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Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

INTERAKCE INHIBITORŮ CYKLIN-DEPENDENTNÍCH KINÁZ S ABC EFLUXNÍMI TRANSPORTÉRY *IN VITRO*: VLIV NA MNOHOČETNOU LÉKOVOU REZISTENCI V PROTINÁDOROVÉ TERAPII

INTERACTIONS OF CYCLIN-DEPENDENT KINASE INHIBITORS WITH ABC EFFLUX TRANSPORTERS IN VITRO: IMPACT ON MULTIDRUG RESISTANCE IN CANCER THERAPY

Dizertační práce

Školitel: Prof. PharmDr. František Štaud, PhD.

Konzultant: PharmDr. Martina Čečková, PhD.

Hradec Králové, 2016

Mgr. Daniela Číhalová

„Prohlašuji, že tato práce je mým původním autorským dílem, které jsem zpracovala samostatně pod vedením svého školitele a konzultanta. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Tato práce nebyla použita k získání jiného či stejného titulu.“

Daniela Číhalová

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Abstrakt

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Katedra Farmakologie a toxikologie

Kandidátka: Mgr. Daniela Číhalová

Školitel: Prof. PharmDr. František Štaud, PhD.

Konzultant: PharmDr. Martina Čečková, PhD.

Název dizertační práce:

Interakce inhibitorů cyklin-dependentních kináz s ABC efluxními transportéry *in vitro*: vliv na mnohočetnou lékovou rezistenci v protinádorové terapii

Cyklin-dependentní kinázy hrají důležitou roli v regulaci buněčného cyklu, jejich zvýšená aktivita však může vést k vývoji nádorových onemocnění. Z tohoto důvodu se tyto enzymy stávají novým racionálním cílem protinádorové terapie, přičemž celá řada látek ze skupiny inhibitorů cyklin-dependentních kináz (CDKI) je momentálně v různých fázích klinického hodnocení.

ABC efluxní transportéry jsou exprimovány ve fyziologických tkáních, kde ovlivňují absorpci, distribuci a exkreci svých substrátů včetně léčiv a významně tak určují jejich farmakokinetické vlastnosti. Na druhou stranu, zvýšená exprese ABC transportérů v nádorových buňkách může přispívat ke vzniku mnohočetné lékové rezistence vůči odlišným protinádorovým léčivům. Tři zástupci této rodiny přitom hrají největší roli, a to ABCB1 (P-glykoprotein), ABCG2 (breast cancer resistance protein) a ABCC1 (multidrug resistance-associated protein 1). Inhibitory a substráty ABC transportérů se mohou při současném podání během léčby různých onemocnění podílet na vzniku farmakokinetických lékových interakcí, které mohou významně pozměnit dispozici léčiv v organismu a ovlivnit tak výsledný účinek léčby či její nežádoucí účinky.

Cílem mé práce bylo objasnit interakce studovaných CDKI s ABC transportéry pomocí *in vitro* metod a zjistit, jestli tyto interakce mohou ovlivnit efektivitu podání konvenčních protinádorových léčiv v lidských nádorových buňkách.

Pomocí akumulačních metod v MDCKII buněčných liniích exprimujících ABC lékové transportéry jsme zjistili, že námi hodnocené CDKI (purvalanol A, olomoucin II, roskovitin, flavopiridol, SNS-032, dinaciclib a palbociclib) jsou inhibitory alespoň jednoho testovaného ABC transportéru, zatímco látka AT-7519 nevykazovala žádný inhibiční efekt. V léčbě nádorových onemocnění jsou léčiva často podávána v kombinaci, aby bylo dosaženo zvýšeného terapeutického účinku a sníženého rizika vzniku mnohočetné lékové rezistence. Pomocí metody kombinačního indexu dle Chou-Talalay jsme v lidských nádorových buněčných liniích prokázali, že souběžné podání CDKI, který má schopnost inhibovat ABC transportér, a cytotoxického substrátu tohoto transportéru povede ke zvýšené nitrobuněčné akumulaci substrátu, potenciaci jeho účinku a synergickému protinádorovému působení. Buněčný model odpovídajících MDCKII buněčných linií jsme použili i pro studium substrátové afinity vybraných CDKI a pomocí transportní metody přes buněčnou monovrstvu jsme identifikovali olomoucin II a dinaciclib jako substráty ABCB1 a ABCG2.

Pomocí *in vitro* metod jsme tak prokázali, že CDKI interagují s ABC transportéry jako inhibitory nebo substráty. V těchto případech se dá předpokládat, že při podání v kombinaci s dalšími léčivy může dojít ke vzniku lékových interakcí. Přitom inhibiční aktivita CDKI vůči ABC transportérům může být využita při překonávání mnohočetné lékové rezistence nádorových buněk.

Abstract

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology and Toxicology

Candidate: Mgr. Daniela Číhalová

Supervisor: Prof. PharmDr. František Štaud, PhD.

Consultant: PharmDr. Martina Čečková, PhD.

Title of doctoral thesis:

Interactions of cyclin-dependent kinase inhibitors with ABC efflux transporters *in vitro*: impact on multidrug resistance in cancer therapy

Cyclin-dependent kinases play an important role in cell cycle regulation and their enhanced activity can lead to the development of various malignancies. Therefore, these kinases have become a rational target for inhibition in cancer therapy and many compounds from the group of cyclin-dependent kinase inhibitors (CDKIs) are being evaluated in clinical trials.

ABC efflux transporters are expressed in physiological tissues, where they influence the absorption, distribution and elimination of their substrates including drugs and determine their pharmacokinetic properties. On the other hand, overexpression of ABC transporters in cancer cells can contribute to the development of multidrug resistance (MDR) against structurally and functionally diverse compounds. Three members of the ABC transporter family play the most prominent role in the development of MDR: ABCB1 (P-glycoprotein), ABCG2 (breast cancer resistance protein) and ABCC1 (multidrug resistance-associated protein 1). Inhibitors and substrates of ABC transporters may participate in drug-drug interactions when administered simultaneously

in the treatment of various diseases, which can significantly affect the drug disposition in the organism and alter the therapeutic outcome or adverse effects.

The aim of my thesis was to elucidate the interactions of the selected CDKIs with ABC transporters using *in vitro* methods and to determine whether these interactions might affect the efficiency of conventionally administered anticancer drugs in human cancer cells.

Using the accumulation method in MDCKII cell lines overexpressing ABC efflux transporters we found that the tested CDKIs (purvalanol A, olomoucine II, roscovitine, flavopiridol, SNS-032, dinaciclib and palbociclib) are all inhibitors of at least one of the ABC transporters, whereas AT-7519 showed no inhibitory potency. In cancer treatment, drugs are often administered in combinations to increase efficacy and limit the risk of MDR. Employing the combination index method of Chou-Talalay in human cancer cell lines, we showed that simultaneous administration of a CDKI with inhibitory potency towards an ABC transporter and a cytotoxic substrate of this transporter can lead to increased intracellular accumulation of the substrate and pronounced synergistic anticancer effect. Applying the corresponding MDCKII cell model and monolayer transport assays, we also studied the substrate affinity of CDKIs toward ABC transporters and identified olomoucine II and dinaciclib as substrates of ABCB1 and ABCG2.

Employing *in vitro* methods we found that CDKIs interact with ABC transporters as inhibitors or substrates. In these cases, drug-drug interactions can occur when the CDKIs are administered simultaneously with other drugs. On the other hand, we also demonstrated that inhibitory activity of CDKIs toward ABC transporters can be exploited to battle the problem of MDR.

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1 Zkratky

ABC	ATP-binding cassette, rodina efluxních transportérů
ABCB1	P-glykoprotein, P-gp, MDR1
ABCC1	multidrug resistance-associated protein 1, MRP1
ABCG2	breast cancer resistance protein, BCRP
CDK	cyklin-dependentní kináza
CDKI	inhibitor cyklin-dependentních kináz
EMA	European Medicines Agency, Evropská léková agentura
FDA	Food and Drug Administration, Úřad pro kontrolu potravin a léčiv
MDCKII	Madin-Darby canine kidney, buněčná linie
MDR	multidrug resistance, mnohočetná léková rezistence
Rb	retinoblastom

Poznámka

V textu mohou být proteiny označeny velkými nebo malými písmeny v návaznosti na současná doporučení, která používají velká písmena u lidských proteinů a malá písmena u zvířecích ekvivalentů.

2 Úvod

Nádorová onemocnění představují jednu z hlavních příčin morbidity a mortality na světě [1]. Každý rok je takové onemocnění nově diagnostikováno u více než 14 miliónu lidí, přičemž 8 miliónů pacientů ročně následkem maligních onemocnění umírá [2]. Za „zlatý standard“ protinádorové terapie jsou stále považována konvenční cytostatika, která vykazují cytotoxický efekt nejčastěji interakcí s DNA nebo jejími prekurzory, což vede k usmrcení nejen nádorových buněk, ale nevýhodně i zdravých, rychle se dělících buněk [3-5]. Dalším závažným limitujícím faktorem pro úspěšnost klasické protinádorové terapie je fenomén lékové rezistence nádorových buněk. K rezistenci vůči cytostatikům může dojít farmakodynamickými mechanismy, které zahrnují např. aktivaci reparace DNA či snížení buněčné apoptotické odpovědi, nebo farmakokinetickými mechanismy, které snižují koncentraci cytostatika v buňce pod účinnou cytotoxickou koncentraci zvýšeným efluxem nebo biotransformací dané látky [6, 7].

Jednou z častých příčin mnohočetné lékové rezistence, tj. rezistence vůči protinádorovým léčivům různých struktur a účinku, je zvýšená exprese efluxních transportérů z rodiny ATP-binding cassette (ABC) v membráně nádorových buněk [8-10]. Obecně jsou tyto ABC transportéry exprimovány v orgánech významně ovlivňujících absorpci, distribuci a exkreci léčiv (např. tenké střevo, mozek, placenta, játra, ledviny) a jsou tedy i častým místem vzniku farmakokinetických lékových interakcí [11, 12]. Z tohoto důvodu vydalo International Transporter Consortium doporučení pro studium interakcí léčiv s ABC transportéry [13], aby se stanovily jejich farmakokinetické a bezpečnostní profily, a instituce jako je Evropská léková agentura (European Medicines Agency, EMA) či americký Úřad pro kontrolu potravin a léčiv (Food and Drug Administration, FDA) následně svými vlastními směrnicemi zdůraznily důležitost *in vitro* metod v tomto výzkumném směru [14, 15]. Na vzniku mnohočetné lékové rezistence se nejvýznamněji podílí především tři zástupci ABC transportérů: ABCB1 (P-glykoprotein, P-gp, MDR1), ABCG2 (breast cancer resistence protein, BCRP) a ABCC1 (multidrug resistence-associated protein 1, MRP1).

Snaha vyvinout nové či zlepšit stávající přístupy k protinádorové farmakoterapii vedla v posledních desetiletích k intenzivnímu vývoji modulátorů ABC lékových transportérů, které by po podání s protinádorovým léčivem ideálně zablokovaly eflux cytostatika z nádorové buňky a tím zvýšily jeho nitrobuněčnou retenci. Tento přístup se však zatím

neosvědčil z důvodu nízké efektivity a vysoké toxicity používaných modulátorů [16]. Po neúspěchu s tradičními modulátory ABC transportérů se zaměření výzkumu posouvá na sloučeniny, které primárně nebyly vyvinuty jako modulátory mnohočetné lékové rezistence, ale jako látky pro cílenou protinádorovou terapii. V poslední době se ukázalo, že kromě těchto modulátorů mohou být ABC transportéry inhibovány i moderními látkami ze skupiny antineoplastik, konkrétně inhibitory tyrozinkináz [17], které jsou svou strukturou i účinkem odlišné od běžně používaných inhibitorů ABC transportérů. Tyto látky tak překonávají rezistenci způsobenou ABC transportéry, mají navíc vlastní cytotoxický účinek a vykazují přesvědčivé výsledky jak *in vitro*, tak *in vivo* a dokonce i v klinických studiích [18-20].

Zcela novou skupinou látek v protinádorové terapii představují inhibitory cyklin-dependentních kináz (CDKI), které inhibují serin/treoninové kinázy regulující buněčný cyklus. Zvýšená aktivita těchto enzymů byla prokázána při vývoji nádorových onemocnění. Z tohoto důvodu se cyklin-dependentní kinázy stávají novým racionálním cílem protinádorové terapie a mnoho látek ze skupiny CDKI je momentálně v různých fázích klinického hodnocení. První z nich, palbociclib, byl přitom v únoru 2015 schválen americkou FDA pro léčbu postmenopauzálních žen s metastazujícím karcinomem prsu [21].

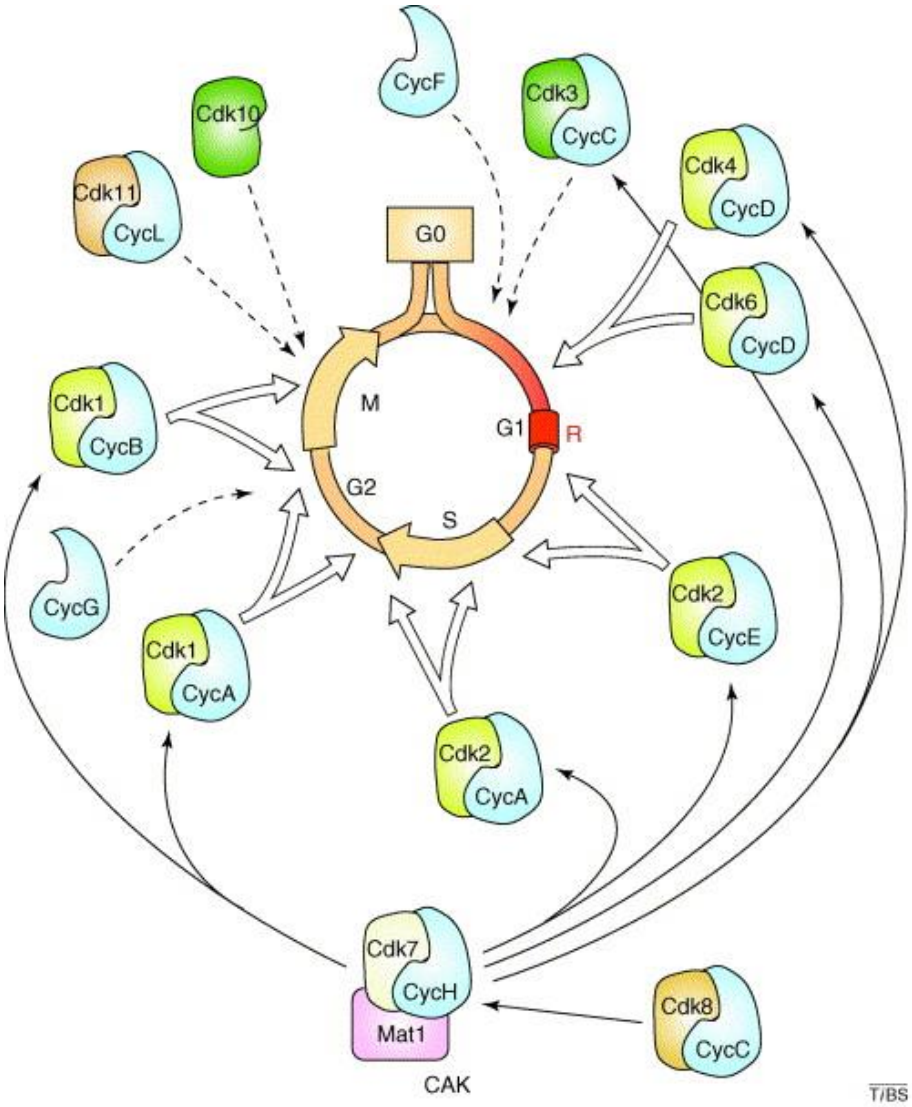
Farmakodynamické a farmakokinetické chování CDKI bylo popsáno během preklinického, příp. i klinického hodnocení, informace o jejich interakcích s ABC transportéry jsou však zatím omezené. Předchozí práce naší výzkumné skupiny prokázaly inhibiční aktivitu CDKI první generace vůči ABCG2 transportéru [22] a lze proto předpokládat, že použití takových CDKI v kombinaci s cytotoxickými substráty inhibovaných ABC transportérů by mohlo vést k výhodnému synergickému efektu obou látek a zlepšit tak efektivitu běžné protinádorové terapie. V případě potvrzení této hypotézy by bylo možné CDKI považovat za látky s duálním účinkem (inhibující množení nádorových buněk aktivitou proti cyklin-dependentním kinázám a zároveň překonávající mnohočetnou lékovou rezistenci při podání s konvenčním cytostatikem). Navíc je nezbytné studovat souvislost mezi CDKI a ABC transportéry i z důvodu včasného vyhodnocení možných farmakokinetických lékových interakcí, které by v průběhu podávání těchto léčiv mohly nastat.

3 Teoretická část

3.1 Cyklin-dependentní kinázy a jejich role ve vývoji nádorových onemocnění

Cyklin-dependentní kinázy (CDK) jsou velkou podrodinou proteinových kináz (konkrétně jsou to serin/treoninové kinázy), které hrají důležitou roli v regulaci fyziologických procesů eukaryotických buněk jako je buněčný cyklus nebo transkripce RNA [23]. Aktivní CDK fosforylují rodinu retinoblastom (Rb) proteinů, což vede k uvolnění transkripčních faktorů nutných pro průběh buněčného cyklu. Katalytická aktivita CDK je regulována interakcí s cykliny nebo inhibitory CDK (CDKI) [24]. Cykliny jsou proteiny, které jsou syntetizovány a degradovány v průběhu buněčného cyklu, slouží jako regulační podjednotky komplexů cyklin-CDK a teprve jejich navázáním na CDK vzniká aktivní komplex [25]. CDK2 nejprve interaguje s cyklinem E před vstupem do S fáze, což navodí syntézu DNA, a poté s cyklinem A během S fáze (obr. 1). Mitóza je následně zahájena vazbou CDK1 na cyklin B [26]. CDK4/CDK6 (po navázání na cyklin D) jsou hlavními regulátory postupu buněčného cyklu z kontrolního bodu G_1 do S fáze potlačením antiproliferačního efektu Rb proteinu [27] a CDK5 má roli ve vývoji neuronů [28]. Exprese CDK6 většinou není ve fyziologických tkáních detekována a její zvýšena aktivita je spojena s vývojem několika druhů nádorových onemocnění [29]. CDK7 a 9 mají svou funkci v regulaci transkripce při fosforylaci RNA-polymerázy II [30]. Zatímco cykliny podporují aktivitu CDK, CDKI mají schopnost potlačit jejich funkci a přispívat tak k negativní regulaci. Fyziologické CDKI jsou rozděleny do dvou skupin na základě struktury a specificity; Ink4 inhibitory primárně blokují funkci CDK4 a CDK6, zatímco Cip/Kip inhibitory jsou méně specifické a široce interferují s aktivitami ostatních kináz [31].

Typickým znakem mnoha nádorových buněk je změna v expresi a/nebo aktivitě CDK, cyklinů či endogenních CDKI, což vede ke ztrátě kontroly nad buněčným cyklem a nekontrolované proliferaci nezávislé na okolních podmínkách [32]. Např. změna v regulaci signální kaskády zahrnující CDK4 a 6, cyklin D, INK4 inhibitory a Rb protein byla popsána u více než 80% lidských nádorů [33, 34]. Rozdílná exprese, aktivita nebo regulace CDK byla také nalezena v souvislosti s virovými infekcemi [35], Alzheimerovou či Parkinsonovou nemocí [36, 37]. V tomto ohledu jsou CDK racionálním terapeutickým cílem pro dosažení normální regulace buněčného cyklu.



Obr. 1. Role komplexů CDK-cyklin v buněčném cyklu savců. Převzato z článku Malumbres et al. [25].

3.2 Cyklin-dependentních kinázy jako cíle protinádorové terapie

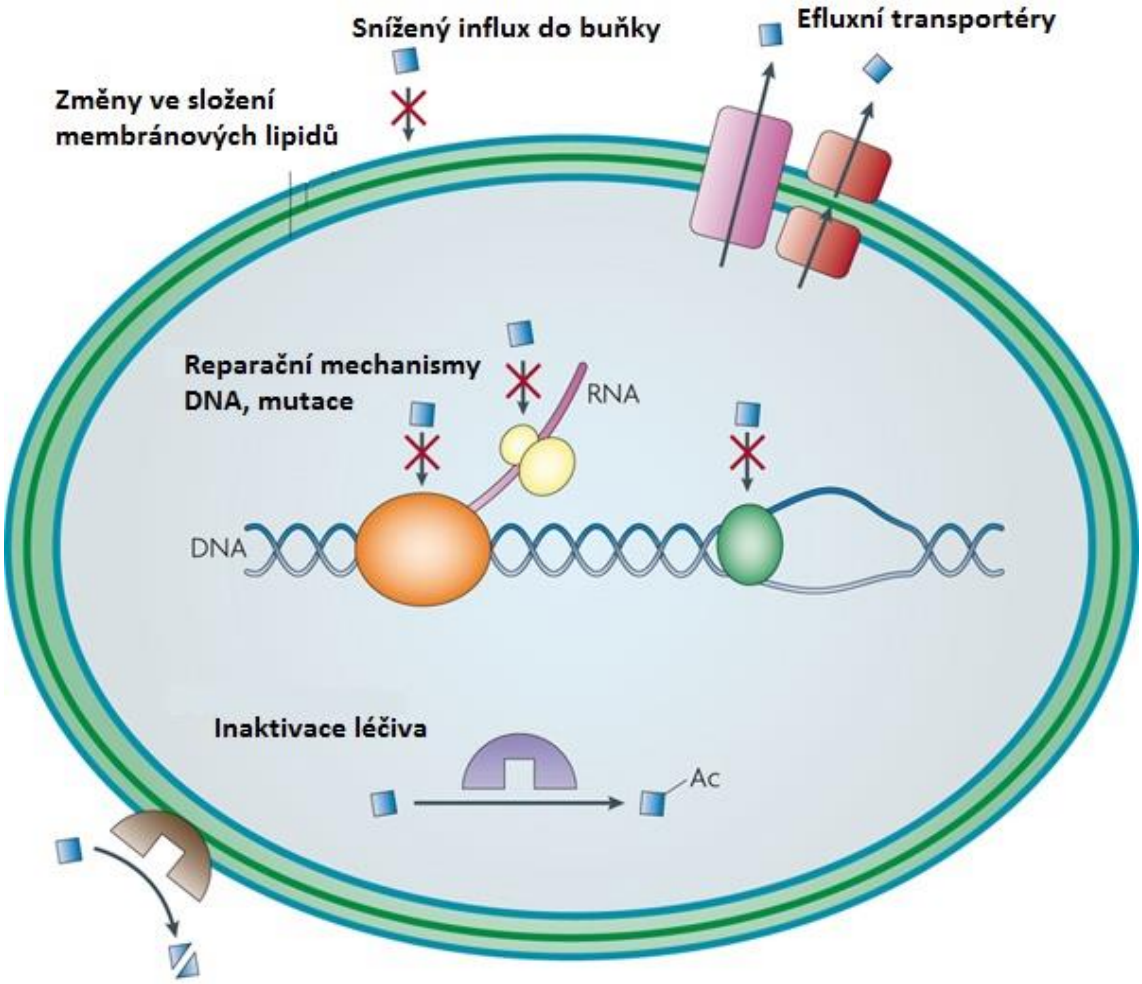
Exprese a aktivita CDK v nádorových buňkách je často zvýšená, nicméně jejich inhibice může navodit buněčnou smrt a vést tak k terapeuticky výhodné apoptóze a lýze nádorové buňky. Z tohoto důvodu se inhibice CDK stává novým přístupem k terapii nádorového bujení a mnohé z nových sloučenin ze skupiny CDKI jsou v preklinickém a klinickém zkoušení. CDKI jsou látky strukturně odlišné a zahrnují puriny, alkaloidy, flavonoidy a jejich analoga [38]. Nejčastěji jsou CDKI kompetitivní inhibitory, které se váží na ATP-vazebné místo, jejichž nevýhodou je však značná nespecifita [39]. První

generace CDKI, kam patří flavopiridol nebo roskovitin, se vyznačuje schopností inhibovat vícero CDK a potýká se s vysokou toxicitou a nežádoucími účinky v klinickém hodnocení, což vede ke snahám vyvíjet nové a specifické CDKI [40, 41]. V posledních letech se nejúspěšněji uplatňují specifické inhibitory CDK4 a 6 [42], především palbociclib, který byl v únoru 2015 jako první CDKI schválen americkou FDA pro použití v léčbě karcinomu prsu u postmenopauzálních žen [21]. Dalších minimálně 20 CDKI bylo hodnoceno v některé z fází klinického zkoušení pro léčbu hematologických malignit i solidních nádorů [43].

3.3 Mnohočetná léková rezistence v terapii nádorových onemocnění

Rozlišujeme dva typy rezistence na léčiva: při primární rezistenci odolávají nádorové buňky účinkům cytostatik již při první léčbě, sekundární (získaná) rezistence vzniká až v průběhu léčby, kdy se z původně citlivé buňky stávají buňky k danému cytostatiku rezistentní [44]. K nejčastějším mechanismům vzniku lékové rezistence v buňkách patří: (1) posun kontrolních bodů buněčného cyklu, (2) změny ve složení membránových lipidů, (3) inhibice apoptózy, (4) snížený influx látky do buňky, (5) zvýšený eflux látky z buňky, (6) indukce DNA-reparačních mechanismů, (7) aktivace detoxikačních systémů v buňce atd. (obr. 2) [45].

Problematickým typem sekundární rezistence je tzv. mnohočetná léková rezistence vůči protinádorovým léčivům různých struktur a účinku. Hlavním mechanismem vzniku tohoto typu rezistence v nádorových buňkách je zvýšená exprese ATP-binding cassette (ABC) efluxních transportérů, které aktivně transportují řadu cytostatik ven z buňky a tím snižují jejich nitrobuněčnou koncentraci a protinádorový účinek [46]. Tento typ rezistence byl klinicky prokázán u mnoha léčiv ze skupiny antineoplastik, včetně nových látek ze skupiny inhibitorů tyrozinových kináz [17], a je tedy pravděpodobné, že postihne i skupinu CDKI.



Obr. 2. Mechanismy vzniku buněčné rezistence. Jedním z hlavních mechanismů vzniku mnohočetné lékové rezistence je zvýšení aktivity či exprese ABC efluxních transportérů, které aktivně transportují řadu cytostatik ven z buňky a tím snižují jejich protinádorový účinek. Modifikováno podle článku Allen et al. [47].

3.4 ABC transportéry

ABC efluxní transportéry představují důležitý mechanismus transportu endogenních látek a xenobiotik přes buněčnou membránu. Tyto proteinové transportéry jsou primárně lokalizovány v plazmatické membráně, kde fungují jako pumpy odstraňující z buňky velké množství látek s odlišnou strukturou a účinkem včetně anorganických aniontů, kovových iontů, peptidů, aminokyselin, cukrů, hydrofobních sloučenin a jejich konjugátů a metabolitů [48]. Transport těchto látek probíhá aktivním procesem, je tedy závislý na zisku energie z hydrolýzy ATP, a může probíhat i proti koncentračnímu gradientu.

Substrátová specifita těchto transportérů, stejně jako jejich exprese a lokalizace v tkáních, se mezi jednotlivými zástupci liší. Obecně jsou ABC transportéry exprimovány v játrech, ledvinách a tenkém střevě, kde ovlivňují farmakokinetiku léčiv (především absorpci, distribuci a exkreci) v systémové cirkulaci a jsou tedy i častým místem vzniku lékových interakcí [11, 12]. Dále se nacházejí v placentě, hematotestikulární či hematoencefalické bariéře, kde hrají důležitou roli v ochraně citlivých tkání před toxickými efekty xenobiotik. Přítomnost ABC transportérů byla také zaznamenána v nádorových buňkách, kde dochází aktivním odstraňováním cytotoxické látky z buňky ke snížení její intracelulární koncentrace, což může vést až k úplné rezistenci. Vysoká exprese ABC transportérů tak může představovat překážku v úspěšné protinádorové terapii [45, 49]. Z celkových 49 lidských ABC transportérů, které byly dosud popsány, hrají v lékové rezistenci nejvýznamnější roli tři zástupci: ABCB1 (P-glykoprotein, P-gp, MDR1), ABCC1 (multidrug resistence-associated protein 1, MRP1) a ABCG2 (breast cancer resistence protein, BCRP) [50].

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

ABCB1, dosud nejlépe prostudovaný ABC transportér, je tvořen jedním polypeptidovým řetězcem 1280 aminokyselin (170 kDa). Skládá se ze dvou homologických polovin s dvanácti transmembránovými a dvěma nukleotid-vázajícími doménami (obr. 3a), které jsou zodpovědné za navázání ATP a jeho následnou hydrolýzu [51]. Typickým znakem ABCB1 je vysoká strukturní diverzita jeho substrátů z různých terapeutických skupin, jejichž jediným společným znakem je amfifilní povaha [52, 53]. Mezi substráty ABCB1 patří i značné množství antineoplastik, na která může v důsledku přítomnosti ABCB1 v membránách nádorových buněk vznikat rezistence.

ABCB1 je exprimován na apikální straně buněčné membrány (obr. 4). Přehled buněk lidského těla, které exprimují ABCB1 je uveden v tabulce 1. Hlavní fyziologickou funkcí ABCB1 je snížená distribuce látek do citlivých tkání (mozek, varlata, plod), snížení biodostupnosti látek po orálním podání (což není vždy žádoucí), transport metabolitů do žluči a moči a jejich exkrece [54]. ABCB1 je také vysoce exprimován v leukemických buňkách a buňkách nádorů prsu, vaječníků, tlustého střeva, ledvin a jater, což vede k nízké klinické odpovědi a špatné prognóze nádorových onemocnění [55-57].

3.4.2 ABCG2 (breast cancer resistance protein, BCRP)

ABCG2 tvoří jeden polypeptidový řetězec s 655 aminokyselinami (72 kDa) a na rozdíl od ABCB1 má pouze šest transmembránových a jednu nukleotid-vázající doménu (obr. 3b). Pro správnou funkci pak tento „poloviční transportér" tvoří dimery a multimery [58]. Podobně jako ABCB1, i ABCG2 hraje roli v mnohočetné lékové rezistenci a transportuje obrovské množství strukturně a chemicky odlišných endogenních i exogenních sloučenin, včetně antineoplastik. ABCG2 a ABCB1 mají mnoho společných substrátů, což je relevantní zejména v gastrointestinálním traktu, kde jejich efluxní aktivita může signifikantně ovlivnit absorpci látek po perorálním podání [59].

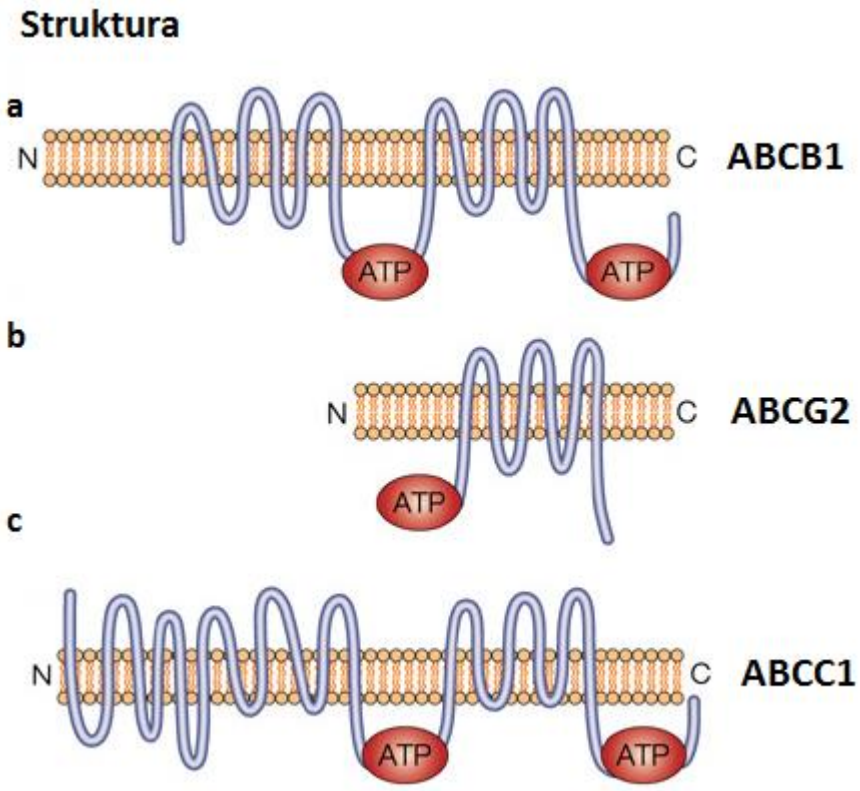
Transportér ABCG2 je stejně jako ABCB1 exprimován na apikální membráně polarizovaných buněk řady fyziologických tkání (tabulka 1, obr. 4). ABCG2 je exprimován v orgánech ovlivňujících farmakokinetiku léčiv a v orgánech, kde má důležitou ochrannou funkci před toxickým působením různých látek. ABCG2 se nachází i v membráně kmenových buněk, kde se podílí na jejich ochraně před toxickými vlivy xenobiotik [60, 61]. ABCG2 přispívá k transportu endogenních substancí (hem, porfyriny, riboflavin) a reguluje tím buněčnou homeostázu [62]. Vysoká exprese ABCG2 způsobuje rezistenci nádorových buněk vůči mitoxantronu, topotekanu a methotrexátu a je spojena se špatnou odpovědí na cytostatickou léčbu především u pacientů s leukémií a rakovinou prsu [63, 64].

3.4.3 ABCC1 (multidrug resistance-associated protein 1, MRP1)

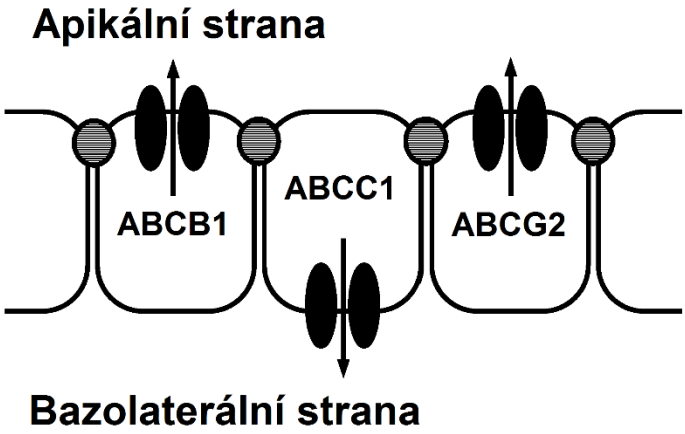
ABCC1 patří do rozsáhlé podrodiny ABCC transportérů a tvoří ho jeden polypeptidový řetězec složený z 1531 aminokyselin (190 kDa). Strukturně je velice podobný ABCB1 s dvanácti transmembránovými a dvěma nukleotid-vázajícími doménami (obr. 3c), ale obsahuje navíc i dalších pět transmembránových segmentů s volným NH_2 koncem [65]. ABCC1 má širokou substrátovou specifitu s vyšší afinitou vůči hydrofobním a aniontovým molekulám, konjugátům glukuronidu a glutathionu a fyziologickým substrátům (foláty, glutathion, konjugáty steroidů, apod.) [66].

Na rozdíl od ABCB1, je transportér ABCC1 často lokalizován na bazolaterální membráně polarizovaných buněčných vrstev (obr. 4) kromě endoteliálních buněk mozku,

kde je jeho lokalizace apikální. Přehled lidských buněk exprimujících ABCC1 je v tabulce 1. ABCC1 je důležitý pro normální funkci buněk (transport endogenních látek a fyziologických substrátů) a pro exkreci metabolitů [67]. Vysoká exprese byla nalezena v nádorech plic, prsu, prostaty, vaječníků, melanomech a leukémiích [68]. Role ABCC1 v nádorové rezistenci je stále diskutabilní; zatímco některé studie prokázaly, že exprese ABCC1 má vliv na prognózu nádorových onemocnění [69, 70], jiné tuto roli zpochybňují, protože nenalezli žádnou korelaci mezi terapeutickou odpovědí a expresí ABCC1 [71, 72].



Obr. 3. Struktura jednotlivých ABC transportérů lokalizovaných v buněčné membráně. Převzato z článku Gottesman et al. [46].



Obr. 4. Lokalizace jednotlivých ABC transportérů v polarizovaném epitelu ledvin, který odpovídá modelové linii MDCKII (Madin-Darby canine kidney). ABCB1 a ABCG2 jsou exprimovány na apikální membráně ledvinných epiteliálních buněk a ABCC1 je naopak exprimován na membráně bazolaterální. Bariéru mezi apikální a bazolaterální membránou tvoří těsné spoje (tight junctions, šedé kruhy). Převzato z článku Schinkel et al. [73].

Tabulka 1. Přehled nejdůležitějších ABC transportérů v mnohočetné lékové rezistenci [12, 13, 50, 73].

Transportér	Lokalizace	Vybrané substráty	Vybrané inhibitory	Poznámky
ABCB1	střevní enterocyty, proximální tubuly ledvin, hepatocyty, endotelové buňky mozku, Sertoliho buňky, placenta, rohovka n*a apikální straně membrány*	digoxin, dexametason, daunorubicin, doxorubicin, irinotecan, vinka alkaloidy, paklitaxel, mitoxantron, topotekan, etoposid, Rhodamin 123, Hoechst 33342 a další	verapamil, cyklosporin A, zosuquidar, elakridar, valspodar, chinidin	• role v ovlivnění absorpce, distribuce a exkrece • lékové interakce klinicky potvrzeny • role v mnohočetné lékové rezistenci
ABCG2	střevní enterocyty, proximální tubuly ledvin, hepatocyty, endotelové buňky mozku, placenta, mléčná žláza, kmenové buňky n*a apikální straně membrány*	prazosin, glyburid, mitoxantron, topotekan, irinotecan, methotrexát, statiny, Hoechst 33342 a další	fumitremorgin C, Ko143, elakridar, novobiocin	• role v ovlivnění absorpce, distribuce a exkrece • klinicky relevantní genetické polymorfismy • lékové interakce klinicky potvrzeny • role v mnohočetné lékové rezistenci
ABCC1	střevní enterocyty, endotelové buňky mozku (apikální lokalizace), myocyty, Sertoliho buňky n*a bazolaterální straně* membrány	daunorubicin, doxorubicin, irinotecan, topotekan, vinka alkaloidy, paklitaxel, methotrexát, mitoxantron, etoposid, Rhodamin 123 a další	MK-571, reversan, probenecid, benzbromaron, sulfinpyrazon, verapamil	• role v ovlivnění distribuce • role v mnohočetné lékové rezistenci

3.5 Přístupy k modulaci mnohočetné lékové rezistence

V chemoterapii se často používají léčiva s cíleným působením a jejich kombinací můžeme docílit maximalizace účinku a aditivního či synergického efektu [74]. Působení kombinace léčiv na několik buněčných drah najednou potencuje protinádorové působení jednotlivých látek a navíc snižuje riziko vzniku lékové rezistence [75]. Tento přístup může pomoci zlepšit efektivitu chemoterapie, není však úplně dostačující. Mnohočetná léková rezistence způsobená zvýšenou expresí ABC transportérů v nádorových buňkách může být překonána několika dalšími metodami jako je např. specifická inhibice transportérů v membráně buněk nebo blokáda signálních drah a transkripčních faktorů, které regulují amplifikaci a zvýšenou expresi těchto transportérů [76]. Výzkum v této oblasti se zaměřil na vývoj tzv. modulátorů, inhibitorů ABC transportérů, které se používají pro zvýšení účinků chemoterapie a biodostupnosti léčiv po perorálním podání.

Jedna z prvních studií prokázala, že verapamil zvyšuje cytotoxicitu vinkristinu a vinblastinu v ABCB1-rezistentní buněčné linii [77]. Slater et al. poté pozorovali, že cyklosporin A kompletně překonal primární rezistenci vůči vinkristinu a daunorubicinu v rezistentní buněčné linii odvozené z lidské akutní leukémie [78]. I další látky schválené pro jiné indikace inhibují aktivitu ABCB1 a překonávají tak rezistenci zprostředkovanou tímto transportérem, nicméně pouze ve vysokých koncentracích a nedají se tak klinicky použít z důvodu své toxicity [79]. Takové látky jsou označovány jako modulátory 1. generace. Začátek éry modulátorů 2. generace byl spojen s objevem látky PSC833 (valspodar, analog cyklosporinu A), který vykazuje až desetinásobně vyšší inhibiční potenci vůči ABCB1 a žádné imunosupresivní účinky [80]. Modulátory prvních dvou generací byly již testovány v rámci klinického zkoušení, jejich použití však nepřineslo očekávaný benefit, protože stále docházelo k výskytu nežádoucích účinků [81-83]. Modulátory 3. generace, jako např. GF120918 (elakridar), LY335979 (zosuquidar) či XR9576 (tariquidar), byly vyvinuty jako selektivní inhibitory ABC transportérů. Tariquidar v nízkých nanomolárních koncentracích zvyšuje citlivost nádorových buněk vůči několika cytostatikům a při jeho podání v kombinaci s doxorubicinem u myší nedochází ke zvýšení toxicity [84]. Během dvou klinických studií na lidech se však ukázalo, že při podávání tariquidaru se zvyšuje toxicita chemoterapie a jeho testování tak bylo zastaveno [85]. Ve vývoji mnohočetné lékové rezistence hrají roli i transportéry ABCC1 a ABCG2. Jejich specifické inhibitory, MK-571 [86], resp. fumitremorgin C

[87], se běžně používají v *in vitro* či *in vivo* experimentech, nicméně nebyly testovány v klinickém hodnocení jako modulátory mnohočetné lékové rezistence.

V poslední době se ukazuje, že kromě těchto modulátorů mohou být ABC transportéry inhibovány i látkami ze skupiny nových protinádorových látek, konkrétně inhibitory tyrozinkináz, které jsou odlišné od běžně používaných inhibitorů ABC transportérů svým účinkem i strukturou. Tyto látky překonávají rezistenci způsobenou ABC transportéry, mají navíc vlastní cytotoxický účinek a vykazují přesvědčivé výsledky jak *in vitro*, tak *in vivo* a některé i v klinických studiích [18, 20]. Novou skupinou kinázových inhibitorů v klinickém zkoušení jsou právě CDKI, které byly důsledně zkoumány z hlediska farmakodynamiky. Jejich farmakokinetické parametry, především pak interakce s ABC transportéry, nebyly dosud podrobně studovány. Jelikož bylo prokázáno, že i některé CDKI jsou schopny inhibovat ABC transportéry [22, 88], dá se předpokládat, že jejich použití v kombinaci se substráty inhibovaných transportérů by mohlo vést k výhodnému synergickému efektu obou látek a zlepšit tak efektivitu běžné protinádorové terapie.

3.6 ABC transportéry a lékové interakce

ABC transportéry mohou svou funkcí ovlivnit farmakokinetiku léčiv, terapeutickou odpověď a nežádoucí účinky. Při podání více látek v kombinaci tak může dojít k lékovým interakcím; jedna látka např. inhibuje efluxní aktivitu transportéru, čímž mění farmakokinetické vlastnosti druhé látky, která je substrátem tohoto transportéru. Klinické studie potvrzují, že existuje řada relevantních farmakokinetických lékových interakcí spojených s ABCB1 či ABCG2 transportéry. Z tohoto důvodu byly na doporučení International Transporter Consortium [13] oba transportéry zařazeny do nových směrnic pro studium lékových interakcí vydaných kontrolními orgány jako jsou FDA [14] a EMA [15]. Tyto směrnice používané v průmyslu, regulačních agenturách a mnohem častěji také ve výzkumu jasně upravují a doporučují postupy studia lékových interakcí a jsou nezbytné pro bezpečné a efektivní podání léčiv.

U ABCB1 je riziko interakcí vysoké vzhledem k velkému množství substrátů z různých farmakologických skupin jako např. antiarytmika, proteázové inhibitory, imunosupresiva, beta blokátory či antineoplastika. Např. společné podání kardioglykosidu digoxinu s inhibitorem ABCB1, chinidinem, zvyšuje biodostupnost

digoxinu a zároveň snižuje jeho eliminaci žlučí a močí, což je zapříčiněno inhibicí ABCB1 v tenkém střevě, játrech a ledvinách [89-91]. Změny farmakokinetických parametrů, včetně clearance digoxinu, byly popsány i po podání společně s dalšími látkami interagujícími s ABCB1 [92, 93]. ABCB1 je transportér, který pravděpodobně nejvíce ovlivňuje přestup xenobiotik přes hematoencefalickou bariéru a zabraňuje přestupu látek do mozku. Studie s morfinem prokázala, že u ABCB1-deficientních myší prochází morfin do mozku více, což vede k vyššímu analgetickému efektu [94]. Stejný efekt má i inhibice ABCB1 pomocí cyklosporinu A či elakridaru [95]. Inhibice ABCG2 je relevantní hlavně v gastrointestinálním traktu. Biodostupnost topotekanu (substrátu ABCG2) se po společném podání s inhibitorem elakridarem dvojnásobně zvýšila [96]. U Abcg2 knockout myší byl po perorálním podání zaznamenán až stonásobný vzestup systémové koncentrace sulfasalazinu ve srovnání s wild-type myšmi [97]. Stejně jako ABCB1, i ABCG2 je exprimován v hematoencefalické bariéře a podílí se tak na ochraně citlivé tkáně mozku [98]. Jelikož oba transportéry přenášejí široké spektrum substrátů, je zřejmé, že mnoho léčiv či doplňků stravy s těmito transportéry interaguje jako substráty, inhibitory či induktory, což vede k vysokému riziku vzájemného ovlivnění farmakokinetiky, efektivity léčby a bezpečnosti při kombinovaném podání [99].

3.7 Přehled inhibitorů cyklin-dependentních kináz studovaných v rámci této dizertační práce

V současné době je mnoho látek ze skupiny CDKI v preklinickém a některé z nich i v klinickém hodnocení. Pro účel studia interakcí s ABC transportéry byly v rámci této dizertační práce vybrány purinové CDKI olomoucin II, purvalanol A a roskovitin, u nichž jsme se zaměřili na doplnění jejich interakčního potenciálu o transportér ABCB1 a navázali jsme tak na podobnou studii naší výzkumné skupiny, kdy jsme studovali interakce CDKI s ABCG2 [22]. Ostatní studované CDKI (flavopiridol, SNS-032, AT-7519, dinaciclib a palbociclib) byly vybrány z toho důvodu, že se již nacházejí v některé z fází klinického hodnocení.

3.7.1 Olomoucin II

Olomoucin II je trisubstituovaný derivát purinu, který inhibuje CDK1, 2, 4, 7 a 9 a jeho cytotoxická aktivita je závislá na expresi tumor supresorového genu p53 v lidských

nádorových buňkách [100-102]. Kromě protinádorové aktivity, vykazuje olomoucin II i antivirový účinek [103]. Je metabolizován cytochromem CYP3A4 a inhibuje aktivitu CYP1A2, CYP2C9 a CYP3A4 [104]. Olomoucin II nebyl nikdy testován v klinických studiích.

Olomoucin II inhiboval transport fluorescenčních a radioaktivně značených substrátů zprostředkovaný transportérem ABCG2, což vedlo k synergickému protinádorovému efektu při podání s antineoplastiky, které jsou zároveň substráty těchto dvou transportérů [22]. Olomoucin II je navíc duálním substrátem ABCB1 i ABCG2, což může vést k jeho omezené akumulaci v nádorových tkáních či lékovým interakcím [105].

3.7.2 Purvalanol A

Purvalanol A, strukturně podobný olomoucinu II, je trisubstituovaný derivát purinu s vysokou selektivitou pro inhibici CDK1 a 2 a žádným efektem vůči dalším proteinovým kinázám. Protinádorová aktivity purvalanolu A byla testována na široké škále 60 lidských nádorových buněčných linií s průměrnou IC_{50} = 2 μM [106, 107]. Stejně jako olomoucin II ani purvalanol A nebyl nikdy testován v klinickém zkoušení.

Purvalanol A inhibuje transport fluorescenčních a radioaktivně značených substrátů zprostředkovaný transportérem ABCG2 v MDCKII-ABCG2 buňkách [22, 108]. Inhibice ABCG2 byla potvrzena na orgánové úrovni s použitím modelu duálně perfundované potkaní placenty, když došlo k výraznému snížení transportu zprostředkovaného placentárním Abcg2 [22]. Schopnost purvalanolu A inhibovat ABCG2 vedla k synergickému efektu v nádorových buňkách při současném podání s chemoterapeutiky, které jsou substráty tohoto transportéru. Značnou výhodou purvalanolu A je to, že není substrátem ABCB1 ani ABCG2, což bylo potvrzeno *in situ*, a nevzniká na něj tedy rezistence spojená s ABC transportéry [105].

3.7.3 Roskovitin

Roskovitin (CYC-202, seliciclib) je trisubstituovaný analog purinu s inhibiční aktivitou vůči CDK1, 2, 5, 7 a 9. Je to nové potenciální léčivo pro léčbu nádorových či virových onemocnění, zánětu a neurodegenerativních onemocnění. Roskovitin

navozuje zástavu buněčného cyklu a s tím spojenou apoptózu v různých nádorových buněčných liniích (průměrná hodnota IC_{50} = 15 μM) [109, 110] i v nádorových štěpech u myší [111, 112]. Momentálně je roskovitin ve fázi I a II klinického hodnocení pro léčbu Cushingovy nemoci (tabulka 2).

Interakcemi roskovitinu s ABC transportéry se vyčerpávajícím způsobem věnovala práce Rajnai a kol. [113]. V této studii roskovitin zvyšoval vanadát-senzitivní ATPázovou aktivitu v membránových veziklech připravených z buněk Sf9 exprimujících lidský transportér ABCB1, což značí, že tato látka je jeho substrátem. Substrátová afinita roskovitinu byla potvrzena i v buňkách MDCKII-ABCB1 pomocí metody transportu přes buněčnou monovrstvu. Dále bylo prokázáno, že roskovitin je inhibitorem lidských transportérů ABCB1, ABCG2 a ABCC1 a že nemá efekt na ABCC2. Rezistence buněk vůči roskovitinu navíc pravděpodobně není způsobená expresí ABCB1 a exprese tohoto transportéru se po podání roskovitinu v buňkách nezvyšuje [113, 114].

3.7.4 Flavopiridol

Flavopiridol (alvocidib, L86-8275) je polosyntetický flavon s vysokou inhibiční aktivitou vůči CDK1, 2, 4, 6, 7 a 9. Patří do první generace CDKI, potlačuje růst mnoha lidských nádorových buněčných linií *in vitro* [115-118] a v preklinických studiích navozuje selektivní apoptózu v brzlíku, slezině a lymfatických uzlinách normální i nádorové tkáně [119]. Flavopiridol je prvním CDKI, který se dostal do klinického hodnocení a momentálně se nachází v jeho druhé fázi pro léčbu solidních nádorů a hlavně hematologických malignit (tabulka 2). Některé klinické studie poukazují na fakt, že flavopiridol může synergicky zvyšovat účinnost jiných chemoterapeutik podaných v kombinaci [120-122].

Zhou a kol. [123] prokázali, že myší Abcg2 a Abcb1 snižují přestup flavopiridolu do mozku. Flavopiridol navíc vykazuje zvýšený transport v bazolaterálně-apikálním směru v MDCK a LLC-PK1 buněčných liniích exprimujících myší nebo lidský ABCB1 a ABCG2, což naznačuje jeho substrátovou afinitu vůči oběma transportérům [123-125]. Přestup flavopiridolu přes střevní bariéru může být také značně omezen z důvodu přítomnosti ABCB1 v tenkém střevě, což bylo prokázáno na buněčné linii Caco-2 [126]. Co se týče rezistence vůči flavopiridolu v nádorových buňkách, je za ni zodpovědný hlavně transportér ABCG2 a z menší míry ABCC1 [88, 124, 127-129]. Flavopiridol

(v mikromolární koncentraci) inhibuje transport substrátů zprostředkovaný ABCB1 a ABCC1, což může vést k synergickému cytotoxickému efektu při podání s odpovídajícími substráty [88, 127].

3.7.5 SNS-032

SNS-032 (BMS-387032) je látka syntetizována primárně jako inhibitor CDK2 s protinádorovou aktivitou vyšší ve srovnání s flavopiridolem [130]. Kromě CDK2 ovšem tato látka silně inhibuje CDK7 a 9 a dále i další CDK. Dosud byla látka SNS-032 hodnocena v klinických studiích I. fáze (tabulka 2), které byly ukončeny z důvodu vysoké toxicity a nežádoucích účinků [131, 132].

V pokusech na buněčné linii Caco-2 vykazovala tato látka vyšší propustnost v bazolaterálně-apikálním směru, což naznačuje, že SNS-032 je substrátem apikálně orientovaného efluxního transportéru ve střevě. Tato informace může pomoci vysvětlit, proč má SNS-032 nízkou biodostupnost po perorálním podání. Další studie na myších modelech prokázaly, že myši s chybějícím transportérem Abcb1 mají vyšší koncentraci SNS-032 v mozku než myši, jejichž buňky tento transportér exprimují [133].

3.7.6 AT-7519

Látka AT-7519 byla primárně vyvinuta jako silný a specifický inhibitor CDK2 (IC_{50} = 47 nM). Kromě CDK2 inhibuje AT-7519 i další CDK (CDK1, 4, 5, 6 a 9) s hodnotou IC_{50} v rozmezí 1 – 200 nM, ale je neaktivní vůči jiným buněčným kinázám [134]. AT-7519 navozuje zástavu buněčného cyklu a apoptózu v řadě lidských nádorových buněčných linií *in vitro* (IC_{50} = 40 – 940 nM) a v myších s lidskými nádorovými štěpy, kde došlo k potlačení růstu subkutánních nádorů brzké i pozdní fáze [135]. AT-7519 je momentálně ve fázích I a II klinických zkoušek pro léčbu různých typů nádorových onemocnění (tabulka 2). Interakce AT-7519 s ABC transportéry nebyly dosud studovány.

3.7.7 Dinaciclib

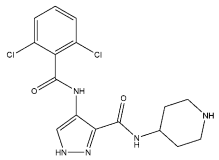
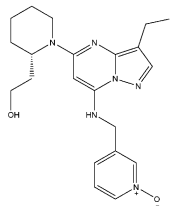
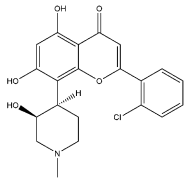
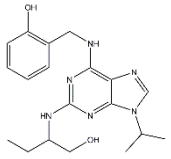
Dinaciclib (MK-7965, SCH727965) je orálně podávaný CDKI se selektivní inhibiční aktivitou vůči CDK1, 2, 5 a 9 [136]. V preklinických studiích inhiboval dinaciclib růst širokého spektra lidských nádorových linií i lidských nádorových štěpů *in vitro* a *in vivo* s vyšší protinádorovou aktivitou ve srovnání se staršími CDKI, flavopiridolem a roskovitinem [137-140]. Přestože je dinaciclib nyní ve třetí fázi klinického hodnocení (tabulka 2), nebyly jeho interakce s ABC transportéry dosud podrobně studovány.

3.7.8 Palbociclib

Palbociclib (PD0332991, IBRANCE®) specificky inhibuje CDK4 a 6 v nanomolárních koncentracích a má zanedbatelný efekt na dalších 36 testovaných proteinových kináz [141, 142]. Tato látka vykazuje silný antiproliferativní účinek proti nádorovým buňkám s vysokou expresí Rb proteinu *in vitro* i *in vivo* [141, 143-146] a byla již testována v klinických studiích pro léčbu různých typů nádorových onemocnění včetně hematologických malignit (tabulka 2) [147, 148]. Nejvyšší terapeutický účinek palbociclibu byl zaznamenán u nádorů prsu [149], což vedlo v únoru 2015 k jeho schválení (jako prvního a zatím jediného CDKI) americkou FDA pro klinické použití v léčbě estrogen-pozitivních karcinomů prsu u postmenopauzálních žen [21].

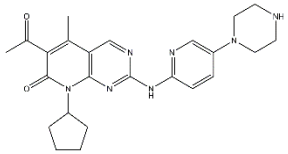
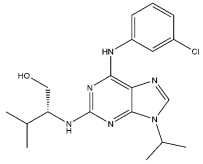
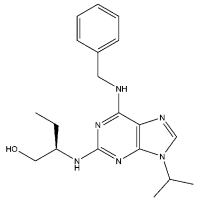
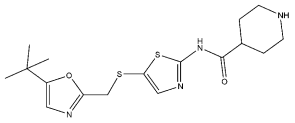
Přestup palbociclibu do mozku je značně omezen transportéry Abcb1 a Abcg2, což bylo prokázáno na modelu knockout myší *in vivo*. *In vitro* transportní studie s využitím koncentračních ekvilibrií v LLC-PK1 a MDCKII buněčných liniích potvrdily, že palbociclib je substrátem myších i lidských transportérů ABCB1 a ABCG2 a inhibitorem myšího Abcg2 [150-152].

Tabulka 2. Přehled studovaných CDKI.

CDKI	Cíl inhibice	Indikace	Fáze klinického hodnocení (celkový počet studií [a])	Chemický vzorec	Známé interakce s ABC transportéry [b]
AT-7519	CDK1, CDK2, CDK4, CDK5, CDK6, CDK9	leukémie, lymfomy, myelomy, solidní nádory	I, II [1, 2] (5)		dosud nebyly popsány žádné interakce
Dinaciclib	CDK1, CDK2, CDK5, CDK9	leukémie, melanomy, myelomy, solidní nádory, nádory prsu a pankreatu	I, II, III [1, 2, 3] (16)		dosud nebyly popsány žádné interakce
Flavopiridol	CDK1, CDK2, CDK4, CDK6, CDK7, CDK9	leukémie, lymfomy, solidní nádory	I, II [1, 2, 3] (61)		ABCB1: S [123. 124. 125] ABCG2: S [123. 125, 127] ABCC1: I [127]
Olomoucin II	CDK1, CDK2, CDK4, CDK7, CDK9	dosud nebyl v klinickém hodnocení	(0)		ABCB1: S [105] ABCG2: S, I [22, 105] ABCC: S [105]

[a] celkový počet studií byl stanoven podle www.clinicaltrials.gov [22]; [1] dokončené, [2] probíhající, [3] přerušené či stáhnuté studie

[b] S – substrát, I – inhibitor

CDKI	Cíl inhibice	Indikace	Fáze klinického hodnocení (celkový počet studií [a])	Chemický vzorec	Známé interakce s ABC transportéry [b]
Palbociclib	CDK4, CDK6	nádory prsu a plic, solidní nádory	I, II, III [1, 2, 3] (66)		ABCB1: S [150, 151, 152] ABCG2: S, I [150, 151, 152]
Purvalanol A	CDK1, CDK2	dosud nebyl v klinickém hodnocení	(0)		ABCG2: S, I [22, 105]
Roskovitin	CDK1, CDK2, CDK5, CDK7, CDK9	Cushingova nemoc, solidní tumory, nádory prsu a plic	I, II [1, 3] (4)		ABCB1: S, I [113] ABCG2: I [22, 113] ABCC1: I [113]
SNS-032	CDK2, CDK7, CDK9	Leukémie, lymfomy, myelomy, solidní nádory	I [1] (2)		ABCB1: S, I [133]

[a] celkový počet studií byl stanoven podle www.clinicaltrials.gov [22]; [1] dokončené, [2] probíhající, [3] přerušené či stáhnuté studie

[b] S – substrát, I – inhibitor

4 Seznam použité literatury

1. WHO, World Cancer Report 2014. 2014, Lyon, France: International Agency for Research on Cancer.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J Clin 2015; 65:5-29.
3. Carvalho C, Santos RX, Cardoso S, Correia S, Oliveira PJ, Santos MS, et al. Doxorubicin: the good, the bad and the ugly effect. Curr Med Chem 2009; 16:3267-85.
4. Guchelaar HJ, ten Napel CH, de Vries EG, Mulder NH. Clinical, toxicological and pharmaceutical aspects of the antineoplastic drug taxol: a review. Clin Oncol (R Coll Radiol) 1994; 6:40-8.
5. Hortobagyi GN. Anthracyclines in the treatment of cancer. An overview. Drugs 1997; 54 Suppl 4:1-7.
6. Vadlapatla RK, Vadlapudi AD, Pal D, Mitra AK. Mechanisms of drug resistance in cancer chemotherapy: coordinated role and regulation of efflux transporters and metabolizing enzymes. Curr Pharm Des 2013; 19:7126-40.
7. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer 2013; 13:714-26.
8. Rochat B. Importance of influx and efflux systems and xenobiotic metabolizing enzymes in intratumoral disposition of anticancer agents. Curr Cancer Drug Targets 2009; 9:652-74.
9. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006; 5:219-34.
10. Noguchi K, Katayama K, Sugimoto Y. Human ABC transporter ABCG2/BCRP expression in chemoresistance: basic and clinical perspectives for molecular cancer therapeutics. Pharmgenomics Pers Med 2014; 7:53-64.
11. Konig J, Muller F, Fromm MF. Transporters and drug-drug interactions: important determinants of drug disposition and effects. Pharmacol Rev 2013; 65:944-66.
12. Szakacs G, Varadi A, Ozvegy-Laczka C, Sarkadi B. The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov Today 2008; 13:379-93.
13. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, et al. Membrane transporters in drug development. Nat Rev Drug Discov 2010; 9:215-36.
14. US Department of Health and Human Services FDA, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research. Guidance for Industry. Drug Interaction Studies - Study Design, Data Analysis, and Implications for Dosing and Labeling. 2006 [citováno 15.12.2015]; dostupné z: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf.
15. European Medicines Agency. Guideline on the investigation of drug interactions. 2012 [citováno 15.12.2015]; dostupné z: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf.
16. Callaghan R, Luk F, Bebawy M. Inhibition of the multidrug resistance P-glycoprotein: time for a change of strategy? Drug Metab Dispos 2014; 42:623-31.

17. Deng J, Shao J, Markowitz JS, An G. ABC transporters in multi-drug resistance and ADME-Tox of small molecule tyrosine kinase inhibitors. Pharm Res 2014; 31:2237-55.
18. Kelly RJ, Robey RW, Chen CC, Draper D, Luchenko V, Barnett D, et al. A pharmacodynamic study of the P-glycoprotein antagonist CBT-1(R) in combination with paclitaxel in solid tumors. Oncologist 2012; 17:512.
19. O'Brien MM, Lacayo NJ, Lum BL, Kshirsagar S, Buck S, Ravindranath Y, et al. Phase I study of valspodar (PSC-833) with mitoxantrone and etoposide in refractory and relapsed pediatric acute leukemia: a report from the Children's Oncology Group. Pediatr Blood Cancer 2010; 54:694-702.
20. Zhang H, Patel A, Ma SL, Li XJ, Zhang YK, Yang PQ, et al. In vitro, in vivo and ex vivo characterization of ibrutinib: a potent inhibitor of the efflux function of the transporter MRP1. Br J Pharmacol 2014; 171:5845-57.
21. Dhillon S. Palbociclib: first global approval. Drugs 2015; 75:543-51.
22. Hofman J, Ahmadimoghaddam D, Hahnova L, Pavek P, Ceckova M, Staud F. Olomoucine II and purvalanol A inhibit ABCG2 transporter in vitro and in situ and synergistically potentiate cytostatic effect of mitoxantrone. Pharmacol Res 2012; 65:312-9.
23. Morgan DO. Cyclin-dependent kinases: engines, clocks, and microprocessors. Annu Rev Cell Dev Biol 1997; 13:261-91.
24. Pavletich NP. Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors. J Mol Biol 1999; 287:821-8.
25. Malumbres M, Barbacid M. Mammalian cyclin-dependent kinases. Trends Biochem Sci 2005; 30:630-41.
26. Golsteyn RM. Cdk1 and Cdk2 complexes (cyclin dependent kinases) in apoptosis: a role beyond the cell cycle. Cancer Lett 2005; 217:129-38.
27. Peters G. The D-type cyclins and their role in tumorigenesis. J Cell Sci Suppl 1994; 18:89-96.
28. Shah K, Lahiri DK. Cdk5 activity in the brain - multiple paths of regulation. J Cell Sci 2014; 127:2391-400.
29. Tadesse S, Yu M, Kumarasiri M, Le BT, Wang S. Targeting CDK6 in Cancer: State of the Art and New Insights. Cell Cycle 2015; 0.
30. Liu Y, Kung C, Fishburn J, Ansari AZ, Shokat KM, Hahn S. Two cyclin-dependent kinases promote RNA polymerase II transcription and formation of the scaffold complex. Mol Cell Biol 2004; 24:1721-35.
31. Nakayama K, Nakayama K. Cip/Kip cyclin-dependent kinase inhibitors: brakes of the cell cycle engine during development. Bioessays 1998; 20:1020-9.
32. MacLachlan TK, Sang N, Giordano A. Cyclins, cyclin-dependent kinases and cdk inhibitors: implications in cell cycle control and cancer. Crit Rev Eukaryot Gene Expr 1995; 5:127-56.
33. Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta 2002; 1602:73-87.
34. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001; 1:222-31.
35. Kashanchi F, Kehn-Hall K. Cyclin dependent kinases as attractive targets to prevent transcription from viral genomes. Curr Pharm Des 2009; 15:2520-32.
36. Chang KH, de Pablo Y, Lee HP, Lee HG, Smith MA, Shah K. Cdk5 is a major regulator of p38 cascade: relevance to neurotoxicity in Alzheimer's disease. J Neurochem 2010; 113:1221-9.

37. Smith PD, Crocker SJ, Jackson-Lewis V, Jordan-Sciutto KL, Hayley S, Mount MP, et al. Cyclin-dependent kinase 5 is a mediator of dopaminergic neuron loss in a mouse model of Parkinson's disease. Proc Natl Acad Sci U S A 2003; 100:13650-5.
38. Sanchez-Martinez C, Gelbert LM, Lallena MJ, de Dios A. Cyclin dependent kinase (CDK) inhibitors as anticancer drugs. Bioorg Med Chem Lett 2015; 25:3420-35.
39. Knockaert M, Greengard P, Meijer L. Pharmacological inhibitors of cyclin-dependent kinases. Trends Pharmacol Sci 2002; 23:417-25.
40. Malumbres M, Pevarello P, Barbacid M, Bischoff JR. CDK inhibitors in cancer therapy: what is next? Trends Pharmacol Sci 2008; 29:16-21.
41. Asghar U, Witkiewicz AK, Turner NC, Knudsen ES. The history and future of targeting cyclin-dependent kinases in cancer therapy. Nat Rev Drug Discov 2015; 14:130-46.
42. Nichols M. New directions for drug-resistant breast cancer: the CDK4/6 inhibitors. Future Med Chem 2015; 7:1473-81.
43. www.clinicaltrials.gov. [citováno 15. 12. 2015].
44. Tsuruo T. Mechanisms of multidrug resistance and implications for therapy. Jpn J Cancer Res 1988; 79:285-96.
45. Gottesman MM. Mechanisms of cancer drug resistance. Annu Rev Med 2002; 53:615-27.
46. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2002; 2:48-58.
47. Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. Call of the wild: antibiotic resistance genes in natural environments. Nat Rev Microbiol 2010; 8:251-9.
48. Linton KJ. Structure and function of ABC transporters. Physiology (Bethesda) 2007; 22:122-30.
49. Wu CP, Hsieh CH, Wu YS. The emergence of drug transporter-mediated multidrug resistance to cancer chemotherapy. Mol Pharm 2011; 8:1996-2011.
50. Dean M, Hamon Y, Chimini G. The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res 2001; 42:1007-17.
51. Li Y, Yuan H, Yang K, Xu W, Tang W, Li X. The structure and functions of P-glycoprotein. Curr Med Chem 2010; 17:786-800.
52. Higgins CF, Gottesman MM. Is the multidrug transporter a flippase? Trends Biochem Sci 1992; 17:18-21.
53. van Helvoort A, Smith AJ, Sprong H, Fritzsche I, Schinkel AH, Borst P, et al. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 1996; 87:507-17.
54. Higgins CF. ABC transporters: physiology, structure and mechanism--an overview. Res Microbiol 2001; 152:205-10.
55. Callaghan R, Crowley E, Potter S, Kerr ID. P-glycoprotein: so many ways to turn it on. J Clin Pharmacol 2008; 48:365-78.
56. Penson RT, Oliva E, Skates SJ, Glyptis T, Fuller AF, Jr., Goodman A, et al. Expression of multidrug resistance-1 protein inversely correlates with paclitaxel response and survival in ovarian cancer patients: a study in serial samples. Gynecol Oncol 2004; 93:98-106.

57. Chan HS, Haddad G, Thorner PS, DeBoer G, Lin YP, Ondrusek N, et al. P-glycoprotein expression as a predictor of the outcome of therapy for neuroblastoma. N Engl J Med 1991; 325:1608-14.
58. Litman T, Brangi M, Hudson E, Fetsch P, Abati A, Ross DD, et al. The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J Cell Sci 2000; 113 (Pt 11):2011-21.
59. Oostendorp RL, Beijnen JH, Schellens JH. The biological and clinical role of drug transporters at the intestinal barrier. Cancer Treat Rev 2009; 35:137-47.
60. Sarkadi B, Orban TI, Szakacs G, Varady G, Schamberger A, Erdei Z, et al. Evaluation of ABCG2 expression in human embryonic stem cells: crossing the same river twice? Stem Cells 2010; 28:174-6.
61. An Y, Ongkeko WM. ABCG2: the key to chemoresistance in cancer stem cells? Expert Opin Drug Metab Toxicol 2009; 5:1529-42.
62. Krishnamurthy P, Xie T, Schuetz JD. The role of transporters in cellular heme and porphyrin homeostasis. Pharmacol Ther 2007; 114:345-58.
63. Noguchi K, Katayama K, Mitsuhashi J, Sugimoto Y. Functions of the breast cancer resistance protein (BCRP/ABCG2) in chemotherapy. Adv Drug Deliv Rev 2009; 61:26-33.
64. Robey RW, Polgar O, Deeken J, To KW, Bates SE. ABCG2: determining its relevance in clinical drug resistance. Cancer Metastasis Rev 2007; 26:39-57.
65. Rosenberg MF, Mao Q, Holzenburg A, Ford RC, Deeley RG, Cole SP. The structure of the multidrug resistance protein 1 (MRP1/ABCC1). crystallization and single-particle analysis. J Biol Chem 2001; 276:16076-82.
66. Cole SP, Deeley RG. Transport of glutathione and glutathione conjugates by MRP1. Trends Pharmacol Sci 2006; 27:438-46.
67. Teodori E, Dei S, Martelli C, Scapecchi S, Gualtieri F. The functions and structure of ABC transporters: implications for the design of new inhibitors of Pgp and MRP1 to control multidrug resistance (MDR). Curr Drug Targets 2006; 7:893-909.
68. Hipfner DR, Deeley RG, Cole SP. Structural, mechanistic and clinical aspects of MRP1. Biochim Biophys Acta 1999; 1461:359-76.
69. Stewart AJ, Canitrot Y, Baracchini E, Dean NM, Deeley RG, Cole SP. Reduction of expression of the multidrug resistance protein (MRP) in human tumor cells by antisense phosphorothioate oligonucleotides. Biochem Pharmacol 1996; 51:461-9.
70. Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, Norris MD. Expression of the multidrug resistance-associated protein (MRP) gene correlates with amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. Cancer Res 1994; 54:5036-40.
71. Nooter K, Brutel de la Riviere G, Look MP, van Wingerden KE, Henzen-Logmans SC, Scheper RJ, et al. The prognostic significance of expression ofthe multidrug resistance-associated protein (MRP) in primary breast cancer. Br J Cancer 1997; 76:486-93.
72. Arts HJ, Katsaros D, de Vries EG, Massobrio M, Genta F, Danese S, et al. Drug resistance-associated markers P-glycoprotein, multidrug resistance-associated protein 1, multidrug resistance-associated protein 2, and lung resistance protein as prognostic factors in ovarian carcinoma. Clin Cancer Res 1999; 5:2798-805.
73. Schinkel AH, Jonker JW. Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. Adv Drug Deliv Rev 2003; 55:3-29.

74. Goldberg RM, Sargent DJ, Morton RF, Fuchs CS, Ramanathan RK, Williamson SK, et al. A randomized controlled trial of fluorouracil plus leucovorin, irinotecan, and oxaliplatin combinations in patients with previously untreated metastatic colorectal cancer. J Clin Oncol 2004; 22:23-30.
75. O'Connor R. A review of mechanisms of circumvention and modulation of chemotherapeutic drug resistance. Curr Cancer Drug Targets 2009; 9:273-80.
76. Shukla S, Wu CP, Ambudkar SV. Development of inhibitors of ATP-binding cassette drug transporters: present status and challenges. Expert Opin Drug Metab Toxicol 2008; 4:205-23.
77. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia in vivo and in vitro through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res 1981; 41:1967-72.
78. Slater LM, Sweet P, Stupecky M, Gupta S. Cyclosporin A reverses vincristine and daunorubicin resistance in acute lymphatic leukemia in vitro. J Clin Invest 1986; 77:1405-8.
79. Raderer M, Scheithauer W. Clinical trials of agents that reverse multidrug resistance. A literature review. Cancer 1993; 72:3553-63.
80. Twentyman PR, Bleehen NM. Resistance modification by PSC-833, a novel non-immunosuppressive cyclosporin [corrected]. Eur J Cancer 1991; 27:1639-42.
81. Benson AB, 3rd, Trump DL, Koeller JM, Egorin MI, Olman EA, Witte RS, et al. Phase I study of vinblastine and verapamil given by concurrent iv infusion. Cancer Treat Rep 1985; 69:795-9.
82. Verweij J, Herweijer H, Oosterom R, van der Burg ME, Planting AS, Seynaeve C, et al. A phase II study of epidoxorubicin in colorectal cancer and the use of cyclosporin-A in an attempt to reverse multidrug resistance. Br J Cancer 1991; 64:361-4.
83. Friedenberg WR, Rue M, Blood EA, Dalton WS, Shustik C, Larson RA, et al. Phase III study of PSC-833 (valspodar) in combination with vincristine, doxorubicin, and dexamethasone (valspodar/VAD) versus VAD alone in patients with recurring or refractory multiple myeloma (E1A95): a trial of the Eastern Cooperative Oncology Group. Cancer 2006; 106:830-8.
84. Mistry P, Stewart AJ, Dangerfield W, Okiji S, Liddle C, Bootle D, et al. In vitro and in vivo reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. Cancer Res 2001; 61:749-58.
85. Fox E, Bates SE. Tariquidar (XR9576): a P-glycoprotein drug efflux pump inhibitor. Expert Rev Anticancer Ther 2007; 7:447-59.
86. Gekeler V, Ise W, Sanders KH, Ulrich WR, Beck J. The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. Biochem Biophys Res Commun 1995; 208:345-52.
87. Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM. Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res 2000; 60:47-50.
88. Hooijberg JH, Broxterman HJ, Scheffer GL, Vrasdonk C, Heijn M, de Jong MC, et al. Potent interaction of flavopiridol with MRP1. Br J Cancer 1999; 81:269-76.
89. Hager WD, Fenster P, Mayersohn M, Perrier D, Graves P, Marcus FI, et al. Digoxin-quinidine interaction Pharmacokinetic evaluation. N Engl J Med 1979; 300:1238-41.
90. Pedersen KE, Christiansen BD, Klitgaard NA, Nielsen-Kudsk F. Effect of quinidine on digoxin bioavailability. Eur J Clin Pharmacol 1983; 24:41-7.

91. Fromm MF, Kim RB, Stein CM, Wilkinson GR, Roden DM. Inhibition of P-glycoprotein-mediated drug transport: A unifying mechanism to explain the interaction between digoxin and quinidine [seecomments]. Circulation 1999; 99:552-7.
92. Fenner KS, Troutman MD, Kempshall S, Cook JA, Ware JA, Smith DA, et al. Drug-drug interactions mediated through P-glycoprotein: clinical relevance and in vitro-in vivo correlation using digoxin as a probe drug. Clin Pharmacol Ther 2009; 85:173-81.
93. Marchetti S, Mazzanti R, Beijnen JH, Schellens JH. Concise review: Clinical relevance of drug drug and herb drug interactions mediated by the ABC transporter ABCB1 (MDR1, P-glycoprotein). Oncologist 2007; 12:927-41.
94. Zong J, Pollack GM. Morphine antinociception is enhanced in mdr1a gene-deficient mice. Pharm Res 2000; 17:749-53.
95. Letrent SP, Pollack GM, Brouwer KR, Brouwer KL. Effects of a potent and specific P-glycoprotein inhibitor on the blood-brain barrier distribution and antinociceptive effect of morphine in the rat. Drug Metab Dispos 1999; 27:827-34.
96. Kruijtzer CM, Beijnen JH, Rosing H, ten Bokkel Huinink WW, Schot M, Jewell RC, et al. Increased oral bioavailability of topotecan in combination with the breast cancer resistance protein and P-glycoprotein inhibitor GF120918. J Clin Oncol 2002; 20:2943-50.
97. Zaher H, Khan AA, Palandra J, Brayman TG, Yu L, Ware JA. Breast cancer resistance protein (Bcrp/abcg2) is a major determinant of sulfasalazine absorption and elimination in the mouse. Mol Pharm 2006; 3:55-61.
98. Enokizono J, Kusuhara H, Ose A, Schinkel AH, Sugiyama Y. Quantitative investigation of the role of breast cancer resistance protein (Bcrp/Abcg2) in limiting brain and testis penetration of xenobiotic compounds. Drug Metab Dispos 2008; 36:995-1002.
99. Zhou SF. Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition. Xenobiotica 2008; 38:802-32.
100. Krystof V, Lenobel R, Havlicek L, Kuzma M, Strnad M. Synthesis and biological activity of olomoucine II. Bioorg Med Chem Lett 2002; 12:3283-6.
101. Krystof V, McNae IW, Walkinshaw MD, Fischer PM, Muller P, Vojtesek B, et al. Antiproliferative activity of olomoucine II, a novel 2,6,9-trisubstituted purine cyclin-dependent kinase inhibitor. Cell Mol Life Sci 2005; 62:1763-71.
102. Paprskarova M, Krystof V, Jorda R, Dzubak P, Hajduch M, Wesierska-Gadek J, et al. Functional p53 in cells contributes to the anticancer effect of the cyclin-dependent kinase inhibitor roscovitine. J Cell Biochem 2009; 107:428-37.
103. Holcakova J, Tomasec P, Bugert JJ, Wang EC, Wilkinson GW, Hrstka R, et al. The inhibitor of cyclin-dependent kinases, olomoucine II, exhibits potent antiviral properties. Antivir Chem Chemother 2010; 20:133-42.
104. Siller M, Anzenbacher P, Anzenbacherova E, Dolezal K, Popa I, Strnad M. Interactions of olomoucine II with human liver microsomal cytochromes P450. Drug Metab Dispos 2009; 37:1198-202.
105. Hofman J, Kucera R, Neumanova Z, Klimes J, Ceckova M, Staud F. Placental passage of olomoucine II, but not purvalanol A, is affected by p-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug resistance-associated proteins (ABCCs). Xenobiotica 2015; 1-8.

106. Gray NS, Wodicka L, Thunnissen AM, Norman TC, Kwon S, Espinoza FH, et al. Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. Science 1998; 281:533-8.
107. Villerbu N, Gaben AM, Redeuilh G, Mester J. Cellular effects of purvalanol A: a specific inhibitor of cyclin-dependent kinase activities. Int J Cancer 2002; 97:761-9.
108. An R, Hagiya Y, Tamura A, Li S, Saito H, Tokushima D, et al. Cellular phototoxicity evoked through the inhibition of human ABC transporter ABCG2 by cyclin-dependent kinase inhibitors in vitro. Pharm Res 2009; 26:449-58.
109. Meijer L, Borgne A, Mulner O, Chong JP, Blow JJ, Inagaki N, et al. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur J Biochem 1997; 243:527-36.
110. McClue SJ, Blake D, Clarke R, Cowan A, Cummings L, Fischer PM, et al. In vitro and in vivo antitumor properties of the cyclin dependent kinase inhibitor CYC202 (R-roscovitine). Int J Cancer 2002; 102:463-8.
111. Appleyard MV, O'Neill MA, Murray KE, Paulin FE, Bray SE, Kernohan NM, et al. Seliciclib (CYC202, R-roscovitine) enhances the antitumor effect of doxorubicin in vivo in a breast cancer xenograft model. Int J Cancer 2009; 124:465-72.
112. Tirado OM, Mateo-Lozano S, Notario V. Roscovitine is an effective inducer of apoptosis of Ewing's sarcoma family tumor cells in vitro and in vivo. Cancer Res 2005; 65:9320-7.
113. Rajnai Z, Mehn D, Beery E, Okyar A, Jani M, Toth GK, et al. ATP-binding cassette B1 transports seliciclib (R-roscovitine), a cyclin-dependent kinase inhibitor. Drug Metab Dispos 2010; 38:2000-6.
114. Wartenberg M, Fischer K, Hescheler J, Sauer H. Modulation of intrinsic P-glycoprotein expression in multicellular prostate tumor spheroids by cell cycle inhibitors. Biochim Biophys Acta 2002; 1589:49-62.
115. Sedlacek H, Czech J, Naik R, Kaur G, Worland P, Losiewicz M, et al. Flavopiridol (L86 8275; NSC 649890), a new kinase inhibitor for tumor therapy. Int J Oncol 1996; 9:1143-68.
116. Drees M, Dengler WA, Roth T, Labonte H, Mayo J, Malspeis L, et al. Flavopiridol (L86-8275): selective antitumor activity in vitro and activity in vivo for prostate carcinoma cells. Clin Cancer Res 1997; 3:273-9.
117. Shapiro GI, Koestner DA, Matranga CB, Rollins BJ. Flavopiridol induces cell cycle arrest and p53-independent apoptosis in non-small cell lung cancer cell lines. Clin Cancer Res 1999; 5:2925-38.
118. Wirger A, Perabo FG, Burgemeister S, Haase L, Schmidt DH, Doehn C, et al. Flavopiridol, an inhibitor of cyclin-dependent kinases, induces growth inhibition and apoptosis in bladder cancer cells in vitro and in vivo. Anticancer Res 2005; 25:4341-7.
119. Arguello F, Alexander M, Sterry JA, Tudor G, Smith EM, Kalavar NT, et al. Flavopiridol induces apoptosis of normal lymphoid cells, causes immunosuppression, and has potent antitumor activity In vivo against human leukemia and lymphoma xenografts. Blood 1998; 91:2482-90.
120. Karp JE, Passaniti A, Gojo I, Kaufmann S, Bible K, Garimella TS, et al. Phase I and pharmacokinetic study of flavopiridol followed by 1-beta-D-arabinofuranosylcytosine and mitoxantrone in relapsed and refractory adult acute leukemias. Clin Cancer Res 2005; 11:8403-12.

121. Karp JE, Smith BD, Levis MJ, Gore SD, Greer J, Hattenburg C, et al. Sequential flavopiridol, cytosine arabinoside, and mitoxantrone: a phase II trial in adults with poor-risk acute myelogenous leukemia. Clin Cancer Res 2007; 13:4467-73.
122. Dispenzieri A, Gertz MA, Lacy MQ, Geyer SM, Fitch TR, Fenton RG, et al. Flavopiridol in patients with relapsed or refractory multiple myeloma: a phase 2 trial with clinical and pharmacodynamic end-points. Haematologica 2006; 91:390-3.
123. Zhou L, Schmidt K, Nelson FR, Zelesky V, Troutman MD, Feng B. The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic acid (PF-407288) in mice. Drug Metab Dispos 2009; 37:946-55.
124. Boerner SA, Tourne ME, Kaufmann SH, Bible KC. Effect of P-glycoprotein on flavopiridol sensitivity. Br J Cancer 2001; 84:1391-6.
125. Kodaira H, Kusuhara H, Ushiki J, Fuse E, Sugiyama Y. Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. J Pharmacol Exp Ther 2010; 333:788-96.
126. Xia B, Liu X, Zhou Q, Feng Q, Li Y, Liu W, et al. Disposition of orally administered a promising chemotherapeutic agent flavopiridol in the intestine. Drug Dev Ind Pharm 2013; 39:845-53.
127. Robey RW, Medina-Perez WY, Nishiyama K, Lahusen T, Miyake K, Litman T, et al. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res 2001; 7:145-52.
128. Bible KC, Boerner SA, Kirkland K, Anderl KL, Bartelt D, Jr., Svingen PA, et al. Characterization of an ovarian carcinoma cell line resistant to cisplatin and flavopiridol. Clin Cancer Res 2000; 6:661-70.
129. Nakanishi T, Karp JE, Tan M, Doyle LA, Peters T, Yang W, et al. Quantitative analysis of breast cancer resistance protein and cellular resistance to flavopiridol in acute leukemia patients. Clin Cancer Res 2003; 9:3320-8.
130. Misra RN, Xiao HY, Kim KS, Lu S, Han WC, Barbosa SA, et al. N-(cycloalkylamino)acyl-2-aminothiazole inhibitors of cyclin-dependent kinase 2. N-[5-[[[5-(1,1-dimethylethyl)-2-oxazolyl]methyl]thio]-2-thiazolyl]-4-piperidinecarboxamide (BMS-387032), a highly efficacious and selective antitumor agent. J Med Chem 2004; 47:1719-28.
131. Heath EI, Bible K, Martell RE, Adelman DC, Lorusso PM. A phase 1 study of SNS-032 (formerly BMS-387032), a potent inhibitor of cyclin-dependent kinases 2, 7 and 9 administered as a single oral dose and weekly infusion in patients with metastatic refractory solid tumors. Invest New Drugs 2008; 26:59-65.
132. Tong WG, Chen R, Plunkett W, Siegel D, Sinha R, Harvey RD, et al. Phase I and pharmacologic study of SNS-032, a potent and selective Cdk2, 7, and 9 inhibitor, in patients with advanced chronic lymphocytic leukemia and multiple myeloma. J Clin Oncol 2010; 28:3015-22.
133. Kamath AV, Chong S, Chang M, Marathe PH. P-glycoprotein plays a role in the oral absorption of BMS-387032, a potent cyclin-dependent kinase 2 inhibitor, in rats. Cancer Chemother Pharmacol 2005; 55:110-6.
134. Wyatt PG, Woodhead AJ, Berdini V, Boulstridge JA, Carr MG, Cross DM, et al. Identification of N-(4-piperidinyl)-4-(2,6-dichlorobenzoylamino)-1H-pyrazole-

3-carboxamide (AT7519), a novel cyclin dependent kinase inhibitor using fragment-based X-ray crystallography and structure based drug design. J Med Chem 2008; 51:4986-99.
135. Squires MS, Feltell RE, Wallis NG, Lewis EJ, Smith DM, Cross DM, et al. Biological characterization of AT7519, a small-molecule inhibitor of cyclin-dependent kinases, in human tumor cell lines. Mol Cancer Ther 2009; 8:324-32.
136. Parry D, Guzi T, Shanahan F, Davis N, Prabhavalkar D, Wiswell D, et al. Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor. Mol Cancer Ther 2010; 9:2344-53.
137. Fu W, Ma L, Chu B, Wang X, Bui MM, Gemmer J, et al. The cyclin-dependent kinase inhibitor SCH 727965 (dinacliclib) induces the apoptosis of osteosarcoma cells. Mol Cancer Ther 2011; 10:1018-27.
138. Gorlick R, Kolb EA, Houghton PJ, Morton CL, Neale G, Keir ST, et al. Initial testing (stage 1) of the cyclin dependent kinase inhibitor SCH 727965 (dinaciclib) by the pediatric preclinical testing program. Pediatr Blood Cancer 2012; 59:1266-74.
139. Gregory GP, Hogg SJ, Kats LM, Vidacs E, Baker AJ, Gilan O, et al. CDK9 inhibition by dinaciclib potently suppresses Mcl-1 to induce durable apoptotic responses in aggressive MYC-driven B-cell lymphoma in vivo. Leukemia 2015; 29:1437-41.
140. Feldmann G, Mishra A, Bisht S, Karikari C, Garrido-Laguna I, Rasheed Z, et al. Cyclin-dependent kinase inhibitor Dinaciclib (SCH727965) inhibits pancreatic cancer growth and progression in murine xenograft models. Cancer Biol Ther 2011; 12:598-609.
141. Fry DW, Harvey PJ, Keller PR, Elliott WL, Meade M, Trachet E, et al. Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. Mol Cancer Ther 2004; 3:1427-38.
142. Toogood PL, Harvey PJ, Repine JT, Sheehan DJ, VanderWel SN, Zhou H, et al. Discovery of a potent and selective inhibitor of cyclin-dependent kinase 4/6. J Med Chem 2005; 48:2388-406.
143. Marzec M, Kasprzycka M, Lai R, Gladden AB, Wlodarski P, Tomczak E, et al. Mantle cell lymphoma cells express predominantly cyclin D1a isoform and are highly sensitive to selective inhibition of CDK4 kinase activity. Blood 2006; 108:1744-50.
144. Saab R, Bills JL, Miceli AP, Anderson CM, Khoury JD, Fry DW, et al. Pharmacologic inhibition of cyclin-dependent kinase 4/6 activity arrests proliferation in myoblasts and rhabdomyosarcoma-derived cells. Mol Cancer Ther 2006; 5:1299-308.
145. Baughn LB, Di Liberto M, Wu K, Toogood PL, Louie T, Gottschalk R, et al. A novel orally active small molecule potently induces G1 arrest in primary myeloma cells and prevents tumor growth by specific inhibition of cyclin-dependent kinase 4/6. Cancer Res 2006; 66:7661-7.
146. Finn RS, Dering J, Conklin D, Kalous O, Cohen DJ, Desai AJ, et al. PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. Breast Cancer Res 2009; 11:R77.
147. Dickson MA. Molecular pathways: CDK4 inhibitors for cancer therapy. Clin Cancer Res 2014; 20:3379-83.
148. Aleem E, Arceci RJ. Targeting cell cycle regulators in hematologic malignancies. Front Cell Dev Biol 2015; 3:16.

149. Cadoo KA, Gucalp A, Traina TA. Palbociclib: an evidence-based review of its potential in the treatment of breast cancer. Breast Cancer (Dove Med Press) 2014; 6:123-33.
150. de Gooijer MC, Zhang P, Thota N, Mayayo-Peralta I, Buil LC, Beijnen JH, et al. P-glycoprotein and breast cancer resistance protein restrict the brain penetration of the CDK4/6 inhibitor palbociclib. Invest New Drugs 2015;
151. Raub TJ, Wishart GN, Kulanthaivel P, Staton BA, Ajamie RT, Sawada GA, et al. Brain Exposure of Two Selective Dual CDK4 and CDK6 Inhibitors and the Antitumor Activity of CDK4 and CDK6 Inhibition in Combination with Temozolomide in an Intracranial Glioblastoma Xenograft. Drug Metab Dispos 2015; 43:1360-71.
152. Parrish KE, Pokorny J, Mittapalli RK, Bakken K, Sarkaria JN, Elmquist WF. Efflux Transporters at the Blood-Brain Barrier Limit Delivery and Efficacy of Cyclin-Dependent Kinase 4/6 Inhibitor Palbociclib (PD-0332991) in an Orthotopic Brain Tumor Model. J Pharmacol Exp Ther 2015; 355:264-71.

5 Cíle práce

Z předchozí kapitoly vyplývá, že CDKI jsou slibnými látkami, které mohou v budoucnu nalézt uplatnění v protinádorové terapii. Znalosti o jejich interakcích s ABC transportéry jsou však dosud velmi omezené, nicméně nezbytné pro jejich uvedení do racionální farmakoterapie. Hlavním cílem této dizertační práce tedy bylo studium farmakokinetických interakcí vybraných CDKI (konkrétně olomoucinu II, purvalanolu A, roskovitinu, flavopiridolu, SNS-032 AT-7519, dinaciclibu a palbociclibu) s ABC lékovými efluxními transportéry. Dílčími cíli práce přitom bylo:

1) zavedení *in vitro* akumulačních metod pro studium interakcí látek s ABCB1, ABCG2 a ABCC1 transportéry s využitím průtokové cytometrie a běžně používaných cytostatik daunorubicinu a mitoxantronu jako fluorescenčních substrátů,
2) studium inhibice ABCB1, ABCG2 a ABCC1 *in vitro* pomocí akumulačních metod a ATPázových esejí,
3) studium substrátové afinity dinaciclibu, jakožto CDKI ve fázi III klinického hodnocení, vůči ABCB1, ABCG2 a ABCC1 *in vitro* pomocí metody transportu přes buněčnou monovrstvu,
4) studium vlivu inhibice ABC transportérů na překonávání lékové rezistence pomocí XTT testu v MDCKII a nádorových buněčných liniích,
5) studium cytotoxického efektu kombinace CDKI s cytostatiky, která jsou substráty inhibovaných ABC transportérů, v modelových MDCKII a nádorových buněčných liniích.

6 Seznam odborných článků publikovaných v zahraničních časopisech s impakt-faktorem, jejich komentář a podíl kandidátky na jednotlivých publikacích

Tato dizertační práce je předkládána jako komentovaný soubor 4 odborných publikací (6.1 – 6.4), z nichž všechny čtyři jsou otištěny v odborných zahraničních časopisech s impakt-faktorem. Předkladatelka je první autorkou tří (6.2 – 6.4) a spoluautorkou jedné z těchto prací. (6.1) Všechny manuskripty jsou původní experimentální práce zaměřené na popis a vyhodnocení interakcí CDKI s ABC transportéry, které se podílejí na vzniku mnohočetné lékové rezistence.

Podíl kandidátky na jednotlivých publikacích je následující:

6.1 Olomoucine II, but not purvalanol A, is transported by breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1).

- kultivace buněčných linií MDCKII-ABCB1, MDCKII-ABCB1 a MDCKII
- pomoc při transportních experimentech: studium transportu purvalanolu A a olomoucinu II přes buněčnou monovrstvu MDCKII buněk

6.2 Purvalanol A, olomoucine II and roscovitine inhibit ABCB1 transporter and synergistically potentiate cytotoxic effects of daunorubicin *in vitro*.

- kultivace buněčných linií MDCKII-ABCB1, MDCKII, HCT-8 a HepG2
- stanovení exprese ABCB1 transportérů v buněčných liniích metodou qRT-PCR
- stanovení inhibiční aktivity testovaných CDKI vůči ABCB1 pomocí akumulačních metod s fluorescenčními substráty; měření akumulace pomocí průtokového cytometru
- stanovení ATPázové aktivity a cytotoxicity jednotlivých CDKI
- stanovení synergického působení CDKI s konvenčními chemoterapeutiky
- analýza dat a sepsání manuskriptu

6.3 Interactions of cyclin-dependent kinase inhibitors AT-7519, flavopiridol and SNS-032 with ABCB1, ABCG2 and ABCC1 transporters and their potential to overcome multidrug resistance *in vitro*.

- kultivace buněčných linií MDCKII-ABCB1, MDCKII-ABCG2, MDCKII-ABCC1, MDCKII, HepG2 a T47D
- stanovení exprese jednotlivých ABC transportérů v buněčných liniích metodou qRT-PCR
- stanovení inhibiční aktivity testovaných CDKI vůči ABCB1, ABCG2 a ABCC1 pomocí akumulačních metod s fluorescenčními substráty; měření akumulace pomocí průtokového cytometru
- stanovení ATPázové aktivity a cytotoxicity jednotlivých CDKI
- stanovení synergického působení CDKI s konvenčními chemoterapeutiky
- analýza dat a sepsání manuskriptu

6.4 Dinaciclib, a cyclin-dependent kinase inhibitor, is a substrate of human ABCB1 and an inhibitor of human ABCC1 *in vitro*.

- kultivace buněčných linií MDCKII-ABCB1, MDCKII-ABCG2, MDCKII-ABCC1, MDCKII a T47D
- studium transportu dinaciclibu přes buněčnou monovrstvu MDCKII buněk
- stanovení inhibiční aktivity dinaciclibu vůči ABCB1, ABCG2 a ABCC1 pomocí akumulačních metod s fluorescenčními substráty; měření akumulace pomocí průtokového cytometru
- stanovení ATPázové aktivity a cytotoxicity dinaciclibu
- stanovení synergického působení dinaciclibu s konvenčními chemoterapeutiky
- analýza dat a sepsání manuskriptu

6.1 Olomoucine II, but not purvalanol A, is transported by breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1)

Hofman J., Kucera R., Cihalova D., Klimes J., Ceckova M., Staud F.

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V návaznosti na informaci, že olomoucin II a purvalanol A inhibují ABCG2 a synergicky potencují antiproliferativní efekt mitoxantronu (substrátu ABCG2) v nádorových buňkách, jsme v této práci zkoumali, jestli jsou olomoucin II a purvalanol A i transportovanými substráty ABCG2 a ABCB1 transportérů.

Za pomocí monovrstev z buněčné linie MDCKII s expresí lidských transportérů ABCB1 nebo ABCG2 jsme ukázali, že olomoucin II je na rozdíl od purvalanolu A substrátem jak ABCB1, tak i ABCG2. Z tohoto důvodu se dá předpokládat, že farmakokinetika olomoucinu II bude ovlivněna ABCB1 a ABCG2 transportními proteiny, což by mohlo vést ke snížené akumulaci této látky v nádorové tkáni a k lékovým interakcím. Farmakokinetika purvalanolu A zůstane na druhou stranu neovlivněna oběma transportéry a je možné, že purvalanol A bude pro léčbu rezistentních nádorů vhodnější než olomoucin II.

Při transportu olomoucinu II z MDCKII buněk docházelo k intenzivní sulfataci a následnému aktivnímu efluxu jeho sulfatovaného metabolitu ven z buněk. Tato informace by měla být brána v potaz při provádění farmakokinetických studií v MDCKII buňkách, především pokud jsou používány radioaktivní substráty; vytvářený sulfatovaný metabolit může kontaminovat vzorky a vést tak ke zkresleným výsledkům při farmakokinetické analýze. S ohledem na chemickou strukturu olomoucinu II a purvalanolu A můžeme říct, že i látky s poměrně vysokou strukturní podobností mohou vykazovat odlišné farmakokinetické chování, např. interakce s ABC transportéry či biotransformačními enzymy.

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Olomoucine II, but Not Purvalanol A, Is Transported by Breast Cancer Resistance Protein (ABCG2) and P-Glycoprotein (ABCB1)

Jakub Hofman[1], Radim Kučera[2], Daniela Cihalova[1], Jiri Klimes[2], Martina Ceckova[1], Frantisek Staud[1]*

1 Department of Pharmacology and Toxicology, Charles University in Prague, Faculty of Pharmacy, Hradec Kralove, Czech Republic, **2** Department of Pharmaceutical Chemistry and Drug Analysis, Charles University in Prague, Faculty of Pharmacy, Hradec Kralove, Czech Republic

Abstract

Purine cyclin-dependent kinase inhibitors have been recognized as promising candidates for the treatment of various cancers; nevertheless, data regarding interaction of these substances with drug efflux transporters is still lacking. Recently, we have demonstrated inhibition of breast cancer resistance protein (ABCG2) by olomoucine II and purvalanol A and shown that these compounds are able to synergistically potentiate the antiproliferative effect of mitoxantrone, an ABCG2 substrate. In this follow up study, we investigated whether olomoucine II and purvalanol A are transported by ABCG2 and ABCB1 (P-glycoprotein). Using monolayers of MDCKII cells stably expressing human ABCB1 or ABCG2, we demonstrated that olomoucine II, but not purvalanol A, is a dual substrate of both ABCG2 and ABCB1. We, therefore, assume that pharmacokinetics of olomoucine II will be affected by both ABCB1 and ABCG2 transport proteins, which might potentially result in limited accumulation of the compound in tumor tissues or lead to drug-drug interactions. Pharmacokinetic behavior of purvalanol A, on the other hand, does not seem to be affected by either ABCG2 or ABCB1, theoretically favoring this drug in the potential treatment of efflux transporter-based multidrug resistant tumors. In addition, we observed intensive sulfatation of olomoucine II in MDCKII cell lines with subsequent active efflux of the metabolite out of the cells. Therefore, care should be taken when performing pharmacokinetic studies in MDCKII cells, especially if radiolabeled substrates are used; the generated sulfated conjugate may largely contaminate pharmacokinetic analysis and result in misleading interpretation. With regard to chemical structures of olomoucine II and purvalanol A, our data emphasize that even drugs with remarkable structure similarity may show different pharmacokinetic behavior such as interactions with ABC transporters or biotransformation enzymes.

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* E-mail: frantisek.staud@faf.cuni.cz

Introduction

Olomoucine II and purvalanol A are potent cyclin-dependent kinase inhibitors (CDKi) that belong to the group of 2,6,9-trisubstituted purine derivatives [1,2]. These compounds effectively stop cellular proliferation, block transcription of essential genes and induce apoptosis [3–5]. For their favorable pharmacodynamic properties, purine CDKi have become modern alternatives in cancer therapy [6,7]. Roscovitine (seliciclib, CYC202), a structural analogue of olomoucine II and purvalanol A, has reached phase II trials for treating various cancers [8,9]. Although olomoucine II and purvalanol A are commonly considered selective for cyclin-dependent kinases, several studies have reported their subordinate intracellular targets from the superfamily of protein kinases, which are inhibited by these compounds in the range of micromolar concentrations [3,10–13]. However, possible interactions with other biological structures, such as drug transporters, have not been properly investigated to date.

ATP-binding cassette transporters (ABC transporters) are membrane proteins that pump many structurally unrelated molecules, including drugs and toxins, out of cells. The most widely studied members of this family, P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2), are abundantly expressed in absorptive and eliminatory organs (e.g. small intestine, liver, kidney) as well as in several blood-tissue barriers (e.g. blood-brain barrier, placenta, blood-testis barrier) playing crucial role in drug disposition [14,15]. In addition, by diminishing intracellular concentrations of chemotherapeutics in cancer cells, ABCB1 and ABCG2 transporters are frequently associated with the multidrug resistance phenomenon [16,17]. Modulation of these transporters is, therefore, of great clinical interest; ABC transporter inhibitors have been investigated for their ability to restore the sensitivity of tumor cells to chemotherapy or to increase oral bioavailability and tissue penetration of ABC transporter substrates [18–20]. Moreover, investigating interactions of novel drug entities with transport proteins is an important issue in drug discovery and development [21].

Recently, we have demonstrated inhibition of ABCG2 by olomoucine II, purvalanol A, bohemine and roscovitine at *in vitro* and *in situ* levels [22]. Olomoucine II and purvalanol A showed

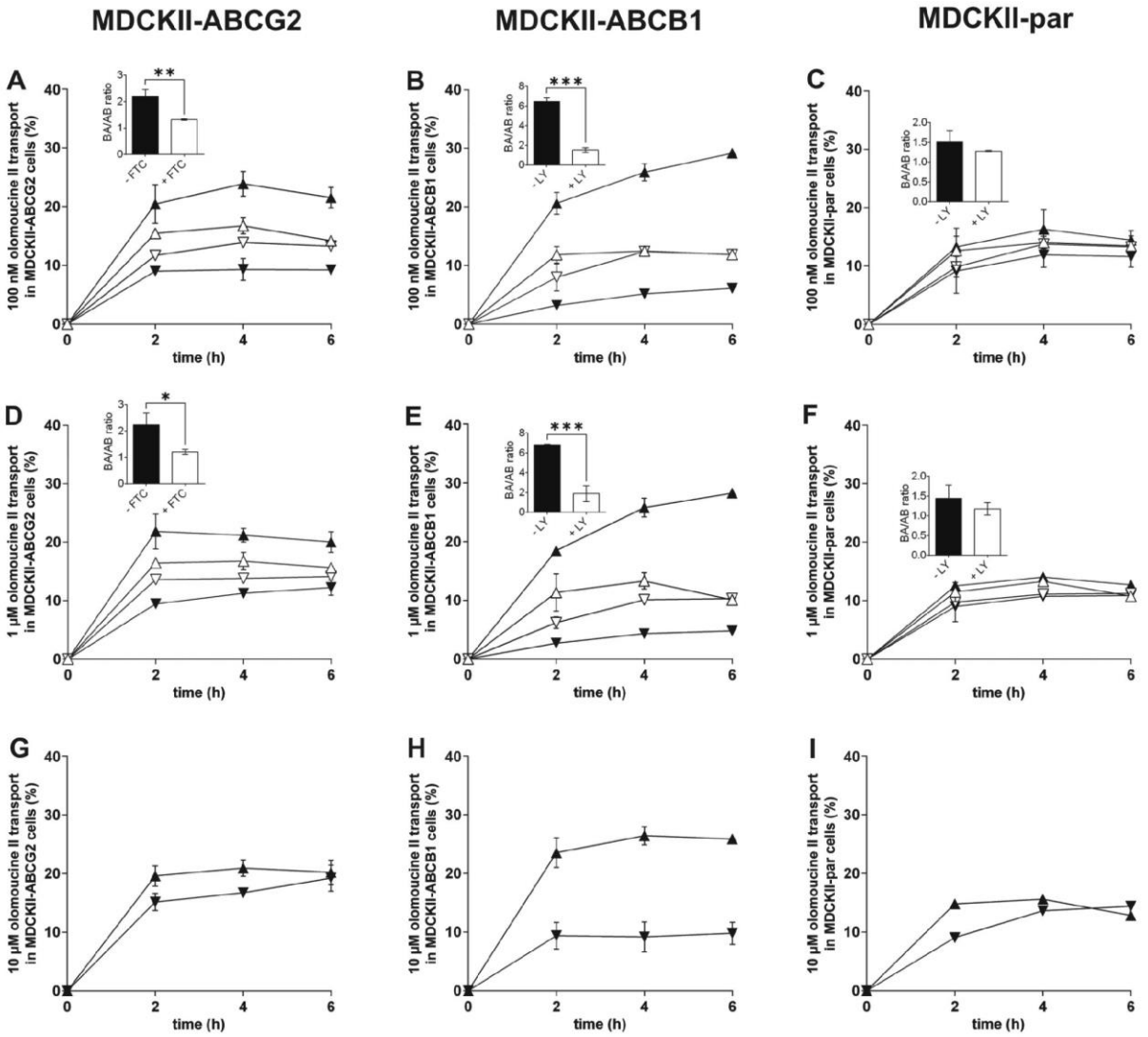

Figure 1. Transport of olomoucine II at concentrations of 100 nM (A, B, C), 1 μM (D, E, F) and 10 μM (G, H, I) across monolayers of MDCKII-ABCG2 (A, D, G), MDCKII-ABCB1 (B, E, H) and MDCKII-par (C, F, I) cells. 5 μM fumitremorgin C (FTC) was used as a specific ABCG2 inhibitor in MDCKII-ABCG2 cells. 1 μM LY335979 (LY) was employed as a specific ABCB1 and endogenous canine Abcb1 inhibitor in MDCKII-ABCB1 and MDCKII-par cells, respectively. Ratios of olomoucine II transport across cell monolayers (olomoucine II transport in basolateral to apical direction divided by transport in apical to basolateral direction) with or without inhibitor were calculated two hours after olomoucine II addition and statistically compared (see insets). Due to the generation of sulfated conjugate of olomoucine II, transport ratios were determined at 2 h interval to reduce the misrepresenting effect of the metabolite. In basolateral to apical transport direction, olomoucine II was added into the basolateral compartment and its concentrations were determined in the apical compartment. In the opposite transport direction, olomoucine II was applied into the apical compartment and its concentrations were analyzed in the basolateral compartment. ▲, basolateral to apical transport without inhibitor; ▼, apical to basolateral transport without inhibitor; △, basolateral to apical transport with inhibitor; ▽, apical to basolateral transport with inhibitor. Data are expressed as means ± SD of three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$.
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comparable or even higher potency than fumitremorgin C, a model specific ABCG2 inhibitor. Moreover, using combination method of Chou-Talalay, we demonstrated that these compounds can synergistically potentiate the antiproliferative effect of mitoxantrone, an ABCG2 substrate, in ABCG2-expressing cell lines [22]. In the present paper, we employed transport assays in MDCKII cells stably expressing ABCG2 or ABCB1 to investigate whether transcellular passage of olomoucine II and purvalanol A is affected by these transporters.

Materials and Methods

Reagents and Chemicals

Olomoucine II and purvalanol A were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific ABCG2 inhibitor, fumitremorgin C, was supplied by Alexis Corporation (Lausanne,

Switzerland). Specific ABCB1 inhibitor, LY335979, was obtained from Toronto Research Chemicals (North York, ON, Canada). Cell culture reagents were obtained from Sigma Aldrich (St. Louis, MO, USA) and from Gibco BRL Life Technologies (Rockville, MD, USA). Fluorescein isothiocyanate labeled dextran was from Sigma-Aldrich (St. Louis, MO, USA). All other compounds and agents were of analytical grade.

Cell Cultures

ABCG2- and ABCB1-transduced MDCKII sublines (MDCKII-ABCG2 and MDCKII-ABCB1), which stably express ABCG2 and ABCB1 protein, respectively, were purchased from dr. Alfred Schinkels lab (The Netherlands Cancer Institute, Amsterdam, The Netherlands). These transduced sublines as well as the parental MDCKII cell line (MDCKII-par) were routinely cultured in complete Dulbecco's modified Eagle's medium with 10% fetal bovine serum. 100 U/ml penicillin and 100 μg/ml streptomycin were used while growing the cells on the membrane inserts. All cells were routinely cultivated in antibiotic-free medium and periodically tested for mycoplasma contamination. Stable expression of ABCB1 and ABCG2 was verified by qRT-PCR method and by daunorubicin and mitoxantrone efflux activity, respectively. Cells from passages 15 to 25 were used in all *in vitro* studies. Dimethyl sulfoxide was applied as a CDKi solvent in concentrations not exceeding 0.1%.

Cellular Monolayer Transport Assay

Transport assays were performed on microporous polycarbonate membrane inserts (3 μm pore size, 24 mm diameter; Transwell 3414, Costar, Cambridge, MA, USA) as described previously [22]. MDCKII-ABCG2, MDCKII-ABCB1 or MDCKII-par cells were seeded at a density of 1×10^6 per insert 72 h before experiment. The medium was replaced after 24 and 48 h of cultivation. One hour before the start of the experiment, the cells were washed with prewarmed 1×phospate buffered saline on both the apical and basal sides and Opti-MEM with or without fumitremorgin C or LY335979 was added into both compartments. At time 0, the experiment was started by replacing the medium with fresh Opti-MEM with or without CDKi and fumitremorgin C or LY335979 in the appropriate chamber. Samples were taken every 2 h from the opposite chambers for the duration of the experiment (6 h). Concentration of CDKi was determined via HPLC/MS analysis. Immediately after the experiment, cellular monolayer integrity was examined using fluorescein isothiocyanate labeled dextran (MW = 40 kDa). Dextran leakage was accepted up to 1% per hour.

HPLC/MS Analysis

HPLC/MS analysis using LC 20A Prominence chromatographic system (Shimadzu, Kyoto, Japan) coupled with LCQ Max advantage mass spectrometer (Thermo Finnigan, San Jose, CA,

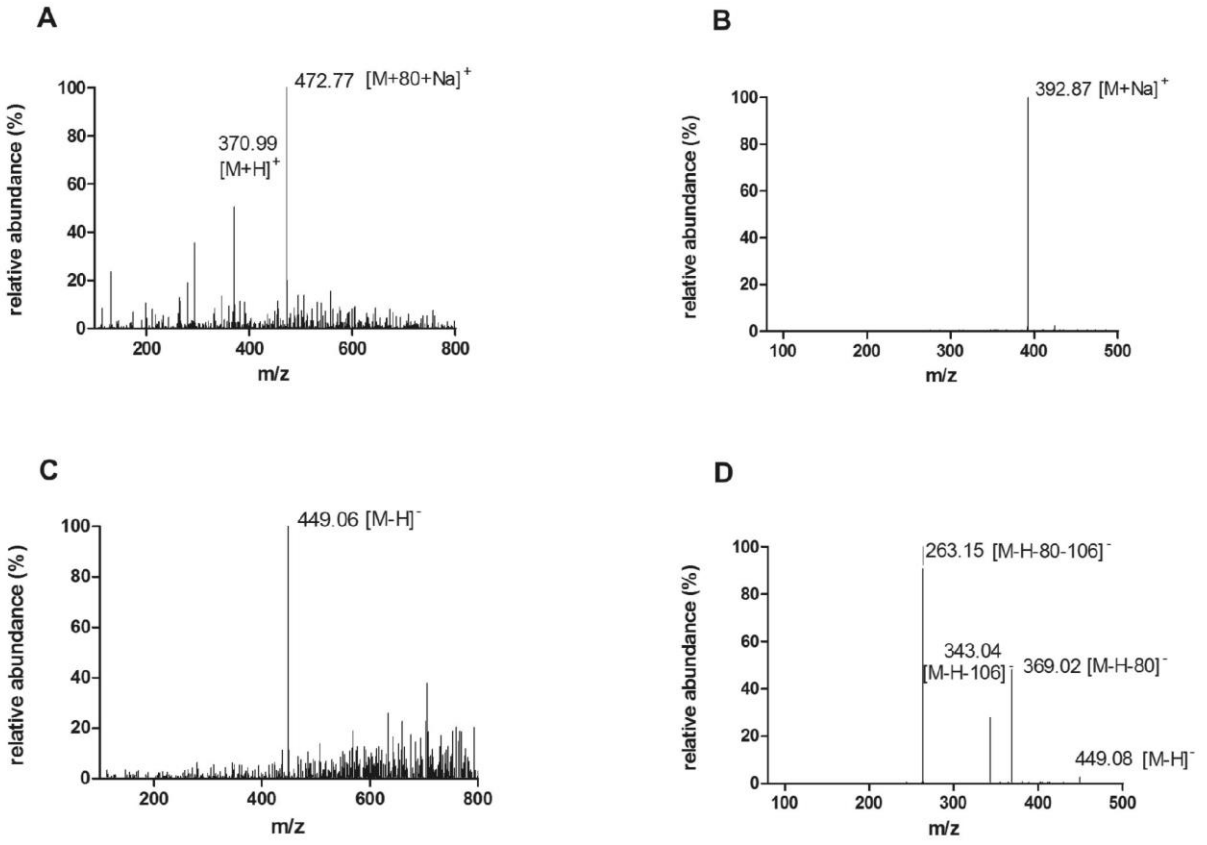

Figure 2. Mass spectra of an unknown peak eluted in the fifth minute of HPLC analysis of olomoucine II transport. (A) spectrum in positive mode, (B) MS^2 in positive mode, (C) negative mode, (D) MS^2 in negative mode. Based on the nominal mass shift (+80 Da) from parent compound and the collision spectra in negative as well as positive mode the compound was identified as a sulfated conjugate of olomoucine II.
doi:10.1371/journal.pone.0075520.g002

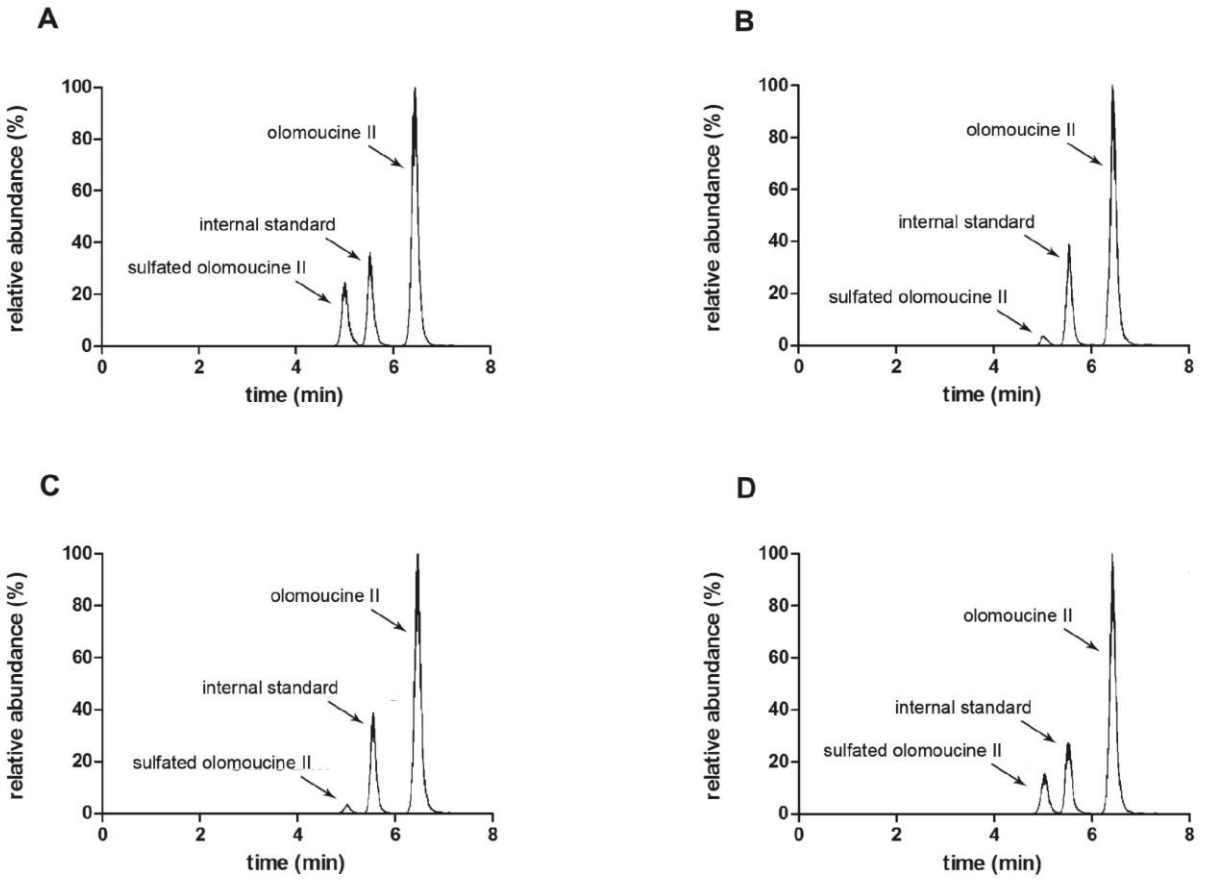

Figure 3. Chromatograms of samples from MDCKII-par cells six hours after olomoucine II addition. (A) olomoucine II was added into apical compartment while olomoucine II and its sulfated conjugate were analyzed in acceptor basolateral compartment, (B) olomoucine II was added into apical compartment while olomoucine II and its sulfated conjugate were analyzed in donor apical compartment, (C) olomoucine II was added into basolateral compartment while olomoucine II and its sulfated conjugate were analyzed in acceptor apical compartment, (D) olomoucine II was added into basolateral compartment while olomoucine II and its sulfated conjugate were analyzed in donor basolateral compartment. This analysis with end point samples was performed for all olomoucine II transport experiments.
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USA) was used for the quantification of olomoucine II and purvalanol A. The separation was performed on a Hypersil GOLD C18 column (100 × 4.6 mm, particle size 3 μm) protected with an OPTI-GUARD 1 mm guard column C18. The mobile phase flow rate was 0.35 ml/min and the column temperature was maintained at 40°C. The data were processed using Xcalibur 2.0 software (Thermo Finnigan, San Jose, CA, USA).

Optimal separation of olomoucine II was achieved in mobile phase containing the mixture of methanol and 0.0125% formic acid (62:38, v/v). Bohemine was added to samples as the internal standard (IS). Retention times were 5.5 and 6.5 min for IS and olomoucine II, respectively. The detector was set as follows: spray voltage of 4.5 kV, capillary temperature of 320°C, sheet and auxiliary gas flows of 30 and 12 arbitrary units, respectively. The chromatograms were recorded in SRM mode using precursor ion at $[M+H]^+$ (*m/z:* 371 olomoucine II and 341 IS) and the product ions 265 (olomoucine II) and 250 (IS) were used for quantification after collision dissociation. The collision energies were 38% and 40% for olomoucine II and IS, respectively. The linearity of the method was evaluated in the range of 5–500 μM ($r^2 = 0.9961$); the method precision and accuracy were evaluated at 500, 100, 10 and 5 nM. The sample stability was evaluated within 94 h.

A mixture of methanol and 0.01% acetic acid (75:25 v/v) was used for the separation of purvalanol A. Roscovitine was utilized as the IS. Retention times were 5.7 and 9.2 min for IS and purvalanol A, respectively. The detector was set as follows: spray voltage of 5.5 kV, capillary temperature of 340°C, sheet and auxiliary gas flows of 28 and 13 arbitrary units, respectively. The chromatograms were recorded in SRM mode using precursor ion at $[M+H]^+$ (*m/z:* 389 purvalanol A and 355 IS) and the product ions 303 (purvalanol A) and 312 (IS) were used for the quantification after collision dissociation. The collision energies were 38% and 40% for purvalanol A and IS, respectively. The linearity of the method was evaluated in the range of 15–384 μM ($r^2 = 0.9925$); the method precision and accuracy were evaluated at 384, 100 and 15 nM. The sample stability was evaluated within 94 hours.

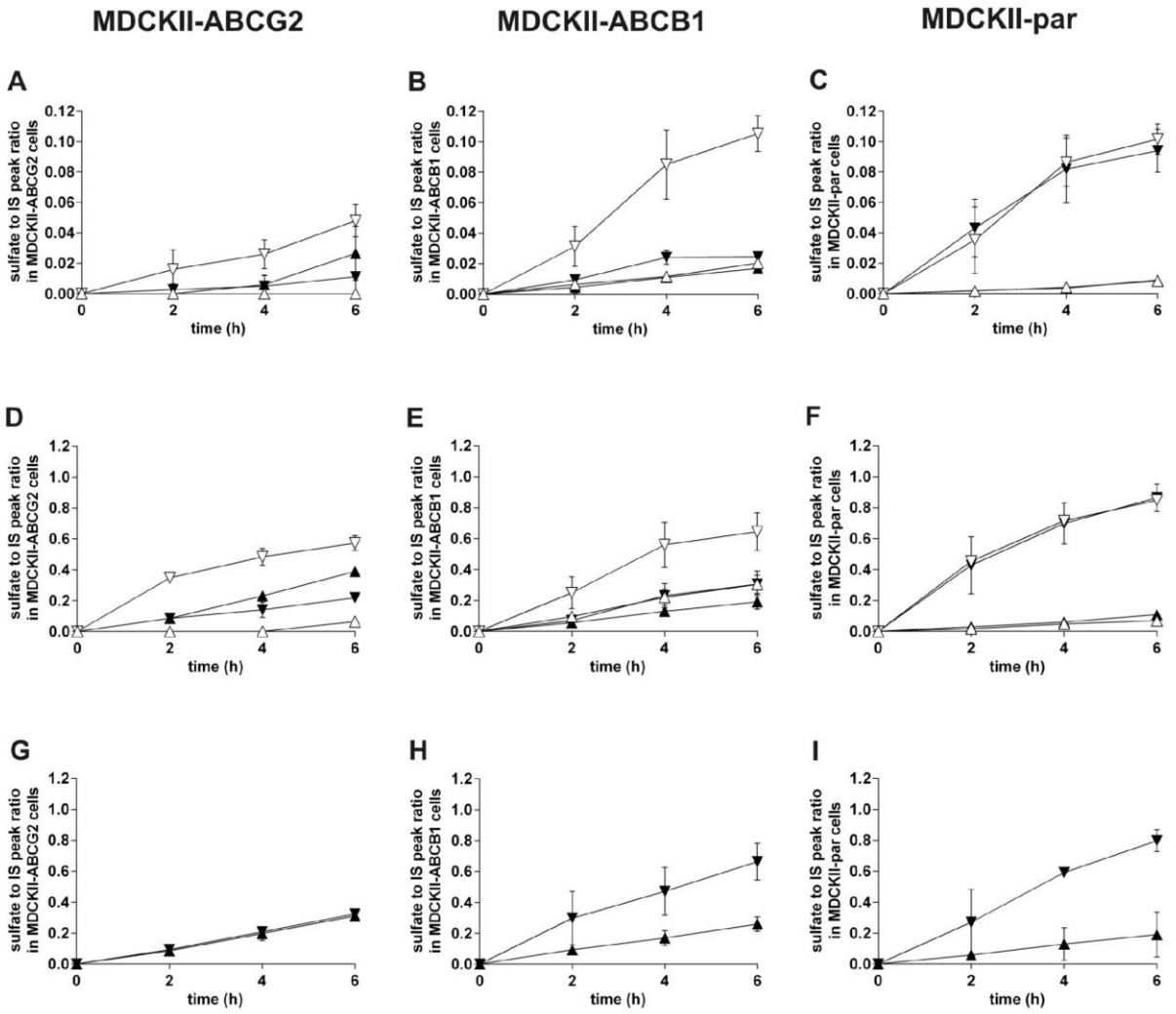

Figure 4. Time-dependent generation of sulfated conjugate of olomoucine II in MDCKII-ABCG2 (A, D, G), MDCKII-ABCB1 (B, E, H) and MDCKII-par (C, F, I) cells and its distribution into the apical and basolateral compartments. Relative quantification of sulfated olomoucine II was calculated as a ratio between peak area of sulfated olomoucine II and the peak area of internal standard (IS). 5 μM fumitremorgin C (FTC), a specific ABCG2 inhibitor, was used in MDCKII-ABCG2 cells for the assessment of possible involvement of ABCG2 in the transport of sulfated metabolite. 1 μM LY335979 (LY) was employed as a specific ABCB1 and endogenous canine Abcb1 inhibitor in MDCKII-ABCB1 and MDCKII-par cells, respectively. Data come from transport experiments with olomoucine II at concentrations of 100 nM (A, B, C), 1 μM (D, E, F) and 10 μM (G, H, I). In basolateral to apical transport direction, olomoucine II was added into the basolateral compartment and its sulfate conjugate was determined in the apical compartment. In the opposite transport direction, olomoucine II was applied into the apical compartment and its sulfated metabolite was analyzed in the basolateral compartment. ▲, transport into apical compartment without inhibitor; ▼, transport into basolateral compartment without inhibitor; △, transport into apical compartment with inhibitor; ▽, transport into basolateral compartment with inhibitor. Values are expressed as means ± SD of three independent experiments.
doi:10.1371/journal.pone.0075520.g004

Statistical Analysis

Student's *t* test was used to assess statistical significance for *in vitro* monolayer transport assays. Differences of $p<0.05$ were considered statistically significant.

Results

Effect of ABCG2 and ABCB1 on the Transepithelial Transport of Olomoucine II *In Vitro*

Transport of olomoucine II by ABCG2 and ABCB1 was tested *in vitro* using transport assays across the polarized monolayers of MDCKII-ABCG2 and MDCKII-ABCB1 cells, respectively. In this method, the transport across the monolayer is greatly accelerated in the basolateral to apical direction, when the compound is a substrate of examined transporter. Based on our

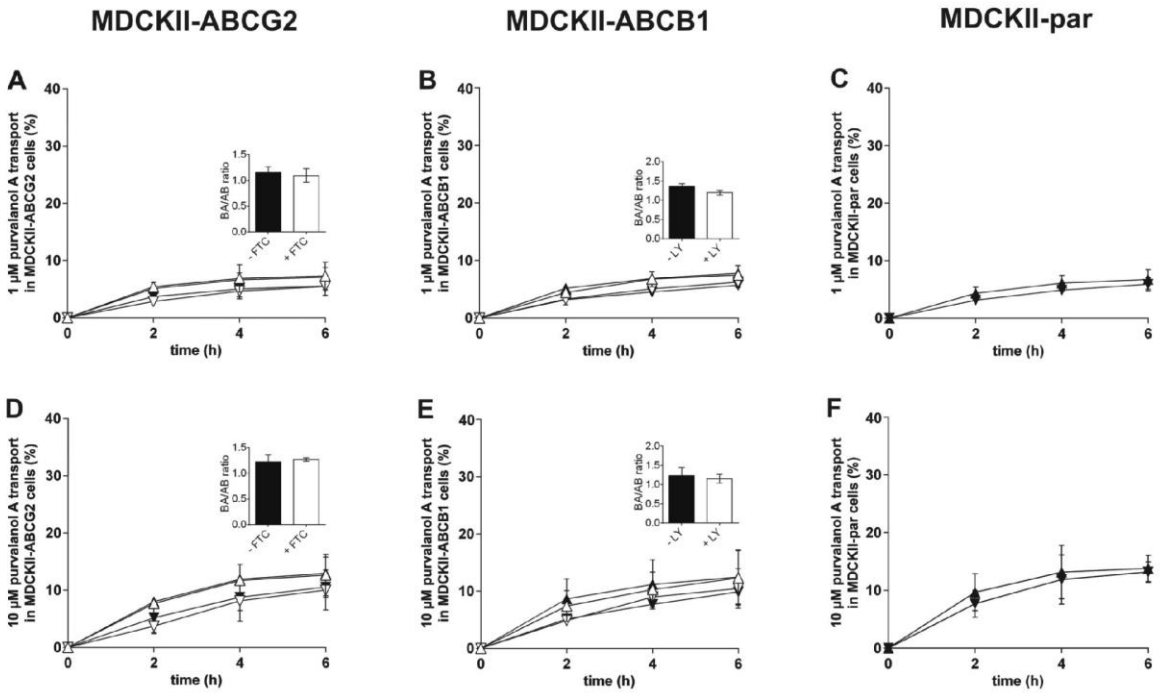

Figure 5. Transport of purvalanol A at concentrations of 1 µM (A, B, C) and 10 µM (D, E, F) across monolayers of MDCKII-ABCG2 (A, D), MDCKII-ABCB1 (B, E) and MDCKII-par (C, F) cells. 5 µM fumitremorgin C (FTC) was used as a specific ABCG2 inhibitor in MDCKII-ABCG2 cells. 1 µM LY335979 (LY) was employed as a specific ABCB1 inhibitor in MDCKII-ABCB1 cells. Ratios of purvalanol A transport across cell monolayers (purvalanol A transport in basolateral to apical direction divided by transport in apical to basolateral direction) with or without inhibitor were calculated and statistically compared (see insets). Transport ratios were determined 6 h after purvalanol A addition. In basolateral to apical transport direction, purvalanol A was added into the basolateral compartment and its concentrations were determined in the apical compartment. In the opposite transport direction, purvalanol A was applied into the apical compartment and its concentrations were analyzed in the basolateral compartment. ▲, basolateral to apical transport without inhibitor; ▼, apical to basolateral transport without inhibitor; △, basolateral to apical transport with inhibitor; ▽, apical to basolateral transport with inhibitor. Data are expressed as means ± SD of three independent experiments.
doi:10.1371/journal.pone.0075520.g005

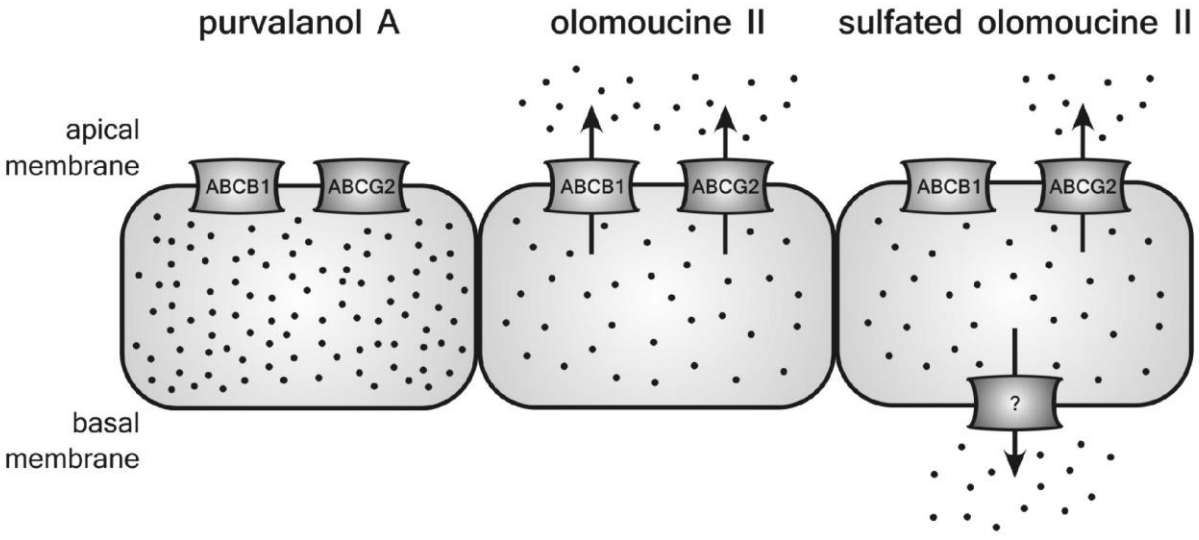

Figure 6. Schematic depiction of olomoucine II and purvalanol A transport in transduced MDCKII cells. Transport pathways for CDKi are indicated according to the results from MDCKII cellular monolayer transport assays. Transporter denoted with interrogation mark is unknown canine transporter.
doi:10.1371/journal.pone.0075520.g006

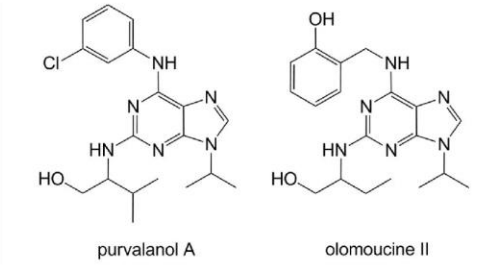

Figure 7. Chemical structures of olomoucine II and purvalanol A.
doi:10.1371/journal.pone.0075520.g007

previous studies [22], three olomoucine II concentrations (100 nM, 1 μM and 10 μM) were tested and transport ratios (*r*; olomoucine II transport in basolateral to apical direction divided by transport in apical to basolateral direction) 2 h after olomoucine II addition were calculated. In contrast to purvalanol A, the interval for data evaluation was shortened due to the generation of sulfated metabolite of olomoucine II (see below).

Using olomoucine II in concentrations of 100 nM or 1 μM, similar *r* values equal to 2.27 and 2.31 were observed in MDCKII-ABCG2 cells, respectively. Fumitremorgin C, a specific ABCG2 inhibitor [23], significantly lowered asymmetry in olomoucine II transport to *r* values of 1.32 and 1.21, respectively, thereby confirming the involvement of ABCG2 in the transport of olomoucine II (Fig. 1A, 1D). When 10 μM olomoucine II was used, *r* decreased to 1.29, indicating transporter saturation (Fig. 1G).

In MDCKII-ABCB1 cells, asymmetry in olomoucine II transport was approximately 3-fold higher ($r = 6.45$ and 6.83 for 100 nM and 1 μM, respectively) in comparison with ABCG2 transduced cells. LY335979, a specific ABCB1 inhibitor [24,25], significantly reduced *r* to values similar to those observed in the case of parent MDCKII cells ($r = 1.48$ and 1.83 for 100 nM and 1 μM, respectively) (Fig. 1B, 1E). At 10 μM concentration, *r* decreased to 2.51 indicating partial saturation of ABCB1 transporter (Fig. 1H). These results clearly demonstrate that olomoucine II is a substrate of ABCB1 *in vitro*.

In MDCKII-par cells, slight transport asymmetry was found with *r* values of 1.45, 1.39 and 1.64 for 100 nM, 1 μM and 10 μM olomoucine II, respectively (Fig. 1C, 1F, 1I). Since MDCKII-par cells express significant amount of endogenous canine Abcb1 [26,27], we investigated its possible role in this basal transport by adding LY335979 inhibitor. However, we did not observe statistically significant changes in *r* values for 100 nM and 1 μM olomoucine II, suggesting no or negligible participation of endogenous canine Abcb1 in olomoucine II transport (Fig. 1C, 1F).

Generation of Sulfated Conjugate of Olomoucine II and its Pharmacokinetic Behavior in MDCKII Cells

In olomoucine II transport experiments, we recorded time-dependent generation of an unknown peak in the fifth minute of HPLC analysis in all three cell lines tested (MDCKII-ABCG2, MDCKII-ABCB1 and MDCKII-par). Based on the MS analysis [28], the compound was identified as a sulfated conjugate of olomoucine II (Fig. 2). Importantly, equilibrium of sulfate conjugate distribution into particular compartments did not significantly differ when the parent compound was added into the basal or apical compartment (Fig. 3). On the other hand, the amount of sulfated metabolite increased with time and caused remarkable distortion of the results of parent compound. Therefore, when analyzing the data of unconjugated olomoucine II, the time interval was shortened to 2 hours in order to reduce the misrepresenting effect of sulfated olomoucine II. Considering ionic nature of sulfated olomoucine II, it is obvious that this compound cannot escape from the cells via passive diffusion but must utilize special transport system(s). Relative quantification of olomoucine II sulfate allowed us to describe its pharmacokinetic behavior in all MDCKII cell sublines.

In MDCKII-ABCG2 cells at 100 nM olomoucine II concentration, the sulfate appearance in apical compartment was markedly higher than that in basolateral compartment. After addition of fumitremorgin C, efflux into apical compartment was fully blocked whereas transport into opposite compartment significantly increased (Fig. 4A). Similar outcome was recorded in the case of 1 μM olomoucine II (Fig. 4D). These data demonstrate the contribution of ABCG2 to the efflux of sulfated olomoucine II through the apical membrane of MDCKII-ABCG2 cells. At 10 μM concentration, we observed identical appearance of sulfated conjugate in both compartments (Fig. 4G), suggesting saturation of ABCG2.

In MDCKII-ABCB1 cells at 100 nM and 1 μM olomoucine II, distribution of sulfated olomoucine II into basolateral and apical compartments was almost identical. This equilibrium was notably changed after LY335979 co-administration which increased appearance of the conjugate in the basolateral, but not apical, compartment (Fig. 4B, 4E). This phenomenon can be explained by LY335979-induced inhibition of ABCB1 which results in higher intracellular concentrations of olomoucine II and, therefore, greater availability of the drug for sulfatation. Generated olomoucine II sulfate is eventually transferred into basolateral compartment, most likely by endogenous canine transporters reported in MDCKII cells [26]. Sulfate distribution ratio after LY335979 addition in MDCKII-ABCB1 cells was almost identical to that observed in MDCKII-par, supporting this hypothesis.

Transport of olomoucine II sulfate in MDCKII-par cells, expressing only endogenous canine transporters, was markedly forced into the basolateral compartment whereas only limited amount reached the apical one. Addition of LY335979 did not affect this asymmetry, excluding the role of canine Abcb1 in the process (Fig. 4C, 4F, 4I). We, therefore, speculate that a transport system located in the basolateral membrane is the key player affecting metabolite distribution in non-transduced cells.

Effect of ABCG2 and ABCB1 on the Transepithelial Transport of Purvalanol A *In Vitro*

Possible involvement of ABCG2 and/or ABCB1 in the transcellular transport of purvalanol A was examined employing cellular monolayer transport assays with MDCKII-ABCG2 and MDCKII-ABCB1 cells, respectively. Based on our previous studies [22], two purvalanol A concentrations (1 μM and 10 μM) were tested and transport ratios (*r*; purvalanol A transport in basolateral to apical direction divided by transport in apical to basolateral direction) at the end of the experiment were calculated.

In contrast to olomoucine II, only negligible asymmetry in purvalanol A transport was observed in MDCKII-ABCG2 cells with *r* of 1.28 and 1.23 for 1 μM and 10 μM, respectively. No changes were recorded after concomitant addition of fumitremorgin C ($r = 1.33$ and 1.26 for 1 μM and 10 μM, respectively) (Fig. 5A, 5D). These results demonstrate that purvalanol A is not an ABCG2 substrate *in vitro*.

Similar results were obtained in MDCKII-ABCB1 cells; only negligible asymmetry in purvalanol A transport was observed,

reaching *r* values of 1.36 and 1.26 for 1 μM and 10 μM, respectively. Addition of LY335979 did not affect *r* values (1.20 and 1.18 for 1 μM and 10 μM, respectively) (Fig. 5B, 5E). These patterns of transport clearly demonstrate that purvalanol A is not an ABCB1 substrate.

As expected, only negligible asymmetry in purvalanol A transport was observed in MDCKII-par cells. 1 μM and 10 μM purvalanol A concentrations yielded *r* values of 1.14 and 1.05, respectively (Fig. 5C, 5F).

Interestingly, no fragments corresponding to sulfated purvalanol A were recorded in MS analysis indicating that, in contrast to olomoucine II, purvalanol A is not subjected to sulfatation in MDCKII cells.

Discussion

Purine CDKi have recently been recognized as promising candidates for the treatment of various cancers [6]. While pharmacodynamic properties of these compounds are relatively well understood, their pharmacokinetic behavior and interactions with other biological structures, such as transport proteins and biotransformation enzymes have not been properly investigated to date. Bachmaier and Miller were the first to observe interactions of purine CDKi with ABC transporters and demonstrated significant inhibition of ABCB1 by roscovitine in bovine brain microvessel endothelial cell monolayers [29]. More recently, An et al. revealed ABCG2 inhibition by purvalanol A, WHI-P180, roscovitine and bohemine employing *in vitro* hematoporphyrin transport across membrane vesicles from insect Sf9 cells transduced with ABCG2 [30]. In our previous work, we observed ABCG2 inhibition by olomoucine II and purvalanol A on *in vitro* as well as *in situ* level. Moreover, using combination method of Chou-Talalay, we demonstrated that these compounds can synergistically potentiate the cytostatic effect of mitoxantrone, an ABCG2 substrate, in ABCG2 expressing cell lines [22].

In the present study, we investigated whether olomoucine II and purvalanol A are transported by ABCG2 and/or ABCB1. To date, only one paper has reported on substrate affinity of purine CDKi toward ABC transporters; using ATPase assay, vesicular transport, Hoechst 33342 and calcein assays, Rajnai et al. [31] demonstrated roscovitine to be a high affinity ABCB1 substrate and suggested that this interaction may be the reason for limited penetration of roscovitine across the blood-brain barrier. In addition, these authors concluded that roscovitine is not transported by ABCG2, multidrug resistance associated protein 1 (ABCC1) and multidrug resistance associated protein 2 (ABCC2). In our current work, using cellular monolayer transport assays with ABCG2 and ABCB1 transduced MDCKII cells, we demonstrate that olomoucine II is a dual substrate of ABCG2 and ABCB1 (Fig. 6). Based on our findings, it is feasible to presume considerable effect of both transporters on the pharmacokinetic behavior of olomoucine II, including absorption, distribution and excretion as well as limited uptake by tumors overexpressing ABCG2 and ABCB1. In addition, drug interactions with other substrates of these transporters must be considered in clinical use.

In contrast to olomoucine II, we show that purvalanol A is not transported by ABCG2 and ABCB1 *in vitro* (Fig. 6). In accordance with these results, we suggest that pharmacokinetic behavior and tumor treating abilities of purvalanol A will not be affected by ABCG2 and/or ABCB1. These findings may, at least partly, explain negligible resistance of ABCG2 overexpressing HeLa-6621 cells to purvalanol observed by Seamon et al. [32].

While investigating transport of olomoucine II across MDCKII monolayers, we detected time-dependent generation of a metabolite that we characterized as a sulfated conjugate of olomoucine II (Fig. 6). Enormous sulfatation capacity of MDCKII cells, significantly exceeding that of human liver, Chang liver and HepG2 cells, has previously been reported by Ng et al. [33]. Since transduced MDCKII cell lines are a well-established and widely used model in drug development for investigation of drug interactions with transport proteins [21], our findings are of great importance for other researchers performing transport or accumulation studies with MDCKII cells, especially if radiolabeled substrates are used. It is very likely that hydrophilic metabolites (sulfates) formed during the experiment will follow transport pathway(s) different from the parent compound and, eventually, may contaminate the pharmacokinetic analysis. Several endogenous canine transporters, such as Abcb1, Abcc1, Abcc2, and Abcc5, have been localized in the MDCKII cells [26,27], of which multidrug resistance associated proteins can transport sulfated metabolites. We, therefore, assume that in our experiments, endogenous canine Abcc1, Abcc2 or Abcc5 transporters might efflux sulfated olomoucine II out of the MDCKII cells.

ABC transporters are well known for their ability to transport a wide variety of structurally unrelated molecules. Considering very similar structures of olomoucine II and purvalanol A (Fig. 7), it is surprising to see strikingly different interactions of both compounds with ABCB1 and ABCG2 proteins; only olomoucine II, but not purvalanol A, is transported by these transporters as observed in this study. However, our results correspond nicely with the studies by Ishikawa et al [34] or Nakagawa et al [35] who prepared several camptothecine analogues and tested them for their ability to circumvent the drug resistance mediated by ABCG2. The authors observed that analogues substituted with hydroxyl group were good ABCG2 substrates whereas replacement of the hydroxyl group with chlorine led to a remarkable reduction in affinity toward ABCG2. Correspondingly, olomoucine II (possessing hydroxyl group on the phenylamine substituent of purine heterocycle) was found to be a substrate of both ABC transporters in our study; on the other hand, purvalanol A (with –OH group replaced by chlorine) (Fig. 7) was not transported by any of the ABC transporters, suggesting that these two substituents play a key role in the recognition of the purine CDKi as ABC transporter substrates. Apart from *in vitro* experiments, we have confirmed identical behavior of both compounds on an organ level *in situ*; in perfused rat placenta, olomoucine II was actively pumped from fetus to mother by placental ABCB1/ABCG2 while purvalanol A showed no interactions with these transporters (data not shown) proposing our findings can be extrapolated beyond the *in vitro* experimental setup. It is thus apparent that structure similarity of particular CDKi cannot be used as a single reliable clue for the prognosis of interactions with ABC transporters.

Conclusions

In conclusion, our data suggest that pharmacokinetic behavior of olomoucine II in the organism will be considerably affected by ABCG2 and ABCB1 transporters as well as by phase II biotransformation enzyme, sulfotransferase. Limited accumulation of olomoucine II in tumors overexpressing ABCG2 and ABCB1 can also be expected. At the same time, overlapping substrate specificity with other drugs may lead to drug-drug interactions on these transporters. In contrast, pharmacokinetic behavior of purvalanol A is not affected by either ABCG2 or ABCB1, theoretically favoring this drug in the treatment of tumors expressing efflux transporters. These facts should be taken into account when introducing these prospective compounds into the clinical area. In addition, care should be taken when performing

pharmacokinetic studies in MDCKII cells, especially if radiolabeled substrates are used; sulfated conjugates formed within the cells may use other transport systems than the parent compound, which can eventually result in misleading interpretation of the pharmacokinetic analysis. With regard to chemical structures of olomoucine II and purvalanol A, our data emphasize that even drugs with remarkable structure similarity may show different pharmacokinetic behavior such as interactions with ABC transporters or biotransformation enzymes.

Author Contributions

Conceived and designed the experiments: FS MC JK. Performed the experiments: JH RK DC. Analyzed the data: JH RK MC FS. Contributed reagents/materials/analysis tools: JH RK MC FS. Wrote the paper: JH RK JK MC FS.

References

1. Krystof V, Lenobel R, Havlicek L, Kuzma M, Strnad M (2002) Synthesis and biological activity of olomoucine II. Bioorg Med Chem Lett 12: 3283–3286.
2. Gray NS, Wodicka L, Thunnissen AM, Norman TC, Kwon S, et al. (1998) Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. Science 281: 533–538.
3. Krystof V, McNae IW, Walkinshaw MD, Fischer PM, Muller P, et al. (2005) Antiproliferative activity of olomoucine II, a novel 2,6,9-trisubstituted purine cyclin-dependent kinase inhibitor. Cell Mol Life Sci 62: 1763–1771.
4. Villerbu N, Gaben AM, Redeuilh G, Mester J (2002) Cellular effects of purvalanol A: a specific inhibitor of cyclin-dependent kinase activities. Int J Cancer 97: 761–769.
5. Monaco EA, Beaman-Hall CM, Mathur A, Vallano ML (2004) Roscovitine, olomoucine, purvalanol: inducers of apoptosis in maturing cerebellar granule neurons. Biochem Pharmacol 67: 1947–1964.
6. Krystof V, Uldrijan S (2010) Cyclin-dependent kinase inhibitors as anticancer drugs. Curr Drug Targets 11: 291–302.
7. Diaz-Padilla I, Siu LL, Duran I (2009) Cyclin-dependent kinase inhibitors as potential targeted anticancer agents. Invest New Drugs.
8. Cicenas J, Valius M (2011) The CDK inhibitors in cancer research and therapy. J Cancer Res Clin Oncol 137: 1409–1418.
9. Aldoss IT, Tashi T, Ganti AK (2009) Seliciclib in malignancies. Expert Opin Investig Drugs 18: 1957–1965.
10. Knockaert M, Gray N, Damiens E, Chang YT, Grellier P, et al. (2000) Intracellular targets of cyclin-dependent kinase inhibitors: identification by affinity chromatography using immobilised inhibitors. Chem Biol 7: 411–422.
11. Knockaert M, Meijer L (2002) Identifying in vivo targets of cyclin-dependent kinase inhibitors by affinity chromatography. Biochem Pharmacol 64: 819–825.
12. Bain J, McLauchlan H, Elliott M, Cohen P (2003) The specificities of protein kinase inhibitors: an update. Biochem J 371: 199–204.
13. Hikita T, Oneyama C, Okada M (2010) Purvalanol A, a CDK inhibitor, effectively suppresses Src-mediated transformation by inhibiting both CDKs and c-Src. Genes Cells 15: 1051–1062.
14. Staud F, Ceckova M, Micuda S, Pavek P (2010) Expression and function of p-glycoprotein in normal tissues: effect on pharmacokinetics. Methods Mol Biol 596: 199–222.
15. Staud F, Pavek P (2005) Breast cancer resistance protein (BCRP/ABCG2). Int J Biochem Cell Biol 37: 720–725.
16. Allen JD, Schinkel AH (2002) Multidrug resistance and pharmacological protection mediated by the breast cancer resistance protein (BCRP/ABCG2). Mol Cancer Ther 1: 427–434.
17. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM (2006) Targeting multidrug resistance in cancer. Nat Rev Drug Discov 5: 219–234.
18. Han B, Zhang JT (2004) Multidrug resistance in cancer chemotherapy and xenobiotic protection mediated by the half ATP-binding cassette transporter ABCG2. Curr Med Chem Anticancer Agents 4: 31–42.
19. Yang K, Wu J, Li X (2008) Recent advances in the research of P-glycoprotein inhibitors. Biosci Trends 2: 137–146.
20. Shukla S, Ohnuma S, Ambudkar SV (2011) Improving cancer chemotherapy with modulators of ABC drug transporters. Curr Drug Targets 12: 621–630.
21. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. (2010) Membrane transporters in drug development. Nat Rev Drug Discov 9: 215–236.
22. Hofman J, Ahmadimoghaddam D, Hahnova L, Pavek P, Ceckova M, et al. (2012) Olomoucine II and purvalanol A inhibit ABCG2 transporter in vitro and in situ and synergistically potentiate cytostatic effect of mitoxantrone. Pharmacol Res 65: 312–319.
23. Rabindran SK, Ross DD, Doyle LA, Yang W, Greenberger LM (2000) Fumitremorgin C reverses multidrug resistance in cells transfected with the breast cancer resistance protein. Cancer Res 60: 47–50.
24. Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, et al. (1996) Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. Cancer Res 56: 4171–4179.
25. Shepard RL, Cao J, Starling JJ, Dantzig AH (2003) Modulation of P-glycoprotein but not MRP1- or BCRP-mediated drug resistance by LY335979. Int J Cancer 103: 121–125.
26. Goh LB, Spears KJ, Yao D, Ayrton A, Morgan P, et al. (2002) Endogenous drug transporters in in vitro and in vivo models for the prediction of drug disposition in man. Biochem Pharmacol 64: 1569–1578.
27. Kuteykin-Teplyakov K, Luna-Tortos C, Ambroziak K, Loscher W (2010) Differences in the expression of endogenous efflux transporters in MDR1-transfected versus wildtype cell lines affect P-glycoprotein mediated drug transport. Br J Pharmacol 160: 1453–1463.
28. Holcapek M, Kolarova L, Nobilis M (2008) High-performance liquid chromatography-tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. Anal Bioanal Chem 391: 59–78.
29. Bachmeier CJ, Miller DW (2005) A fluorometric screening assay for drug efflux transporter activity in the blood-brain barrier. Pharm Res 22: 113–121.
30. An R, Hagiya Y, Tamura A, Li S, Saito H, et al. (2009) Cellular phototoxicity evoked through the inhibition of human ABC transporter ABCG2 by cyclin-dependent kinase inhibitors in vitro. Pharm Res 26: 449–458.
31. Rajnai Z, Mehn D, Beery E, Okyar A, Jani M, et al. (2010) ATP-binding cassette B1 transports seliciclib (R-roscovitine), a cyclin-dependent kinase inhibitor. Drug Metab Dispos 38: 2000–2006.
32. Seamon JA, Rugg CA, Emanuel S, Calcagno AM, Ambudkar SV, et al. (2006) Role of the ABCG2 drug transporter in the resistance and oral bioavailability of a potent cyclin-dependent kinase/Aurora kinase inhibitor. Mol Cancer Ther 5: 2459–2467.
33. Ng KH, Lim BG, Wong KP (2003) Sulfate conjugating and transport functions of MDCK distal tubular cells. Kidney Int 63: 976–986.
34. Ishikawa T, Ikegami Y, Sano K, Nakagawa H, Sawada S (2006) Transport mechanism-based drug molecular design: novel camptothecin analogues to circumvent ABCG2-associated drug resistance of human tumor cells. Curr Pharm Des 12: 313–325.
35. Nakagawa H, Saito H, Ikegami Y, Aida-Hyugaji S, Sawada S, et al. (2006) Molecular modeling of new camptothecin analogues to circumvent ABCG2-mediated drug resistance in cancer. Cancer Lett 234: 81–89.

6.2 Purvalanol A, olomoucine II and roscovitine inhibit ABCB1 transporter and synergistically potentiate cytotoxic effects of daunorubicin *in vitro*

Cihalova D., Hofman J., Ceckova M., Staud F.

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V této publikaci jsme se věnovali objasnění interakcí purinových CDKI (purvalanolu A, olomoucinu II a roskovitinu) s transportérem ABCB1, abychom doplnili jejich interakční profil. Flavopiridol a SNS-032 doplnily škálu studovaných CDKI o látky, které se již nacházejí v klinickém hodnocení.

Čtyři z námi studovaných látek inhibovaly eflux dvou různých substrátů ABCB1 v buněčné linii MDCKII-ABCB1: nejvyšší inhibiční aktivitu vykazoval olomoucin II, následován roskovitinem, purvalanolem A a flavopiridolem. Látka SNS-032 inhibovala pouze ABCB1-zprostředkovaný transport látky Hoechst 33342. Purvalanol A, SNS-032 a flavopiridol snižovaly stimulovanou ATPázovou aktivitu v membránových veziklech s expresí lidského ABCB1, zatímco olomoucin II a roskovitin nejen snižovaly stimulovanou aktivitu, ale signifikantně zvyšovaly bazální ATPázovou aktivitu, což naznačuje, že obě tyto látky jsou nejen inhibitory ale i substráty ABCB1. Dále jsme ukázali, že nejsilnější inhibitory ABCB1 (purvalanol A, olomoucin II a roskovitin) synergicky potencují antiproliferativní efekt daunorubicinu, což je běžně používané chemoterapeutikum a také substrát ABCB1, v MDCKII-ABCB1 buňkách i v lidských nádorových buněčných liniích HCT-8 a HepG2. Tento výrazný synergismus je alespoň z části způsoben (i) inhibicí ABCB1 transportéru pomocí CDKI vedoucí ke zvýšené buněčné akumulaci daunorubicinu a (ii) přirozenou cytotoxickou aktivitou CDKI.

Naše výsledky naznačují, že podání testovaných CDKI s protinádorovými léčivy, které jsou zároveň substráty ABCB1, může vést k výraznému snížení dávek obou léčiv v kombinaci při léčbě nádorů s expresí ABCB1.

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Purvalanol A, Olomoucine II and Roscovitine Inhibit ABCB1 Transporter and Synergistically Potentiate Cytotoxic Effects of Daunorubicin In Vitro

Daniela Cihalova, Jakub Hofman, Martina Ceckova, Frantisek Staud*

Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Hradec Kralove, Czech Republic

Abstract

Cyclin-dependent kinase inhibitors (CDKi) have high potential applicability in anticancer therapy, but various aspects of their pharmacokinetics, especially their interactions with drug efflux transporters, have not yet been evaluated in detail. Thus, we investigated interactions of five CDKi (purvalanol A, olomoucine II, roscovitine, flavopiridol and SNS-032) with the ABCB1 transporter. Four of the compounds inhibited efflux of two ABCB1 substrates, Hoechst 33342 and daunorubicin, in MDCKII-ABCB1 cells: Olomoucine II most strongly, followed by roscovitine, purvalanol A, and flavopiridol. SNS-032 inhibited ABCB1-mediated efflux of Hoechst 33342 but not daunorubicin. In addition, purvalanol A, SNS-032 and flavopiridol lowered the stimulated ATPase activity in ABCB1 membrane preparations, while olomoucine II and roscovitine not only inhibited the stimulated ATPase but also significantly activated the basal ABCB1 ATPase, suggesting that these two CDKi are ABCB1 substrates. We further revealed that the strongest ABCB1 inhibitors (purvalanol A, olomoucine II and roscovitine) synergistically potentiate the antiproliferative effect of daunorubicin, a commonly used anticancer drug and ABCB1 substrate, in MDCKII-ABCB1 cells as well as in human carcinoma HCT-8 and HepG2 cells. We suggest that this pronounced synergism is at least partly caused by (i) CDKi-mediated inhibition of ABCB1 transporter leading to increased intracellular retention of daunorubicin and (ii) native cytotoxic activity of the CDKi. Our results indicate that co-administration of the tested CDKi with anticancer drugs that are ABCB1 substrates may allow significant dose reduction in the treatment of ABCB1-expressing tumors.

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* E-mail: frantisek.staud@faf.cuni.cz

Introduction

Drug efflux transporters from the family of ATP-binding cassette (ABC) transport proteins, such as ABCB1 (P-glycoprotein, MDR1), ABCG2 (breast cancer resistance protein, BCRP), and ABCCs (multidrug resistance associated proteins, MRPs) mediate membrane transport of many endogenous substrates as well as xenobiotics. Abundantly expressed in tumor cells as well as physiological tissues, they play important roles in drug disposition, tissue protection and cancer resistance [1,2,3], thereby affecting pharmacokinetic/pharmacodynamic properties of many clinically used drugs [4]. The importance of identifying interactions of novel therapeutic agents with membrane drug transporters has recently been emphasized by regulatory agencies and many recommendations and decision trees for elucidating these interactions have been proposed [5,6].

ABCB1 is the most extensively studied drug efflux transporter [7,8]. Utilizing energy from ATP hydrolysis, it actively pumps structurally diverse compounds, including anticancer drugs, out of cells [9]. Two distinct drug binding and transport sites have been identified in ABCB1: the R- and H-sites, which bind rhodamine 123 and Hoechst 33342, respectively [10]. ABCB1 has become an attractive molecular target and inhibitors of this efflux transporter are being sought to increase the bioavailability of drugs after oral administration [11] or overcome drug resistance and sensitize cancer cells [12,13].

Cyclin-dependent kinases (CDK) play important roles in the control of cell cycle progression and transcription. Thus, abnormalities in their regulation and expression can cause pathogenic changes resulting in various malignancies, and suppression of their activities by CDK inhibitors (CDKi) is a promising approach in cancer therapy [14,15,16,17]. Several of these compounds are currently undergoing preclinical and clinical trials. Considerable attention has been devoted to their pharmacodynamic properties, but various pharmacokinetic aspects, especially their interactions with drug efflux transporters, have not yet been evaluated in detail.

In our previous studies we examined interactions of the prototypical purine CDKi olomoucine II and its derivative purvalanol A, with ABCG2, another important ABC transporter [18,19]. The results revealed that these two compounds can inhibit ABCG2 in vitro and in situ and synergistically potentiate the antiproliferative effect of mitoxantrone in ABCG2-expressing cells. The aim of the study presented here was to characterize the inhibitory effect of several CDKi on the efflux activity of ABCB1. The selected set included olomoucine II, purvalanol A, roscovitine (another olomoucine II-derived drug), and the two most exten-

Table 1. Number of ABCB1 transcripts per μg of total RNA in each of the cell lines.

	MDCKII-parent	MDCKII-ABCB1	HCT-8	HepG2
ABCB1 Transcripts (10^4)	ND	3100±288 [a]	35.2±9.65	153±16.9

Presented data are means ± SD of three experiments performed in triplicate. [a] Significantly different from ABCB1 expression in HCT-8 and HepG2 cell line ($P<0.001$) as analyzed by ANOVA followed by Bonferronis test. ND, no transcripts detected.
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sively studied CDKi that are currently undergoing clinical trials for treating various cancers: flavopiridol and SNS-032 [20,21,22]. To assess the ability of these compounds to inhibit ABCB1 transport activity, we examined their effects on the in vitro accumulation of Hoechst 33342 and daunorubicin (well established ABCB1 substrates that bind to the H- and R-sites of ABCB1, respectively) in MDCKII cells transduced with human ABCB1. We then further characterized these interactions by examining their ATPase activation and inhibition effects in ABCB1-overexpressing membrane vesicles. Moreover, as CDKi appear to be more clinically successful when co-administered with other cytotoxic agents [23], we hypothesized that interactive effects of the drugs on the ABCB1 transporter in tumor cells might intensify anticancer potency and strongly affect the outcome of treatments. To test this hypothesis, we applied each of the CDKi in combination with daunorubicin to ABCB1-expressing cells, both genetically modified and cancer-derived, to evaluate whether CDKi can synergistically potentiate daunorubicin's cytotoxic effects.

Materials and Methods

Chemicals

Hoechst 33342 (HOE), daunorubicin (DNR), XTT sodium salt (XTT), phenazine methosulfate (PMS), purvalanol A and roscovitine (R-enantiomer) were purchased from Sigma Aldrich (St. Louis, MO, USA). ABCB1 inhibitor LY335979 (LY) was supplied by Toronto Research Chemicals (North York, ON, Canada). Olomoucin II was obtained from Merck (Darmstadt, Germany), flavopiridol and SNS-032 were purchased from SelleckChem (Houston, TX, USA). Cell culture reagents were supplied by Sigma Aldrich (St. Louis, MO, USA) and Gibco BRL Life Technologies (Rockville, MD, USA). An ABCB1 PREDEASY ATPase kit was purchased from Solvo Biotechnology (Szeged, Hungary).

Cell lines

MDCKII cells transduced with the human ABCB1 gene (MDCKII-ABCB1) that stably express the ABCB1 transporter, and the parental MDCKII cell line, were obtained from Prof. Piet Borst and Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Both cell lines were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. For in vitro drug combination studies we also used human ileocecal adenocarcinoma HCT-8 and human liver carcinoma HepG2 cell lines, both of which express ABCB1 [24]. The HCT-8 cells were purchased from the European Collection of Cell Cultures (HPA, Salisbury, Wiltshire, UK) and cultured in RPMI medium supplemented with 10% horse serum and 1 mM sodium pyruvate. The HepG2 cells, obtained from the American Type Culture Collection (LGC Promochem, Teddington, Middlesex, UK), were grown in minimal essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10% FBS. Cells from passages 5 to 30 were used in all in vitro studies. Dimethyl sulfoxide was applied as a CDKi solvent in concentrations not exceeding 0.5% (1% in ATPase assays).

Absolute qRT-PCR quantification of ABCB1 transcripts in the cell lines

To evaluate and compare ABCB1 transcript levels in the cell lines used in this study, we used absolute real time RT-PCR quantification, as follows. Total RNA was isolated from each of

Table 2. Dose reduction index (DRI) values for drug combinations scheduled after 72 h of simultaneous treatment.

Cell line	Drugs		Concentration ratio	DRI at Fa					
				0.5		0.75		0.9	
	I	II	I:II	I	II	I	II	I	II
MDCKII-ABCB1	DNR	Purvalanol A	1:0.9	3.76±0.07	4.03±0.08	4.56±0.07	3.73±0.06	5.54±0.06	3.45±0.04
		Olomoucine II	1:0.7	2.68±0.26	2.83±0.27	3.32±0.22	2.84±0.19	4.12±0.21	2.86±0.10
		Roscovitine	1:0.8	3.06±0.03	3.20±0.03	3.80±0.03	3.08±0.02	4.72±0.05	2.96±0.03
MDCKII parent	DNR	Purvalanol A	1:11.7	2.25±0.03	2.41±0.03	2.84±0.05	2.40±0.04	3.60±0.09	2.39±0.06
		Olomoucine II	1:9.2	2.38±0.08	2.51±0.08	3.07±0.06	2.63±0.05	3.96±0.03	2.75±0.02
		Roscovitine	1:12.3	ND	ND	2.74±0.06	2.29±0.05	3.57±0.09	2.37±0.06
HCT-8	DNR	Purvalanol A	1:17.7	ND	ND	3.12±0.03	1.77±0.01	4.36±0.13	1.68±0.05
		Olomoucine II	1:6.3	3.11±0.13	3.04±0.13	4.14 ±0.08	2.98±0.06	5.45±0.02	2.92±0.05
		Roscovitine	1:17.0	2.63±0.02	2.49±0.02	3.96±0.05	2.29±0.03	5.96±0.17	2.11±0.06
HepG2	DNR	Purvalanol A	1:43.0	ND	ND	ND	ND	4.05±0.08	1.68±0.03
		Olomoucine II	1:20.5	ND	ND	2.55±0.03	2.08±0.03	4.68±0.09	2.38±0.04
		Roscovitine	1:47.0	ND	ND	2.72±0.02	2.51±0.02	5.02±0.03	2.56±0.02

ND, not determined.
doi:10.1371/journal.pone.0083467.t002

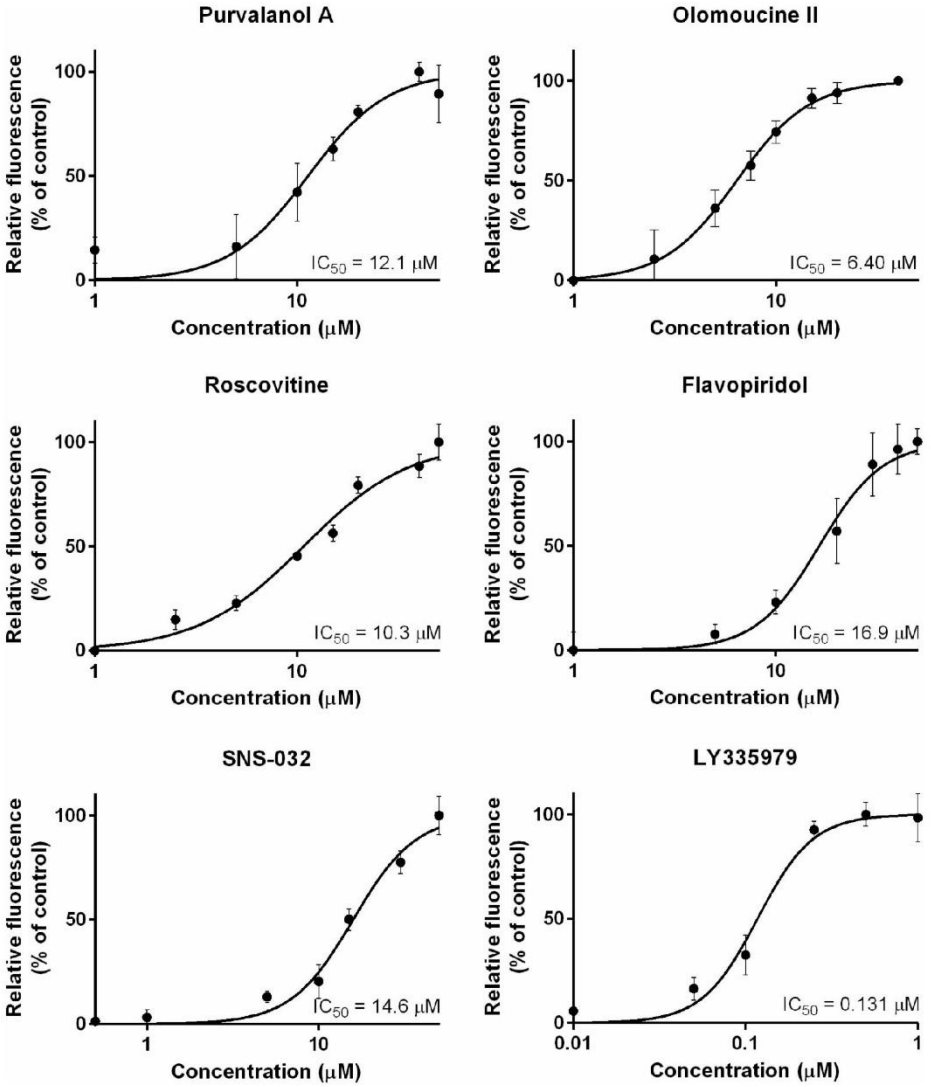

Figure 1. Effects of CDKi and the model ABCB1 inhibitor LY on ABCB1-mediated efflux of HOE in MDCKII-ABCB1 cells. 0% and 100% respectively indicate the fluorescence of unaffected control cells and the maximal fluorescence observed in assays with a particular CDKi. Presented data are means ± SD obtained from three independent experiments performed in triplicate.
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the cell lines grown in culture flasks using TriReagent (Molecular Research Centre, Cincinnati, OH, USA) according to the manufacturer's instructions. We measured the UV absorbance of the isolated RNA spectrophotometrically at 260 nm to determine its concentration and at 280 nm to check its purity from the 260/208 nm absorbance ratio using a NanoDrop spectrometer (Thermo Scientific, Wilmington, DE, USA). cDNA was prepared from 2 μg portions of the extracted total RNA with MMLV transcriptase using oligo(dT)18VN nucleotides and porcine RNase inhibitor (Tetro cDNA Synthesis Kit, Bioline, London, UK). We then amplified cDNA (from 40 ng of transcribed RNA) by real-time PCR using an iCycler (BioRad, Hercules, CA, USA) and 2× Probe Master Mix (Generi Biotech, Hradec Kralove, Czech Republic) in pre-designed PCR assays for ABCB1 (hABCB1_Q2, Generi Biotech, Hradec Kralove, Czech Republic). For absolute quantification, pCR plasmids (Generi Biotech, Hradec Kralove, Czech Republic) hosting subcloned PCR products of ABCB1 were used as PCR standards. Each sample and standard was amplified in triplicate, by incubation at 95°C for 3 min followed by 40 cycles of 95°C for 10 s and 60°C for 20 s. Standard curves were generated by preparing and amplifying seven decimal dilutions of the ABCB1 pCR plasmid, yielding copy numbers ranging from 2.5×10^{1} to 2.5×10^{7} copies per 20 μl reaction mixture. The resulting real-time amplification curves were analyzed, and threshold (Ct) values subtracted, using iCycler iQ 3.0 software (BioRad, Cincinnati, OH, USA). Excel software (Microsoft, Seattle, WA, USA) was used for all other calculations and the absolute number of cDNA copies in each sample was calculated from the generated calibration curves.

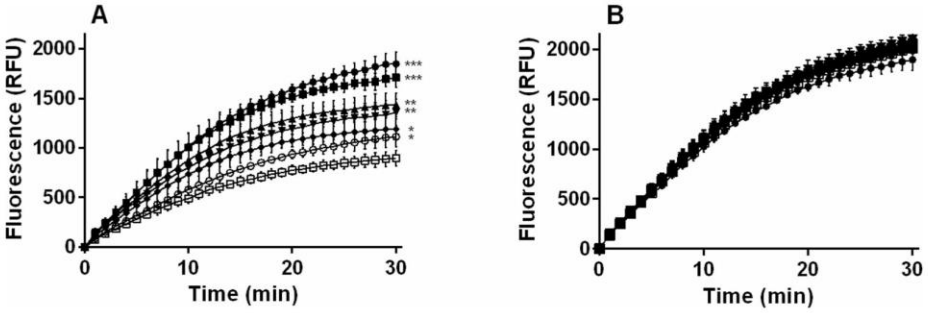

Figure 2. Time-dependent accumulation of HOE in MDCKII cells. MDCKII-ABCB1 (A) and MDCKII parent (B) cells were incubated in the absence (control □) or presence of purvalanol A (■), roscovitine (▲), olomoucine II (▼) flavopiridol (◆) or SNS-032 (○) at their respective IC_{50} concentrations. A potent ABCB1 inhibitor, LY (●), was used as a positive control for ABCB1 inhibition. Presented data are means ± SD obtained from three independent experiments performed in triplicate. The statistical significance (*$P<0.05$; **$P<0.01$; ***$P<0.001$) of differences in HOE levels detected in treated and control cells was determined using unpaired *t* tests.
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HOE accumulation

To investigate the inhibitory activity of CDKi on ABCB1, the intracellular accumulation of HOE, a fluorescent ABCB1 substrate [25], was examined in both MDCKII-ABCB1 and MDCKII parent cell lines in the presence and absence of the tested compounds. The reduction in fluorescence intensity in ABCB1-transduced cells indicates the efflux activity of the ABCB1 transporter, its inhibition increases HOE accumulation and thus the intracellular fluorescence.

The assay was conducted as previously described [18] and optimized for application in ABCB1-expressing cells. Briefly, MDCKII-ABCB1 and MDCKII parent cells were seeded at 5×10^4 cells per well on a 96-well culture plate and used for accumulation experiments after 24 h cultivation. The medium was removed and cells were washed twice with prewarmed phosphate buffered saline (PBS) at pH 7.4. They were then preincubated for 30 min (at 37°C in 5% CO_2) with or without individual CDKi or LY (the potent ABCB1 inhibitor LY335979, [26]), each at eight selected concentrations. HOE was then added to 8 µM final concentration and fluorescence at 465 nm resulting from excitation at 350 nm was measured in 1 min intervals for 30 min using an Infinite 200 instrument (Tecan, Männedorf, Switzerland). The end-point fluorescence at t = 30 min (after subtracting autofluorescence values of untreated cells) was used to calculate (IC_{50}), the concentration of the tested CDKi providing 50% of its maximum inhibitory activity. For curve fitting and IC_{50} calculations, GraphPad Prism 5.04 (GraphPad Software Inc., La Jolla, CA, USA) was employed. As the tested CDKi did not reach the same maximum inhibitory levels (due to their cytotoxicity), the degree of ABCB1-mediated efflux inhibition by the individual compounds was assessed from their respective IC_{50} values.

DNR accumulation

The accumulation of DNR, another fluorescent substrate of ABCB1 [27] that is known to bind to a site distinct from that of HOE [10,28], was measured in an ABCB1-overexpressing cell line and compared to its accumulation in a control cell line lacking the transporter, using a previously published flow cytometry method [29], with slight modifications. Briefly, MDCKII-ABCB1 and MDCKII parent cells were seeded at 1.5×10^5 cells per well on a 12-well plate 24 h before the experiment. The medium was removed and the cells were washed with prewarmed PBS. The cells were then preincubated in Opti-MEM, with or without CDKi or LY at 37°C in 5% CO_2 for 30 min. DNR was then added to a final concentration of 2 µM and the cells were incubated under the same conditions for a further 60 min. Accumulation was stopped by cooling the samples on ice, removing the medium and washing twice with ice-cold PBS. The cells were then detached from the plates with 10× trypsin-EDTA, resuspended in PBS with 2% FBS and transferred to Eppendorf tubes, which were placed on ice until analysis. The intracellular DNR fluorescence of individual cells was analyzed using a C6 flow cytometer (Accuri, Ann Arbor, USA) with a 488 nm/585 nm excitation/emission filter and recorded as histograms. The median fluorescence (MF) intensity of 10,000 measured cells was used to compare the fluorescence resulting from each of the treatments. Viable cells (typically 60–80% of those measured) were gated based on forward and side scatter plots. The validity of the method was verified in control experiments using 7-aminoactinomycin D as a viability marker. The MF intensity of untreated cells was subtracted from fluorescence values obtained for all the measured samples. 1 µM LY was selected as a positive control because it can potently inhibit the ABCB1 efflux transporter.

To quantify the inhibitory effect of each tested compound on the ABCB1 transporter in the MDCKII-ABCB1 cell line, the ratio between the MF intensity with or without inhibitor was calculated and normalized to the effect in the parental MDCKII cell line according to the following equation [30]:

$$\text{Inhibition ratio} = \frac{MF_{\text{over expressing cell line with test compound}}/MF_{\text{over expressing cell line without test compound}}}{MF_{\text{parental cell line with test compound}}/MF_{\text{parental cell line without test compound}}}$$

ABCB1-ATPase assay

The drug efflux function of ABCB1 is linked to hydrolysis of ATP by ATPase, which is stimulated in the presence of ABCB1 substrates. In the activation assay, transported substrates can stimulate baseline vanadate-sensitive ATPase activity, whereas in the inhibition assay, which is carried out in the presence of a known activator of the transporter, inhibitors may reduce the maximal vanadate-sensitive ATPase activity. ATPase activity was measured by assessing the amount of phosphate liberated from ATP by the ABCB1 transporter using the PREDEASY ATPase kit for ABCB1 according to the manufacturer's instructions. For this purpose, Sf9 cell membranes (4 µg protein per well) were mixed

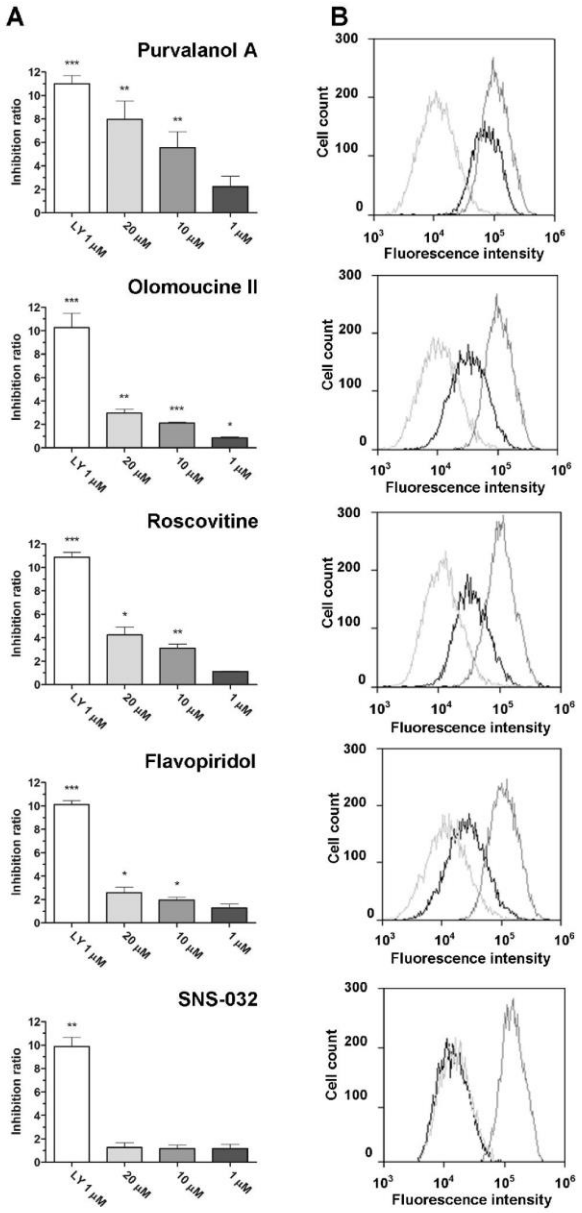

Figure 3. Effect of the tested CDKi on intracellular accumulation of DNR in MDCKII-ABCB1 cells. (A) Inhibition, expressed as the ratio between the median fluorescence with and without the indicated inhibitors during the efflux period, normalized to the effect in the parental cell line MDCKII (see *Materials and Methods* for details). LY (1 μM) was used as a model inhibitor. Presented data are means ± SD obtained from three independent experiments performed in duplicate. *P* values of differences between observed inhibition ratios and the null hypothetical value of 1 (no ABCB1 inhibition) were determined by unpaired two-tailed *t* tests: **P*<0.05; ***P*<0.01; ****P*<0.001. (B) Representative histograms of data obtained in assays with each compound at 20 μM: control with no inhibitor (light grey), tested compound (black), 1 μM LY (dark grey).
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with each of the test compounds (singly) in solutions with concentrations ranging from 140 nM to 300 μM, then incubated at 37°C for 10 min in the presence or absence of 1.2 mM sodium orthovanadate. The reaction was started by adding 10 mM ATP solution (magnesium salt) to the reaction mixture, stopped 10 min later, and the absorbance at 590 nm was measured after a further 30 min incubation (GeniosPlus apparatus; Tecan, Männedorf, Switzerland). The ATPase activity in each sample was determined as the difference in liberated amounts of phosphate measured in the presence and absence of 1.2 mM sodium orthovanadate. Phosphate standards were prepared in each plate and verapamil served as a positive control for ABCB1 stimulation. The results are expressed as vanadate-sensitive ATPase activities.

Cytotoxicity assay

1×10^4 MDCKII-ABCB1, 1×10^4 MDCKII parent, 2×10^4 HCT-8, or 2×10^4 HepG2 cells were grown in 96-well culture plates and incubated for 24 h. Individual CDKi diluted with growth medium were added to the exponentially growing cells and the resulting mixtures were incubated for 72 h at 37°C, 5% CO_2. Cytotoxicity was then assessed using the XTT assay as follows: cells were incubated with 0.167 mg/mL XTT and 4 μM PMS in Opti-MEM for 2 h. The absorbance of the soluble formazan released was measured at 470 nm on a microplate reader (Tecan, Männedorf, Switzerland). The median effective antiproliferative concentrations (EC_{50}) of the compounds were calculated using GraphPad Prism 5.04.

Drug combinations

The combination index (CI) method of Chou-Talalay, based on the median-effect equation, was used to calculate combined drug effects. This approach offers quantitative definitions for additive, synergistic and antagonistic effects (CI values of 0.9–1.1, <0.9, and >1.1, respectively) [31]. Combination experiments were performed in a constant-ratio experimental design as recommended for the most efficient data analysis [32], and the generated data were used to quantify dose-reduction indices (DRI) for pairs of the tested drugs. DRI represents the fold-change of a focal effect when individual agents are used simultaneously relative to their separate effects, and their activity is synergistic if DRI > 1. The three ABCB1 inhibitors identified as most potent (purvalanol A, roscovitine and olomoucine II) in our accumulation experiments were combined with DNR, a commonly used anticancer drug and ABCB1 substrate. The XTT cytotoxicity assay was used to measure the cell viability in four cell lines (MDCKII-ABCB1, MDCKII parent, HCT-8 and HepG2) in the presence of the CDKi and DNR both singly and in combination, at constant concentration ratios, ranging from 0.1 to 1.5 multiples of their respective, predetermined EC_{50} values. The data acquired from these drug combination experiments were analyzed using CompuSyn ver. 3.0.1 software (ComboSyn Inc., Paramus, NJ, USA).

Statistical analysis

Data are presented as means ± SD. Between-treatment differences, calculated using ANOVA or Student's t test implemented in GraphPad Prism 5.04, are considered significant if $P<0.05$.

Results

Expression of ABCB1 mRNA in the cell lines

Expression of the gene encoding human ABCB1 transporter was quantified in all cell lines used in this study. Levels of ABCB1

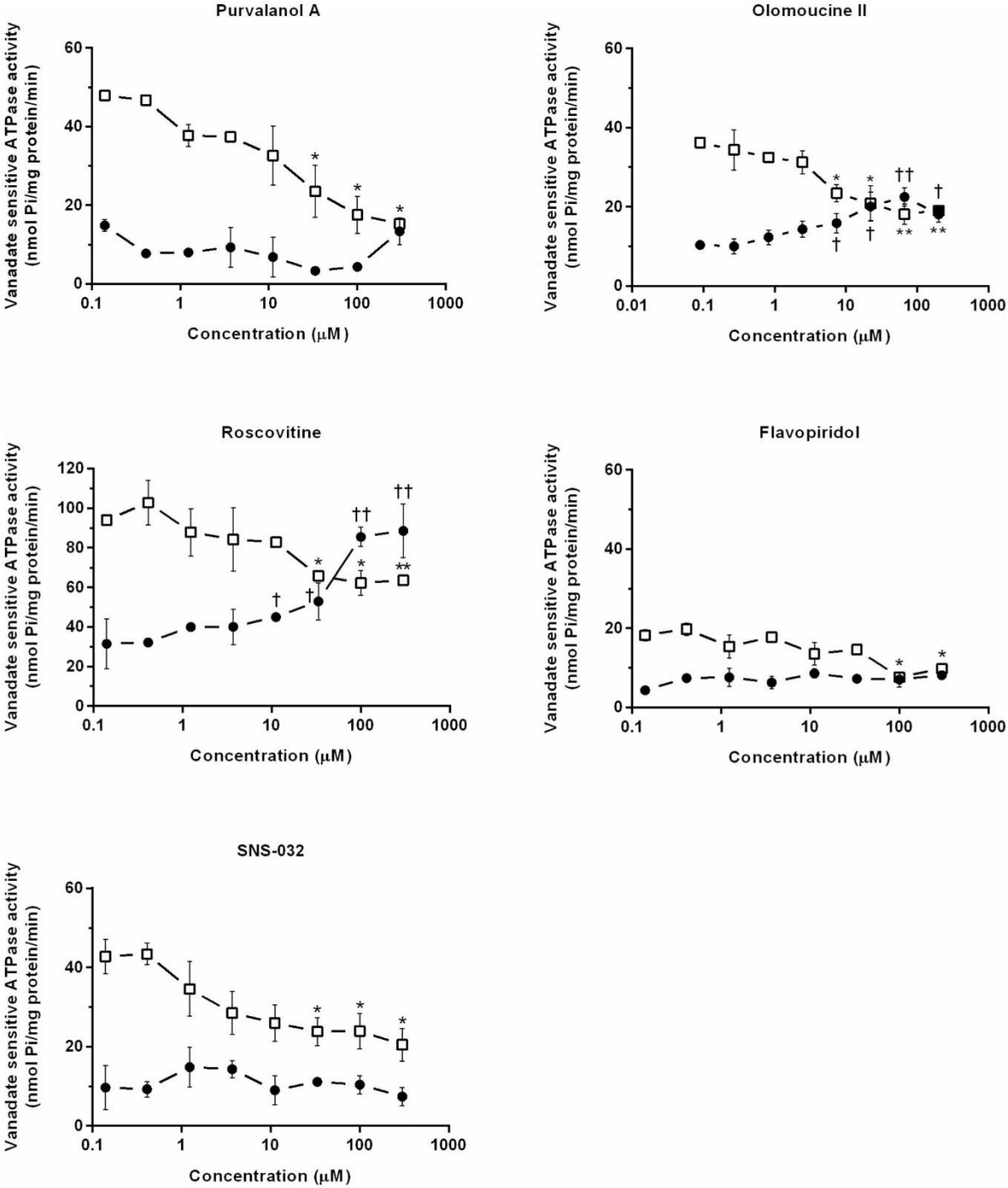

Figure 4. Effects of CDKi on the ATPase activity of ABCB1-Sf9 membrane preparations. Vanadate-sensitive ATPase activity in the presence of purvalanol A, olomoucine II, roscovitine, flavopiridol, or SNS-032 in activation (●) and inhibition (□) experiments. Presented data are means ± SD representative of at least two experiments performed in duplicate. The significance of differences linked to the absence and presence of CDKi in the basal activity of the transporter in activation assays (†$P<0.05$; ††$P<0.01$; †††$P<0.001$) and the activity of the activated transporter in inhibition assays (*$P<0.05$; **$P<0.01$; ***$P<0.001$) were determined using unpaired *t* tests.
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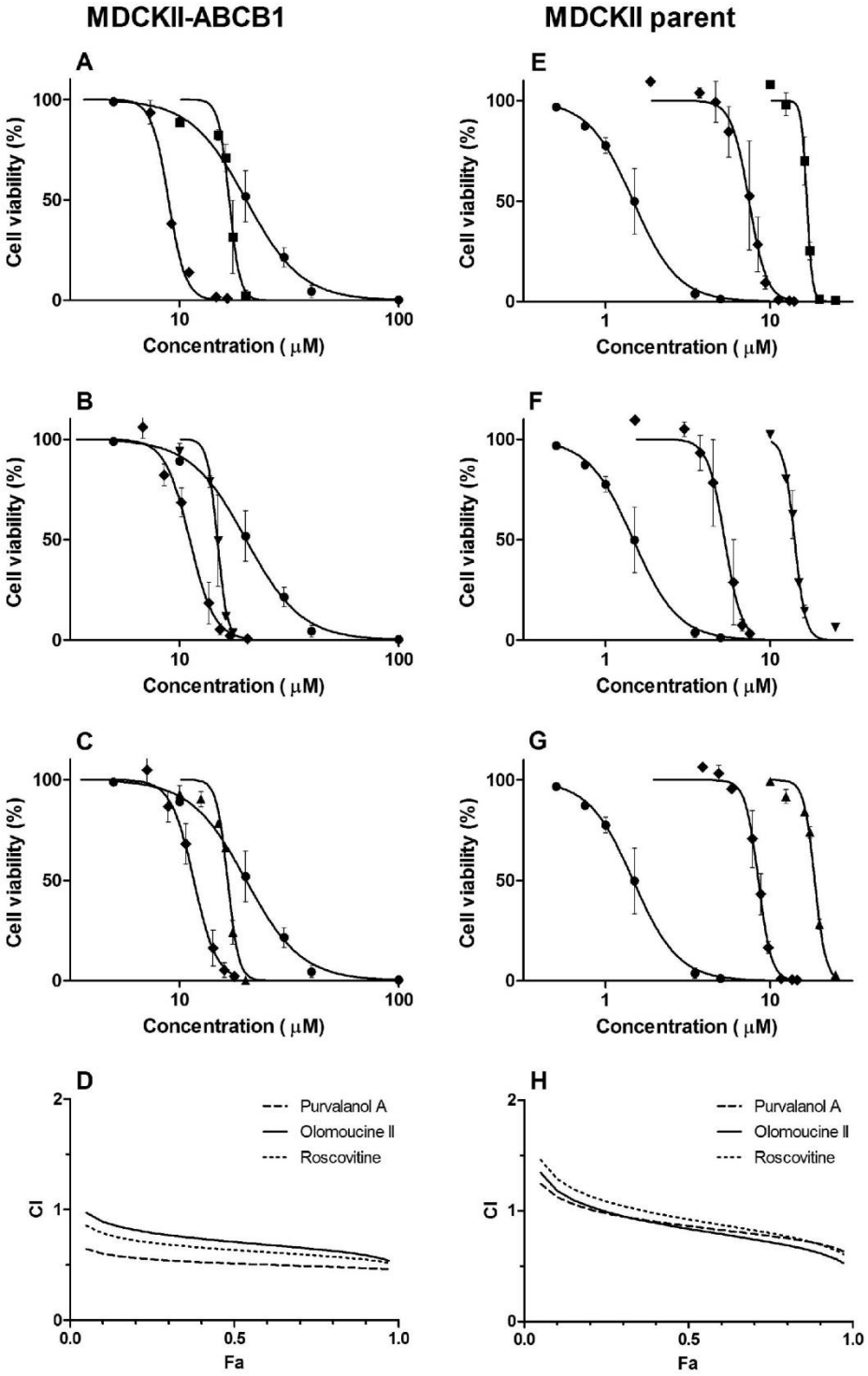

Figure 5. Cytotoxicity and combination experiments in MDCKII-ABCB1 and parental cell lines. Cytotoxic effect of (A, E) purvalanol A (■), (B, F) olomoucine II (▼), (C, G) roscovitine (▲) or daunorubicin (●) and their combination (◆) on MDCKII-ABCB1 or MDCKII parent cells. For combinations, concentrations corresponding to particular points in the graph are sums of concentrations of the individual drugs administered in fixed concentration ratios (Table 2), based on the ratio of their respective EC_{50} values. Presented data are means ± SD obtained from at least three independent experiments performed in triplicate. (D, H) The cytotoxic effect (combination index, CI, plot) of CDKi and daunorubicin combinations on MDCKII-ABCB1 or MDCKII parent cells, obtained using CompuSyn software. Fractional effects (Fa) were calculated from the cell viability values of

individual compounds; Fa = 0 means no antiproliferative effect, Fa = 1 means 100% antiproliferative effect. CI = 0.9–1.1 indicates additive effect, CI <0.9 synergism and CI > 1.1 antagonism.
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transcripts were highest in the MDCKII-ABCB1 line: more than an order of magnitude higher than in HepG2 and HCT-8 cells. As expected, no transcripts of the human ABCB1 gene were detected in the parental MDCKII cell line (Table 1).

Effect of CDKi on ABCB1-mediated efflux of HOE from MDCKII-ABCB1 cells

All tested CDKi inhibited ABCB1-mediated efflux of HOE in MDCKII-ABCB1 cells (Fig. 1), with potency declining in the following order: olomoucine II > roscovitine > purvalanol A > SNS-032 > flavopiridol (IC_{50} = 6.4, 10.3, 12.1, 14.6 and 16.9 μM, respectively). However, all of the compounds were much less potent than the model ABCB1 inhibitor, LY (IC_{50}, 0.131 μM). When the CDKi were applied at their respective IC_{50} concentrations, purvalanol A inhibited HOE efflux from MDCKII-ABCB1 cells most strongly (86% as strongly as LY) followed by roscovitine, olomoucine II, flavopiridol and SNS-032 (57%, 48%, 31% and just 23% as strongly as LY, respectively). The accumulation of HOE in the MDCKII parent cell line was unaffected by addition of the CDKi (Fig. 2).

Effect of CDKi on ABCB1-mediated efflux of DNR from MDCKII-ABCB1 cells

Based on the results of the HOE efflux experiments, the CDKi were each applied at three concentrations (1, 10 and 20 μM) to investigate their effects on the ABCB1-mediated efflux of DNR. LY (1 μM) was applied as a positive control for ABCB1 inhibition. At CDKi concentrations above 20 μM and LY concentrations above 1 μM cells drifted out of the gates, thus the resulting data were not included in the analysis.

All of the tested CDKi inhibited DNR efflux dose-dependently across the applied range, 1 - 20 μM, but less strongly than LY (Fig. 3). At 1 μM they showed at most slight inhibitory activity, in accordance with the results of our HOE accumulation studies. However, at the highest concentration (20 μM), four CDKi exhibited significant ($P<0.05$) ABCB1 inhibition, declining in the order purvalanol A > roscovitine > olomoucine II > flavopiridol. In contrast to its observed inhibitory effect in HOE accumulation assays, SNS-032 did not inhibit DNR accumulation at any tested concentration.

Effects of CDKi on ATPase activity in ABCB1-containing membrane preparations

To further characterize interactions of the CDKi with ABCB1, we tested their modulatory effects on vanadate-sensitive ATPase activity in isolated insect Sf9 cell membranes overexpressing human ABCB1. In the inhibition study, purvalanol A, olomoucine II and roscovitine considerably and dose-dependently reduced the verapamil-stimulated vanadate-sensitive ATPase activity of ABCB1 while flavopiridol and SNS-032 only slightly reduced it at the highest tested concentration. In the ATPase activation assay, roscovitine and olomoucine II (but not purvalanol A, flavopiridol or SNS-032) increased the baseline vanadate-sensitive ATPase activity of ABCB1 (Fig. 4).

Determination of synergistic antiproliferative activity of CDKi and DNR in combination

To assess whether the tested CDKi can synergistically potentiate the effect of another concomitantly administered cytotoxic compound that is known to be an ABCB1 substrate, we employed the combination index method of Chou-Talalay. The CI values obtained from applications of purvalanol A, olomoucine II and roscovitine in combination with DNR to the MDCKII-ABCB1 cell line fell in the synergistic category of drug combination effects across almost the whole fraction of cells affected (Fa) range (Fig. 5A-D). In contrast, significantly weaker synergistic effects were observed in the MDCKII parent cell line, where combinations of purvalanol A, olomoucine II and roscovitine with DNR only displayed synergistic effects when the Fa exceeded 0.4, 0.4 and 0.45, respectively (Fig. 5E-H). The calculated DRI indicate that the presence of purvalanol A, olomoucine II or roscovitine allows 4.6-, 3.3- or 3.8-fold reductions in the DNR doses required to reach an Fa of 0.75 in MDCKII-ABCB1 cells (Table 2).

In the HCT-8 cell line, combinations of DNR with olomoucine II, roscovitine and purvalanol A showed synergistic cytotoxic effects at Fa >0.1, >0.3 and >0.75, respectively (Fig. 6A-D). Corresponding Fa values for synergism in the HepG2 cell line were 0.75, 0.65 and 0.9, respectively (Fig. 6E-H).

Discussion

CDKi are a promising class of anticancer [15,16] and antiviral [33,34,35] drugs. The cell cycle-related effects of these compounds have been intensively researched, but their interactions with drug efflux transporters have not been previously evaluated in detail. Thus, in the presented study we employed several experimental approaches to elucidate interactions of five CDKi (purvalanol A, olomoucine II, roscovitine, flavopiridol, and SNS-032) with the ABCB1 transporter in vitro.

Using accumulation assays in MDCKII-ABCB1 cells, we show that all tested CDKi inhibit the ABCB1 transporter. We demonstrate that olomoucine II, roscovitine, purvalanol A and flavopiridol can inhibit ABCB1-mediated efflux of both HOE and DNR, indicating that the drugs can interact with the H- as well as R-site of the ABCB1 transporter. In contrast, SNS-032 selectively inhibited ABCB1-mediated transport of HOE, but not DNR, suggesting that this compound interacts with efflux activity of the H-site, but not R-site, of ABCB1. Preferential affinity of substrates and inhibitors for either of the two ABCB1 binding sites is thus an important factor to consider when investigating and predicting ABCB1-mediated drug-drug interactions, as recently demonstrated by Wang et al. [36].

To further characterize the interactions of CDKi with ABCB1 we examined their effects on the activities of ATPase in Sf9 membranes overexpressing human ABCB1. All the tested substances decreased activation of ABCB1 ATPase, confirming that they interact with the ABCB1 transporter. Our results also provide the first indications that olomoucine II is an ABCB1 substrate and inhibitor, as well confirming previous observations that roscovitine has these characteristics [37]. In contrast, purvalanol A, flavopiridol and SNS-032 can be classified as non-substrates of ABCB1 as they did not affect the ATPase activity. Interestingly, flavopiridol has recently been shown to be transported by mouse Abcb1 [38,39]. Similarly, higher levels of SNS-032 have been detected in brains of Abcb1 knockout mice than in wild type mice, suggesting that SNS-032 is a substrate of mouse P-glycoprotein [40]. We believe these discrepancies may be due to interspecies differences, in accordance with observations recently reviewed by Chu et al. [41].

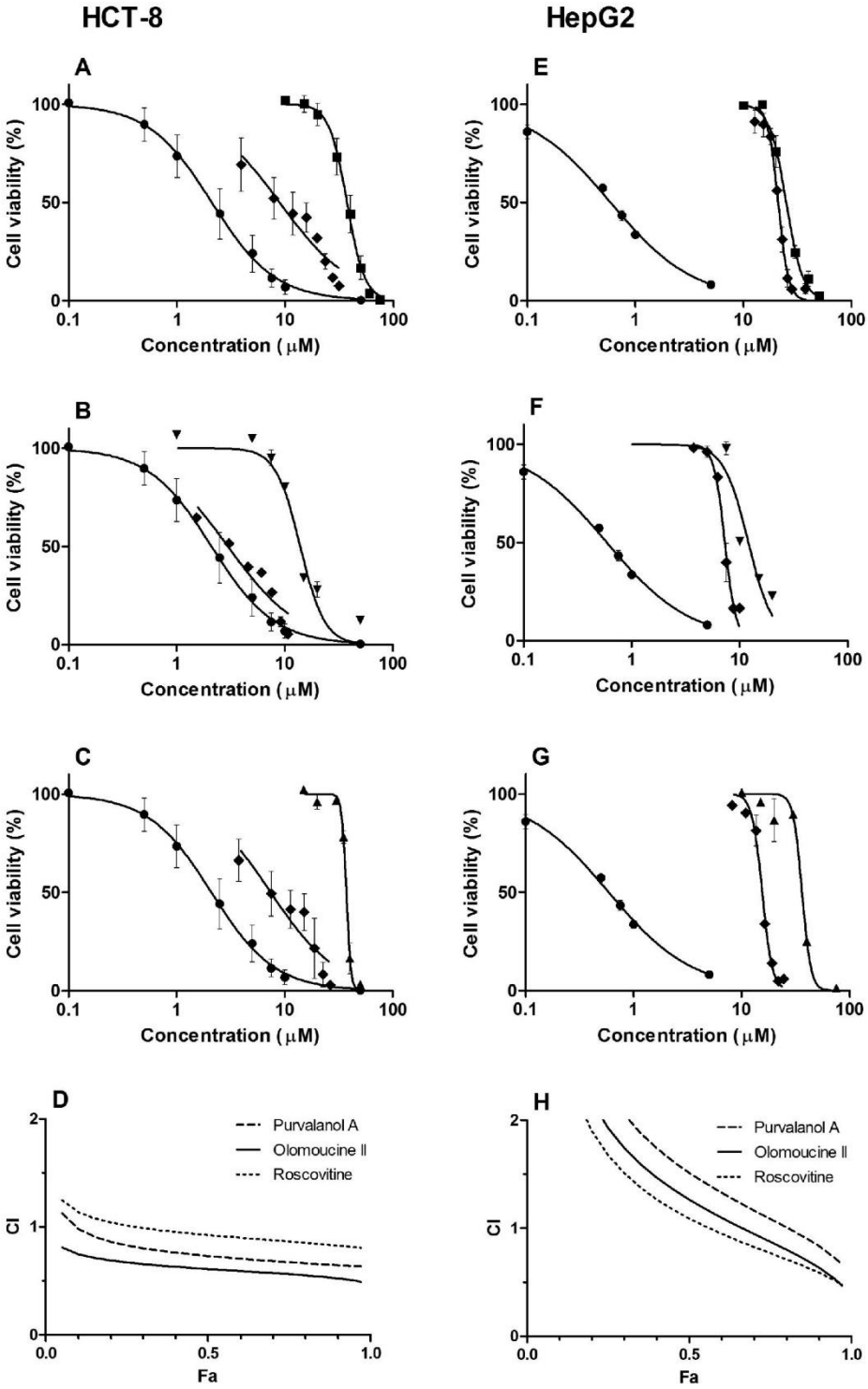

Figure 6. Cytotoxicity and combination experiments in HCT-8 and HepG2 cell lines. Cytotoxic effect of (A, E) purvalanol A (■), (B, F) olomoucine II (▼), (C, G) roscovitine (▲) or daunorubicin (●) and their combination (◆) on HCT-8 cells or HepG2. For combinations, concentrations corresponding to particular points in the graph are sums of concentrations of the individual drugs administered in fixed concentration ratios (Table 2), based on the ratio of their respective EC_{50} values. Presented data are means ± SD obtained from at least three independent experiments performed in triplicate. (D, H) The cytotoxic effect (combination index, CI, plot) of CDKi and daunorubicin combinations on MDCKII-ABCB1 or MDCKII

parent cells, obtained using CompuSyn software. Fractional effects (Fa) were calculated from the cell viability values of individual compounds; Fa = 0 means no antiproliferative effect, Fa = 1 means 100% antiproliferative effect. CI = 0.9–1.1 indicates additive effect, CI <0.9 synergism and CI >1.1 antagonism.
doi:10.1371/journal.pone.0083467.g006

In cancer treatment, drugs are frequently administered in various combinations to increase their therapeutic effects, reduce toxicity, and minimize the induction of drug resistance [32,42]. Here we hypothesized that simultaneous administration of ABCB1-inhibiting CDKi with another cytotoxic agent that is an ABCB1 substrate might have synergistic antiproliferative effects. To test this hypothesis, we applied each of the three ABCB1 inhibitors that were most potent in our accumulation experiments (purvalanol A, roscovitine or olomoucine II) in combination with DNR (a commonly used anticancer drug and ABCB1 substrate) to several ABCB1-transduced or human tumor-derived cell lines. The CDKi-DNR combinations had significantly more pronounced synergistic effects on MDCKII-ABCB1 cells than on parental MDCKII cells. Thus, the synergistic effect of these combinations is clearly directly related to the expression of ABCB1. We postulate that CDKi increases the intracellular accumulation of DNR by inhibiting ABCB1, thus increasing its cytotoxic effect. Moreover, purvalanol A, olomoucine II and roscovitine also contribute to the cytostatic effect by their own cooperative proapoptotic activity.

In addition to genetically modified cells, two human carcinoma cell lines (HCT-8 and HepG2, derived from ileocecal adenocarcinoma and Caucasian hepatocyte carcinoma, respectively) were included in these studies since they abundantly express ABCB1 [24] and represent more clinically relevant settings than MDCK cells lines. We observed synergistic effects of CDKi and DNR combinations in both carcinoma cell lines, but weaker than those detected in MDCKII-ABCB1 cells, probably because expression of ABCB1 mRNA is an order of magnitude weaker in HCT-8 and HepG2 cells than in MDCKII-ABCB1 cells (Table 1). However, other factors such as the biotransformation of intracellularly accumulated DNR [43] or activities of other efflux transporters [44] may also affect the strength of the synergistic effects in various cells.

The synergistic activity of roscovitine, purvalanol A or olomoucine II in combination with DNR could offer a promising strategy in cancer treatment. There have been several reports on the synergistic effects of combinations of roscovitine with various cytotoxic agents, including paclitaxel [45], vinblastine, 5-fluorouracil and taxol [46] in vitro and others, e.g. doxorubicin, in vivo [47]. The synergistic activity is often attributed to reductions in survivin levels [45,46], leading to increased induction of apoptosis. However, Appleyard et al. [47] observed no changes in p53 or survivin levels following combined applications of roscovitine and doxorubicin in a breast cancer xenograft model, suggesting that cell cycle arrest rather than apoptosis is the main mechanism of the enhanced antitumor effect. We provide here the first indications that the synergistic effect of DNR and CDKi might be at least partly due to interactive effects of the drugs on the ABCB1 transporter.

In conclusion, this is the first demonstration of the ability of five CDKi – purvalanol A, olomoucine II, roscovitine, flavopiridol, and SNS-032 – to inhibit ABCB1-mediated efflux, which can have a considerable impact on the pharmacokinetic behavior of simultaneously administered ABCB1 substrates. Identification of ABCB1 modulators is of great clinical interest, as these compounds are capable of reversing drug resistance and improving cancer chemotherapy [13]. The CDKi tested in our study act as such modulators and moreover contribute to a positive therapeutic outcome through their own cytotoxic activity. Thus, they have an advantage over "plain" ABCB1 modulators that only inhibit the efflux transporter without any other anticancer effect. As observed, administration of purvalanol A, olomoucine II, or roscovitine in combination with a cytostatic ABCB1 substrate has synergistic antiproliferative effects in ABCB1-expressing cells. Simultaneous administration of CDKi and ABCB1 substrates in the treatment of ABCB1-expressing tumors could, therefore, allow significant dose reductions of both concomitantly administered compounds (Table 2) and thus decrease their cumulative side effects and toxicity. We believe that our findings could be beneficial for further considerations of CDKi in pharmacotherapy, especially in cancer treatment as these compounds could have novel applications in circumventing multidrug resistance.

Author Contributions

Conceived and designed the experiments: FS MC. Performed the experiments: DC JH. Analyzed the data: DC MC FS. Contributed reagents/materials/analysis tools: FS. Wrote the paper: DC MC FS.

References

1. Bodo A, Bakos E, Szeri F, Varadi A, Sarkadi B (2003) The role of multidrug transporters in drug availability, metabolism and toxicity. Toxicol Lett 140–141: 133-143.
2. Staud F, Pavek P (2005) Breast cancer resistance protein (BCRP/ABCG2). Int J Biochem Cell Biol 37: 720–725.
3. Staud F, Ceckova M, Micuda S, Pavek P (2010) Expression and Function of P-Glycoprotein in Normal Tissues: Effect on Pharmacokinetics. In: Zhou J, editor. Multi-Drug Resistance in Cancer: Humana Press. pp. 199–222.
4. Szakacs G, Varadi A, Ozvegy-Laczka C, Sarkadi B (2008) The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug Discov Today 13: 379–393.
5. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. (2010) Membrane transporters in drug development. Nat Rev Drug Discov 9: 215–236.
6. Zamek-Gliszczynski MJ, Hoffmaster KA, Tweedie DJ, Giacomini KM, Hillgren KM (2012) Highlights from the International Transporter Consortium second workshop. Clin Pharmacol Ther 92: 553–556.
7. Li Y, Yuan H, Yang K, Xu W, Tang W, et al. (2010) The structure and functions of P-glycoprotein. Curr Med Chem 17: 786–800.
8. Staud F, Ceckova M, Micuda S, Pavek P (2010) Expression and function of p-glycoprotein in normal tissues: effect on pharmacokinetics. Methods Mol Biol 596: 199–222.
9. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, et al. (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39: 361–398.
10. Shapiro AB, Ling V (1997) Positively cooperative sites for drug transport by P-glycoprotein with distinct drug specificities. Eur J Biochem 250: 130–137.
11. Fromm MF (2000) P-glycoprotein: a defense mechanism limiting oral bioavailability and CNS accumulation of drugs. Int J Clin Pharmacol Ther 38: 69–74.
12. Caceres G, Robey RW, Sokol L, McGraw KL, Clark J, et al. (2012) HG-829 is a potent noncompetitive inhibitor of the ATP-binding cassette multidrug resistance transporter ABCB1. Cancer Res 72: 4204–4213.
13. Shukla S, Ohnuma S, Ambudkar SV (2011) Improving cancer chemotherapy with modulators of ABC drug transporters. Curr Drug Targets 12: 621–630.
14. Dai Y, Grant S (2003) Cyclin-dependent kinase inhibitors. Curr Opin Pharmacol 3: 362–370.
15. Krystof V, Uldrijan S (2010) Cyclin-dependent kinase inhibitors as anticancer drugs. Curr Drug Targets 11: 291–302.
16. Cicenas J, Valius M (2011) The CDK inhibitors in cancer research and therapy. J Cancer Res Clin Oncol 137: 1409–1418.
17. Shapiro GI (2006) Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol 24: 1770–1783.

18. Hofman J, Ahmadimoghaddam D, Hahnova L, Pavek P, Ceckova M, et al. (2012) Olomoucine II and purvalanol A inhibit ABCG2 transporter in vitro and in situ and synergistically potentiate cytostatic effect of mitoxantrone. Pharmacol Res 65: 312–319.
19. Hofman J, Kucera R, Cihalova D, Klimes J, Ceckova M, et al. (2013) Olomoucine II, but not Purvalanol A, is transported by Breast Cancer Resistance Protein (ABCG2) and P-glycoprotein (ABCB1). PLOS One: In press.
20. Le Tourneau C, Faivre S, Laurence V, Delbaldo C, Vera K, et al. (2010) Phase I evaluation of seliciclib (R-roscovitine), a novel oral cyclin-dependent kinase inhibitor, in patients with advanced malignancies. Eur J Cancer 46: 3243–3250.
21. Lin TS, Ruppert AS, Johnson AJ, Fischer B, Heerema NA, et al. (2009) Phase II study of flavopiridol in relapsed chronic lymphocytic leukemia demonstrating high response rates in genetically high-risk disease. J Clin Oncol 27: 6012–6018.
22. Tong WG, Chen R, Plunkett W, Siegel D, Sinha R, et al. (2010) Phase I and pharmacologic study of SNS-032, a potent and selective Cdk2, 7, and 9 inhibitor, in patients with advanced chronic lymphocytic leukemia and multiple myeloma. J Clin Oncol 28: 3015–3022.
23. Wang LM, Ren DM (2010) Flavopiridol, the first cyclin-dependent kinase inhibitor: recent advances in combination chemotherapy. Mini Rev Med Chem 10: 1058–1070.
24. Ceckova M, Libra A, Pavek P, Nachtigal P, Brabec M, et al. (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.
25. Lalande ME, Ling V, Miller RG (1981) Hoechst 33342 dye uptake as a probe of membrane permeability changes in mammalian cells. Proc Natl Acad Sci U S A 78: 363–367.
26. Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, et al. (1996) Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. Cancer Res 56: 4171–4179.
27. Spoelstra EC, Westerhoff HV, Dekker H, Lankelma J (1992) Kinetics of daunorubicin transport by P-glycoprotein of intact cancer cells. Eur J Biochem 207: 567–579.
28. Tang F, Ouyang H, Yang JZ, Borchardt RT (2004) Bidirectional transport of rhodamine 123 and Hoechst 33342, fluorescence probes of the binding sites on P-glycoprotein, across MDCK-MDR1 cell monolayers. J Pharm Sci 93: 1185–1194.
29. Wang EJ, Casciano CN, Clement RP, Johnson WW (2000) In vitro flow cytometry method to quantitatively assess inhibitors of P-glycoprotein. Drug Metab Dispos 28: 522–528.
30. Weiss J, Rose J, Storch CH, Ketabi-Kiyanvash N, Sauer A, et al. (2007) Modulation of human BCRP (ABCG2) activity by anti-HIV drugs. J Antimicrob Chemother 59: 238–245.
31. Chou TC, Talalay P (1984) Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. Adv Enzyme Regul 22: 27–55.
32. Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58: 621–681.
33. Guendel I, Agbottah ET, Kehn-Hall K, Kashanchi F (2010) Inhibition of human immunodeficiency virus type-1 by cdk inhibitors. AIDS Res Ther 7: 7.
34. Schang LM, St Vincent MR, Lacasse JJ (2006) Five years of progress on cyclin-dependent kinases and other cellular proteins as potential targets for antiviral drugs. Antivir Chem Chemother 17: 293–320.
35. Holcakova J, Tomasec P, Bugert JJ, Wang EC, Wilkinson GW, et al. (2010) The inhibitor of cyclin-dependent kinases, olomoucine II, exhibits potent antiviral properties. Antivir Chem Chemother 20: 133–142.
36. Wang E-j, Casciano CN, Clement RP, Johnson WW (2001) Active transport of fluorescent P-glycoprotein substrates: evaluation as markers and interaction with inhibitors. Biochem Biophys Res Commun 289: 580–585.
37. Rajnai Z, Mehn D, Beery E, Okyar A, Jani M, et al. (2010) ATP-binding cassette B1 transports seliciclib (R-roscovitine), a cyclin-dependent kinase inhibitor. Drug Metab Dispos 38: 2000–2006.
38. Zhou L, Schmidt K, Nelson FR, Zelesky V, Troutman MD, et al. (2009) The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic acid (PF-407288) in mice. Drug Metab Dispos 37: 946–955.
39. Kodaira H, Kusuhara H, Ushiki J, Fuse E, Sugiyama Y (2010) Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. J Pharmacol Exp Ther 333: 788–796.
40. Kamath AV, Chong S, Chang M, Marathe PH (2005) P-glycoprotein plays a role in the oral absorption of BMS-387032, a potent cyclin-dependent kinase 2 inhibitor, in rats. Cancer Chemother Pharmacol 55: 110–116.
41. Chu X, Bleasby K, Evers R (2013) Species differences in drug transporters and implications for translating preclinical findings to humans. Expert Opin Drug Metab Toxicol 9: 237–252.
42. O'Connor R (2009) A review of mechanisms of circumvention and modulation of chemotherapeutic drug resistance. Curr Cancer Drug Targets 9: 273–280.
43. Riddick DS, Lee C, Ramji S, Chinje EC, Cowen RL, et al. (2005) Cancer chemotherapy and drug metabolism. Drug Metab Dispos 33: 1083–1096.
44. Cole SP, Sparks KE, Fraser K, Loe DW, Grant CE, et al. (1994) Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 54: 5902–5910.
45. Coley HM, Shotton CF, Thomas H (2007) Seliciclib (CYC202; r-roscovitine) in combination with cytotoxic agents in human uterine sarcoma cell lines. Anticancer Res 27: 273–278.
46. Abaza MS, Bahman AM, Al-Attiyah RJ (2008) Roscovitine synergizes with conventional chemo-therapeutic drugs to induce efficient apoptosis of human colorectal cancer cells. World J Gastroenterol 14: 5162–5175.
47. Appleyard MV, O'Neill MA, Murray KE, Paulin FE, Bray SE, et al. (2009) Seliciclib (CYC202, R-roscovitine) enhances the antitumor effect of doxorubicin in vivo in a breast cancer xenograft model. Int J Cancer 124: 465–472.

6.3 Interactions of cyclin-dependent kinase inhibitors AT-7519, flavopiridol and SNS-032 with ABCB1, ABCG2 and ABCC1 transporters and their potential to overcome multidrug resistance *in vitro*

Cihalova D., Staud F., Ceckova M.

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V této práci jsme studovali interakce CDKI AT-7519, flavopiridolu a SNS-032 s ABC transportéry a zkompletovali jsme tak interakční profil těchto látek s transportéry ABCB1, ABCG2 i ABCC1.

Inhibiční aktivitu CDKI vůči jednotlivým transportérům jsme hodnotili pomocí akumulačních metod s fluorescenčními substráty v MDCKII buněčných liniích s expresí lidských transportérů ABCB1, ABCG2 nebo ABCC1. Rezistence buněk s expresí transportérů vůči testovaným CDKI byla hodnocena pomocí XTT antiproliferativního testu. Případné interakce byly rovněž potvrzeny metodou měření ATPázové aktivity v membránových veziklech. Následně byla také provedena analýza kombinačního indexu v lidských nádorových buněčných liniích, HepG2 a T47D.

Flavopiridol signifikantně inhiboval transportéry ABCG2 a ABCC1. Látka SNS-032 také snižovala eflux ABCG2, zatímco AT-7519 nevykazoval inhibiční aktivitu vůči žádnému transportéru. Jak flavopiridol, tak SNS-032 docílily v nádorových buňkách synergického antiproliferativního efektu v kombinaci s relevantními substráty ABC transportérů, jakými jsou např. daunorubicin a topotekan. Zjistili jsme také, že ABCB1 způsobuje rezistenci vůči AT-7519 a SNS-032. Na druhou stranu, transportéry ABCG2 a ABCC1 mohou být zodpovědné za vznik rezistence vůči flavopiridolu.

Tato data poskytují detailní informace o interakcích flavopiridolu, SNS-032 a AT-7519 s ABC transportéry, což může pomoci k podrobnému popsání farmakokinetiky a toxicity těchto látek. Navíc jsme ukázali schopnost flavopiridolu a SNS-032 překonávat mnohočetnou lékovou rezistenci, čehož by mohlo být využito v protinádorové terapii.

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ORIGINAL ARTICLE

Interactions of cyclin-dependent kinase inhibitors AT-7519, flavopiridol and SNS-032 with ABCB1, ABCG2 and ABCC1 transporters and their potential to overcome multidrug resistance in vitro

Daniela Cihalova[1] · Frantisek Staud[1] · Martina Ceckova[1]

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Abstract
Purpose ATP-binding cassette (ABC) transporters play an important role in multidrug resistance (MDR) toward anticancer drugs. Here, we evaluated interactions of cyclin-dependent kinase inhibitors (CDKi) AT-7519, flavopiridol and SNS-032 with the following ABC transporters in vitro: P-glycoprotein (ABCB1), breast cancer resistance protein (ABCG2) and multidrug resistance-associated protein 1 (ABCC1).
Methods Inhibitory potency of studied CDKi to the transporters was evaluated by accumulation assays using fluorescent substrates and MDCKII cells overexpressing human ABCB1, ABCG2 or ABCC1. Resistance of transporter-expressing cells to the CDKi was evaluated by XTT proliferation assay. Observed interactions of CDKi were verified by ATPase assay in ABC transporter-expressing Sf9 membrane vesicles. Combination index analysis was additionally performed in ABC transporter-expressing cancer cell lines, HepG2 and T47D.
Results Flavopiridol showed a significant inhibitory potency toward ABCG2 and ABCC1. SNS-032 also decreased ABCG2-mediated efflux, while AT-7519 failed to inhibit ABCB1, ABCG2 or ABCC1. Both flavopiridol and SNS-032 showed synergistic antiproliferative effects in combination with relevant ABC transporter substrates such as daunorubicin and topotecan in cancer cells. ABCB1 was found to confer significant resistance to AT-7519 and SNS-032, but not to flavopiridol. In contrast, ABCG2 and ABCC1 conferred resistance to flavopiridol, but not to AT-7519 and SNS-032.
Conclusion Our data provide detailed information on interactions of flavopiridol, SNS-032 and AT-7519 with ABC transporters, which may help elucidate the pharmacokinetic behavior and toxicity of these compounds. Moreover, we show the ability of flavopiridol and SNS-032, but not AT-7519, to overcome ABC transporter-mediated MDR.

Keywords Cyclin-dependent kinase inhibitor · Multidrug resistance · ABC transporter · AT-7519 · Flavopiridol · SNS-032

Abbreviations

ABC	ATP-binding cassette
ABCB1	P-glycoprotein
ABCC1	Multidrug resistance-associated protein 1
ABCG2	Breast cancer resistance protein
CDK	Cyclin-dependent kinase
CDKi	Cyclin-dependent kinase inhibitor
CI	Combination index
DNR	Daunorubicin
DRI	Dose-reduction index
EC_{50}	Median effective antiproliferative concentration
MDCKII	Madin–Darby canine kidney
MDR	Multidrug resistance
MFI	Median fluorescence intensity
MIT	Mitoxantrone
NEM-SG	*N*-ethylmaleimide-glutathione
PMS	Phenazine methosulfate

✉ Martina Ceckova
martina.ceckova@faf.cuni.cz

Daniela Cihalova
cihad6aa@faf.cuni.cz

Frantisek Staud
frantisek.staud@faf.cuni.cz

[1] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Hradec Kralove, Czech Republic

RF	Resistance factor
TOP	Topotecan
XTT	XTT sodium salt

Introduction

Multidrug resistance (MDR) is one of the major causes of failure in cancer chemotherapy. This phenomenon occurs when cancer cells become resistant to different anticancer drugs and are able to survive treatment by a multitude of structurally and functionally unrelated chemotherapeutics. Overexpression of ATP-binding cassette (ABC) transporters, such as ABCB1 (P-glycoprotein, P-gp), ABCG2 (breast cancer resistance protein, BCRP) and ABCC1 (multidrug resistance-associated protein 1, MRP1), in tumor cells is one of the most important mechanisms of MDR as these efflux transporters recognize different chemotherapeutic agents and transport them out of the cell, thereby decreasing their intracellular accumulation [1, 2]. These transporters have become attractive molecular targets, and great effort has been made to find their inhibitors (MDR modulators) in order to increase bioavailability after oral administration, to enhance tissue penetration of transported drugs and to overcome drug resistance in cancer cells [3–5]. Several generations of ABC transporter modulators have failed to fulfill their expectations in the clinical area so far [6, 7]. However, MDR research has recently been directed to examining modulatory effects of compounds that were not originally designed for reversing multidrug resistance, such as tyrosine kinase inhibitors (TKI) [8]. TKI have been shown to block or antagonize ABC transporters in vitro and in vivo, and preclinical data indicate that TKI are effective in overcoming MDR when used with standard anticancer drugs [9, 10]. Small molecule kinase inhibitors may therefore represent a new approach in ABC transporter modulation.

Another novel group of kinase inhibitors is targeted toward cyclin-dependent kinases (CDKs), which are serine/threonine protein kinases regulating the progression of the cell through the cell cycle and RNA transcription. CDKs are regulated by positive phosphorylation by CDK-activating kinase [11], as well as by negative phosphorylation by endogenous Cip/Kip or INK inhibitors [12, 13]. Because of their critical role in cell cycle progression, cellular transcription and apoptosis, CDKs are major targets for deregulation in different human tumors [14, 15]. This fact has led to the development of CDK inhibitors (CDKi) as an effective method for controlling tumor growth and hence a potential therapeutic tool for cancer treatment [16, 17]. Small molecule CDKi have been shown to be highly effective against the activity of several CDKs, causing significant cell cycle arrest and apoptosis in many cancers; some of these compounds have entered clinical trials, and among the most advanced are AT-7519, flavopiridol (alvocidib) and SNS-032 (BMS-387032). These CDKi have been tested for several indications, including solid and hematological malignancies, either as single agents or in combination with other chemotherapeutics. AT-7519 is currently undergoing phase II clinical trials for the treatment of leukemia and lymphoma (clinicaltrials.gov, NCT01627054 and NCT01652144, respectively). Flavopiridol has shown clinical activity in chronic lymphocytic leukemia [18] and ovarian carcinoma [19] in phase II clinical trials, and SNS-032 is reported to be in phase I development in metastatic refractory solid tumors and B cell malignancies [20, 21].

These new CDKi clearly offer a possibility of improved therapy for cancer patients. However, data on their drug–drug interactions are insufficient, although crucial for a thorough understanding of their pharmacokinetic behavior or the behavior of other simultaneously administered compounds. Currently, only a few studies have examined the effect of these three CDKi in cell lines or animal models that overexpress ABCB1, ABCG2 or ABCC1 transporters. In our previous study, we demonstrated the inhibitory effect of several CDKi, including flavopiridol and SNS-032, toward the ABCB1 transporter [22]. In the present work, we aimed to comprehensively investigate the in vitro effect of three promising CDKi, AT-7519, flavopiridol or SNS-032, on the efflux activity of not only ABCB1, but also ABCG2 and ABCC1 transporters in MDCKII cells and to determine whether the inhibiting compounds can potentiate the efficacy of other conventional antineoplastic drugs in cancer cells through these interactions. Moreover, we also evaluated the causative role of the ABC transporters in cellular resistance to the CDKi.

Materials and methods

Materials

AT-7519 was obtained from Axon Medchem (Groningen, the Netherlands). Flavopiridol and SNS-032 were supplied by SelleckChem (Houston, TX, USA). Daunorubicin (DNR), mitoxantrone (MIT), XTT sodium salt (XTT), phenazine methosulfate (PMS) and ABCC1 inhibitor MK-571 were purchased from Sigma-Aldrich (St. Louis, MO, USA). ABCB1 inhibitor LY335979 was obtained from Toronto Research Chemicals (North York, ON, Canada) and ABCG2 inhibitor Ko143 was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Cell culture reagents were supplied by Sigma-Aldrich (St. Louis, MO, USA) and Gibco BRL Life Technologies (Rockville, MD, USA). ABCB1, ABCG2 and ABCC1 PREDEASY™

ATPase kits (SB MDR1/P-gp, SB BCRP HAM Sf9 and SB MRP1, respectively) were purchased from Solvo Biotechnology (Szeged, Hungary).

Cell culture

Madin–Darby canine kidney (MDCKII) cell lines transduced with the human transporters ABCB1 (MDCKII-ABCB1), ABCG2 (MDCKII-ABCG2) or ABCC1 (MDCKII-ABCC1) which stably express ABCB1, ABCG2 or ABCC1 transporter, respectively, and the MDCKII parent cell line were obtained from Prof. Piet Borst and Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, the Netherlands). The stable expression of ABC transporters was verified by RT-PCR (see Sect. 3.4). The cell lines were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS. For in vitro drug combination studies, we also used human liver carcinoma HepG2 and human ductal breast carcinoma T47D cell lines. HepG2 cells were purchased from American Type Culture Collection (LGC Promochem, Teddington, Middlesex, UK) and were grown in minimal essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 10 % FBS. T47D cells, obtained from the European Collection of Cell Cultures (PHE, Salisbury, Wiltshire, UK), were cultured in DMEM without phenol red, supplemented with 10 % FBS and 2 mM L-glutamine. Dimethyl sulfoxide was applied as a solvent in concentrations not exceeding 0.5 % (1 % in ATPase assays).

RNA isolation and RT-PCR for ABCB1, ABCG2 and ABCC1 expression in cell lines

Total RNA was isolated from confluent monolayers of ABC transporter-expressing MDCKII, MDCKII parent, HepG2 and T47D cells using the TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Each cell line has been sampled at least in triplicate. RNA was dissolved in DEPC-treated water, and concentration and purity of each sample were determined spectrophotometrically from A260/A280 measurements (NanoDrop, Thermo Scientific, Wilmington, DE, USA). Integrity of RNA was also checked by agarose gel electrophoresis. cDNA was prepared from 1 µg extracted total RNA by MMLV reverse transcriptase using oligo(dT) VN nucleotides (gb Reverse Transcription Kit, Generi Biotech, Hradec Kralove, Czech Republic). PCR analysis was performed on QuantStudio 6 (Life Technologies). cDNA (40 ng) was amplified using 2 × Probe Master Mix (Generi Biotech, Hradec Kralove, Czech Republic) and predesigned PCR assays for ABC transporters: *ABCB1*, *ABCG2* and *ABCC1* (hABCB1_Q2, hABCG2_Q2 and hABCC1_Q2, Generi Biotech, Hradec Kralove, Czech Republic) and reference genes: *HPRT* and *β2-microglobulin* (hHPRT_Q2 and hB2M_Q2, Generi Biotech, Hradec Kralove, Czech Republic). The temperature profile was 95 °C for 3 min and 35 repeats of a cycle consisting of 95 °C for 10 s and 60 °C for 20 s. C_t values for particular samples were noted, and PCR products were additionally separated by agarose gel electrophoresis and visualized by UV (Bio-Rad Laboratories). Mean C_t values of HPRT and β2-microglobulin were used as normalizing controls for each sample of carcinoma cells to calculate ΔC_t values. Normalizing genes have been chosen based on previous experiments showing no statistically significant difference in C_t values for HPRT and β2-microglobulin between the HepG2 and T47D cells.

XTT cell proliferation assays

A total of 1×10^4 MDCKII-ABCB1, MDCKII-ABCG2, MDCKII-ABCC1 or MDCKII parent, 2×10^4 HepG2 or 1.5×10^4 T47D cells were grown in 96-well culture plates and incubated for 24 h. Individual CDKi diluted with growth medium were added to the exponentially growing cells, and the resulting mixtures were incubated for 72 h at 37 °C, 5 % CO_2. Cell viability was assessed using the XTT assay as follows: Cells were incubated with 0.167 mg/mL XTT with 4 µM PMS in Opti-MEM for 2 h. The absorbance of the soluble formazan released was measured at 470 nm on a microplate reader (Tecan, Männedorf, Switzerland). The median effective antiproliferative concentrations (EC_{50}) of the compounds were calculated using GraphPad Prism 6.00. To determine the influence of ABC transporters on growth inhibition, resistance factor (RF) was calculated by dividing the EC_{50} value of ABC transporter-overexpressing cell line by the EC_{50} value of the respective parental cell line; RF therefore represents a fold increase in resistance caused by the presence of a particular ABC transporter [23]. As an indirect method to assess whether the tested CDKi are substrates of ABCB1, ABCG2 or ABCC1, cell proliferation assays were repeated with the addition of model inhibitors of the three transporters (1 µM LY335979, 1 µM Ko143 and 25 µM MK-571 for ABCB1, ABCG2 and ABCC1, respectively) to abolish the potential influence of ABC transporter on the resistance.

DNR and MIT accumulation assays

The effect of CDKi on the intracellular DNR accumulation in MDCKII-ABCB1 and MDCKII-ABCC1, and MIT accumulation in MDCKII-ABCG2 cells was examined by flow cytometry (Accuri C6, Accuri, Ann Arbor, USA). Parental MDCKII cells were analyzed as a control using both substrates separately. Cells were seeded at a density of 1.5×10^5 on a 12-well plate 24 h before experiment and

treated with five concentrations of CDKi, solvent (0.5 % DMSO, control) or Opti-MEM (untreated control) for 30 min at 37 °C, 5 % CO_2. DNR or MIT was then added to a final concentration of 2 or 1 μM, respectively, and the cells were incubated under the same conditions for a further 60 min. Accumulation was stopped by cooling the samples on ice and washing twice with ice-cold PBS. The cells were detached with 10× trypsin–EDTA and resuspended in PBS with 2 % FBS. The intracellular accumulation of individual cells was measured with an excitation/emission filter of 488/585 nm for DNR and 488/670 nm for MIT and recorded as histograms. As positive controls for ABCB1, ABCG2 and ABCC1 inhibition, 1 μM LY335979, 1 μM Ko143 and 50 μM MK-571 were used, respectively. Viable cells were gated based on forward and side scatter plots. The median fluorescence intensity (MFI) of 10,000 measured cells was used to compare the fluorescence resulting from each of the treatments. Relative values were identified by dividing the MFI of each measurement by that of untreated control cells.

ABCB1, ABCG2 and ABCC1 ATPase assays

Membrane preparations overexpressing ABC transporters show vanadate-sensitive ATPase activity that is modulated by interacting compounds. In the activation assay, transported substrates can stimulate baseline vanadate-sensitive ATPase activity, whereas in the inhibition assay, which is carried out in the presence of a known activator of the transporter, inhibitors may reduce the maximally stimulated vanadate-sensitive ATPase activity [24]. ATPase activity was measured by assessing the amount of phosphate liberated from ATP by the ABCB1, ABCG2 or ABCC1 transporters using the PREDEASY™ ATPase Kit for a corresponding transporter according to the manufacturer's instructions. For this purpose, Sf9 cell membranes (4 μg protein per well) were mixed with each of the test compounds in solutions, with concentrations ranging from 0.14 to 300 μM, and then incubated at 37 °C for 10 min in the presence or absence of 1.2 mM sodium orthovanadate. ATPase reaction was started by adding 10 mM ATP magnesium salt to the reaction mixture and stopped 10 min later, and the absorbance at 590 nm was measured after a further 30-min incubation (Tecan, Männedorf, Switzerland). ATPase activity in each sample was determined as the difference in liberated amounts of phosphate measured in the presence and absence of 1.2 mM sodium orthovanadate. Phosphate standards were prepared in each plate; verapamil, sulfasalazine and *N*-ethylmaleimide-glutathione (NEM-SG) served as positive controls for ABCB1, ABCG2 and ABCC1 stimulation, respectively, as provided by the manufacturer. Results are expressed as vanadate-sensitive ATPase activities.

Drug combination studies

The combination index (CI) method of Chou–Talalay, based on the median-effect equation, was used to calculate combined drug effects; this approach offers quantitative definition for additive, synergistic and antagonistic effects (CI values of 0.9–1.1, <0.9 and >1.1, respectively) [25]. Data generated from the CI method were used to quantify dose-reduction indices (DRI) for pairs of the tested drugs. DRI represents the fold change of a focal effect when individual agents are used simultaneously relative to their separate effects, and their activity is synergistic if DRI > 1. CDKi exhibiting inhibitory activity on ABC transporters (flavopiridol and SNS-032) were combined with DNR or topotecan (TOP), commonly used anticancer drugs and ABC transporter substrates. The XTT cytotoxicity assay was used to measure the cell viability in the tested cell lines, i.e., HepG2 and T47D, in the presence of the CDKi and DNR/TOP both alone and in combination, at constant concentration ratios, ranging from 0.1 to 2 multiples of the respective, predetermined EC_{50} values. Data acquired from these drug combination experiments were analyzed using CompuSyn version 3.0.1 software (ComboSyn Inc., Paramus, NJ, USA).

Statistical analysis

Data are presented as mean ± SD of at least three independent experiments. Statistical significance was determined using two-tailed unpaired Student's *t* test or one-way ANOVA implemented in GraphPad Prism 6.00, and $P < 0.05$ is considered significant.

Results

Role of ABCB1, ABCG2 and ABCC1 in chemoresistance to CDKi

To determine the possible role of ABC transporters in causing cellular resistance to AT-7519, flavopiridol and SNS-032, we examined the antiproliferative effect of the CDKi in ABCB1-, ABCG2- and ABCC1-overexpressing MDCKII and control MDCKII parent cell lines using XTT assay. Chemotherapeutic agents, DNR and TOP, were also included in the XTT assays to verify the role of particular ABC transporters in cellular resistance to these drugs and to justify further employment of these ABC transporter substrates in combination studies (see Sects. 2.7 and 3.5). Mean EC_{50} values and RF are shown in Table 1. MDCKII-ABCB1 cells were significantly more resistant to both AT-7519 and SNS-032, with RF values of 17 and 13, respectively, compared to the parental cell line. In contrast,

Table 1 Resistance to CDKi, DNR and TOP conferred by human ABC transporters

Drug[a]	MDCKII	MDCKII-ABCB1		MDCKII-ABCG2		MDCKII-ABCC1	
	EC_{50} (μM)	EC_{50} (μM)[b]	RF[c]	EC_{50} (μM)	RF	EC_{50} (μM)	RF
AT	1.9 ± 0.038	33 ± 1.8***	17	2.1 ± 0.11	1.1	2.3 ± 0.28	1.2
+LY	0.41 ± 0.013	0.50 ± 0.041	1.2	–	–	–	–
FLA	0.16 ± 0.011	0.15 ± 0.0080	0.94	0.36 ± 0.030***	2.3	0.23 ± 0.014**	1.4
+Ko	0.15 ± 0.0088	–	–	0.14 ± 0.020	0.93	–	–
+MK	0.21 ± 0.0068	–	–	–	–	0.15 ± 0.041	0.71
SNS	1.5 ± 0.018	20 ± 1.5***	13	1.4 ± 0.16	0.93	1.0 ± 0.11	0.67
+LY	0.27 ± 0.068	0.18 ± 0.036	0.67	–	–	–	–
DNR	0.69 ± 0.083	7.6 ± 0.64***	11	0.87 ± 0.19	1.3	3.8 ± 0.26***	5.5
TOP	3.3 ± 0.20	7.8 ± 1.2**	2.4	13 ± 1.3***	3.9	17 ± 1.6***	5.2

[a] *AT* AT-7519, *FLA* flavopiridol, *LY* LY335979, *Ko* Ko143, *MK* MK-571, *SNS* SNS-032, *DNR* daunorubicin, *TOP* topotecan

[b] EC_{50} values from XTT assays are presented as mean ± SD of three independent experiments performed in triplicate. Statistical significance was calculated for ABC transporter-overexpressing cells compared to parental cells by unpaired *t* test (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)

[c] RF were calculated by dividing the EC_{50} values of ABC transporter-overexpressing cells by the EC_{50} values of MDCKII parental cells

neither ABCG2 nor ABCC1 was able to confer resistance to AT-7519 or SNS-032 over the tested concentration range in the respective overexpressing MDCKII cell lines. On the other hand, we observed that MDCKII-ABCG2 and MDCKII-ABCC1 cells were significantly more resistant to flavopiridol, with RF values of 2.3 and 1.4, respectively, compared to the drug-sensitive parental cell line, while no effect of ABCB1 on flavopiridol resistance was observed in MDCKII-ABCB1 cells.

To confirm the causative role of ABC transporters in the resistance to studied CDKi, cell proliferation assays were repeated for a combination of CDKi with model inhibitors of ABC transporters in transporter-overexpressing MDCKII cells. Indeed, we have observed a reversal of the resistance to AT-7519 and SNS-032 in the presence of 1 μM LY335979 in MDCKII-ABCB1, and to flavopiridol in the presence of 1 μM Ko143 or 25 μM MK-571 in MDCKII-ABCG2 and MDCKII-ABCC1 cells, respectively (Table 1).

Inhibitory effect of CDKi on the transporter-mediated efflux of DNR and MIT

DNR, a fluorescent substrate of ABCB1 and ABCC1, was used to determine the effect of CDKi on ABCB1- and ABCC1-mediated efflux. AT-7519 revealed no significant effect on intracellular accumulation of DNR in MDCKII-ABCB1 or MDCKII-ABCC1 cells (Fig. 1a, b). Employing MDCKII-ABCC1 cells, flavopiridol treatment enhanced the intracellular accumulation of DNR in a dose-dependent manner, showing significant inhibitory potency at 1 μM concentration. At concentrations of 10 μM and higher, flavopiridol caused the maximal transporter inhibitory effect (2.3-, 2.6- and 2.6-fold increase in the presence of 10, 30 and 50 μM flavopiridol, respectively), which was comparable to the activity of a model inhibitor, MK-571 (2.4-fold increase in DNR accumulation). No significant effect was observed in the MDCKII parental cell line. SNS-032 did not show significant effect on the DNR accumulation in MDCKII-ABCC1 cells over the tested concentration range (1–50 μM) (Fig. 1b).

The fluorescent ABCG2 substrate MIT was used to investigate the influence of studied CDKi on ABCG2-mediated efflux. The intracellular accumulation of MIT in MDCKII-ABCG2 cells was significantly increased by flavopiridol and SNS-032, while no effect on MIT accumulation was observed in the control parental cell line. In the presence of 10, 30 and 50 μM flavopiridol, the intracellular accumulation of MIT was enhanced to 3.5-, 4.2- and 4.2-fold, respectively, while 50 μM SNS-032 increased the intracellular accumulation of MIT in MDCKII-ABCG2 cells 1.9-fold. In contrast, AT-7519 did not significantly modify the intracellular accumulation of MIT in MDCKII-ABCG2, indicating a lack of inhibitory potency to the ABCG2 transporter (Fig. 1c).

ATPase assay

To further characterize interactions of CDKi with ABC transporters, we tested the modulatory effects of the drugs on vanadate-sensitive ATPase activity in isolated insect Sf9 cell membranes overexpressing human ABCB1, ABCG2 or ABCC1. In the ATPase inhibition study, flavopiridol and SNS-032 lowered the sulfasalazine-stimulated vanadate-sensitive ATPase activity of ABCG2 in a dose-dependent manner at a concentration of 300 μM, while AT-7519 did not lower the stimulated activity at all. Similarly, AT-7519 did not show any decrease in verapamil-stimulated ATPase

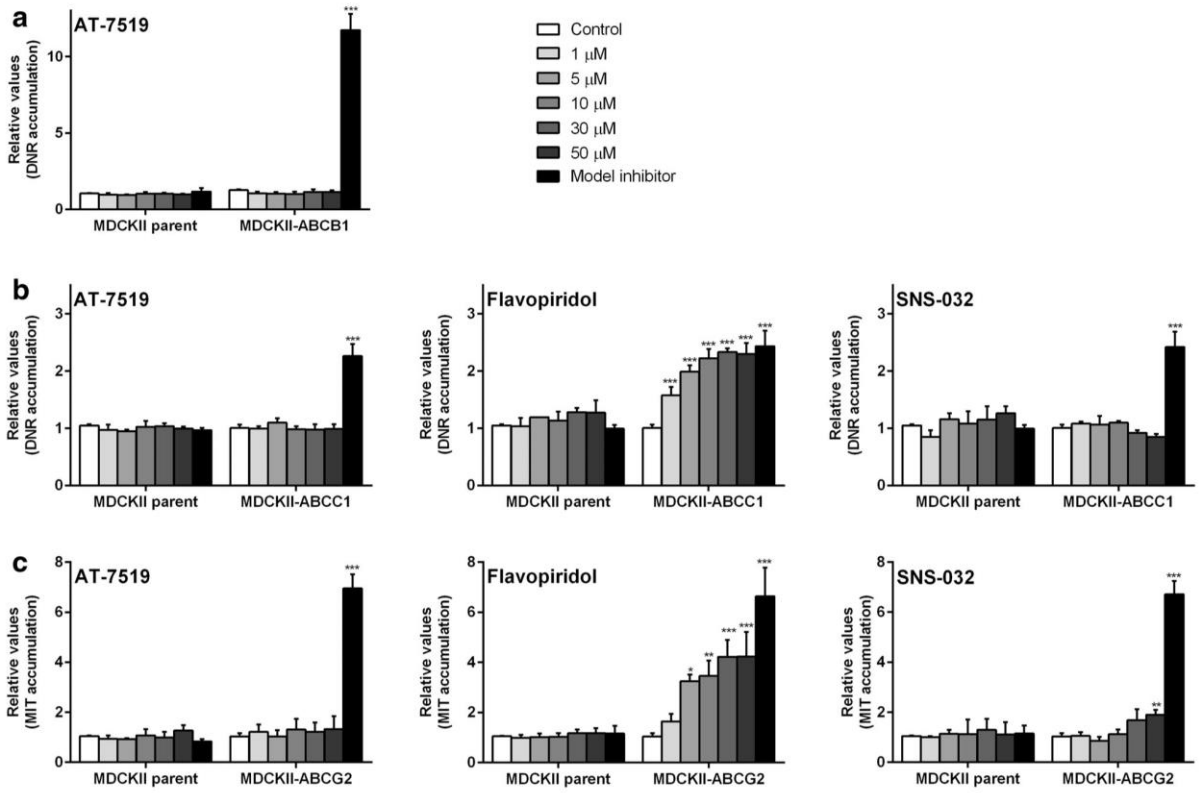

Fig. 1 Effects of AT-7519, flavopiridol and SNS-032 on the intracellular accumulation of DNR in MDCKII-ABCB1 cells (**a**), DNR in MDCKII-ABCC1 cells (**b**) and MIT in MDCKII-ABCG2 cells (**c**). Results are presented as fold changes in fluorescence intensity compared to untreated control cells. LY335979 (1 μM), MK-571 (50 μM) and Ko143 (1 μM) are used as model inhibitors for ABCB1, ABCC1 and ABCG2 inhibition, respectively. Data represent mean ± SD of three independent experiments performed in duplicate. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ determined by one-way ANOVA followed by Dunnet's test

in ABCB1 membrane preparations. Flavopiridol (300 μM) significantly reduced the NEM-SG-stimulated vanadate-sensitive ATPase activity of ABCC1, while AT-7519 and SNS-032 showed no effect. In the ATPase activation assays, only flavopiridol (but not AT-7519 and SNS-032) significantly increased the baseline vanadate-sensitive ATPase activity of ABCG2 at concentrations from 10 to 100 μM, while no other effects of CDKi on baseline ABCB1 or ABCC1 ATPase activity were observed (Fig. 2).

Expression of ABCB1, ABCG2 and ABCC1 in cell lines

RT-PCR was performed to verify the expression of individual transporters in the carcinoma cell lines employed in subsequent combination studies. MDCKII stably expressing ABCB1, ABCG2 or ABCC1 were used as positive controls, while MDCKII parent cells served as negative control. We confirmed that hepatocarcinoma HepG2 cells express all of the studied transporters, while the expression of ABCB1 in breast carcinoma T47D cell line was too low and not detectable using agarose gel electrophoresis of PCR products (Fig. 3a).

Based on the observed differences between normalized C_t values reached for ABC transporters in HepG2 and T47D cells (Fig. 3b) and comparison of particular gene expressions calculated as $2^{\Delta Ct}$, we could estimate ABCB1 and ABCG2 expression being approximately 800- and 18-fold higher, respectively, in HepG2 than in T47D cells, while ABCC1 mRNA expression is approximately fivefold higher in T47D cell line compared to HepG2.

Drug combination studies

Combination studies were performed to assess the ability of the CDKi, which showed inhibitory effect on ABC transporters in the accumulation studies, to sensitize cancer cells to selected cytotoxic substrates of the relevant ABC transporters. The growth inhibitory effect of flavopiridol and SNS-032 treatment alone was therefore assessed and compared to combination treatment

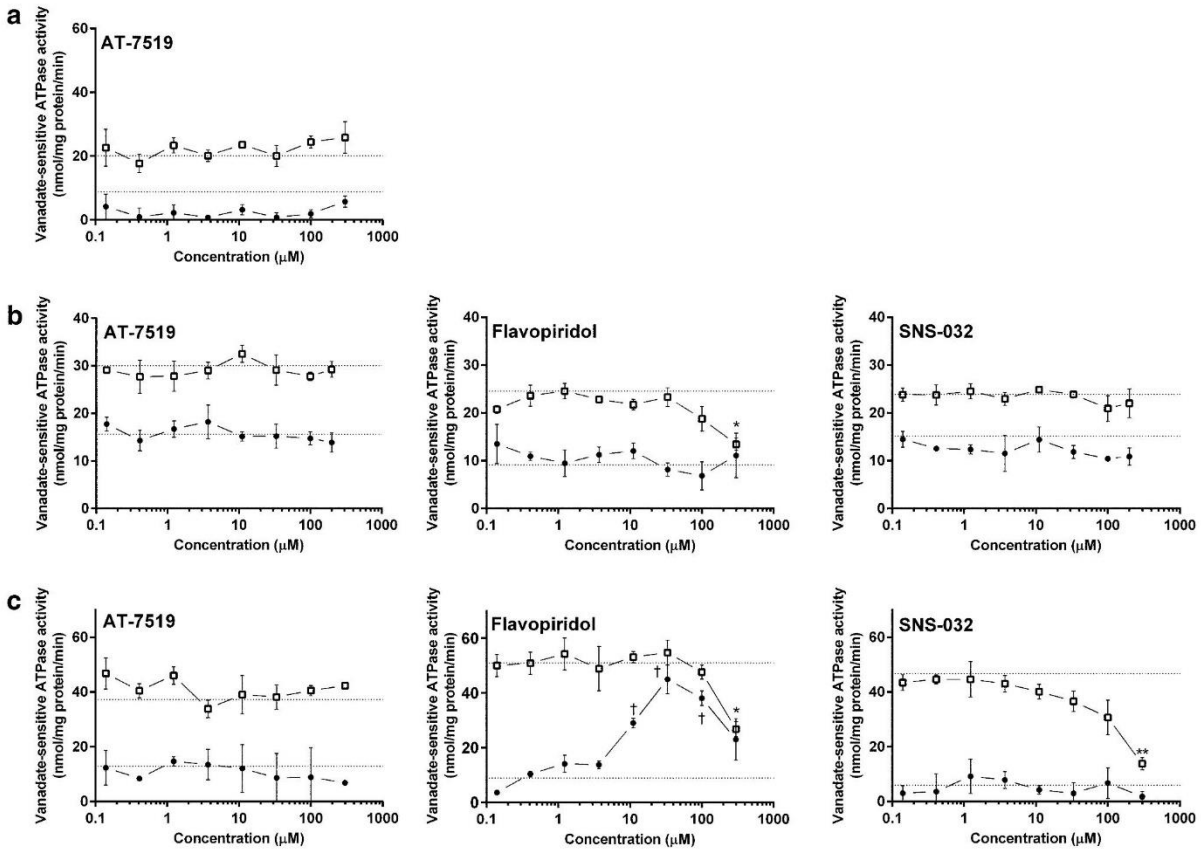

Fig. 2 Effect of CDKi on the ATPase activity of ABCB1-Sf9 (**a**), ABCC1-Sf9 (**b**) and ABCG2-Sf9 (**c**) membrane preparations. Vanadate-sensitive activity in the presence of flavopiridol, AT-7519 or SNS-032 in activation (*filled circle*) and inhibition (*empty square*) experiments. *Lower dotted line* represents baseline vanadate-sensitive ATPase and *upper dotted line* represents activated ATPase in all graphs. Presented data are mean ± SD representative of three experiments performed in duplicate. Statistical significance of differences between control and CDKi-treated samples in activation assays ($^{\dagger}P < 0.05$) and inhibition assays ($^{*}P < 0.05$; $^{**}P < 0.01$) is determined using unpaired two-tailed *t* test

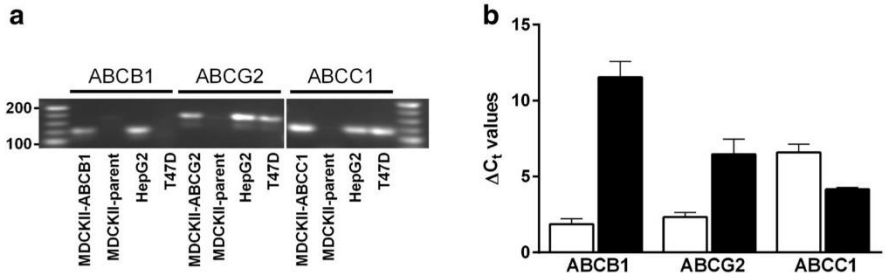

Fig. 3 mRNA expression of human *ABCB1*, *ABCG2* and *ABCC1* in human ABC transporter-overexpressing MDCKII, parent MDCKII, HepG2 and T47D cell lines. Amplicon sizes: ABCB1 (122 bp), ABCG2 (165 bp), ABCC1 (118 bp) (**a**); data are representative of three similar independent experiments. ΔC_t values for human *ABCB1*, *ABCG2* and *ABCC1* in HepG2 (*white columns*) and T47D (*black columns*) normalized to mean C_t values for *HPRT* and *β2-microglobulin* in both cell lines are shown; data represent mean ± SD of three independent samples (**b**)

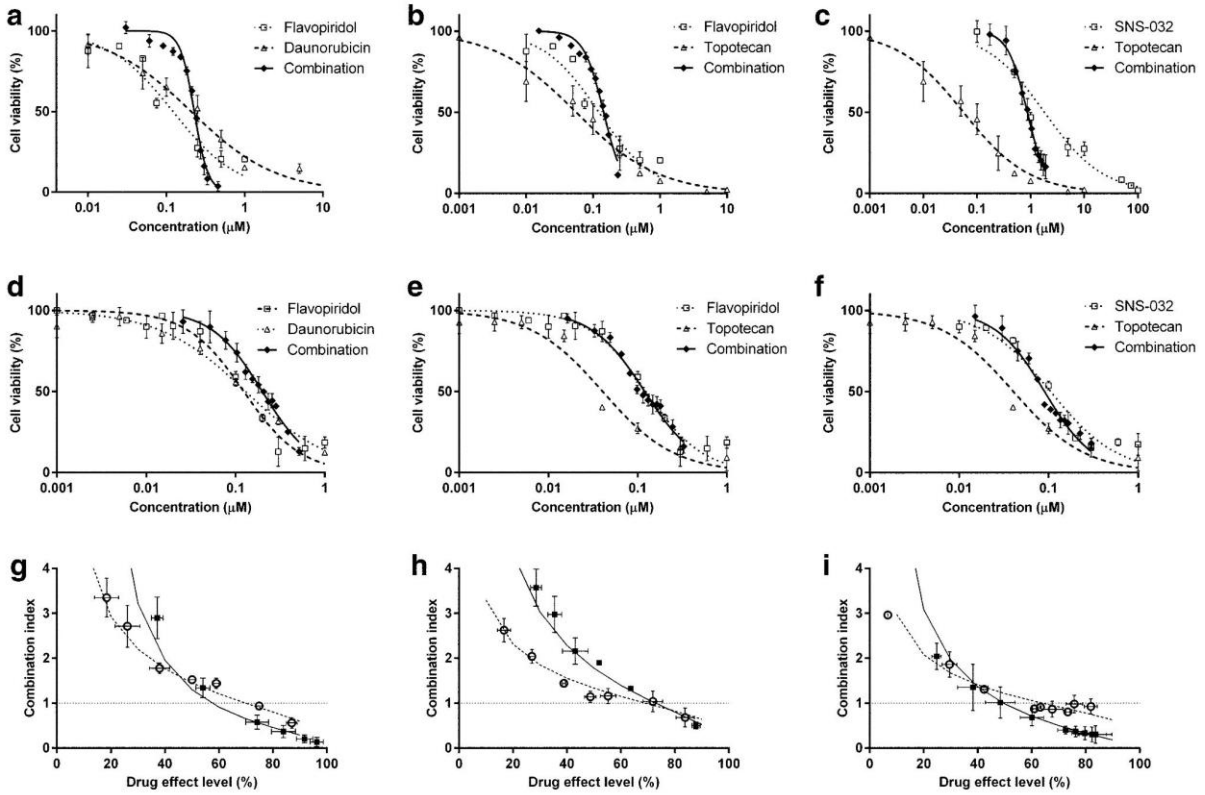

Fig. 4 Cytotoxic effect of flavopiridol and daunorubicin (**a**, **d**), flavopiridol and topotecan (**b**, **e**) and SNS-032 and topotecan (**c**, **f**) in HepG2 (**a**, **b**, **c**) and T47D (**d**, **e**, **f**) cells. The *r* values from the median-effect plots calculated by CompuSyn for single drugs and their combinations fall in the range of 0.95–0.99. Combination index analysis of flavopiridol combined with DNR (**g**) or TOP (**h**) and SNS-032 combined with TOP (**i**) in HepG2 (*filled square*) and T47D (*empty circle*) cell lines. *Lines* represent computer-simulated CI plots in HepG2 (*full line*) and T47D (*dashed line*) cell lines. The concentration ratio was based on the EC_{50} ratio of individual drugs (Table 1). CI values <0.9, 0.9–1.1 and >1.1 indicate synergism, additivity and antagonism, respectively. Drug effect levels (%) are calculated from the cell viability values and correspond to the proportional amount of cells affected by the drug combination; 0 % means no antiproliferative effect, and 100 % means absolute antiproliferative effect. Data are presented as mean ± SD of three independent experiments performed in triplicate

with DNR (ABCB1 and ABCC1 substrate) and/or TOP (ABCB1, ABCG2 and ABCC1 substrate) using the combination index method of Chou–Talalay. In all cases, combination treatments yielded greater growth inhibition than DNR/TOP alone. CI values are shown in Fig. 4, and the results are summarized in Table 2. Combinations in which synergism was observed (CI < 0.9) were used to calculate DRI.

Flavopiridol inhibited all three studied transporters and was therefore combined with both DNR and TOP. The combination of DNR with flavopiridol showed synergistic cytotoxicity at drug effect levels >70 % in HepG2 and >75 % in T47D cell line. When combined with TOP, the synergistic effect was also reached at drug effect levels >75 % in HepG2 and >80 % in T47D cells.

SNS-032 was combined with TOP due to its inhibitory effect on ABCG2 only. This combination yielded synergistic activity at drug effect levels >55 % in HepG2 and >75 % in T47D.

Discussion

Multidrug resistance to various chemotherapeutic agents and drug interactions with anticancer drugs represent a considerable problem in the effectiveness and safety of cancer treatment [26–28]. These interactions may also potentially alter the pharmacokinetic and pharmacodynamic behavior of the compounds, as well as behavior of other simultaneously administered drugs. As CDKi represent a novel group of anticancer agents [17], their interaction with major ABC transporters associated with MDR needs to be elucidated. We have previously reported on the potential of first-generation CDKi to interact with ABCB1 and ABCG2

Table 2 Combination index (CI) and dose-reduction index (DRI) values for drug combinations based on XTT assays scheduled after 72 h of simultaneous treatment in HepG2 and T47D cells

Drug[a]		EC_{50} (μM)		EC_{50} ratio	CI		DRI			
I	II	I	II	I: II	75 % effect	90 % effect	75 % effect		90 % effect	
							I	II	I	II
HepG2										
DNR	FLA	0.19 ± 0.030	0.12 ± 0.024	1:0.63	0.55 ± 0.022	0.29 ± 0.033	10 ± 0.40	2.2 ± 0.088	56 ± 6.2	3.7 ± 0.41
TOP	FLA	0.045 ± 0.0051	0.12 ± 0.024	1:2.7	0.83 ± 0.18	0.47 ± 0.094	3.8 ± 0.96	1.8 ± 0.46	9.7 ± 2.2	2.9 ± 0.66
TOP	SNS	0.045 ± 0.0051	1.7 ± 0.27	1:38	0.41 ± 0.087	0.18 ± 0.084	5.2 ± 0.98	4.8 ± 0.91	13 ± 4.9	12 ± 4.3
T47D										
DNR	FLA	0.13 ± 0.0073	0.12 ± 0.0067	1:0.92	0.90 ± 0.081	0.60 ± 0.19	ND[b]	ND	2.8 ± 0.76	5.3 ± 1.4
TOP	FLA	0.041 ± 0.0026	0.12 ± 0.0067	1:2.9	0.91 ± 0.14	0.64 ± 0.15	ND	ND	2.4 ± 0.61	5.5 ± 1.4
TOP	SNS	0.041 ± 0.0026	0.11 ± 0.010	1:2.7	0.85 ± 0.068	0.63 ± 0.098	1.8 ± 0.13	3.6 ± 0.27	2.1 ± 0.33	6.9 ± 1.0

[a] *DNR* daunorubicin, *FLA* flavopiridol, *SNS* SNS-032, *TOP* topotecan

[b] ND, DRI not determined for non-synergistic combinations

transporters [29, 30]. Our recent work has demonstrated the inhibitory potency of several CDKi, including flavopiridol and SNS-032, on ABCB1-mediated efflux [22]; here, we aim to complete the interaction profile of flavopiridol and SNS-032 with the three major ABC transporters associated with MDR. We include also AT-7519 as a novel CDKi with perspective use in cancer therapy and evaluate the interactions of these drugs with ABCB1, ABCG2 and ABCC1 in detail.

Zhou et al. [31] demonstrated that mouse ABCG2 and ABCB1 limit the brain penetration of flavopiridol in mice with greater transport associated with ABCG2. Based on experiments in Caco-2 cell monolayers, it has recently been suggested that ABCB1 could be involved in transmembrane transport of flavopiridol [32]. Nevertheless, among the ABC transporters, mainly ABCG2 [33, 34] and to a lower extent ABCC1 [35] rather than ABCB1 seem to confer resistance to flavopiridol in cancer cells. In our assays, we directly addressed the contribution of ABC transporters to cellular resistance of particular CDKi and found that ABCB1 did not confer resistance to flavopiridol in MDCKII-ABCB1 cells, confirming our previous data based on ATPase assays [22]. MDCKII-ABCG2 cells were more resistant to flavopiridol compared to parental MDCKII cells, whereas ABCC1 function only slightly (but significantly) modified the cytotoxic effect of this drug.

SNS-032 has been revealed as a substrate of ABCB1 in pharmacokinetic studies in rats [36], and ABCB1 expression has been suggested as an important SNS-032 resistance mechanism in neuroblastoma [37]. In our assays, we confirmed the major role of ABCB1 in SNS-032 resistance showing significantly increased viability in MDCKII-ABCB1 cell line compared to the parental MDCKII cells, with no such effect in ABCG2- or ABCC1-expressing cells. Interestingly, AT-7519 follows the same resistance pattern as SNS-032; in our proliferation assays, MDCKII-ABCB1 cells show a significantly higher resistance to AT-7519 than MDCKII-ABCG2, MDCKII-ABCC1 and MDCKII parental cells. Our data therefore indicate that ABCB1 confers resistance to AT-7519 and SNS-032, but not to flavopiridol, while ABCG2 and ABCC1 are causative of chemoresistance to flavopiridol, but not to AT-7519 and SNS-032.

Besides being subjected to ABC transporter-mediated efflux, many drugs can act as "modulators" overcoming MDR by inhibition of the drug transporters [38]. It was reported previously that flavopiridol was able to inhibit ABCB1- and ABCC1-mediated efflux in ABCB1- or ABCC1-overexpressing cells, respectively, when applied at micromolar concentrations [22, 33, 35]. Previous studies, including ours, also showed the ability of SNS-032 to inhibit the transport in ABCB1-expressing cells [22, 37].

Using accumulation assays in transporter-overexpressing MDCKII cells, we demonstrate here that flavopiridol can inhibit ABCG2-mediated efflux of MIT as well as ABCC1-mediated efflux of DNR even when applied at a low 1 μM concentration. Moreover, flavopiridol was able to achieve the maximal inhibition of ABCC1-mediated DNR efflux comparable to MK-571, a model ABCC1 inhibitor applied at 50 μM. These data indicate that flavopiridol is able to reverse not only ABCB1-mediated resistance, but also the resistance caused by ABCG2 and ABCC1 transporters. SNS-032 inhibited ABCG2-mediated transport of MIT but at high concentrations (50 μM) only. In contrast, AT-7519 had no effect on either ABCB1-, ABCG2- or ABCC1-mediated transport of the fluorescent substrates.

Our previous studies showed that flavopiridol and SNS-032 significantly reduced the activated ATPase of ABCB1 at concentrations higher than 100 μM, but neither of these

two compounds was able to increase the baseline vanadate-sensitive ATPase in ABCB1 membrane vesicles [22]. In this study, we examined the effects of CDKi on the transporter-related ATPase activities in Sf9 membranes overexpressing human ABCB1 (AT-7519 only), ABCG2 or ABCC1. Flavopiridol decreased the activated ATPase of both ABCG2 and ABCC1 ATPase, confirming its interaction with both transporters. We also found that flavopiridol stimulated ABCG2 baseline ATPase activity, which provides further indications that flavopiridol is a substrate of ABCG2. SNS-032 was able to significantly decrease the activated ABCG2 ATPase, albeit at concentrations higher than 100 μM only, while it had no effect on the baseline ABCG2 ATPase activity and neither did it affect ABCC1 ATPase-stimulated or baseline activity. These data confirm that SNS-032 interacts with ABCG2, but not with ABCC1, as observed in our accumulation assays as well as cytotoxicity studies. In the case of AT-7519, the activation and inhibition mode of ATPase assays did not show any measurable effect on either ABCG2 or ABCC1 ATPase activities up to 300 μM concentration, providing first indications that AT-7519 does not interact with ABCG2 or ABCC1 transporters. Interestingly, AT-7519 failed to affect ABCB1-linked ATPase activity in this assay, which contradicts our cytotoxicity results. This phenomenon can be attributed to the fact that the value of resistance factor obtained by measuring cytotoxicity in MDCKII cells does not necessarily correlate with ABC transporter substrates and also to the fact that ATPase assays can provide false-negative results [39].

In undergoing clinical trials, CDKi are being evaluated not only as single agents but also in combination with other chemotherapeutics, with the aim of yielding synergistic activity [40–42], increasing their therapeutic effects [19, 43] and the survival of cancer patients [44]. We have previously suggested that ABC transporter inhibition may represent one of the mechanisms underlying the onset of synergistic effects of CDKi combined with conventional anticancer drugs [22, 29]. Therefore, here we also addressed whether ABC transporter-inhibiting compounds, flavopiridol and SNS-032, can potentiate the cytotoxic effects of anticancer agents in vitro. We applied CDKi in combination with topoisomerase inhibitors, DNR or TOP (commonly used anticancer drugs and ABC transporter substrates), to human cancer cell lines expressing ABC transporters [22, 45, 46], i.e., HepG2 and T47D, derived from hepatocyte carcinoma and ductal breast carcinoma, respectively. Flavopiridol inhibited all studied ABC transporters and was therefore combined with both DNR and TOP, while SNS-032 was combined with TOP due to its inhibitory potency toward ABCG2 only. We found that all studied CDKi inhibited the growth of both HepG2 and T47D cells in a dose-dependent manner and showed synergism when administered in combination with DNR or TOP. The combination of flavopiridol with daunorubicin exhibited a higher synergistic effect in the HepG2 cell line compared to T47D. As daunorubicin is a stronger substrate of ABCB1 than ABCC1, we believe that the lacking expression of ABCB1 in T47D cells may be the reason for higher CI values and thus lower synergistic effects in that cell line. Based on our inhibitory studies and resistance profile of TOP, flavopiridol could be expected to contribute to the synergistic effect mainly by inhibition of ABCG2- and ABCC1-mediated efflux of TOP. Both transporters are expressed at comparable levels in HepG2 and T47D cells, which might be the reason for the similar synergistic effect exhibited in both cell lines. SNS-032 in combination with topotecan yielded synergistic effects in both cell lines as well, with a more prominent effect in the cell line with higher ABCG2 expression (HepG2). It is obvious that CDKi can function as modulators of apoptosis induced by other cytotoxic agents [47, 48] but, as we show here, they are also able to reverse MDR by inhibition of ABC transporters. CDKi may therefore represent a new class of ABC transporter modulators and, in combination with other anticancer agents, could also become a promising strategy to overcome resistance in ABC transporter-expressing tumors.

In summary, we were able to determine and complete the interaction profiles of AT-7519, flavopiridol and SNS-032 with ABCB1, ABCG2 and ABCC1 multidrug transporters, which is an important part of their preclinical testing phase. Our results indicate that flavopiridol is able to reverse ABC transporter-mediated MDR and thus increase the intracellular concentrations of substrate chemotherapeutic drugs yielding synergistic antiproliferative effect. We also show here for the first time that ABCB1 can cause resistance to AT-7519 and SNS-032, but not to flavopiridol, while ABCG2 and ABCC1 confer resistance to flavopiridol, but not to AT-7519 and SNS-032. Based on these results, we conclude that all tested CDKi may play an important role in transporter-mediated interactions, pharmacokinetics, tissue distribution and drug resistance, and all therapies should be adjusted accordingly.

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References

1. Choi YH, Yu AM (2014) ABC transporters in multidrug resistance and pharmacokinetics, and strategies for drug development. Curr Pharm Des 20:793–807

2. Wu CP, Hsieh CH, Wu YS (2011) The emergence of drug transporter-mediated multidrug resistance to cancer chemotherapy. Mol Pharm 8:1996–2011
3. Shukla S, Wu CP, Ambudkar SV (2008) Development of inhibitors of ATP-binding cassette drug transporters: present status and challenges. Expert Opin Drug Metab Toxicol 4:205–223
4. Shukla S, Ohnuma S, Ambudkar SV (2010) Improving cancer chemotherapy with modulators of ABC drug transporters. Curr Drug Targets 12:621–630
5. Yang AK, Zhou ZW, Wei MQ, Liu JP, Zhou SF (2010) Modulators of multidrug resistance associated proteins in the management of anticancer and antimicrobial drug resistance and the treatment of inflammatory diseases. Curr Top Med Chem 10:1732–1756
6. Coley HM (2010) Overcoming multidrug resistance in cancer: clinical studies of p-glycoprotein inhibitors. Methods Mol Biol 596:341–358
7. Robey RW, Ierano C, Zhan Z, Bates SE (2011) The challenge of exploiting ABCG2 in the clinic. Curr Pharm Biotechnol 12:595–608
8. Kathawala RJ, Gupta P, Ashby CR Jr, Chen ZS (2015) The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. Drug Resist Updat 18:1–17
9. Eadie LN, Hughes TP, White DL (2014) Interaction of the efflux transporters ABCB1 and ABCG2 with imatinib, nilotinib, and dasatinib. Clin Pharmacol Ther 95:294–306
10. Anreddy N, Gupta P, Kathawala RJ, Patel A, Wurpel JN, Chen ZS (2014) Tyrosine kinase inhibitors as reversal agents for ABC transporter mediated drug resistance. Molecules 19:13848–13877
11. Lolli G, Johnson LN (2005) CAK-cyclin-dependent activating kinase: a key kinase in cell cycle control and a target for drugs? Cell Cycle 4:572–577
12. Morgan DO (1995) Principles of CDK regulation. Nature 374:131–134
13. Pavletich NP (1999) Mechanisms of cyclin-dependent kinase regulation: structures of Cdks, their cyclin activators, and Cip and INK4 inhibitors. J Mol Biol 287:821–828
14. Malumbres M, Barbacid M (2009) Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer 9:153–166
15. Canavese M, Santo L, Raje N (2012) Cyclin dependent kinases in cancer: potential for therapeutic intervention. Cancer Biol Ther 13:451–457
16. Cicenas J, Valius M (2011) The CDK inhibitors in cancer research and therapy. J Cancer Res Clin Oncol 137:1409–1418
17. Gallorini M, Cataldi A, di Giacomo V (2012) Cyclin-dependent kinase modulators and cancer therapy. BioDrugs 26:377–391
18. Lin TS, Ruppert AS, Johnson AJ, Fischer B, Heerema NA, Andritsos LA, Blum KA, Flynn JM, Jones JA, Hu W, Moran ME, Mitchell SM, Smith LL, Wagner AJ, Raymond CA, Schaaf LJ, Phelps MA, Villalona-Calero MA, Grever MR, Byrd JC (2009) Phase II study of flavopiridol in relapsed chronic lymphocytic leukemia demonstrating high response rates in genetically high-risk disease. J Clin Oncol 27:6012–6018
19. Bible KC, Peethambaram PP, Oberg AL, Maples W, Groteluschen DL, Boente M, Burton JK, Gomez Dahl LC, Tibodeau JD, Isham CR, Maguire JL, Shridhar V, Kukla AK, Voll KJ, Mauer MJ, Colevas AD, Wright J, Doyle LA, Erlichman C (2012) A phase 2 trial of flavopiridol (Alvocidib) and cisplatin in platin-resistant ovarian and primary peritoneal carcinoma: MC0261. Gynecol Oncol 127:55–62
20. Heath EI, Bible K, Martell RE, Adelman DC, Lorusso PM (2008) A phase 1 study of SNS-032 (formerly BMS-387032), a potent inhibitor of cyclin-dependent kinases 2, 7 and 9 administered as a single oral dose and weekly infusion in patients with metastatic refractory solid tumors. Invest New Drugs 26:59–65
21. Tong WG, Chen R, Plunkett W, Siegel D, Sinha R, Harvey RD, Badros AZ, Popplewell L, Coutre S, Fox JA, Mahadocon K, Chen T, Kegley P, Hoch U, Wierda WG (2010) Phase I and pharmacologic study of SNS-032, a potent and selective Cdk2, 7, and 9 inhibitor, in patients with advanced chronic lymphocytic leukemia and multiple myeloma. J Clin Oncol 28:3015–3022
22. Cihalova D, Hofman J, Ceckova M, Staud F (2013) Purvalanol A, olomoucine II and roscovitine inhibit ABCB1 transporter and synergistically potentiate cytotoxic effects of daunorubicin in vitro. PLoS ONE 8:e83467
23. Cui Y, Konig J, Buchholz JK, Spring H, Leier I, Keppler D (1999) Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells. Mol Pharmacol 55:929–937
24. Sarkadi B, Price EM, Boucher RC, Germann UA, Scarborough GA (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J Biol Chem 267:4854–4858
25. Chou TC (2006) Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol Rev 58:621–681
26. Gottesman MM, Fojo T, Bates SE (2002) Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2:48–58
27. Xia CQ, Smith PG (2012) Drug efflux transporters and multidrug resistance in acute leukemia: therapeutic impact and novel approaches to mediation. Mol Pharmacol 82:1008–1021
28. Scripture CD, Figg WD (2006) Drug interactions in cancer therapy. Nat Rev Cancer 6:546–558
29. Hofman J, Ahmadimoghaddam D, Hahnova L, Pavek P, Ceckova M, Staud F (2012) Olomoucine II and purvalanol A inhibit ABCG2 transporter in vitro and in situ and synergistically potentiate cytostatic effect of mitoxantrone. Pharmacol Res 65:312–319
30. Hofman J, Kucera R, Cihalova D, Klimes J, Ceckova M, Staud F (2013) Olomoucine II, but not purvalanol A, is transported by breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1). PLoS ONE 8:e75520
31. Zhou L, Schmidt K, Nelson FR, Zelesky V, Troutman MD, Feng B (2009) The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic acid (PF-407288) in mice. Drug Metab Dispos 37:946–955
32. Xia B, Liu X, Zhou Q, Feng Q, Li Y, Liu W, Liu Z (2013) Disposition of orally administered a promising chemotherapeutic agent flavopiridol in the intestine. Drug Dev Ind Pharm 39:845–853
33. Robey RW, Medina-Perez WY, Nishiyama K, Lahusen T, Miyake K, Litman T, Senderowicz AM, Ross DD, Bates SE (2001) Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res 7:145–152
34. Nakanishi T, Karp JE, Tan M, Doyle LA, Peters T, Yang W, Wei D, Ross DD (2003) Quantitative analysis of breast cancer resistance protein and cellular resistance to flavopiridol in acute leukemia patients. Clin Cancer Res 9:3320–3328
35. Hooijberg JH, Broxterman HJ, Scheffer GL, Vrasdonk C, Heijn M, de Jong MC, Scheper RJ, Lankelma J, Pinedo HM (1999) Potent interaction of flavopiridol with MRP1. Br J Cancer 81:269–276
36. Kamath AV, Chong S, Chang M, Marathe PH (2005) P-glycoprotein plays a role in the oral absorption of BMS-387032, a potent cyclin-dependent kinase 2 inhibitor, in rats. Cancer Chemother Pharmacol 55:110–116
37. Loschmann N, Michaelis M, Rothweiler F, Zehner R, Cinatl J, Voges Y, Sharifi M, Riecken K, Meyer J, von Deimling A,

Fichtner I, Ghafourian T, Westermann F, Cinatl J Jr (2013) Testing of SNS-032 in a panel of human neuroblastoma cell lines with acquired resistance to a broad range of drugs. Transl Oncol 6:685–696
38. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM (2006) Targeting multidrug resistance in cancer. Nat Rev Drug Discov 5:219–234
39. Glavinas H, Mehn D, Jani M, Oosterhuis B, Heredi-Szabo K, Krajcsi P (2008) Utilization of membrane vesicle preparations to study drug-ABC transporter interactions. Expert Opin Drug Metab Toxicol 4:721–732
40. Baumann KH, Kim H, Rinke J, Plaum T, Wagner U, Reinartz S (2013) Effects of alvocidib and carboplatin on ovarian cancer cells in vitro. Exp Oncol 35:168–173
41. Nagaria TS, Williams JL, Leduc C, Squire JA, Greer PA, Sangrar W (2013) Flavopiridol synergizes with sorafenib to induce cytotoxicity and potentiate antitumorigenic activity in EGFR/HER-2 and mutant RAS/RAF breast cancer model systems. Neoplasia 15:939–951
42. Walsby E, Lazenby M, Pepper C, Burnett AK (2011) The cyclin-dependent kinase inhibitor SNS-032 has single agent activity in AML cells and is highly synergistic with cytarabine. Leukemia 25:411–419
43. Karp JE, Garrett-Mayer E, Estey EH, Rudek MA, Smith BD, Greer JM, Drye DM, Mackey K, Dorcy KS, Gore SD, Levis MJ, McDevitt MA, Carraway HE, Pratz KW, Gladstone DE, Showel MM, Othus M, Doyle LA, Wright JJ, Pagel JM (2012) Randomized phase II study of two schedules of flavopiridol given as timed sequential therapy with cytosine arabinoside and mitoxantrone for adults with newly diagnosed, poor-risk acute myelogenous leukemia. Haematologica 97:1736–1742
44. Carrick S, Parker S, Wilcken N, Ghersi D, Marzo M, Simes J (2005) Single agent versus combination chemotherapy for metastatic breast cancer. Cochrane Database Syst Rev CD003372
45. Saxena M, Stephens MA, Pathak H, Rangarajan A (2011) Transcription factors that mediate epithelial-mesenchymal transition lead to multidrug resistance by upregulating ABC transporters. Cell Death Dis 2:e179
46. Hilgendorf C, Ahlin G, Seithel A, Artursson P, Ungell AL, Karlsson J (2007) Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. Drug Metab Dispos 35:1333–1340
47. Rathos MJ, Joshi K, Khanwalkar H, Manohar SM, Joshi KS (2012) Molecular evidence for increased antitumor activity of gemcitabine in combination with a cyclin-dependent kinase inhibitor, P276-00 in pancreatic cancers. J Transl Med 10:161
48. Rosato RR, Almenara JA, Maggio SC, Atadja P, Craig R, Vrana J, Dent P, Grant S (2005) Potentiation of the lethality of the histone deacetylase inhibitor LAQ824 by the cyclin-dependent kinase inhibitor roscovitine in human leukemia cells. Mol Cancer Ther 4:1772–1785

6.4 Dinaciclib, a cyclin-dependent kinase inhibitor, is a substrate of human ABCB1 and ABCG2 and an inhibitor of human ABCC1 *in vitro*

Cihalova D., Ceckova M., Kucera R., Klimes J., Staud F.

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Dinaciclib je relativně nový CDKI s významnou aktivitou proti různým typům nádorových onemocnění *in vitro* a *in vivo*. ABC efluxní transportéry hrají důležitou roli v dispozici léčiv a jsou zodpovědné za vznik mnohočetné lékové rezistence v nádorových buňkách. Inhibitory a substráty těchto transportérů se mohou podílet na vzniku farmakokinetických lékových interakcí, které mohou měnit lékovou dispozici během farmakoterapie. Abychom odhadli taková rizika spojená s podáváním dinaciclibu, vyhodnotili jsme jeho možný efekt na efluxní aktivitu ABCB1, ABCC1 a ABCG2 transportérů *in vitro* pomocí následujících metodik: transporty přes buněčnou monovrstvu, XTT buněčná proliferace, měření ATPázové aktivity a intracelulární buněčné akumulace.

Naše výsledky ukazují, že poměr transportu dinaciclibu byl několikanásobně vyšší při transportu v buněčných monovrstvách MDCKII-ABCB1 a MDCKII-ABCG2 ve srovnání s parentními MDCKII buňkami, což značí, že dinaciclib je transportovaným substrátem ABCB1 a ABCG2. Navíc, zvýšená exprese transportérů ABCB1, ABCG2 a ABCC1 zvyšuje rezistenci vůči dinaciclibu v MDCKII buňkách. Dinaciclib snižoval stimulovanou aktivitu ABCB1, ABCG2 a ABCC1 v ATPázových esejích, což dále potvrzuje jeho interakci se všemi testovanými transportéry. Dinaciclib také signifikantně inhiboval eflux daunorubicinu zprostředkovaný ABCC1 (IC_{50} = 18 μM). Inhibice ABCC1 dále vedla k synergickému efektu dinaciclibu v kombinaci s dalšími protinádorovými látkami při aplikaci v buňkách MDCKII-ABCC1 i lidské nádorové buněčné linii T47D.

Naše výsledky tedy naznačují, že ABC transportéry mohou výrazně ovlivnit transport dinaciclibu přes buněčné membrány, což může vést k lékovým interakcím.

Zároveň, mohou být tyto lékové interakce dinaciclibu využity v terapii nádorových onemocnění za předpokladu, že dojde ke kombinaci této látky s protinádorovými léčivy, která jsou ABCC1 substráty.

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Dinaciclib, a cyclin-dependent kinase inhibitor, is a substrate of human ABCB1 and ABCG2 and an inhibitor of human ABCC1 *in vitro*

Daniela Cihalova[a], Martina Ceckova[a], Radim Kucera[b], Jiri Klimes[b], Frantisek Staud[a,*]

[a] Department of Pharmacology and Toxicology, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, 500 05 Hradec Kralove, Czech Republic
[b] Department of Pharmaceutical Chemistry and Drug Analysis, Faculty of Pharmacy in Hradec Kralove, Charles University in Prague, Heyrovskeho 1203, 500 05 Hradec Kralove, Czech Republic

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ABSTRACT

Dinaciclib is a novel cyclin-dependent kinase inhibitor (CDKI) with significant activity against various cancers *in vitro* and *in vivo*. ABC efflux transporters play an important role in drug disposition and are responsible for multidrug resistance in cancer cells. Inhibitors and substrates of these transporters may participate in pharmacokinetic drug–drug interactions (DDIs) that alter drug disposition during pharmacotherapy. To assess such risks associated with dinaciclib we evaluated its possible effects on efflux activities of ABCB1, ABCC1 and ABCG2 transporters *in vitro*. Monolayer transport, XTT cell proliferation, ATPase and intracellular accumulation assays were employed. Here, we show that the transport ratio of dinaciclib was far higher across monolayers of MDCKII-ABCB1 and MDCKII-ABCG2 cells than across MDCKII parental cell layers, demonstrating that dinaciclib is a substrate of ABCB1 and ABCG2. In addition, overexpression of ABCB1, ABCG2 and ABCC1 conferred resistance to dinaciclib in MDCKII cells. In ATPase assays, dinaciclib decreased stimulated ATPase activity of ABCB1, ABCG2 and ABCC1, confirming it has interactive potential toward all three transporters. Moreover, dinaciclib significantly inhibited ABCC1-mediated efflux of daunorubicin (EC_{50} = 18 μM). The inhibition of ABCC1 further led to a synergistic effect of dinaciclib in both MDCKII-ABCC1 and human cancer T47D cells, when applied in combination with anticancer drugs. Taken together, our results suggest that ABC transporters can substantially affect dinaciclib transport across cellular membranes, leading to DDIs. The DDIs of dinaciclib with ABCC1 substrate chemotherapeutics might be exploited in novel cancer therapies.

1. Introduction

Cyclin-dependent kinases (CDKs) are critical regulators of cell cycle progression, and deregulation of their function has been detected in multiple human cancers. Thus, they are attractive targets for cancer treatment and new CDK inhibitors (CDKIs) with favorable pharmacological profiles and minimum adverse effects are being intensively sought. Several small-molecule CDKIs have entered clinical trials [1,2] and a CDK4/6 inhibitor, palbociclib, has been recently approved by the US Food and Drug Administration (FDA) for use in initial endocrine-based therapy for postmenopausal women with estrogen-positive, human epidermal growth factor receptor 2-negative advanced breast cancer [3].

Dinaciclib (MK-7965, SCH727965) is an orally administered small-molecule CDKI that selectively inhibits important members of the CDK family (CDK1, CDK2, CDK5 and CDK9) at nanomolar concentrations [4]. In preclinical studies dinaciclib has shown excellent anticancer efficacy, surpassing that of older CDKIs (*e.g.*, flavopiridol and roscovitine), inhibiting the growth of a broad

Abbreviations: AB, apical-to-basolateral; ABC, ATP-binding cassette; ABCB1, P-glycoprotein; ABCC1, multidrug resistance-associated protein 1; ABCG2, breast cancer resistance protein; ADME, absorption, distribution, metabolism and excretion; BA, basolateral-to-apical; CDK, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; CI, combination index; DDI, drug–drug interactions; DMEM, Dulbecco's modified Eagle's medium; DNR, daunorubicin; EC_{50}, half maximal effective concentration(s); EMA, the European Medicines Agency; FBS, fetal bovine serum; FDA, the Food and Drug Administration; IC_{50}, half maximal inhibitory concentration(s); IS, internal standard; ITC, International Transporter Consortium; MDCKII, Madin–Darby canine kidney; MDR, multidrug resistance; MFI, median fluorescence intensity; MIT, mitoxantrone; NEM-SG, *N*-ethylmaleimide-glutathione; PBS, phosphate-buffered saline; PMS, phenazine methosulfate; *r*, transport ratio; RF, resistance factor; SD, standard deviation; TKI, tyrosine kinase inhibitor; TOP, topotecan; XTT, sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt.

* Corresponding author. Fax: +420 495067170.
E-mail addresses: cihad6aa@faf.cuni.cz (D. Cihalova), martina.ceckova@faf.cuni.cz (M. Ceckova), radim.kucera@faf.cuni.cz (R. Kucera), jiri.klimes@faf.cuni.cz (J. Klimes), frantisek.staud@faf.cuni.cz (F. Staud).

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spectrum of human cancer cell lines both *in vitro* and in *in vivo* xenograft models [4–8]. In addition, 16 clinical trials have been initiated to evaluate its effects, as a single agent or in combination with other anticancer drugs, in patients with hematologic malignancies or solid tumors (clinicaltrials.gov, accessed July 2015). So far, its safety, tolerability and pharmacokinetics have been assessed in phase 1 clinical studies [9,10] and phase 2 studies have evaluated its efficacy relative to erlotinib and capecitabine in patients with non-small cell lung cancer [11] and advanced breast cancer [12], respectively. Dinaciclib also reportedly has encouraging single agent activity in patients with relapsed multiple myeloma [13]. Several other clinical studies, including a phase 3 clinical trial (clinicaltrials.gov, ID: NCT01580228), have evaluated therapeutic effects of dinaciclib in the treatment of refractory chronic lymphocytic leukemia.

ATP-binding cassette (ABC) efflux transporters drive substrates across biological membranes, even against concentration gradients, using energy from ATP hydrolysis. They are expressed in the liver, kidneys and small intestine, where they modulate absorption, distribution, metabolism and excretion (ADME) of their substrates. ABC transporters protectively limit the entry of xenobiotics into the testes, placenta and blood-brain barrier, and thus may control drug penetration to particularly sensitive organs [14]. In addition to normal tissues, ABC drug transporters are abundantly expressed in cancer cells where they participate in the development of multidrug resistance (MDR). This poses a major obstacle in cancer chemotherapy, as ABC transporters can actively efflux structurally and functionally diverse anticancer drugs, diminishing their intracellular concentrations. Three members of the ABC transporter family contribute most significantly to MDR: ABCB1 (P-glycoprotein, P-gp), ABCG2 (breast cancer resistance protein, BCRP) and ABCC1 (multidrug resistance-associated protein, MRP1) [15,16]. Inhibitors and substrates of ABC transporters may participate in pharmacokinetic drug-drug interactions (DDIs) that substantially change drug disposition during pharmacotherapy and affect both the therapeutic efficacy and the severity of adverse effects. Therefore, the International Transporter Consortium (ITC) emphasizes that such interactions must be considered in order to help determine their pharmacokinetic, safety and efficacy profiles [17]. Following these recommendations, both the FDA and the European Medicines Agency (EMA) have issued new guidelines for drug interaction studies, emphasizing the requirements for *in vitro* methods in drug transporter interaction assessment [18,19].

In vitro methods, such as ATPase, cellular uptake and monolayer transport assays, are currently cornerstones for evaluating molecular-level transporter interactions [17,20]. Employing these methods, it has been shown that several CDKIs can interact with ABC transporters by either inhibiting their efflux activity [21,22] or being transported as substrates [23,24]. In these cases, transporter-mediated pharmacokinetic DDIs can occur when the CDKIs are applied in combination with other therapeutic agents [25].

Understanding dinaciclib interactions with ABC transporters is important for determining potential pharmacokinetic DDIs involving the drug, however, to date, no data on ABC transporter substrate specificity has been reported. Thus, in the study reported here we explored interactions of dinaciclib with ABCB1, ABCG2 and ABCC1 transporters *in vitro*. We also explored the potential ability of these ABC transporters to confer dinaciclib resistance upon cells.

2. Materials and methods

2.1. Chemicals

Dinaciclib was obtained from Axon Medchem (Groningen, the Netherlands). Daunorubicin (DNR), mitoxantrone (MIT), topotecan (TOP), sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt (XTT), phenazine methosulfate (PMS), ABCC1 inhibitor MK-571, dual ABCB1/ABCG2 inhibitor GF120918, fluorescein-isothiocyanate-labeled dextran, roscovitine and HPLC grade solvents (methanol, acetic acid) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The ABCB1 inhibitor LY335979 was obtained from Toronto Research Chemicals (North York, ON, Canada) and the ABCG2 inhibitor Ko143 from Enzo Life Sciences (Farmingdale, NY, USA). Cell culture reagents (media, sera, L-glutamine, DMSO, buffers, trypsin-EDTA) were supplied by Sigma–Aldrich (St. Louis, MO, USA) and Opti-MEM® from Gibco BRL Life Technologies (Rockville, MD, USA). ABCB1, ABCG2 and ABCC1 PREDEASY™ ATPase kits (SB MDR1/P-gp, SB BCRP HAM Sf9 and SB MRP1, respectively) were purchased from Solvo Biotechnology (Szeged, Hungary). Transwell inserts were obtained from Corning Inc. (Corning, NY, USA).

2.2. Cell culture

MDCKII (Madin–Darby canine kidney) cell lines transduced for stable expression of human transporters ABCB1 (MDCKII-ABCB1), ABCC1 (MDCKII-ABCC1) or ABCG2 (MDCKII-ABCG2) as well as the MDCKII-parent cell line, were obtained from Prof. Piet Borst and Dr. Alfred Schinkel (The Netherlands Cancer Institute, Amsterdam, the Netherlands). The cell lines were grown in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). These cell lines were used for monolayer transport, XTT antiproliferative and intracellular accumulation assays. For *in vitro* drug combination studies, we also used human ductal breast carcinoma T47D cells, obtained from the European Collection of Cell Culture (PHE, Salisbury, Wiltshire, UK). This cell line was cultured in DMEM without phenol red, supplemented with 10% FBS and 2 mM L-glutamine. DMSO was applied as a solvent in concentrations not exceeding 0.5% (1% in ATPase assays).

2.3. MDCKII monolayer transport assay

MDCKII-ABCB1, MDCKII-ABCC1, MDCKII-ABCG2 or MDCKII-parent cells were seeded on microporous polycarbonate membrane inserts (3 μm pore size, 24 mm diameter; Costar, Cambridge, MA, USA) at a density of 1×10^6 per insert 72 h before experiments. The medium was replaced after 24 and 48 h of cultivation. The cells were then washed with 1× phosphate-buffered saline (PBS) on both the apical and basal sides and preincubated for 1 h in Opti-MEM® with or without inhibitors. The appropriate ABC transporter inhibitor (1 μM LY335979, 25 μM MK-571 or 1 μM Ko143 for ABCB1, ABCC1 or ABCG2 inhibition, respectively) was present in both compartments during the preincubation and the transport experiment, at a concentration known to efficiently inhibit the corresponding transporter. To inhibit any endogenous transporter activity, appropriate inhibitors were also added (1 μM of the ABCB1 inhibitor LY335979 to MDCKII-ABCG2 cultures, 1 μM of the ABCG2 inhibitor Ko143 to MDCKII-ABCB1 cultures, and 2 μM of the dual ABCB1/ABCG2 inhibitor GF120918 to MDCKII-ABCC1 and parent cell cultures). The experiments were started (time = 0) by replacing the medium with fresh Opti-MEM® containing dinaciclib, with or without inhibitor, in the appropriate chambers. Samples were taken from opposite compartments after 2, 4 and 6 h, then the concentration of transported dinaciclib was determined by HPLC–MS/MS analysis. Immediately after each experiment, cellular monolayer integrity was examined using fluorescein isothiocyanate-labeled dextran (MW = 40 kDa). Dextran leakage up to 1% per hour was accepted. Dinaciclib transport in parental and ABC transporter-expressing MDCKII cells was assayed and

transport ratios (r), defined as the dinaciclib transport rate in the basolateral-to-apical (BA) direction divided by the rate in the apical-to-basolateral (AB) direction, were calculated from the observed concentrations at 6 h. In these assays dinaciclib was applied at 100 nM (the lowest concentration allowing sensitive analysis of samples above the detection limits of the HPLC–MS/MS method and instruments, as described in Section 2.4).

2.4. HPLC–MS/MS analysis

Dinaciclib was quantified by HPLC–MS/MS using an LC 20A Prominence chromatograph (Shimadzu, Kyoto, Japan), equipped with an OPTI-GUARD 1 mm C18 guard column (Sigma–Aldrich, St. Louis, MO, USA) and a Hypersil GOLD C18 column (100 × 4.6 mm, particle size 3 μm; Pragolab, Prague, Czech Republic), coupled to a LCQ Max advantage mass spectrometer (Thermo Finnigan, San Jose, CA, USA). The mobile phase flow rate was 0.4 mL/min and the column temperature was maintained at 40 °C. The acquired MS data were processed using Xcalibur 2.0 software (Thermo Finnigan, San Jose, CA, USA).

Following optimization of conditions to separate dinaciclib from Opti-MEM® components and inhibitors (GF120918, MK-571, LY335979, Ko143) a mobile phase consisting of methanol and 0.1% acetic acid (75:25, v/v) was used in all analyses reported here. Roscovitine was added to samples as an internal standard (IS). Retention times were 4.1 and 4.7 min for dinaciclib and the IS, respectively. ESI probe settings were: source voltage 4.5 kV, capillary temperature 340 °C, sheet and auxiliary gas flows 60 and 15 arbitrary units, respectively. The tandem MS was operated in SRM mode, using the molecular ion $[M+H]^+$ (m/z 397 for dinaciclib and 355 for the IS) as precursor ion and product ions with m/z ratios of 240, 268, 285, 379 (dinaciclib) and 313 (IS) for quantifying dinaciclib after collision dissociation. The collision energies were 38% and 40% for dinaciclib and IS, respectively. The linearity of the signals was evaluated in the range of 2.5–500 nM ($r^2 = 0.9994$); the method precision and accuracy were evaluated at analyte concentrations of 500, 50, 10 and 2.5 nM. Samples were found to be stable for at least 40 h.

2.5. XTT cell proliferation assays

The XTT assay was used to assess effects of dinaciclib and ABC transporter substrates on cell viability and growth. MDCKII-ABCB1, MDCKII-ABCC1, MDCKII-ABCG2 or MDCKII-parent cells were seeded in 96-well culture plates at a density of 1×10^4 cells and incubated for 24 h. Test compounds diluted with growth medium were added to the exponentially growing cells and the resulting mixtures were incubated for 72 h at 37 °C, 5% CO_2. XTT (0.167 mg/mL) mixed with 4 μM PMS in Opti-MEM® was then added to the cultures. After a further 2 h incubation the absorbance of the soluble formazan released was measured at 470 nm using a microplate reader (Tecan, Männedorf, Switzerland), and the half maximal inhibitory concentrations (IC_{50}) of the drugs were calculated using GraphPad Prism 6.00. To determine the influence of ABC transporters on antiproliferative activity of dinaciclib, resistance factors (RFs) were calculated by dividing the IC_{50} value for each ABC transporter-overexpressing cell line by the value for the correspondingly-treated parental cell line. Thus, the RFs represent fold-increases in resistance caused by the presence of specific ABC transporters [26]. To indirectly assess whether cellular resistance to dinaciclib can be caused by ABCB1, ABCC1 or ABCG2, the cell proliferation assays were repeated with the addition of model inhibitors of the three transporters (1 μM LY335979, 25 μM MK-571 and 1 μM Ko143 for ABCB1, ABCC1 and ABCG2, respectively) to abolish the potential influence of ABC transporters on the resistance.

2.6. ABCB1, ABCC1 and ABCG2 ATPase assays

Preparations of membranes with overexpressed ABC transporters show vanadate-sensitive ATPase activity that is modulated by interacting compounds. In the activation assay, increases in this ATPase activity associated with increases in substrate transport are measured, while in the inhibition assay reductions in the activity associated with the presence of a known activator of the transporter are measured [27]. ATPase activity was measured by assessing the amount of phosphate liberated from ATP by the ABCB1, ABCC1 or ABCG2 transporters using the corresponding PREDEASY ATPase kits according to the manufacturer's instructions. For this purpose, Sf9 cell membranes (4 μg protein per well) were mixed with dinaciclib at concentrations ranging from 14 nM to 300 μM, then incubated at 37 °C for 10 min in the presence or absence of 1.2 mM sodium orthovanadate. The ATPase reaction was started by adding 10 mM ATP magnesium salt to the reaction mixture, stopped 10 min later, and the absorbance at 590 nm was measured after 30 min incubation using the Tecan microplate reader. The ATPase activity in each sample was determined as the difference in liberated amounts of phosphate measured in the presence and absence of 1.2 mM sodium orthovanadate. Phosphate standards were prepared in each plate, using verapamil, *N*-ethylmaleimide-glutathione (NEM-SG) and sulfasalazine as positive controls for ABCB1, ABCC1 and ABCG2 stimulation, respectively. The results are expressed as vanadate-sensitive ATPase activities.

2.7. DNR and MIT accumulation assays

Effects of dinaciclib on intracellular DNR accumulation in MDCKII-ABCB1 and MDCKII-ABCC1 cells, and MIT accumulation in MDCKII-ABCG2 cells, were examined using a C6 flow cytometer (Accuri, Ann Arbor, USA). MDCKII-parent cells were analyzed as controls for accumulation of each substrate. Cells were seeded at a density of 1.5×10^5 on a 12-well plate 24 h before each experiment and treated with dinaciclib at five concentrations, solvent (0.5% DMSO) or Opti-MEM® (untreated control) for 30 min at 37 °C, 5% CO_2. DNR or MIT was then added to a final concentration of 2 μM or 1 μM, respectively, and the cells were incubated under the same conditions for a further 60 min. Accumulation was stopped by cooling the samples on ice and washing twice with ice-cold PBS. The cells were detached with 10× trypsin-EDTA and resuspended in PBS with 2% FBS. Levels of DNR and MIT in individual cells were measured using 488/585 and 488/670 nm excitation/emission filters, respectively, and recorded as histograms. 1 μM LY335979, 50 μM MK-571 and 1 μM Ko143 were used as positive controls for ABCB1, ABCC1 and ABCG2 inhibition, respectively. All inhibitors were used at concentrations that are known to efficiently inhibit the corresponding transporter. Viable cells were gated based on forward and side scatter plots. The median fluorescence intensity (MFI) of 10 000 measured cells was used to compare the fluorescence resulting from each of the treatments. The relative values were identified by dividing the MFI of each measurement by that of untreated control cells. Where applicable, the half maximal effective concentration (EC_{50}) for ABC transporter inhibition was calculated using GraphPad Prism 6.00.

2.8. Drug combination assays

Combination indices (CIs, derived from the median-effect equation) were calculated to assess combined effects of dinaciclib and DNR or TOP, commonly used anticancer drugs and ABCC1 transporter substrates. CIs provide quantitatively defined indications of additive, synergistic and antagonistic effects, when CI = 0.9–1.1, <0.9, and >1.1, respectively [28]. The XTT cytotoxicity

assay was used to measure the viability of the tested cells (MDCKII-parent, MDCKII-ABCC1 and T47D) in the presence of dinaciclib and DNR/TOP both alone and in combination, at constant concentration ratios ranging from 0.1 to 2 multiples of the respective, predetermined IC_{50} values. The data acquired from these drug combination experiments were analyzed using CompuSyn ver. 3.0.1 software (ComboSyn Inc., Paramus, NJ, USA).

2.9. Statistical analysis

Data are presented as means ± standard deviations (SDs) obtained from at least three independent experiments. Statistical significance was determined using two-tailed unpaired Student's *t* tests and one-way ANOVA implemented in GraphPad Prism 6.00, and differences were considered significant if $P < 0.05$.

3. Results

3.1. Effects of ABCB1, ABCC1 and ABCG2 on transepithelial transport of dinaciclib in vitro

To evaluate whether dinaciclib is a substrate of the tested transporters, we assayed its transport *in vitro* across polarized monolayers of MDCKII-ABCB1, MDCKII-ABCC1, MDCKII-ABCG2 and MDCKII-parent cells. In MDCKII cells, ABCB1 and ABCG2 are localized apically, therefore, in this method, the transport of a substrate across the monolayer is greatly accelerated in the basolateral-to-apical direction. ABCC1, on the other hand, is localized basolaterally, accelerating transport of its substrates in apical-to-basolateral direction.

Under our test conditions, the BA to AB transport ratio (*r*) of dinaciclib (applied at 100 nM in all assays of this variable) in MDCKII-ABCB1 cells was 30.8. Presence of the model ABCB1 inhibitor LY335979 strongly reduced the ratio to 1.12 (Fig. 1A), confirming the involvement of ABCB1 in dinaciclib transport. Similarly, in MDCKII-ABCG2 cells, the transport ratios in the absence and presence of the model ABCG2 inhibitor Ko143 were 7.84 and 1.33, respectively (Fig. 1B), indicating that dinaciclib is also transported by ABCG2. The transport ratio in MDCKII-ABCC1 cells was 0.91 (Fig. 1C), significantly lower than the ratio, under the same conditions, in MDCKII-parent cells (1.29) (Fig. 1D). Presence of the ABCC1 inhibitor MK-571 significantly altered the transport asymmetry in MDCKII-ABCC1 cells, resulting in a transport ratio similar to that recorded for parental cells (1.20). This suggests that ABCC1 might also contribute to the transport of dinaciclib.

3.2. Effects of dinaciclib on the viability of MDCKII cell lines

To determine effects of ABC transporters on the antiproliferative effects of dinaciclib, the substance was used in XTT assays with ABCB1-, ABCC1- and ABCG2-overexpressing MDCKII and MDCKII-parent cell lines. The respective IC_{50} values of dinaciclib and calculated RFs are shown in Table 1. MDCKII-ABCB1 cells were significantly more resistant to dinaciclib than the parental MDCKII

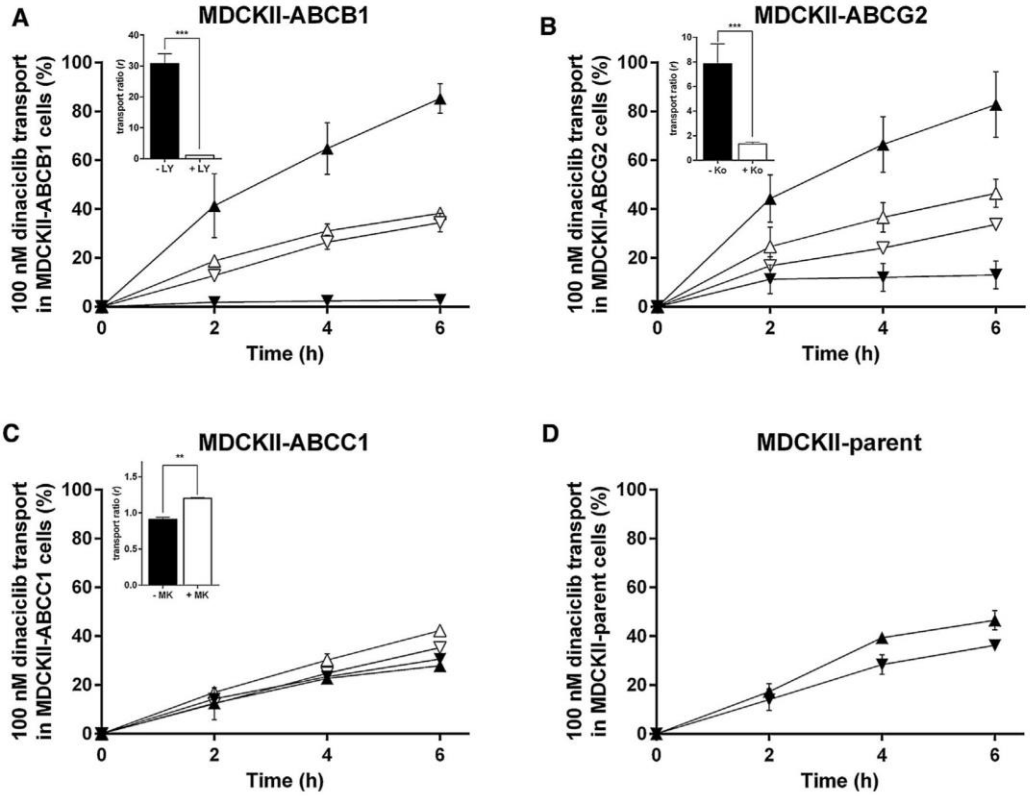

Fig. 1. Transport of dinaciclib (100 nM) across monolayers of MDCKII-ABCB1 (A), MDCKII-ABCG2 (B), MDCKII-ABCC1 (C) and MDCKII-parent cells (D). ▲, basolateral-to-apical transport without inhibitor; ▼ apical-to-basolateral transport without inhibitor; △, basolateral-to-apical transport with inhibitor; ▽, apical-to-basolateral transport with inhibitor; 1 μM LY335979 (LY), 1 μM Ko143 (Ko) and 25 μM MK-571 (MK) were used as model inhibitors of ABCB1, ABCG2 and ABCC1, respectively, in the corresponding overexpressing cell line. Ratios of dinaciclib transport across cell monolayers (dinaciclib transport in basolateral-to-apical direction divided by transport in apical-to-basolateral direction) with or without inhibitor were calculated using data acquired 6 h after dinaciclib addition. Data are presented as means ± SDs obtained from at least three independent experiments. Asterisks indicate the statistical significance of differences between *r* values in the absence and presence of the inhibitors ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Table 1
Antiproliferative IC_{50} values of dinaciclib in MDCKII cells in the absence and presence of ABC transporter inhibitors.

Drug[a]	MDCKII	MDCKII-ABCB1		MDCKII-ABCC1		MDCKII-ABCG2	
	IC_{50} (μM)	IC_{50} (μM)	RF[b]	IC_{50} (μM)	RF	IC_{50} (μM)	RF
DIN	0.024 ± 0.0049	0.16 ± 0.054*	6.7	0.088 ± 0.014**	3.7	0.070 ± 0.086**	2.9
+LY	0.016 ± 0.00039	0.017 ± 0.0018	1.1	–	–	–	–
+MK	0.023 ± 0.0023	–	–	0.031 ± 0.0042	1.3	–	–
+Ko	0.029 ± 0.0022	–	–	–	–	0.036 ± 0.0072	1.2

–: not applicable.
[a] DIN, dinaciclib; LY, LY335979 (1 μM); MK, MK-571 (25 μM); Ko, Ko143 (1 μM).
[b] RF values were calculated by dividing the IC_{50} values for ABC transporter-overexpressing cells by those for parental cells. Asterisks indicate the significance of differences between ABC transporter-overexpressing cells and parental cells, as determined by unpaired *t* tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

cell line (RF = 6.7), indicating that ABCB1 mediates dinaciclib efflux. Similarly, both ABCC1 and ABCG2 conferred resistance to dinaciclib in MDCKII-ABCC1 and MDCKII-ABCG2 cell lines, with RF values of 3.7 and 2.9, respectively. To confirm the effect of ABC transporters on sensitivity to dinaciclib, the cell proliferation assays were repeated with combinations of dinaciclib with model inhibitors of the overexpressed transporters. In each case the inhibitors significantly reduced the cells' resistance to dinaciclib: 1 μM LY335979, 25 μM MK-571 and 1 μM Ko143 reducing the RFs of MDCKII-ABCB1, MDCKII-ABCC1 and MDCKII-ABCG2 cells to 1.1, 1.3 and 1.2, respectively, which further indicates the role of ABCB1, ABCG2 and ABCC1 in the resistance to dinaciclib.

3.3. ATPase assays

To further characterize whether dinaciclib interacts with ABC transporter-associated ATPase activity, we tested its modulatory effects on vanadate-sensitive ATPase in isolated Sf9 cell membranes overexpressing human ABCB1, ABCC1 and ABCG2. In the ATPase activation assay, dinaciclib did not stimulate increases in vanadate-sensitive ATPase activity of the tested transporters. However, in the inhibition study, dinaciclib significantly lowered the stimulated vanadate-sensitive ATPase activity of all three transporters at concentrations ≥100 μM, strongly confirming that dinaciclib interacts with ABCB1, ABCC1 and ABCG2 (Fig. 2).

3.4. Effects of dinaciclib on ABC transporter-mediated efflux of fluorescent substrates

To determine whether dinaciclib can inhibit ABC transporter-mediated efflux we tested its effect on the accumulation of known substrates in MDCKII cells. DNR, a fluorescent substrate of ABCB1 and ABCC1, was used to determine the effect of dinaciclib on ABCB1- and ABCC1-mediated effluxes. In MDCKII-ABCB1 cells, we observed no significant effect of dinaciclib on intracellular accumulation of DNR (Fig. 3A). However, it significantly and dose-dependently enhanced DNR accumulation in MDCKII-ABCC1 cells (1.5-, 1.9- and 2.4-fold at 10, 30 and 50 μM, respectively) with an EC_{50} value of 18 ± 5.9 μM (Fig. 3B). Indeed, at the highest tested concentration (50 μM) its inhibitory effect was similar to that of a model ABCC1 inhibitor, MK-571, applied at the same concentration (a 2.5-fold increase in DNR accumulation). In addition, dinaciclib significantly increased the intracellular accumulation of MIT, a fluorescent substrate, in MDCKII-ABCG2 cells, but only at the highest tested concentration (50 μM). At this concentration it induced a 2.5-fold increase in MIT accumulation (Fig. 3C), substantially weaker than the response to the ABCG2 model inhibitor, Ko143, at just 1 μM (a 6.5-fold increase). No effect of dinaciclib on DNR or MIT accumulation was observed in the control MDCKII-parent cells. Our results suggest that dinaciclib can inhibit ABCC1-mediated efflux and may be able to reverse ABCC1-mediated MDR.

3.5. Drug combination assays

Since dinaciclib potently inhibited ABCC1-mediated efflux of DNR, combination studies were performed to assess its ability to sensitize ABCC1-expressing cells to selected cytotoxic ABCC1 transporter substrates through ABCC1 inhibition. Employing the XTT assay, the antiproliferative effect of dinaciclib alone was assessed and compared to the effect of concomitant treatments of dinaciclib with DNR and TOP. The combination indices (CIs) obtained for combinations of dinaciclib with DNR or TOP in MDCKII-ABCC1 cells were lower than 0.9 across the entire range of drug effect levels, indicating that these drugs have synergistic antiproliferative effects (Table 2). In contrast, the combinations of dinaciclib with TOP and DNR had significantly weaker and no synergistic effects, respectively, on the control (MDCKII-parent) cells (Fig. 4A and B).

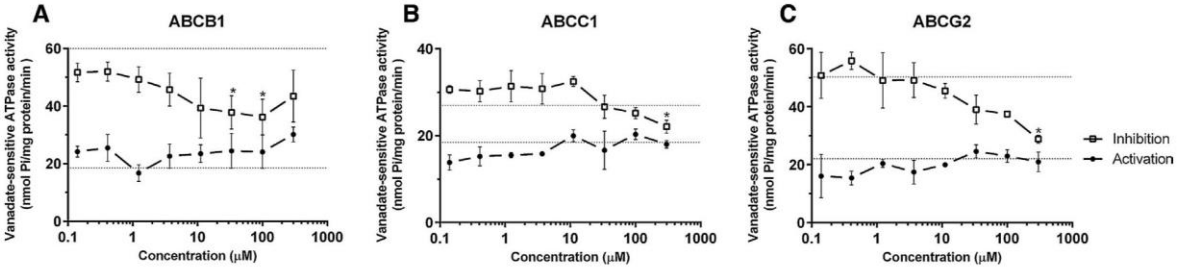

Fig. 2. Effects of dinaciclib on the ATPase activity of ABCB1-Sf9 (A), ABCC1-Sf9 (B) and ABCG2-Sf9 (C) membrane preparations. Vanadate-sensitive activity in the presence of dinaciclib in activation (●) and inhibition (□) experiments. Lower dotted line represents baseline vanadate-sensitive ATPase and upper dotted line represents activated ATPase triggered by a reference substrate in all graphs. In the inhibition and activation assays, reductions in the stimulated ATPase activity (indicating interaction of the drug with transporter's ATPase) and increases in baseline ATPase activity (indicating the drug as a transporter substrate) were measured, respectively. Data are presented as means ± SDs obtained from three independent experiments. Statistically significant differences between stimulated control and dinaciclib-treated samples in inhibition assays (*$P < 0.05$) were determined using unpaired *t* tests.

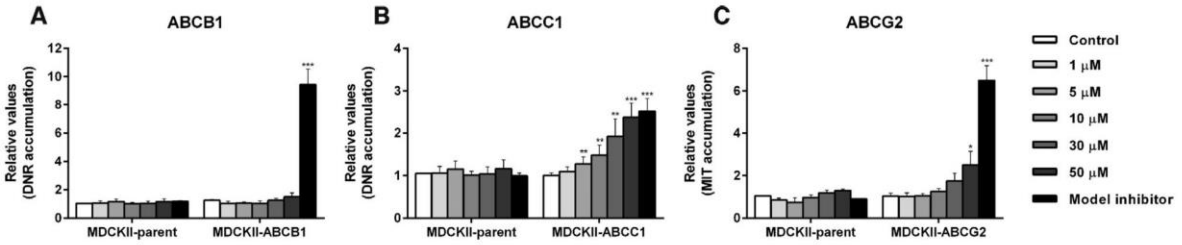

Fig. 3. Effects of dinaciclib on the intracellular accumulation of DNR in MDCKII-ABCB1 cells (A) or MDCKII-ABCC1 cells (B) and MIT in MDCKII-ABCG2 cells (C). The results represent fold changes in median fluorescence intensity compared to signals obtained with untreated controls. LY335979 (1 μM), MK-571 (50 μM) and Ko143 (1 μM) were used as model inhibitors for ABCB1, ABCC1 and ABCG2 inhibition, respectively. Data represent means ± SDs obtained from three independent experiments. Asterisks indicate the significance of differences, relative to controls, determined by unpaired *t* tests ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Table 2
Combination indices (CI) for drug combinations assessed after 72 h of simultaneous treatment in MDCKII-parent, MDCKII-ABCC1 and T47D cells.

Drug[a]		IC_{50} (μM)		Ratio	CI	
I	II	I	II	I:II	75% effect	90% effect
MDCKII-parent						
DNR	DIN	0.69 ± 0.083	0.024 ± 0.0049	1:0.034	1.3 ± 0.17	1.2 ± 0.22
TOP		3.3 ± 0.20		1:0.00073	0.97 ± 0.033	0.81 ± 0.10
MDCKII-ABCC1						
DNR	DIN	3.8 ± 0.26	0.088 ± 0.014	1:0.023	0.50 ± 0.010	0.44 ± 0.017
TOP		17 ± 1.6		1:0.00052	0.53 ± 0.0071	0.44 ± 0.019
T47D						
DNR	DIN	0.13 ± 0.0073	0.011 ± 0.0024	1:0.085	0.93 ± 0.21	0.59 ± 0.20
TOP		0.041 ± 0.0026		1:0.27	0.96 ± 0.21	0.88 ± 0.30

[a] DIN, dinaciclib; DNR, daunorubicin; TOP, topotecan.

In the ABCC1-expressing T47D human ductal breast carcinoma cells, the dinaciclib with DNR and TOP combinations showed synergistic cytotoxicity at drug effect levels of >80% and >90%, respectively (Fig. 4C and D).

4. Discussion

Evaluation of new molecules that interact with membrane-bound drug transporters is an integral part of drug development and regulatory review [29]. *In vitro* studies using ABC transporter-overexpressing polarized epithelial cell lines are considered a critical first step in the assessment of drug interactions with these transporters [18–20]. Dinaciclib, currently in phase 3 clinical trials, is an attractive potential cancer therapeutic because it inhibits several key CDKs. In the study reported here, we used MDCKII cells transduced with human ABC transporters to explore interactions of dinaciclib with ABCB1, ABCC1 and ABCG2 transporters *in vitro*.

According to the ITC white paper [17], a compound is considered a potential substrate of apically localized transporters if its transport ratio is ≥2 (*i.e.*, transport of the drug is at least twice as high in the transporter-driven direction than in the opposite direction), provided that the epithelial cell system used expresses the studied transporter and a model inhibitor reduces its transport by ≥50%. Dinaciclib exhibited high transport ratios when applied to ABCB1- and ABCG2-expressing MDCKII cell monolayers (30.8 and 7.84, respectively), and addition of model inhibitors significantly decreased these ratios, by 96% and 83%, respectively. Based on these findings, we conclude that dinaciclib can be a substrate of both ABCB1 and ABCG2 transporters. As ABCC1 is localized basolaterally in MDCKII-ABCC1 cells, the transfer of ABCC1 substrates should be greatly accelerated in apical-to-basolateral direction rather than in the opposite (basolateral-to-apical) direction. We observed a significantly lower transport ratio of dinaciclib in MDCKII-ABCC1 cells (0.91) than in MDCKII-parent cells (1.29), with significantly accentuated transport in apical-to-basolateral direction, and significant (26%) inhibition of dinaciclib transport in this direction by an ABCC1 inhibitor (MK-571). These findings indicate that ABCC1 contributes to the transport of dinaciclib. However, as the ITC does not provide substrate criteria for basolaterally localized transporters, we cannot conclusively consider dinaciclib an ABCC1 substrate.

We have also shown that overexpression of all three studied transporters (ABCB1, ABCC1 and ABCG2) confers resistance to dinaciclib. In all cases the transduced cells were significantly (2.9- to 6.7-fold) more resistant to dinaciclib than the control parental cell line. Furthermore, co-administration with selective inhibitors resulted in complete reversal of the resistance in the corresponding cell lines. These results suggest that efflux of dinaciclib mediated by ABC transporters should reduce its antiproliferative effects. Moreover, application of dinaciclib in a broad range of concentrations significantly decreased activated ATPase activities in the ATPase inhibition assays, which is a typical feature of transporter inhibitors and slowly transported substrates [30].

Since ABC transporters, particularly ABCB1 and ABCG2, constitute an effective pharmacological barrier by restricting the passage of drugs through membranes [14], dinaciclib will probably have low permeability to sensitive tissues and organs, *e.g.*, brain and fetal tissues. Its interactions with ABC transporters will also presumably affect its disposition in the body, and the transporters may present important sites for pharmacokinetic DDI if it is used in combination with other therapeutics.

Moreover, as mechanisms of resistance to dinaciclib have not been previously addressed in detail, it should be noted that ABC transporters could diminish the accumulation of dinaciclib in cancer cells, leading to the development of drug resistance and subsequent failure of anticancer therapy. Recent data have

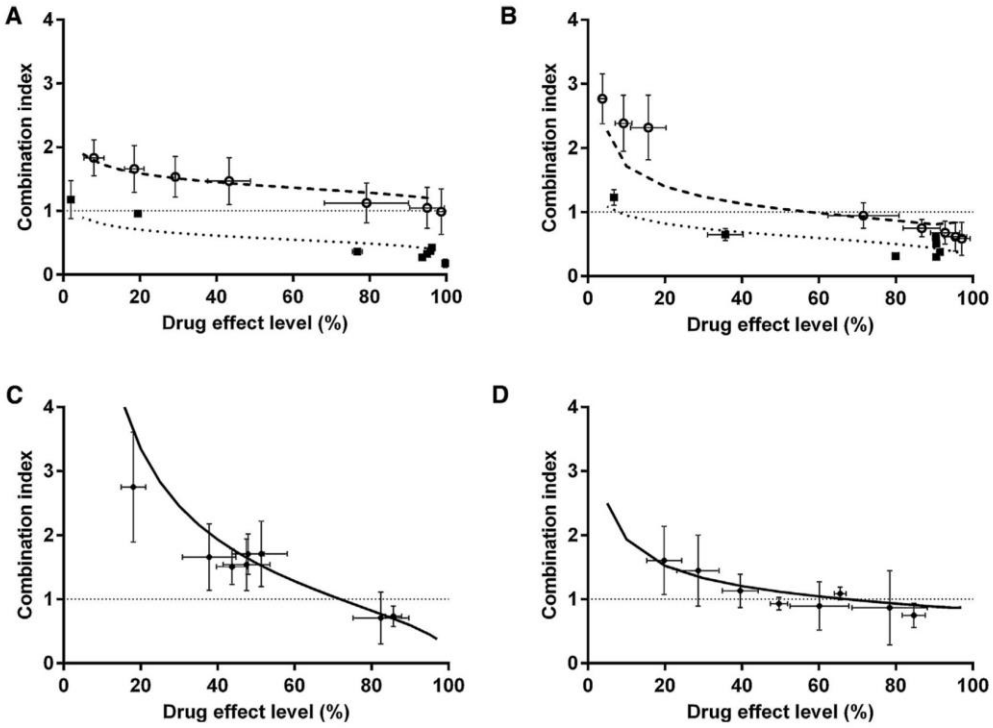

Fig. 4. Combination indices for applications of dinaciclib with DNR (A and C) and TOP (B and D) in MDCKII-parent (○), MDCKII-ABCC1 (■) and T47D (◆) cells. Lines represent computer-simulated CI plots in MDCKII-parent (dashed line), MDCKII-ABCC1 (dotted line) and T47D (full line) cells, while symbols represent experimental data points. The concentration ratio was based on the IC_{50} ratio of individual drugs. CI values < 0.9 indicate synergism, values = 0.9–1.1 indicate additivity, and values > 1.1 indicate antagonism. Drug effect levels were calculated from the cell viability values and correspond to the proportions of cells affected by the drug combination: 0 and 100% indicate no and absolute antiproliferative effects, respectively. Data are presented as means ± SDs obtained from three independent experiments.

indicated that cancer cells can develop resistance toward dinaciclib, but the evaluated mechanisms did not include ABC transporter expression [31]. Furthermore, other known resistance mechanisms, like upregulation of antiapoptotic Mcl-1, are unlikely as dinaciclib has been shown to inhibit Mcl-1 transcription, leading to significant apoptotic cell death [32–35]. Thus, the findings presented here appear to be the first indications that ABCB1-, ABCG2- and ABCC1-mediated transport is the causative mechanism of cellular resistance to dinaciclib.

Several studies have convincingly shown that tyrosine kinase inhibitors (TKIs) can inhibit ABC transporters and modulate MDR, both *in vitro* and *in vivo* [36–39]. Similarly, we have recently demonstrated that several novel small molecule protein kinase inhibitors can modulate MDR [21,40,41]. In the present study, we demonstrated that dinaciclib can effectively inhibit ABCC1-mediated efflux of DNR (EC_{50} = 18 μM), indicating that this drug may be able to reverse ABCC1-mediated MDR. Interestingly, we observed weak and no inhibitory effects of dinaciclib on ABCG2- and ABCB1-mediated effluxes, respectively.

The new novel kinase inhibitors are believed to have high anticancer potential, especially in combination with conventional chemotherapeutics, because they can circumvent compensatory mechanisms that are generally activated when CDKIs interrupt cell functions [42,43]. Dinaciclib has already been found to potently synergize with cisplatin in preclinical models of ovarian cancer, corroborating potential benefits of combinational therapy [44]. Several other combinations of dinaciclib and other drugs, *e.g.*, rituximab or epirubicine, are being evaluated in ongoing trials (clinicaltrials.gov, ID: NCT01650727 and NCT01624441, respectively). Therefore, we investigated whether dinaciclib could potentiate the cytotoxic effects of other anticancer drugs through ABCC1 transporter inhibition *in vitro*. We hypothesized that by inhibiting ABCC1-mediated effluxes of DNR and TOP, dinaciclib could increase intracellular accumulation of these drugs and enhance their cytotoxic effects. Accordingly, synergistic effects were observed when combinations of dinaciclib with DNR and TOP were applied to ABCC1-transduced and parental MDCKII cells as well as human tumor-derived cells expressing ABCC1. Furthermore, combinations of dinaciclib with DNR or TOP had significantly more pronounced synergistic effects in MDCKII-ABCC1 than in MDCKII-parent cells, suggesting the effects are directly related to ABCC1 expression. We believe that inhibition of ABCC1 was also responsible for the synergistic effects of these combinations in human ductal breast carcinoma T47D cells, as they abundantly express ABCC1 [41,45]. Exploiting the synergistic activity of dinaciclib and ABCC1 transporter substrates could therefore offer a promising clinical strategy, particularly for treating resistant tumors.

In conclusion, we have provided the first demonstration that dinaciclib is a substrate of ABCB1 and ABCG2 transporters, and an inhibitor of ABCC1 transporter. These pharmacokinetic features should be considered before bringing dinaciclib into clinical practice as they may result in DDI in normal tissues and/or multidrug resistance in cancer cells. On the other hand, we also show that DDI associated with dinaciclib could be beneficially exploited in cancer treatment if combined with other cytostatic substrates of ABCC1.

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References

[1] G.I. Shapiro, Cyclin-dependent kinase pathways as targets for cancer treatment, J. Clin. Oncol. 24 (2006) 1770–1783.
[2] G. Mariaule, P. Belmont, Cyclin-dependent kinase inhibitors as marketed anticancer drugs: where are we now? A short survey, Molecules 19 (2014) 14366–14382.
[3] S. Dhillon, Palbociclib: first global approval, Drugs 75 (2015) 543–551.
[4] D. Parry, T. Guzi, F. Shanahan, N. Davis, D. Prabhavalkar, D. Wiswell, et al., Dinaciclib (SCH 727965), a novel and potent cyclin-dependent kinase inhibitor, Mol. Cancer Ther. 9 (2010) 2344–2353.
[5] W. Fu, L. Ma, B. Chu, X. Wang, M.M. Bui, J. Gemmer, et al., The cyclin-dependent kinase inhibitor SCH 727965 (dinacliclib) induces the apoptosis of osteosarcoma cells, Mol. Cancer Ther. 10 (2011) 1018–1027.
[6] C. Abdullah, X. Wang, D. Becker, Expression analysis and molecular targeting of cyclin-dependent kinases in advanced melanoma, Cell Cycle 10 (2011) 977–988.
[7] G.P. Gregory, S.J. Hogg, L.M. Kats, E. Vidacs, A.J. Baker, O. Gilan, et al., CDK9 inhibition by dinaciclib potently suppresses Mcl-1 to induce durable apoptotic responses in aggressive MYC-driven B-cell lymphoma in vivo, Leukemia 29 (2014) 1437–1441.
[8] R. Gorlick, E.A. Kolb, P.J. Houghton, C.L. Morton, G. Neale, S.T. Keir, et al., Initial testing (stage 1) of the cyclin dependent kinase inhibitor SCH 727965 (dinaciclib) by the pediatric preclinical testing program, Pediatr. Blood Cancer 59 (2012) 1266–1274.
[9] D. Zhang, M. Mita, G.I. Shapiro, J. Poon, K. Small, A. Tzontcheva, et al., Effect of aprepitant on the pharmacokinetics of the cyclin-dependent kinase inhibitor dinaciclib in patients with advanced malignancies, Cancer Chemother. Pharmacol. 70 (2012) 891–898.
[10] J.J. Nemunaitis, K.A. Small, P. Kirschmeier, D. Zhang, Y. Zhu, Y.M. Jou, et al., A first-in-human, phase 1, dose-escalation study of dinaciclib, a novel cyclin-dependent kinase inhibitor, administered weekly in subjects with advanced malignancies, J. Transl. Med. 11 (2013) 259.
[11] J.J. Stephenson, J. Nemunaitis, A.A. Joy, J.C. Martin, Y.M. Jou, D. Zhang, et al., Randomized phase 2 study of the cyclin-dependent kinase inhibitor dinaciclib (MK-7965) versus erlotinib in patients with non-small cell lung cancer, Lung Cancer 83 (2014) 219–223.
[12] M.M. Mita, A.A. Joy, A. Mita, K. Sankhala, Y.M. Jou, D. Zhang, et al., Randomized phase II trial of the cyclin-dependent kinase inhibitor dinaciclib (MK-7965) versus capecitabine in patients with advanced breast cancer, Clin. Breast Cancer 14 (2014) 169–176.
[13] S.K. Kumar, B. LaPlant, W.J. Chng, J. Zonder, N. Callander, R. Fonseca, et al., Dinaciclib, a novel CDK inhibitor, demonstrates encouraging single-agent activity in patients with relapsed multiple myeloma, Blood 125 (2015) 443–448.
[14] G. Szakacs, A. Varadi, C. Ozvegy-Laczka, B. Sarkadi, The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox), Drug Discov. Today 13 (2008) 379–393.
[15] M. Dean, Y. Hamon, G. Chimini, The human ATP-binding cassette (ABC) transporter superfamily, J. Lipid Res. 42 (2001) 1007–1017.
[16] C.P. Wu, C.H. Hsieh, Y.S. Wu, The emergence of drug transporter-mediated multidrug resistance to cancer chemotherapy, Mol. Pharm. 8 (2011) 1996–2011.
[17] T. International, C. ransporter, G. onsortium, K.M. iacomini, S.M. Huang, D.J. Tweedie, L.Z. Benet, K.L. Brouwer, et al., Membrane transporters in drug development, Nat. Rev. Drug Discov. 9 (2010) 215–236.
[18] US Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research, Center for Biologics Evaluation and Research, 2006. Guidance for Industry. Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling [cited 2015 July], available from: http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf.
[19] European Medicines Agency, 2012. Guideline on the Investigation of Drug Interactions [cited 2015 June], available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf.
[20] M. Jani, P. Krajcsi, In vitro methods in drug transporter interaction assessment, Drug Discov. Today Technol. 12 (2014) e105–e112.
[21] D. Cihalova, J. Hofman, M. Ceckova, F. Staud, Purvalanol A, olomoucine II and roscovitine inhibit ABCB1 transporter and synergistically potentiate cytotoxic effects of daunorubicin in vitro, PLoS One 8 (2013) e83467.
[22] R.W. Robey, W.Y. Medina-Perez, K. Nishiyama, T. Lahusen, K. Miyake, T. Litman, et al., Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells, Clin. Cancer Res. 7 (2001) 145–152.
[23] Z. Rajnai, D. Mehn, E. Beery, A. Okyar, M. Jani, G.K. Toth, et al., ATP-binding cassette B1 transports seliciclib (R-roscovitine), a cyclin-dependent kinase inhibitor, Drug Metab. Dispos. 38 (2010) 2000–2006.
[24] J. Hofman, R. Kucera, D. Cihalova, J. Klimes, M. Ceckova, F. Staud, Olomoucine II, but not purvalanol A, is transported by breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1), PLoS One 8 (2013) e75520.
[25] F. Muller, M.F. Fromm, Transporter-mediated drug–drug interactions, Pharmacogenomics 12 (2011) 1017–1037.
[26] Y. Cui, J. Konig, J.K. Buchholz, H. Spring, I. Leier, D. Keppler, Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells, Mol. Pharmacol. 55 (1999) 929–937.
[27] B. Sarkadi, E.M. Price, R.C. Boucher, U.A. Germann, G.A. Scarborough, Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase, J. Biol. Chem. 267 (1992) 4854–4858.
[28] T.C. Chou, Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies, Pharmacol. Rev. 58 (2006) 621–681.
[29] L. Zhang, K.S. Reynolds, P. Zhao, S.M. Huang, Drug interactions evaluation: an integrated part of risk assessment of therapeutics, Toxicol. Appl. Pharmacol. 243 (2010) 134–145.
[30] H. Glavinas, D. Mehn, M. Jani, B. Oosterhuis, K. Heredi-Szabo, P. Krajcsi, Utilization of membrane vesicle preparations to study drug–ABC transporter interactions, Expert Opin. Drug Metab. Toxicol. 4 (2008) 721–732.
[31] G. Feldmann, A. Mishra, S. Bisht, C. Karikari, I. Garrido-Laguna, Z. Rasheed, et al., Cyclin-dependent kinase inhibitor dinaciclib (SCH727965) inhibits pancreatic cancer growth and progression in murine xenograft models, Cancer Biol. Ther. 12 (2011) 598–609.
[32] D.J. Bates, B.L. Salerni, C.H. Lowrey, A. Eastman, Vinblastine sensitizes leukemia cells to cyclin-dependent kinase inhibitors, inducing acute cell cycle phase-independent apoptosis, Cancer Biol. Ther. 12 (2011) 314–325.
[33] A.J. Johnson, Y.Y. Yeh, L.L. Smith, A.J. Wagner, J. Hessler, S. Gupta, et al., The novel cyclin-dependent kinase inhibitor dinaciclib (SCH727965) promotes apoptosis and abrogates microenvironmental cytokine protection in chronic lymphocytic leukemia cells, Leukemia 26 (2012) 2554–2557.
[34] B.M. Desai, J. Villanueva, T.T. Nguyen, M. Lioni, M. Xiao, J. Kong, et al., The anti-melanoma activity of dinaciclib, a cyclin-dependent kinase inhibitor, is dependent on p53 signaling, PLoS One 8 (2013) e59588.
[35] R.N. Booher, H. Hatch, B.M. Dolinski, T. Nguyen, L. Harmonay, A.S. Al-Assaad, et al., MCL1 and BCL-xL levels in solid tumors are predictive of dinaciclib-induced apoptosis, PLoS One 9 (2014) e108371.
[36] N. Anreddy, P. Gupta, R.J. Kathawala, A. Patel, J.N. Wurpel, Z.S. Chen, Tyrosine kinase inhibitors as reversal agents for ABC transporter mediated drug resistance, Molecules 19 (2014) 13848–13877.
[37] L.N. Eadie, T.P. Hughes, D.L. White, Interaction of the efflux transporters ABCB1 and ABCG2 with imatinib, nilotinib, and dasatinib, Clin. Pharmacol. Ther. 95 (2014) 294–306.
[38] R.J. Kelly, R.W. Robey, C.C. Chen, D. Draper, V. Luchenko, D. Barnett, et al., A pharmacodynamic study of the P-glycoprotein antagonist CBT-1(R) in combination with paclitaxel in solid tumors, Oncologist 17 (2012) 512.
[39] H. Zhang, A. Patel, S.L. Ma, X.J. Li, Y.K. Zhang, P.Q. Yang, et al., In vitro, in vivo and ex vivo characterization of ibrutinib: a potent inhibitor of the efflux function of the transporter MRP1, Br. J. Pharmacol. 171 (2014) 5845–5857.
[40] J. Hofman, D. Ahmadimoghaddam, L. Hahnova, P. Pavek, M. Ceckova, F. Staud, Olomoucine II and purvalanol A inhibit ABCG2 transporter in vitro and in situ and synergistically potentiate cytostatic effect of mitoxantrone, Pharmacol. Res. 65 (2012) 312–319.
[41] D. Cihalova, F. Staud, M. Ceckova, Interactions of cyclin-dependent kinase inhibitors AT-7519, flavopiridol and SNS-032 with ABCB1, ABCG2 and ABCC1 transporters and their potential to overcome multidrug resistance in vitro, Cancer Chemother. Pharmacol. 76 (2015) 105–116.
[42] L.M. Wang, D.M. Ren, Flavopiridol, the first cyclin-dependent kinase inhibitor: recent advances in combination chemotherapy, Mini Rev. Med. Chem. 10 (2010) 1058–1070.
[43] E. Aleem, R.J. Arceci, Targeting cell cycle regulators in hematologic malignancies, Front. Cell Dev. Biol. 3 (2015) 16.
[44] X.X. Chen, F.F. Xie, X.J. Zhu, F. Lin, S.S. Pan, L.H. Gong, et al., Cyclin-dependent kinase inhibitor dinaciclib potently synergizes with cisplatin in preclinical models of ovarian cancer, Oncotarget 6 (2015) 14926–14939.
[45] I.F. Faneyte, P.M. Kristel, M.J. van de Vijver, Multidrug resistance associated genes MRP1, MRP2 and MRP3 in primary and anthracycline exposed breast cancer, Anticancer Res. 24 (2004) 2931–2939.

7 Závěr

V rámci této dizertační práce jsme prokázali interakce nových protinádorových léčiv ze skupiny CDKI s ABC lékovými transportéry, a to jak ve smyslu inhibice jejich efluxní funkce, tak i substrátové afinity.

První práce, na které jsem spolupracovala, měla za úkol zjistit, zda jsou purinové CDKI olomoucin II a purvalanol A substráty lékových transportérů ABCB1 a ABCG2. Pomocí metody transportu přes polarizovanou buněčnou monovrstvu MDCKII buněčných linií jsme jako první zjistili, že olomoucin II je substrátem obou testovaných transportérů, zatímco purvalanol A není transportován ABCB1 ani ABCG2. Z těchto výsledků vyplývá, že farmakokinetika olomoucinu II může být po podání do organismu významně ovlivněna z důvodu přítomnosti efluxních transportérů ABCB1 a ABCG2 ve fyziologických bariérách. V nádorových buňkách s vysokou expresí obou transportérů může navíc dojít ke snížené akumulaci olomoucinu II a k následnému selhání protinádorové terapie. Při podání této látky v kombinaci s dalšími substráty či inhibitory ABCB1 či ABCG2 může dojít ke vzniku lékových interakcích a tento fakt by měl být brán v potaz při případném zavádění olomoucinu II do klinické praxe. Na druhou stranu, farmakokinetiku purvalanolu A transportéry ABCB1 a ABCG2 neovlivní, což zvýhodňuje tuto látku pro použití v terapii rezistentních nádorů. Z těchto výsledků je zřejmé, že i chemicky velmi podobné látky jako jsou olomoucin II a purvalanol A, mohou mít odlišný interakční potenciál vůči ABC transportérům či biotransformačním enzymům. Při stanovení koncentrace olomoucinu II pomocí metody HPLC-MS jsme navíc pozorovali, že v buňkách dochází k jeho metabolizaci, konkrétně k sulfataci, což může vést ke kontaminaci vzorků farmakokinetické analýzy při jiném než chromatografickém stanovení (především při měření akumulace radioaktivně značených látek, kdy není brán v potaz vznik metabolitu) a špatné interpretaci výsledků.

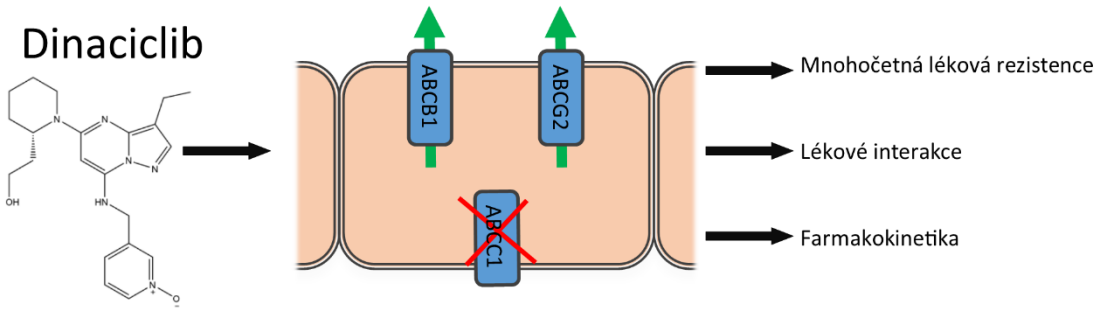
Ve své první vlastní experimentální práci jsem navazovala na původní práci našeho kolektivu, která ukázala, že CDKI olomoucin II, purvalanol A a roskovitin inhibují lékový efluxní transportér ABCG2 a že olomoucin II je na rozdíl od purvalanolu A substrátem ABCB1 i ABCG2. Podobnými metodami jsme na modelové buněčné linii MDCKII-ABCB1 ukázali, že tyto látky a navíc i další CDKI flavopiridol a SNS-032 snižují eflux fluorescenčních substrátů zprostředkovaný ABCB1, což může ovlivnit farmakokinetické chování společně podaných substrátů tohoto transportérů.

Olomoucin II vykazoval nejsilnější inhibiční aktivitu vůči ABCB1, následován roskovitinem, purvalanolem A, flavopiridolem a SNS-032. Inhibice byla potvrzená i snížením ATPázové aktivity ABCB1 po aplikaci jednotlivých látek. Jediný olomoucin II ATPázovou aktivitu ABCB1 i zvyšoval, což dokazuje, že je i substrátem ABCB1. Dále jsme ukázali, že nejsilnější inhibitory ABCB1 (olomoucin II, purvalanol A a roskovitin) synergicky potencují cytotoxický efekt konvenčních protinádorových léčiv, substrátů ABCB1, v modelové MDCKII-ABCB1 a nádorových HCT-8 a HepG2 buněčných liniích. Tento synergický efekt je v tomto případě dán vlastní cytotoxickou a protinádorovou aktivitou CDKI a navíc zvýšenou akumulací daunorubicinu v buňce, způsobenou specifickou inhibicí ABCB1. Podání obou léčiv v kombinaci pak může vést ke snížení kumulativních dávek jednotlivých látek a ke snížení rizika vzniku mnohočetné lékové rezistence a nežádoucích účinků při terapii nádorů s vysokou expresí ABCB1.

Cílem naší další práce bylo stanovit interakční profil dalších CDKI, konkrétně AT-7519, flavopiridolu a SNS-032, které se již nacházejí v některé z fází klinického hodnocení, s ABC transportéry podílejícími se na vzniku mnohočetné lékové rezistence. Na modelové linii MDCKII, exprimující jednotlivé transportéry jsme zjistili, že transportér ABCB1 nepřispívá ke vzniku rezistence vůči flavopiridolu, nicméně se účastní transportu AT-7519 a SNS-032. ABCG2 a ABCC1 na druhou stranu mohou způsobit rezistenci vůči flavopiridolu, ale už ne vůči AT-7519 a SNS-032. Flavopiridol a SNS 032 inhibují transport substrátů ABCG2 a flavopiridol navíc vykazuje inhibiční schopnost vůči ABCC1 s aktivitou srovnatelnou s modelovým inhibitorem ABCC1, MK-571. Jelikož se CDKI v klinickém zkoušení používají i v kombinaci s dalšími protinádorovými léčivy, bylo naším dalším cílem stanovení synergického efektu látek podaných v kombinaci. Látky s inhibiční aktivitou vůči ABCB1 či ABCG2, flavopiridol a SNS-032, byly aplikovány spolu v daunorubicinem či topotekanem, což jsou substráty ABCB1 a/nebo ABCG2, nádorovým buněčným liniím HepG2 a T47D. Ve všech případech došlo k synergickému protinádorovému působení a zlepšení efektivity podání jednotlivých látek. CDKI zde fungují jako modulátory apoptózy navozené daunorubicinem a topotekanem, a navíc překonávají mnohočetnou lékovou rezistenci inhibicí ABC transportérů.

Má poslední prvoautorská práce se zabývala studiem lékových interakcí ABC transportérů s CDKI dinaciclibem, který je již ve fázi III klinického hodnocení pro terapii hematologických malignit. Znalosti těchto interakcí je totiž nezbytnou součástí vývoje

nové terapeutické látky a optimálního začlenění do klinické praxe. Dle doporučení ITC je látka substrátem ABCB1 či ABCG2, pokud je transportní poměr (podíl transportu v bazolaterálně-apikálním směru k transportu ve směru opačném) vyšší než 2 v systému epiteliálních buněk s expresí daného transportéru. Pomocí experimentů v MDCKII buněčných liniích jsme zjistili, že transportní poměr dinaciclibu je 30,8 v MDCKII-ABCB1 a 7,84 v MDCKII-ABCG2 buňkách, což značí, že tato látka je transportovaným substrátem obou transportérů. Z našich dalších experimentů je zřejmé, že transportéry ABCB1, ABCG2 i ABCC1 mohou navodit rezistenci vůči dinaciclibu jeho aktivním transportem ven z buňky, což může představovat značnou překážku v úspěšné protinádorové terapii. Prostup dinaciclibu přes fyziologické bariéry může být zpomalen a farmakokinetika této látky po podání do organismu může být ABC transportéry značně ovlivněna. Hrozí zde i riziko četných lékových interakcí při podání s dalšími látkami. Interakčního potenciálu dinaciclibu se však dá i využít především z toho důvodu, že tato látka je inhibitorem ABCC1. V návaznosti na podávání dinaciclibu v kombinaci s dalšími léčivy v klinickém zkoušení jsme podávali dinaciclib v kombinaci s ABCC1 substráty, daunorubicinem a topotekanem. Na lidských nádorových buněčných liniích jsme zjistili, že dinaciclib je schopen překonávat mnohočetnou lékovou rezistenci vůči daným substrátům, zvýšit jejich akumulaci v buňce a synergicky potencovat cytotoxický efekt těchto protinádorových léčiv (obr. 5).

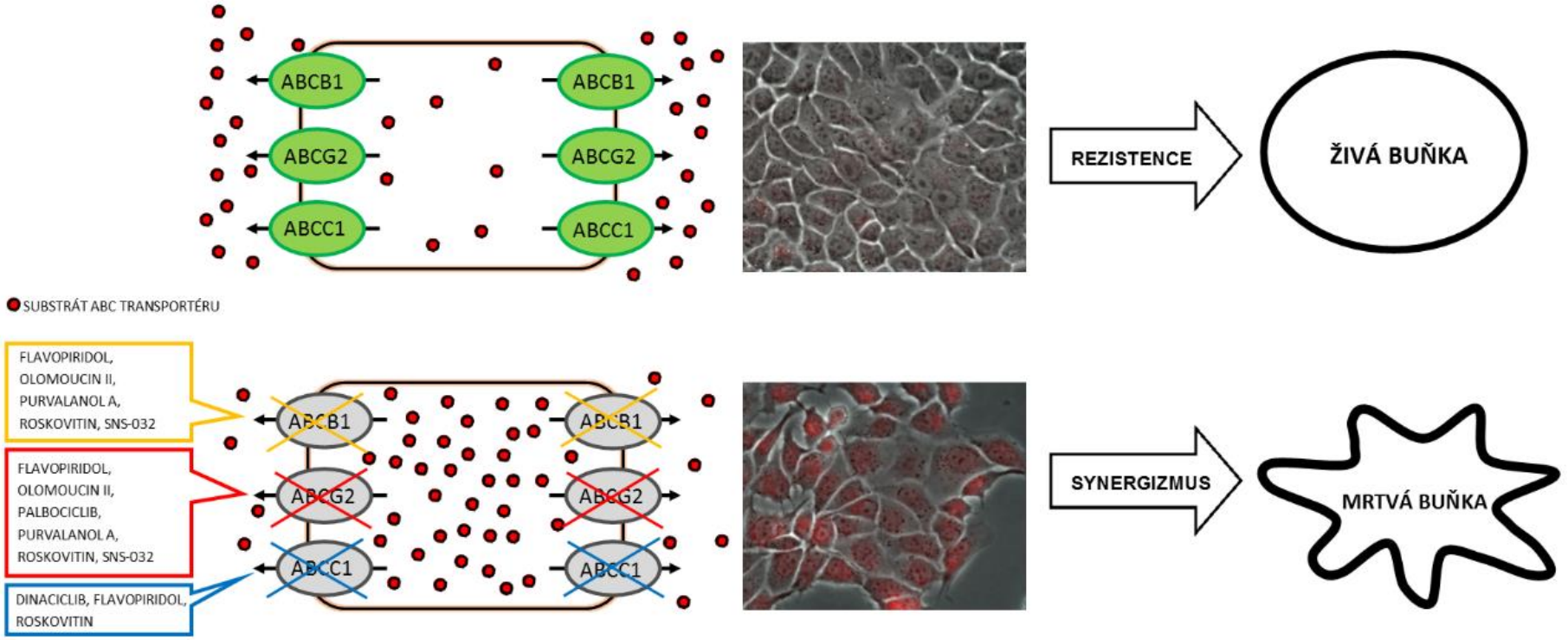


Obr. 5. Schématické znázornění interakce dinaciclibu s ABC transportéry.

Výše uvedené vlastnosti CDKI mohou zvýšit riziko lékových interakcí při uvedení do klinické praxe, ale ve většině případů mohou také zvýhodnit použití těchto látek jako nových protinádorových léčiv. CDKI působí jako léčiva s duálním účinkem: (i) zástava

buněčného dělení inhibicí CDK a (ii) schopnost překonávat mnohočetnou lékovou rezistenci nádorových buněk při podání s konvenčními cytostatiky (obr. 6).

Tato práce tak významně rozšiřuje současnou znalost farmakokinetických vlastností CDKI a potvrzuje jejich významný potenciál v terapii nádorových onemocnění.



Obr. 6. Přehled CDKI s inhibiční aktivitou vůči ABC transportérům. Současné podání takového CDKI s konvenčním cytostatikem vede ke zvýšené nitrobuněčné koncentraci tohoto cytostatika a synergickému efektu kombinované protinádorové terapie.

8 Seznam odborných publikací

8.1 Odborné články publikované v zahraničních časopisech s impakt-faktorem

1. Hofman J., Kucera R., Cihalova D., Klimes J., Ceckova M., Staud F.: **Olomoucine II, but not purvalanol A, is transported by breast cancer resistance protein (ABCG2) and P-glycoprotein (ABCB1).** Plos One 2013;8(10):e75520.

   IF_{2013} 3,534

2. Cihalova D., Hofman J., Ceckova M., Staud F.: **Purvalanol A, olomoucine II and roscovitine inhibit ABCB1 transporter and synergistically potentiate cytotoxic effects of daunorubicin *in vitro*.** Plos One 2013;8(12):e83467.

   IF_{2013} 3,534

3. Cihalova D., Staud F., Ceckova M.**: Interactions of cyclin-dependent kinase inhibitors AT-7519, flavopiridol and SNS-032 with ABCB1, ABCG2 and ABCC1 transporters and their potential to overcome multidrug resistance *in vitro*.** Cancer Chemotherapy and Pharmacology 2015;76(1):105-16.

   IF_{2014} 2,769

4. Cihalova D., Ceckova M., Kucera R., Klimes J., Staud F.: **Dinaciclib, a cyclin-dependent kinase inhibitor, is a substrate of human ABCB1 and ABCG2 and an inhibitor of human ABCC1 *in vitro*.** Biochemical Pharmacology 2015 98:465-72.

   IF_{2014} 5,009

8.2 Posterové prezentace a abstrakty publikované ve sbornících

1. Cihalova D., Hofman J., Ceckova M., Staud F.: **Inhibition of P-glycoprotein efflux activity by cyclin-dependent kinase inhibitors.**

   62. Farmakologické dni, 25. – 27. června 2012, Košice, Slovenská republika

2. Hofman J., Cihalova D., Kucera R., Klimes J., Staud F.: **Substrate affinity of olomoucine II and purvalanol A for ABCG2 and ABCB1 transporters *in vitro* and *in situ*.**

   62. Farmakologické dni, 25. – 27. června 2012, Košice, Slovenská republika

3. Cihalova D., Ceckova M., Staud F.: **Cyclin-dependent kinase inhibitors, purvalanol A, olomoucine II and roscovitine, enhance daunorubicin cytotoxicity in multidrug resistant cell lines via P-glycoprotein inhibition.**

   Toxcon, 19. – 21. června 2013, Hradec Králové, Česká republika

4. Ceckova M., Cihalova D., Hofman J., Urbanek L., Krystof V., Staud F.: **Novel cyclin-dependent kinase inhibitors BA-12 and BP-14 are able to modulate multidrug resistance through inhibition of ABCB1 and ABCG2 efflux transporters.**

   BioMedical Transporters, 11. – 15. srpna 2013, St. Moritz, Švýcarsko

5. Cihalova D., Ceckova M., Staud F.: **Cyclin-dependent kinase inhibitors, AT-7519, dinaciclib, PD 0332991, and SNS-032, inhibit ABCB1 and ABCG2 transporters.**

   63. Farmakologické dny, 11. – 13. září 2013, Olomouc, Česká republika

6. Cihalova D., Ceckova M., Staud F.: **Cyclin-dependent kinase inhibitors, flavopiridol, PD 0332991 and roscovitine, inhibit ABCB1 transporter *in vitro* and synergistically potentiate the cytotoxic effect of daunorubicin in MDCKII-ABCB1 cell line.**

   10th International ISSX Meeting, 29. září – 3. října 2013, Toronto, Kanada

7. Cihalova D., Ceckova M., Staud F.: **Flavopiridol (Alvocidib), a cyclin-dependent kinase inhibitor, displays modulatory activity of ABC transporters and synergistically potentiates the cytotoxic effect of daunorubicin and topotecan in cancer cell lines *in vitro*.**

   5th FEBS Special Meeting 2014, ABC Proteins: From Multidrug Resistance to Genetic Diseases, 8. – 14. března 2014, Innsbruck, Rakousko

8. Cihalova D., Ceckova M., Staud F.: **Interaction of the cyclin-dependent kinase inhibitors, alvocidib, AT-7519 and SNS-032, with ABCG2.**

   Solvo Meet the experts, Transporter conference 2014, 1. – 4. dubna 2014, Budapešť, Maďarsko

9. Cihalova D., Ceckova M., van den Heuvel J. J. M. W., Russel F. G. M., Staud F.: **Development of a fluorescence-based assay for drug interactions with human ABC transporters in HEK293 membrane vesicles.**

   64. Farmakologické dni, 25. – 27. června 2014, Martin, Slovenská republika

10. Cihalova D., Ceckova M., Staud F.: **Cyclin-dependent kinase inhibitors enhance cytotoxicity of daunorubicin and topotecan in cancer cell lines.**

    Toxcon, 24. – 26. září 2014, Stará Lesná, Slovenská republika

11. Číhalová D., Čečková M., Štaud F.: **Inhibitory cyklin-dependentních kináz: Vliv na mnohočetnou lékovou rezistenci.**

    XV. Mezioborové setkání mladých biologů, biochemiků a chemiků, 12. – 15. května 2015, Milovy, Česká republika

12. Sorf A., Cihalova D., Weinelt F., Ceckova M., Staud F.: **A study of interactions of cyclin-dependent kinase inhibitors AZD5438 and R547 with ABC drug efflux transporters ABCB1, ABCG2 and ABCC1.**

    Toxcon, 27. – 29. května 2015, Brno, Česká republika

13. Cihalova D., Ceckova M., Staud F.: **ABCB1, ABCC1 and ABCG2 modulate transport and pharmacokinetics of dinaciclib *in vitro*.**

    13th European ISSX Meeting, 22. – 25. června 2015, Glasgow, Velká Británie

14. Sorf A., Cihalova D., Ceckova M., Staud F.: **Interactions of cyclin-dependent kinase inhibitors, ribociclib, AZD5438 and R547, with drug efflux transporters ABCB1, ABCG2 and ABCC1.**

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

15. Sorf A., Cihalova D., Ceckova M., Staud F.: **Cyclin-dependent kinase inhibitors AZD5438 and R547, but not ribociclib, modulate ABCC1 activity *in vitro*.**

    V4 International Conference: Analytical Cytometry VIII, 3. – 6. října 2015, Olomouc, Česká republika

8.3 Ústní prezentace

1. Číhalová D., Hofman J., Čečková M., Štaud F.: **Inhibice efluxní aktivity P-glykoproteinu inhibitory cyklin-dependentních kináz**.

   62. Farmakologické dni, 25. – 27. června 2012, Košice, Slovenská republika

2. Cihalova D., Hofman J., Ceckova M., Staud F.: **Pharmacokinetic interactions of cyclin-dependent kinase inhibitors with P-glycoprotein *in vitro*.**

   3. Postgraduální a 1. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 29. – 30. ledna 2013, Hradec Králové, Česká republika

3. Cihalova D., Ceckova M., Staud F.: **Interactions of dinaciclib and palbociclib with ABC transporters associated with multidrug resistance.**

   4. Postgraduální a 2. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 28. – 29. ledna 2014, Hradec Králové, Česká republika

4. Cihalova D., Ceckova M., van den Heuvel J. J. M. W., Russel F. G. M., Staud F.: **Development of a fluorescence-based assay for drug interactions with human ABC transporters in HEK293 membrane vesicles.**

   64. Farmakologické dni, 25. – 27. června 2014, Martin, Slovenská republika

5. Cihalova D., Ceckova M., Staud F.: **PD 0332991 reverses ABC transporter-mediated multidrug resistance and synergizes with cancer chemotherapeutics in MDCKII and MCF-7 cell lines.**

   5. Postgraduální a 3. Postdoktorandská vědecká konference Farmaceutické fakulty Univerzity Karlovy, 3. – 4. února 2015, Hradec Králové, Česká republika

9 Ocenění

Za posterovou prezentaci s názvem „Flavopiridol (Alvocidib), a cyclin-dependent kinase inhibitor, displays modulatory activity of ABC transporters and synergistically potentiates the cytotoxic effect of daunorubicin and topotecan in cancer cell lines *in vitro*“ byla udělena cena **„ABC2014 Young Investigator Award“**.

Cihalova D., Ceckova M., Staud F.

5th FEBS Special Meeting 2014, ABC Proteins: From Multidrug Resistance to Genetic Diseases, 8. – 14. března 2014, Innsbruck, Rakousko

Posterová prezentace s názvem „ABCB1, ABCC1 and ABCG2 modulate transport and pharmacokinetics of dinaciclib *in vitro*“ byla vybrána mezi šest finalistů v soutěži **„Predoctoral Poster Awards Competition“**.

Cihalova D., Ceckova M., Staud F.

13th European ISSX Meeting, 22. – 25. června 2015, Glasgow, Velká Británie