The renal handling of amphotericin B and amphotericin B/deoxycholate and potential renal drug-drug interactions with selected antivirals

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Abstract

Amphotericin B (AmB) is excreted via the renal excretion route. This excretion process may result in nephrotoxicity. However, relevant information on the precise renal excretion mechanisms is not available. The aim of the study was to analyze possible interaction of AmB or its prodrug AmB deoxycholate (Amb/DOC) with the typical renal transporters for organic anions (OCTs) and organic cations (OCTs), using cellular and organ models. The relevant transport systems were then investigated in terms of the drug-drug interaction of AmB/DOC with potentially concomitantly used antivirals. To analyze renal excretion mechanisms of [3H]AmB, perfused rat kidney was employed. HeLa and MDCK II cells transiently transfected with hOAT1 or hOCT2 were used as the cellular models. A significant tubular
secretion of AmB was demonstrated in the perfused rat kidney. The performed cellular studies confirmed the active transport of AmB into cells. AmB did not interact with hOAT1 but strongly inhibited hOCT2. In contrast, AmB/DOC inhibited both hOAT1 and hOCT2. On the other hand, \[^{3}H\]AmB cellular uptake by hOAT1 and hOCT2 was not found. AmB/DOC interacted significantly with adefovir, tenofovir, and cidofovir in hOAT1 transfected cells at supratherapeutic concentrations. In conclusion, a significant potency of AmB and AmB/DOC to inhibit the studied transporters was demonstrated. The found secretion of AmB in the renal tubules is likely not related to the studied transporters, since the drug was not proven to be a substrate for them. Drug-drug interactions of AmB and the used antivirals on the studied transporters are not probable.

INTRODUCTION

Amphotericin B (AmB) is a natural antibiotic belonging to the polyene group. The drug has been a mainstay of antifungal therapy for treating life-threatening fungal infections since the 1960s. The drug disrupts biological membranes by binding non-specifically to ergosterol in fungal cells and cholesterol in mammalian cells (1-2). Despite the development of new antifungal drugs such as azoles and echinocandins, AmB is still considered a treatment option in select cases of severe or life-threatening invasive fungal systemic fungal diseases caused by selected fungal pathogens (2-3). Moreover, the increased numbers of immunocompromised patients with AIDS, transplant patients as well as patients with cancer undergoing intensive chemotherapy with a risk of serious fungal infections accentuate the significance of the drug in clinical practice (3-4). In its pure form, AmB has little solubility in aqueous solutions at physiological pH, thus requiring complexing with another agent serving as a detergent. The first such agent used to increase the solubility of AmB in the water was sodium deoxycholate (DOC) (2,16). To
improve tolerability profile and enhance efficacy, several new preparations containing AmB
complexed with other lipids such as cholesterol or phospholipids have been introduced into
therapy. Nevertheless, for various reasons, the convention formulation of AmB with DOC
also remains in general use (3, 6, 7). Specially, the higher effectiveness and/or economic
advantage of AmB/DOC in comparison with the more recently introduced lipid preparations
have been found in some patient groups (8-9).

One of the limiting factors regarding the clinical use of AmB is nephrotoxicity, the
potential for which is highest in conventional AmB/DOC preparations. Although the new
lipid formulations of AmB have a lower frequency of nephrotoxicity in comparison with the
AmB/DOC, they all exhibit this adverse effect in a considerable number of patients (10-12).
AmB most likely produces renal injury by a variety of mechanisms, however the exact
nephrotoxic mechanism remains an open question (2, 13). Since concentration of AmB in
particular segments of the kidney may be an important factor in its nephrotoxic action, the
processes potentially determining distribution of the drug within the kidney should be studied
thoroughly.

Disposition of AmB within the kidney may be significantly determined by the
transporters mediating transmembrane transport of drugs into renal cells. However, no
detailed information exists on the renal excretion mechanism/s of AmB and its interactions
with the renal membrane transporters potentially involved in AmB transmembrane transport.
Since AmB contains in its molecule both acidic and basic structural components, the drug
could have a potential to interact with transporters for organic anions (OATs) and/or organic
cations (OCTs). In addition, DOC, a component of conventional amphotericin preparation,
also has a potential for such interactions. Among the OATs and OCTs found in the human
kidney the most abundant renal expression has been proven for OAT1 and OCT2,
respectively (14-15). Both transporters are localized in the kidney in the basolateral
membrane of the proximal tubular cells (14, 16), and they mediate the uptake of their substrates into the tubular cells (16-17). These renal influx transporters may be responsible not only for the renal toxicity of the drugs but also for potential drug-drug interactions, which may result in changes of renal excretion parameters and the modification of pharmacokinetics (18-19).

This investigation was aimed at interactions of AmB with selected membrane transporters potentially responsible for transmembrane transport of the drug in the kidney. The study evaluated in vitro interactions of two main human renal influx transporters for organic acids and bases, hOAT1 and hOCT2, with the most nephrotoxic form of AmB, AmB complexed by DOC, and with both components of the preparation, i.e. free AmB or DOC. The study also included an evaluation of the involvement of energy-dependent processes (active transport) in the renal cellular uptake of AmB. To confirm the findings determined by the use of cellular models and to reveal the renal excretion mechanism of AmB, we evaluated the renal elimination parameters of the drug as well as the contribution of OATs and OCTs to renal elimination; for this perfused rat kidney was used. At the relevant transporters, we also tried to demonstrate in vitro possible drug-drug interactions of AmB with selected potentially concomitantly administered antivirals.

MATERIAL AND METHODS

Chemicals. [3H]-Amphotericin B ([3H]AmB; specific radioactivity 10 Ci/mmol), and [3H]-methyl-4-phenylpyridinium acetate ([3H]MPP⁺; specific radioactivity 80 Ci/mmol) were obtained from American Radiolabeled Chemicals (St. Louis, USA). [3H]-p-aminohippuric acid ([3H]PAH; specific radioactivity 4.56 Ci/mmol) was purchased from Perkin Elmer (Waltham, USA). Adenine-2,8-[3H]-adefovir, ([3H]adefovir, specific radioactivity 12.1
Ci/mmol), [adenine-2,8-^3H]-tenofovir ([^3H]tenofovir, specific radioactivity 7.8 Ci/mmol) and [5^-^3H]-cidofovir ([^3H]cidofovir, specific radioactivity 24 Ci/mmol), were obtained from Moravek Biochemicals (Brea, USA). Nonlabeled amphotericin B, AmB/Doc, tetrathylammonium (TEA), cidofovir hydrate and probenecid (p-[dipropylsulfamoyl] benzoic acid; PB) were obtained from Sigma Aldrich (St. Louis, USA). Adefovir was a generous gift from Dr. Janeba from the Institute of Organic Chemistry and Biochemistry ASCR, Czech Republic. Tenofovir was obtained from Santa Cruz Biotechnology (California, USA). Human OAT1 (SLC22A6) expression plasmid, transcript variant 2, catalog number: RC207579; human OCT2 (SLC22A2) expression plasmid, catalog number: RC207921 and pCMV6-Entry vector (empty vector), catalog number: PS100001 were obtained from OriGene Technologies (Rockville, USA).

**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 12 - 20) were routinely cultured in 75 cm^2_2 cell culture flasks in a humidified atmosphere containing 5% CO_2_ at 37°C in 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.

**MDCK II cell culture.** 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 6 - 13) were routinely cultured in 75 cm^2_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.

**HK-2 cell culture.** Human kidney cell line (HK-2) was obtained from American Type Culture Collection (ATCC, Manassas, USA). The HK-2 cells (passage 11) were routinely
cultured in 75 cm² 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% CO₂ at 37°C. Confluent cells were split every 4 days using 0.25% trypsin/EDTA solution.

**Transfection.** According to preliminary function tests, HeLa cells were selected as the optimal model for transient transfection with hOAT1. Similarly, MDCKII cells were identified as the most useful cell line for transfection with hOCT2. The HeLa cells were seeded at a density of 7×10⁴ cells per well in 24-well plates. The MDCK II cells were seeded at a density of 2×10⁵ cells per well in 24-well plates. The following day the cells were transiently transfected with appropriate plasmid coding for the studied transporter or empty vector pCMV6-Entry (pCMV6, empty vector) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) and Opti-MEM (Invitrogen, Carlsbad, USA) according to the manufacturer’s protocol. The cells transiently transfected with the appropriate empty vector served as a control (mock cells). The overexpression of the studied transporters and the proper function of the cell model was confirmed as described previously (20).

**Inhibitor and accumulation studies in the transfected cells.** Transport experiments were carried out as described previously (20). HeLa cells transiently transfected with hOAT1 or MDCK II cells transiently transfected with hOCT2 were used for the experiments. The transport solution contained 130 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂·6H₂O, 5 mM glucose and 10 mM HEPES, pH 7.4. The cells were washed with transport solution and preincubated for 10 min at 37°C. The tested radioactive substances were added to the cell monolayers and incubated 2 min. The effectiveness of the transfection was proven using specific substrate for hOATs, [³H]PAH (1 µM) or for hOCT2, [³H]MPP⁺ (1 µM). The rate of inhibition of the intracellular accumulation of the specific substrates induced by gradually increasing concentrations of AmB/DOC (dissolved in distilled water), AmB (dissolved in
dimethyl sulfoxide) or DOC (dissolved in distilled water) was used as a measure of affinity to
the transporter. $[^3H]$Adefovir (0.1 µM), $[^3H]$tenofovir (0.5 µM) or $[^3H]$cidofovir (70 µM) in
combination with an increasing concentration of AmB/DOC were used in the interaction
experiments in hOAT1 transfected cells. Accumulation of radiolabeled substrate into the
transfected cells was measured in comparison with the control (mock cells) treated with the
same amount of the solvent. The incubation was stopped by washing the cells with an ice-cold
solution containing 137 mM NaCl and 10 mM HEPES, pH 7.4. The cells were disintegrated
with 0.1 mL TritonX 0.5 % in 100 mM NaOH for 60 min. Finally, the radioactivity of the
samples in a scintillation solution (Sigma-Aldrich, St. Louis, USA) was measured on the beta-
spectrometer Tri-Carb 2900TR (Perkin Elmer, Shelton, USA). All the values were
standardized against cell protein content. The affinity results expressed as inhibitory
concentration IC$_{50}$ necessary to decrease the accumulation of radiolabeled substrate to 50%
were calculated using the nonlinear regression analysis software GraphPad Prism (version 6).

**Temperature-dependent accumulation study.** The HK-2 cells were seeded at a density
of 1×10$^4$ cells per well in 24-well plates. The confluent cells were washed and preincubated in
a transport solution at 2°C or 37°C as described above. $[^3H]$AmB (1 µM) was incubated with
the cells at 4°C or 37°C for 0, 30 and 60 min. The incubation was stopped by washing the
cells with an ice-cold solution. Samples in which the added substance was immediately
washed out (0 min) were used as a control of non-specific binding to the membrane, and
served as a background of radioactivity. The radioactivity of the disintegrated cells was
measured on a beta-spectrometer. All the values were standardized against cell protein
content.

**Perfused rat kidney.** Male Wistar rats (Konarovice, Czech Republic) weighing 300-390
g were used for the perfusion experiments. The rats were housed under standard conditions
(tap water and standard diet, light cycle 12/12 h). The perfusion was performed according to
the procedure described previously (21): in brief, the rat kidney was perfused with 100 ml of Krebs-Henseleit buffer 7.4 containing glucose (5.6 mmol/L), bovine serum albumin, fraction V (6%), washed rat erythrocytes (5-6%) and a mixture of amino acids and metabolic substrates. The kidney was perfused via the renal artery under a recirculation regimen at 37°C and a constant pressure of 14.5 kPa (110 mmHg). Following an equilibration period of 30 min, [3H]AmB was added in a mixture with nonlabeled AmB into the perfusion circuit as a bolus dose (total starting concentration in the perfusate 0.1 µM) and the kidney was perfused for an interval of 70 min. Urine samples were collected every 10 min and midpoint samples of perfusate were obtained. Polyfructosan (Laevosan, Linz, Austria) was used as a marker of glomerular filtration rate (GFR) at a starting concentration of 4 mg/ml. The concentrations of polyfructosan in the perfusate and urine were determined colorimetrically (22).

To evaluate the participation of OATs in the renal excretion of AmB at the organ level, an experiment with an addition of the OATs inhibitor probenecid was performed under the same conditions. The inhibitor was added to the perfusate as a bolus at a starting concentration of 500 µM. Similarly, to reveal the potential contribution of renal OCTs to the renal excretion of AmB, experiments using TEA, an inhibitor of OCTs, were carried out (starting concentration in the perfusate of 500 µM). The used inhibitors of transporters were introduced into the perfusion system 10 min before adding the AmB.

Excretion of the drug in the perfused rat kidney was characterized by total renal clearance (\( CL_{TR} = \) urinary radioactivity x volume of urine / radioactivity in the perfusate), by the filtered amount/filtration clearance of the compounds (\( CL_{GF} = \) GFR x free fraction of the drug in the perfusate), and by net tubular secretion/secretion clearance (\( CL_{S} = CL_{TR} - CL_{GF} \)).

The binding of AmB to the proteins in the perfusion medium was determined by ultrafiltration across a semipermeable membrane (Priesvit, Chemosvit, Slovak Republic) for 30 min at 1600g at 37 °C.
All the experiments with animals were carried out in compliance with the respective Czech laws concerning animal protection. The experimental project was approved by the Ethical Committee of the Pharmaceutical Faculty, Charles University in Prague, as well as by the Ministry of Education, Youth and Sport, Czech Republic (decision number 5407/2010-30).

**Statistical analysis.** The experimental data found in the individual experimental groups were compared using a two way ANOVA test using GraphPad Prism software (version 6). Differences between groups were considered statistically significant at the p-value <0.05. The number of repetitions for the performed experiments is presented in the legend of each figure and table.

**RESULTS**

**Temperature-dependent accumulation study.** To test whether active processes contribute to the cellular transport of AmB, human kidney cells (HK-2) were incubated in the presence of radiolabeled AmB in three increasing incubation time intervals (0, 30, 60 min) using two different temperatures (4°C and 37°C). The accumulation of AmB in the cells incubated at 4°C was significantly lower than in the cells incubated at 37°C. The accumulation of AmB at 4°C was decreased by approximately 60-70%, (Fig. 1).

**Interaction with OAT1 and OCT2.** To investigate the affinity of AmB and AmB/DOC to the tested SLC transporters, the cells transiently transfected with hOAT1 or hOCT2 were incubated with their respective substrates in combination with unlabeled tested substances. In the study, AmB revealed no interaction with hOAT1, but AmB/DOC strongly inhibited the hOAT1 mediated transport of [³H]PAH (Fig. 2a, 2c). The DOC alone displayed also a significant potency to interact with hOAT1 (Fig. 2e). Since DOC alone was proven to be a
relatively strong inhibitor of hOAT1 and AmB had no significant effect on this transporter the inhibitory effect of AmB/DOC on hOAT1 was caused almost exclusively by the latter component. Even the inhibitory potency of AmB/DOC and DOC was very similar the effect is not equivalent since the found IC50 of AmB/DOC for hOAT1 was not highly but significantly lower (p = 0.029) than that of DOC alone. Interestingly, strong interactions of both AmB and AmB/DOC were observed under the used conditions in hOCT2 transfected cells (Fig. 2b, 2d).

In contrast with this finding, the inhibitory effect of DOC on the uptake of MPP+ was very low (Fig. 2f). A series of accumulation studies aimed at the substrate specificity of AmB showed no significant differences in the accumulation of AmB in hOCT2 or hOAT1 transfected cells compared with the mock cells (Fig. 3). Therefore, AmB seems to not be a substrate for hOCT2 or hOAT1, but is a strong inhibitor of hOCT2. In summary, AmB/DOC showed in vitro a strong inhibitory effect on both hOAT1 and hOCT2 in transiently transfected cells (Fig. 2c, 2d) which is caused by AmB in hOCT2 and by DOC in hOAT1.

To evaluate the possible interactions of drug formulation AmB/DOC with antivirals that are known substrates of hOAT1, various concentrations AmB/DOC were co-incubated with [3H]-labeled adefovir, tenofovir and cidofovir in HeLa cells transfected with hOAT1. The results demonstrated considerable interactions of AmB/DOC with all tested antiviral agents in vitro (Fig. 4).

Handling in the perfused rat kidney. The [3H]Amphotericin renal excretion parameters found using the perfused rat kidney are presented in Table 1. The clearance values are related to the renal clearance of polyfructosan as a measure of glomerular filtration rate. The free fraction of amphotericin B in the perfusion medium is also included in Table 1. No significant differences were found between amphotericin B renal clearance values in the perfused rat kidneys with and without the presence of the OATs inhibitor probenecid in the perfusion medium (Table 1). The difference between amphotericin B renal clearance with and without
the addition of TEA into the perfusate was also not statistically different, but a significantly
different AmB free fraction in the perfusate was found (Table 1). The value of filtration
clearance of AmB was lower than the total clearance in all groups. The values of AmB
secretion clearance in all three experimental groups showed a similar contribution of renal
secretion to the renal excretion of the drug in the perfused rat kidney (Table 1).

**DISCUSSION**

Organic anion transporters are responsible for the membrane transport of anionic
compounds at the renal basolateral membranes. The complex AmB/DOC significantly
interacted with hOAT1. Whereas the pure AmB did not interact with this transporter, sodium
deoxycholate (DOC) exerted a significant interaction with hOAT1. According to the
literature, PAH uptake by rat OAT1 (rOAT1) was significantly inhibited by deoxycholic acid
in HEK293 cells transiently expressing rOAT1 (23). Thus, the deoxycholate part of the
complex AmB/DOC is most likely responsible for this interaction. The accumulation study
confirmed that AmB is not a substrate of hOAT1 and hOCT2; the large molecular weight of
AmB (924.08) probably prevents penetration via SLC transporters. These findings seem to be
in accordance with a rule suggested by some authors that ideal candidates for transport by
OATs should have a molecular size of up to 500 daltons (24-25).

A relatively strong interaction of both AmB and AmB/DOC was proven with hOCT2
in transfected MDCK II cells at 37°C and pH 7.4. At physiological pH levels, both charges
(positive and negative) carried by amino and carboxyl groups can be found in the AmB
molecule according to specialized software (Advanced Chemistry Development, Inc.,
Toronto, Canada). It remains unclear why AmB does not interact with hOAT1, while the
hOCT2 was affected very strongly. An explanation may be the spatial arrangement of atoms
in the molecule of AmB. A positive charged segment of AmB may help preferably direct the
molecule toward the hOCT2.

The accumulation of AmB in human kidney cells was inhibited by a low incubation temperature, a finding which demonstrates the contribution of active transport processes to the cellular uptake of AmB. Similarly, it has been documented that the incubation of AmB at 5°C resulted in a drastic decrease in the uptake of AmB in Chinese hamster ovary cells (CHO) compared to the uptake at 37°C (26). These authors also found that the internalization of AmB in CHO cells was mediated by endocytosis. Since our cellular experiments show that neither OAT1 nor OCT2 are able to transport AmB into cells, active endocytosis may be considered at least one of the uptake mechanisms involved in AmB transport into cells.

Neither probenecid, an inhibitor of OATs, nor TEA, an inhibitor of OCTs, changed the renal clearance of AmB in the perfused rat kidney significantly in comparison to the perfusion with AmB alone. Such a finding is in good agreement with the performed cellular experiments using transfected models, in which no significant influx of AmB by hOAT1 and hOCT2 was demonstrated. The used concentrations of the inhibitors have been found to inhibit transport via the studied transporters efficiently both in cell and organ experimental models (27-30). Based on these facts, the finding of no distinct effect of the inhibitors at effective inhibitory concentrations on the renal excretion parameters of AmB showed the insignificance of OATs and OCTs regarding AmB renal excretion in the perfused rat kidney.

The effect of the inhibitors of OATs and OCTs in the perfused kidney may reveal the possible partition of the particular transporters in a less specific way than the cellular experiments in the transfected cell lines. Since highly specific inhibitors for the individual types of OATs or OCTs are not available, the possible inhibition by the used inhibitors showed in the perfused kidney only an interaction with OATs or OCTs as a group. In addition, the used inhibitors may inhibit other groups of renal transporters which could potentially transport organic anions or cations. An undetectable inhibition of AmB excretion...
in the perfusion experiments may mean that the other SLC transporters expressed in the kidney and inhibited by the studied inhibitors such as OAT3 or OCTN2 (16) also do not contribute substantially to the handling of AmB in the kidney.

Although the renal clearance values for AmB were slightly lower than the values of glomerular filtration rate as indicated by polyfructosan clearance, after correction for binding to proteins in the perfusion medium, a considerable contribution of the renal secretion of AmB was revealed. The performed analysis of the excretion parameters of AmB in the perfused rat kidney showed that the amount of AmB excreted in the urine was higher than that filtered in the kidney. These data suggest that active transport in the renal tubules may contribute to AmB renal excretion. Therefore, it could be valuable to consider the potential involvement of efflux drug transporters localized in the kidney (16) such as MDR1/P-glycoprotein, BCRP or MRPs in AmB excretion. Unfortunately, relevant experimental data on AmB interaction with efflux transporters are very scarce. One study documented the drug-drug interactions of AmB on P-glycoprotein in the GIT. The authors demonstrated that AmB treatment significantly increased the level of P-glycoprotein, which resulted in a decrease of the oral bioavailability of cyclosporine A in rats (31). Additional information on AmB interactions with efflux transporters, however, is not available, therefore, further studies should be performed.

AmB binding to bovine albumin in the used perfusion medium was about 50%. No other data on binding of AmB to bovine plasma proteins are available in the published scientific literature. In comparison, the bound fraction of AmB in human and rat plasma is higher than 90% (32). Nevertheless, AmB is in in vivo conditions bound not only to albumin but also in a substantial amount to plasma lipoproteins (33-34). This fact may explain the observed lower binding in the perfusate, since no plasma lipoproteins were added into the perfusion system. AmB binding to proteins in the kidneys perfused with the OCT inhibitor...
TEA was somewhat lower in comparison to the other experimental groups. An explanation for this may be a competitive interaction of AmB with TEA, since the used concentration of TEA (500 µM) was substantially higher than that of AmB (0.1 µM).

According to in vitro studies, AmB may dissociate in the water from every AmB/lipid preparation regardless of the lipid used to complex AmB (5, 35). Such a process may also be seen in body fluids. The free (noncomplexed) AmB is then distributed within body tissues and organs, including the kidney. Since we have found that interactions with the studied transporters are dependent on the form of AmB, the ratio of free and complexed AmB in the blood or urine could be an important factor in determining interactions with the transporters in vivo. However, no information on this ratio based on a direct measurement in vivo is available. Nevertheless, mainly the found inhibitory effect of AmB on hOCT2 should be considered since the IC50 of free AmB is the lowest among the studied drug-transporter interactions (Fig. 2). Moreover, free AmB is probably present in the organism following AmB/DOC administration in all situations. However, no clinically relevant drug-drug interaction of AmB caused by an effect on membrane transport mechanisms has been described in the existing literature (1, 36). The clinical drug-drug interactions involving AmB found in clinical conditions so far have been suggested to be based only on the toxicological effects of the drug which are additive to those of other drugs (e.g. cyclosporine, tacrolimus, digoxin, aminoglycosides), or which reduce elimination of concomitantly administered agents (5-flucytosine) (1, 36). If we consider that the total concentrations of AmB in plasma following regular therapeutic doses have been declared to be at maximum between 1-5 µM (5, 37), the found effective inhibitory concentrations starting at about 1-2 µM (Fig. 2) may suggest the potential of AmB to affect interactions of other drugs with OCT2 in the organism. However, the above mentioned high plasma binding in vivo (32-34) is likely to result in much lower effective concentrations of free AmB in real conditions. This could explain the
lack of clinical observations of pharmacokinetic drug-drug interactions of AmB based on hOCT2 under regular conditions. Probably, the clinical significance of the drug-drug interactions at hOCT2 can be expected only in case of extremely high plasma concentrations of AmB/DOC caused by overdosing or intoxication. On the other hand, animal and human biodistribution studies have found AmB concentrations in the organs such as the kidney or liver to be much higher than in the blood (5, 37). It is necessary to determine in further in vivo experiments whether this tissue accumulation of AmB may result in a significant effect on transport of other drugs via hOCT2. In addition, AmB may also interact in the organism with other OCT isoforms such as OCT1 since the substrate specificity of OCTs may usually overlap considerably (38, 39). Because OCT1 is an important hepatic carrier mediating transmembrane transport of many drugs (38, 39) further studies may be also aimed at an analysis of AmB transport interactions in the liver.

Based on our finding that AmB/DOC acts as an inhibitor of hOAT1, we focused on the drug-drug interaction studies of AmB/DOC with three known substrates of OATs, adefovir, tenofovir and cidofovir (40-41) eliminated by the kidney. The used incubation concentrations of adefovir, tenofovir and cidofovir corresponded to therapeutic plasma levels achieved in humans (42-44). A considerable in vitro interaction with hOAT1 between AmB/DOC and all three antiviral agents was demonstrated (Fig. 4). Because AmB/DOC inhibitory concentrations were higher by several orders of magnitude than the therapeutic ones, the probability of this drug-drug interactions at hOAT1 in vivo could be evaluated as minimal. This finding is in accordance with the absence of any pharmacokinetic interactions between AmB and the studied antivirals in the available clinical literature (1, 36). The obtained in vitro results regarding hOAT1 led us to discontinue further experiments on the contribution of the individual components of AmB/DOC to the observed drug-drug interaction at hOAT1, as such studies could be expected to be clinically irrelevant.
The main renal OATs and OCTs are similarly expressed in human and rat kidney tissue; OCT2 is highly expressed in both (28, 45). Similarly, OAT1 ranks among the main OATs found both in human and rat kidneys (46). To describe AmB renal handling in a more complex way it is therefore essential to combine experimental data obtained both in cells expressing human transporters and perfused rat kidney. The concordance in the results found in the transfected cells and perfused kidney show that there is most likely no significant interspecies difference in the renal handling of AmB by the tested transporters in these two species.

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REFERENCES


**Figure caption:**

**Fig. 1** Temperature-dependent accumulation of [3H]AmB in HK-2 cells. [3H]AmB (1 μM) was incubated at 37°C and 4°C in two different increasing time intervals in triplicates. The value of [3H]AmB accumulation at the 0 min time interval was subtracted. Each column represents the mean ± SD of the part of accumulated dose of [3H]AmB.

**Fig. 2** Effect of AmB and AmB/DOC on the accumulation of typical substrates of the studied transporters. The HeLa cells transiently transfected with the expressing vector for hOAT1 and MDCK II cells transiently transfected with hOCT2 were incubated with 1 μM [3H]PAH (2a, 2c, 2e) and 1 μM [3H]MPP+ (2b, 2d, 2f) in the presence of gradually increasing concentrations of AmB, AmB/DOC or DOC (0 – 500 μM) for 2 min at 37°C in triplicates. Each point represents the mean ± SD of the part of accumulated dose of radiotracer. The IC50 value represents the inhibitory concentration of the tested compound calculated using nonlinear regression analysis. The value of radiotracer accumulation in the mock cells was subtracted.

**Fig. 3** Uptake of [3H]AmB in hOCT2 and hOAT1 cells. 1 μM [3H]AmB was incubated in hOCT2 (3a) and hOAT1 (3b) transfected cells or mock cells at 37°C in triplicates. 1 μM

Fig. 4 Effect of AmB/DOC on adefovir, tenofovir and cidofovir accumulation mediated by hOAT1 in HeLa cells. The accumulation of[^3]H]adefovir (0.1 μM) (4a),[^3]H]tenofovir (0.5 μM) (4b) and[^3]H]cidofovir (70 μM) (4c) was inhibited by AmB (0 - 345 μM) for a 2 min period in HeLa cells transiently transfected with hOAT1 in triplicates. Each point represents the mean ± SD of the part of accumulated dose of radiotracer. The IC50 value represents the inhibitory concentration of AmB/DOC calculated using nonlinear regression analysis. The value of radiotracer accumulation in mock cells was subtracted.
Table 1. Comparison of [3H]AmB excretion parameters in the perfused rat kidney without and with addition of probenecid (500 µM) or TEA (500 µM) into the perfusion circuit (mean ± SD)

<table>
<thead>
<tr>
<th>Excretion parameter</th>
<th>AmB (n=5)</th>
<th>AmB + probenecid (n=5)</th>
<th>AmB + TEA (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total renal clearance (ml/min/g)</td>
<td>0.316 ± 0.036</td>
<td>0.341 ± 0.078</td>
<td>0.362 ± 0.068</td>
</tr>
<tr>
<td>Glomerular filtration rate (ml/min/g)</td>
<td>0.401 ± 0.075</td>
<td>0.387 ± 0.058</td>
<td>0.423 ± 0.095</td>
</tr>
<tr>
<td>Free fraction in perfusate</td>
<td>0.488 ± 0.024</td>
<td>0.532 ± 0.077</td>
<td>0.667 ± 0.117*</td>
</tr>
<tr>
<td>Filtration clearance (ml/min/g)</td>
<td>0.195 ± 0.033</td>
<td>0.205 ± 0.038</td>
<td>0.282 ± 0.096</td>
</tr>
<tr>
<td>Secretion clearance (ml/min/g)</td>
<td>0.121 ± 0.035</td>
<td>0.136 ± 0.049</td>
<td>0.077 ± 0.046</td>
</tr>
</tbody>
</table>

* Statistically significant difference in comparison with the AmB group at the p-value <0.05
AmB uptake (%D/mg protein)

Time (min)

30

60

AmB uptake (%D/mg protein)

37°C

4°C
Table 1. Comparison of [³H]AmB excretion parameters in the perfused rat kidney without and with addition of probenecid (500 µM) or TEA (500 µM) into the perfusion circuit (mean ± SD)

<table>
<thead>
<tr>
<th>Excretion parameter</th>
<th>AmB (n=5)</th>
<th>AmB + probenecid (n=5)</th>
<th>AmB + TEA (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total renal clearance (ml/min/g)</td>
<td>0.316 ± 0.036</td>
<td>0.341 ± 0.078</td>
<td>0.362 ± 0.068</td>
</tr>
<tr>
<td>Glomerular filtration rate (ml/min/g)</td>
<td>0.401 ± 0.075</td>
<td>0.387 ± 0.058</td>
<td>0.423 ± 0.095</td>
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