Differential Interaction of Dantrolene, Glafenine, Nalidixic Acid, and Prazosin with Human Organic Anion Transporters 1 and 3

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ABSTRACT

In renal proximal tubule cells, the organic anion transporters 1 and 3 (OAT1 and OAT3) in the basolateral membrane and the multidrug resistance-associated protein 4 (MRP4) in the apical membrane share substrates and co-operate in renal drug secretion. We hypothesized that recently identified MRP4 inhibitors dantrolene, glafenine, nalidixic acid, and prazosin also interact with human OAT1 and/or OAT3 stably transfected in human embryonic kidney 293 cells. These four drugs were tested as possible inhibitors of *p*-[³H]aminohippurate (PAH) and [¹⁴C]glutamate uptake by OAT1, and of [³H]estrone-3-sulfate (ES) uptake by OAT3. In addition, we explored whether these drugs decrease the equilibrium distribution of radiolabeled PAH, glutamate, or ES, an approach intended to indirectly suggest drug/substrate exchange through OAT1 and OAT3. With OAT3, a dose-dependent inhibition of [³H]ES uptake and a

downward shift in [³H]ES equilibrium were observed, indicating that all four drugs bind to OAT3 and may possibly be translocated. In contrast, the interaction with OAT1 was more complex. With [¹⁴C]glutamate as substrate, all four drugs inhibited uptake but only glafenine and nalidixic acid shifted glutamate equilibrium. Using [³H]PAH as a substrate of OAT1, nalidixic acid inhibited but dantrolene, glafenine, and prazosin stimulated uptake. Nalidixic acid decreased equilibrium content of [³H]PAH, suggesting that it may possibly be exchanged by OAT1. Taken together, OAT1 and OAT3 interact with the MRP4 inhibitors dantrolene, glafenine, nalidixic acid, and prazosin, indicating overlapping specificities. At OAT1, more than one binding site must be assumed to explain substrate and drug-dependent stimulation and inhibition of transport activity.

Introduction

Efficient renal drug elimination involves active secretion in proximal tubules. Thereby, transporters in the basolateral and luminal (brush-border) membrane co-operate in taking up a drug from blood and delivering it to the primary urine. For anionic, i.e., negatively charged, compounds, the organic anion transporters 1 (OAT1; SLC22A6) and OAT3 (SLC22A8) are the main uptake transporters, functioning physiologically as organic anion/ α -ketoglutarate exchangers (reviewed in: VanWert et al., 2010; Pelis and Wright, 2011; Burckhardt, 2012; Morrissey et al., 2013; Nigam et al., 2015), whereas the ATP-driven multidrug resistance-associated proteins MRP2 (ABCC2) and MRP4 (ABCC4) accomplish efflux into the tubule lumen (Masereeuw and Russel, 2010). Co-operation in transcellular drug secretion requires that OATs and MRPs exhibit overlapping specificities for their substrates.

In an ongoing search for specific inhibitors of MRP4, which confers resistance to tumor cells to a variety of antineoplastic agents (for review: Russel et al., 2008; Keppler, 2011; Wen et al., 2015), the FDA-approved drugs dantrolene, glafenine,

nalidixic acid, and prazosin were identified, being even more selective inhibitors than the commonly used leukotriene 4 receptor antagonist MK-571 (Cheung et al., 2015).

Dantrolene, glafenine, nalidixic acid, and prazosin are structurally unrelated and are, at physiologic pH, mainly uncharged (glafenine, prazosin), zwitterionic (dantrolene) or negatively charged (nalidixic acid) (Table 1). In addition, these drugs serve various therapeutic purposes. Dantrolene is the only drug available to treat malignant hyperthermia (Krause et al., 2004; Rosenberg et al., 2015). Glafenine is a nonsteroidal anti-inflammatory agent that was taken off the market (Withdrawal of glafenine, 1992). Nalidixic acid is a quinolone derivative with a wide antibacterial spectrum (Hiraoka et al., 2003; Fabrega et al., 2009), and the antihypertensive prazosin is an α_1 -adrenergic receptor antagonist (Batty et al., 2016; Digne-Malcolm et al., 2016). Owing to their different charges, it was not clear a priori whether these newly identified inhibitors of MRP4 are shared with OAT1 and/or OAT3, although these latter transporters do not exclusively interact with organic anions but also accept some organic cations such as cimetidine (Tahara et al., 2005).

The interaction of a test compound with a transporter can be investigated by several experimental approaches. Most commonly, the potential of test compounds to inhibit the uptake of a reference substrate is determined. Test compound

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ABBREVIATIONS: ES, estrone-3-sulfate; HEK, human embryonic kidney; MK-571, 5-(3-(2-(7-chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamiyl-4,6-dithiaoctanoic acid; MRI, mammalian Ringer solution; PAH, *p*-aminohippurate; OAT, organic anion transporter.

TABLE 1

Chemical structures and percentage of the ionic forms at pH 7.4 as calculated by MarvinSketch

Structural Formula of the FDA-Approved Drug	Uncharged/Zwitterion	Cation	Anion
<p>Dantrolene: ryanodine receptor antagonist, muscle relaxant</p>	87.19%	None	12.81%
<p>Glafenine: nonsteroidal anti-inflammatory drug</p>	90.67%	9.33%	None
<p>Nalidixic acid: antimicrobial agent with a limited spectrum</p>	3.19%	None	96.56%
<p>Prazosin: selective α_1-adrenergic receptor agonist</p>	58.94%	41.06%	None

and radiolabeled reference substrate are offered from the same side (“*cis*”) to cells expressing the transporter under investigation. If an interaction takes place, the test compound *cis*-inhibits substrate uptake. Inhibition, however, does not prove transport of the test compound. To directly demonstrate translocation, the test compound must be available in radio- or fluorescence-labeled form or be detectable by mass spectroscopy. Indirect evidence of translocation is provided by *trans*-stimulation experiments in which the labeled substrate is usually offered from the outside and the test agent from the inside of the cell. However, this type of experiment relies on the efficient uptake of test compounds before the addition of the labeled reference substrate. Whether this prior uptake took place is not known in many cases.

Since dantrolene, glafenine, nalidixic acid, and prazosin are not available in radiolabeled form, we employed a technique previously called *competitive counter flow* (Harper and Wright, 2013). In this setting, cells are equilibrated with their radiolabeled reference substrate. Thereafter, a test compound is added to the medium in the continuous presence of the labeled reference substrate. If the test compound enters the cell via the transporter under investigation, it will force the efflux of radiolabeled reference substrate by exchange (antiport). Thereby, the equilibrium distribution of the reference substrate is distorted, leading to a decrease in its intracellular content. The energy for this downward shift of equilibrium is provided by flux-coupling of drug influx driving substrate efflux. Since OAT1 and OAT3 physiologically operate as antiporters, this technique appeared

applicable to indirectly test for translocation of dantrolene, glafenine, nalidixic acid, and prazosin by these transporters.

It turned out that all four drugs *cis*-inhibited OAT3-driven estrone-3-sulfate (ES) uptake and downward shifted the ES equilibrium. With OAT1, dantrolene, glafenine, and prazosin *cis*-stimulated *p*-aminohippurate (PAH) uptake but *cis*-inhibited glutarate uptake, suggesting a complex interaction with this transporter. The anionic nalidixic acid *cis*-inhibited PAH as well as glutarate uptake. A downward shift in equilibrium was observed only with nalidixic acid, suggesting its exchange with intracellular PAH or glutarate through OAT1.

Material and Methods

Reagents and Chemicals. All chemicals were of analytical grade and purchased from Sigma-Aldrich (Taufkirchen, Germany) or Appli-Chem (Darmstadt, Germany). Stocks of drugs were prepared in dimethyl sulfoxide, the final concentration of which in the individual experiments did not exceed 0.8%. For dantrolene, glafenine, nalidixic acid, and prazosin, the individual amount of charged or uncharged form was calculated by the free software MarvinSketch 6.3.0 (ChemAxon Ltd., Cambridge, MA) and is displayed in Table 1. The [^3H]-labeled compounds *p*-aminohippurate and estrone-3-sulfate were from Perkin Elmer (Rodgau, Germany), and [^{14}C]glutarate was from Biotrend (Cologne, Germany).

Cell Culture and Transport Experiments. Human embryonic kidney (HEK)293 cells stably transfected with human OAT1 and OAT3 (PortaCellTec Biosciences GmbH, Goettingen, Germany) or vector (pcDNA5) were used. Cell cultures were grown in high-glucose Dulbecco's modified Eagle's medium (Life Technologies GmbH, Darmstadt, Germany), supplemented with 10% fetal calf serum (European Union approved origin; Life Technologies GmbH), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin in an atmosphere of 95% air and 5% CO_2 at 37°C. Cells were harvested and plated into 24-well polylysine-coated plastic dishes (Sarstedt, Nuembrecht, Germany) at a density of 2×10^5 cells/well. After 72 hours of incubation, cells were washed twice with 0.5 ml mammalian Ringer solution (MRi) containing in millimolar concentrations (mM): 130 NaCl, 4 KCl, 1 CaCl_2 , 1 MgSO_4 , 1 NaH_2PO_4 , 20 HEPES, and 18 glucose at pH 7.4. For transport experiments, cells were incubated at 37°C in MRi that contained the labeled substrate at the concentration indicated in the respective figure legends. For *cis*-inhibition experiments and determinations of IC_{50} values, the [^3H]- or [^{14}C]-labeled substrate and the potential inhibitor were applied simultaneously in the same well. In equilibrium shift experiments, cells were first allowed to accumulate the labeled substrate to an equilibrium. Afterward, cells were exposed to MRi containing the labeled reference substrate at the same concentration as during the preincubation in the absence or presence of test substrates. In all experiments, uptake was terminated at the time indicated in the figure legends by removal of the medium and followed immediately by three washes with 0.5 ml ice-cold phosphate-buffered saline. Cells were lysed in 0.5 ml 1 N NaOH by gentle shaking for 120 minutes, and the [^3H]- or [^{14}C]-content was determined by liquid scintillation counting (Tricarb 2810; Perkin Elmer). Protein quantification was performed according to Bradford (1976).

Data Analysis. Data are presented as means $\pm$ S.D., with calculations of standard deviations made on the basis of the number of separate experiments conducted on cells of three different cell passages. One-way analysis of variance was used to test the effect of possible substrates. IC_{50} , EC_{50} , and K_m values were obtained by a fit of dose-response curves by nonlinear regression (SigmaPlot Version 13; Systat Software, San Jose, CA).

Results

Implications of Dantrolene, Glafenine, Nalidixic Acid, and Prazosin on OAT3. In OAT3-transfected HEK293 cells, dantrolene, glafenine, nalidixic acid, and prazosin inhibited the

5-minute uptake of 10 nM [^3H]ES in a concentration-dependent manner with IC_{50} values of 0.30 ± 0.03 (Fig. 1A, ●), 5.31 ± 0.74 (○), 24.87 ± 1.48 (▼), and 29.84 ± 8.46 μM (△), respectively (*cis*-inhibition). Whereas dantrolene was most effective and dantrolene concentrations exceeding 10 μM reduced ES uptake close to that observed in pcDNA5-transfected HEK293 cells, inhibition of ES uptake by glafenine, nalidixic acid, and prazosin was partial, including an apparent inhibitor-insensitive part of ES uptake. When 250 μM concentration of these drugs was added to cells pre-equilibrated for 60 minutes with 10 nM [^3H]ES, cellular content of [^3H]ES was significantly decreased at 10 minutes despite the continuous presence of 10 nM [^3H]ES in the medium (downward shift in equilibrium). The effectiveness of decrease followed the order: dantrolene > glafenine > nalidixic

Fig. 1. Impact of dantrolene, glafenine, nalidixic acid, and prazosin on OAT3. (A) Inhibition pattern. OAT3- and pcDNA5-transfected HEK293 cells were incubated for 5 minutes at 37°C in MRi containing 10 nM [^3H]estrone-sulfate (ES) and in addition the following inhibitor concentrations: dantrolene (●) 0.1, 0.2, 0.5, 1, 2, 5, 10 μM ; glafenine (○), nalidixic acid (▼), and prazosin (△) 2, 5, 10, 20, 50, 100, 200 μM . For better comparison, the OAT3-mediated uptake in the absence of the putative inhibitors was set to 100%. (B) Equilibrium shift assay. OAT3-transfected HEK293 cells were incubated for 60 minutes at 37°C in MRi containing 10 nM [^3H]ES. After a washout, cells were incubated again in MRi containing 10 nM [^3H]ES minus or plus unlabeled 250 μM of ES, dantrolene, glafenine, nalidixic acid, or prazosin. Ten minutes later, the cellular [^3H]ES content was determined. The content in pcDNA-transfected HEK293 cells was subtracted from that measured in OAT1-transfected HEK293 cells. Data are a mean obtained from three cell passages with triplicate determinations each and were normalized. * $P < 0.05$, *** $P < 0.001$ with respect to none.

acid > prazosin (Fig. 1B). Thereby, dantrolene reduced the intracellular [^3H]ES content to values similar to those achieved by 250 μM unlabeled ES, serving as a control.

Stimulation and Inhibition of OAT1 by Dantrolene, Glafenine, Nalidixic Acid, and Prazosin. In OAT1-transfected HEK293 cells, dantrolene, glafenine, nalidixic acid, and prazosin showed, dependent on the reference substrate used, either an increase, a decrease, or no change in their 5-minute substrate uptake. With 0.25 μM [^3H]PAH as substrate and dantrolene concentrations of 1–200 μM , an increase in the uptake of PAH was observed that saturated at approximately 250% of the uptake in the absence of dantrolene (Fig. 2A, ●). The concentration for the half-maximal stimulation of [^3H]PAH uptake (EC_{50}) was calculated to be $1.89 \pm 0.25 \mu\text{M}$ dantrolene. In contrast, the 5-minute uptake of 5 μM [^{14}C]glutarate was inhibited by dantrolene in a concentration-dependent manner with an IC_{50} of $78.3 \pm 21.7 \mu\text{M}$ for the dantrolene-sensitive part. Performing similar experiments with glafenine, nalidixic acid, and prazosin revealed the results presented in Fig. 2, B–D, respectively. Glafenine evoked a biphasic response when [^3H]PAH was the reference substrate (Fig. 2B, ●); low concentrations stimulated and higher concentrations inhibited PAH uptake. Using [^{14}C]glutarate as substrate (Fig. 2B, ○), an inhibition with an IC_{50} of $54.5 \pm 8.9 \mu\text{M}$ was observed. Nalidixic acid resulted in an inhibition of PAH as well as of glutarate uptake with IC_{50} values of 110.6 ± 52.2 (Fig. 2C, ●) and $10.8 \pm 1.7 \mu\text{M}$ (Fig. 2C, ○), respectively. Prazosin increased [^3H]PAH uptake with an EC_{50} of $4.89 \pm 1.98 \mu\text{M}$ (Fig. 2D, ●) with no inhibition of [^{14}C]glutarate uptake up to prazosin concentrations of 200 μM (Fig. 2D, ○).

The experiments shown in Fig. 2 were performed with different substrate concentrations (0.5 μM [^3H]PAH, 5 μM

[^{14}C]glutarate) to overcome the low specific radioactivity of [^{14}C]glutarate. Decreasing [^{14}C]glutarate to 0.5 μM did not change the results, i.e., 100 μM dantrolene still inhibited glutarate uptake (data not shown). Likewise, increasing [^3H]PAH concentration from 0.5 to 10 μM did not abolish the stimulating effect of 100 μM dantrolene on PAH uptake (data not shown). Thus, the differential effects of dantrolene and, by inference, of the other drugs on PAH and glutarate uptake are not the result of different substrate concentrations but indicate a substrate-dependent effect.

Shift in Equilibrium Using Glutarate as a Reference Substrate of OAT1. After a 5-minute incubation time, [^{14}C]glutarate uptake into OAT1-transfected cells tended to saturate with increasing glutarate concentrations (Fig. 3A, ●). In pcDNA5-transfected cells, [^{14}C]glutarate uptake rose linearly with glutarate concentration (○). OAT1-dependent uptake was obtained by subtracting [^{14}C]glutarate in pcDNA5- from that in OAT1-transfected cells (▼). From the OAT1-mediated uptake, a K_m of $65.2 \pm 7.6 \mu\text{M}$ was calculated for glutarate on the basis of Michaelis-Menten kinetics. As an internal control, a K_m for the uptake of [^3H]PAH was determined on the same batch of cells. After correction for non-OAT1-mediated PAH uptake, a K_m of $57.0 \pm 3.4 \mu\text{M}$ was found (data not shown). Hence, the K_m values for glutarate and PAH turned out to be similar in our cells.

Uptake of 5 μM [^{14}C]glutarate reached an equilibrium at incubation times exceeding 30 minutes (Fig. 3B, ●). Uptake of [^{14}C]glutarate into pcDNA5-transfected HEK293 cells increased linearly (Fig. 3B, ○) but was at all times much smaller than uptake into OAT1-transfected cells. After a 60-minute equilibration in MRI containing 5 μM [^{14}C]glutarate, OAT1- and pcDNA5-transfected HEK293 cells were transferred in

Fig. 2. Differential effects of drugs on OAT1-mediated PAH and glutarate transport. The 5-minute uptakes of 0.25 μM [^3H]PAH (●) and of 5 μM [^{14}C]glutarate (○) in response to increasing concentrations of (A) dantrolene, (B) glafenine, (C) nalidixic acid, and (D) prazosin were determined. Uptakes of glutarate and PAH in pcDNA5-transfected HEK293 cells were subtracted from the uptakes in OAT1-transfected HEK293 cells (OAT1-mediated reference substrate uptake). Data are means obtained from three consecutive cell passages, each run in triplicate.

Fig. 3. Evaluation of experimental conditions for equilibrium shifts, using glutarate as a reference substrate for OAT1. (A) Determination of a Michaelis-Menten (K_m) constant for glutarate. In OAT1- (●) and pcDNA5-transfected HEK293 cells (○), the 5-minute uptake of glutarate was measured using the following glutarate concentrations (in μM): 0.5, 1, 2, 5, 10, 20, 50, 100. The concentrations 10–100 μM were applied as a mixture of 5 μM [^{14}C]glutarate supplemented with 5, 15, 45, or 95 μM unlabeled glutarate, respectively. Concentrations of 0.5, 1, 2, and 5 μM glutarate contained only [^{14}C]glutarate. The inverted solid triangle (▼) represents the OAT1-mediated part of glutarate uptake from which the K_m was calculated. (B) Time course of glutarate uptake. The uptake of 5 μM [^{14}C]glutarate was followed over 120 minutes in OAT1- (●) and pcDNA5-transfected HEK293 cells (○). (C) Time course of [^{14}C]glutarate equilibrium shift of OAT1- and

MRi containing again 5 μM [^{14}C]glutarate but now in the absence and presence of 250 μM unlabeled glutarate. A time-dependent decrease in cellular [^{14}C]glutarate content was detected upon application of 250 μM unlabeled glutarate in MRi (Fig. 3C, ○), which stabilized at times beyond 2 minutes. The [^{14}C]glutarate content at 10 minutes was $35.9 \pm 2.7\%$ of the [^{14}C]glutarate content in the absence of 250 μM glutarate. The sole application of MRi containing 5 μM [^{14}C]glutarate decreased the [^{14}C]glutarate content by less than 20% within 10 minutes (Fig. 3C, ●). The substantial decrease in cellular [^{14}C]glutarate content in the continuous presence of [^{14}C]glutarate in MRi plus external unlabeled glutarate indicates a downward shift in equilibrium most probably resulting from an exchange of extracellular unlabeled glutarate against intracellular [^{14}C]glutarate through OAT1.

Equilibrium Shifts Induced by Substrates and Drugs Using Glutarate as a Reference Substrate of OAT1. In these experiments, cellular [^{14}C]glutarate was measured in the absence and presence of test substances at 10 minutes. Addition of the OAT1 substrates 250 μM PAH or 250 μM glutarate decreased the 10-minute glutarate content in OAT1-transfected HEK293 cells to $35.8 \pm 11.0\%$ or $34.8 \pm 4.4\%$ of the value in the absence of glutarate or PAH (Fig. 4A, gray bars). Probenecid and succinate (both at 250 μM) also reduced the 10-minute glutarate content. In pcDNA5-transfected cells, [^{14}C]glutarate uptake was rather stimulated by extracellular glutarate, PAH, succinate, and probenecid but it remained negligible with respect to uptake into OAT1-transfected cells. The reason for this apparent stimulation in pcDNA5-transfected cells is unknown.

Applied to OAT1-expressing cells after preincubation with [^{14}C]glutarate, 250 μM dantrolene slightly decreased 10-minute glutarate content, and 250 μM prazosin tended to increase it, but both effects did not reach statistical significance (Fig. 4B, gray bars). In contrast, nalidixic acid and glafenine both reduced 10-minute glutarate content by $74.3 \pm 1.0\%$ and $43.1 \pm 16.9\%$, respectively. Again, in pcDNA5-transfected HEK293 cells, the drugs rather increased the 10-minute glutarate content (Fig. 4B, black bars). Correcting [^{14}C]glutarate content in OAT1-expressing cells by that in pcDNA5-cells revealed significant equilibrium shifts induced by glafenine and nalidixic acid but not by dantrolene and prazosin (Fig. 4C).

Shifts in Equilibrium with PAH as a Reference Substrate for OAT1. In OAT1-transfected HEK293 cells, time-dependent uptake of [^3H]PAH already leveled off at 5 minutes (Fig. 5A, ●), indicating a fast equilibration. In pcDNA5-transfected HEK293 cells, the uptake 0.25 μM [^3H]PAH increased less steeply and tended to level off at incubation times beyond 30 minutes (○). OAT1-mediated PAH uptake, i.e., uptake into OAT1- minus uptake into pcDNA5-transfected cells, showed a maximum at about 3 minutes of incubation (Fig. 5A, inset).

For the further experiments, 10 minutes of pre-equilibration with [^3H]PAH were used. After this time, cells were transferred to MRi containing 0.25 μM [^3H]PAH in the absence and presence of 250 μM unlabeled PAH, and changes in the content

pcDNA5-transfected HEK293 cells in the absence (OAT1: ●; pcDNA5: ▼) and presence of 250 μM glutarate (OAT1: ○; pcDNA5: △). After a 60-minute equilibration in 5 μM [^{14}C]glutarate, cells were transferred to MRi containing again 5 μM [^{14}C]glutarate without or with 250 μM unlabeled glutarate, and the decay in cellular [^{14}C]glutarate content was followed with time. Data shown in (A), (B), and (C) are a mean obtained on three consecutive cell passages, each run in triplicate.

Fig. 4. Equilibrium shifts performed with established OAT1 substrates and drugs. OAT1- and pcDNA5-transfected HEK293 cells were incubated for 60 minutes at 37°C in MRi containing 5 μ M [14 C]glutarate. Afterward, cells were incubated for 10 minutes in 5 μ M [14 C]glutarate minus or plus 250 μ M (A) glutarate, PAH, succinate, and probenecid or (B) dantrolene, glafenine, nalidixic acid, or prazosin. (C) The OAT1-mediated 10-minute glutarate content was achieved by subtraction of the 10-minute glutarate content measured in pcDNA5- from that in OAT1-transfected HEK293 cells. Data presented in (A), (B), and (C) are means obtained from three consecutive cell passages, each run in triplicate. Statistically significant differences from OAT1-transfected HEK293 cells not exposed to test compounds (none);

of [3 H]PAH were monitored as a function of time (Fig. 5B). The PAH content in pcDNA5-transfected HEK293 cells increased slightly with time, independent of whether unlabeled PAH was present in MRi or not (Fig. 5B, ∇ , Δ). In OAT1-transfected HEK293 cells, application of 0.25 μ M [3 H]PAH in MRi evoked only small changes in PAH content with time (Fig. 5B, $\bullet$). However, when 250 μ M unlabeled PAH was added, the cellular [3 H]PAH content decreased, showed a minimum at 2 minutes, and increased slightly with time afterward (Fig. 5B, $\circ$), revealing a downward shift in equilibrium most probably attributable to PAH/[3 H]PAH exchange through OAT1.

Equilibrium Shifts Induced by Substrates and Drugs Using PAH as a Reference Substrate for OAT1. Measuring the 2-minute PAH content in the absence and presence of extracellular 250 μ M PAH, glutarate, or succinate revealed a decrease in [3 H]PAH content by PAH and glutarate but not by succinate (Fig. 6A, gray bars). In pcDNA5-transfected HEK293 cells (Fig. 6A, black bars), the [3 H]PAH content increased rather than decreased by application of PAH, glutarate, and succinate. Dantrolene and prazosin (250 μ M) increased the 2-minute [3 H]PAH content compared with none, but glafenine and nalidixic acid did not significantly affect the 2-minute PAH content (Fig. 6B). In pcDNA5-transfected HEK293 cells, the 2-minute PAH content was significantly higher in the presence of the drugs than in their absence (Fig. 6B, black bars). Calculation of the 2-minute OAT1-dependent [3 H]PAH content, i.e., uptake into OAT1- minus uptake into pcDNA5-transfected cells, revealed a significant decrease by nalidixic acid but not by dantrolene, glafenine, and prazosin (Fig. 6C).

Discussion

Secretion of organic anions in renal proximal tubules involves uptake across the basolateral membrane and release across the luminal membrane. Uptake is mainly accomplished by two transporters of wide and overlapping substrate specificities, organic anion transporters 1 and 3 (OAT1 and OAT3; gene names SLC22A6 and SLC22A8; reviewed in VanWert et al., 2010; Pelis and Wright, 2011; Burckhardt, 2012; Morrissey et al., 2013; Nigam et al., 2015). These OATs take up organic anions from the blood in exchange against intracellular α -ketoglutarate. Since a high intracellular α -ketoglutarate concentration is maintained by metabolism and by sodium-driven uptake across luminal and basolateral membranes (Dantzer, 2002), organic anion transport across the basolateral membrane is continuously poised in the uptake direction. Multidrug resistance-associated protein 4 (MRP4; gene name ABCC4) is an ATP-driven export pump that is, among other transporters, involved in the release of organic anions into the primary urine (reviewed in Russel et al., 2008; Keppler, 2011; Wen et al., 2015). ATP hydrolysis provides the driving force for organic anion efflux across the luminal membrane.

When OAT1/3 in the basolateral membrane and MRP4 in the luminal membrane are to co-operate efficiently in proximal tubular anion secretion, it is mandatory that their spectrum of transported substrates overlaps broadly. Hence,

*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$; n.s., not significant. Statistically significant differences compared to pcDNA5-transfected HEK293 cells (none); # $P < 0.05$.

Fig. 5. Evaluation of experimental conditions for equilibrium shifts using PAH as a reference substrate for OAT1. (A) Time course of PAH uptake. The uptake of $0.25 \mu\text{M}$ [^3H]PAH uptake was followed over 30 minutes in OAT1- (●) and pcDNA5-transfected HEK293 cells (○). The inset shows the OAT1-mediated PAH uptake. (B) Time course of intracellular PAH content of OAT1- and pcDNA5-transfected HEK293 cells in the absence (OAT1: ●; pcDNA5: ▼) and presence of $250 \mu\text{M}$ PAH (OAT1: ○; pcDNA5: △). After 10-minute equilibration in $0.25 \mu\text{M}$ [^3H]PAH, cells were transferred to MRI containing again $0.25 \mu\text{M}$ [^3H]PAH without and with $250 \mu\text{M}$ unlabeled PAH and the change in cellular [^3H]PAH content was followed with time. Data shown in (A) and (B) are means obtained on three consecutive cell passages, each run in triplicate.

we reasoned that the recently detected MRP4 inhibitors dantrolene, glafenine, nalidixic acid, and prazosin (Cheung et al., 2015) could interact with OAT1 and OAT3 as well. To demonstrate interaction we used two types of experiments: *cis*-inhibition studies and equilibrium shift assays on HEK293 cells stably expressing human OAT1 or OAT3. Given the unavailability of labeled drugs, the latter type of experiment was performed to obtain an indirect indication, though not a proof, of drug translocation by OAT1 and OAT3. If an extracellularly added drug decreases the amount of intracellularly labeled reference substrate in the continuous presence of extracellularly labeled reference substrate, i.e., shifts the equilibrium to lower values, an exchange (antiport) of drug against reference substrate may have taken place at the transporter under investigation.

With OAT3, straightforward results were obtained using dantrolene, glafenine, nalidixic acid, and prazosin as test substances. In *cis*-inhibition experiments, all compounds inhibited ES uptake dose dependently, indicating a hitherto unknown interaction with human OAT3. Dantrolene, with an IC_{50} of

Fig. 6. Equilibrium shifts induced by established OAT1 substrates and drugs. OAT1- and pcDNA5-transfected HEK293 cells were incubated for 10 minutes at 37°C in MRI containing $0.25 \mu\text{M}$ [^3H]PAH. Afterward, cells were incubated for 2 minutes in $0.25 \mu\text{M}$ [^3H]PAH minus or plus $250 \mu\text{M}$ (A) PAH, glutarate, and succinate or (B) dantrolene, glafenine, nalidixic acid, or prazosin. (C) Subtraction of the 2-minute PAH content observed in pcDNA5- from that observed in OAT1-transfected HEK293 cells revealed the OAT1-mediated PAH content. Data presented in (A), (B), and (C) are a mean obtained on three consecutive cell passages, each run in triplicate. Statistically significant differences from OAT1-transfected HEK293 cells not exposed to test compounds (none) *** $P < 0.001$, * $P < 0.05$, n.s.: not significant. Statistically significant differences from pcDNA5-transfected HEK293 cells (none); # $P < 0.05$.

TABLE 2

Comparison of the interaction of dantrolene, glafenine, nalidixic acid, and prazosin with OAT1, OAT3, and MRP4

Shown are maximum plasma concentrations, $C_{\max}$, in $\mu\text{g/ml}$ as reported in the literature and recalculated in μM , and the IC_{50} (inhibition), or EC_{50} (stimulation; values labeled with *) values determined in this study for OAT1 with PAH or glutarate as substrates and OAT3 with estrone-3-sulfate as substrate. The last two columns show the concentration of compounds that inhibited apoptosis induced by 6-mercaptopurine or ISN-38 in MRP4-transfected HEK293 cells.

Compound	MW	C _{max}	IC ₅₀ /EC ₅₀ [*]			IC ₅₀		
			OAT1 PAH	OAT1 Glutarate	OAT3 ES	MRP4 ^a 6-MP	MRP4 ^c ISN-38	
	<i>g/mol</i>	<i>μg/ml</i>	<i>μM</i>	<i>μM</i>	<i>μM</i>	<i>μM</i>	<i>μM</i>	
Dantrolene	314.25	9.0 ± 4.6 ^b	28.6	1.89 ± 0.25*	78.3 ± 21.7	0.30 ± 0.03	5.29	4.2
Glafenine	372.80	0.7–2.2 ^c	1.8–5.9	Biphasic	54.5 ± 8.9	5.31 ± 0.74	12.3	5.7
Nalidixic acid	232.24	20–40 ^d	86.1–172.3	110.6 ± 52.2	10.8 ± 1.7	24.87 ± 1.48	9.2	13.3
Prazosin	383.40	0.017 ^e , <0.150 ^f	0.04; 0.39	4.89 ± 1.98*	>>200	29.84 ± 8.46	11.0	7.2

^aCheung et al., 2015.^bRyanodex (data sheet).^cVermerie et al., 1992.^dNalidixic acid (Drugs.com).^eGuelen et al., 1990.^fJaillon, 1980.

0.3 μM , turned out to be a very potent inhibitor, followed by glafenine (5.3 μM), nalidixic acid (24.9 μM), and prazosin (29.8 μM). For comparison, MK571 inhibited OAT3- and MRP4-mediated substrate uptake with an IC_{50} of 1.6 μM (Henjakovic et al., 2015) and 10 μM (Reid et al., 2003), respectively. It appears that the net charge of the compounds does not play an important role in OAT3 selectivity for these drugs. At physiologic pH, only 12.9% of dantrolene is net negatively charged, and 96.6% of nalidixic acid is present in the anionic form; yet dantrolene was a more potent inhibitor than nalidixic acid. Prazosin and nalidixic acid were equipotent inhibitors despite the fact that prazosin is partially cationic (41.1%) and nalidixic acid is nearly completely anionic. The previously described interaction of OAT3 with the cationic H_2 receptor antagonist cimetidine is in line with the assumption that this transporter is able to interact with organic anions, zwitterions, and cations (Tahara et al., 2005). In equilibrium shift assays, unlabeled ES was most and prazosin least effective in decreasing the cellular [^3H]ES content, suggesting that extracellular ES and possibly also the drugs exchanged for intracellular [^3H]ES via OAT3. Alternatively, the drugs only inhibited OAT3 and left an as yet undefined [^3H]ES efflux transporter unchanged.

Testing the drugs on OAT1 revealed results that are more difficult to interpret. Using [^{14}C]glutarate as a substrate, nalidixic acid (IC_{50} 10.8 μM), glafenine (54.5 μM), and dantrolene (78.3 μM) inhibited OAT1 activity. Prazosin had no effect. The rank order of inhibition is distinct from that on OAT3 (see above) and the IC_{50} values tended to be higher (see Table 2). When [^3H]PAH was used as a substrate, dantrolene and prazosin *cis*-stimulated uptake, and glafenine showed a biphasic effect (*cis*-stimulation followed by *cis*-inhibition at higher concentrations). Nalidixic acid inhibited [^3H]PAH uptake with an IC_{50} of 110 μM .

Stimulation of [^3H]PAH uptake by dantrolene occurred with an EC_{50} of 1.89 μM and a maximum stimulation was reached at 10 μM . Thereby, the EC_{50} was considerably lower than the IC_{50} for the inhibition of [^{14}C]glutarate uptake, suggesting two independent actions of dantrolene on OAT1. Prazosin stimulated [^3H]PAH uptake with an EC_{50} of 4.9 μM and did not inhibit [^{14}C]glutarate uptake at all, again indicating that stimulation of [^3H]PAH uptake and inhibition of [^{14}C]glutarate uptake imply different sites at OAT1. In our OAT1-transfected HEK293 cells, the K_m for PAH (57 μM) and glutarate (65.2 μM)

were undistinguishable. Hence, the differential effect cannot be the result of different affinities of OAT1 for PAH and glutarate but indicates a reference substrate-specific effect.

Substrate-dependent *cis*-stimulation and *cis*-inhibition was recently reported for OATP1B3-expressing CHO cells: The green tea constituent epigallocatechin gallate (EGCG) dose dependently stimulated [^3H]ES uptake, inhibited Fluo-3 uptake, and left estrogen-17 β glucuronide uptake unchanged (Roth et al., 2011). The authors reasoned that this behavior is indicative of multiple binding sites and/or different transport modes of OATP1B3. Likewise, we assume also for OAT1 multiple binding sites with different transport modes and kinetics. This may be a hallmark of polyspecific transporters that accept a wide variety of compounds for transport (see also results on the organic cation transporters; Egenberger et al., 2012; Belzer et al., 2013; Ingraham et al., 2014; Hotchkiss et al., 2015). Possibly, OAT1 has at least two binding sites: one for translocation of PAH and another one for allosteric *cis*-stimulation by dantrolene, glafenine, or prazosin. Nalidixic acid and, at higher concentrations, glafenine may displace PAH from the transport site, leading to a *cis*-inhibition. In case of glutarate transport, all drugs were inhibitory. As a divalent anion with spatially separated charges, glutarate may bind to the “PAH transport site” and to an additional transport site that may partially overlap with the allosteric binding site for drugs. In consequence, the drugs would displace glutarate and thus *cis*-inhibit transport.

The equilibrium content of [^{14}C]glutarate in OAT1-expressing cells was decreased by PAH $\sim$ glutarate $>>$ succinate. This sequence fits well with the affinity of OAT1 toward these translocated substrates (Kaufhold et al., 2011). In equilibrium shift assays, glafenine and nalidixic acid decreased intracellular [^{14}C]glutarate content, whereas only nalidixic acid also reduced intracellular [^3H]PAH content. Dantrolene and prazosin were without effect. It is, therefore, possible that nalidixic acid exchanges with intracellular labeled substrates through OAT1. Alternatively, nalidixic acid may inhibit influx without disturbing efflux of [^3H]PAH or [^{14}C]glutarate through an undefined transporter. Whereas PAH may well be pumped back out by MRP4 (Smeets et al., 2004) that is endogenously expressed in HEK cells (Cheung et al., 2015; Henjakovic et al., 2015), this may not be the case for glutarate, for which an efflux transporter is not known.

Table 2 summarizes our findings and compares them with published results on MRP4. Dantrolene, glafenine, nalidixic acid, and prazosin inhibited OAT3 as well as MRP4, indicating overlapping inhibitor specificities. Dantrolene had a more than 10-fold higher affinity, and nalidixic acid and prazosin a 2- to 3-fold lower affinity for OAT3 than for MRP4. With the exception of prazosin, the maximum plasma concentrations are either in the range (glafenine) or above (dantrolene, nalidixic acid) the IC₅₀ values. All four drugs shifted the equilibrium distribution of ES, suggesting that OAT3 may take up dantrolene, glafenine, nalidixic acid, and prazosin across the basolateral membrane and MRP4 may efflux these compounds into the urine. A finite proof of translocation of these drugs by OAT3 and MRP4, however, awaits further experimentation. The IC₅₀ values obtained with glutarate as a reference substrate for OAT1 were higher than (dantrolene, glafenine, prazosin) or in the range of (nalidixic acid) those obtained with MRP4. Thus, although there is some overlapping specificity, OAT1 may in vivo play a role, if at all, only in the transport of nalidixic acid.

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Authorship Contributions

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