Transport of Lamivudine (3TC) and High-Affinity Interaction of Nucleoside Reverse Transcriptase Inhibitors With Human Organic Cation Transporters 1, 2, and 3

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ABSTRACT

Nucleoside reverse transcriptase inhibitors (NRTIs) need to enter cells to act against the human immunodeficiency virus-1 (HIV-1). Human organic cation transporters (hOCT1-3) are expressed and active in CD4+ T cells, the main target of HIV-1, and have been associated with antiviral uptake in different tissues. In this study, we examined whether NRTIs interact and are substrates of hOCT in cells stably expressing these transporters. Using [3H]MPP+, we found a high-affinity interaction between abacavir (ABC, <0.08 nM), azidothymidine (AZT, <0.4 nM), tenofovir (TDF, <1.0 nM), and emtricitabine (FTC, <2.5 nM) and hOCTs. Using a wide range of concentrations of lamivudine (3TC), we determined two different binding sites for hOCTs: a high-affinity site ($K_{d1} = 12.3-15.4$ pM) and a low-affinity site ($K_{d2} = 1.9-3.4$ mM). Measuring direct uptake of [3H]3TC and inhibition with hOCT substrates, we identified 3TC as a novel substrate for hOCT1, 2, and 3, with hOCT1 as the most efficient transporter ($K_m = 1.25 \pm 0.1$ mM; $V_{max} = 10.40 \pm 0.32$ nmol·mg prot$^{-1}$·min$^{-1}$; $V_{max}/K_m = 8.32 \pm 0.40$ μl·mg prot$^{-1}$·min$^{-1}$). In drug-drug interaction experiments, we analyzed cis-inhibition of [3H]3TC uptake by ABC and AZT and found that 40-50% was inhibited at low concentrations of the drugs ($K_i = 22-500$ pM). These data reveal that NRTIs experience a high-affinity interaction with hOCTs, suggesting a putative role for these drugs as modulators of hOCT activity. Finally, 3TC is a novel substrate for hOCTs and the inhibition of its uptake at low concentrations of ABC and AZT could have implications for the pharmacokinetics of 3TC.
INTRODUCTION

Nucleoside reverse transcriptase inhibitors (NRTIs) are important drugs in the treatment of infection by the human immunodeficiency virus-1 (HIV-1). The most widely used today are lamivudine (3TC), abacavir (ABC), azidothymidine (AZT), emtricitabine (FTC), and tenofovir (TDF). Today, long-term efficacy, lack of resistance, and appearance and avoidance of toxicity are the main therapeutic challenges. Inadequate suppression of HIV-1 replication remains a major limitation to successful treatment. Moreover, failure of antiretroviral therapy involves a complex interplay of many factors including poor adherence, virological resistance, and pharmacological issues such as protein binding and cellular resistance (Cinatl et al., 1994; Shehu-Xhilaga et al., 2005). As for virological resistance, it is known that reduced entry or increased efflux of anti-HIV drugs could compromise intracellular drug levels, thus favoring the emergence of resistant viruses (Fridland et al., 2000; Turriziani and Antonelli, 2004).

The most important biochemical and pharmacological features that influence intracellular drug concentrations (in addition to oral bioavailability, plasma protein binding, and physiochemical properties of the drug) are the expression and activity of efficient antiviral influx and efflux transporters, specifically for those drugs incapable of freely crossing cell membranes. Given the role played by specific NRTI uptake and efflux transporters (such as P-glycoprotein and multidrug resistance proteins) in the membrane of CD4+ T cells (main targets for HIV-1) and hepatocytes, and in the renal epithelial cells involved in the metabolism and excretion of drugs and xenobiotics, it is important to elucidate the possible involvement of these transporter proteins in drug-drug interactions and toxicity mechanisms (Ford et al., 2004; Koepsell, 2004; McRae et al., 2006).

The expression and activity of a wide variety of efflux transporters of NRTIs and other antiretroviral drugs and their association with intracellular drug levels are well described in the literature (Janneh et al., 2007; Kock et al., 2007). Interestingly, specific polymorphisms in efflux transporters and in metabolizing enzymes have been associated with changes in the...
plasma levels of some anti-HIV drugs (Owen et al., 2006; Rodriguez Novoa et al., 2006; Rotger et al., 2007).

As for influx transporters, some members of \( SLC28 \) and \( SLC29 \) have been associated with NRTI uptake and with anticancer nucleoside analogs (Pastor-Anglada et al., 2004; Errasti-Murugarren et al., 2007), although none of the functionally expressed members of \( SLC28 \) or \( SLC29 \) gene families seem to be involved in the uptake of AZT in immune cells (Purcet et al., 2006; Minuesa et al., 2008). Polyspecific organic anion, cation, and carnitine transporters of the \( SLC22 \) family have also been associated with the uptake of some nucleoside and nucleotide analogs and antiviral drugs. Specifically, human organic anion transporters (hOAT) 1 and 3 have been reported to be involved in the uptake of TDF in renal epithelial cells (Cihlar et al., 2001; Uwai et al., 2007). Our previous findings that hOATs are not expressed in mmune cells are particularly interesting (Purcet et al., 2006). As for human organic cation transporters (hOCTs), no specific member has yet been described as an NRTI transporter in immune cells, even though it has been suggested an organic cation transport for AZT and 3TC uptake in microglia and the renal brush-border membrane, respectively (Takubo et al., 2000a; Hong et al., 2001). As we had previously found that hOCTs are well expressed and functionally active in immune cells and highly upregulated after activation of CD4+ T cells (Minuesa et al., 2008), we were interested in studying the role of hOCTs in the uptake of NRTIs, the possible cross-inhibition between them, and whether these drugs interact and therefore inhibit the physiological function of hOCTs.

We present a detailed study of the interaction between NRTIs and hOCT, the uptake properties of these transporter proteins in relation to 3TC, and the drug-drug interaction between 3TC, ABC, and AZT. We used a stably transfected system of Chinese hamster ovarian (CHO-) cells to elucidate the role of the transporter proteins in pharmacological efficacy, drug-drug interactions, and toxicity mechanisms in excretion tissues.
METHODS

Reagents

N-methyl-4-phenylpyridinium (MPP+) ([H3C-3H], 85 Ci/mmol) was purchased from Biotrend (Köln, Germany). (1S, cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol sulfate (abacavir sulfate; ABC) ([3H], 0.5 Ci/mmol), (-)-beta-L-2',3'-dideoxy-3'-thiacytidine (lamivudine; 3TC) ([5.3H (N)]; 9 Ci/mmol), 3'-azido-3'-deoxycytidine (azidothymidine; AZT) ([H3C-3H]; 12.7 Ci/mmol) and 1,1-[Dimethyl]biguanide hydrochloride (metformin) (Dimethyl-[14C]; 112 mCi/mmol) were purchased from Hartmann Analytic (Braunschweig, Germany).

The substrates and inhibitors of organic cation transport tetraethylammonium (TEA), tetrabutylammonium (TBuA), N-methyl-4-phenylpyridinium (MPP+), ranitidine (Rani), atropine (Atrop), 1,1'-diethyl-2,2'-cyanine iodide (D-22) were obtained from Sigma-Aldrich (Taufkirchen, Germany). The remaining reagents used for uptake measurements were also purchased from Sigma-Aldrich unless indicated in the text.

3TC, AZT, ABC, FTC, and TDF were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), USA.

Stable transfection of hOCT in CHO-cells and cell culture

The hOCT1 (GenBank accession numbers X98322), hOCT2 (X98333), and hOCT3 cDNAs (AJ001417, kindly provided by Dr. V. Ganapathy, Augusta, Georgia, USA) were recloned into the pcDNA5/FRT/TO vector (Invitrogen, Karlsruhe, Germany). The eukaryotic expression vectors and an empty vector (pcDNA5)—used as a control in uptake experiments—were then transfected into the Flp-In-CHO cell line (Invitrogen) using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's recommendations and selected for positive clones with 600 µg/mL hygromycin B (PAA Laboratories, Pasching, Austria). The cell lines with the highest transport activity were chosen for further study and routinely cultured.
in F-12 (HAMs) medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum in the presence of 300 µg/mL hygromycin B. They were maintained at 37ºC in a humidified atmosphere containing 5% CO₂.

“Short-time” uptake measurements

In order to determine the IC₅₀ values of the NRTIs and to assess which of the NRTIs were substrates of hOCTs, [³H]MPP+ and [³H]3TC uptake in CHO-hOCTs and/or CHO-pcDNA5 (empty vector) cells was measured after 1 second (MPP+) and 15 seconds (3TC) incubations. Briefly, after detaching cells with a soft EDTA/HEPES/NaHCO₃ buffer (0.02% / 10 mM / 28 mM) and resuspending them in a transport solution (PBS 1x with 0.5 mM MgCl₂ and 1 mM CaCl₂, pH 7.4) at 10⁸ cells/mL, 90 µL (9x10⁶ cells) was placed at the bottom of four 2-mL tubes (Sardstedt, Nümbrecht, Germany) and shaken in a water bath at 37ºC. The uptake measurement was then made tube by tube: 10 µL of radioactive solution (containing the appropriate concentration of the corresponding substrate or inhibitors) was placed on the inner wall of each tube approximately 1 cm above the cells. Uptake measurement was started by vortexing the tube and enabling the radioactive solution and the cells to be mixed, and immediately stopped with 1 mL of stop buffer (cold PBS 1x plus 100 µM quinine solution). The incubation time was determined using a metronome for 1 sec measurements, or a timer for 15 sec measurements. After two centrifugation/washing steps with stop buffer, cells were lysed and solubilized with 200 µL of guanidine thiocyanate (4M), mixed with 2 mL of scintillation liquid and put into the scintillation counter to determine the levels of intracellular radioactivity. The advantage of performing this “short-time” uptake measurement lies in the reduction of the passive diffusion of the radiolabeled substrates used (MPP+, 3TC, ABC, or AZT). For time-course measurements, we used the same procedure described above with incubation times extended up to 3 min. To measure uptake at 0 s incubation, ice-cold stop solution was added to the cells first, and radioactive substrates were added thereafter.
Kinetic and statistical analysis

For the IC\textsubscript{50} and K\textsubscript{i} analyses, data were fitted to the Hill equation (1) \[ V = \frac{V_0}{1 + (I/IC_{50})^n} \], where \( V \) is the uptake of [\textsuperscript{3}H]MPP\textsuperscript{+} (1 sec) or [\textsuperscript{3}H]3TC (15 sec) in the presence of the inhibitor (a specific NRTI), \( V_0 \) is the uptake of [\textsuperscript{3}H]MPP\textsuperscript{+} (1 sec) or [\textsuperscript{3}H]3TC (15 sec) in the absence of the inhibitor, \( I \) is the inhibitor concentration (nM), and \( n \) is the Hill coefficient. The kinetics graphs (uptake velocity of 3TC vs. substrate concentration) were fitted using the classic Michaelis-Menten equation (2) \[ V_0 = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \], where \( V_0 \) and \( V_{\text{max}} \) represent initial and maximal transport velocity, respectively (in pmol·mg prot\textsuperscript{-1}·min\textsuperscript{-1}), \([S]\) the initial substrate concentration (\( \mu \)M), and \( K_m \) the substrate concentration at half maximal transport velocity (\( \mu \)M). We also used the equation to calculate the quotient \( V_{\text{max}}/K_m \), which represents the transport efficiency. A paired \( t \) test was used for the statistical comparison of experimental data. We also compared which of the fittings for 3TC inhibition curves (two-binding-site competition vs. one-binding-site competition) was best.

The kinetic and statistical analyses were carried out using GraphPad Prism v4.0 and v5.0 software (GraphPad Software Inc., San Diego, California, USA).
RESULTS

High-affinity interaction of NRTIs with hOCTs. Our first objective was to study the interaction of the currently most used NRTIs with the three subtypes of hOCTs we had previously cloned and stably expressed in CHO cells. To do so, we performed 1-second measurements of $[^3]$H]MPP+ (12.5 nM) uptake (within the linearity range; Supplemental Data - Figure S4) in the presence of different low-range concentrations (from $10^{-3}$-$10^4$ nM) of ABC, AZT, FTC, and TDF. All tested NRTIs showed a high-affinity interaction with the three subtypes of hOCTs (Figure 1). For hOCT1, FTC showed the highest affinity ($IC_{50} = 0.020$ nM), followed by ABC, AZT and TDF (Table 1). The mean percentage of MPP+ transport inhibition ranged from 45% to 60%, with a higher value for ABC (>70% percent inhibition). For hOCT2, all NRTIs also showed a high-affinity interaction: ABC had the highest interaction value ($IC_{50} = 0.041$ nM), followed by AZT, TDF, and FTC (Table 1). For hOCT2, the percentage of MPP+ uptake inhibition was no higher than 60% for all drugs. Finally, for hOCT3, all NRTIs again showed high values and a notable percentage of MPP+ uptake inhibition. The drug that interacted with the highest affinity was TDF ($IC_{50} = 0.005$ nM), followed by ABC, AZT, and FTC (Table 1).

Interaction between lamivudine (3TC) and hOCT1, 2, and 3: identification of high- and low-affinity binding sites. The existence of two different binding sites with hOCTs for the substrates TEA, choline, and MPP+ and three binding sites for the nontransported inhibitor TBuA have been described elsewhere (Gorbunov et al., 2008). Due to the widespread prescription of 3TC and previous evidence that it could be a substrate for hOCTs (Takubo et al., 2000a; Takubo et al., 2002), we focused on the interaction between 3TC and hOCTs, by studying the inhibitory effect of a wide range of concentrations of 3TC on radiolabeled MPP+ uptake. The inhibition of MPP+ uptake clearly followed a biphasic curve (Figure 2). At low concentrations of nonlabeled 3TC, the inhibition was clear for the three hOCT subtypes, but did not reach more than 35-40% (Figure 2). Interestingly, the cells were incubated with concentrations of 3TC greater than 10 μM and inhibition increased to 80-85%. In all cases, the
$K_d$ values for the high-affinity binding site were in the pM range (*Table 2*), whereas the $K_d$ values for the low-affinity binding site were in the mM range (*Table 2*). A comparison between fitting the data by one-binding-site competition versus two-binding-site competition indicated that the latter was preferred in all cases, with statistical significance ($p<0.0001$).

To determine whether high and low affinity inhibition by 3TC is due to an interaction with previously described high and low-affinity MPP+ binding sites in OCT1 (Gorbunov et al., 2008) we tested the interaction of 3TC using three different MPP+ concentrations (12.5 nM, 125 nM and 5 µM) (*Figure 3*). Results showed, that $IC_{50}$ values for high affinity binding site increased at higher concentrations of substrate (MPP+) whereas $IC_{50}$ values for low-affinity binding site remained unchanged (*Table 3*).

**hOCTs show a saturable time-course and facilitate transport of 3TC.** We then tested the uptake of radiolabeled 3TC in CHO cell lines stably expressing hOCTs in comparison with the uptake in CHO cells stably expressing the empty vector (pcDNA5). We found a substantial difference between uptake rates: while CHO-pcDNA5 cells transported very low quantities of 3TC showing a nonsaturable and linear uptake, CHO-hOCT1, -hOCT2 and -hOCT3 cell lines showed a saturable uptake (up to 60-90 seconds) and high transport of 3TC (*Figure 4*). $[^{3}H]3TC$ uptake was proved to be linear for hOCT1 during the first 15 seconds (inset *Figure 4a*).

The same time-course experiments performed with radiolabeled ABC and AZT showed some differences in CHO-hOCT1 versus CHO-pcDNA5 for AZT, and in CHO-hOCT1 and -hOCT3 for ABC. Nevertheless, when performing $[^{3}H]ABC$ or $[^{3}H]AZT$ uptake inhibition with hOCTs substrates, we concluded that these proteins were not involved in transport (*Supplemental Data – Figures S1 and S2*).

**3TC uptake can be inhibited either by hOCT substrates and their inhibitors or by nonradiolabeled 3TC.** After demonstrating that 3TC was taken up by the three hOCTs, we assessed whether this uptake could be inhibited by substrates and inhibitors of hOCTs and by the nonradiolabeled drug. We therefore performed radiolabeled 3TC uptake in CHO-hOCT cell
lines inhibited by the substrate MPP+ (2mM), by the low-affinity inhibitors TBuA (2mM), Rani (2mM), and Atrop (2mM), by the high-affinity inhibitor D-22 (200μM), and by nonradiolabeled 3TC (2 mM). The inhibition reached for hOCT1 and hOCT2 uptake was greater than for hOCT3 uptake (Figure 5), although they were all statistically significant (p<0.005). In the case of hOCT1, the highest inhibition was found in the presence of Rani, Atrop and D-22. For hOCT2, the highest inhibition was found with TBuA followed by Rani, although the remaining drugs also inhibited at similar level. hOCT3 showed the lowest inhibition: 60% with D-22 and Rani, and nearly 50% with the remaining compounds. Nevertheless, inhibition was statistically significant in all cases and showed that 3TC was a substrate for hOCTs.

These results were confirmed by uptake experiments in Xenopus laevis oocytes injected with cRNA of hOCT1 and hOCT2. Thus, the uptake of [3H]3TC and [3H]MPP+ was inhibited by 2 mM 3TC and 1 mM MPP+ respectively, while the uptake of [3H]ABC and [3H]AZT was not affected by 2 mM ABC and AZT respectively (Supplemental Data – Figure S3).

Transport kinetics revealed hOCT1 to be the most efficient 3TC transporter of hOCTs. To kinetically characterize the uptake of 3TC by the three hOCT subtypes and compare the efficacy of transport and affinity for the substrate, we performed 3TC uptake at increasing concentrations of the drug (Figure 6). All three hOCTs showed saturable transport kinetics and followed a Michaelis-Menten curve for 3TC uptake, with $K_m$ in the same order of magnitude (mM range) and only slight differences in $V_{max}$ values (Table 4). Specifically, hOCT1 showed the highest affinity for the 3TC substrate, with a saturation curve with an estimated $K_m$ of 1.25 ± 0.1 mM and a $V_{max}$ of 10.40 ± 0.32 nmol·mg prot⁻¹·min⁻¹, followed by hOCT2 and hOCT3. Moreover, hOCT1 also showed the highest efficiency of transport with a $V_{max}/K_m$ quotient of 8.33 ± 0.40 μl·mg prot⁻¹·min⁻¹, that is, 2-fold higher than hOCT2 and hOCT3, which showed the same transport efficacy (4.10 ± 0.30 vs. 4.30 ± 0.30 μl·mg prot⁻¹·min⁻¹, respectively).

Finally, we aimed to compare the transport efficiency of 3TC with MPP+ (model substrate of hOCTs) and metformin (an anti-diabetic drug recently discovered as hOCT1 and hOCT2 substrate), a drug with a $K_m$ also in the mM range, in our cell system. To do so, we
performed kinetic curves with both drugs and we found a very high $V_{\text{max}}/K_m$ for MPP+ (293.5 µl·mg prot⁻¹·min⁻¹) as expected, but a 5-fold times lower transport efficiency for metformin (1.7 µl·mg prot⁻¹·min⁻¹) than the one found for 3TC (Supplemental Data – Figure S4).

**Abacavir (ABC) and azidothymidine (AZT) inhibit 3TC uptake at very low concentrations.** Finally, as we had seen that all the NRTIs interacted with a high affinity with hOCTs (Figure 1) and that 3TC was a substrate for these transporters, we wanted to explore whether other NRTIs usually taken in combination with 3TC during HAART could inhibit 3TC uptake in our stably transfected cell system (Figure 7). Using low concentrations of ABC and AZT (up to 10 µM), we found inhibition of hOCT-mediated transport of 3TC with $K_i$ ranging from 6.2 ± 4.1 pM (ABC inhibition for hOCT2-mediated transport) to 330 ± 280 pM (ABC inhibition for hOCT1-mediated transport) (Table 5). Importantly, both NRTIs showed considerably different affinities for the transporter subtypes: as an example, ABC had a more than 50-fold greater affinity for hOCT2 and 5-fold greater affinity for hOCT3 than for hOCT1. Nevertheless, for both drugs, no inhibition was more than 45-55% at the highest concentration tested.
DISCUSSION

The present study assesses the interaction of NRTIs with hOCTs and cell uptake transport of three of the most widely prescribed NRTIs: 3TC, ABC, and AZT. The study shows that (1) 3TC is a substrate for hOCTs, being hOCT1 the most efficient transporter, (2) all tested NRTIs interact with hOCTs with a high affinity and transporter activity is inhibited by 50-60%, and (3) ABC and AZT (NRTIs frequently co-administered with 3TC) inhibit by up to 50% the transport of 3TC through hOCTs at low concentrations.

Even though some drug uptake transporters have been described in renal epithelial cells and hepatocytes, it is still unknown which ones are implicated in NRTI transport across biplasma membranes of immune cells. The SLC22 gene family, which encodes for hOATs and hOCTs proteins, has been associated with the uptake of antiviral drugs used in HIV and other viral infections (Chen and Nelson, 2000; Takeda et al., 2002). We previously determined that hOCTs are highly expressed, active, and upregulated in immune cells (including CD4+ T cells, main targets of HIV) (Minuesa et al., 2008). Here, we firstly studied the interaction of hOCTs with NRTIs finding a high affinity ($IC_{50}$ in the pM range) interaction in all cases. This means that all NRTIs (with $C_{max}$ in plasma ranging from 2-10 µM) could physiologically inhibit the in vivo function of the three hOCT subtypes tested and potentially inhibit the transport of xenobiotics or endogenous substrates (Koepsell et al., 2007). The expression and activity of hOCTs is increasingly important in the clinical response to drugs such as metformin (used to treat type 2 diabetes), imatinib (used against chronic myeloid leukemia), or cisplatin and oxaliplatin (used in chemotherapy against some solid tumors) (Yonezawa et al., 2006; Zhang et al., 2006; Shu et al., 2007; Wang et al., 2008). Consequently, this high-affinity interaction of NRTIs with hOCTs could play a role in drug-drug interactions.

To our knowledge, this high-affinity interaction of NRTIs with hOCTs is a new finding. Other authors have described the interaction of protease inhibitors (PIs) and the anti-infective drugs pentamidine and trimethoprim with hOCTs, but the $IC_{50}$ were much higher (Zhang et al., 2000; Jung et al., 2008). The inhibition effect of both NRTIs and PIs at different concentrations
could have implications for clinical outcome in combinations of these drug families during highly active antiretroviral therapy (HAART). Nevertheless, further studies are necessary before we can draw conclusions on the possible antagonistic effects of specific drug combinations.

3TC is frequently included in HAART regimens and is one of the first choices in the treatment of therapy-naïve patients. Previous studies had suggested that it could be a substrate for OCTs (Takubo et al., 2000a). Therefore, we tested the interaction of the three hOCT subtypes with a wide range of concentrations of 3TC. Interestingly, we found a two-interaction-site inhibition curve with a first, high-affinity binding site in the pM range, and a second, low-affinity binding site in the mM range. The presence of two-binding-site interactions has recently been shown for choline, TEA, and MPP+ for rat OCT1 (rOCT1) in epifluorescence measurements performed in *Xenopus* oocytes (Gorbunov et al., 2008). Of note, high-affinity binding site for 3TC in hOCT1 showed a similar $K_d$ value than the one for MPP+ in rOCT1. The existence of a high-affinity MPP+ binding site in hOCT1, with a $K_d$ in the pM range, has recently also been demonstrated (Moaddel et al., 2005). Previously, we showed that high-affinity binding of the nontransported inhibitor TBuA inhibited MPP+-uptake by an rOCT1 mutant but not by the wild-type transporter (Gorbunov et al., 2008). This study provides evidence that binding of transported or nontransported compounds to high-affinity sites may lead to the inhibition of human wild-type OCTs as all NRTIs investigated partially inhibited uptake of 3TC and/or MPP+ by the hOCTs. Functional, molecular and structural characterization of rOCTs has provided evidence that OCTs contain a large binding cleft that switches from an outward-facing to an inward-facing conformation during the transport cycle (Volk et al., 2003; Gorboulev et al., 2005). The high-affinity binding sites for organic cations are probably located outside the innermost cavity of the outward-facing binding cleft (Gorbunov et al., 2008). The functional role of the high-affinity binding sites remains unclear. Assuming a rate constant for association ($K_{on}$) lower than $1\times10^9$ M$^{-1}$·S$^{-1}$, the $K_d$ values (pM) found for NRTIs, suggest half time for dissociation in the order of hours (Corzo, 2006). Thus, these sites would be occupied during a long time in the presence of low concentrations of individual ligands and might modulate the substrate selectivity for transport and/or the transport velocity. In
addition, in the presence of other ligands (eg. NRTIs), these would release the previously bound molecules by either direct competition at the same binding site or by allosteric effects on other high-affinity binding sites nearby. In competition experiments, when we increased [3H]MPP+ concentrations, IC\textsubscript{50} values for 3TC at high-affinity site experimented a significant shift, an evidence of direct competition between MPP+ and 3TC at these sites.

The similarity between the low affinity IC\textsubscript{50} value for 3TC inhibition and the Km value for 3TC uptake suggests that the low-affinity inhibition site of 3TC is identical with the transport site for 3TC. Despite this, this site may not overlap largely with the low-affinity binding site for MPP+ due to the fact that no competition between MPP+ and 3TC was found at low-affinity binding site of 3TC. In accordance, recent data from our laboratories suggest that different hOCT-transported compounds may have partially different binding regions in the low-affinity binding site (H. Koepsell, unpublished data).

Few studies have investigated the role of organic anion and cation transporter proteins in the uptake of antiretroviral drugs (Cihlar et al., 2001; Uwai et al., 2007). In our study, the direct uptake of [3H]NRTIs indicated that 3TC is a substrate for hOCTs, whereas ABC and AZT are not (Supplementary Figures S1 and S2). Although AZT influx involves a 40-50% protein-associated mechanism in T lymphocytes (Purcet et al., 2006), hOCTs do not seem to be relevant for uptake. For 3TC, the percentage of inhibition with substrates of hOCT, the kinetic parameters (Km, V\text{max}), and the transport efficiency (V\text{max}/Km) allow us to conclude that hOCT1 is the best transporter, although hOCT2 and hOCT3 can also participate in its uptake. A recent study has described 3TC and zalcitabine as substrates for hOCT1 and hOCT2, but shows only one interaction site with an IC\textsubscript{50} in the low µM range (Jung et al., 2008). In relation to kinetic parameters, both studies showed the same transport efficiency values and agreed on the fact that hOCT1 was the most efficient transporter.

Even though our Km values for 3TC are in the mM range (1.25-2.14 mM) and the transport efficiency of OCTs for MPP+ is much higher than that for 3TC (Supplementary Figure S4), the transport of 3TC by OCTs could be relevant in vivo. This assertion is supported by the fact that genetic polymorphisms in hOCT1 which transports metformin with a Km of 2.42
mM play a role in modulating the clinical response to the drug by influencing plasma disposition and pharmacokinetics (Shu et al., 2007). Moreover, in our system, hOCT1 showed a transport efficiency for metformin 5-fold times lower than the efficiency of hOCT1 for 3TC.

Finally, we wanted to assess the issue of drug-drug interactions, since some NRTIs are frequently co-administered. Two co-formulations are frequently prescribed as first-line antiretroviral regimens for HIV-1 infection: Kivexa® (ABC and 3TC), and Truvada®, (FTC and TDF), both in combination with either a PI or non-NRTI. In our study, we focused on ABC and AZT taken separately at low concentrations. As expected, both NRTIs inhibited 3TC uptake by up to 50%. The implications for clinical practice are important, as both hOCT1 and hOCT2 are highly expressed in the kidney and 3TC is mainly eliminated via the kidneys (Epivir® drug information sheet, GlaxoSmithKline). ABC and/or AZT could inhibit hOCTs function as a modulator of 3TC renal clearance and pharmacokinetics. This inhibitory phenomenon of 3TC renal clearance and the maintenance of higher levels of 3TC in plasma have been previously described for trimethoprim, a drug widely used against Pneumocystis jiroveci pneumonia in HIV+ patients (Sweeney et al., 1995; Takubo et al., 2000b). Moreover, subsequent clinical trials have confirmed that this interaction might be clinically relevant (Moore et al., 1996). The effect of trimethoprim on the clearance of 3TC, emtricitabine and apricitabine -with similar chemical structures to 3TC- have been confirmed in rat kidney (Nakatani-Freshwater et al., 2006). Extended studies to clarify the role of hOCTs in the uptake of other NRTIs with similar structures to 3TC, are under way in our laboratories.

To conclude, this study provides evidence of hOCTs as important determinants of 3TC intracellular and plasma concentrations, as all three hOCT subtypes transport 3TC and are expressed in both immune cells and excretion tissues. The finding that 3TC is a substrate of hOCTs and that NRTIs are high-affinity inhibitors of hOCT function provides new insights into drug-drug interactions. Due to the co-administration of ABC and AZT with 3TC in HAART, these observations could have important implications for clinical practice, especially with regard to 3TC clearance and pharmacokinetics.
ACKNOWLEDGMENTS

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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Use of NRTIs for cis-inhibition of \[^3\text{H}\]MPP\(^+\) uptake in CHO-hOCT1, -hOCT2 and -hOCT3 cell lines. Abacavir (ABC) (■) and azidothymidine (AZT) (□) at different low-range concentrations (10\(^{-3}\), 10\(^{-2}\), 0.1, 1.0, 10, 10\(^2\), 10\(^3\), 10\(^4\) nM) were used to perform cis-inhibition of 1 sec \[^3\text{H}\]MPP\(^+\) uptake (12.5 nM) by CHO-hOCT1 (a), -hOCT2 (b), and -hOCT3 (c) transfected cells at 37ºC. Likewise, emtricitabine (FTC) (▲) and tenofovir (TDF) (△) at the same low-range concentrations (10\(^{-3}\)-10\(^4\) nM) were used to perform cis-inhibition of 1 sec \[^3\text{H}\]MPP\(^+\) uptake (12.5 nM) by CHO-hOCT1 (c), -hOCT2 (d), and -hOCT3 (f) transfected cells at 37ºC. \(IC_{50}\) values and SEM from at least three independent experiments with each point performed in quadruplicate are shown in Table 1.

Figure 2. Two-site interaction of lamivudine (3TC) with hOCT1, 2 and 3. Cis-inhibition of \[^3\text{H}\]MPP\(^+\) (12.5 nM) uptake in CHO-hOCT1, -hOCT2, and -hOCT3 cells was performed in the presence of a wide-ranging concentration of 3TC (low and high concentration range from 10\(^3\) to 10\(^4\) pM and 10\(^1\) to 10\(^5\) µM, respectively). Low-affinity (pM) and high-affinity (mM) \(K_d\) values obtained by fitting the data to the two site-competition model and the \(P\) values for the statistical comparison between the two-site model and the one-site model are presented in Table 2.

Figure 3. Competition experiments of MPP\(^+\) uptake with high-affinity (pM) and low-affinity (mM) binding sites of 3TC in CHO-hOCT1. Cis-inhibition of \[^3\text{H}\]MPP\(^+\) uptake at three different concentrations (12.5 nM ○, 125 nM ● and 5 µM ◦) in CHO-hOCT1 was performed in (a) the presence of low 3TC concentrations (10\(^3\) to 10\(^4\) pM) and (b) high 3TC concentrations (10\(^1\) to 10\(^5\) µM). Data are expressed as the mean percentage of inhibition +/-one-half the range of the two mean uptake values of two independent experiments with each point performed in quadruplicate values are shown in Table 3.
Figure 4. Time-courses of [3H]3TC uptake in CHO-hOCT1, -hOCT2, -hOCT3 and -pcDNA5 (empty vector) cells. [3H]3TC uptake (156.25 nM) at 37ºC was performed at different times (1, 5, 15, 30, 60, 120, 180 and 300 sec) in CHO-hOCT1 (a), -hOCT2 (b) and -hOCT3 (c) (black circles ●) to assess the linearity and behavior of 3TC uptake and the involvement of each transporter subtype (compared with control cells -CHO-pcDNA5, in white circles ○). Inset graph in panel a represent CHO-hOCT1 [3H]3TC uptake values for the first 15-seconds of transport. Data are expressed as the mean uptake (pmol·mg prot⁻¹) +/- one-half the range of the two mean uptake values of two independent experiments with each point performed in quadruplicate. Error bars are not shown if the deviation was smaller than the size of the symbol.

Figure 5. Inhibition of [3H]3TC uptake by hOCT substrates and inhibitors in CHO-hOCT1, -hOCT2, and -hOCT3 cells. [3H]3TC uptake (156.25 nM) at 15 sec (linear range) was performed in the absence (Ctrl) or presence of 2 mM TBuA (TEA, in case of hOCT3), MPP+, ranitidine, atropine, nonradiolabeled 3TC and 200 μM D-22 at 37ºC. Results are expressed as the percentage of transport (normalized by the uptake in control cells) and are represented as the mean ± SEM of three independent experiments with each point performed in quadruplicate. Statistical significance was assessed using a paired t test (***, p<0.001).

Figure 6. Kinetics of the uptake of 3TC by hOCT1, hOCT2, and hOCT3. CHO-hOCT1 (a), -hOCT2 (b), and -hOCT3 (c) were incubated with [3H]3TC (156.25 nM) for 15 sec (linear range) in the presence of increasing concentrations of nonradiolabeled 3TC (10⁻⁴, 10⁻³, 10⁻², 10⁻¹, 0.25, 0.5, 1.0, 2.0, 5.0 mM) at 37ºC. Kinetic parameters (Kₘ and Vₘₐₓ) were estimated by fitting hOCT-specific uptake rates to a Michaelis-Menten nonlinear equation (Table 4). Data represent the mean (in nmol·mg prot⁻¹·min⁻¹) ± SEM from three independent experiments with each point performed in quadruplicate. Error bars are not shown if the S.E.M. values were smaller than the size of the symbol.
Figure 7. Use of abacavir (ABC) and azidothymidine (AZT) for cis-inhibition of $[^3]H3TC$ uptake in CHO-hOCT1, -hOCT2, and -hOCT3 cell lines. Different low-range concentrations ($10^{-3}, 10^{-2}, 10^{-1}, 1.0, 10^{2}, 10^{3}, 10^{4}$ nM) of abacavir (ABC) were used to perform cis-inhibition of 15 sec $[^3]H3TC$ uptake (156.25 nM) by CHO-hOCT1 (a), -hOCT2 (b), and -hOCT3 (c) transfected cells at 37ºC. The same low-range concentrations ($10^{-3}-10^{4}$ nM) of azidothymidine (AZT) were used to perform cis-inhibition of $[^3]H3TC$ uptake (156.25 nM) by CHO-hOCT1 (d), -hOCT2 (e), and -hOCT3 (f) transfected cells at 37ºC. The results are expressed as the percentage of inhibition (mean ± SEM) from at least three independent experiments with each point performed in quadruplicate. Inhibition constant ($K_i$) values were obtained by fitting the data to the Hill Inhibition equation (Table 5).
**Tables**

Table 1. $IC_{50}$ values (nM) for inhibition of $[^3H]MPP^+$ uptake by NRTIs in CHO-hOCT1, -hOCT2, -hOCT3 cell lines. Data is shown as $IC_{50}$ values ± SEM from at least three independent experiments with each point performed in quadruplicate.

<table>
<thead>
<tr>
<th>NRTIs</th>
<th>hOCT1</th>
<th>hOCT2</th>
<th>hOCT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>0.072 ± 0.033</td>
<td>0.041 ± 0.020</td>
<td>0.050 ± 0.029</td>
</tr>
<tr>
<td>AZT</td>
<td>0.155 ± 0.111</td>
<td>0.270 ± 0.105</td>
<td>0.396 ± 0.270</td>
</tr>
<tr>
<td>FTC</td>
<td>0.020 ± 0.006</td>
<td>2.400 ± 1.162</td>
<td>0.530 ± 0.260</td>
</tr>
<tr>
<td>TDF</td>
<td>0.854 ± 0.012</td>
<td>0.566 ± 0.549</td>
<td>0.005 ± 0.003</td>
</tr>
</tbody>
</table>
Table 2. $K_d$ for high-affinity binding site (pM) and low-affinity binding site (mM) and $P$ values for statistical comparison of two-binding versus one-binding site fitting of inhibition curve shown in Figure 2. Data is shown as $K_d$ values ± SEM from at least three independent experiments with each point performed in quadruplicate.

<table>
<thead>
<tr>
<th></th>
<th>High-affinity binding site</th>
<th>Low-affinity binding site</th>
<th>Two-binding vs. One-binding-site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{d1}$ (pM)</td>
<td>$K_{d2}$ (mM)</td>
<td></td>
</tr>
<tr>
<td>hOCT1</td>
<td>12.30 ± 0.25</td>
<td>1.90 ± 0.14</td>
<td>$P&lt;0.0001$</td>
</tr>
<tr>
<td>hOCT2</td>
<td>8.13 ± 0.17</td>
<td>3.45 ± 0.11</td>
<td>$P&lt;0.0001$</td>
</tr>
<tr>
<td>hOCT3</td>
<td>15.40 ± 0.23</td>
<td>2.40 ± 0.13</td>
<td>$P&lt;0.0001$</td>
</tr>
</tbody>
</table>
Table 3. *IC₅₀* values for high-affinity binding site (pM) and low-affinity binding site (mM) for inhibition of [³H]MPP⁺ uptake (0.0125, 0.125 µM or 5.0 µM) by 3TC in CHO-hOCT1.

The statistical comparison between reference *IC₅₀* value ([MPP⁺] at 0.0125 µM) and the other two MPP⁺ concentrations was done (*, *p*<0.05).

<table>
<thead>
<tr>
<th>[MPP⁺] (µM)</th>
<th>High-affinity binding site (pM)</th>
<th>Low-affinity binding site (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0125</td>
<td>12.3 ± 0.25</td>
<td>1.90 ± 0.14</td>
</tr>
<tr>
<td>0.125</td>
<td>145.5 ± 2.15*</td>
<td>1.12 ± 0.07</td>
</tr>
<tr>
<td>5.0</td>
<td>2127.5 ± 38.5*</td>
<td>1.42 ± 0.08</td>
</tr>
</tbody>
</table>

* *p*<0.05, *IC₅₀* different from the reference one (0.0125 µM MPP⁺)
Table 4. Kinetic parameters of 3TC uptake in CHO-hOCT1, -hOCT2 and -hOCT3. The $K_m$ and $V_{max}$ values were estimated by fitting data of Figure 6 to a Michaelis-Menten nonlinear equation. Values are mean ± SEM of at least three experiments.

<table>
<thead>
<tr>
<th></th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (nmol·mg prot⁻¹·min⁻¹)</th>
<th>$V_{max}/K_m$ (µl·mg prot⁻¹·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hOCT1</td>
<td>1.25 ± 0.10</td>
<td>10.04 ± 0.32</td>
<td>8.03 ± 0.40</td>
</tr>
<tr>
<td>hOCT2</td>
<td>1.90 ± 0.25</td>
<td>7.80 ± 0.45</td>
<td>4.10 ± 0.30</td>
</tr>
<tr>
<td>hOCT3</td>
<td>2.14 ± 0.24</td>
<td>9.27 ± 0.50</td>
<td>4.30 ± 0.31</td>
</tr>
</tbody>
</table>
Table 5. $K_i$ values of ABC and AZT inhibition of [3H]3TC uptake in CHO-hOCT1, -hOCT2 and -hOCT3. Data is shown as $K_i$ values ± SEM from at least three independent experiments with each point performed in quadruplicate.

<table>
<thead>
<tr>
<th>NRTIs</th>
<th>$K_i$ (nM)</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>hOCT1</td>
<td>hOCT2</td>
<td>hOCT3</td>
</tr>
<tr>
<td>ABC</td>
<td>0.330 ± 0.280</td>
<td>0.006 ± 0.004</td>
<td>0.070 ± 0.045</td>
</tr>
<tr>
<td>AZT</td>
<td>0.021 ± 0.016</td>
<td>0.205 ± 0.098</td>
<td>0.030 ± 0.021</td>
</tr>
</tbody>
</table>
Figure 1

(a) hOCT1  
(b) hOCT2  
(c) hOCT3

- Log M [NRTI]
- [% transport]
- [HMP]+ uptake

- ABC
- AZT

- FTC
- TDF
Figure 2

a

\[ ^{\text{H}} \text{MPP}^+ \text{ uptake (\% transport)} \]

\[ \log M [3TC] \]

b

\[ ^{\text{H}} \text{MPP}^+ \text{ uptake (\% transport)} \]

\[ \log M [3TC] \]

c

\[ ^{\text{H}} \text{MPP}^+ \text{ uptake (\% transport)} \]

\[ \log M [3TC] \]
Figure 4

(a) 

- CHO-hOCT1
- CHO-pcDNA5

[3H]3TC uptake (pmol/mg prot)

Time (sec)

(b) 

- CHO-hOCT2
- CHO-pcDNA5

[3H]3TC uptake (pmol/mg prot)

Time (sec)

(c) 

- CHO-hOCT3
- CHO-pcDNA5

[3H]3TC uptake (pmol/mg prot)

Time (sec)
Figure 6

(a) 

(b) 

(c) 

$[^{3}H]3T C$ uptake (nmols/mg prot$^{-1}$ min$^{-1}$)

[3TC] (mM)
Figure 7

ABC

\[ ^3 \text{H} \text{JTC} \text{ uptake} \ (\% \text{ transport}) \]

\[ \text{Log M [ABC]} \]

AZT

\[ ^3 \text{H} \text{JTC} \text{ uptake} \ (\% \text{ transport}) \]

\[ \text{Log M [AZT]} \]
Supplemental Data - Figure S1

a) hOCT1

![Graph showing [3H]ABC uptake (pmol/mg prot) for CHO-hOCT1 and CHO-pcDNA5 over time (sec).]

b) hOCT2

![Graph showing [3H]ABC uptake (pmol/mg prot) for CHO-hOCT2 and CHO-pcDNA5 over time (sec).]

c) hOCT3

![Graph showing [3H]ABC uptake (pmol/mg prot) for CHO-hOCT3 and CHO-pcDNA5 over time (sec).]

d) hOCT1

![Bar graph showing [3H]ABC uptake (% transport) for different treatments (Ctrl, TBuA, MPP+, Rani, Atrop, D-22, ABC).]

e) hOCT2

![Bar graph showing [3H]ABC uptake (% transport) for different treatments (Ctrl, TBuA, MPP+, Rani, Atrop, D-22, ABC).]

f) hOCT3

![Bar graph showing [3H]ABC uptake (% transport) for different treatments (Ctrl, TEA, MPP+, Rani, Atrop, D-22, ABC).]
V_{max} = 4.08 \pm 0.225 \text{ nmol-mg prot}^{-1} \cdot \text{min}^{-1}
K_m = 2.39 \pm 0.22 \text{ mM}
V_{max}/K_m = 1.7 \pm 0.01 \mu\text{l-mg prot}^{-1} \cdot \text{min}^{-1}
Supplemental Data - Figure S2

a) hOCT1

b) hOCT2

c) hOCT3

Minuesa et al.
Correction to “Transport of Lamivudine [(–)-β-L-2’,3’-Dideoxy-3’-thiacytidine] and High-Affinity Interaction of Nucleoside Reverse Transcriptase Inhibitors with Human Organic Cation Transporters 1, 2, and 3”

In the above article [Minuesa G, Volk C, Molina-Arcas M, Gorboulev V, Erkizia I, Arndt P, Clotet B, Pastor-Anglada M, Koepsell H, and Martinez-Picado J (2009) J Pharmacol Exp Ther 329:252–261], Fig. 5 and the unit of measure in the last column of Table 4 were printed incorrectly. The correct versions appear below.

The printer and authors regret this error and apologize for any confusion or inconvenience it may have caused.

The online version has been corrected in departure from the print version.

![Fig. 5. Inhibition of [3H]3TC uptake by hOCT substrates and inhibitors in CHO-hOCT1 (a), -hOCT2 (b), and -hOCT3 cells (c). [3H]3TC uptake (156.25 nM) at 15 s (linear range) was performed in the absence (Ctrl) or presence of 2 mM TBuA (TEA, in the case of hOCT3), MPP⁺, ranitidine, atropine, nonradiolabeled 3TC, and 200 mM D-22 at 37°C. Results are expressed as the percentage of transport (normalized by the uptake in control cells) and are represented as the mean ± S.E.M. of three independent experiments, with each point performed in quadruplicate. Statistical significance was assessed using a paired Student’s t test (***, p < 0.001).](image)

<table>
<thead>
<tr>
<th></th>
<th>Km</th>
<th>Vmax</th>
<th>Vmax/Km</th>
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</thead>
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<tr>
<td></td>
<td>mM</td>
<td>nmol/mg protein/min</td>
<td>µl/mg protein/min</td>
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