Renal Handling of Amphotericin B and Amphotericin B-Deoxycholate and Potential Renal Drug-Drug Interactions with Selected Antivirals

František Trejtnar, Jana Mandíková, Jana Kočíncová, Marie Volková

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

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 the possible interaction of AmB or its prodrug AmB deoxycholate (AmB-DOC) with the typical renal organic anion transporters (OATs) and organic cation transporters (OCTs), using cellular and organ models. The relevant transport systems were then investigated in terms of the drug–drug interactions of AmB-DOC with antivirals that might potentially be used concomitantly. To analyze the renal excretion mechanisms of [3H]AmB, perfused rat kidney was employed. HeLa and MDCK II cells transiently transfected with human OAT1 (hOAT1) or hOCT2 were used as the cellular models. A significant tubular secretion of AmB was demonstrated in the perfused rat kidney. The cellular studies performed 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. However, [3H]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, the significant potency of AmB and AmB-DOC for inhibiting the transporters was demonstrated in this study. The secretion of AmB in the renal tubules is likely not related to the transporters here, since the drug was not proven to be a substrate for them. Drug–drug interactions of AmB and the antivirals used in this study on the investigated transporters are not probable.
of AmB with selected membrane transporters potentially responsible for transmembrane transport of the drug in the kidney. The study evaluated the in vitro interactions of two main human renal influx transporters for organic acids and bases, human OAT1 (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 and the contributions of OATs and OCTs to renal elimination; for these, perfused rat kidney was used. At the relevant transporters, we also tried to demonstrate in vitro possible drug-drug interactions of AmB with selected antivirals that might potentially be used concomitantly.

MATERIALS 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, MO, USA). [3H]Aminopterin (specific radioactivity, 4.56 Ci/mmol) was purchased from PerkinElmer (Waltham, MA, USA). [2,8-3H]Adenine ([3H]Ado) (specific radioactivity, 12.1 Ci/mmol), [adenosine-2,8-3H]adenosine ([3H]ADO) (specific radioactivity, 12.1 Ci/mmol), [adenine-2,8-3H]tenofovir ([3H]Teno) (specific radioactivity, 7.8 Ci/mmol), and [5-3H]cidofovir ([3H]Cido) (specific radioactivity, 24 Ci/mmol), were obtained from Moravek Biochemicals (Brea, CA, USA). Adefovir was a generous gift from Z. Benecid (p-aminobenzene-sulfamoyl)benzoic acid) (PB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Tenofovir was obtained from Moravek Biochemicals (Brea, CA, 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, MD, USA).

HeLa cell culture. The human cervical epithelioid carcinoma cell line (HeLa) was purchased from the European Collection of Cell Culture (Salisbury, United Kingdom). The cells (passages 12 to 20) were routinely cultured in 75-cm² culture flasks in a humidified atmosphere containing 5% CO₂ at 37°C. The cells were dissociated with trypsin–EDTA 0.25%. HeLa cells were seeded at a density of 7 × 10⁴ cells per well in 24-well plates. The MDCK I cells were seeded at a density of 2 × 10⁵ cells per well in 24-well plates. On the following day, the cells were transfected with the appropriate plasmid coding for the transporter or empty vector and incubated with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Invitrogen) according to the manufacturer’s protocol. The cells transiently transfected with the appropriate empty vector served as controls (mock cells). The overexpression of the transporters studied and the proper function of the cell model were confirmed as described previously (19).

Inhibitory and accumulation studies in the transfected cells. Transport experiments were carried out as described previously (19). 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, 5.6 mM HEPES, 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 radioactive substances to be tested were added to the cell monolayers and incubated for 2 min. The effectiveness of the transfection was proven using a standard substrate for hOATs (1 μM [3H]PAH) or hOCT2 (1 μM [3H]MPP+). The rate of inhibition of the intracellular accumulation of the typical 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]Adeovir (0.1 μM), [3H]Tenofovir (0.5 μM), or [3H]Cidofovir (70 μM) in combination with an increasing concentration of AmB-DOC was used in the interaction experiments in hOAT1-transfected cells. Accumulation of radiolabeled substrate in the transfected cells was measured in comparison with that in 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 of 0.5% Triton X-100 in 100 mM NaOH for 60 min. Finally, the radioactivity of the samples in a scintillation solution (Sima-Aldrich) was measured on a Tri-Carb 2900TR beta spectrometer (PerkinElmer, Shelton, CT, USA). All of the values were standardized against the cell protein content. The affinity results, expressed as the inhibitory concentration necessary to decrease the accumulation of radiolabeled substrate to 50% (IC₅₀), 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 × 10⁵ 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 for nonspecific binding to the membrane and served as a background for radioactivity. The radioactivity of the disintegrated cells was measured on a beta spectrometer. All of the values were standardized against the cell protein content.

Perfused rat kidney. Male Wistar rats (Konarovicv, Czech Republic) weighing 300 to 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 (20). In brief, the rat kidney was perfused with 100 ml of Krebs-Henseleit buffer (pH 7.4) containing glucose (5.6 mmol/liter), bovine serum albumin, fraction V (6%), washed rat erythrocytes (5 to 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 mm Hg). 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 of 0.1 μM), and the kidney was perfused for an interval of 70 min. Urine samples were collected every 10 min, and mid-
point samples of perfusate were obtained. Polyfructosan (Laevovan, 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 perfuse and urine were determined colorimetrically (21).

To evaluate the participation of OATs in the renal excretion of AmB at the organ level, an experiment with addition of the OAT inhibitor probenecid was performed under the same conditions. The inhibitor was added to the perfuse 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 perfuse of 500 μM). The inhibitors of transporters were introduced into the perfusion system 10 min before addition of the AmB.

Excretion of the drug in the perfused rat kidney was characterized by total renal clearance (CL_{TR} = urinary radioactivity × volume of urine/radioactivity in the perfusate), by the filtered amount/filtration clearance of the compounds (CL_{CF} = GFR × free fraction of the drug in the perfuse), and by net tubular secretion/secretion clearance (CL_{S} = CL_{TR} – CL_{CF}). 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 1,600 × g 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 from the individual experimental groups were compared by a two-way analysis of variance (ANOVA) test using GraphPad Prism software (version 6). Differences between groups were considered statistically significant at a \( P \) value of <0.05. The numbers of repetitions performed for the experiments are 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 radio-labeled AmB for three increasing incubation time intervals (0, 30, and 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 that in the cells incubated at 37°C. The accumulation of AmB at 4°C was decreased by approximately 60 to 70% (Fig. 1).

Interaction with OAT1 and OCT2. To investigate the affinity of AmB and AmB-DOC to the tested solute carrier (SLC) transporters, the cells transiently transfected with hOAT1 or hOCT2 were incubated with their respective substrates in combination with unlabeled test substances. In this study, AmB revealed no interaction with hOAT1, but AmB-DOC strongly inhibited the hOAT1-mediated transport of [3H]PAH (Fig. 2a and c). DOC alone also displayed significant potency for interaction 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 though the inhibitory potencies of AmB-DOC and DOC were very similar, the effects are not equivalent since the IC_{50} for AmB-DOC for hOAT1 was significantly, although not greatly, lower (\( P = 0.029 \)) than that for DOC alone. Interestingly, strong interactions of both AmB and AmB-DOC in hOCT2-transfected cells were observed under the conditions used (Fig. 2b and d). 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 to determine the substrate specificity of AmB showed no significant differences in the accumulation of AmB in hOCT2- or hOAT1-transfected cells compared with that in 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 a strong inhibitory effect in vitro on both hOAT1 and hOCT2 in transiently transfected cells (Fig. 2c and d), which is caused by AmB for hOCT2 and by DOC for hOAT1.

To evaluate the possible interactions of the drug formulation AmB-DOC with antivirals that are known substrates of hOAT1, various concentrations of AmB-DOC were coincubated 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 or without the presence of the OAT inhibitor probenecid in the perfusion medium (Table 1). The difference between amphotericin B renal clearance with and without the addition of TEA in the perfusate was also not statistically different, but a significantly different AmB free fraction in the perfusate was found (Table 1). The filtration clearance value for AmB was lower than the total clearance in all groups. The AmB secretion clearance values showed a contribution of tubular secretion to the total renal excretion of the drug in the perfused rat kidney in all three experimental groups (Table 1).

DISCUSSION
Organic anion transporters are responsible for the membrane transport of anionic compounds at the renal basolateral membranes. The AmB-DOC combination significantly interacted
with hOAT1. Whereas pure AmB did not interact with this transporter, sodium deoxycholate (DOC) interacted significantly with hOAT1. According to the literature, PAH uptake by rat OAT1 (rOAT1) was significantly inhibited by deoxycholic acid in HEK293 cells transiently expressing rOAT1 (22). 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 or 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 Da (23,24).

A relatively strong interaction of both AmB and AmB-DOC with hOCT2 in transfected MDCK II cells at 37°C and pH 7.4 was proven. 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 hOCT2 was affected very strongly. An explanation may be the spatial arrangement of atoms in the molecule of AmB. A positively 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 up-

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**FIG 2** Effect of AmB and AmB-DOC on the accumulation of typical substrates of the transporters in this study. The HeLa cells transiently transfected with the expressing vector for hOAT1 and the MDCK II cells transiently transfected with hOCT2 were incubated with 1 μM [3H]PAH (a, c, and e) or 1 μM [3H]MPP⁺ (b, d, and f) in the presence of gradually increasing concentrations of AmB, AmB-DOC, or DOC (0 to 500 μM) for 2 min at 37°C in triplicate experiments. Each point represents the mean ± SD of the part of the accumulated dose of the radiotracer. The IC₅₀ value represents the inhibitory concentration of the tested compound calculated using nonlinear regression analysis. The value for radiotracer accumulation in the mock cells was subtracted.
take 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 (CHO) cells compared to the uptake at 37°C (25). 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 is 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 cellular experiments performed using transfected models, in which no significant influx of AmB by hOAT1 and hOCT2 was demonstrated. The concentrations of the inhibitors used here have been found to inhibit transport via the transporters efficiently in both cell and organ experimental models (26–29). 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 rat kidney...
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 in the perfused kidney by the inhibitors used here showed only an interaction with OATs or OCTs as a group. In addition, the inhibitors may inhibit other groups of potential renal transporters of 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, such as OAT3 or OCTN2 (5), which are concomitantly inhibited by the used transport inhibitors, 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 glomerular filtration rates 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 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 by the kidney. These data suggest that active transport in the renal tubules may contribute to AmB renal excretion. Therefore, it might be valuable to consider the potential involvement of efflux drug transporters localized in the kidney (5), such as multidrug resistance protein 1 (MDR1)/P-glycoprotein, breast cancer resistance protein (BCRP), or multidrug resistance-associated proteins (MRPs) in AmB excretion. Unfortunately, relevant experimental data on AmB interactions with efflux transporters are very scarce. One study documented the drug-drug interactions of AmB and P-glycoprotein in the gastrointestinal tract (GIT). The authors demonstrated that AmB treatment significantly increased the level of P-glycoprotein, which resulted in a decrease in the oral bioavailability of cyclosporine in rats (30). 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 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 fractions of AmB in human and rat plasma are higher than 90% (31). Nevertheless, AmB under in vivo conditions bound not only to albumin but also in a substantial amount to plasma lipoproteins (32, 33). This fact may explain the observed lower binding in the perfusate, since no plasma lipoproteins were added to the perfusion system. AmB binding to proteins in kidneys perfused with the OCT inhibitor TEA was somewhat lower than that found in other experimental groups. An explanation may be a competitive interaction of AmB with TEA, since the concentration of TEA (500 μM) was substantially higher than that of AmB (0.1 μM).

According to in vitro studies, AmB may dissociate from every AmB-lipid preparation in water regardless of the lipid used to complex AmB (34, 35). Such a process may also be seen in body fluids. The free (noncomplexed) AmB is then distributed within the body tissues and organs, including the kidney. Since we have found that interactions with the 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 the interactions with the transporters in vivo. However, no information on this ratio based on a direct measurement in vivo is available. Nevertheless, mainly the inhibitory effect of AmB on hOCT2 should be considered since the IC50 of free AmB is the lowest among the drug-transporter interactions found (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 with 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, and aminoglycosides) or which reduce the 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 found to be at a maximum between 1 and 5 μM (34, 37), the effective inhibitory concentrations starting at about 1 to 2 μM in this study (Fig. 2) may suggest the potential of AmB to affect the interactions of other drugs with OCT2 in the organism. However, the above-mentioned high plasma binding in vivo (31–33) is likely to result in much lower effective concentrations of free AmB under real conditions. This could explain the lack of clinical observations of pharmacokinetic drug-drug drug interactions for AmB based on hOCT2 under regular conditions. Probably, the clinical significance of the drug-drug interactions on 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 biokinetic studies have found that AmB concentrations in organs such as the kidney or liver are much higher than those in the blood (34, 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 specificities of OCTs usually overlap considerably (38, 39). Because OCT1 is an important hepatic carrier mediating the transmembrane transport of many drugs (38, 39), further studies to analyze of AmB transport interactions in the liver might also be performed.

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 eliminated by the kid-

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

a Values are means ± SD.

b Statistically significant difference in comparison with the AmB group at P < 0.05.
ney: adefovir, tenofovir, and cidofovir (40, 41). The incubation concentrations of adefovir, tenofovir, and cidofovir corresponded to the therapeutic plasma levels achieved in humans (42–44). Considerable in vitro interactions between AmB-DOC and all three antiviral agents at hOAT1 were demonstrated (Fig. 4). Because AmB-DOC inhibitory concentrations were higher by several orders of magnitude than the therapeutic concentrations, the probability of these drug-drug interactions at hOAT1 can be considered to be minimal in in vivo conditions. This finding is in accordance with the absence of any pharmacokinetic interactions between AmB and antivirals studied in the available clinical literature (1, 36). Our 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 with hOAT1, as such studies might be expected to be clinically irrelevant.

The main renal OATs and OCTs are similarly expressed in human and rat kidney tissues; OCT2 is highly expressed in both (27, 45). Similarly, OAT1 ranks among the main OATs found in human and rat kidneys (46). To describe AmB renal handling in a more complex way, it is therefore essential to combine the experimental data obtained in both cells expressing human transporters and the perfused rat kidney. The concordance in the results found in the transfected cells and the perfused kidney shows that there is most likely no significant interspecies difference in the renal handling of AmB by the transporters tested in these two species.

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