Potent and Selective Inhibition of Plasma Membrane Monoamine Transporter by HIV Protease Inhibitors

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ABSTRACT

Plasma membrane monoamine transporter (PMAT) is a major uptake-2 monoamine transporter that shares extensive substrate and inhibitor overlap with organic cation transporters 1–3 (OCT1–3). Currently, there are no PMAT-specific inhibitors available that can be used in *in vitro* and *in vivo* studies to differentiate between PMAT and OCT activities. In this study, we showed that IDT307 (4-(4-(dimethylamino)phenyl)-1-methylpyridinium iodide), a fluorescent analog of 1-methyl-4-phenylpyridinium (MPP⁺), is a transportable substrate for PMAT and that IDT307-based fluorescence assay can be used to rapidly identify and characterize PMAT inhibitors. Using the fluorescent substrate-based assays, we analyzed the interactions of eight human immunodeficiency virus (HIV) protease inhibitors (PIs) with human PMAT and OCT1–3 in human embryonic kidney 293 (HEK293) cells stably transfected with individual transporters.

Our data revealed that PMAT and OCTs exhibit distinct sensitivity and inhibition patterns toward HIV PIs. PMAT is most sensitive to PI inhibition whereas OCT2 and OCT3 are resistant. OCT1 showed an intermediate sensitivity and a distinct inhibition profile from PMAT. Importantly, lopinavir is a potent PMAT inhibitor and exhibited >120 fold selectivity toward PMAT ($IC_{50} = 1.4 \pm 0.2 \mu M$) over OCT1 ($IC_{50} = 174 \pm 40 \mu M$). Lopinavir has no inhibitory effect on OCT2 or OCT3 at maximal tested concentrations. Lopinavir also exhibited no or much weaker interactions with uptake-1 monoamine transporters. Together, our results reveal that PMAT and OCTs have distinct specificity exemplified by their differential interaction with HIV PIs. Further, we demonstrate that lopinavir can be used as a selective PMAT inhibitor to differentiate PMAT-mediated monoamine and organic cation transport from those mediated by OCT1–3.

Introduction

The monoamines, including serotonin (5-HT) and catecholamines (dopamine, epinephrine, norepinephrine), are a group of important neurotransmitters and neurohormones. The actions of released monoamine neurotransmitters are terminated by cellular uptake mediated by specific plasma membrane monoamine transporters (PMATs). Predominantly expressed in monoaminergic neurons, the Na⁺- and Cl[−]-dependent, high-affinity serotonin transporter (SERT), dopamine transporter (DAT), and norepinephrine transporter (NET) are the classic transporters responsible for clearing released transmitters from the neurosynaptic cleft (Blakely et al., 1994; Torres et al., 2003). These transporters, occasionally referred to as uptake-1, are the major targets for many psychostimulants, antidepressants, and neurotoxins (Blakely et al., 1994; Torres et al., 2003).

Besides uptake-1, cellular uptake of monoamines also occurs via Na⁺- and Cl[−]-independent mechanisms mediated by a group of low-affinity, high-capacity transporters known as uptake-2 (Daws, 2009; Duan and Wang, 2010). We and others have previously demonstrated

that PMAT and the organic cation transporter 3 (OCT3) are the two major uptake-2 transporters in central nervous system (Wu et al., 1998; Engel et al., 2004; Duan and Wang, 2010). OCT1 and OCT2, which are predominantly expressed in liver and kidney, may represent additional uptake-2 transporters in other cell types (Koepsell et al., 2007; Daws, 2009). These uptake-2 transporters may support monoamine uptake in cells lacking uptake-1 transporters and play a compensatory role in monoamine clearance when uptake-1 is compromised by pharmacologic inhibition (Engel et al., 2004; Zhou et al., 2007a; Daws, 2009). Indeed, recent studies provided further *in vivo* evidence that PMAT and/or OCT3 are involved in biogenic amine signaling pathways (Cui et al., 2009; Duan and Wang, 2013; Horton et al., 2013; Yoshikawa et al., 2013), and both transporters have been proposed as novel targets for neuropsychiatric and neurodegenerative disorders (Engel et al., 2004; Zhou et al., 2007a; Cui et al., 2009; Daws, 2009; Horton et al., 2013).

Among the uptake-2 transporters, PMAT is most highly expressed in the brain and likely represents an important uptake-2 transporter for 5-HT and dopamine in the central nervous system (Engel et al., 2004; Duan and Wang, 2010). Unlike uptake-1 transporters, which are confined to monoaminergic neurons, PMAT and OCT3 are broadly expressed in both monoaminergic and non-monoaminergic neurons in

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ABBREVIATIONS: ASP⁺, 4-(4-(diethylamino)styryl)-N-methylpyridinium; CDD, Collaborative Drug Discovery (database); D22, decynium-22 (1,1'-diethyl-2,2'-cyanine iodide); DAT, dopamine transporter; GBR12935, 1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl) homopiperazine; GI, gastrointestinal; HEK293, human embryonic kidney 293 cell line; HIV, human immunodeficiency virus; 5-HT, serotonin; IDT307, 4-(4-(dimethylamino)phenyl)-1-methylpyridinium iodide; MATE, multidrug and toxin extrusion proteins; MPP⁺, 1-methyl-4-phenylpyridinium; NET, norepinephrine transporter; OCT, organic cation transporter; PI, protease inhibitor; PMAT, plasma membrane monoamine transporter; RFU, relative fluorescence unit; SAR, structure-activity relationship; SERT, serotonin transporter.

many brain areas (Engel et al., 2004; Dahlin et al., 2007; Gasser et al., 2009). Expression of PMAT and OCT3 in astroglial cells was also reported (Cui et al., 2009; Yoshikawa et al., 2013). Moreover, PMAT and OCTs transport a broad range of biogenic amines and share extensive overlap in substrate and inhibitor profile (Engel and Wang, 2005; Duan and Wang, 2010), making it difficult to distinguish their specific contribution to monoamine uptake processes in ex vivo and in vivo studies.

Although several compounds, including decynium 22 (D22) and type II cations (e.g., quinine, rhodamine123), have been shown to inhibit PMAT at low micromolar concentrations, all inhibitors are nonselective toward PMAT and OCTs (Engel and Wang, 2005). For example, D22, which has been widely used in cell, tissue, and animal studies to block uptake-2 activities (Duan and Wang, 2010, 2013; Horton et al., 2013), inhibits PMAT and OCT3 with equal potency ($K_i \sim 0.1 \mu\text{M}$) (Hayer-Zillgen et al., 2002; Engel and Wang, 2005). Corticosterone is more selective toward the OCTs with reported IC_{50} values in 0.29–34 μM for human OCT1–3 and a K_i of 450 μM for PMAT (Hayer-Zillgen et al., 2002; Engel and Wang, 2005). Due to the lack of a specific PMAT inhibitor, sensitivity to D22, but not corticosterone, is currently used as an indirect method to discern PMAT activity from those of the OCTs (Baganz et al., 2008; Duan and Wang, 2010, 2013; Hosford et al., 2015). Therefore, PMAT-specific inhibitors are highly desirable for further assessment of the role of this transporter in monoamine clearance in tissue preparations and in animal models in vivo.

In this study, we first developed and validated a fluorescence method to enable rapid identification and characterization of potential PMAT and OCT inhibitors based on the use of fluorescent analogs of the well-established PMAT and OCT substrate 1-methyl-4-phenylpyridinium (MPP+) (Engel et al., 2004; Koepsell et al., 2007). Using previously developed pharmacophore models for PMAT (Ho et al., 2011), we predicted PMAT interaction with human immunodeficiency virus (HIV) protease inhibitors (PIs). We then characterized the potency and specificity of a panel of HIV PIs with the human PMAT and OCT1–3 transporters. Our results revealed potent inhibition of PMAT by HIV PIs and identified lopinavir as a selective inhibitor for PMAT.

Materials and Methods

Materials. An IDT307 [4-(4-(dimethylamino)phenyl)-1-methylpyridinium iodide]-based neurotransmitter uptake kit was purchased from Molecular Devices (Sunnyvale, CA). ASP+ [4-(4-(diethylamino)styryl)-*N*-methylpyridinium] and trypan blue were purchased from Life Technologies (Carlsbad, CA). The HIV PIs indinavir, amprenavir, nelfinavir, atazanavir, tipranavir, lopinavir, ritonavir, and saquinavir, originally obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, were gifts from Dr. Jashvant Unadkat at the University of Washington. [^3H]Ritonavir (25.3 Ci/mmol) was purchased from Movarek Biochemicals (Brea, CA) and [^3H]5-HT (28 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). All other chemicals were from Sigma-Aldrich (St. Louis, MO). Cell culture media and reagents were from Life Technologies (Carlsbad, CA).

Cell Lines and Cell Culture. Fip-in human embryonic kidney 293 (HEK293) cell lines stably expressing human PMAT and OCT3 at isogenic locations were previously generated (Duan and Wang, 2010). Quantitative mRNA analysis showed that the PMAT and OCT3 transcripts were expressed at similar levels in the stably transfected HEK293 cell lines (Duan and Wang, 2010). To generate Fip-in HEK293 cell lines stably expressing human OCT1 and OCT2, a full-length human OCT1 clone was amplified from human liver cDNA, and full-length human OCT2 clone was amplified from human kidney cDNA. The cDNAs were subcloned into the pcDNA5/FRT vector, and their sequences were verified by DNA sequencing. Stable cells were generated by hygromycin selection as previously described elsewhere (Duan and Wang, 2010). Fip-in HEK293 cell lines stably expressing human SERT, DAT, and NET were previously generated using

a similar procedure (Duan and Wang, 2013). All cell lines were cultured and maintained in a 37°C humidified incubator with 5% CO_2 . For better attachment of cells, all cell culture plastic surfaces were pretreated with 0.01% poly L-ornithine (MW 30,000 ~70,000) in phosphate-buffered saline solution before plating.

Fluorescence Microscopy. To visualize cellular uptake of IDT307 and ASP+, confluent cell cultures were washed once with Dulbecco's phosphate-buffered saline, then incubated at 37°C with IDT307 or ASP+ cocktail in the presence or absence of an inhibitor for 10 minutes. The cells were then observed under a Zeiss Axiovert 200 fluorescent microscope (Carl Zeiss, Thornwood, NY). Fluorescein isothiocyanate and rhodamine excitation/emission filters were used for IDT307 and ASP+, respectively.

Fluorescent Substrate Uptake Cocktails. For the IDT307-based assay, a commercial assay kit was purchased from Molecular Devices, and each vial of the assay kit was reconstituted in 10 ml of uptake buffer (1X Hank's balanced salt solution and 20 mM HEPES, pH 7.4) before use. This kit was originally designed for inhibitor assays for the high-affinity monoamine transporters (DAT, SERT, NET) (Schwartz et al., 2003). The solution contains a membrane impermeable dye to extinguish the extracellular fluorescence of untransported substrate. Therefore, only intracellular fluorescence will be detected when the fluorescent substrate is taken up into cells.

For the APS+ based assay, the uptake cocktail consisted of 2 μM ASP+ and 10 μM trypan blue in uptake buffer. The K_m value of ASP+ for OCT3 was determined to be $28 \pm 1.0 \mu\text{M}$ (data not shown), which is much greater than the final ASP+ concentration (1.0 μM) used in our inhibition studies. Similarly, in the presence of trypan blue, a membrane impermeable dye, extracellular ASP+ fluorescence is quenched. Titration experiments indicated that at the molar ratio of 5:1 of trypan blue/ASP+, over 99% of ASP+ fluorescence was suppressed (data not shown). At the concentration used, trypan blue has no effect on PMAT or OCT3-mediated [^3H]5-HT uptake (data not shown). Therefore, this ratio was used in the ASP+ uptake assay cocktail.

All compounds were prepared in uptake buffer at 2X the final concentration. Whenever needed to increase solubility, dimethylsulfoxide was added at up to 3% in the final solution without affecting the fluorescent substrate uptake. HIV PIs have limited solubility, and the maximum final concentrations that can be achieved in our assays are around 50 μM .

Fluorescent Substrate Uptake Protocol. Cell-based fluorescent uptake assays were performed in 96-well plate format. Cells were plated at a density of 90,000/well in 96-well plate and grown overnight. The uptake assay was performed at 37°C. All reagents were prewarmed to 37°C. Immediately before the assay, the culture medium was aspirated away. Cells were washed once with 100 μl of uptake buffer and aspirated again. Then, 100 μl of prepared inhibitor solution were transferred to the wells and preincubated with the cells for 10 minutes at 37°C.

Uptake was then initiated by adding 100 μl of ASP+ based or IDT307-based cocktail solution. The relative fluorescence unit (RFU) was recorded for each well immediately after adding the fluorescent substrates (time 0) and at the end of the uptake period. Specific uptake was calculated by subtracting the fluorescence readings at time 0 from the end point ($\text{RFU}_{\text{end}} - \text{RFU}_{\text{time0}}$). Fluorescence measurements were performed from a bottom-read position in a PerkinElmer Wallac 1420 Multilabel Counter, capable of precise temperature control and kinetic measurements. For ASP+, the excitation/emission wavelengths are 475 nm/609 nm. For IDT307, the excitation/emission wavelengths are 440 nm/520 nm. The excitation and emission filters are configured within 10 nm of these wavelengths.

Virtual Screening of PMAT Inhibitors. The pharmacophore models for PMAT interaction with inhibitors were generated previously (Ho et al., 2011). The Collaborative Drug Discovery (CDD) database (Burlingame, CA. www.collaborativedrug.com) is an open-access database containing 2815 drugs approved by the U.S. Food and Drug Administration (Pan et al., 2013). Drugs from the CDD database were virtually screened with the "Ligand Profiler" protocol in Discovery Studio (version 3.0; Accelrys, San Diego, CA) using the generated pharmacophore models as templates as described previously elsewhere (Pan et al., 2011, 2013).

Determination of IC_{50} Values for Chemical Inhibitors. Fluorescent substrate-based assays were performed in the presence of serial dilutions of the compounds under study. The degree of inhibition was expressed as the percentage of specific uptake RFU in the presence of an inhibitor normalized by uptake RFU in the absence of any inhibitors (control). IC_{50} values were obtained

Fig. 1. Uptake of ASP+ and IDT307 by OCT3 and PMAT into transporter-transfected cells. Flp-in pcDNA5, OCT3, and PMAT cells were incubated with ASP+ or IDT307 uptake cocktails in the absence or presence of the inhibitor quinine (200 μ M). Fluorescence from intracellular IDT307 or ASP+ was visualized with a fluorescent microscope.

by fitting concentration-dependent uptake data to the log(inhibitor) versus response (four parameters) model in GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA) using the following equation: $Y = \text{Bottom} + [\text{Top} - \text{Bottom}] / [1 + (C/IC_{50})^\gamma]$, where Y is uptake RFU in percentage, Bottom is the

residual uptake at maximal transporter inhibition, Top is transporter uptake without inhibition, C is the inhibitor concentration, IC_{50} is the fitted IC_{50} value, and γ is the Hill coefficient.

Radiotracer Uptake Assays. Cells were plated in 24-well plates and allowed to grow for 2 to 3 days to reach 80%–90% confluence. Transport assays were performed at 37°C in Krebs-Ringer-HEPES buffer containing known concentrations of substrates with radiolabeled tracer compounds. Uptake was terminated by washing the cells 3 times with ice-cold Krebs-Ringer-HEPES buffer. Cells were then solubilized with 0.5 ml of 1 M NaOH at 37°C for 2 hours, and neutralized with 0.5 ml of 1 M HCl; 0.4 ml of the lysates was used for liquid scintillation counting. Protein concentrations in the lysates were measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL), and the uptake in each well was normalized to its protein content. All uptake assays were performed in triplicate.

Data Analysis. Data points with error bars indicate mean $\pm$ S.D. for independent triplicates. All experiments were repeated 2–3 times. Where applicable, P values were obtained through Student's t test.

Results

ASP+ and IDT307 as Fluorescent Substrates for OCTs and PMAT. ASP+, a fluorescent analog of MPP+, was previously shown to be a substrate for OCT1 and OCT2; and ASP+-based fluorescence assays have been well used for OCT1 and OCT2 (Ciarimboli et al., 2005; Mason et al., 2005; Kido et al., 2011). To efficiently characterize the interaction of PIs with PMAT and OCT3, we set out to develop fluorescent substrate-based assays for PMAT and OCT3. In these assays, a membrane impermeable dye was used to quench extracellular

Fig. 2. Time-dependent uptake of (A) IDT307 by PMAT and (B–D) ASP+ by OCT1–3. Flp-in pcDNA5, PMAT, and OCT1–3 cells were incubated with IDT307 and ASP+ uptake cocktails. The RFU readings were recorded as described in *Materials and Methods* every 20 seconds for up to 30 minutes. Fluorescence signals in the absence or presence of the inhibitors quinine (200 μ M, for PMAT and OCT3), desipramine (100 μ M, for OCT1), and cimetidine (200 μ M, for OCT2) were compared with pcDNA5 cells as the negative control.

fluorescence, and only intracellular fluorescence is detected when the fluorescent substrate is taken up into cells. When incubated with the ASP+ uptake cocktail, OCT3 cells displayed increased intracellular red fluorescence, which was completely blocked by a nonselective inhibitor quinine (Fig. 1A), indicating ASP+ is a substrate for OCT3. However, we could not detect any significant increase in ASP+ fluorescence in PMAT cells either by fluorescence microscopy or spectrometry (data not shown), indicating PMAT does not transport ASP+.

We next tested whether IDT307, a fluorescent substrate for high-affinity monoamine transporters including SERT, DAT, and NET (Beikmann et al., 2013), is a transportable substrate for PMAT. The green fluorescence signal of IDT307 was readily detectable in PMAT-expressing cells and was completely blocked by quinine (Fig. 1B), demonstrating that IDT307 is a true substrate for PMAT. IDT307 is also transported by OCT1–3 (data not shown). IDT307 and ASP+ are structural analogs, and preliminary studies with selected inhibitors suggested IC_{50} values obtained with the two fluorescent substrates were very similar. Due to the much higher cost of the commercial kit, we chose to use the ASP+ assay for OCT1–3.

Time-Dependent Uptake of ASP+ and IDT307 by OCT1–3 and PMAT. To choose an optimal time point for uptake assays, we examined the time-dependent uptake of IDT307 and ASP+ in control and HEK cells stably expressing human PMAT and OCT1–3. As shown in Fig. 2, the RFU in transporter-expressing cells increased progressively with time and were much higher than the RFU in control cells. Coincubation with known inhibitors for these transporters (desipramine for OCT1, cimetidine for OCT2, quinine for OCT3 and PMAT) decreased the fluorescence signals to almost baseline levels. The times for the

fluorescence signals to begin to plateau in each cell line varied between 5 and 20 minutes. To maximize the signal-to-noise ratio while still maintaining initial uptake rates, we chose to use 10 minutes of incubation for PMAT and OCT2 and 5 minutes of incubation for OCT1 and OCT3 in all following uptake and inhibition assays.

Validation of ASP+ and IDT307-Based Inhibition Assays. The development of the IDT307-based fluorescence assay allowed us to rapidly characterize transporter-inhibitor interaction for PMAT on a mix-and-read format on 96-well plates. The IC_{50} values determined by this method showed minimal plate-to-plate or day-to-day variability (data not shown). Next, inhibition studies were performed with eight published PMAT inhibitors using the fluorescence-based assay (Fig. 3A). All compounds exhibited concentration-dependent inhibition in the assay. Notably the Hill slopes for phenylethylamine and phenylbutylamine were significantly less steep than for the other compounds, indicating possible allosteric effects (Fig. 3A).

The obtained IC_{50} values correlated well ($R^2 = 0.91$) with previously reported IC_{50} values determined by traditional radiotracer assays (Fig. 3B). Similarly, IC_{50} values determined for seven known OCT3 inhibitors using the ASP+ assay also showed a good correlation ($R^2 = 0.86$) with the literature-reported values using radiotracer assays (Fig. 3, C and D). These data suggest that our assays are well suited for characterization of PMAT and OCT3 inhibitors.

Virtual Screening Identify Several HIV PIs as Potential PMAT Inhibitors. Using computer-aided modeling, we previously generated three-dimensional pharmacophore models based on data from a series of known PMAT inhibitors and noninhibitors (Ho et al., 2011). These pharmacophore models are characterized by 1 hydrogen bond donor

Fig. 3. Concentration-dependent inhibition of PMAT (A) and OCT3 (C) by known inhibitors determined with the IDT307 and ASP+ assays. Correlation analysis of $LogIC_{50}$ values from PMAT/IDT307 (B) and OCT3/ASP+ (D) assays with reported values from the literature using radiotracer uptake assays.

and 2–3 hydrophobic features with a separation distance between 5.20 Å and 7.02 Å. Using the pharmacophore models, we performed virtual screening of the CDD database containing 2815 commercial drugs. HIV PIs, including saquinavir, indinavir, lopinavir, nelfinavir, amprenavir, atazanavir, ritonavir, and tipranavir, were among the compounds predicted to be most potent PMAT inhibitors ($IC_{50} < 10 \mu M$). Based on these data, we set out to investigate whether PIs are true inhibitors for PMAT and whether they also interact with OCTs.

Selective Interaction of HIV Protease Inhibitors with PMAT and OCT1–3. We next investigated whether HIV PIs are good PMAT inhibitors as predicted by virtual screening. A panel of eight HIV protease inhibitors, including indinavir, amprenavir, nelfinavir, atazanavir, tipranavir, lopinavir, ritonavir, and saquinavir, were examined using the IDT307 fluorescent assay for PMAT (Fig. 4A). Their interactions with OCT1–3 were examined using the ASP+ assays (Fig. 4, B–D). The IC_{50} values of various PI toward each transporter are summarized in Table 1.

With the exception of indinavir, all other seven PIs showed significant inhibition on PMAT. Among them, lopinavir was revealed as the most potent inhibitor for PMAT ($IC_{50} = 1.4 \mu M$). Ritonavir, saquinavir, and tipranavir also potently inhibited PMAT with IC_{50} ranging from 6.0 to $8.9 \mu M$. In contrast to the marked inhibitory effect of PIs toward PMAT, OCTs are more resistant to PI inhibition. Except a weak interaction with ritonavir, OCT2 and OCT3 showed no significant interaction with other PIs.

OCT1 is more sensitive to PIs than OCT2 and OCT3 and interacted with six PIs with IC_{50} ranging from 11 to $60 \mu M$. However, the inhibition pattern of OCT1 was different from that of PMAT. Lopinavir, saquinavir, and tipranavir are more potent inhibitors for PMAT than for

OCT1. Of particular note, lopinavir displayed >120 -fold selectivity toward PMAT versus OCT1, and showed no inhibition toward OCT2 and OCT3. The data strongly suggest lopinavir is a potent and selective PMAT inhibitor that can be used to pharmacologically discern PMAT activities from those of the OCTs.

Inhibition of PMAT and OCT-Mediated [3H]5-HT Uptake by HIV Protease Inhibitors. To further confirm the interactions between HIV PIs and PMAT, OCT1–3, we conducted single concentration inhibition studies using [3H]5-HT as the substrate. Based on the IC_{50} values (Table 1), a concentration of $20 \mu M$ was used for lopinavir while $50 \mu M$ was used for all other HIV PIs. As shown in Fig. 5, PMAT and OCT1 showed differential sensitivity to HIV PIs whereas OCT2 and OCT3 displayed a general resistance to the PIs. The nonselective inhibitor D22 ($20 \mu M$) universally inhibited PMAT- and OCT-mediated 5-HT uptake to baseline levels. In contrast, lopinavir ($20 \mu M$) potently and selectively inhibited PMAT-mediated 5-HT uptake to the baseline level. Lopinavir had no significant inhibitory effect on OCT1–3 at $20 \mu M$.

Effects of Lopinavir on SERT, DAT, and NET. We then evaluated the interactions between lopinavir, the most potent and selective PMAT inhibitor, with the high-affinity monoamine transporters SERT, DAT, and NET. Inhibition studies using the IDT307 kit showed that lopinavir ($10 \mu M$) did not significantly inhibit DAT and NET, but exhibited a moderate inhibitory effect on SERT (Fig. 6A). Detailed analysis with [3H]5-HT further revealed an IC_{50} value of $36.3 \pm 8.5 \mu M$ for SERT, suggesting that lopinavir is ~ 26 -fold more selective toward PMAT than for SERT (Fig. 6B).

Ritonavir Is a Possible Substrate for PMAT. The potent interaction between PMAT and HIV PIs raised a question of whether PIs

Fig. 4. Concentration-dependent inhibition of PMAT (A), OCT1 (B), OCT2 (C), and OCT3 (D) by HIV PIs determined with IDT307 or ASP+ assays. $LogIC_{50}$ values for HIV PIs were obtained by nonlinear regression analyses as described in *Materials and Methods*. Only curves with $R^2 > 0.9$ are shown.

TABLE 1

IC₅₀ values of HIV PIs against PMAT and OCT1–3

Concentration-dependent inhibition experiments were conducted on PMAT and OCT1–3 with IDT307 or ASP+ assays. IC₅₀ values were determined by nonlinear regression fit as described in *Materials and Methods*. Shown are the mean $\pm$ S.D. values from three independent kinetic experiments.

HIV PI	IC ₅₀ (μ M)			
	PMAT	OCT1	OCT2	OCT3
Indinavir	NI	33.4 $\pm$ 6.8 ^a	NI	NI
Amprenavir	51.5 $\pm$ 8.8 ^a	40.4 $\pm$ 7.3 ^a	NI	NI
Nelfinavir	44.6 $\pm$ 8.0 ^a	60.5 $\pm$ 10.9 ^a	NI	NI
Atazanavir	49.5 $\pm$ 7.5 ^a	NI	NI	NI
Tipranavir	8.9 $\pm$ 2.8	38.0 $\pm$ 5.8 ^a	NI	NI
Lopinavir	1.4 $\pm$ 0.2	174 $\pm$ 40.1 ^a	NI	NI
Ritonavir	6.0 $\pm$ 0.5	11.2 $\pm$ 3.5	78.9 $\pm$ 19.7 ^a	59.8 $\pm$ 9.3 ^a
Saquinavir	7.0 $\pm$ 1.0	44.8 $\pm$ 12.0 ^a	NI	NI

NI, no significant concentration-dependent inhibition.

^a Partial inhibition at the highest concentration tested (50 μ M) due to limited drug solubility.

are also a transportable substrate for PMAT. We thus performed uptake studies using a commercially available radiolabeled PI, [³H]ritonavir. Compared with control (pcDNA5-transfected) cells, PMAT-expressing cells showed a time-dependent increase in [³H]-ritonavir uptake (Fig. 7A). The increased uptake was abolished by D22 and GBR12935 (1-[2-(diphenylmethoxy)ethyl]-4-(3-phenylpropyl) homopiperazine), two known inhibitors for PMAT (Fig. 7B). In contrast, the uptake-2 transporter OCT3 did not show ritonavir uptake above baseline level (Fig. 7B). Together these data suggest that ritonavir is likely to be a transportable substrate of PMAT but not OCT3.

Discussion

A growing body of evidence suggests that the uptake-2 transporter PMAT plays an important role in brain monoamine uptake and may represent a novel pharmacologic target for monoamine-related disorders such as depression (Engel et al., 2004; Zhou et al., 2007b; Daws, 2009; Horton et al., 2013). However, further investigation of the physiologic and pharmacologic functions of PMAT is hampered by the lack of specific inhibitors that can be used to distinguish PMAT activity from those of OCTs. In this study, we developed a novel fluorescence-substrate assay for PMAT and characterized the interaction and specificity of HIV PIs toward PMAT and OCT1–3. These efforts have led to the identification of lopinavir as a selective PMAT inhibitor.

It has been hitherto unknown whether HIV PIs inhibit PMAT and OCT3, the two major uptake-2 transporters. Although two studies have described the interaction of HIV PIs with OCT1 and OCT2 (Zhang et al., 2000; Jung et al., 2008), only a few PIs were analyzed. Here we comprehensively analyzed the interaction of eight HIV PIs with human PMAT and OCT1–3 isogenic expressed in HEK293 cells. We found that PMAT and OCTs demonstrated distinct sensitivity and inhibition patterns toward the HIV PIs. PMAT is generally more sensitive to PI inhibition than the OCTs (Fig. 4 and Table 1). Importantly, our data suggested that lopinavir is a selective PMAT inhibitor that can be used to differentiate PMAT-mediated monoamine uptake from those mediated by the OCTs.

Coexpressed in many brain areas, PMAT and OCT3 are capable of clearing released monoamine neurotransmitters from the extracellular space. Both transporters are resistant to uptake-1 inhibitors such as the selective serotonin reuptake inhibitors (SSRIs) (Wu et al., 1998; Koepsell et al., 2007; Zhou et al., 2007a). Emerging evidence suggests that these transporters could play a significant role in regulating monoamine neurotransmission and may represent novel targets for new antidepressant drugs (Engel et al., 2004; Zhou et al., 2007b; Daws,

2009; Horton et al., 2013). For example, in SERT knockout mice, the nonselective OCT3 and PMAT inhibitor D22 diminished hippocampal 5-HT clearance and exerted antidepressant effects in these animals (Engel et al., 2004; Zhou et al., 2007b; Daws, 2009; Horton et al., 2013). In rat brains, D22 increased extracellular 5-HT levels in the dorsomedial hypothalamus (Feng et al., 2005) and potentiated 5-HT signal in nucleus tractus solitarius where the SERT inhibitor citalopram had no effect (Hosford et al., 2015).

However, D22 is nonselective and has equal inhibition potency toward PMAT and OCT3. To further dissect the specific contribution of PMAT and OCT3, the investigators had to use the OCT-specific inhibitor corticosterone. Sensitivity to D22 but not corticosterone is interpreted as potential involvement of PMAT (Baganz et al., 2008; Duan and Wang, 2010; Duan and Wang, 2013; Hosford et al., 2015). However, this approach is indirect and is under the assumption that there was no other D22-sensitive, corticosterone-insensitive transporter(s). Furthermore, corticosterone is a steroid hormone that can elicit complex physiologic responses, which will limit its use in vivo studies.

The identification of lopinavir as a PMAT, but not OCT, inhibitor would allow direct assessment of the contribution of PMAT to overall uptake-2 activity in isolated brain tissues or in situ or in vivo studies. Lopinavir also showed a good selectivity toward PMAT over the high-affinity uptake-1 transporters, and only weakly inhibited SERT (Fig. 6). This can be differentiated by using a low inhibitor concentration (e.g., 10 μ M) or further controlled by the well-established selective serotonin reuptake inhibitors to which PMAT is resistant (Zhou et al., 2007a). Recently, a genetic knockout mouse model of PMAT has been developed (Duan and Wang, 2013). The combined use of lopinavir in wild-type and PMAT knockout mice should provide definitive information regarding the role of this transporter in regulating monoamine signaling in vivo.

HIV PIs are known inhibitors or substrates for several efflux transporters including P-glycoprotein and breast cancer resistant protein (BCRP) (Lee et al., 1998; Gupta et al., 2004). PIs, including lopinavir, are also known to interact with the OAT polypeptides (Annaert et al., 2010; Kis et al., 2010). Nevertheless, these transporters do not transport biogenic amines or organic cations, and they have substrate profiles completely different from that of PMAT and OCTs.

It is currently unknown whether HIV PIs interact with the multidrug and toxin extrusion proteins (MATE1, MATE2-K). However, the

Fig. 5. Inhibition of PMAT and OCT1–3 uptake of [³H]5-HT by HIV PIs. Uptake of [³H]5-HT (1 μ M, 1 μ Ci/ml) was determined in the absence or presence of HIV PIs (20 μ M for lopinavir, 50 μ M for others). Uptake in the presence of the nonselective inhibitor D22 (20 μ M) was included as the positive control. Data represent the mean $\pm$ S.D. for triplicates. **P* < 0.02 compared with no inhibitor control.

Fig. 6. (A) Effect of lopinavir (10 μ M) toward SERT, DAT, and NET as determined by IDT307-based fluorescence assay. Data represent the mean $\pm$ S.D. for triplicates. * $P < 0.05$ compared with the control (no inhibitor) group. (B) Concentration-dependent inhibition of SERT-mediated uptake of [3 H]5-HT by lopinavir. The logIC₅₀ value was obtained by nonlinear regression analyses as described in *Materials and Methods*.

MATEs mainly function as efflux (not uptake) transporters for organic cations under physiologic conditions. Currently, there is no evidence to support a role of the MATEs in mediating monoamine uptake. Therefore, lopinavir should be a useful inhibitor to differentiate PMAT-mediated monoamine or organic cation uptake processes from those mediated by OCT1–3.

From a chemical and structural perspective, it would be interesting to know why lopinavir would be a selective PMAT inhibitor whereas other HIV PIs are less effective. However, our preliminary structure-activity relationship (SAR) analysis failed to identify any prominent physicochemical or structural feature that could clearly account for the selectivity of lopinavir. This is due to the limited data set as well as the complex chemical structures of the HIV PIs, which were not designed to rationally alter functional groups to probe the SAR of PMAT. Nevertheless, a comparison of the three-dimensional structures of these HIV PIs revealed that lopinavir has one of the highest molecular volumes among the tested HIV PI set, suggesting that the overall geometry of the molecule could play a role in its selective interaction with PMAT. There is probably a point of interaction for lopinavir with PMAT, or conversely a steric hindrance with the OCTs that drives the overall selectivity. Further molecular docking analysis is needed once the crystal structures of these membrane transporters are available to understand the true SAR between the HIV PIs and their selectivity toward PMAT.

HIV PIs are large molecular weight peptidomimetics that are highly (>98%) bound to plasma proteins (Gimenez et al., 2004). In humans, the maximum circulating concentrations (C_{max}) of HIV PIs in the plasma at steady state are typically in the low micromolar range (2–16 μ M). The free plasma concentrations of HIV PIs are at least 10- to 100-fold below their IC₅₀ values for PMAT, suggesting that they may not

significantly interfere with the physiologic function of PMAT at systemic sites. However in the gastrointestinal (GI) tract, orally administered HIV PIs may reach much higher concentrations, leading to potential inhibition of PMAT expressed on the luminal surface of the GI tract (Zhou et al., 2007c). Although the physiologic function of PMAT in the GI tract is unclear, 5-HT plays major role in regulating GI function (Gershon and Tack, 2007; Spiller, 2008). More than 90% of the body's total 5-HT is synthesized and stored in the intestine, and 5-HT is released in large quantity to regulate gut motility and secretory functions (Gershon and Tack, 2007; Spiller, 2008). Therefore, it is reasonable to speculate that PMAT may be involved in 5-HT clearance and signaling the gut; and that inhibition of PMAT by HIV inhibitors may partly contribute to some of their side effects in the GI tract. In addition, uptake studies with radiolabeled ritonavir indicate that HIV PIs may also be transported by PMAT (Fig. 6), suggesting that PMAT may play a role in the absorption and disposition of these antiviral drugs.

Fig. 7. (A) Time-dependent uptake of [3 H]-ritonavir by PMAT. The pcDNA5 and PMAT cells were incubated with [3 H]-ritonavir (500 nM, 0.5 μ Ci/ml) for up to 20 minutes. Specific uptake by PMAT was determined by subtracting pcDNA5 from the PMAT uptake activity. (B) Specific uptake (5 minutes) of [3 H]-ritonavir (500 nM, 0.5 μ Ci/ml) by PMAT in the absence or presence of the inhibitors D22 (10 μ M) and GBR12935 (100 μ M). Data represent the mean $\pm$ S.D. for triplicates. * $P < 0.05$ compared with pcDNA5 cells.

Lastly, our studies showed that the IDT307, a fluorescent analog of MPP⁺, is a transportable substrate for PMAT. The other fluorescent analog ASP⁺, however, is not recognized by PMAT. OCT1–3 transport both ASP⁺ and IDT307. Using these fluorescent substrates and an extracellular quenching dye, we have developed rapid assays that allow screening PMAT or OCT3 inhibitors in a rapid mix-and-read format. Good correlations were observed between the IC₅₀ values of inhibitors determined using the fluorescent substrate-based assays and radiolabeled tracer-based uptake assays. The IDT307 and ASP⁺ based uptake assays thus have the potential to be further developed for high-throughput screening of novel inhibitors for uptake-2 monoamine transporters.

In summary, we have developed a fluorescent substrate-based assay that could be used for rapid identification and analysis of PMAT inhibitors. Our analysis with HIV PIs revealed that PMAT is much more sensitive to PI inhibition than the OCTs, and that lopinavir can be used as a selective PMAT inhibitor to differentiate PMAT-mediated monoamine and organic cation transport from those mediated by the OCT1–3.

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Authorship Contributions

Participated in research design: Duan, Swaan, Wang.

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