell line was purchased from the European Cell Culture Collection (Salisbury, Wiltshire, UK). Cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (Karbanova et al., 2017). The cells were cultured at 37°C under an atmosphere containing 5% CO_2.

Animals. Pregnant Wistar rats were purchased from MediTox s.r.o. (Konarovice, Czech Republic) and maintained under 12/12-hour day/night standard conditions with water and chow pellets ad libitum. Experiments were performed on day 21 of gestation (counted from the day when copulation plug was found). Overnight-fasted rats were anesthetized by administering a dose of 40 mg of pentobarbital/kg bodyweight (Nembutal; Abbott Laboratories) into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (Charles University in Prague, Prague, Czech Republic) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (2011) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Sample Collection of Human and Rat Placentas. Third-trimester placentas were obtained from uncomplicated pregnancies (n = 14) following elective cesarean section at term (between weeks 38 and 41 of gestation). First-trimester placentas (n = 7) were acquired from voluntary interruption of physiologically ongoing pregnancies between weeks 9 and 13 of gestation as described previously (Ahmadimoghaddam et al., 2013). All participants provided written informed consent. Rat term placentas were collected from five rats on day 21 of pregnancy (n = 5). The samples were frozen in liquid nitrogen immediately after surgery and then stored at −80°C until analysis.

RNA Isolation and Reverse Transcription. Total RNA was isolated from weighed tissue samples or directly from BeWo cells using Tri-Reagent solution purchased from Molecular Research Centre (Cincinnati, OH) according to the manufacturer's instructions. The purity of the isolated RNA was checked by the A260/A280 ratio, and RNA integrity was confirmed by electrophoresis on 1% agarose gel. The concentration of RNA was calculated by A260 measurement. RNA was converted into cDNA using the gb Reverse Transcription Kit from Generi Biotech s.r.o. (Hradec Kralove, Czech Republic) on a T100 Thermal Cycler (Bio-Rad, Hercules, CA).

Qualitative End-Point PCR Analysis. End-point PCR was carried out in BeWo cells and samples of rat/human term placentas to verify expression of target genes in our experimental models. cDNA (25 ng) was amplified in a 20-μl reaction volume using MyTaq Red DNA Polymerase (cat. no. BIO-21108; Bioline, Taunton, MA) according to the manufacturer's instructions using a Bio-Rad T100 Thermal Cycler. For amplification of human *SLC29A1* and *SLC29A2* in human placentas and BeWo cells, we used primers designed by Yamamoto et al. (2007) that provide amplicons of 512 and 470 bp, respectively (Yamamoto et al., 2007). PCR analysis of rat placental samples was performed using 5′-CCAAGAGGAGGAAGAGAGGAATC-3′ and 5′-TAAAGAGGGAGGG-CAGGTAGTG-3′ as the forward and reverse primers, respectively, for *SLC29A1*, and 5′-CCCACAGACACCTTCAACTTCA-3′ and 5′-GTGCTGTAGGTA-GAAGGCATGGT-3′ as the forward and reverse primers for *SLC29A2*, providing amplicons of 400 and 302 bp, respectively. The PCR cycling conditions used were 95°C for 3 minutes followed by 50 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 45 seconds, followed by 10 minutes at 72°C. Amplicons were analyzed on 1.5% agarose gel labeled with GelRed Nucleic Acid Stain (Biotium, Hayward, CA) using the HyperLadderTM 100 bp DNA length marker (Bioline).

Quantitative PCR Analysis. Quantitative PCR analysis of *SLC29A1* and *SLC29A2* expression in rat term placentas, BeWo cells, and first- and third-trimester human placentas was performed using QuantStudio 6 (Thermo Fisher Scientific, Waltham, MA). cDNA (25 ng) prepared from rat tissue and BeWo cells was analyzed in 20-μl reaction volumes in a 96-well plate. Human placental cDNA (10 ng) was amplified in a 384-well plate, with total reaction volumes of 10 μl per well. PCR was performed using the TaqMan Universal Master Mix II without uracil-DNA glycosylase (Thermo Fisher Scientific) and predesigned TaqMan Real Time Expression PCR assays for human (*h*) or rat (*r*) *SLC29A1* (Hs01085704_g1c, Rn01648953_m1) and *SLC29A2* (Hs00155426_m1, Rn01479421_m1). For greater precision during the mRNA quantification of

target genes, data were normalized against the geometric mean of expression of two previously identified TaqMan housekeeping genes: *B2M* (Hs00187842_m1) and *GAPDH* (Hs02758991_g1) for human samples, and *Ywhaz* (Rn00755072_m1) and *Gapdh* (Rn01775763_g1) for rat samples (Ahmadimoghaddam et al., 2013; Cerveny et al., 2016). Stable expression of both reference genes was verified before beginning the quantitative analysis. Each sample was amplified in triplicate using the following PCR cycling profile: 95°C for 3 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. Analyses of amplification efficiency (Pfaffl, 2001) for *SLC29A1* and *SLC29A2* yielded values of ≈2-fold increase per PCR cycle (data not shown), making it possible to compare the expression data for the two genes. Expression levels are reported in arbitrary units and were derived by normalizing the expression of the target gene against that of the reference genes (Radilova et al., 2009).

In Vitro Accumulation Studies in BeWo Cells. For uptake experiments, BeWo cells were seeded at a density of 3.5×10^5 on 24-well culture plates (TPP Techno Plastic Products, Trasadingen, Switzerland) and cultured for 3 days until confluence with daily medium replacement. Uptake experiments were performed as previously described, with modifications (Yamamoto et al., 2007; Karbanova et al., 2017). In brief, experiments were performed in 0.25 ml of control (Na^+-containing) buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM Tris) or Na^+-free buffer (Na^+ was replaced by *N*-methyl-D-glucamine). Two model substrates of nucleoside transporters, [^{3}H]-adenosine (17.4 nM) and [^{3}H]-thymidine (5 nM), were used as positive controls (Molina-Arcas et al., 2009) at a final activity of 0.4 μCi/ml. Time-dependent uptakes of [^{3}H]-adenosine (17.4 nM), [^{3}H]-thymidine (5 nM), and [^{3}H]-abacavir (10 μM) in both Na^+-containing and Na^+-free buffers were assessed. Based on time-course data, the effect of NBMPR (0.1 or 100 μM) or uridine (5 mM) as control inhibitors on [^{3}H]-adenosine (17.4 nM) and [^{3}H]-abacavir (10 μM) accumulation was subsequently evaluated over 5- and 1-minute intervals, respectively. Before the start of the experiment, cells were preincubated for 10 minutes in the respective buffer with or without the aforementioned inhibitors. Accumulation was stopped by quick aspiration of the radioactivity-containing buffer. Then, cells were washed twice with 0.75 ml of buffer containing the appropriate inhibitor, after which the cells were lysed in 0.02% SDS. The intracellular concentrations of radioisotopes were determined and normalized against the protein content (Pierce BCA Protein Assay Kit; Thermo Fisher Scientific).

Ex Vivo Accumulation Studies in Fresh Villous Fragments from Human Placenta. Ex vivo analysis of [^{3}H]-abacavir uptake by the human placenta was performed using the method of accumulation in fresh villous fragments of human placentas at term (Atkinson et al., 2006; Greenwood and Sibley, 2006; Neumanova et al., 2015; Karbanova et al., 2017). In brief, placentas were collected at term after uncomplicated pregnancies (after 38–40 weeks of gestation) from women at St. Mary's Hospital (Manchester, UK) or from the University Hospital (Hradec Kralove, Czech Republic) after receiving the women's written informed consent as approved by the Local Research Ethics Committees (REC 12/NW/0574 and 201006S15P, respectively). Small fragments of villous tissue were dissected within 30 minutes of delivery, appropriately washed in either Na^+-containing Tyrode's buffer (135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 5.6 mM glucose, pH 7.4) or Na^+-free Tyrode's buffer (Na^+ was replaced by choline chloride), and then tied to hooks. Before the experiments, the fragments were stabilized for 30 minutes in a 1:1 mixture of Na^+-containing or Na^+-free Tyrode's buffer and Dulbecco's modified Eagle's medium (4 ml). Initially, we determined the time courses of [^{3}H]-adenosine (0.5 μCi/ml; 21.8 nM), [^{3}H]-thymidine (0.5 μCi/ml; 6 nM), and [^{3}H]-abacavir (0.5 μCi/ml, 5 μM) uptakes in control buffer with/without NBMPR (100 μM) or in the absence of Na^+ to confirm ENT and CNT activity in placental fresh villous fragments. Based on the time-course data, we next investigated the effect of selected inhibitors (NBMPR at 100 and 0.1 μM, or uridine at 5 mM) on [^{3}H]-adenosine and [^{3}H]-abacavir uptake after 5 minutes of incubation. To terminate accumulation and remove extracellularly bound isotope, fragments were vigorously washed twice in an excess of ice-cold Tyrode's buffer containing the appropriate inhibitor for 15 seconds. Villous fragments were subsequently stored in distilled water for 18 hours, and the quantity of radioisotope released was determined. The fragments were then removed from the water and dissolved in 0.3 M NaOH for 10 hours at 37°C, after which fragment protein content was quantified using the Bradford protein assay (Bio-Rad). Finally, the NaOH lysate was analyzed for tissue-bound radioisotopes; samples were excluded from further analysis if the lysate's radioisotope content was above 1% of the amount of tracer added.

Preparation of MVM Vesicles and Uptake Assays. MVM vesicles were used to directly analyze the role of ENTs in [^{3}H]-abacavir transport across the apical plasma membrane of the human syncytiotrophoblast layer. Human placentas were obtained as described in the previous paragraph. MVM vesicles were isolated by Mg^{2+} precipitation and differential centrifugation (Glazier et al., 1988; Ceckova et al., 2016). The final MVM pellet was resuspended in intravesicular buffer (290 mM sucrose, 5 mM HEPES, and 5 mM Tris, pH 7.4), vesiculated by passing 15 times through a 25-gauge needle, stored at 4°C, and used within 3 days of isolation or frozen at −80°C and equilibrated to room temperature on the day of the experiment. Comparable uptake rate in fresh and thawed vesicles was verified before initiation of uptake experiments. The MVM protein concentration was determined using the BCA assay. Optimal purity was confirmed by measuring the enrichment of MVM alkaline phosphatase activity related to the placental homogenate, whereas vesicle orientation was evaluated by comparing specific alkaline phosphatase activity upon addition of detergent, as described previously (Glazier et al., 1988). The alkaline phosphatase enrichment factor was 25.2 ± 6.39 (mean ± S.D., $n = 8$), and percentage of physiologically orientated vesicles was 87.2% ± 8.79%, documenting that potential contamination of the MVM vesicles with basal plasma membrane and/or intracellular membranes was negligible (Glazier et al., 1988; Mahendran et al., 1994; Godfrey et al., 1998; Harrington et al., 1999), and thus, the quality of MVM vesicles was sufficient for functional study of apically localized placental ENTs and CNTs. Uptake of [^{3}H]-adenosine (3.3 μCi/ml; 0.145 μM), [^{3}H]-thymidine (3.3 μCi/ml; 1 μM), and [^{3}H]-abacavir (0.5 μCi/ml; 5 μM) into MVM vesicles was measured at room temperature using a rapid vacuum filtration approach (Glazier and Sibley, 2006). MVM vesicles (10–20 mg protein/ml) were equilibrated to room temperature (21°C–25°C) prior to uptake. Ten microliters of MVM was preincubated for 10 minutes with or without inhibitor (0.1, 100 μM NBMPR; 1 mM uridine; or 1 mM adenosine) in extravesicular buffer (145 mM NaCl, 5 mM HEPES, and 5 mM Tris, pH 7.4; for Na^+-free buffer, KCl was used instead of NaCl). Uptake of [^{3}H]-adenosine, [^{3}H]-thymidine, or [^{3}H]-abacavir was initiated by adding the substrate diluted in extravesicular buffer to the preincubated MVM vesicles. Uptake was halted after defined time points by adding 2 ml of ice-cold stopping buffer (130 mM NaCl, 10 mM Na_2HPO_4, 4.2 mM KCl, 1.2 mM $MgSO_4$, 0.75 mM $CaCl_2$, pH 7.4) with 100 μM NBMPR where appropriate and subsequent filtering through a 0.45-μm mixed cellulose ester filter (MF-Millipore membrane filter HAWP02500; MilliporeSigma, Burlington, MA) under vacuum. Filters were washed with 10 ml of stopping buffer, and the filter-associated radioactivity was determined. No protein controls (in which the MVM vesicle protein was replaced with intravesicular buffer) were analyzed in parallel to determine tracer binding to the filter, which was subtracted from the total vesicle count. Unspecific binding of all three compounds to the plasma membrane was excluded by measuring time zero uptakes, showing comparable values to the no-protein controls.

In Situ Dual Perfusion of the Rat Term Placenta. Transplacental transport of [^{3}H]-abacavir was measured using dually perfused rat term placentas in open- and closed-circuit setups as described previously (Neumanova et al., 2014, 2015, 2016; Ceckova et al., 2016; Karbanova et al., 2017; Cerveny et al., 2018). At the end of each experiment, the placenta was perfused with radioactivity-free buffer for 10 minutes, then excised from the uterine tissue and dissolved in Solvable tissue solubilizer (PerkinElmer, Waltham, MA), after which tissue-bound [^{3}H]-abacavir was determined.

An open-circuit perfusion system was used to assess the effect of NBMPR (0.1 and 100 μM) or uridine (5 mM) on maternal-to-fetal (M→F) and fetal-to-maternal (F→M) clearances of [^{3}H]-abacavir at an activity of 0.06 μCi/ml, corresponding to a concentration of 300 nM. The appropriate inhibitor was added to both reservoirs immediately after successful surgery, and [^{3}H]-abacavir was added to either the maternal (M→F studies) or the fetal (F→M studies) reservoir. After a 5-minute stabilization period, sample collection was started (this experimental time point was designated 0 minutes). Fetal effluent samples were collected at 5-minute intervals and placed in preweighed vials, after which [^{3}H]-abacavir concentration was determined and the transplacental clearance was calculated as described previously (Neumanova et al., 2014, 2015).

A closed-circuit (recirculation) perfusion system was used to further study the involvement of ENTs in transplacental abacavir transport. The maternal and fetal

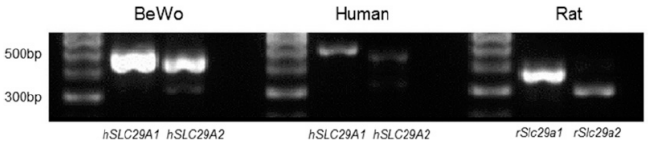

Fig. 1. End-point PCR analysis of *hSLC29A1*/*rSlc29a1* and *hSLC29A2*/*rSlc29a2* mRNA expression in BeWo cells and term human and rat placentas. The expected sizes of the PCR products were 512 bp for *hSLC29A1*, 470 bp for *hSLC29A2*, 400 bp for *rSlc29a1*, and 302 bp for *rSlc29a2*.

sides of the placenta were infused with equal concentrations of [^{3}H]-abacavir (0.06 μCi/ml, 300 nM). NBMPR (0.1 μM) was then added into both compartments, and after a 5-minute stabilization period, the fetal perfusate (10 ml) was recirculated for 60 minutes. Samples (250 μl) were then collected every 10 minutes from the maternal and fetal reservoirs.

Radioisotope Analysis. The concentrations of [^{3}H]-adenosine, [^{3}H]-thymidine, and [^{3}H]-abacavir in experimental samples were measured by liquid scintillation counting (Tri-Carb 2910 TR; PerkinElmer). Tested concentrations of [^{3}H]-adenosine, [^{3}H]-thymidine, and [^{3}H]-abacavir differed among experimental procedures, as the lowest possible concentrations providing sufficient activity measurable in respective experimental systems were used; the low concentrations prevented transporter saturation, guaranteeing method sensitivity. The radioisotopes have been previously used to investigate their interactions with membrane transporters (Pan et al., 2007; Shaik et al., 2007; Chishu et al., 2008; Giri et al., 2008; Neumanova et al., 2015; Karbanova et al., 2017). The radioisotope quantitation of [^{3}H]-adenosine and [^{3}H]-thymidine is complicated by its extensive placental metabolism (Dancis et al., 1993; Acevedo et al., 1995). Since the major metabolites are not transported by ENTs (Osses et al., 1996), it can be assumed that these had a negligible impact on the uptake studies.

Statistical Analyses. Quantitative PCR data were processed using the nonparametric unpaired Mann-Whitney test or the parametric unpaired two-tailed Student's *t* test where appropriate. Results collected from accumulation studies in BeWo cells and dual-perfusion studies on rat placentas were processed by parametric unpaired two-tailed Student's *t* test and one-way analysis of variance after a post hoc Dunnett's multiple comparison test. Uptake studies on fresh villous fragments and vesicles prepared from human placentas were analyzed using the nonparametric unpaired Kruskal-Wallis test following Dunn's multiple comparison test and multiple and nonparametric Wilcoxon matched-pairs signed rank tests, respectively. All statistical calculations were performed with GraphPad Prism 7.04 (GraphPad Software, La Jolla, CA).

Results

End-Point PCR Analysis of *SLC29A1* and *SLC29A2* mRNA Expression in BeWo Cells Human and Rat Term Placentas. We first confirmed the expression of *SLC29A1* and *SLC29A2* mRNA (encoding ENT1 and ENT2, respectively) in BeWo cells and human and rat term placentas. Amplicons specific for the primer pairs used (512 bp for *hSLC29A1* and 470 bp for *hSLC29A2*; 400 bp for *rSlc29a1* and 302 bp for *rSlc29a2*) were detected in all tested samples (Fig. 1).

In Vitro Investigation of Nucleoside Transporters' Role in [^{3}H]-Abacavir Uptake Using BeWo Cells. BeWo cells were used as a simple in vitro model of the placental barrier to investigate potential roles of ENTs and CNTs in the transplacental kinetics of abacavir. We first analyzed time-dependent uptake of [^{3}H]-adenosine (17.4 nM), [^{3}H]-thymidine (5 nM), and [^{3}H]-abacavir (10 μM) (Fig. 2, A–C), showing a significant effect of Na^+ depletion on [^{3}H]-adenosine and [^{3}H]-abacavir.

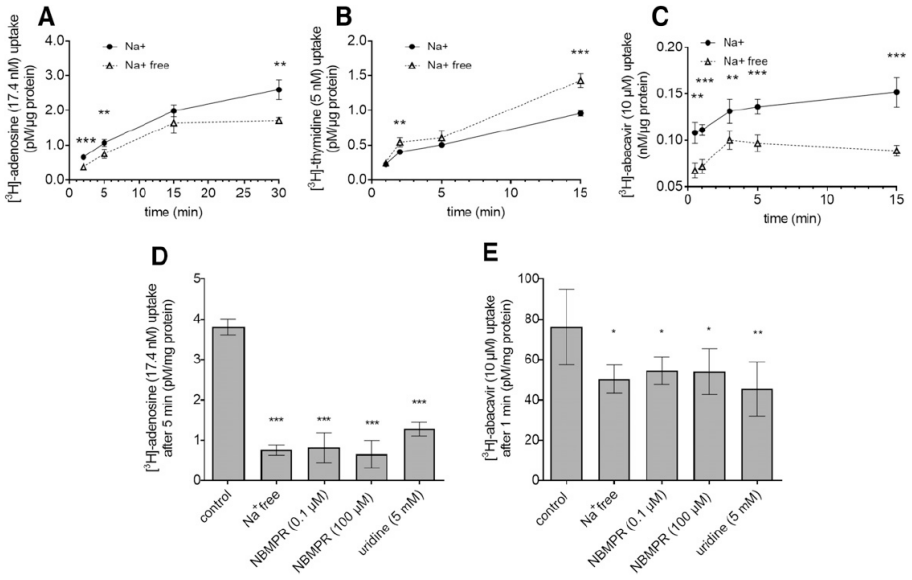

Fig. 2. In vitro accumulation study in choriocarcinoma-derived BeWo cells. Time-dependent uptakes of [^{3}H]-adenosine (17.4 nM) (A), [^{3}H]-thymidine (5 nM) (B), and [^{3}H]-abacavir (10 μM) (C) in control (Na^+-containing) buffer and under Na^+ depletion showed that [^{3}H]-adenosine and [^{3}H]-abacavir, but not [^{3}H]-thymidine, intracellular concentrations were reduced by Na^+ depletion. NBMPR (0.1 and 100 μM), uridine (5 mM), and Na^+ depletion significantly reduced intracellular concentrations of [^{3}H]-adenosine (D) and [^{3}H]-abacavir (E) over 5- and 1-minute accumulations, respectively. Each value is reported in picomolar per microgram protein as the mean ± S.D.; n = 4 for time-dependent uptakes and n = 5 for accumulation at selected time points. The parametric unpaired two-tailed Student's *t* test (A–C) and one-way analysis of variance with Dunnett's post hoc test (D and E) were used to evaluate the difference from control samples. Statistical significance is denoted (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).

Subsequently, NBMPR (0.1 and 100 μM), uridine (5 mM), and Na^+ depletion were shown to reduce [^{3}H]-adenosine uptake over 5 minutes of incubation by more than 70% relative to an uninhibited control (Fig. 2D). These results confirmed the activity of ENT1 and CNTs in BeWo cells. All tested inhibitors affected 1-minute accumulation of [^{3}H]-abacavir in a similar but less-pronounced fashion, reducing its uptake by around 30% (Fig. 2E), suggesting a contribution of ENT1 and other Na^+-dependent transporters, most likely CNTs, to [^{3}H]-abacavir membrane transfer.

Ex Vivo Analysis of Nucleoside Transporters' Role in [^{3}H]-Abacavir Uptake by Human Fresh Villous Placental Fragments. We performed ex vivo accumulation studies with fresh villous fragments isolated from the human placenta to evaluate the role of ENTs and CNTs in abacavir uptake. We observed that the placental fragments exhibited a time-dependent increase in [^{3}H]-adenosine (21.8 nM) and [^{3}H]-thymidine (6 nM) accumulation without any significant effect of Na^+ depletion (Fig. 3, A and B). This suggests no or negligible activity of CNTs in the apical membrane of the term placenta. NBMPR (100 μM) reduced time-dependent accumulation of both model substrates, confirming ENT activity (Fig. 3, A and B). Over a 5-minute [^{3}H]-adenosine accumulation period, the effect of treatment with NBMPR at 0.1 μM was similar to that for treatment at 100 μM (Fig. 3D), indicating that, of the ENTs, ENT1 is a dominant adenosine placental transporter; in contrast, uridine (5 mM) had no significant effect (Fig. 3D).

The accumulation of [^{3}H]-abacavir was also time-dependent, although it was insensitive to both the presence of NBMPR (100 μM) and depletion of Na^+ (Fig. 3C). Moreover, upon a 5-minute incubation period, none of the tested inhibitors revealed a significant effect on [^{3}H]-abacavir uptake into fresh villous fragments after 5 minutes of incubation (Fig. 3E).

Investigation of Nucleoside Transporters' Effects on [^{3}H]-Abacavir Uptake into Human Placental MVM Vesicles. Because of the discrepancies between our in vitro and ex vivo results, we evaluated the role of ENTs and CNTs in [^{3}H]-abacavir uptake directly in MVM vesicles. [^{3}H]-Adenosine (0.145 μM), [^{3}H]-thymidine (1 μM), and [^{3}H]-abacavir (5 μM) showed time-dependent accumulation into MVM vesicles (Fig. 4, A–C). As observed in villous fragments, the uptakes were insensitive to Na^+ depletion, confirming no or negligible functional expression of CNTs in the apical membrane of the term placenta. Addition of NBMPR (0.1 or 100 μM) or uridine (1 mM) caused significant reduction in the 1-minute accumulation of [^{3}H]-adenosine (Fig. 4D), indicating the functional activity of ENTs in MVM vesicles. A similar inhibition pattern was observed in the case of [^{3}H]-abacavir (Fig. 4E) over 1-minute uptake. Furthermore, we tested the inhibitory potential of adenosine (1 mM) in [^{3}H]-abacavir accumulation, revealing an effect comparable to uridine (1 mM). A significant effect of 100 μM NBMPR on [^{3}H]-abacavir uptake into MVM vesicles was also observed over 5- and 10-minute incubations (Fig. 4C).

In Situ Open-Circuit Perfusion of the Rat Term Placenta; Effect of ENTs on Transplacental [^{3}H]-Abacavir Clearance. Because uptake studies performed in vitro and using MVM vesicles had indicated that ENTs may drive placental [^{3}H]-abacavir uptake, we investigated the ability of rat Ent(s) to facilitate abacavir transfer across the placental barrier. Treatment with NBMPR (0.1, 100 μM) or uridine (5 mM)

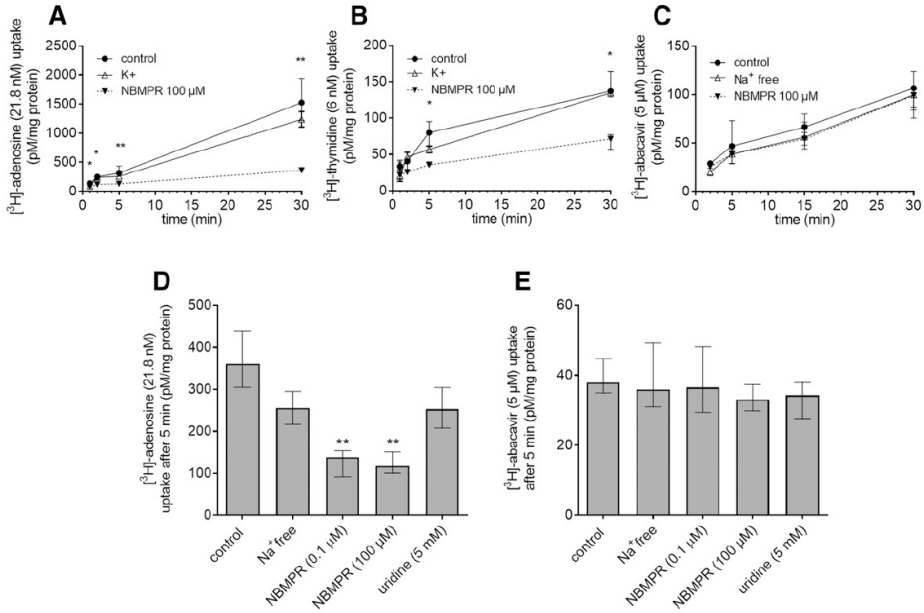

Fig. 3. Ex vivo accumulation studies in human placental fresh villous fragments. Time-dependent uptakes of [^{3}H]-adenosine (21.8 nM) (A), [^{3}H]-thymidine (6 nM) (B), and [^{3}H]-abacavir (5 μM) (C) in control (Na^+-containing) buffer, under Na^+ depletion, or in the presence of NBMPR (100 μM) showed that exposure to NBMPR (100 μM) resulted in decreased intracellular accumulation of [^{3}H]-adenosine and [^{3}H]-thymidine, but not [^{3}H]-abacavir, whereas Na^+ depletion did not exhibit any effect. (D) Over a 5-minute accumulation, uptake of [^{3}H]-adenosine was significantly slowed by the inhibitor NBMPR (0.1 and 100 μM). (E) None of the tested inhibitors significantly affected accumulation of [^{3}H]-abacavir. Data are presented as the median of picomolar per microgram protein with interquartile range; $n \geq 3$ for time-dependent accumulation and $n = 6$ for evaluation of effects of selected nucleoside transporters' inhibitors at 5-minute time point (n = number of placenta donors). Statistical significance was evaluated using the nonparametric Kruskal-Wallis test followed by post hoc Dunn's multiple comparison test. Significant differences relative to the control are labeled (*$P \leq 0.05$; **$P \leq 0.01$).

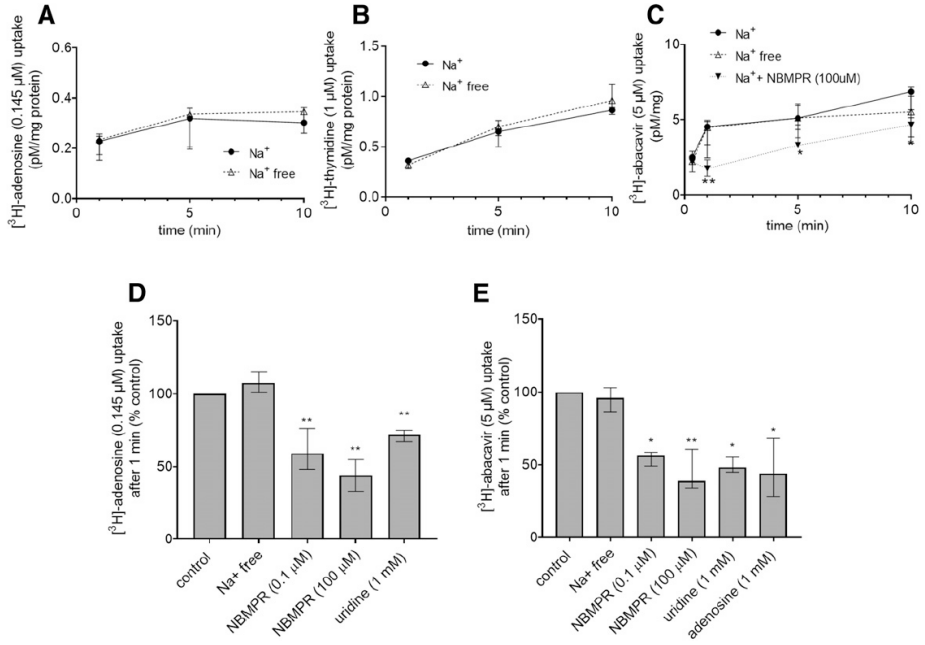

Fig. 4. Ex vivo accumulation studies in human MVM vesicles isolated from human term placentas. Time-dependent uptake studies with [^{3}H]-adenosine (0.145 μM) (A), [^{3}H]-thymidine (1 μM) (B), and [^{3}H]-abacavir (5 μM) (C) in control (Na^+-containing) buffer, under Na^+ depletion, or in the presence of NBMPR (100 μM) showed that Na^+ depletion did not cause any significant effect on [^{3}H]-adenosine, [^{3}H]-thymidine, or [^{3}H]-abacavir, and NBMPR (100 μM) significantly slowed [^{3}H]-abacavir accumulation. One-minute accumulation of [^{3}H]-adenosine (D) and 5 μM [^{3}H]-abacavir (E) was significantly decreased in the presence of NBMPR (0.1 or 100 μM) or uridine (1 mM). Additionally, adenosine (1 mM) showed a significant effect on [^{3}H]-abacavir uptake. The data are presented as the median with interquartile range ($n \geq 3$ for time-dependent uptakes and $n = 9$ for 1-minute accumulation studies). Significance was evaluated by multiple nonparametric Wilcoxon matched-pairs signed rank tests; significant differences relative to the control are denoted ($^*P \leq 0.05$; $^{**}P \leq 0.01$).

caused similar and significant changes in total [^{3}H]-abacavir transplacental clearance, reducing it by ≈50% in both the M→F (Fig. 5A) and F→M (Fig. 5B) directions. This strongly suggests that Ent1 does participate in abacavir transport across the rat term placenta into fetal circulation. In all cases, the proportion of placental tissue-bound [^{3}H]-abacavir was below 1% (data not shown).

In Situ Closed-Circuit Perfusion of the Rat Term Placenta; Effect of NBMPR (0.1 μM) on Fetal-To-Maternal Transport of [^{3}H]-Abacavir at Equilibrium. To further study transplacental abacavir transport, both sides of the placenta were perfused with the concentration of [^{3}H]-abacavir (300 nM) used in the closed-circuit experimental setup, and we analyzed the time course of [^{3}H]-abacavir fetal concentration (Fig. 6A). The fetal-to-maternal concentration ratio after 60 minutes of recirculation was subsequently quantified (Fig. 6B). NBMPR at a concentration of 0.1 μM significantly slowed F→M transport of [^{3}H]-abacavir (Fig. 6A), increasing the fetal-to-maternal concentration ratio (Fig. 6B). This suggests that NBMPR (0.1 μM) might inhibit Ent1- or Ent1-like uptake of [^{3}H]-abacavir on the basal membrane of the syncytiotrophoblast in the rat term placenta, thus abolishing F→M transfer of [^{3}H]-abacavir against the concentration gradient in rats.

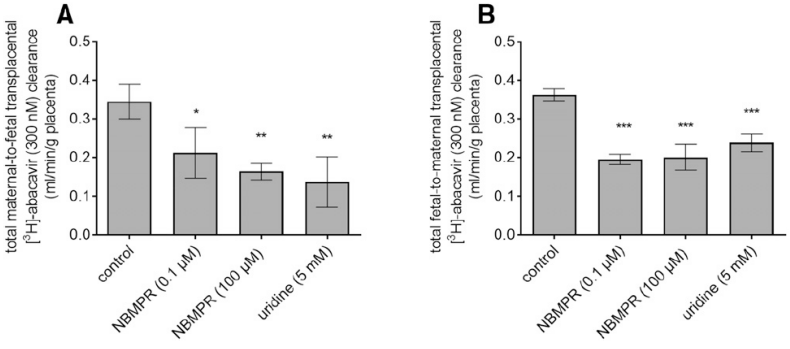

Fig. 5. Open-circuit perfusion experiments; effect of ENTs on transplacental clearance of [^{3}H]-abacavir (0.06 μCi/ml, 300 nM) in the M→F (A) and F→M (B) directions. Both NBMPR (at concentrations of 0.1 or 100 μM) and uridine (5 mM) significantly reduced transplacental [^{3}H]-abacavir clearance in both directions, implying Ent1 facilitates abacavir passage across the placenta. The proportion of [^{3}H]-abacavir bound to placental tissues was below 1% in all cases (data not shown). Data are presented as the mean ± S.D., $n = 4$. Significance was evaluated by one-way analysis of variance followed by post hoc Dunnett's multiple comparison test; statistically significant differences from the control are denoted ($^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$).

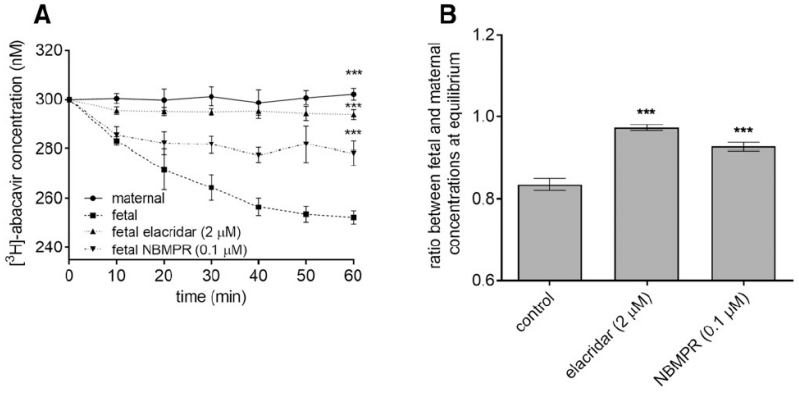

Fig. 6. Closed-circuit perfusion experiments; effect of NBMPR (0.1 μM) on fetal-to-maternal transport of [^{3}H]-abacavir (300 nM) at equilibrium. We analyzed the time course of the fetal [^{3}H]-abacavir concentration (A) and used the fetal-to-maternal concentration ratio to quantify the drug's transport after 60 minutes of recirculation (B). Adding NBMPR (0.1 μM) to both compartments significantly slowed the overall [^{3}H]-abacavir transfer against the concentration gradient in the maternal-to-fetal direction, leading to a significant increase in the fetal-to-maternal concentration ratio after 60 minutes. Results are presented as the mean ± S.D. Significance was evaluated by one-way analysis of variance followed by post hoc Dunnett's multiple comparison test; significant differences relative to the fetal concentration (A) or results for control animals (B) are denoted (***$P \leq 0.001$) and are based on n = 3 measurements in all cases.

Quantitative Reverse-Transcription PCR Analysis of *hSLC29A1/rSlc29a1* and *hSLC29A2/rSlc29a2* Expression in First- and Third-Trimester Human Placentas, Rat Term Placentas, and BeWo Cells. To date, mRNA expression of placental *SLC29A/Slc29a* transporters has been quantitatively assessed only in term placentas of rats (Leazer and Klaassen, 2003; Nishimura et al., 2012), not in BeWo cells or human first-/third-trimester placentas. The normalized expression of *hSLC29A1* in first- and third-trimester human placentas was significantly greater than that of *hSLC29A2* (by a factor of around 30); the level of *hSLC29A2* mRNA was below the limit of detection in two samples of both first- and third-trimester placentas (Fig. 7A). For both *hSLC29A1* and *hSLC29A2*, the level of mRNA in the first-trimester placenta was similar to that in the third. However, both genes exhibited considerable interindividual variability; in the case of *hSLC29A2*, the observed levels varied over two orders of magnitude (Fig. 7A). The expression of *rSlc29a1* in the rat term placentas was 15-fold stronger than that of *rSlc29a2* (Fig. 7B), further confirming the published data, and the expression of *hSLC29A1* in BeWo cells was 40-fold higher than that of *hSLC29A2* (Fig. 7C).

Discussion

To fully assess the safety profile of pharmacotherapy in pregnant women, it is important to understand factors potentially affecting transplacental pharmacokinetics (Brownbill et al., 2016). As abacavir is a nucleoside analog, our study is the first attempt to investigate the role of nucleoside transporters in its transplacental transfer.

We first confirmed the presence of *SLC29A1* and *SLC29A2* mRNA in the BeWo cell line and human/rat term placentas (Fig. 1). Subsequently, we used the accumulation method in BeWo cells, a well established in vitro model for studying drug interactions with placental ABCB1 and ABCG2 transporters (Utoguchi et al., 2000; Ceckova et al., 2006) as well as with SLC transporters (Boumah et al., 1992; Mani et al., 1998; Yamamoto et al., 2007; Nabekura et al., 2015; Karbanova et al., 2017; Ma et al., 2017). The presence of functional ENTs in BeWo cells has previously been confirmed by binding assays with NBMPR and by observing the effect of NBMPR on the accumulation of thymidine (Boumah et al., 1992; Mani et al., 1998; Karbanova et al., 2017). Recently, we have shown that our clone of BeWo expresses *SLC28A2* and lower levels of *SLC28A3* mRNA (Jiraskova et al., 2018). Here, we observed the effect of Na^+ depletion on [^{3}H]-adenosine (CNT2 and CNT3 substrate) but not [^{3}H]-thymidine (CNT1 and CNT3 substrate) (Molina-Arcas et al., 2009) (Fig. 2, A and B). Therefore, we suggest the functional expression of only CNT2 in BeWo cells. As [^{3}H]-abacavir uptake into BeWo cells was sensitive to Na^+ depletion (Fig. 2, C and E), we concluded that CNT2 may contribute to this phenomenon. Subsequently, we used [^{3}H]-adenosine to study the effects of particular

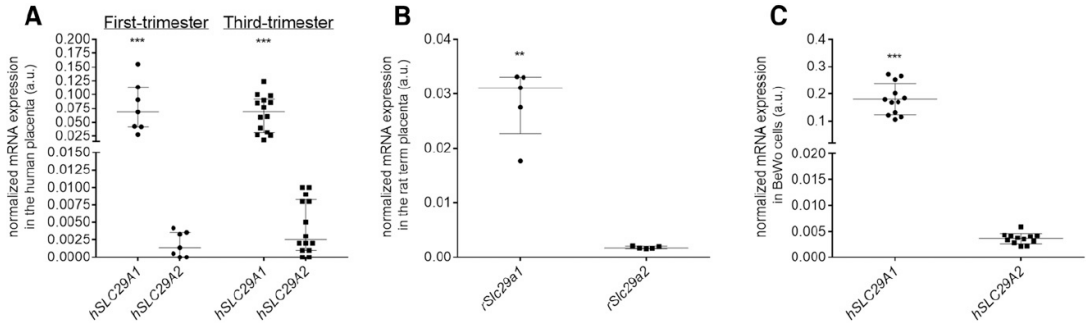

Fig. 7. Quantitative reverse-transcription PCR analysis of *hSLC29A1/rSlc29a1* and *hSLC29A2/rSlc29a2* mRNA expression in first- and third-trimester human placentas (A), rat term placentas (B), and BeWo cells (C). Gene expression levels were normalized against the geometric mean expression of two housekeeping genes: *GAPDH* and *B2M* for human samples, and *Gapdh* and *Ywhaz* for rat samples. Data are reported in arbitrary units (a.u.) as the median with interquartile range (A and B) or mean ± S.D. (C); n = 14 for third-trimester human placentas and BeWo samples, n = 7 for first-trimester human placentas, and n = 5 for rat placentas. Statistical significance for human and rat placenta samples was evaluated using the nonparametric unpaired Mann-Whitney test (**$P \leq 0.01$; ***$P \leq 0.001$). The parametric unpaired two-tailed Student's t test (***$P \leq 0.001$) was used to evaluate significance for BeWo cells.

inhibitors because its structure resembles abacavir more closely than does that of thymidine. NBMPR at both tested concentrations (0.1 and 100 μM) and uridine (5 mM) induced comparable reductions in [^{3}H]-adenosine uptake into BeWo cells (Fig. 2D), indicating that the observed effect was solely due to ENT1. Time-dependent uptake and a similar pattern of NBMPR effects were observed with [^{3}H]-abacavir, suggesting that abacavir uptake into BeWo cells is driven by ENT1 without any detectable contribution of ENT2 (Fig. 2, C and E).

To further study the nucleoside transporter-mediated placental uptake of abacavir, we investigated its accumulation into fresh villous fragments that have been previously used to study human placental amino acid transport (Greenwood and Sibley, 2006) and the interactions of abacavir with ABCB1 and ABCG2 (Neumanova et al., 2015) and zidovudine/emtricitabine with ENTs (Karbanova et al., 2017). We validated this experimental model using [^{3}H]-adenosine and [^{3}H]-thymidine, observing time-dependent uptakes. Significant sensitivity to treatment with 100 μM NBMPR confirmed functional ENT expression; however, we did not see any effect of Na^+ depletion (Fig. 3, A and B). Five-minute experiments with [^{3}H]-adenosine in the presence of NBMPR (0.1 μM) reinforced the conclusion that placental [^{3}H]-adenosine uptake is substantially driven by ENT1 (Fig. 3D). The overall influence of the tested inhibitors on [^{3}H]-abacavir accumulation was negligible (Fig. 3E). Lack of a significant role of ENTs on [^{3}H]-abacavir uptake in villous fragments might be caused by a lower affinity of abacavir to ENT1 compared with adenosine and/or higher lipophilicity (abacavir log P = 1.2 vs. adenosine log P = −1.05), potentially leading to increased uptake into other cell types (e.g., macrophages, endothelium, and/or smooth muscle cells) (Greenwood and Sibley, 2006).

Given the discrepancy between results in villous fragments and BeWo cells, we performed accumulation studies directly in human placenta-derived MVM vesicles (Glazier and Sibley, 2006). This method has previously been used to investigate the role of nucleoside transporters in placental nucleoside uptake (Barros et al., 1991; Errasti-Murugarren et al., 2011). We observed time-dependent [^{3}H]-adenosine, [^{3}H]-thymidine, and [^{3}H]-abacavir uptakes, and by comparing the inhibitory potencies of two NBMPR concentrations (0.1 and 100 μM), we confirmed ENT1-mediated uptake of [^{3}H]-adenosine and [^{3}H]-abacavir by the MVM vesicles (Fig. 4). On the other hand, the uptakes were insensitive to Na^+ depletion (Fig. 4), which is in line with previous findings drawn from studies in human (Barros et al., 1991) and rat MVM vesicles (Nishimura et al., 2012) and our observations in villous fragments (Fig. 3).

The different effects of Na^+ depletion in BeWo cells and ex vivo human placental models can be explained by a three-orders-of-magnitude higher expression of *SLC28A2* (coding for CNT2) in BeWo cells when compared with the human placenta (Jiraskova et al., 2018). Moreover, the lack of CNT effect seems to be in accordance with the observation by Govindarajan et al. (2007), who did not detect any protein expression of CNTs in the trophoblast layer of the term placenta. Because the levels of CNTs increase over the course of gestation (Jiraskova et al., 2018), we do not hypothesize that CNTs are involved in maternal-placental transfer in earlier phases of gestation either.

In the next step, we used an in situ model based on dually perfused rat placentas in open- and closed-circuit setups to evaluate the overall contribution of Ent(s) to transplacental abacavir kinetics at the organ level. Dual perfusion of the rat term placenta is an established and well

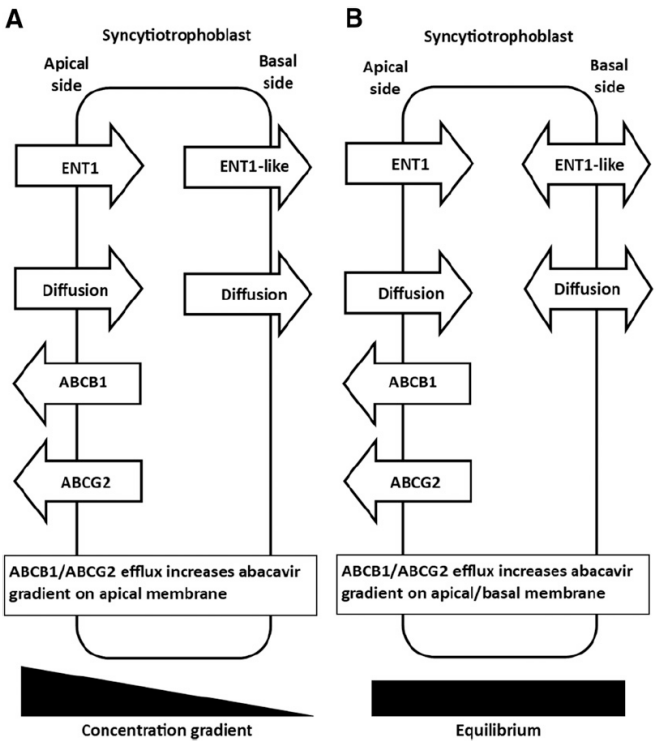

Fig. 8. Hypothesized role of ENT1, ENT1-like, ABCB1, ABCG2, and passive diffusion in the transfer of abacavir across the syncytiotrophoblast when the maternal abacavir concentration is higher than the fetal concentration (A) and at equilibrium (B). In the former case, ENT1 and passive diffusion transfer abacavir unidirectionally from the maternal to the fetal circulation, outweighing the efflux activity of ABCB1 and ABCG2. At equilibrium, ABCB1/ABCG2 activity probably creates transient concentration gradients on both poles of the syncytiotrophoblast that may drive passive abacavir uptake (via passive diffusion and diffusion facilitated by ENT1 on the apical membrane and ENT1-like on the basal membrane) back into the syncytiotrophoblast. However, when the intracellular concentration exceeds the fetal concentration, we assume that ENT1-like- and passive diffusion–mediated transport act in opposite directions. We thus speculate that passive transport of abacavir is unidirectional on the apical membrane but may be bidirectional on the basal membrane.

justified method that has been used to investigate the interactions of placental ABC and SLC transporters with various drugs, including abacavir (Ahmadimoghaddam et al., 2012; Ahmadimoghaddam and Staud, 2013; Neumanova et al., 2014, 2015, 2016; Ceckova et al., 2016). ENTs in the placenta of Wistar rats have been functionally characterized by analyzing adenosine uptake from the maternal blood circulation into the feto-placental unit and into MVM vesicles prepared from the rat placenta (Nishimura et al., 2012). In the open-circuit setup, all tested inhibitors substantially reduced the total clearance of [^{3}H]-abacavir in the M→F and F→M directions in a comparable manner (Fig. 5). These results suggest that ENT1 is an important transporter for more than just placental uptake, and that it also mediates the transport of abacavir into fetal circulation. The effect of Na^+ depletion could not be investigated in this model, as this condition deleteriously affected the rat term placenta, causing edema and high pressure during the experiment. In closed-circuit dual-perfusion experiments, NBMPR (0.1 μM) significantly reduced fetal-to-maternal transport of [^{3}H]-abacavir (Fig. 6). This was surprising because inhibiting apically localized Ent1 should have the opposite effect, further reducing the abacavir concentration in fetal circulation. However, our observation seems to be consistent with previous reports describing Ent1-like activity on the basal membrane of the syncytiotrophoblast (Barros et al., 1995; Govindarajan et al., 2007; Errasti-Murugarren et al., 2011). When considering our previously published data (Neumanova et al., 2015) and the data presented here, we hypothesize cross-talk among placental ABCB1, ABCG2, and ENT1 that might differ at concentration equilibrium and when the maternal abacavir concentration is higher than the fetal concentration (Fig. 8).

In the next step, we quantified the mRNA expression in the placental models/tissues showing that *hSLC29A1* is the dominantly transcribed ENT in BeWo cells and in the first- and third-trimester human placenta (Fig. 7), and we confirmed a previously reported expression profile of *rSlc29a* expression in rat term placenta (Leazer and Klaassen, 2003; Nishimura et al., 2012). Low *hSLC29A2* placental expression may thus hamper the capability of our experimental models to detect abacavir interactions with ENT2. In contrast to findings for other placental transporters (e.g., ABCB1, ABCG2, and CNTs) (Gil et al., 2005; Meyer zu Schwabedissen et al., 2006; Jiraskova et al., 2018), the expression of *hSLC29A1* and *hSLC29A2* did not change in the course of gestation (Fig. 7A). On the other hand, considerable interindividual variability was observed for both genes (Fig. 7A). Because the mRNA seems to correlate with ENT1 function, as evidenced in pharmacoresistance studies (Giovannetti et al., 2006; Marcé et al., 2006; Tsujie et al., 2007; Eto et al., 2013), we hypothesize that the observed placental mRNA variability might be reflected in protein/function level. Therefore, it may represent a potential reason for differences in reported cord-to-maternal blood concentration ratios (Chappuy et al., 2004; Best et al., 2006; Fauchet et al., 2014).

This study provides the first evidence that ENT1 is the dominant placental ENT isoform showing significant uptake of nucleosides, whereas ENT2 and CNTs do not exhibit any activity on the apical side of the syncytiotrophoblast in the term placenta. We also showed for the first time that the expression of *SLC29A1* and *SLC29A2* mRNA is comparable in the first- and third-trimester placenta, although there is substantial interindividual variability in the expression of both genes. Drug-drug interactions (e.g., with suggested substrates of ENT ribavirin) and role of interindividual variability in placental ENT1 expression in drug disposition into fetal circulation of ENT substrates should be further investigated to guarantee safe and effective abacavir-based combination therapies in pregnancy.

Acknowledgments

We thank Dr. Marian Kacerovsky (Department of Obstetrics and Gynecology, University Hospital in Hradec Kralove) for providing us with human placentas, and Martina Hudeckova for help with the human placenta collection and sampling. We also thank Dana Souckova and Renata Exnarova for their skillful assistance with the perfusion experiments.

Authorship Contributions

Participated in research design: Cerveny, Ptackova, Ceckova, Karahoda, Greenwood, Glazier, Staud.

Conducted experiments: Cerveny, Ptackova, Ceckova, Karahoda, Karbanova, Jiraskova.

Performed data analysis: Cerveny, Ptackova, Ceckova, Karahoda, Greenwood, Glazier.

Wrote or contributed to the writing of the manuscript: Cerveny, Ptackova, Ceckova, Karahoda, Greenwood, Glazier, Staud.

References

Acevedo CG, Rojas S, Ramirez M, and Bravo I (1995) Transport and metabolism of adenosine in the perfused human placenta. *Placenta* **16**:611–622.

Ahmadimoghaddam D, Hofman J, Zemankova L, Nachtigal P, Dolezelova E, Cerveny L, Ceckova M, Micuda S, and Staud F (2012) Synchronized activity of organic cation transporter 3 (Oct3/Slc22a3) and multidrug and toxin extrusion 1 (Mate1/Slc47a1) transporter in transplacental passage of MPP+ in rat. *Toxicol Sci* **128**:471–481.

Ahmadimoghaddam D and Staud F (2013) Transfer of metformin across the rat placenta is mediated by organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) protein. *Reprod Toxicol* **39**:17–22.

Ahmadimoghaddam D, Zemankova L, Nachtigal P, Dolezelova E, Neumanova Z, Cerveny L, Ceckova M, Kacerovský M, Micuda S, and Staud F (2013) Organic cation transporter 3 (OCT3/SLC22A3) and multidrug and toxin extrusion 1 (MATE1/SLC47A1) transporter in the placenta and fetal tissues: expression profile and fetus protective role at different stages of gestation. *Biol Reprod* **88**:55.

Atkinson DE, Sibley CP, Fairbairn LJ, and Greenwood SL (2006) MDR1 P-gp expression and activity in intact human placental tissue; upregulation by retroviral transduction. *Placenta* **27**: 707–714.

Barros LF, Bustamante JC, Yudilevich DL, and Jarvis SM (1991) Adenosine transport and nitrobenzylthioinosine binding in human placental membrane vesicles from brush-border and basal sides of the trophoblast. *J Membr Biol* **119**:151–161.

Barros LF, Yudilevich DL, Jarvis SM, Beaumont N, Young JD, and Baldwin SA (1995) Immunolocalisation of nucleoside transporters in human placental trophoblast and endothelial cells: evidence for multiple transporter isoforms. *Pflugers Arch* **429**:394–399.

Best BM, Mirochnick M, Capparelli EV, Stek A, Burchett SK, Holland DT, Read JS, Smith E, Hu C, Spector SA, et al.; PACTG P1026s Study Team (2006) Impact of pregnancy on abacavir pharmacokinetics. *AIDS* **20**:553–560.

Boumah CE, Hogue DL, and Cass CE (1992) Expression of high levels of nitrobenzylthioinosine-sensitive nucleoside transport in cultured human choriocarcinoma (BeWo) cells. *Biochem J* **288**: 987–996.

Brownbill P, Chernyavsky I, Bottalico B, Desoye G, Hansson S, Kenna G, Knudsen LE, Markert UR, Powles-Glover N, Schneider H, et al. (2016) An international network (PlaNet) to evaluate a human placental testing platform for chemicals safety testing in pregnancy. *Reprod Toxicol* **64**: 191–202.

Ceckova M, Libra A, Pavek P, Nachtigal P, Brabec M, Fuchs R, and Staud F (2006) Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo. *Clin Exp Pharmacol Physiol* **33**:58–65.

Ceckova M, Reznicek J, Ptackova Z, Cerveny L, Müller F, Kacerovsky M, Fromm MF, Glazier JD, and Staud F (2016) Role of ABC and solute carrier transporters in the placental transport of lamivudine. *Antimicrob Agents Chemother* **60**:5563–5572.

Cerveny L, Neumanova Z, Karbanova S, Havlova I, and Staud F (2016) Long-term administration of tenofovir or emtricitabine to pregnant rats; effect on Abcb1a, Abcb1b and Abcg2 expression in the placenta and in maternal and fetal organs. *J Pharm Pharmacol* **68**:84–92.

Cerveny L, Ptackova Z, Durisova M, and Staud F (2018) Interactions of protease inhibitors atazanavir and ritonavir with ABCB1, ABCG2, and ABCC2 transporters: effect on transplacental disposition in rats. *Reprod Toxicol* **79**:57–65.

Chappuy H, Tréluyer JM, Jullien V, Dimet J, Rey E, Fouché M, Firtion G, Pons G, and Mandelbrot L (2004) Maternal-fetal transfer and amniotic fluid accumulation of nucleoside analogue reverse transcriptase inhibitors in human immunodeficiency virus-infected pregnant women. *Antimicrob Agents Chemother* **48**:4332–4336.

Chishu T, Sai Y, Nishimura T, Sato K, Kose N, and Nakashima E (2008) Potential of various drugs to inhibit nucleoside uptake in rat syncytiotrophoblast cell line, TR-TBT 18d-1. *Placenta* **29**: 461–467.

Committee for the Update of the Guide for the Care and Use of Laboratory Animals (2011) *Guide for the Care and Use of Laboratory Animals Eighth Edition*. The National Academies Press, Washington D.C., ISBN 978-0-309-15401-7.

Dancis J, Lee J, Mendoza S, and Liebes L (1993) Nucleoside transport by perfused human placenta. *Placenta* **14**:547–554.

Errasti-Murugarren E, Díaz P, Godoy V, Riquelme G, and Pastor-Anglada M (2011) Expression and distribution of nucleoside transporter proteins in the human syncytiotrophoblast. *Mol Pharmacol* **80**:809–817.

Eto K, Kawakami H, Kuwatani M, Kudo T, Abe Y, Kawahata S, Takasawa A, Fukuoka M, Matsuno Y, Asaka M, et al. (2013) Human equilibrative nucleoside transporter 1 and Notch3 can predict gemcitabine effects in patients with unresectable pancreatic cancer. *Br J Cancer* **108**:1488–1494.

Fauchet F, Treluyer JM, Préta LH, Valade E, Pannier E, Urien S, and Hirt D (2014) Population pharmacokinetics of abacavir in pregnant women. *Antimicrob Agents Chemother* **58**:6287–6289.

Gil S, Saura R, Forestier F, and Farinotti R (2005) P-glycoprotein expression of the human placenta during pregnancy. *Placenta* **26**:268–270.

Giovannetti E, Del Tacca M, Mey V, Funel N, Nannizzi S, Ricci S, Orlandini C, Boggi U, Campani D, Del Chiaro M, et al. (2006) Transcription analysis of human equilibrative nucleoside

transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res* **66**:3928–3935.
Giri N, Shaik N, Pan G, Terasaki T, Mukai C, Kitagaki S, Miyakoshi N, and Elmquist WF (2008) Investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) on pharmacokinetics and central nervous system penetration of abacavir and zidovudine in the mouse. *Drug Metab Dispos* **36**:1476–1484.
Glazier JD, Jones CJ, and Sibley CP (1988) Purification and Na+ uptake by human placental microvillus membrane vesicles prepared by three different methods. *Biochim Biophys Acta* **945**: 127–134.
Glazier JD and Sibley CP (2006) In vitro methods for studying human placental amino acid transport: placental plasma membrane vesicles. *Methods Mol Med* **122**:241–252.
Godfrey KM, Matthews N, Glazier J, Jackson A, Wilman C, and Sibley CP (1998) Neutral amino acid uptake by the microvillous plasma membrane of the human placenta is inversely related to fetal size at birth in normal pregnancy. *J Clin Endocrinol Metab* **83**:3320–3326.
Govindarajan R, Bakken AH, Hudkins KL, Lai Y, Casado FJ, Pastor-Anglada M, Tse CM, Hayashi J, and Unadkat JD (2007) In situ hybridization and immunolocalization of concentrative and equilibrative nucleoside transporters in the human intestine, liver, kidneys, and placenta. *Am J Physiol Regul Integr Comp Physiol* **293**:R1809–R1822.
Greenwood SL and Sibley CP (2006) In vitro methods for studying human placental amino acid transport placental villous fragments. *Methods Mol Med* **122**:253–264.
Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, and Baldwin SA (1997) Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. *Biochem J* **328**:739–743.
Harrington B, Glazier J, D'Souza S, and Sibley C (1999) System A amino acid transporter activity in human placental microvillous membrane vesicles in relation to various anthropometric measurements in appropriate and small for gestational age babies. *Pediatr Res* **45**:810–814.
Hong M, Schlichter L, and Bendayan R (2000) A Na(+)-dependent nucleoside transporter in microglia. *J Pharmacol Exp Ther* **292**:366–374.
Jiraskova L, Cerveny L, Karbanova S, Ptackova Z, and Staud F (2018) Expression of concentrative nucleoside transporters (SLC28A) in the human placenta: effects of gestation age and prototype differentiation-affecting agents. *Mol Pharm* **15**:2732–2741.
Karbanova S, Cerveny L, Ceckova M, Ptackova Z, Jiraskova L, Greenwood S, and Staud F (2017) Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine. *Placenta* **60**:86–92.
Kis O, Robillard K, Chan GN, and Bendayan R (2010) The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters. *Trends Pharmacol Sci* **31**:22–35.
Leazer TM and Klaassen CD (2003) The presence of xenobiotic transporters in rat placenta. *Drug Metab Dispos* **31**:153–167.
Li RW, Yang C, Chan SW, Hoi MP, Lee SM, Kwan YW, and Leung GP (2015) Relaxation effect of abacavir on rat basilar arteries. *PLoS One* **10**:e0123043.
Ma Z, Yang X, Jiang T, Bai M, Zheng C, Zeng S, Sun D, and Jiang H (2017) Multiple SLC and ABC transporters contribute to the placental transfer of entecavir. *Drug Metab Dispos* **45**: 269–278.
Mahendran D, Byrne S, Donnai P, D'Souza SW, Glazier JD, Jones CJ, and Sibley CP (1994) Na+ transport, H+ concentration gradient dissipation, and system A amino acid transporter activity in purified microvillous plasma membrane isolated from first-trimester human placenta: comparison with the term microvillous membrane. *Am J Obstet Gynecol* **171**:1534–1540.
Mani RS, Hammond JR, Marjan JM, Graham KA, Young JD, Baldwin SA, and Cass CE (1998) Demonstration of equilibrative nucleoside transporters (hENT1 and hENT2) in nuclear envelopes of cultured human choriocarcinoma (BeWo) cells by functional reconstitution in proteoliposomes. *J Biol Chem* **273**:30818–30825.
Mao Q (2008) BCRP/ABCG2 in the placenta: expression, function and regulation. *Pharm Res* **25**: 1244–1255.
Marcé S, Molina-Arcas M, Villamor N, Casado FJ, Campo E, Pastor-Anglada M, and Colomer D (2006) Expression of human equilibrative nucleoside transporter 1 (hENT1) and its correlation with gemcitabine uptake and cytotoxicity in mantle cell lymphoma. *Haematologica* **91**:895–902.
Meyer zu Schwabedissen HE, Grube M, Dreisbach A, Jedlitschky G, Meissner K, Linnemann K, Fusch C, Ritter CA, Völker U, and Kroemer HK (2006) Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP). *Drug Metab Dispos* **34**:524–533.
Molina-Arcas M, Casado FJ, and Pastor-Anglada M (2009) Nucleoside transporter proteins. *Curr Vasc Pharmacol* **7**:426–434.
Nabekura T, Kawasaki T, Kamiya Y, and Uwai Y (2015) Effects of antiviral drugs on organic anion transport in human placental BeWo cells. *Antimicrob Agents Chemother* **59**:7666–7670.
Neumanova Z, Cerveny L, Ceckova M, and Staud F (2014) Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta. *AIDS* **28**:9–17.
Neumanova Z, Cerveny L, Ceckova M, and Staud F (2016) Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine. *Biopharm Drug Dispos* **37**:28–38.
Neumanova Z, Cerveny L, Greenwood SL, Ceckova M, and Staud F (2015) Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir. *Reprod Toxicol* **57**:176–182.
Nishimura T, Chishu T, Tomi M, Nakamura R, Sato K, Kose N, Sai Y, and Nakashima E (2012) Mechanism of nucleoside uptake in rat placenta and induction of placental CNT2 in experimental diabetes. *Drug Metab Pharmacokinet* **27**:439–446.
Nishimura T, Tanaka J, Tomi M, Seki Y, Kose N, Sai Y, and Nakashima E (2011) Enhancement of zidovudine transfer to molt-4 cells, a human t-cell model, by dehydroepiandrosterone sulfate. *J Pharm Sci* **100**:3959–3967.
Osses N, Pearson JD, Yudilevich DL, and Jarvis SM (1996) Hypoxanthine enters human vascular endothelial cells (ECV 304) via the nitrobenzylthioinosine-insensitive equilibrative nucleoside transporter. *Biochem J* **317**:843–848.
Pan G, Giri N, and Elmquist WF (2007) Abcg2/Bcrp1 mediates the polarized transport of antiretroviral nucleosides abacavir and zidovudine. *Drug Metab Dispos* **35**:1165–1173.
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**:e45.
Radilova H, Libra A, Holasova S, Safarova M, Viskova A, Kunc F, and Buncek M (2009) COX-1 is coupled with mPGES-1 and ABCC4 in human cervix cancer cells. *Mol Cell Biochem* **330**:131–140.
Sai Y, Nishimura T, Shimpo S, Chishu T, Sato K, Kose N, Terasaki T, Mukai C, Kitagaki S, Miyakoshi N, et al. (2008) Characterization of the mechanism of zidovudine uptake by rat conditionally immortalized syncytiotrophoblast cell line TR-TBT. *Pharm Res* **25**:1647–1653.
Shaik N, Giri N, Pan G, and Elmquist WF (2007) P-glycoprotein-mediated active efflux of the anti-HIV1 nucleoside abacavir limits cellular accumulation and brain distribution. *Drug Metab Dispos* **35**:2076–2085.
Staud F and Ceckova M (2015) Regulation of drug transporter expression and function in the placenta. *Expert Opin Drug Metab Toxicol* **11**:533–555.
Staud F, Cerveny L, and Ceckova M (2012) Pharmacotherapy in pregnancy; effect of ABC and SLC transporters on drug transport across the placenta and fetal drug exposure. *J Drug Target* **20**:736–763.
Thomas SH and Yates LM (2012) Prescribing without evidence - pregnancy. *Br J Clin Pharmacol* **74**:691–697.
Tsujie M, Nakamori S, Nakahira S, Takahashi Y, Hayashi N, Okami J, Nagano H, Dono K, Umeshita K, Sakon M, et al. (2007) Human equilibrative nucleoside transporter 1, as a predictor of 5-fluorouracil resistance in human pancreatic cancer. *Anticancer Res* **27** (4B):2241–2249.
Utoguchi N, Chandorkar GA, Avery M, and Audus KL (2000) Functional expression of P-glycoprotein in primary cultures of human cytotrophoblasts and BeWo cells. *Reprod Toxicol* **14**:217–224.
Yamamoto T, Kuniki K, Takekuma Y, Hirano T, Iseki K, and Sugawara M (2007) Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters. *Eur J Pharmacol* **557**:1–8.

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Publikace VI: S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) is not a selective inhibitor of equilibrative nucleoside transporters but also blocks efflux activity of breast cancer resistance protein.

Pharm Res (2020) 37:58
https://doi.org/10.1007/s11095-020-2782-5

RESEARCH PAPER

S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) is Not a Selective Inhibitor of Equilibrative Nucleoside Transporters but Also Blocks Efflux Activity of Breast Cancer Resistance Protein

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Received: 25 November 2019 / Accepted: 10 February 2020

ABSTRACT

Purpose S-(4-Nitrobenzyl)-6-thioinosine (NBMPR) is routinely used at concentrations of 0.10 μM and 0.10 mM to specifically inhibit transport of nucleosides mediated by equilibrative nucleoside transporters 1 (ENT1) and 2 (ENT2), respectively. We recently showed that NBMPR (0.10 mM) might also inhibit placental active efflux of [^{3}H]zidovudine and [^{3}H]tenofovir disoproxil fumarate. Here we test the hypothesis that NBMPR abolishes the activity of P-glycoprotein (ABCB1) and/or breast cancer resistance protein (ABCG2).

Methods We performed accumulation assays with Hoechst 33342 (a model dual substrate of ABCB1 and ABCG2) and bi-directional transport studies with the ABCG2 substrate [^{3}H]glyburide in transduced MDCKII cells, accumulation studies in choriocarcinoma-derived BeWo cells, and *in situ* dual perfusions of rat term placenta with glyburide.

Results NBMPR inhibited Hoechst 33342 accumulation in MDCKII-ABCG2 cells (IC_{50} = 53 μM) but not in MDCKII-ABCB1 and MDCKII-parental cells. NBMPR (0.10 mM) also inhibited bi-directional [^{3}H]glyburide transport across monolayers of MDCKII-ABCG2 cells and blocked ABCG2-mediated [^{3}H]glyburide efflux by rat term placenta *in situ*.

Conclusion NBMPR at a concentration of 0.10 mM abolishes ABCG2 activity. Researchers using NBMPR to evaluate the effect of ENTs on pharmacokinetics must therefore interpret their results carefully if studying compounds that are substrates of both ENTs and ABCG2.

KEY WORDS breast cancer resistance protein · equilibrative nucleoside transporters · inhibition · NBMPR · selectivity

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Published online: 21 February 2020

ABBREVIATIONS

AB	Apical-to-basolateral direction
ABC	ATP-binding cassette
ABCB1	P-glycoprotein
ABCG2	Breast cancer resistance protein
BA	Basolateral-to-apical direction
DMSO	Dimethyl sulfoxide
ENT	Equilibrative nucleoside transporter
MDCKII cells	Madin-Darby canine kidney cells II;
NBMPR	S-(4-Nitrobenzyl)-6-thioinosine
NBTI	S-(4-Nitrobenzyl)-6-thioinosine
SLC	Solute carrier
Tenofovir DF	Tenofovir disoproxil fumarate

INTRODUCTION

The purine nucleoside analogue S-(4-Nitrobenzyl)-6-thioinosine (NBMPR; NBTI) was reported in 1972 as a specific inhibitor of equilibrative nucleoside transporters (ENTs; *SLC29A*). Since then, it has been used in hundreds of studies on the functional activity of ENTs in experimental physiology and pharmacology (e.g. (1–9).

ENTs are members of nucleoside transporter family; four transporters in this class (ENT1–4) have been described to date (10). ENT1–3 are ubiquitous proteins belonging to the solute carrier (SLC) transporter superfamily that mediate bi-directional facilitated diffusion of nucleosides in tissues to maintain nucleoside homeostasis (11–13). In addition to their physiological role, they (especially ENT1 and ENT2) affect the pharmacokinetics of nucleoside-derived anti(retro)viral and antitumor agents (4,8,10,14). ENT4 is a pH-dependent, evolutionarily divergent SLC transporter that transports adenosine and organic cations, and is known as the plasma membrane monoamine transporter (15).

ENT1–3 can be functionally distinguished based on their sensitivity to NBMPR: ENT1 is the "NBMPR-sensitive" transporter and is inhibited by NBMPR at nanomolar concentrations. ENT2, the "NBMPR-insensitive" transporter, is inhibited by micromolar concentrations of NBMPR, while ENT3 is unaffected by NBMPR (10). NBMPR inhibits human, murine, and rat orthologues of ENTs and is experimentally used at concentrations of 0.10 μM and 0.10 mM (3,4,9,16–21). If treatment with 0.10 μM NBMPR reduces the cellular uptake of nucleosides, this outcome is attributed to specific inhibition of ENT1. Any further reduction in nucleoside uptake induced by 0.10 mM NBMPR is attributed to ENT2 inhibition (3–5,14,18).

We recently investigated the roles of ENTs (3,4,14) and ATP-binding cassette (ABC) transporters, P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) (4,22–25), in the placental pharmacokinetics of nucleoside-derived antivirals. Unexpectedly, we observed that 0.10 mM NBMPR blocks active fetal-to-maternal transport of zidovudine and tenofovir disoproxil fumarate (tenofovir DF). Because both zidovudine and tenofovir DF are substrates of placental ABCB1 and ABCG2 (23,24), we hypothesized that NBMPR inhibits the protective/efflux activity of ABCB1 and/or ABCG2 in the placenta.

Here, we present *in vitro* accumulation and transport studies and *in situ* experiments using dually perfused rat placenta conducted to assess the inhibitory effect of NBMPR on the efflux activity of ABCB1 and ABCG2 transporters.

MATERIALS AND METHODS

Chemicals

The inhibitor NBMPR, the ABCB1 and ABCG2 substrate Hoechst 33342, dimethyl sulfoxide (DMSO), and cell culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). The model ABCB1 inhibitor LY335979 was obtained from Toronto Research Chemicals (North York, ON, Canada), the ABCG2 inhibitor Ko143 (26) was obtained from Enzo Life Sciences (Farmingdale, NY, USA), and the competitive nucleoside transporter inhibitor uridine (10) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Opti-MEM was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Radiolabeled [^{3}H]glyburide (activity 8.5 Ci/mmol), a model substrate of ABCG2 (27), was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). The radiolabeled model nucleoside transporter substrate [^{3}H]adenosine (activity 23 Ci/mmol) (10,14) and the radiolabeled antiretrovirals [^{3}H]zidovudine (activity 6.7 Ci/mmol) and [^{3}H]tenofovir DF (activities 3.8 and 1.2 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA, USA). The scintillation solution for radioisotope detection was supplied by Sigma-Aldrich (St. Louis, MO, USA).

Cell Lines

Madin-Darby canine kidney (MDCKII) cells II transfected for expression of human ABCB1 and human ABCG2, as well as MDCKII-parental cells, were obtained from Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). MDCKII cells were grown in complete Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). Cells of the human choriocarcinoma line BeWo were obtained from the European Cell Culture Collection (ECACC; Salisbury, UK), and were grown in Ham's F-12 medium (Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum. Cells were routinely cultured in antibiotic-free media and incubated in a humidified incubator at 37°C in an atmosphere containing 5.0% CO_2. All experiments were performed using cells from passages 5 to 25. DMSO was used as the solvent for the compounds tested in the cellular experiments, at concentrations not exceeding 0.20%. DMSO exhibited no undesirable effects when applied at this concentration in control experiments.

Animals

Pregnant Wistar rats were obtained from Meditox s.r.o. (Konarovice, Czech Republic) and Velaz (Prague, Czech Republic). They were maintained under standard conditions (12-h/12-h day/night, water and pellets *ad libitum*). Experiments were performed on the 21st day of gestation. Fasted rats were anesthetized by i.v. administration of 40 mg/kg pentobarbital into the tail vein. All experiments were approved by the Ethical Committee of the Faculty of Pharmacy in Hradec Kralove (MSMT-4312/2015–8; Charles University, Czech Republic) and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (1996) (28) and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (29).

Hoechst 33342 Accumulation Assays

To study the inhibitory potential of NBMPR towards ABCG2 and ABCB1, a Hoechst 33342 (8.0 μM) accumulation assay was performed in MDCKII cells as described previously (26). The cells were preincubated over 15 min in Opti-MEM medium containing NBMPR at concentrations ranging from 0.10 μM (the standard concentration used to inhibit ENT1) to 0.20 mM (twice the concentration typically used to inhibit both ENT1 and ENT2) and then incubated over 30 min in

the presence of both Hoechst 33342 and NBMPR at respective concentration. The effect of NBMPR was also compared to that of the specific ABCG2 inhibitor Ko143 (2.0 μM). Additional assays were performed using MDCKII-parental cells to evaluate the effect of endogenous canine transporters in the MDCKII line.

Bi-Directional Transport across MDCKII-ABCG2 Monolayers

Based on the results of the accumulation assays, the movement of the ABCG2 substrate [^{3}H]glyburide across monolayers of MDCKII-ABCG2 cells grown on semipermeable membranes was measured using a modified variant of a previously reported protocol (26). Cells (6.0×10^5) were seeded and cultured for 72 h on microporous membrane inserts (3402, 3.0 μm pores, 12 mm diameter, Costar, Cambridge, MA, USA) until a confluent monolayer was established. The monolayer was then pre-incubated for 30 min with 0.10 mM NBMPR or 2.0 μM Ko143 in Opti-MEM medium, after which the experiment was initiated by adding 22 nM [^{3}H]glyburide (corresponding to a final activity of 0.12 μCi) to the donor compartments. Samples (50 μl) were then collected from the opposite compartments after 1, 2, and 4 h. Because [^{3}H]glyburide has also been suggested to be a weak ABCB1 substrate (30,31), LY335979 (1.0 μM) was added to the experimental media to abolish the activity of canine ABCB1. The medium was immediately removed at the end of the incubation period, and the cells were washed twice with ice-cold PBS. The inserts were then excised and the cells were lysed in 0.020% SDS to determine the level of intracellular radioactivity. Leakage of FITC-dextran (0.50 mg/ml) (Sigma Aldrich, St. Louis, MO, USA) was analyzed over the course of the transport experiments; leakage rates of up to 1.0% per hour were considered acceptable. The concentration of [^{3}H]glyburide was determined and the ratio (r) between basolateral-to-apical (BA) and apical-to-basolateral (AB) transport after 4 h was calculated in the presence and absence of the inhibitors, as described previously (16,23–26).

In Vitro Effects of NT Inhibitors on [^{3}H]Tenofovir DF Uptake by BeWo Cells

Uptake experiments using [^{3}H]tenofovir DF were performed as described previously (3,4,8,14). Briefly, sets of cells were pre-incubated for 10 min in Na^+-containing buffer at 37°C, with or without an inhibitor (0.10 mM NBMPR or 5.0 mM uridine), or in Na^+-free buffer (3,4,14,32). Each set was then incubated in 0.25 ml of buffer containing [^{3}H]tenofovir DF (0.26 μM; final activity of radioisotopes 1.0 μCi/ml) under otherwise identical conditions. [^{3}H]tenofovir DF uptake was halted after 5 min by rapidly aspiring the radioactivity-containing buffer, washing twice with 0.75 ml portions of the pre-incubation buffer (with or without inhibitor), then lysing the cells in 0.020% SDS. [^{3}H]adenosine at a concentration corresponding to a final activity of 0.40 μCi (18 nM) was used to evaluate the activity of nucleoside transporters in BeWo cells, as described previously (3,14).

In Situ Dual Perfusion of the Rat Placenta in a Closed Circuit Setup

Both maternal and fetal sides of the placenta were infused with non-saturating concentrations of [^{3}H]glyburide (7.0 nM), [^{3}H]zidovudine (9.0 nM), or [^{3}H]tenofovir DF (50 nM) (in all cases, the radioisotope activity was 0.060 μCi/ml) and after a brief stabilization period, the fetal perfusate (10 ml) was recirculated for 60 min. Samples (0.10 ml) were collected every 10 min from the maternal and fetal reservoirs, and the concentrations of the tested compounds were measured. This experimental setup ensures a steady concentration on the maternal side of the placenta and enables measurement of changes in analyte concentrations in the fetal circulation. The extent of substrate transfer across the placenta can then be quantified in terms of the fetal/maternal ratio (calculated after 60 min recirculation); any net transfer of the substrate from one side to the other implies transport against a concentration gradient and thus provides evidence of active transport.

Radioisotope Analyses

We quantified radioisotopes in experimental samples by liquid scintillation counting using a Tri-Carb 2910 TR instrument (PerkinElmer, Waltham, MA, USA), as described previously (3,4,14,16,23–25). The concentrations of radioisotopes used in the tests depended on the experimental system; in all cases, the lowest concentration providing sufficient measurable activity was used to minimize the risk of transporter saturation and maximize the method's sensitivity.

Statistical Analyses

All data are presented as means ± SD from at least three independent experiments. Statistical analyses were performed using GraphPad Prism software version 8.2.1 (GraphPad Software Inc., La Jolla, CA, USA). The probability of the null hypothesis (P) was calculated using Student's t test or one-way *ANOVA* followed by Dunnett's multiple comparisons test or Turkey's multiple comparisons test, and results were considered significant if $P < 0.05$.

RESULTS

NBMPR (0.10 mM) Inhibits Active Fetal-to-Maternal Transport of the Nucleoside-Derived Antivirals [^{3}H] Zidovudine and [^{3}H]Tenofovir DF

Dual perfusion of rat term placenta in a closed circuit setup (with identical maternal and fetal concentrations at the start of the experiment) is a method for measuring the contributions of active membrane transporters to placental pharmacokinetics. Changes in drug concentrations in the fetal perfusate can be attributed to the involvement of active transporters in the placental net transfer; if all such transporters are inhibited, the ratio of fetal and to maternal concentrations (F/M) will be approximately one. NBMPR (0.10 mM) increased the F/M ratio of [^{3}H]tenofovir DF (50 nM) from 0.50 to 0.90, while F/M ratio of [^{3}H]zidovudine (9.0 nM) was raised from 0.70 to 1.0 (Fig. 1).

Inhibitors of Nucleoside Transporters Did Not Affect [^{3}H]Tenofovir DF Uptake by BeWo Cells

NBMPR may block vectorial fetal-to-maternal transfer of nucleoside-derived drugs controlled by ENTs in the fetus-facing (basal) membrane and/or ABC efflux transporters in the mother-facing (apical) membrane (14). Zidovudine is not transported by ENTs (3,19,20), but it is a proposed substrate of other transporters (33) including ABCB1 and ABCG2 (24). So, it can be hypothesized that decreased fetal-to-maternal transport in the presence of NBMPR (0.10 mM) can be caused by inhibition of placental ABCB1 and/or ABCG2. However, the interactions of nucleoside transporters with tenofovir DF have not previously been investigated. We therefore performed accumulation studies using radiolabeled tenofovir DF in the BeWo cell line (3,4,8,14). After 5 min incubations at 37°C, neither NBMPR (0.10 mM) nor uridine (5.0 mM) significantly reduced the uptake of [^{3}H]tenofovir DF (Fig. 2), indicating that ENTs do not significantly facilitate uptake of this compound by BeWo cells. To evaluate the potential role of influx concentrative nucleoside transporters in the net transfer of [^{3}H]tenofovir DF, we also measured its uptake under Na^+-free conditions (14). The absence of Na^+ had no effect on [^{3}H]tenofovir DF uptake by BeWo cells, indicating that uptake of this compound is not mediated by concentrative nucleoside transporters. Experiments were also performed using the general nucleoside transporter substrate [^{3}H]adenosine (17 nM) as a positive control. [^{3}H]adenosine uptake was significantly sensitive to all of the tested treatments, demonstrating that ENT1, ENT2, and concentrative nucleoside transporter 2 were all expressed and functional in the BeWo cells (Fig. 2) (14).

NBMPR Enhances Hoechst 33342 Accumulation in MDCKII-ABCG2 Cells

We performed a Hoechst 33342 accumulation assay to test the inhibition of ABCB1 and ABCG2 by NBMPR. No significant differences in Hoechst 33342 accumulation were observed in the control MDCKII-parental cell line, suggesting that its transport is not influenced by any endogenous transporters that might be affected by NBMPR or the model inhibitors Ko143 (2.0 μM) and LY335979 (1.0 μM) (Fig. 3a). Changes in Hoechst 33342 accumulation were also not observed in MDCKII-ABCB1 cells exposed to increasing concentrations of NBMPR (Fig. 3b), but the ABCB1 inhibitor LY335979 (1.0 μM) had significant effects. It can therefore be concluded that NBMPR does not inhibit ABCB1. In MDCKII-ABCG2 cells, NBMPR concentrations above 50 μM caused significant increases in Hoechst 33342 accumulation. NBMPR at a concentration of 0.10 mM inhibited ABCG2 by 69% (Fig. 3c; the level of inhibition in this case is expressed as a percentage of that induced by treatment with 2.0 μM Ko143). The calculated IC_{50} for NBMPR was 53 μM (Fig. 3d). These data indicate that NBMPR is a potent ABCG2 inhibitor at concentrations routinely used for specific inhibition of ENT2.

NBMPR Abolishes Transport of [^{3}H]Glyburide across MDCKII-ABCG2 Monolayers

To further validate the results of the accumulation assays, we tested the transport of the reported preferential ABCG2 substrate [^{3}H]glyburide (22 nM) (27) across MDCKII-ABCG2 monolayers. The transport ratio (*r*) of [^{3}H]glyburide after 4 h was 2.6 (Fig. 4). Adding the model ABCG2 inhibitor Ko143 (2.0 μM) reduced *r* to 1.1, indicating that active [^{3}H]glyburide transport was fully inhibited (Fig. 4a, c). The application of NBMPR (0.10 mM) also significantly reduced *r*, causing it to fall to 1.3 (Fig. 4b, c). However, the inhibitory effect of NBMPR was clearly weaker than that of Ko143 (2.0 μM) (Fig. 4c).

NBMPR Limits the Active Fetal-to-Maternal Efflux of [^{3}H]Glyburide in Closed Circuit Perfused Rat Placenta

To determine whether the results obtained using *in vitro* methods are consistent with organ-level outcomes, we performed *in situ* experiments using dually perfused rat term placentas in a closed circuit setup. In this system, [^{3}H]glyburide was transported against the concentration gradient in the fetal-to-maternal direction, causing its concentration in the fetal perfusate to fall from 7.0 nM at the start of the experiment to 4.9 nM after 60 min (Fig. 5a). 0.10 mM NBMPR significantly inhibited this active fetal-to-maternal transfer of [^{3}H]glyburide across the rat term placenta (Fig. 5b).

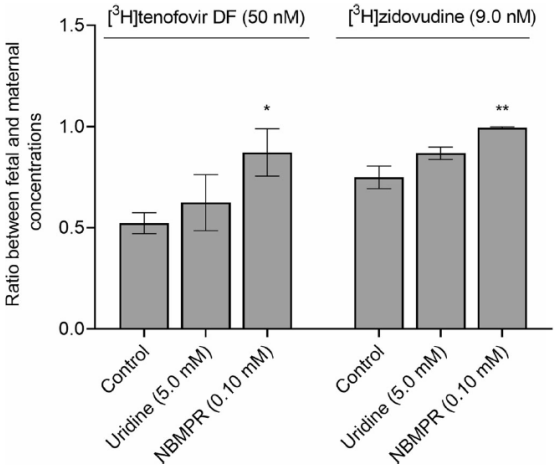

Fig. 1 Effect of NBMPR on fetal-to-maternal active transport of the known ABCB1/ABCG2 substrates [^{3}H]zidovudine and [^{3}H]tenofovir DF. NBMPR (0.10 mM) increased the fetal-to-maternal (F/M) concentration ratio after 60 min recirculation of the fetal perfusate whereas uridine (5.0 mM) had no detectable effect. Columns represent means ± SD from three independent experiments. Statistical significance was evaluated using one-way *ANOVA* followed by Dunnett's multiple comparisons test; values differing significantly from controls are labeled * ($P < 0.05$) or ** ($P < 0.01$).

DISCUSSION

We recently showed that maternal-to-fetal and fetal-to-maternal transfer of zidovudine across the rat term placenta is not mediated by ABCB1/ABCG2 and ENTs when a concentration gradient exists (3,24). Conversely, ABCB1/ABCG2 mediated active zidovudine transport from the fetal compartment under conditions of concentration equilibrium (24). In addition, we demonstrated that the placental kinetics of tenofovir DF are significantly controlled by ABCB1/ABCG2 under both concentration gradient and equilibrium conditions (23). In a parallel study, 0.10 mM NBMPR was surprisingly found to inhibit active fetal-to-maternal transfer of both [^{3}H]zidovudine and [^{3}H]tenofovir DF (Fig. 1). The inhibitory effect of NBMPR (0.10 mM) was comparable to that caused by a model dual ABCB1/ABCG2 inhibitor elacridar. Therefore, we hypothesized that NBMPR inhibits ABCB1 and/or ABCG2.

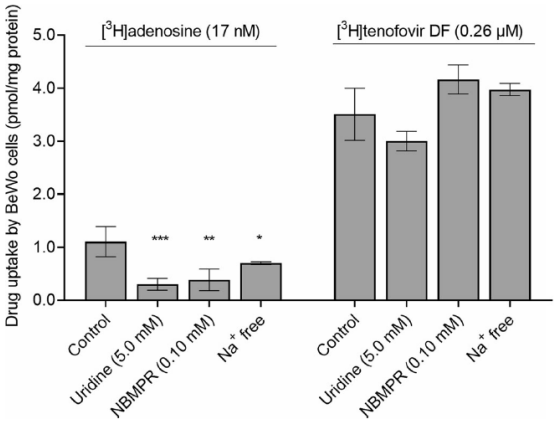

Fig. 2 Effect of NBMPR, uridine, and Na^+-free conditions on [^{3}H]tenofovir DF uptake by BeWo cells. Neither NBMPR (0.10 mM), uridine (5.0 mM), nor Na^+ depletion affected the uptake of [^{3}H]tenofovir DF (0.26 μM) by BeWo cells over 5 min. [^{3}H]adenosine (17 nM) was used as a positive control. Columns represent means ± SD from three independent experiments. Statistical significance was evaluated using one-way *ANOVA* followed by Dunnett's multiple comparisons test; values differing significantly from controls are labeled * ($P < 0.05$); ** ($P < 0.01$), or *** ($P < 0.001$).

The *in situ* experimental model system based on dually perfused Wistar rat placentas in a closed-circuit setup is an established and thoroughly validated method for evaluating drug interactions with placental ABC and SLC transporters (3,4,14,16,22–25). The observed effect of NBMPR (0.10 mM) on [^{3}H]zidovudine and [^{3}H]tenofovir DF placental transfer could be due to inhibition of ABCB1/ABCG2-mediated efflux on the maternal side or a reduction in ENT-mediated influx on the fetal side (16). However, because placental ENTs do not transport zidovudine (3,19,20), we believe the effect of NBMPR (0.10 mM) is due to ABCB1/ABCG2 inhibition. To our knowledge, the interactions of tenofovir DF with nucleoside transporters have not been studied previously. Here, we show that both NBMPR and uridine (an inhibitor of all nucleoside transporter types) does not reduce the accumulation of [^{3}H]tenofovir DF in an *in vitro* model based on BeWo cells with high ENT expression (3,4,8,14,32,34). BeWo cells also functionally express ABCG2 transporter (35). The inhibitory effect of NBMPR on [^{3}H]tenofovir DF in BeWo cells was not detected likely due to short incubation period (5 min) that was selected based on previously published protocols used to evaluate transport activity of nucleoside transporters (3,4,8,14). Longer incubation periods (> 30 min) are required to investigate potency of drugs to abolish activity of ABC transporters in uptake studies (26,36–39) Additionally, the uptake of [^{3}H]tenofovir DF was not affected by treatment with Na^+ depletion in the experimental media, suggesting that concentrative nucleoside transporters do not contribute to placental tenofovir DF uptake (14,32). This indicates that tenofovir DF is not transported via ENTs. We also performed accumulation assays with [^{3}H]tenofovir DF at 4.0°C to inhibit active influx transporters, including concentrative nucleoside transporters, whose enhanced activity may counteract inhibited ENTs (4). This did not affect the intracellular concentration of [^{3}H]tenofovir DF (data not shown) concluding that there are likely no active influx mechanisms mediating tenofovir DF uptake by BeWo cells. These results suggest that

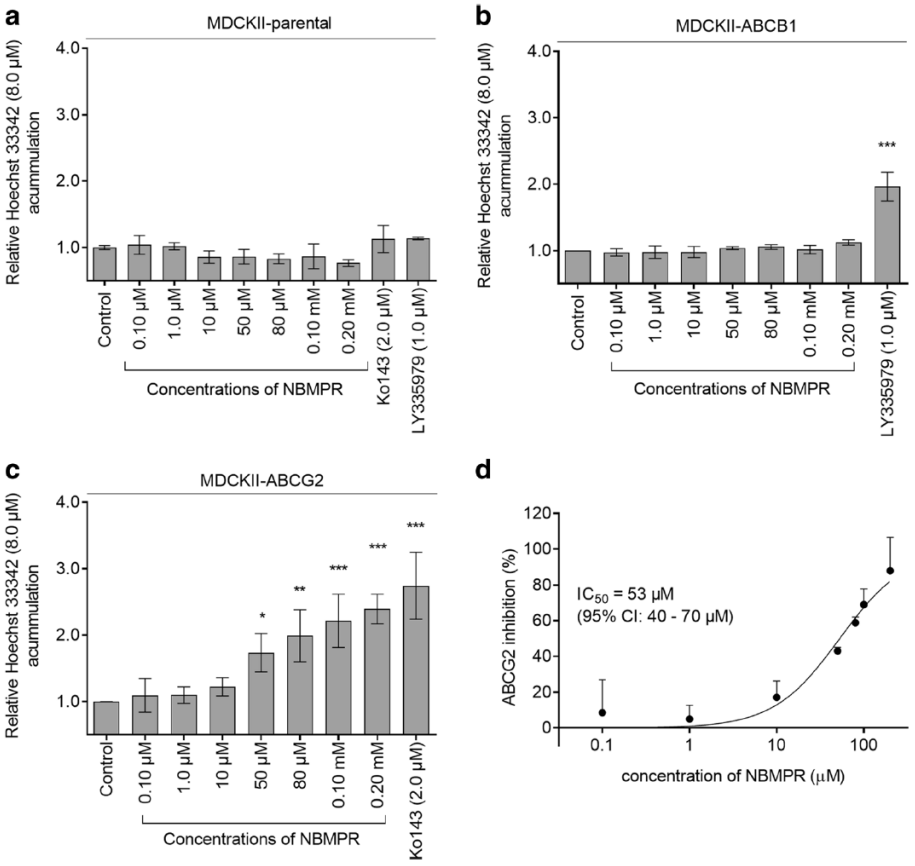

Fig. 3 The effect of NBMPR on accumulation of the dual ABCB1/ABCG2 substrate Hoechst 33342 in MDCKII-parental (**a**), MDCKII-ABCB1 (**b**), and MDCKII-ABCG2 (**c**) cells. Ko143 (2.0 μM) and LY335979 (1.0 μM) were used as positive control inhibitors of ABCG2 and ABCB1, respectively. Columns represent means ± SD from at least three independent experiments. The data were statistically analyzed by one-way *ANOVA* followed by Dunnett's multiple comparisons test. Differences were considered significant when $P < 0.05$; *, **, and *** indicate values that differ significantly from the corresponding controls at the $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels, respectively. The IC_{50} for ABCG2 inhibition by NBMPR was calculated using non-linear regression analysis with sigmoidal Hill kinetics; treatment with 2.0 μM Ko143 was assumed to cause 100% inhibition (**d**).

NBMPR inhibits either ABCB1/ABCG2 in the apical membrane of the syncytiotrophoblast, or as-yet unknown influx mechanisms in the basal membrane, or both. We therefore investigated its potential to inhibit ABCB1 and ABCG2.

In line with studies on the ability of antitumor drugs to reverse multidrug resistance mediated by efflux ABC transporter activity in tumor tissue (26,37,40) we performed an accumulation study using Hoechst 33342 – a fluorescent model dual substrate of ABCB1/ABCG2 – in MDCKII-ABCB1, MDCKII-ABCG2, and MDCKII-parental cells. We showed that NBMPR significantly increased Hoechst 33342 accumulation only in MDCKII-ABCG2 cells, with a relative IC_{50} value of 53 µM (Fig. 3). To confirm this finding, we evaluated the ability of NBMPR (0.10 mM) to inhibit bi-directional transport of [^{3}H]glyburide, an antidiabetic drug that was previously identified as a preferential ABCG2 substrate (39) and was tested at non-saturating concentration of 22 nM. In keeping with the report of Zhou *et al.* (2008) (39), we observed a transport ratio (*r*) of 2.6. The asymmetry in AB and BA transport was fully abolished by the specific ABCG2 inhibitor Ko143 (2.0 µM) and by NBMPR at 0.10 mM. However, Ko143 (2.0 µM) was the stronger inhibitor (Fig. 4). These findings demonstrate that NBMPR (0.10 mM) inhibits ABCG2 *in vitro*.

A dual perfusion study with [^{3}H]glyburide (7.0 nM) was performed to test the potential of NBMPR (0.10 mM) to inhibit ABCG2-controlled active transfer at the organ level. Cygalova *et al.* (2009) demonstrated that fumitremorgin C

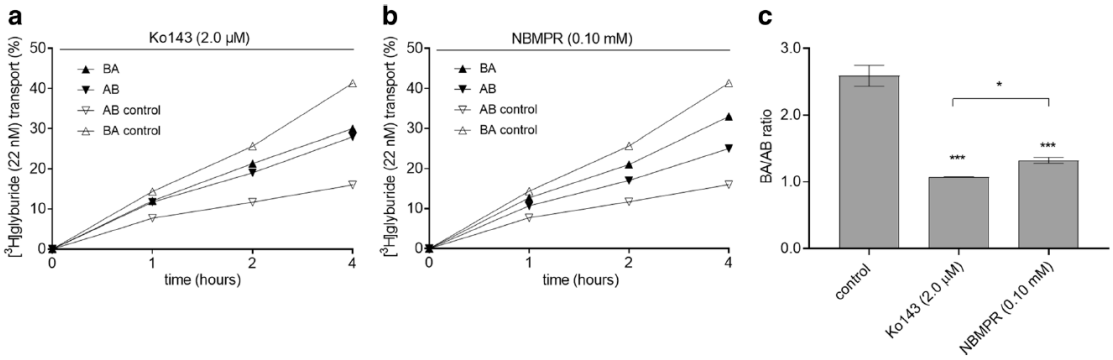

Fig. 4 NBMPR inhibits active membrane transport of [^{3}H]glyburide across MDCKII-ABCG2 monolayers. Basolateral-to-apical (BA) and apical-to-basolateral (AB) transport rates were determined for [^{3}H]glyburide (22 nM) with or without the model ABCG2 inhibitor Ko143 at a concentration of 2.0 μM (**a**) and 0.10 mM NBMPR (**b**). Each data point represents the mean ± SD of three independent experiments. Transport ratios (*r*) were calculated as the ratio of BA transport to AB transport after 4 h (**c**). The effects of each inhibitor were compared using one-way *ANOVA* followed by Turkey's multiple comparisons test. Results differing significantly from those for controls are labeled * ($P < 0.05$) or *** ($P < 0.001$).

and GF120918 both have significant inhibitory effects, confirming the role of ABCG2 in fetal-to-maternal glyburide transfer in rats (27). In accordance with previous reports (27), we observed a significant decline in [^{3}H]glyburide concentration in the fetal perfusate after 60 min. Fetal-to-maternal transfer was fully abolished by adding NBMPR (0.10 mM), providing further evidence that this compound inhibits ABCG2 when applied at 0.10 mM. Glyburide is also transported by human OATP2B1 (41) and zidovudine is a suggested substrate of OAT4 (42). Both transporters are localized in basal membrane of syncytiotrophoblast where they are believed to mediate cellular uptake. Therefore, it cannot be ruled out that inhibition of these transporters also contributes, to some extent, to the observed increase in F/M ratio of [^{3}H]glyburide and/or [^{3}H]zidovudine, respectively.

CONCLUSION

Over last two decades, several nucleoside-derived drugs transported by both ENTs and ABCG2 have been described (10,16,25,43,44). NBMPR has been used as a 'selective' inhibitor of ENTs in many studies on the contributions of ENT-mediated uptake to the cytotoxicity of nucleoside-derived anticancer drugs (e.g. (45,46). Here we present the first evidence that NBMPR at a concentration of 0.10 mM inhibits not only ENT1/ENT2-mediated transport of nucleosides/nucleosides analogues but also ABCG2 transport activity. This effect should be borne in mind when designing experiments and quantifying the contributions of ENTs and ABCG2 to the membrane transport of compounds that are "dual substrates" of these transporter types in tissues expressing high levels of

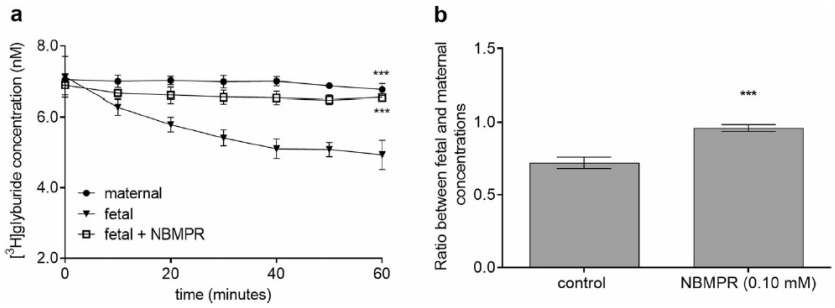

Fig. 5 Results obtained by dual perfusion of rat term placenta with [^{3}H]glyburide (7.0 nM) in a closed-circuit setup. The plots show the decline in the concentration of [^{3}H]glyburide over the course of 60 min of fetal perfusate recirculation and the effect of NBMPR (**a**). NBMPR (0.10 mM) significantly increased the concentration ratio between fetal and maternal (F/M) circulation after 60 min of fetal perfusate recirculation (**b**). Data are presented as means ± SD from three independent experiments. The concentration of [^{3}H]glyburide in the maternal compartment and in the fetal compartment in the presence of NBMPR was compared to that in the fetal compartment without inhibition (control) by one-way *ANOVA* followed by Dunnett's multiple comparisons test. The significance of the increase in the F/M ratio in the presence of NBMPR (0.10 mM) was evaluated using Student's unpaired *t*-test. In both subfigures, the label *** indicates a result that differs significantly from the control value at the $P < 0.001$ level.

both ENTs and ABCG2, such as the placental syncytiotrophoblast, hepatocytes, and intestinal tissues.

ACKNOWLEDGMENTS AND DISCLOSURES

We would like to thank Assoc. Prof. PharmDr. Martina Ceckova for her valuable comments regarding the accumulation studies in MDCKII cells. This study was supported by the Czech Science Foundation (grant no. GACR 17-16169S), and the EFSA-CDN project (No. CZ.02.1.01/0.0/0.0/16_019/0000841), which is co-funded by the ERDF. The authors declare no conflict of interest.

CONTRIBUTION OF AUTHORS

Ales Sorf and Sara Karbanova contributed equally to this work.

REFERENCES

1. Crawford CR, Ng CY, Noel LD, Belt JA. Nucleoside transport in L1210 murine leukemia cells. Evidence for three transporters. J Biol Chem. 1990;265(17):9732–6.
2. Jarvis SM, Young JD. Nucleoside transport in rat erythrocytes: two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and p-chloromercuriphenyl sulfonate. J Membr Biol. 1986;93(1):1–10.
3. Karbanova S, Cerveny L, Ceckova M, Ptackova Z, Jiraskova L, Greenwood S, et al. Role of nucleoside transporters in transplacental pharmacokinetics of nucleoside reverse transcriptase inhibitors zidovudine and emtricitabine. Placenta. 2017;60:86–92.
4. Karbanova S, Cerveny L, Jiraskova L, Karahoda R, Ceckova M, Ptackova Z, et al. Transport of ribavirin across the rat and human placental barrier: roles of nucleoside and ATP-binding cassette drug efflux transporters. Biochem Pharmacol. 2019;163:60–70.
5. Nishimura T, Sano Y, Takahashi Y, Noguchi S, Uchida Y, Takagi A, et al. Quantification of ENT1 and ENT2 proteins at the placental barrier and Contribution of these transporters to ribavirin uptake. J Pharm Sci. 2019;108(12):3917–22.
6. Paterson AR, Lau EY, Dahlig E, Cass CE. A common basis for inhibition of nucleoside transport by dipyridamole and nitrobenzylthioinosine? Mol Pharmacol. 1980;18(1):40–4.
7. Pickard MA, Paterson AR. Use of 4-nitrobenzylthioinosine in the measurement of rates of nucleoside transport in human erythrocytes. Can J Biochem. 1972;50(7):839–40.
8. Yamamoto T, Kuniki K, Takekuma Y, Hirano T, Iseki K, Sugawara M. Ribavirin uptake by cultured human choriocarcinoma (BeWo) cells and Xenopus laevis oocytes expressing recombinant plasma membrane human nucleoside transporters. Eur J Pharmacol. 2007;557(1):1–8.
9. Yao SY, Ng AM, Muzyka WR, Griffiths M, Cass CE, Baldwin SA, et al. Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. J Biol Chem. 1997;272(45):28423–30.
10. Molina-Arcas M, Casado FJ, Pastor-Anglada M. Nucleoside transporter proteins. Curr Vasc Pharmacol. 2009;7(4):426–34.
11. Pastor-Anglada M, Perez-Torras S. Nucleoside transporter proteins as biomarkers of drug responsiveness and drug targets. Front Pharmacol. 2015;6:13.
12. Pastor-Anglada M, Perez-Torras S. Emerging roles of nucleoside transporters. Front Pharmacol. 2018;9:606.
13. Pastor-Anglada M, Urtasun N, Perez-Torras S. Intestinal nucleoside transporters: function, expression, and regulation. Compr Physiol. 2018;8(3):1003–17.
14. Cerveny L, Ptackova Z, Ceckova M, Karahoda R, Karbanova S, Jiraskova L, et al. Equilibrative nucleoside transporter 1 (ENT1, SLC29A1) facilitates transfer of the antiretroviral drug Abacavir across the placenta. Drug Metab Dispos. 2018;46(11):1817–26.
15. Barnes K, Dobrzynski H, Foppolo S, Beal PR, Ismat F, Scullion ER, et al. Distribution and functional characterization of equilibrative nucleoside transporter-4, a novel cardiac adenosine transporter activated at acidic pH. Circ Res. 2006;99(5):510–9.
16. Cerveny L, Ptackova Z, Durisova M, Staud F. Interactions of protease inhibitors atazanavir and ritonavir with ABCB1, ABCG2, and ABCC2 transporters: effect on transplacental disposition in rats. Reprod Toxicol. 2018;79:57–65.
17. Chishu T, Sai Y, Nishimura T, Sato K, Kose N, Nakashima E. Potential of various drugs to inhibit nucleoside uptake in rat syncytiotrophoblast cell line, TR-TBT 18d-1. Placenta. 2008;29(5):461–7.
18. Nishimura T, Chishu T, Tomi M, Nakamura R, Sato K, Kose N, et al. Mechanism of nucleoside uptake in rat placenta and induction of placental CNT2 in experimental diabetes. Drug Metabolism and Pharmacokinetics. 2012;27(4):439–46.
19. Nishimura T, Tanaka J, Tomi M, Seki Y, Kose N, Sai Y, et al. Enhancement of zidovudine transfer to molt-4 cells, a human t-cell model, by dehydroepiandrosterone sulfate. J Pharm Sci. 2011;100(9):3959–67.
20. Sai Y, Nishimura T, Shimpo S, Chishu T, Sato K, Kose N, et al. Characterization of the mechanism of zidovudine uptake by rat conditionally immortalized syncytiotrophoblast cell line TR-TBT. Pharm Res. 2008;25(7):1647–53.
21. Sundaram M, Yao SY, Ingram JC, Berry ZA, Abidi F, Cass CE, et al. Topology of a human equilibrative, nitrobenzylthioinosine (NBMPR)-sensitive nucleoside transporter (hENT1) implicated in the cellular uptake of adenosine and anti-cancer drugs. J Biol Chem. 2001;276(48):45270–5.
22. Ceckova M, Reznicek J, Ptackova Z, Cerveny L, Muller F, Kacerovsky M, et al. Role of ABC and solute carrier transporters in the placental transport of lamivudine. Antimicrob Agents Chemother. 2016;60(9):5563–72.
23. Neumanova Z, Cerveny L, Ceckova M, Staud F. Interactions of tenofovir and tenofovir disoproxil fumarate with drug efflux transporters ABCB1, ABCG2, and ABCC2; role in transport across the placenta. AIDS. 2014;28(1):9–17.
24. Neumanova Z, Cerveny L, Ceckova M, Staud F. Role of ABCB1, ABCG2, ABCC2 and ABCC5 transporters in placental passage of zidovudine. Biopharm Drug Dispos. 2016;37(1):28–38.
25. Neumanova Z, Cerveny L, Greenwood SL, Ceckova M, Staud F. Effect of drug efflux transporters on placental transport of antiretroviral agent abacavir. Reprod Toxicol. 2015;57:176–82.
26. Sorf A, Hofman J, Kucera R, Staud F, Ceckova M. Ribociclib shows potential for pharmacokinetic drug-drug interactions being a substrate of ABCB1 and potent inhibitor of ABCB1, ABCG2 and CYP450 isoforms in vitro. Biochem Pharmacol. 2018;154:10–7.
27. Cygalova LH, Hofman J, Ceckova M, Staud F. Transplacental pharmacokinetics of glyburide, rhodamine 123, and BODIPY FL prazosin: effect of drug efflux transporters and lipid solubility. J Pharmacol Exp Ther. 2009;331(3):1118–25.
28. Guide for the care and use of laboratory animals Eighth Edition. Washington D.C.: The National Academies Press, ISBN 978-0-309-15400-0.

29. Council of Europe. European convention for the protection of vertebrate animals used for experimental and other scientific purposes. European treaty series no. 123. Council of Europe, Strasbourg, France. 1986 March 18. Available from https://www.coe.int/en/web/conventions/full-list/-/conventions/treaty/123.
30. Golstein PE, Boom A, van Geffel J, Jacobs P, Masereel B, Beauwens R. P-glycoprotein inhibition by glibenclamide and related compounds. Pflugers Arch. 1999;437(5):652–60.
31. Hemauer SJ, Patrikeeva SL, Nanovskaya TN, Hankins GD, Ahmed MS. Role of human placental apical membrane transporters in the efflux of glyburide, rosiglitazone, and metformin. Am J Obstet Gynecol. 2010;202(4):383 e381–7.
32. Jiraskova L, Cerveny L, Karbanova S, Ptackova Z, Staud F. Expression of concentrative nucleoside transporters (SLC28A) in the human placenta: effects of gestation age and prototype differentiation-affecting agents. Mol Pharm. 2018;15(7):2732–41.
33. Kis O, Robillard K, Chan GN, Bendayan R. The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters. Trends Pharmacol Sci. 2010;31(1):22–35.
34. Mani RS, Hammond JR, Marjan JM, Graham KA, Young JD, Baldwin SA, et al. Demonstration of equilibrative nucleoside transporters (hENT1 and hENT2) in nuclear envelopes of cultured human choriocarcinoma (BeWo) cells by functional reconstitution in proteoliposomes. J Biol Chem. 1998;273(46):30818–25.
35. Ceckova M, Libra A, Pavek P, Nachtigal P, Brabec M, Fuchs R, et al. Expression and functional activity of breast cancer resistance protein (BCRP, ABCG2) transporter in the human choriocarcinoma cell line BeWo. Clin Exp Pharmacol Physiol. 2006;33(1–2):58–65.
36. Cerveny L, Pavek P, Malakova J, Staud F, Fendrich Z. Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. Epilepsia. 2006;47(3):461–8.
37. Sorf A, Novotna E, Hofman J, Morell A, Staud F, Wsol V, et al. Cyclin-dependent kinase inhibitors AZD5438 and R547 show potential for enhancing efficacy of daunorubicin-based anticancer therapy: interaction with carbonyl-reducing enzymes and ABC transporters. Biochem Pharmacol. 2019;163:290–8.
38. Staud F, Vackova Z, Pospechova K, Pavek P, Ceckova M, Libra A, et al. Expression and transport activity of breast cancer resistance protein (Bcrp/Abcg2) in dually perfused rat placenta and HRP-1 cell line. J Pharmacol Exp Ther. 2006;319(1):53–62.
39. Zhou L, Naraharisetti SB, Wang H, Unadkat JD, Hebert MF, Mao Q. The breast cancer resistance protein (Bcrp1/Abcg2) limits fetal distribution of glyburide in the pregnant mouse: an obstetric-fetal pharmacology research unit network and University of Washington Specialized Center of research study. Mol Pharmacol. 2008;73(3):949–59.
40. Hofman J, Sorf A, Vagiannis D, Sucha S, Novotna E, Kammerer S, et al. Interactions of Alectinib with human ATP-binding cassette drug efflux transporters and cytochrome P450 biotransformation enzymes: effect on pharmacokinetic multidrug resistance. Drug Metab Dispos. 2019;47(7):699–709.
41. Satoh H, Yamashita F, Tsujimoto M, Murakami H, Koyabu N, Ohtani H, et al. Citrus juices inhibit the function of human organic anion-transporting polypeptide OATP-B. Drug Metab Dispos. 2005;33(4):518–23.
42. Rizwan AN, Burckhardt G. Organic anion transporters of the SLC22 family: biopharmaceutical, physiological, and pathological roles. Pharm Res. 2007;24(3):450–70.
43. Takenaka K, Morgan JA, Scheffer GL, Adachi M, Stewart CF, Sun D, et al. Substrate overlap between Mrp4 and Abcg2/Bcrp affects purine analogue drug cytotoxicity and tissue distribution. Cancer Res. 2007;67(14):6965–72.
44. Nagai S, Takenaka K, Nachagari D, Rose C, Domoney K, Sun D, et al. Deoxycytidine kinase modulates the impact of the ABC transporter ABCG2 on clofarabine cytotoxicity. Cancer Res. 2011;71(5):1781–91.
45. Stumpel DJ, Schneider P, Pieters R, Stam RW. The potential of clofarabine in MLL-rearranged infant acute lymphoblastic leukaemia. Eur J Cancer. 2015;51(14):2008–21.
46. Macanas-Pirard P, Broekhuizen R, Gonzalez A, Oyanadel C, Ernst D, Garcia P, et al. Resistance of leukemia cells to cytarabine chemotherapy is mediated by bone marrow stroma, involves cell-surface equilibrative nucleoside transporter-1 removal and correlates with patient outcome. Oncotarget. 2017;8(14):23073–86.