Rigid Adenine Nucleoside Derivatives as Novel Modulators of the Human Sodium Symporters for Dopamine and Norepinephrine

Aaron Janowsky, Dilip K. Tosh, Amy J. Eshleman, and Kenneth A. Jacobson

Abstract

Thirty-two congeneric rigid adenine nucleoside derivatives containing a North (N)-methanocarba ribose substitution and a 2-arylthienyl group either enhanced (up to 760% of control) or inhibited [125I]RTI-55 (dextrorphanyl)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (RTI-55) binding at the human dopamine (DA) transporter (DAT) and inhibited DA uptake. Several nucleosides also enhanced [3H]mazindol [(c)-5-(4-chlorophenyl)-3,5-dihydro-2H-imidazo[2,1-β]isoindol-5-ol] binding to the DAT. The combination of binding enhancement and functional inhibition suggests possible allosteric interaction with the tropans. The structure-activity relationship of this novel class of DAT ligands was explored: small Nβ-substitution (methyl or ethyl) was favored, while the N1 of the adenine ring was essential. Effective terminal aryl groups include thien-2-yl (compounds 9 and 16), with EC50 values of 35.1 and 9.1 nM, respectively, in [125I]RTI-55 binding enhancement, and 3,4-difluorophenyl as in the most potent DA uptake inhibitor (compound 6) with an IC50 value of 92 nM (3-fold more potent than cocaine), but not nitrogen heterocycles. Several compounds inhibited or enhanced binding at the norepinephrine transporter (NET) and serotonin transporter (SERT) and inhibited function in the micromolar range; truncation at the 4'-position in compound 23 allowed for weak inhibition of the SERT. We have not yet eliminated adenosine receptor affinity from this class of DAT modulators, but we identified modifications that remove DAT inhibition as an off-target effect of potent adenosine receptor agonists. Thus, we have identified a new class of allosteric DAT ligands, rigidified adenosine derivatives, and explored their initial structural requirements. They display a very atypical pharmacological profile, i.e., either enhancement by increasing affinity or inhibition of radioligand binding at the DAT, and in some cases the NET and SERT, and inhibition of neurotransmitter uptake.

Introduction

The dopamine (DA) transporter (DAT) (i.e., SLC6A3) is a sodium-coupled symporter that clears, and thereby inactivates, an extracellular neurotransmitter after its release (Kilty et al., 1991; Torres et al., 2003; Rice and Cragg, 2008). Defects in the function, regulation, or expression of the DAT are implicated in various psychiatric disorders, including attention deficit hyperactivity disorder, depression, mood disorders, and addiction (Laasko et al., 2000, Hansen et al., 2014). Blockade of DA uptake is one of the main mechanisms of action of drugs of abuse such as cocaine (Fig. 1A) (Sekine et al., 2003). A subjective cocaine-induced high is directly proportional to the occupancy of the DAT (Volkow et al., 1997). Attention deficit hyperactivity disorder—associated sequence variants and early onset Parkinson’s disease-associated mutations of the DAT have been identified (Koldso et al., 2013).

The structure of the integral membrane protein DAT was initially deduced by analysis of its amino acid sequence, and by analogy to other proteins in the large family of solute carrier (SLC) transporters, such as the γ-aminobutyric acid transporter (Vaughan and Kuhar, 1996). The neurotransmitters—the norepinephrine transporter (NET) (i.e., SLC6A2) (Axelrod et al., 1961; Wang et al., 2012) and the serotonin transporter (SERT) (i.e., SLC6A4) (Felts et al., 2014)—belong to the same structural family (also known as neurotransmitter sodium symporters) and have much commonality of ligand structures with DAT. Recently, the structure of the Drosophila melanogaster DAT was determined by X-ray crystallography (Penmatsa et al., 2013), and interactions between specific amino acid residues and the substituents of psychostimulants were described (Wang et al., 2015). Having 12 transmembrane

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ABSTRACT

Thirty-two congeneric rigid adenine nucleoside derivatives containing a North (N)-methanocarba ribose substitution and a 2-arylthienyl group either enhanced (up to 760% of control) or inhibited [125I]RTI-55 (dextrorphanyl)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (RTI-55) binding at the human dopamine (DA) transporter (DAT) and inhibited DA uptake. Several nucleosides also enhanced [3H]mazindol [(c)-5-(4-chlorophenyl)-3,5-dihydro-2H-imidazo[2,1-β]isoindol-5-ol] binding to the DAT. The combination of binding enhancement and functional inhibition suggests possible allosteric interaction with the tropans. The structure-activity relationship of this novel class of DAT ligands was explored: small Nβ-substitution (methyl or ethyl) was favored, while the N1 of the adenine ring was essential. Effective terminal aryl groups include thien-2-yl (compounds 9 and 16), with EC50 values of 35.1 and 9.1 nM, respectively, in [125I]RTI-55 binding enhancement, and 3,4-difluorophenyl as in the most potent DA uptake inhibitor (compound 6) with an IC50 value of 92 nM (3-fold more potent than cocaine), but not nitrogen heterocycles. Several compounds inhibited or enhanced binding at the norepinephrine transporter (NET) and serotonin transporter (SERT) and inhibited function in the micromolar range; truncation at the 4'-position in compound 23 allowed for weak inhibition of the SERT. We have not yet eliminated adenosine receptor affinity from this class of DAT modulators, but we identified modifications that remove DAT inhibition as an off-target effect of potent adenosine receptor agonists. Thus, we have identified a new class of allosteric DAT ligands, rigidified adenosine derivatives, and explored their initial structural requirements. They display a very atypical pharmacological profile, i.e., either enhancement by increasing affinity or inhibition of radioligand binding at the DAT, and in some cases the NET and SERT, and inhibition of neurotransmitter uptake.

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The structure of the integral membrane protein DAT was initially deduced by analysis of its amino acid sequence, and by analogy to other proteins in the large family of solute carrier (SLC) transporters, such as the γ-aminobutyric acid transporter (Vaughan and Kuhar, 1996). The neurotransmitters—the norepinephrine transporter (NET) (i.e., SLC6A2) (Axelrod et al., 1961; Wang et al., 2012) and the serotonin transporter (SERT) (i.e., SLC6A4) (Felts et al., 2014)—belong to the same structural family (also known as neurotransmitter sodium symporters) and have much commonality of ligand structures with DAT. Recently, the structure of the Drosophila melanogaster DAT was determined by X-ray crystallography (Penmatsa et al., 2013), and interactions between specific amino acid residues and the substituents of psychostimulants were described (Wang et al., 2015). Having 12 transmembrane
domains, the DAT first binds sodium ions at the extracellular side before binding DA, and then both are internalized and released on the cytosolic side.

Various ligand tools are available for the study of the DAT, such as the tropane radioligands methyl (1R,2S,3S)-3-(4-fluorophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (WIN 35,487) and methyl (1R,2S,3S)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate (RTI-55), which are analogs of cocaine (Little et al., 1993; Carroll et al., 2004; Schmitt et al., 2013). Mazindol [(6S)-5-(4-chlorophenyl)-3,5-dihydro-2H-imidazo[2,1-a]isoindol-5-ol] is another ligand in a different structural class that binds to the DAT and other transporters and blocks neurotransmitter uptake (Severinsen et al., 2014).

Adenosine analogs are under development as potential therapeutic agents for treating chronic neuropathic pain and other diseases (Tosh et al., 2012a; Borea et al., 2015; Little et al., 2015). Among these potent adenosine receptor (AR) agonists are the 9-riboside N6-(3-iodobenzyl)adenosine-5-N-methylcarboxamide (IB-MECA; Stoilov et al., 2014) and the carbocyclic (19S,29R,39S,49R,59S)-49-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1(methylaminocarbonyl)-bicyclo[3.1.0]hexane-2,3-dirol MRS1898 (Fig. 1B), which bind selectively to the A3AR subtype and reduce pain in the chronic constriction injury model and other models of prolonged pain (Chen et al., 2012). Recently, we enlarged the set of conformationally constrained A3AR agonists like (19S,29R,39S,49R,59S)-49-{2-chloro-6-[(3-chlorophenylmethyl)amino]purin-9-yl}-1(methylaminocarbonyl)-bicycle[3.1.0]hexane-2,3-dirol that contain, in place of the natural D-ribose, a sterically rigidified bicyclic bicyclo[3.1.0]hexane (methanocarba) ring system, which maintains a receptor-preferred North (N)-methanocarba conformation (Tosh et al., 2012a). These adenosine derivatives optionally contain a rigid extension at the C2 position, consisting of an arylethynyl group, which enhances A3AR selectivity. In the process of derisking these compounds for possible development as clinical candidates, the National Institute of Mental Health Psychoactive Drug Screening Program (PDSP) (Besnard et al., 2012) conducted broad screening at receptors, ion channels, and transporters. It was noted that some of the analogs that were potent A3AR agonists bound to off-target receptors, such as serotonergic (5HT2B and 5HT2C) and α2- or β3-adrenergic receptors (Paolietta et al., 2014). Furthermore, members of the series were found to modulate radioligand binding to the human DAT (hDAT), although these results were not included in our previous report. Here, we have characterized at the DAT, NET, and SERT the activity of (N)-methanocarba adenosine derivatives and correlated these activities with structure. Both binding and functional activities of this new class of DAT ligands have been characterized.

Materials and Methods

Materials

FetalClone and bovine calf serum were purchased from HyClone (Logan, UT). Most other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All growth media included 100 units/ml penicillin and 100 mg/ml streptomycin. Most Food and Drug Administration–scheduled substances were obtained from the National Institute on Drug Abuse drug supply program. All radioligands and [3H]neurotransmitters were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA) except [3H]dihydroetabenzene (DHTB), which was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Biogenic Amine Transporters

Inhibition of [125I]RTI-55 binding to—and [3H]neurotransmitter uptake by—the hDAT, human SERT (hSERT), or human NET (hNET) in clonal cells have been described previously (Eshleman et al., 1999). Briefly, human embryonic kidney (HEK)-293 cells expressing the recombinant hDAT (HEK-hDAT), hSERT (HEK-hSERT), or hNET (HEK-hNET) were used. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% FetalClone, 5% bovine calf serum, and 2 µg puromycin/ml (HEK-hDAT and HEK-hSERT) or 10% FetalClone and 300 µg G418/ml (HEK-hNET). The [125I]RTI-55 (40–80 pM final concentration) and [3H]mazindol binding assays were...
conducted with duplicate determinations using a total particulate membrane preparation. The \([^{3}H]mazindol\) assay was conducted with modification of the \([^{125}I]\)RTI-55 binding methods, including 1–3 nM \([^{3}H]mazindol\) for the hDAT and hNET assays and 10–13 nM \([^{3}H]mazindol\) in 0.5 ml volume in the hSERT assays. RTI-55 was used to define nonspecific \([^{3}H]mazindol\) binding. The uptake assays were conducted with duplicate determinations and initiated by the addition of \([^{3}H]DA, \([^{3}H]5\)-hydroxytryptamine, or \([^{3}H]noradrenaline\) (20 nM final concentration) to intact detached cells. Assays were terminated by filtration using a Wallac 96-well harvester through Perkin Elmer filtermat A filters presoaked in 0.05% polyethyleneimine (\([^{3}H]neurotransmitter uptake assays). Scintillation fluid was added to the filters, and radioactivity retained on the filters was determined using a Perkin Elmer microbeta plate counter.

\[ [^{125}I]\)RTI-55 Saturation Binding Assays.\] Saturation binding assays were conducted to determine whether the nucleoside derivative–induced enhancement of \([^{125}I]\)RTI-55 binding was due to an increase in affinity or an increase in the \(B_{\text{max}}\) value. The methods for \([^{125}I]\)RTI-55 saturation binding to HEK-hDAT membranes have been reported previously (Eshleman et al., 1999). Briefly, saturation binding experiments were conducted in triplicate by diluting the specific activity of \([^{125}I]\)RTI-55 with unlabeled RTI-55 ranging in concentration from 0.036 to 16.6 nM. Buffer or compounds 6, 16, or mazindol at the indicated concentrations was added prior to the addition of HEK-hDAT membranes. Protein concentrations ranged from 2.7 to 7.8 μg. GraphPad Prism software (San Diego, CA) was used to analyze the saturation curves to yield the \(K_{d}\) and \(B_{\text{max}}\) values.

Cocaine Antagonism Assay. A cocaine antagonist is expected to shift the dose-response curve for cocaine in the \([^{3}H]DA\) uptake assay to the right without having an effect on uptake by itself. Control cocaine curves, in the presence of 0.1% dimethylsulfoxide and three- to six- cocaine dose-response curves in the presence of selected concentrations of the test compounds, were conducted. Nine concentrations of cocaine ranging from 21.6 nM to 10 μM were used. Cocaine IC\(_{50}\) values were calculated using GraphPad Prism.

HEK-hDAT \([^{3}H]DA\) Release Assay. The methods for characterizing drug-induced release of preloaded \([^{3}H]DA\) from HEK-hDAT cells have been described previously (Eshleman et al., 2013). Drugs were perfused for 22 minutes during the assays.

Vesicular Monoamine Transporter 2 (VMAT2)

Inhibition of \([^{3}H]DTTB\) binding to human VMAT2 in clonal cells has been previously described (Eshleman et al., 1999). Inhibition of \([^{3}H]ketanserin binding to human VMAT2 was conducted in an identical fashion except that DHTB (10 μM) was used to define nonspecific binding.

Data and Statistical Analyses

At least three independent competition experiments were conducted. GraphPAD Prism was used to analyze the data, with the IC\(_{50}\) values converted to \(K_{d}\) values using the equation \(K_{d} = IC_{50}/(1 + ([\text{drug}]/\text{IC}_{50}\text{drug}]),\) where \([\text{drug}]\) is the concentration of the labeled ligand used in the binding assays (Cheng and Prusoff, 1973). The \(K_{d}\) values used in the equation for \([^{125}I]\)RTI are as previously reported (Eshleman et al., 1999), and the \(K_{d}\) values for \([^{3}H]imazindol\) were 54, 82, and 3.9 nM for the hDAT, hSERT, and hNET, respectively. When a drug enhanced the radioligand binding, an EC\(_{50}\) value was determined. Differences in affinities, potencies, or \(B_{\text{max}}\) values were assessed by one-way analysis of variance using the logarithms of the \(K_{d}\) values for the test compounds. Dunnett’s multiple comparison test was used to compare the effects of the test compounds with the control values.

Results

Various sterically constrained adenine nucleoside derivatives (Supplemental Material; Supplemental Table 1) (Table 1) have been synthesized and studied for their potent binding to the A\(_{1}\)AR (compound 2) (Tosh et al., 2012b) or A\(_{2}\)AR (compound 1, 3–9, 14–19, 21–28, 31, and 32) (Tosh et al., 2012a, 2014, 2015a,b). Many of these A\(_{2}\)AR agonists reduce chronic neuropathic pain in a phenotypic screen, and the AR binding affinities of the previously reported nucleosides are provided (Supplemental Material; Supplemental Table 2). These rigid nucleosides were tested at 10 μM for inhibition or enhancement of radioligand binding at the DAT, NET, and SERT in a preliminary screen by the PDSP (Bensard et al., 2012). A large fraction of those compounds tested either inhibited or enhanced the binding of radioligand at the DAT (Supplemental Table 1), with negligible activity at the NET and SERT. Six compounds were found to enhance DAT binding using radiolabeled methyl (1R,2S,3S)-3-(4-fluorophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate, which was confirmed in full concentration-response curves performed by the PDSP (Supplemental Material). Based on these findings, additional compounds (10–13, 20, 29, and 30) were synthesized to explore the structure-activity relationship (SAR) at these three transporters. The synthetic procedures and the AR activity of the latter set of compounds are reported elsewhere (Tosh et al., 2015a).

The enlarged set of nucleoside analogs can be categorized according to the substitution at the C2 position as follows: simple C2 derivatives (H, compound 21, or Cl, compounds 1 and 2); aryalkyne derivatives (5'-methylamides 3–20 and 4’-truncated compounds 22–24); and triazole derivatives (compounds 25–31). The truncated derivatives were included because 4’-truncation tends to convert selective A\(_{2}\)AR agonists into selective antagonists (Tosh et al., 2012c). The triazole linker was explored as a substitute for the ethynyl group that would maintain key interactions with the A\(_{2}\)AR agonists (Tosh et al., 2015b). The expanded set of compounds was initially tested for modulation of binding of \([^{125}I]\)RTI-55 binding to HEK cell membranes expressing the hDAT, hNET, or hSERT.

Many of the nucleosides, particularly those bearing an extended C2 substituent potentially modulated DAT binding (Fig. 2, A and B; Supplemental Fig. 1). Compounds containing both a C2-fluorophenylalkyne and a N\(_{6}\)-(3-chlorobenzyl) group (compounds 3 and 4) were weakly inhibiting or inactive at the DAT. Only one related compound, 2-chlorophenyl analog 5, displayed a measurable \(K_{d}\) value in \([^{125}I]\)RTI-55 binding inhibition of 2.65 μM. Binding inhibition at the DAT by N\(_{6}\)-(3-chlorobenzyl) derivatives, i.e., the truncated nucleoside (compound 22) and triazole (compound 31), was found using the radioligand \([^{125}I]\)RTI-55. The 2-aryalkynyl-N\(_{6}\)-methyl derivatives included numerous analogs that interacted with the DAT. However, inhibition by the N\(_{6}\)-methyl derivative (compound 17) was not observed using \([^{125}I]\)RTI-55. Furthermore, numerous analogs greatly enhanced the binding of \([^{125}I]\)RTI-55 (40–80 pM) to the DAT: 2-arylene alkynyl 9, 14, 16, 19; truncated derivatives (compounds 23 and 24); and triazole derivative (compound 26). The most potent enhancers (all N\(_{6}\)-methyl or ethyl) were (EC\(_{50}\) in nM): 5-bromothien-2-yl 16 (9.1) > 5-chlorothien-2-yl 9 (35.1) > 3,4-difluorophenyl 6 (70) > 5-chlorothien-2-yl 11 (300) > thien-2-yl 8 (446) derivatives. Arranged according to maximal enhancement of binding, the order was as follows (percentage of enhancement): compound 11 (760), compound 6 (690), compound 9 (550), compound 8 (387), compound 12 (268), compound 13 (251), and compound 16 (217). Other
TABLE 1
Structures and modulation of binding and activity at the hDAT ([125I]RTI-55, unless noted) of (N)-methanocarba adenosine derivatives
The derivatives include simple C2 derivatives (compounds 1, 2 and 21), alkyne compounds 3–20 (5-amides) and 22–24 (truncated), and triazole derivatives (compounds 25–32). Each compound that produced an effect is associated with data for inhibition or enhancement, in separate columns. —, no effect.

Compounds 1, 3–9 and 11–19, and 10

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<th>R^1</th>
<th>R^2 or R^3</th>
<th>DAT Binding Inhibition, R^b K_i (nM)</th>
<th>DAT Binding Enhancement, *EC_{50} (nM)</th>
<th>Dopamine Uptake Assay IC_{50} (nM)</th>
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<th>DAT Binding Enhancement, $^b$ EC$_{50}$</th>
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<td>![Image](218x87 to 279x115)</td>
<td>—</td>
<td>&gt;7100 (9), 136.2% ± 8.0%</td>
<td>&gt;10,000 (2)</td>
</tr>
<tr>
<td>27</td>
<td>CH₃</td>
<td>![Image](218x87 to 279x115)</td>
<td>(Ki 4.48 μM)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>—</td>
<td>ND</td>
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<tr>
<td>28</td>
<td>CH₃</td>
<td>![Image](218x87 to 279x115)</td>
<td>a</td>
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</table>

<sup>a</sup> Inhibition; <sup>b</sup> Enhancement; <sup>c</sup> EC₅₀; <sup>d</sup> Ki; <sup>e</sup> %; <sup>f</sup> Ki 4.48 μM.
compounds that enhanced were weaker in potency (＞1 μM) and in maximal enhancement (＜200%).

Various N^6^-substituted derivatives in the 2-arylalkyne 5'-methylamide series contained a common 5-chlorothien-2-yl ethynyl group, which allowed comparison of the effects of various N^6^- groups. Di-methyl substitution of the N^6^- amine in compound 10 in the 5-chlorothien-2-yl series eliminated the potent interaction with the DAT observed with the corresponding monomethyl derivative (compound 9). Enlargement of the N^6^-methyl group to ethyl in compound 11 maintained enhancement of binding (EC_{50} value of 300 ± 150 nM); however, this EC_{50} was 9-fold weaker in comparison with compound 9. The N^6^-propyl 12 and N^6^-cyclobutylmethyl (compound 13) analogs were less efficacious in enhancing DAT binding at 10 μM; further enlargement of the N^6^- group in cyclopropylmethyl (compound 14) and cyclobutylmethyl (compound 15) analogs successively reduced and eliminated DAT interaction. Therefore, in the alkyne series enlargement of the N^6^- group progressively disfavored interaction with the DAT. The 5-bromoethen-2-yl-N^-methyl analog (compound 16) enhanced DAT binding with 4-fold higher potency (EC_{50} value of 9.1 ± 1.7 nM) and less than half of the percent enhancement in comparison with the corresponding 5-chlorothien-2-yl analog (compound 9). Substitution of the terminal aryl group in the 2-arylalkynyl-N^6^-methyl series with N-heterocycles in compounds 17–19 greatly decreased the degree of interaction with the DAT, with pyrazine (compound 17) and pyrimidine (compound 18) being essentially inactive. Replacement of the N1-nitrogen in the 1-deaza analog (compound 20) eliminated DAT interaction, which is a major difference compared with the corresponding adenine derivative (compound 9). 4'-Truncation in compounds 21–24 resulted in maintaining weak interaction with the DAT (μM inhibition or enhancement), but only when a 2-arylalkynyl group was present. The 2-triazole derivatives (compounds 25–32) were either weak or inactive in modulating DAT binding. With N^6^-methyl, the ability of the triazole derivatives to interact with the DAT depended on the terminal aryl group, with 3,4-difluorophenyl (compound 26) and fur-2-yl (compound 27) being weakly permissive, but with 5-chlorothien-2-yl (compound 28) being nonpermissive toward inhibition of DAT binding. Upon enlargement of the N^6^- group in the 5-chlorothien-2-yl triazole series (compounds 29–31) weak binding inhibition was observed only with the largest group, i.e., N^6^-3-chlorobenzyl (compound 31).

Six compounds (6, 7, 8, 9, 16, and 19) that enhanced [125I]RTI-55 binding were also evaluated in [3H]mazindol binding to the DAT, and a substantial binding enhancement was found (Fig. 2, C and D). This tetracyclic radioligand is not of the same structural class as the cocaine-related [125I]RTI-55, but it binds in the central substrate binding site (Severinsen et al., 2014).

Functional activity at the DAT was determined using [3H]DA uptake (Fig. 6A). In general, both the inhibitors and the enhancers of radioligand binding at the DAT inhibited the uptake of DA, while those compounds that lacked activity in the binding assay had no functional effect on DA uptake. The most potent inhibitors of DA uptake (all binding enhancers) were the following (IC_{50} in nM): compound 6 (92), compound 9 (253), compound 11 (206), and compound 16 (229) (Figs. 1 and 6A). All drugs completely inhibited [3H]DA uptake. All four compounds shared N^6^-methyl or ethyl substitution in the 2-arylalkyne 5'-methylamide series and differed mainly in the terminal aryl substitution, and all enhanced radioligand binding. Three of these four binding enhancers contained a 5-halothien-2-yl group. In the same functional assay cocaine and mazindol had IC_{50} values of 250 ± 35 and 13.9 ± 1.6 nM, respectively. The DA uptake inhibition among DAT binding inhibitors, such as compound 5, was weak at best and the IC_{50} values could not be determined.
Binding activity of the nucleoside derivatives was also measured at the hNET (Fig. 3) and hSERT (Fig. 4). There was mostly negligible activity at the SERT in the \( ^{\text{[125]I}} \)RTI-55 binding (Fig. 4, A and B) and \( ^{\text{[3H]}} \)mazindol binding (Fig. 4, C and D) assays, but the truncated 2-chlorophenyl derivative (compound 23) inhibited SERT binding using \( ^{\text{[125]I}} \)RTI-55 with a \( K_i \) value of 5.30 ± 0.92 \( \mu \text{M} \) (\( n = 6 \)) (Fig. 4A) and serotonin uptake with an \( IC_{50} \) value of 5.5 ± 1.9 \( \mu \text{M} \) (\( n = 3 \)). In the same uptake assay cocaine and mazindol had \( IC_{50} \) values of 307 ± 22 and 40.4 ± 9.1 nM, respectively. Compound 5 inhibited SERT \( ^{\text{[125]I}} \)RTI-55 binding with a \( K_i \) value of 6.71 ± 0.55 \( \mu \text{M} \) (\( n = 5 \)) (Fig. 4B), but the effect on serotonin uptake was insignificant.

There was more widespread interaction of the nucleoside derivatives at the NET (Fig. 3) than at the SERT (Fig. 4), with several compounds modulating binding. The \( N^6 \)-methyl derivatives that enhanced binding of \( ^{\text{[125]I}} \)RTI-55 at the hNET were the following (\( EC_{50} \) in \( \mu \text{M} \), percentage of control, \( n = 5 \) to 6): compound 6 (1.76 ± 0.64, 371% ± 67%), compound 9 (1.18 ± 0.36, 386% ± 73%), and compound 16 (0.670 ± 0.200, 285% ± 22%), while compound 5 inhibited binding \( IC_{50} \) of 2.45 ± 0.59 \( \mu \text{M} \). Some derivatives with \( N^6 \) substituents larger than methyl enhanced binding of \( ^{\text{[125]I}} \)RTI-55 at the hNET but with small maximal enhancement (\( EC_{50} \) in \( \mu \text{M} \), percentage of control, \( n = 3 \)): compound 12 (0.90 ± 0.32, 159% ± 1%) and compound 13 (0.40 ± 0.10, 134% ± 13%). The \( N^6 \)-ethyl derivative (compound 11) weakly enhanced binding at the hNET, with an \( EC_{50} \) value of 4.1 ± 1.2 \( \mu \text{M} \), up to 373% ± 15% of control (\( n = 6 \)). Curiously, enhancement of NET binding by compounds 6, 9, and 16 was not observed using \( ^{\text{[3H]}} \)mazindol as the radioligand (Fig. 3, C and D). The greatest functional inhibition at the NET was seen with the 5-bromothien-2-yl derivative (compound 16) \( IC_{50} \) value of 6.11 ± 0.57 \( \mu \text{M} \). This was the only compound that modulated NET binding and also had a significant functional effect on \( ^{\text{[3H]}} \)norepinephrine uptake at 10 \( \mu \text{M} \). In the same functional assay cocaine and mazindol had \( IC_{50} \) values of 230 ± 22 and 1.31 ± 0.12 nM, respectively. Compounds 2, 4, 7, 8, 17, 19, 20, 22–26, 31, and 32 at 10 \( \mu \text{M} \) were determined to have no significant effect on \( ^{\text{[125]I}} \)RTI-55 binding at the hNET or hSERT. Furthermore, compounds 1, 3, 10, 14, 15, 18, 21, and 27–30 at 10 \( \mu \text{M} \) were inactive in screening at the DAT, NET, and SERT in the PDSP screening.

To determine potential mechanisms for enhancement of \( ^{\text{[125]I}} \)RTI-55 binding to the hDAT, we conducted saturation isotherm binding in the presence of increasing concentrations of compounds 9 and 16. The data in Table 2 and Fig. 5 indicate that compounds 9 and 16 increase the affinity of the hDAT for the radioligand (one-way analysis of variance followed by Dunnett’s multiple comparisons test). However, no significant change in the \( B_{\text{max}} \) value was induced by either compound. Thus, the substantial increase in binding at a fixed, low concentration of the radioligand can be explained as an allosteric enhancement of the affinity of the radioligand.

The effects of the nucleoside derivative (compound 9) on inhibition of \( ^{\text{[3H]}} \)DA uptake by cocaine are shown in Fig. 6B. A leftward shift of the cocaine concentration-response curve is observed in the presence of increasing, fixed concentrations of compound 9 (1 nM to 1 \( \mu \text{M} \)). The average cocaine \( IC_{50} \) values (nM, mean ± S.E.M.) in the presence of 0, 1 nM, 10 nM, 100 nM, 300 nM, and 1 \( \mu \text{M} \) of compound 9 were 507 ± 57, 479 ± 78, 614 ± 82, 460 ± 150, 182 ± 73 (\( P < 0.05 \), one-way analysis of variance followed by Dunnett’s multiple comparisons test), and 35 ± 11 (\( P < 0.001 \), respectively. Furthermore the maximal uptake is decreased in the presence of high nanomolar concentrations of compound 9. There is no significant
enhancement of DA uptake or a rightward shift of the cocaine concentration-response curve by compound 9.

The inability of the nucleoside derivatives (compounds 2 and 8) to induce release of preloaded [3H]DA is shown in Fig. 6C.

Although these two derivatives had differing effects on binding and uptake—wherein compound 2 had no effect on binding or uptake, while compound 8 enhanced [125I]RTI-55 binding and inhibited [3H]DA uptake—neither compound induced release.

Fig. 4. Lack of drug-induced enhancement of [125I]RTI-55 binding and [3H]mazindol binding to HEK-hSERT cell membranes. (A) [125I]RTI-55 binding of compounds 2, 6, 8, 9, and 22; (B) [125I]RTI-55 binding of compounds 4, 5, 7, 16, 17, and 19; (C) [3H]mazindol binding of compounds 2, 6, 8, 9, and 22; (D) [3H]mazindol binding of compounds 4, 5, 7, 16, 17, and 19 (n = 3–7, except for drugs with no effect, where n = 2).
The ability to induce release is strong evidence that a drug is a substrate for the hDAT, as shown by the ability of methamphetamine to induce robust release (Fig. 6C).

All of the nucleosides tested (compounds 2, 4–9, 11, 16, 17, 19, 20, 22–26, 31, and 32) were shown to have no effect on [3H]DHTB or [3H]ketanserin binding to VMAT2 (SLC18A2) expressed in HEK cells (Eshleman et al., 2013) (Supplemental Fig. 2). In the [3H]DHTB binding assay 2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-1,2,3,4,5,6,7-hexahydrobenzo[a]chinolizine, a potent VMAT2 inhibitor, had a $K_i$ value of 50.4 ± 1.8 nM and in the [3H]ketanserin binding assay ketanserin had a $K_i$ value of 13.9 ± 3.8 nM.

Discussion

Multiple binding sites on the DAT have already been identified (Schmitt et al., 2013). In some cases DAT ligands of diverse structure are overlapping in their protein binding sites. The sites of either benztrpine inhibitors or cocaine overlap with the DA site (Beuming et al., 2008; Bisgaard et al., 2011). Allosteric ligands of the DAT have been detected by pharmacological methods (Rothman et al., 2009). Photoaffinity labeling of the DAT by a cocaine analog and molecular modeling have established its binding site location (Dahal et al., 2014). The combination of binding enhancement and functional inhibition by the nucleoside derivatives suggests possible allosteric binding with respect to the tropanes. The finding that these compounds enhance the affinity of the hDAT for RTI-55 further supports an allosteric mechanism for the interaction of the nucleoside derivatives with the hDAT. Furthermore, the enhancement of binding applies to two distinct probe molecules, RTI-55 and mazindol. Furthermore, the enhancement of binding by some drugs suggests that they are interacting with different transporter residues compared with residues involved in drug-induced transporter inhibition. Additionally, drug-induced conformational changes that increase binding but decrease uptake cannot be ruled out.

The interaction of nucleoside derivatives at the DAT and NET proteins is novel. None of the previously reported structural classes of small molecule modulators of these proteins resembles nucleosides. The phenomenon of enhanced binding at the hDAT of radioligands that are derived from the structure of cocaine is unprecedented (for a review, see Reith et al., 2015). This phenomenon was observed using radiolabeled tropanes, which bind with high affinity to the DAT, NET, and SERT, and radiolabeled mazindol, which like the nucleoside analogs, binds to the hDAT and NET with greater affinity than to the hSERT (Eshleman et al., 1999; Severinsen et al., 2014). At the same time, these rigid nucleosides inhibited DA uptake in a similar fashion to cocaine, suggesting an allosteric interaction with respect to the tropane binding site. Furthermore, those related nucleoside derivatives that inhibit radioligand binding at the DAT are likely to bind to the same site on the DAT as the binding enhancers, based on the close similarity of structure. Therefore, it is likely that even the inhibitors of DAT binding in this structural class are allosteric with respect to cocaine. It is also likely that the enhancement of NET binding observed for a few compounds is the result of nucleoside binding at a similar site on this transporter. The NET and DAT are on the same subfamily of SLC transporters, and there is a high degree of homology between them. Modeling has demonstrated commonality of binding of the same ligand families at different transporters (Koldso et al., 2013). Recently, four diverse classes of antidepressant drugs were crystallized in complex with a bacterial homologue of biogenic amine transporters, which serves as a model for various transport proteins. Common binding regions.
were located in association with transmembrane helices 1, 3, 6, and 8, and a mechanism for how this impedes function of the transporters by conformationally locking the helices with respect to each other was proposed (Wang et al., 2013). It will be interesting to see how the nucleoside ligands fit in the DAT, NET, and SERT proteins to inhibit transport and to modulate binding affinity in a complex manner.

The SAR of the nucleoside derivatives at the DAT and other SLC transporters is summarized in Fig. 7. This SAR of adenosine derivatives at the DAT diverges greatly from the SAR of the same compound set at ARs. For example, potent A3AR agonist N6-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide does not interact with the DAT (<10% inhibition at 10 μM; PDSP). Because the (N)-methanocarba nucleoside analogs found here to interact with the DAT are generally also potent AR ligands, we have not yet identified a nucleoside derivative with selectivity for the DAT over various receptor sites. That will be the objective of future exploration of this phenomenon, by further defining the SAR at the DAT. The dimethyl analog (compound 10) was initially intended for this purpose because it is known that disubstitution of the exocyclic amine of adenosine derivatives is generally not tolerated in AR binding (Jacobson and Gao, 2006). However, this compound proved to be inactive at the DAT. Therefore, the requirements for adenosine derivatives to interact with the DAT include at least one amine hydrogen, and if substituted on the N6 group, with small alkyl monosubstitution being most favorable. We have identified the 1-deaza modification and bulky N6 substitution as means of eliminating DAT interaction as an undesirable off-target effect in potent A3AR agonists. However, there might be situations where inhibition of neurotransmitter uptake and modulation of the A3AR agonist might be synergistic, for example, in neuropsychiatric disorders. The SERT and A3AR agonist are colocalized in midbrain serotonergic neurons (Zhu et al., 2011). We do not know if adenosine derivatives containing a native ribose ring rather than the rigid methanocarba bicyclic system will interact similarly at the DAT. The rigid ring system may eventually aid in the structural analysis of protein binding in a systematic fashion, as was done with off-target receptors (Paoletta et al., 2014).
function in the μM range. Truncation at the 4′-position in compound 23 allowed for weak inhibition of the SERT. Studies aimed at determining the mode of binding of these atypical ligands to the family of neurotransmitter sodium symporters, and additional structural modification will be performed in follow-up studies (see the (Supplemental Material of the PSDP screening results, which include off-target activities other than transporters, and (Supplemental Table 2 of the AR affinities of previously reported nucleosides).

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Authorship Contributions

Participated in research design: Janowsky, Jacobson.
Conducted experiments: Ehleman, Tosh.
Contributed new reagents or analytic tools: Jacobson, Tosh.
Performed data analysis: Ehleman, Janowsky.
Wrote or contributed to writing of the manuscript: Janowsky, Ehleman, Jacobson.

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