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Interactions with selected drug renal transporters and transporter-mediated cytotoxicity in antiviral agents from the group of acyclic nucleoside phosphonates

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ABSTRACT

Members of acyclic nucleoside phosphonates (ANPs) possess antiviral and antiproliferative activities. However, several clinically important ANPs may cause renal injury, most likely due to their active accumulation in the renal tubular cells. The goal of this study was to investigate *in vitro* relationships between the affinity of several structurally related potent ANPs to selected human transporters and their cytotoxicity. SLC (solute carrier family) transporters (hOAT1, hOCT2, hCNT2, hCNT3) and ABC (ATP-binding cassette) transporters (MDR1, BCRP), which are typically expressed in the kidney, were included in the study. The transport and toxic parameters of the tested compounds were compared to those of two clinically approved ANPs, adefovir and tenofovir. Transport studies with transiently transfected cells were used as the main method in the experiments. Most of the ANPs studied showed the potency to interact with hOAT1. GS-9191, a double prodrug of PMEG, displayed an affinity for hOAT1 comparable with that of adefovir and tenofovir. No significant interaction of the tested ANPs with hOCT2, hCNT2 and hCNT3 was observed. Only GS-9191 was found to be a strong inhibitor for both MDR1 and BCRP. PMEO-DAPy showed the potency to interact with MDR1. Most of the tested substances caused a significant decrease in cellular viability in the cells transfected with hOAT1. Only with the exclusion of GS-9191, a relatively lipophilic compound, did the *in vitro* cytotoxicity of the ANPs closely correspond to their potential to interact with hOAT1. The increased cytotoxicity of the studied ANPs found in OAT1 transfected cells was effectively reduced by OAT inhibitors probenecid and quercetin. The higher cytotoxicity of the compounds with affinity to hOAT1 proved in the inhibitory experiments evidences that ANPs are not only inhibitors but also substrates of hOAT1. Any clear relationship between the potency of ANPs to inhibit the studied efflux transporters and their cytotoxicity was not demonstrated. In conclusion, the study documented that among the studied transporters hOAT1 seems to be the decisive determinant for renal handling in most of the tested ANPs. This transporter may also play an important role in the mechanism of their potential cytotoxic effects. These facts are in good accordance with previous findings in the clinically used ANPs.

1. Introduction

Acyclic nucleoside phosphonates (ANPs) are nucleotide analogs originated at the Antonin Holy Laboratory at IOCB (Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic) that have been shown to possess broad spectrum antiviral, cytostatic and antiproliferative activities. ANPs such as tenofovir, adefovir or cidofovir have been licensed for the treatment of various DNA virus and retrovirus infections (De Clercq, 2007a,b; Holy, 2006). Their characteristic phosphonate group is unique in that it is attached to the nucleoside analog in the formation of phosphonomethyl ether and thus can withstand an attack by esterases and other catabolic enzymes (De Clercq, 2007a,b). ANPs are excreted predominantly

Abbreviations: ABC, ATP-binding cassette; ANPs, acyclic nucleoside phosphonates; BCRP/ABCG2, breast cancer resistance protein; CNTs, concentrative nucleoside transporters; DMEM, Dulbecco's modified Eagle's medium; EMEM, Eagle's minimum essential medium; hCNT2, human concentrative nucleoside transporter 2; hCNT3, human concentrative nucleoside transporter 3; HeLa, human cervical epitheloid carcinoma cell line; hOAT1, human organic anion transporter 1; hOCT2, human organic cation transporter 2; IC_{50}, inhibitory concentration to reduce substrate accumulation to 50%; MDCK II, Madin–Darby canine kidney II cell line; MPP^+, 1-methyl-4-phenylpyridinium; MRPs, multidrug-resistance proteins; OATs, organic anion transporters; OCTs, organic cation transporters; PAH, *para*-aminohippuric acid; RFU, relative fluorescence units; SLC, solute carrier family.

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by the kidney using tubular secretion mechanisms (Cihlar et al., 1999). The transport of ANPs in the renal proximal tubules is mediated specifically by uptake at the basolateral membrane and efflux at the luminal membrane. Treatments that enhance accumulation, such as those that block efflux, may cause a change of excretion equilibrium and thus result in renal damage (Cundy et al., 1996; De Clercq, 2011; Izzedine et al., 2005).

A group of several multispecific drug transporters from the solute carrier family (SLC) such as organic anion and cation transporters (OATs/OCTs), nucleoside transporters (NTs) and numerous others may be an important entrance pathway into cells, and could significantly contribute to the cellular accumulation of antiviral agents. Uptake in the renal tubular cells mediated by these transporters may be potentially responsible for the renal toxicity detected in several ANPs (Cihlar et al., 2009; Klaassen and Aleksunes, 2010; Minuesa et al., 2011). It has been proven that cidofovir and adefovir as well as other antiviral nucleoside phosphonate analogs PMEG and PMEDAP are high-affinity substrates for human organic anion transporter 1 (hOAT1) (Cihlar et al., 1999). Related to toxicity, the expression of hOAT1 has been observed to induce the cytotoxicity of adefovir and cidofovir in Chinese hamster ovary cells stably transfected with hOAT1 (Ho et al., 2000). Transporters from the ABC (ATP-binding cassette) superfamily, multidrug resistance P glycoprotein (PgP/MDR1/ABCB1) and breast cancer resistance protein (BCRP/ABCG2) localized to the luminal membrane of the proximal tubular cells have been shown to mediate the efflux of xenobiotics in proximal tubules (Eilers et al., 2008; Van de Ven et al., 2009; Sarkadi et al., 2006). The goal of this study was to investigate *in vitro* if other members of ANPs with potent antiviral activity may demonstrate a similar affinity to renal drug transporters as the commonly used antivirals adefovir and tenofovir, or if other uptake and efflux transporters abundantly expressed in the kidney may potentially play a role in renal accumulation of these compounds. The study presents data on the interactions of five ANPs from groups of PME, 2-(phosphonomethoxy)ethyl and HPMP, 3-hydroxy-2-(phosphonomethoxy)propyl compounds (Fig. 1), respectively, with selected human uptake (hOAT1, hOCT2, hCNT2, hCNT3) and efflux (ABCB1, ABCG2) transporters, which are typically expressed in the kidney. Our work shows possible relationship between the potency of the agents to interact with the studied transporters and their cytotoxicity. In addition, we have tried to consider the possible significance of liphophilicity in the interaction of the studied agents with the relevant transporters.

2. Materials and methods

2.1. Chemical reagents used

PMEG, 9-[2-(phosphonomethoxy)ethyl]guanine; cPrPMEDAP, 9-(2-phosphonylmethoxyethyl)-N(6)-cyclopropyl-2,6-diaminopurine, GS-9191, (L-phenylalanine, N,N′-[[[2-[2-amino-6-(cyclopropylamino)-9H-purine-9-yl]ethoxy]methyl] phosphinylidene]bis-, bis(2-methylpropyl) ester), (*S*)-HPMPA, (*S*)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine; PMEO-DAPy, 2,4-diamino-6-[(2-phosphonomethoxy)ethyl]purine were synthesized by the Institute of Organic Chemistry and Biochemistry AS CR, Prague, Czech Republic. For the experiments, GS-9191 and PMEG were dissolved in dimethyl sulfoxide, cPrPMEDAP, (*S*)-HPMPA and PMEO-DAPy in water and diluted with the incubation medium. Quercetin in the form of dihydrate and probenecid were obtained from Sigma–Aldrich (St. Louis, USA).

[5-^{3}H]-uridine ([^{3}H]-uridine) were obtained from Moravek Biochemicals (Brea, USA), p-[glycyl-2-^{3}H]-aminohippuric acid ([^{3}H]-PAH) was purchased from Perkin Elmer (Waltham, USA). Methyl-4-phenylpyridinium acetate, N-[methyl-^{3}H] ([^{3}H]-MPP$^+$) was obtained from American Radiolabeled Chemicals (St. Louis, USA). Hoechst 33342 and the specific ABCG2 inhibitor KO143 were purchased from Sigma–Aldrich. The specific ABCB1 inhibitor LY335979 was obtained from Karl-Schmid (Munich, Germany).

OAT1 (SLC22A6) expression plasmid, transcript variant 2, cat. number: RC207579; OCT2 (SLC22A2) expression plasmid, cat. number: RC207921; CNT2 (SLC28A2) expression plasmid, cat. number: RC211308; CNT3 (SLC28A3) expression plasmid, transcript variant 2, cat. number: RC210847 and pCMV6-Entry vector, cat. number: PS100001 were obtained from OriGene Technologies (Rockville, USA).

2.2. HeLa cell culture

The human cervical epithelioid carcinoma cell line (HeLa) was purchased from the European Collection of Cell Culture (Salisbury, UK). The cells (passages 15–25, 28–30) were routinely cultured in 75 cm^2 cell culture flasks in a humidified atmosphere containing 5% CO_2 at 37 °C in cell culture Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 1% nonessential amino acids and 2 mM L-glutamine. Confluent cells were split every 4 days using 0.25% trypsin/EDTA solution.

2.3. MDCK II cell cultures

The Madin–Darby canine kidney cell line (MDCK II) was purchased from the European Collection of Cell Culture (Salisbury, UK). The MDCK II cells (passages 11–20), ABCG2-transduced MDCK II subline (passages 5–11), which stably expresses ABCG2 protein (a breast cancer resistance protein) and ABCB1-transduced MDCK II subline (passages 5–11), which stably expresses ABCB1 protein (kindly provided by Assoc. Prof. Petr Pavek) were routinely cultured in 75 cm^2 cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with high glucose, 2 mM L-glutamine and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37 °C. Confluent cells were split every 4 days using 0.25% trypsin/EDTA solution.

2.4. Transient transfection

The HeLa cells were seeded at a density 7×10^4 cells/well in 24-well plates. The MDCK II cells were seeded at a density 2×10^5 cells/well in 24-well plates. The following day the cells were transiently transfected with the appropriate plasmid expressing either the studied influx transporter or the empty vector (pCMV6) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) and Opti-MEM (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Transport assays in the HeLa cells transiently transfected with hOAT1 were performed 48 h after transfection. Transport assays in the MDCK II cells transiently transfected with hOCT2, hCNT2 or hCNT3 were performed 24 h after transfection. The cells transiently transfected with empty vector served as the control.

2.5. Interactions with SLC transporters

Interactions of the tested ANPs with the SLC transporters were carried out in 24-well plates as described previously (Errasti-Murugarren et al., 2010). The transport solution contained 130 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 5 mM glucose and 10 mM HEPES, pH 7.4. The cultivation medium was removed and the cells were washed with transport solution and preincubated for 10 min at 37 °C. The prototypical substrate or tested radioactive substance dissolved in transport solution was added in triplicates to the cell monolayers and incubated for 2 min. [^{3}H]*para*-aminohippuric acid ([^{3}H]PAH) was used as a specific substrate for hOAT1, [^{3}H]methyl-4-phenylpyridinium ([^{3}H]MPP$^+$) for hOCT2 and [^{3}H]uridine for CNTs. The rate of inhibition of the intracellular accumulation of the radioactive substrates induced by gradually increasing concentrations of PMEG, cPrPMEDAP, (*S*)-HPMPA or PMEO-DAPy (0–1000 μM) or GS-9191 in concentration (0–250 μM) was used as a measure of the affinity to the transporter. The incubation was terminated by washing the cells twice with an ice-cold solution containing 137 mM NaCl and 10 mM HEPES, pH 7.4. The cells were disintegrated with 0.1 mL of TritonX 0.5% in 100 mM NaOH for 60 min. The radiotracer accumulation in the cells transfected with empty vector was subtracted. After disintegration, cell protein content was measured using the BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). Finally, the radioactivity of the samples in scintillation solution (Sigma–Aldrich, St. Louis, USA) was measured with a beta counter (Tri-Carb 2900TR; Perkin Elmer, Shelton, USA). All the values were standardized against protein content, which was determined using the bicinchonic acid method. The results on affinity were expressed as inhibitory concentration IC_{50} calculated with a nonlinear regression analysis using GraphPad Prism software (version 6).

2.6. Interactions with ABC transporters

Interactions of the tested ANPs with the selected ABC transporters were studied using stably transfected MDCK II cells. MDCK-ABCG2 and MDCK-ABCB1 (2×10^5) cells were seeded on a 24-well plate 24 h before the experiment. The cell culture medium was removed and the cells were washed twice with pre-warmed transport medium containing NaCl 130 mM, KCl 4 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, glucose 5 mM, HEPES 10 mM, pH 7.4. The cells were then preincubated in transport medium with a three-point concentration scale of ANPs at 37 °C in 5% CO_2 for 30 min before Hoechst 33342 (8 μM) was added. Specific ABCG2 inhibitor KO143 as well as LY335979 as a specific inhibitor of ABCB1 were used as the comparators for inhibition. Relative fluorescence units (RFU) in bottom mode (excitation = 360 nm, emission = 465 nm) were measured immediately after the addition of Hoechst 33342 in 1 min intervals for 31 min at a constant temperature of 37 °C (Genios Plus, Tecan, Salzburg, Austria).