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# Rigid adenine nucleoside derivatives as novel modulators of the human sodium symporters for dopamine and norepinephrine

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**Nonstandard Abbreviations**: AR, adenosine receptor; IB-MECA, $N^6$-(3-iodobenzyl)adenosine-5'-*N*-methylcarboxamide; MRS1898, (1'S,2'R,3'S,4'R,5'S)-4'-{2-

chloro-6-[(3-chlorophenylmethyl)amino]purin-9-yl}-1 (methylaminocarbonyl)-bicyclo[3.1.0]hexane-2,3-diol; HEK 293, human embryonic kidney 293; mazindol, (±)-5-(4-chlorophenyl)-3,5-dihydro-2*H*-imidazo[2,1-*a*]isoindol-5-ol; METH, methamphetamine; VMAT2, vesicular monoamine transporter 2 (SLC18A2); DAT, dopamine transporter (SLC6A3); SERT, serotonin transporter (SLC6A4); NET, norepinephrine transporter (SLC6A2); Ro4-1284, 2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-1,2,3,4,5,6,7-hexahydrobenzo[a]chinolizine; RTI-55, methyl (1*R*,2*S*,3*S*)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate; WIN35,428, methyl (1*R*,2*S*,3*S*)-3-(4-fluorophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate; DHTB, dihydrotetrabenezine.

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## Abstract

Thirty two congeneric rigid adenine nucleoside derivatives containing a (N)-methanocarba ribose substitution and a 2-arylethynyl group either enhanced (up to 760% of control) or inhibited [$^{125}$I]RTI-55 binding at the human dopamine (DA) transporter (DAT) and inhibited DA uptake. Several nucleosides also enhanced [$^{3}$H]mazindol binding to DAT. The combination of binding enhancement and functional inhibition suggests possible allosteric interaction with the tropanes. The structure-activity relationship (SAR) of this novel class of DAT ligands was explored: small $N^6$-substition (methyl or ethyl) was favored, while the N1 of the adenine ring was essential. Effective terminal aryl groups include thien-2-yl (**9** and **16**, with $EC_{50}$s in [$^{125}$I]RTI-55 binding enhancement of 35.1 and 9.1 nM, respectively) and 3,4-difluorophenyl (as in the most potent DA uptake inhibitor **6** with $IC_{50}$ of 92 nM, 3-fold more potent than cocaine), but not nitrogen heterocycles. Several compounds inhibited or enhanced binding at NET and SERT and inhibited function in the micromolar range. Truncation at the 4'-position in **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 DAT, and in some cases NET and SERT, and inhibition of neurotransmitter uptake.

## INTRODUCTION

The dopamine transporter (DAT, SLC6A3) is a sodium-coupled symporter that clears, and thereby inactivates, extracellular neurotransmitter after its release (Torres et al., 2003; Kilty et al., 1991; Rice and Cragg, 2008). Defects in the function, regulation or expression of DAT are implicated in various psychiatric disorders, including attention deficit hyperactivity disorder (ADHD), depression, mood disorders and addiction (Laasko et al., 2000). Blockade of dopamine (DA) uptake is one of the main mechanisms of action of drugs of abuse such as cocaine (Figure 1A) (Sekine et al., 2003). A subjective cocaine-induced 'high' is directly proportional to the occupancy of DAT (Volkow et al., 1997). ADHD-associated sequence variants and early-onset Parkinson's disease-associated mutations of DAT have been identified (Koldsø 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 carriers (SLC) transporters, such as the γ-aminobutyric acid (GABA) transporter (Vaughan and Kuhar, 1996). Transporters of the neurotransmitters norepinephrine (NET, SLC6A2) (Axelrod et al., 1961; Wang et al., 2012) and serotonin (SERT, SLC6A4) (Felts et al., 2014) belong to the same structural family (also known as neurotransmitter sodium symporters, NSSs) and have much commonality of ligand structures with DAT. Recently, the structure of 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 was described (Wang et al., 2015). Having twelve transmembrane domains

(TMs), 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 DAT, such as the tropane radioligands WIN35,428 and RTI-55, which are analogues of cocaine (Little et al., 1993; Carroll et al., 2004; Schmitt et al., 2013). Mazindol is another ligand in a different structural class that binds to DAT and other transporters and blocks neurotransmitter uptake (Severinsen et al., 2014).

Adenosine analogues are under development as potential therapeutic agents for treating chronic neuropathic pain and other diseases (Tosh et al., 2012b; Little et al., 2015; Borea et al., 2015). Among these potent adenosine receptor (AR) agonists, are the 9-riboside IB-MECA (Stoilov et al., 2014) and the carbocyclic MRS1898 (Figure 1B) that bind selectively to the $A_3$AR subtype and reduce pain in the chronic constriction injury (CCI) model and other models of prolonged pain (Chen et al., 2012). Recently, we enlarged the set of conformationally constrained $A_3$AR agonists like MRS1898 that contain, in place of the natural D-ribose, a sterically rigidified bicyclic [3.1.0]-bicyclohexane (methanocarba) ring system, which maintains a receptor-preferred North (N) conformation (Tosh et al., 2012b). These adenosine derivatives optionally contain a rigid extension at the C2 position, consisting of an arylethynyl group, which enhances $A_3$AR selectivity. In the process of de-risking these compounds for possible development as clinical candidates, the National Institute of Mental Health Psychoactive Drug Screening Program (PSDP) (Besnard et al., 2012) conducted broad screening at receptors, ion channels and transporters. It was noted

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that some of the analogues that were potent $A_3$AR agonists bound to off-target receptors, such as serotonergic ($5HT_{2B}$ and $5HT_{2C}$) and $alpha_2$- or $beta_3$-adrenergic receptors (Paoletta et al., 2014). Furthermore, members of the series were found to modulate radioligand binding to the human (h) DAT, although these results were not included in our previous report. Here, we have characterized at 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.



## METHODS

### Materials.

FetalClone and bovine calf serum (BCS) 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 (NIDA) drug supply program. All radioligands and [$^3$H]neurotransmitters were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA) except [$^3$H]DHTB, which was purchased from American Radiolabeled Chemicals (St. Louis, MO).

### Biogenic amine transporters

Inhibition of [$^{125}$I]RTI-55 binding to, and [$^3$H]neurotransmitter uptake by, hDAT, hSERT or 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 DMEM 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). [$^{125}$I]RTI-55 (40–80 pM final concentration) and [$^{3}$H]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 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-HT, or [$^{3}$H]NE (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% polyethylenimine ([$^{3}$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 Bmax. The methods for [$^{125}$I]RTI-55 saturation binding to HEK-hDAT membranes were 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 2.7-7.8 µg. GraphPad Prism software (San Diego, CA) was used to analyze saturation curves to yield Kd and Bmax values.

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**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% DMSO and three-to-six cocaine dose-response curves in the presence of selected concentrations of the test compound 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 (San Diego, CA).

**HEK-hDAT [$^3$H]DA release assay:** The methods for characterizing drug-induced release of pre-loaded [$^3$H]DA from HEK-hDAT cells have been described previously (Eshleman et al., 2013). Drugs were perfused for 22 min during the assays.

**Vesicular monoamine transporter (VMAT2)**

Inhibition of [$^3$H]DHTB binding to the hVMAT2 in clonal cells has been previously described (Eshleman et al., 1999). Inhibition of [$^3$H]ketanserin binding to the hVMAT2 was conducted in an identical fashion except that DHTB (10 μM) was used to define non-specific binding.

**Data and statistical analyses.**

At least three independent competition experiments were conducted. GraphPAD Prism was used to analyze the data, with $IC_{50}$ values converted to Ki values using the equation ($Ki = IC_{50}/(1+ ([drug*]/Kd\ drug*))$), where [drug*] is the concentration of the labeled ligand used in the binding assays (Cheng and Prusoff, 1975). Kd values used in the equation for [$^{125}$I]RTI are as reported (Eshleman et al., 1999), and Kd values for [$^3$H]mazindol were 54, 82 and 3.9 nM for hDAT, hSERT and hNET, respectively. When a drug enhanced radioligand binding, an $EC_{50}$ value was determined. Differences in affinities, potencies or Bmax values were

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assessed by one way ANOVA using the logarithms of the Ki values for test compounds. Dunnett's multiple comparison test was used to compare the effects of test compounds with control values.

## RESULTS

Various sterically constrained adenine nucleoside derivatives (Table 1, and Table S1, Supporting information) have been synthesized and studied for their potent binding to the $A_1$AR (compound **2**) (Tosh et al., 2012a) or $A_3$AR (compounds **1**, **3** – **9**, **14** – **19**, **21** – **28**, **31**, and **32**) (Tosh et al., 2012b, 2014, 2105). Many of these $A_3$AR agonists reduce chronic neuropathic pain in a phenotypic screen, and AR binding affinities of the previously reported nucleosides are provided (Table S2, Supporting information). These rigid nucleosides were tested at 10 μM for inhibition of radioligand binding at DAT, NET and SERT in a preliminary screen by the PDSP (Besnard et al., 2012). A large fraction of those compounds tested either inhibited or enhanced the binding of radioligand at DAT (Table S1), with negligible activity at NET and SERT. Six compounds were found to enhance DAT binding using radiolabeled WIN35,428, which was confirmed in full concentration-response curves performed by the PDSP (Supporting information). 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., 2015b).

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The enlarged set of nucleoside analogues can be categorized according to the substitution at the C2 position: simple C2 derivatives (H, **21** or Cl, **1** and **2**); arylalkyne derivatives (5'-methylamides **3**-**20** and 4'-truncated **22**-**24**); and triazole derivatives (**25**-**31**). The truncated derivatives were included because 4'-truncation tends to convert selective $A_3$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 $A_3$AR (Tosh et al., 2015a). The expanded set of compounds was initially tested for modulation of binding of [$^{125}$I]RTI-55 binding to HEK cell membranes expressing the human DAT, NET or SERT.

Many of the nucleosides, particularly those bearing an extended C2 substituent potently modulated DAT binding (Figure 2A, 2B, Supplemental Figure 1). Compounds containing both a C2-fluorophenylalkyne and a $N^6$-(3-chlorobenzyl) group (**3**, **4**) were weakly inhibiting or inactive at DAT. Only one related compound, 2-chlorophenyl analogue **5**, displayed a measurable Ki value in [$^{125}$I]RTI-55 binding inhibition of 2.65 μM. Binding inhibition at DAT by $N^6$-(3-chlorobenzyl) derivatives, i.e. truncated nucleoside **22** and triazole **31**, was found using the radioligand [$^{125}$I]RTI-55. 2-Arylalkynyl-$N^6$-methyl derivatives included numerous analogues that interacted with DAT. However, inhibition by $N^6$-methyl derivative **17** was not observed using [$^{125}$I]RTI-55. Furthermore, numerous analogues greatly enhanced the binding of [$^{125}$I]RTI-55 (40-80 pM) to DAT: 2-arylalkyne 5'-methylamides **6** - **9**, **11**-**13,** **16** and **19**, truncated derivatives **23** and **24** and triazole derivative **26**. The most potent enhancers (all $N^6$-methyl or ethyl) were ($EC_{50}$, 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

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was (% enhancement): **11** (760), **6** (690), **9** (550), **8** (387), **12** (268), **13** (251) and **16** (217). Other 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-ylethynyl group, which allowed comparison of the effects of various $N^6$ groups. Di-methyl substitution of the $N^6$ amine in **10** in the 5-chlorothien-2-yl series eliminated the potent interaction with DAT observed with the corresponding monomethyl derivative **9**. Enlargement of the $N^6$-methyl group to ethyl in **11** maintained enhancement of binding ($EC_{50}$ 300±150 nM), although this $EC_{50}$ was 9-fold weaker in comparison to **9**. The $N^6$-propyl **12** and $N^6$-cyclobutyl **13** analogues were less efficacious in enhancing DAT binding at 10 μM. Further enlargement of the $N^6$ group in cyclopropylmethyl **14** and cyclobutylmethyl **15** analogues successively reduced and eliminated DAT interaction. Therefore, in the alkyne series enlargement of the $N^6$ group progressively disfavored interaction with DAT. The 5-bromothien-2-yl-$N^6$-methyl analogue **16** enhanced DAT binding with 4-fold higher potency ($EC_{50}$ 9.1±1.7 nM) and less than half of the percent enhancement in comparison to the corresponding 5-chlorothien-2-yl analogue **9**. Substitution of the terminal aryl group in the 2-arylalkynyl-$N^6$-methyl series with N-heterocycles in **17**-**19** greatly decreased the degree of interaction with DAT, with pyrazine **17** and pyrimidine **18** being essentially inactive. Replacement of the N1-nitrogen in 1-deaza analogue **20** eliminated DAT interaction, which is a major difference compared to the corresponding adenine derivative **9**. 4'-Truncation in compounds **21** – **24** resulted in maintaining weak interaction with DAT (μM inhibition or enhancement), but only when a 2-

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arylalkynyl group was present. The 2-triazole derivatives **25** – **32** were either weak or inactive in modulating DAT binding. With $N^6$-methyl, the ability of the triazole derivatives to interact with DAT depended on the terminal aryl group, with 3,4-difluorophenyl **26** and fur-2-yl **27** being weakly permissive, but 5-chlorothien-2-yl **28** being nonpermissive toward inhibition of DAT binding. Upon enlargement of the $N^6$ group in the 5-chlorothien-2-yl triazole series **29** – **31**, weak binding inhibition was observed only with the largest group, i.e. $N^6$-3-chlorobenzyl **31**.

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

Functional activity at DAT was determined using [$^3$H]DA uptake (Figure 6A). In general, both the inhibitors and the enhancers of radioligand binding at 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 ($IC_{50}$, nM): **6** (92), **9** (253), **11** (206) and **16** (229) (Figures 1, 6A). All drugs completely inhibited [$^3$H]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

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250±35 and 13.9±1.6 nM, respectively. The DA uptake inhibition among DAT binding inhibitors, such as **5**, was weak at best, and $IC_{50}$ values could not be determined.

Binding activity of the nucleoside derivatives was also measured at hNET (Figure 3) and hSERT (Figure 4). There was mostly negligible activity at SERT in [$^{125}$I]RTI-55 binding (Figure 4A, 4B) and [$^{3}$H]mazindol binding (Figure 4C, 4D) assays, but truncated 2-chlorophenyl derivative **23** inhibited SERT binding using [$^{125}$I]RTI-55 with a $K_i$ value of 5.30±0.92 μM (n = 6, Figure 4A) and serotonin uptake with an $IC_{50}$ of 5.5±1.9 μM (n = 3). In the same uptake assay cocaine and mazindol had $IC_{50}$ values of 307±49 and 40.4± 9.1 nM, respectively. Compound **5** inhibited SERT [$^{125}$I]RTI-55 binding with a $K_i$ value of 6.71±0.55 μM (n = 5, Figure 4B), but the effect on serotonin uptake was insignificant.

There was more widespread interaction of the nucleoside derivatives at NET (Fig. 3) than at SERT (Fig. 4), with several compounds modulating binding. $N^6$-Methyl derivatives that enhanced binding of [$^{125}$I]RTI-55 at hNET were ($EC_{50}$ in μM, % of control, n = 5-6): **6** (1.76±0.64, 371±67%), **9** (1.18±0.36, 386±73%), and **16** (0.670±0.200, 285±22%), while compound **5** inhibited binding ($IC_{50}$ 2.45 ± 0.59 μM). Some derivatives with $N^6$ substituents larger than methyl enhanced binding of [$^{125}$I]RTI-55 at hNET but with small maximal enhancement ($EC_{50}$ in μM, % of control, n = 3): **12** (0.90±0.32, 159±1%), **13** (0.40±0.10, 134±13%). $N^6$-Ethyl derivative **11** weakly enhanced binding at hNET, with $EC_{50}$ of 4.1±1.2 μM, up to 373±15% of control (n = 6). Curiously, enhancement of NET binding by **6**, **9** and **16** was not observed using [$^{3}$H]mazindol as radioligand (Figure 3C,3D). The greatest functional inhibition at NET was seen with 5-bromothien-2-yl derivative **16** ($IC_{50}$ 6.11±0.57

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μM). This was the only compound that modulated NET binding and also had a significant functional effect on [$^3$H]NE uptake at 10 μM. 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 μM were determined to have no significant effect on [$^{125}$I]RTI-55 binding at hNET or hSERT. Furthermore, compounds **1**, **3**, **10**, **14**, **15**, **18**, **21** and **27 - 30** at 10 μM were inactive in screening at DAT, NET and SERT in PDSP screening.

To determine potential mechanisms for enhancement of [$^{125}$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 Figure 5 indicate that Compounds **9** and **16** increase the affinity of the hDAT for the radioligand (one-way ANOVA followed by Dunnett's multiple comparisons test). However, no significant change in the Bmax was induced by either compound. Thus, the substantial increase in binding at a fixed, low concentration of radioligand can be explained as an allosteric enhancement of the affinity of the radioligand.

Effects of the nucleoside derivative **9** on inhibition of [$^3$H]DA uptake by cocaine are shown in Figure 6B. A leftward shift of the cocaine concentration-response curve is observed in the presence of increasing, fixed concentrations of **9** (1 nM to 1 μM). Average cocaine $IC_{50}$ values (nM, mean ± sem) in the presence of 0, 1 nM, 10 nM, 100 nM, 300 nM and 1 μM compound **9** were 507±57, 479 ± 78, 614±82, 460±150, 182±73 ($p<0.05$, one way ANOVA followed by Dunnett's multiple comparison), and 35±11 ($p<0.001$), respectively. Furthermore the maximal uptake is decreased in the presence of high nanomolar

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concentrations of **9**. There is no significant enhancement of DA uptake or a rightward shift of the cocaine concentration-response curve by **9**.

The inability of the nucleoside derivatives **2** and **8** to induce release of pre-loaded [$^{3}$H]DA is shown in Figure 6C. Although these two derivatives had differing effects on binding and uptake, wherein compound **2** had no effect on binding or uptake, while **8** enhanced [$^{125}$I]RTI-55 binding and inhibited [$^{3}$H]DA uptake, neither compound induced release. The ability to induce release is strong evidence that a drug is a substrate for hDAT, as shown by the ability of METH to induce robust release (Figure 6C).

All of the nucleosides tested (**2**, **4 - 9**, **11**, **16**, **17**, **19, 20, 22-26, 31, 32**) were shown to have no effect on [$^{3}$H]DHTB or [$^{3}$H]ketanserin binding to vesicular monoamine transporter 2 (SLC18A2, VMAT2) expressed in HEK cells (Eshleman et al., 2013) (Supplemental figure 2). In the [$^{3}$H]DHTB binding assay Ro4-1284, a potent VMAT2 inhibitor, had a $K_i$ value of 50.4 ± 1.8 nM and in the [$^{3}$H]ketanserin binding assay, ketanserin had a $K_i$ value of 13.9 ± 3.8 nM.

## DISCUSSION

Multiple binding sites on DAT have already been identified (Schmitt et la., 2013). In some cases DAT ligands of diverse structure are overlapping in their protein binding sites. The sites of either benztropine inhibitors or cocaine overlap with the DA site (Beuming et al., 2008; Bisgaard et al., 2011). Allosteric ligands of DAT have been detected by

pharmacological methods (Rothman et al, 2009). Photoaffinity labeling of DAT by a cocaine analogue 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 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. Further, the enhancement of binding by some drugs suggests that they are interacting with different transporter residues compared to 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 transport 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 hDAT of radioligands that are derived from the structure of cocaine is unprecedented (see Reith et al., 2015 for review). This phenomenon was observed using radiolabeled tropanes, which bind with high affinity to DAT, NET and SERT, and radiolabeled mazindol, which like the nucleoside analogues, binds to hDAT and hNET with greater affinity than to 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 cocaine binding site. Furthermore, those related nucleoside derivatives that inhibit radioligand binding at DAT are likely to bind to the same site on 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. 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 (Koldsø et al., 2013). Recently, four diverse classes of antidepressant drugs were crystallized in complex with LeuBAT, which serves as a model for various transport proteins. Common binding regions were located in association with transmembrane helices (TMs) 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.

SAR of the nucleoside derivatives at DAT and other transporters is summarized in Figure 6. This SAR of adenosine derivatives at DAT diverges greatly from the SAR of the same compound set at ARs. For example, potent $A_3$AR agonist IB-MECA does not interact with DAT (<10% inhibition at 10 μM, PDSP). Because the (N)-methanocarba nucleoside analogues found here to interact with DAT are generally also potent AR ligands, we have not yet identified a nucleoside derivative with selectivity for DAT over various receptor sites. That will be the objective of future exploration of this phenomenon, by further defining the SAR at DAT. The dimethyl analogue **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 DAT. Therefore, the requirements for adenosine derivatives to interact with DAT include at least one NH, and if substituted on the $N^6$ group, with small alkyl monosubstitution being most favorable. We have identified the 1-deaza modification and bulky $N^6$ substitution as means of eliminating DAT interaction as an undesirable off-target effect in potent $A_3$AR agonists. However, there might be situations where inhibition of neurotransmitter uptake and modulation of the $A_3$AR might be synergistic, for example neuropsychiatric disorders. SERT and the $A_3$AR 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 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., 2012).

In conclusion, we have identified a new class of ligands, i.e. rigidified adenosine derivatives containing a (N)-methanocarba ribose substitution and a 2-arylethynyl group, which appear to be moderately selective modulators of DAT within the NSS family. They alternately enhanced or inhibited binding of two diverse radioligands at the hDAT. The combination of binding enhancement and inhibition of DA uptake suggests possible allosteric binding with respect to cocaine analogues. Given the structural similarities within this family it is likely that there is a conserved binding site for this nucleoside series. The structure-activity relationship (SAR) of this novel class of DAT ligands was explored; the adenine N1 was essential and terminal aryl groups that promote DAT interaction include thien-2-yl and 3,4-difluorophenyl, but not nitrogen heterocycles. Several compounds also inhibited or enhanced

binding at NET and SERT and inhibited function in the μM range. Truncation at the 4′-position in **23** allowed for weak inhibition of the SERT. Studies aimed at determining the mode of binding of these atypical ligands to the family of NSSs, and additional structural modification will be performed in follow-up studies.

Supporting information available. PSDP screening results (including off-target activities other than transporters) and table of AR affinity of previously reported nucleosides.

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## AUTHORSHIP CONTRIBUTIONS

*Participated in research design*: A.J., K.A.J.

*Conducted experiments*: A.J.E., D. K. T.

*Contributed new reagents or analytical tools*: K.A. J., D.K.T.

*Performed data analysis*: A.J.E., A.J.

*Wrote or contributed to writing of the manuscript:* A.J., A.J.E., K.A.J.

## References

Axelrod J, Whitby LG, and Hertting G (1961) Effect of psychotropic drugs on the uptake of H3-norepinephrine by tissues. *Science* **133**:383–384.

Besnard J, Ruda GF, Setola V, Abecassis K, Rodriguiz RM, Huang XP, Norval S, Sassano MF, Shin AI, Webster LA, Simeons FR, Stojanovski L, Prat A, Seidah NG, Constam DB, Bickerton GR, Read KD, Wetsel WC, Gilbert IH, Roth BL, and Hopkins AL (2012) Nature 492(7428):215-220.

Beuming T, Kniazeff J, Bergmann ML, Shi L, Gracia L, Raniszewska K, Newman AH, Javitch JA, Weinstein H, Gether U and Loland CJ (2008) The binding sites for cocaine and dopamine in the dopamine transporter overlap. *Nature Neurosci* **11**:780-789.

Bisgaard H, M. Andreas B. Larsen, Sonia Mazier, Thijs Beuming, Amy Hauck Newman, Harel Weinstein, Lei Shi, Claus J. Loland, and Ulrik Gether (2011) The binding sites for benztropines and dopamine in the dopamine transporter overlap. *Neuropharmacology* **60**:182-190.

Borea PA, Varani K, Vincenzi F, Baraldi PG, Tabrizi MA, Merighi S, and Gessi S (2015) The $A_3$ adenosine receptor: history and perspectives. *Pharmacol Rev* 67(1):74-102.

Carroll FI, Runyon SP, Abraham P *et al* (2004) Monoamine Transporter Binding, Locomotor Activity, and Drug Discrimination Properties of 3-(4-Substituted-phenyl)tropane-2-carboxylic Acid Methyl Ester Isomers. *J Med Chem* **47**:6401-6409.

Chen Z, Janes K, Chen C, Doyle T, Tosh DK, Jacobson KA, and Salvemini D (2012) Controlling murine and rat chronic pain through $A_3$ adenosine receptor activation. *FASEB J* **26**:1855-1865.

Cheng Y, and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of an inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22:3099–3108.

Dahal RA, Pramod AB, Sharma B, Krout D, Foster JD, Cha JH, Cao J, Newman AH, Lever JR, Vaughan RA, and Henry LK (2014) Computational and biochemical docking of the irreversible cocaine analog RTI 82 directly demonstrates ligand positioning in the dopamine transporter central substrate-binding site. *J Biol Chem* 2014, **289**:29712-29727

Eshleman AJ, Carmolli M, Cumbay M, Martens CR, Neve KA, and Janowsky A (1999) Characteristics of drug interactions with recombinant biogenic amine transporters expressed in the same cell type. *J Pharmacol Exp Ther* **289**:877–885.

Eshleman AJ, Wolfrum KM, Hatfield MG, Johnson RA, Murphy KV, and Janowsky A (2013) Substituted methcathinones differ in transporter and receptor interactions. *Biochem Pharmacol* **85**:1803–1815.

Felts, B., Pramod, A.B., Sandtner, W., (...), Sitte, H.H., and Henry, L.K. (2014) The two Na+ sites in the human serotonin transporter play distinct roles in the ion coupling and electrogenicity of transport. *J Biol Chem* **289**:1825-1840.

Gao ZG and Jacobson KA (2006) Keynote review: allosterism in membrane receptors. *Drug Discov Today* **11**:191-202.

Hansen FH, Skjørringe T, Yasmeen S, Arends NV, Sahai MA, Erreger K, Andreassen TF, Holy M, Hamilton PJ, Neergheen V, Karlsborg M, Newman AH, Pope S, Heales SJ, Friberg L, Law I, Pinborg LH, Sitte HH, Loland C, Shi L, Weinstein H, Galli A, Hjermind LE, Møller LB, and Gether U (2014) Missense dopamine transporter mutations associate with adult parkinsonism and ADHD. *J Clin Invest* **124**:3107–3120. doi:10.1172/JCI73778.

Kilty J, Lorang D, and Amara S (1991) Cloning and expression of a cocaine-sensitive rat dopamine transporter. *Science* **254**:578–579.

Koldsø H, Christiansen AB, Sinning S, and Schiøtt B (2013) Comparative Modeling of the Human Monoamine Transporters: Similarities in Substrate Binding. *ACS Chem. Neurosci.* **4**:295–309.

Laakso A, Vilkman H, Alakare B, Haaparanta M et al. (2000) Striatal dopamine transporter binding in neuroleptic-naive patients with schizophrenia studied with positron emission tomography. *Am J Psychiatry* **157**:269-271.

Little KY, Kirkman JA, Carroll FI, Breese GR, and Duncan GE (1993) [$^{125}$I]RTI-55 binding to cocaine-sensitive dopaminergic and serotonergic uptake sites in the human brain. *J Neurochem* **61**:1996-2006.

Little JW, Ford A, Symons-Liguori AM, Chen Z, Janes K, Doyle T, Xie J, Luongo L, Tosh DK, Maione S, Bannister K, Dickenson A, Vanderah TW, Porreca F, Jacobson KA, and Salvemini D (2015) Endogenous adenosine $A_3$ receptor activation selectively alleviates persistent pain states. *Brain* **138**:28-35.

Paoletta S, Tosh DK, Salvemini D, and Jacobson KA (2014) Structural probing of off-target G protein-coupled receptor activities within a series of adenosine/adenine congeners. *PLoS ONE* **9**: e97858.

Penmatsa A, Wang K, and Gouaux E (2013) X-ray structure of dopamine transporter elucidates antidepressant mechanism. *Nature* **503**:85–90.

Reith ME, Blough BE, Hong WC, Jones KT, Schmitt KC, Baumann MH, Partilla JS, Rothman RB, and Katz JL (2015) Behavioral, biological, and chemical perspectives on atypical agents targeting the dopamine transporter. *Drug Alc Dep* 147:1-19.

Rothman RB, Dersch CM, Ananthan S, and Partilla JS (2009) Studies of the biogenic amine transporters. 13. Identification of "agonist" and "antagonist" allosteric modulators of amphetamine-induced dopamine release. *J Pharmacol Exp Ther* **329**:718–728.

Schmitt KC, Rothman RB, and Reith ME (2013) Nonclassical pharmacology of the dopamine transporter: atypical inhibitors, allosteric modulators, and partial substrates. *J. Pharmacol Exp Ther* **364**:2-10.

Sekine Y et al. (2003) Association of dopamine transporter loss in the orbitofrontal and dorsolateral prefrontal cortices with methamphetamine-related psychiatric symptoms. *Am J Psychiatry* **160**:1699–1701.

Severinsen K, Koldsø H, Thorup KA, Schjøth-Eskesen C, Møller PT, Wiborg O, Jensen HH, Sinning S, and Schiøtt B (2014) Binding of mazindol and analogs to the human serotonin and dopamine transporters. *Mol Pharmacol* **85**:208-217.

Stoilov RM, Licheva RN, Mihaylova MK, Reitblat T, Dimitrov EA, Shimbova KM, Bhatia G, Pispati A, Gurman-Balbir A, Bagaria BR, Oparanov BA, Fishman S, Harpaz Z, Farbstein M, Cohen S, Bristol D, Michael H Silverman MH, and Fishman P, (2014) Therapeutic Effect of Oral CF101 in Patients with Rheumatoid Arthritis: A Randomized, Double-blind, Placebo-controlled Phase II Study. *Immunome Res* **11**:1, http://dx.doi.org/10.4172/1745-7580.1000087.

Torres G, Gainetdinov R, and Caron M (2003) Plasma membrane monoamine transporters: structure, regulation and function. *Nat Rev Neurosci* **4**:13–25.

Tosh DK, Paoletta S, Deflorian F, Phan K, Moss SM, Gao ZG, Jiang X, and Jacobson KA (2012a) Structural sweet spot for $A_1$ adenosine receptor activation by truncated (N)-

methanocarba nucleosides: Receptor docking and potent anticonvulsant activity. *J Med Chem* **55**:8075–8090.

Tosh DK, Deflorian F, Phan K, Gao ZG, Wan TC, Gizewski E, Auchampach JA, and Jacobson KA (2012b) Structure-guided design of $A_3$ adenosine receptor-selective nucleosides: Combination of 2-arylethynyl and bicyclo[3.1.0]hexane substitutions. *J Med Chem* **55**:4847-4860.

Tosh DK, Paoletta S, Phan K, Gao ZG, Jiang X and Jacobson KA (2012c) Truncated nucleosides as $A_3$ adenosine receptor ligands: Combined 2-arylethynyl and bicyclohexane substitutions. *ACS Med Chem Lett* **3**:596-601.

Tosh DK, Finley A, Paoletta S, Moss SM, Gao ZG, Gizewski E, Auchampach J, Salvemini D, and Jacobson KA (2014) In vivo phenotypic screening for treating chronic neuropathic pain: Modification of C2-arylethynyl group of conformationally constrained $A_3$ adenosine receptor agonists. *J Med Chem* **57**:9901-9914.

Tosh DK, Paoletta S, Chen Z, Crane S, Lloyd J, Gao ZG, Gizewski E, Auchampach JA, Salvemini D, and Jacobson KA (2015a) Structure-based design, synthesis by click chemistry and in vivo activity of highly selective $A_3$ adenosine receptor agonists. *Med Chem Comm* **6**:555-563.

Tosh, D.K., Crane, S., Chen, Z., Paoletta, S., Gao, Z.G., Gizewski, E., Auchampach, J.A., Salvemini, D., Jacobson, K.A. (2015b) Rigidified $A_3$ adenosine receptor agonists: 1-Deaza modification maintains high in vivo efficacy. *ACS Med Chem Lett* **6**:804–808.

Volkow ND, Wang GJ, Fischman MW, Foltin RW, Fowler JS, Abumrad NN, Vitkun S, Logan J, Gatley SJ, Pappas N, Hitzemann R, and Shea CE (1997) Relationship between subjective effects of cocaine and dopamine transporter occupancy. *Nature* **386**:827-830.

Vaughan R and Kuhar M (1996) Dopamine transporter ligand binding domains. Structural and functional properties revealed by limited proteolysis. *J Biol Chem* **271**:21672–21680.

Wang, C.-I.A. et al. (2012) A second extracellular site is required for norepinephrine transport by the human norepinephrine transporter. Mol Pharmacol 82:898-909.

Wang H, Goehring A, Wang KH, Penmatsa A, Ressler R, and Gouaux E (2013) Structural basis for action by diverse antidepressants on biogenic amine transporters. Nature **503**: 141-145.

Wang KH, Penamasta A, and Gouaux E (2015) Neurotransmitter and psychostimulant recognition by the dopamine transporter. Nature **521:**322-327.

Zhu CB, Lindler KM, Campbell NG, Sutcliffe JS, Hewlett WA, et al. (2011) Colocalization and Regulated Physical Association of Presynaptic Serotonin Transporters with $A_3$ Adenosine Receptors. *Mol Pharmacol* **80**:458-465.

## Footnotes

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## FIGURE LEGENDS

**Figure 1.** Chemical structures of DAT (A) and $A_3$AR (B) ligands referred to in the text.

**Figure 2.** Drug-induced enhancement or inhibition of [$^{125}$I]RTI-55 binding and [$^{3}$H]mazindol binding to HEK-hDAT cell membranes. Assays were conducted as described in methods. n=3-9 independent experiments conducted in duplicate, except compounds with no effect where n=2. A. [$^{125}$I]RTI-55 binding of compounds **2**, **6**, **8**, **9** and **22**, B. [$^{125}$I]RTI-55 binding of compounds **4**, **5**, **7**, **16**, **17** and **19**, C. [$^{3}$H]mazindol binding of compounds **2**, **6**, **8**, **9** and **22**, D. [$^{3}$H]mazindol binding of compounds **4**, **5**, **7**, **16**, **17** and **19**. N = 3-8 independent experiments, except for drugs with no effect, n = 2.

**Figure 3.** Drug-induced enhancement of [$^{125}$I]RTI-55 binding to HEK-hNET cell membranes is not observed with [$^{3}$H]mazindol binding to HEK-hNET cell membranes. n=2-6 independent experiments conducted in duplicate, except compounds with no effect where n=2. A. [$^{125}$I]RTI-55 binding of compounds **2**, **6**, **8**, **9** and **22**, B. [$^{125}$I]RTI-55 binding of compounds **4**, **5**, **7**, **16**, **17** and **19**, C. [$^{3}$H]mazindol binding of compounds **2**, **6**, **8**, **9** and **22**, D. [$^{3}$H]mazindol binding of compounds **4**, **5**, **7**, **16**, **17** and **19**. N=3-10, except for drugs with no effect, n=2.

**Figure 4.** Lack of drug-induced enhancement of [$^{125}$I]RTI-55 binding and [$^{3}$H]mazindol binding to HEK-hSERT cell membranes. A. [$^{125}$I]RTI-55 binding of compounds **2**, **6**, **8**, **9**

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and **22**, B. [$^{125}$I]RTI-55 binding of compounds **4**, **5**, **7**, **16**, **17** and **19**. C. [$^{3}$H]mazindol binding of compounds **2**, **6**, **8**, **9** and **22**, D. [$^{3}$H]mazindol binding of compounds **4**, **5**, **7**, **16**, **17** and **19**. N = 3-7 independent experiments, except for drugs with no effect, n = 2.

**Figure 5.** Scatchard plots of [$^{125}$I]RTI-55 binding in the presence of **9** or **16**. Saturation [$^{125}$I]RTI-55 binding experiments were conducted in the absence or presence of varying concentrations of **9** (A) or **16** (B), as described in methods. Data shown are from a representative experiment, conducted in triplicate, that was replicated at least 2 times with similar results. In Scatchard analysis, the (-)reciprocal of the slope of the line is an estimate of the Kd value for the radioligand. The steeper slope in the presence of higher concentrations of drugs indicates a lower Kd and increased affinity.

**Figure 6.** Effect of compounds on HEK-hDAT functional assays: [$^{3}$H]DA uptake and [$^{3}$H]DA release. A. Effect of compounds, 6, 9, 11, 16 on [$^{3}$H]DA uptake into HEK-hDAT cells. n=3-5. B. Effect of Compound **9** (1 nM to 1 µM) on cocaine-induced inhibition of [$^{3}$H]DA uptake into HEK-hDAT cells. n=2-5 for each concentration of **9**. C. Lack of effect of Compound 2 (0.3 and 10 µM) and Compound 8 (0.3 and 10 µM), compared to methamphetamine (METH) (1 and 10 µM), on pre-loaded [$^{3}$H]DA release from HEK-hDAT cells. Data are from a representative experiment that was repeated with similar results.

**Figure 7.** Summary of SAR of the nucleoside derivatives at DAT and other SLC transporters. The structure of 3,4-difluorophenyl derivative **6** is shown with substitutions leading to thienyl derivatives **9** and **16**, and when truncated, to 2-chlorophenyl derivative **23**. Colored

regions correspond to structural features on the most potent modulators, which both enhance the binding of tropane radioligands and inhibit DA uptake. The colors correspond to: 5 (blue), $N^6$ (green), and C2-terminal aryl (yellow) substituents.

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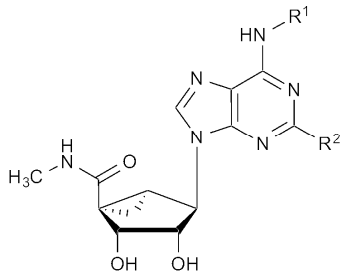
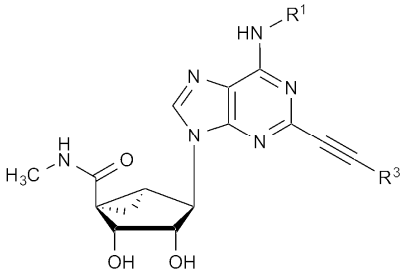
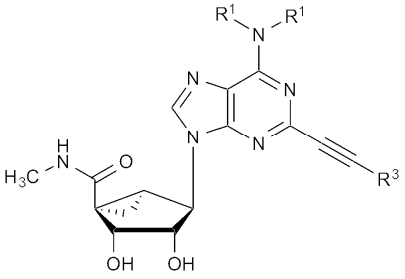
**Table 1.** Structures and modulation of binding and activity at hDAT[a] ([$^{125}$I]RTI-55, unless noted) of (N)-methanocarba adenosine derivatives, including simple C2 derivatives **1**, **2** and **21**, alkyne compounds **3 - 20** (5′-amides) and **22 – 24** (truncated), and triazole derivatives **25-32**. Each compound that produced an effect is associated with data for inhibition or for enhancement, in separate columns.



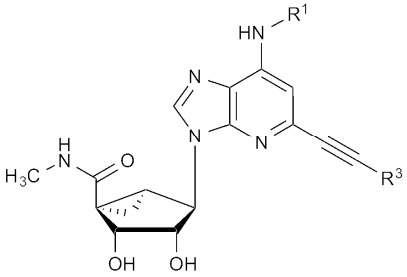
**1**



**3 – 9** and **11 – 19**



**10**



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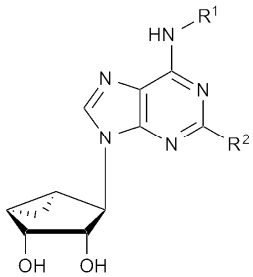
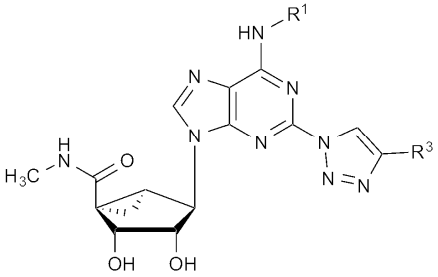
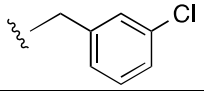
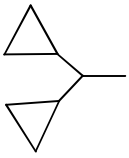
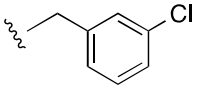
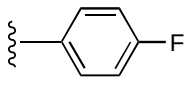
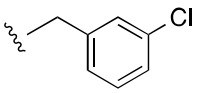
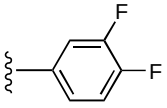


**2**, **21 – 24**



**25 – 32**

| **No.**[c] | $R^1$ | $R^2$ or $R^3$ | DAT Binding Inhibition,[a] $K_i$, nM | DAT Binding Enhancement,[a] $EC_{50}$, nM, or *% of control* | Dopamine Uptake Assay $IC_{50}$, nM[b] |
|---|---|---|---|---|---|
| **1** |  | Cl | c | c | ND |
| **2** |  | Cl | >10,000 (4) | - | >10,000 (3) |
| **3** |  |  | c | c | ND |
| **4** |  |  | >2500 [c] (5) | - | >10,000 (2) |

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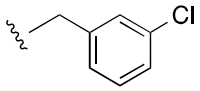
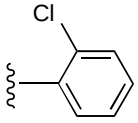
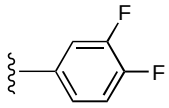
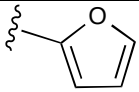
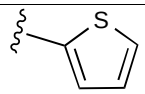
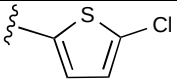
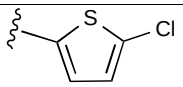
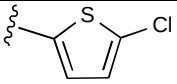
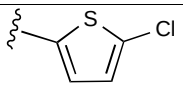
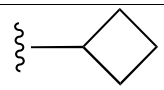
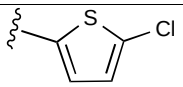
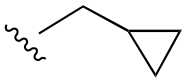
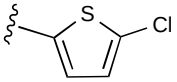
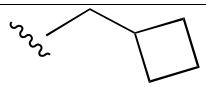
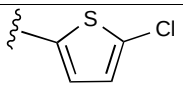
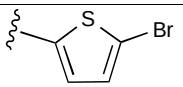
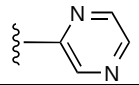
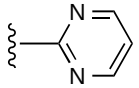
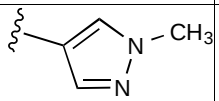
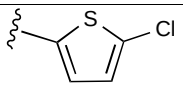
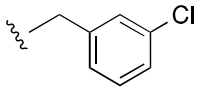
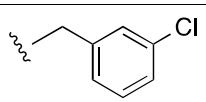
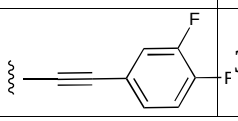
| | | | | | |
|---|---|---|---|---|---|
| **5** |  |  | 2650±300 [d] (6) | - | >10,000 (2) |
| **6** | $CH_3$ |  | - | 70±26 [e] (4), *690±180%* | 92±16 (5) |
| **7** | $CH_3$ |  | - | 540±160 [e] (5), *194±12%* | >9500 (3) |
| **8** | $CH_3$ |  | - | 446±76 (8), *387±48%* | 3170±380 (5) |
| **9** | $CH_3$ |  | - | 35.1±8.4 [e] (5), *550±110%* | 253±92 (5) |
| **10** | N,N-di-$CH_3$ |  | c | c | ND |
| **11** | $C_2H_5$ |  | - | 300±150 [e] (6), *760±260%* | 206±61 (4) |
| **12** | $(CH_2)_2CH_3$ |  | - | 1120±220 [e] (4), *268±32%* | >8700 (4) |
| **13** |  |  | - | 560±190 [e] (6), *251±30%* | >6600 (4) |
| **14** |  |  | c | c | ND |
| **15** |  |  | c | c | ND |
| **16** | $CH_3$ |  | - | 9.1±1.7 [e] (4), *217±24%* | 229±30 (3) |
| **17** | $CH_3$ |  | >10,000 [d] (2) | >10,000 [d] (2) | >10,000 (2) |
| **18** | $CH_3$ |  | c | c | ND |
| **19** | $CH_3$ |  | - | 3370±820 [e] (3), *163±14%* | >10,000 (2) |
| **20** | $CH_3$ |  | >10,000 [c] (2) | - | >10,000 (2) |
| **21** |  | H | c | c | ND |
| **22** |  |  | 3950±900 [c] (3) | - | >10,000 (3) |

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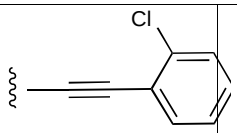
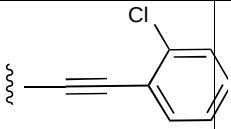
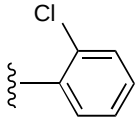
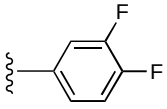
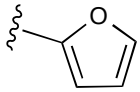
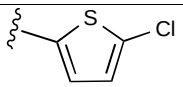
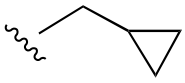
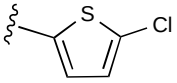
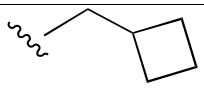
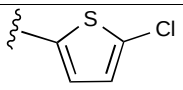
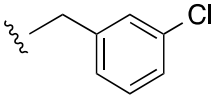
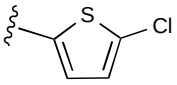
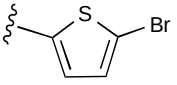
| | | | | | |
|---|---|---|---|---|---|
| **23** | $CH_3$ |  | - | 4110±540 (3), *277±36%* | >10,000 (2) |
| **24** | $C_2H_5$ |  | - | >7700 (9), *163.2±8.6%* | >10,000 (2) |
| **25** | $CH_3$ |  | >10,000 (4) | >10,000 (4) | >10,000 (2) |
| **26** | $CH_3$ |  | - | >7100 (9), *136.2±8.0%* | >10,000 (2) |
| **27** | $CH_3$ |  | ($K_i$ 4.48 μM) [d] | - | ND |
| **28** | $CH_3$ |  | c | c | ND |
| **29** |  |  | c | c | ND |
| **30** |  |  | c | c | ND |
| **31** |  |  | 7300±1700 [d] (3) | - | >10,000 (2) |
| **32** | $CH_3$ |  | >10,000 (2) | - | >10,000 (2) |

[a] Modulation of [$^{125}$I]RTI-55 binding (inhibition, enhancement or undetermined effect). Values are expressed as the mean ± SEM of n values (in parentheses).

[b] Inhibition of DA uptake in HEK cells expressing DAT. Values are expressed as the mean ± SEM of n values (in parentheses). AR affinities are given for reported compounds in Table S2 (Supporting information)

[c] Found to have negligible or no modulation of [$^3$H]WIN35,428 binding at hDAT by PDSP (see Table S1, Supporting information).

[d] Found to inhibit [$^3$H]WIN35,428 binding at hDAT by PDSP (see Table S1, Supporting information).

[e] Found to enhance [$^3$H]WIN35,428 binding at hDAT by PDSP (see Table S1, Supporting information).

[f] **6**, MRS5676; **9**, MRS5980; **11**, MRS7135; **16**, MRS7036

ND – not determined.

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Table 2: Compounds **9** and **16** increase affinity, and mazindol decreases affinity, of hDAT for [$^{125}$I]RTI-55. None of the treatment conditions had a significant effect on $B_{max}$ values.

| Treatment (Compound, conc.) | Ki (nM) (95% Confidence interval) | Bmax (fmol/mg protein) ± sem |
|---|---|---|
| Control | 1.31 (0.92-1.89) | 7040 ± 590 |
| **9**, 10 nM | 0.902 (0.58-1.39) | 7820 ± 1170 |
| **9**, 30 nM | 0.60 (0.29-1.23)* | 6960 ± 520 |
| **9**, 100 nM | 0.39 (0.15-1.01)*** | 7280 ± 540 |
| **9**, 300 nM | 0.53 (0.42-0.67)* | 7730 ± 880 |
| | | |
| Control | 1.20 (0.96-1.50) | 6460 ± 420 |
| **16**, 30 nM | 1.09 (0.50-2.38) | 7240 ± 1560 |
| **16**, 100 nM | 0.94 (0.51-1.72) | 6700 ± 410 |
| **16**, 300 nM | 0.60 (0.38-0.95)* | 7390 ± 770 |
| **16**, 1 µM | 0.58 (0.33-1.00)** | 7140 ± 580 |
| | | |
| Control | 1.24 (0.99-1.54) | 6510 ± 350 |
| Mazindol, 10 nM | 2.30 (1.66-3.18)* | 6310 ± 1150 |
| Mazindol, 30 nM | 2.77 (0.67-1.14)** | 6360 ± 480 |

*, **, *** $p<0.05$, 0.01, 0.001. All Bmax p values > 0.05.
One way ANOVA, Dunnett's multiple comparisons test.
N=3-12.

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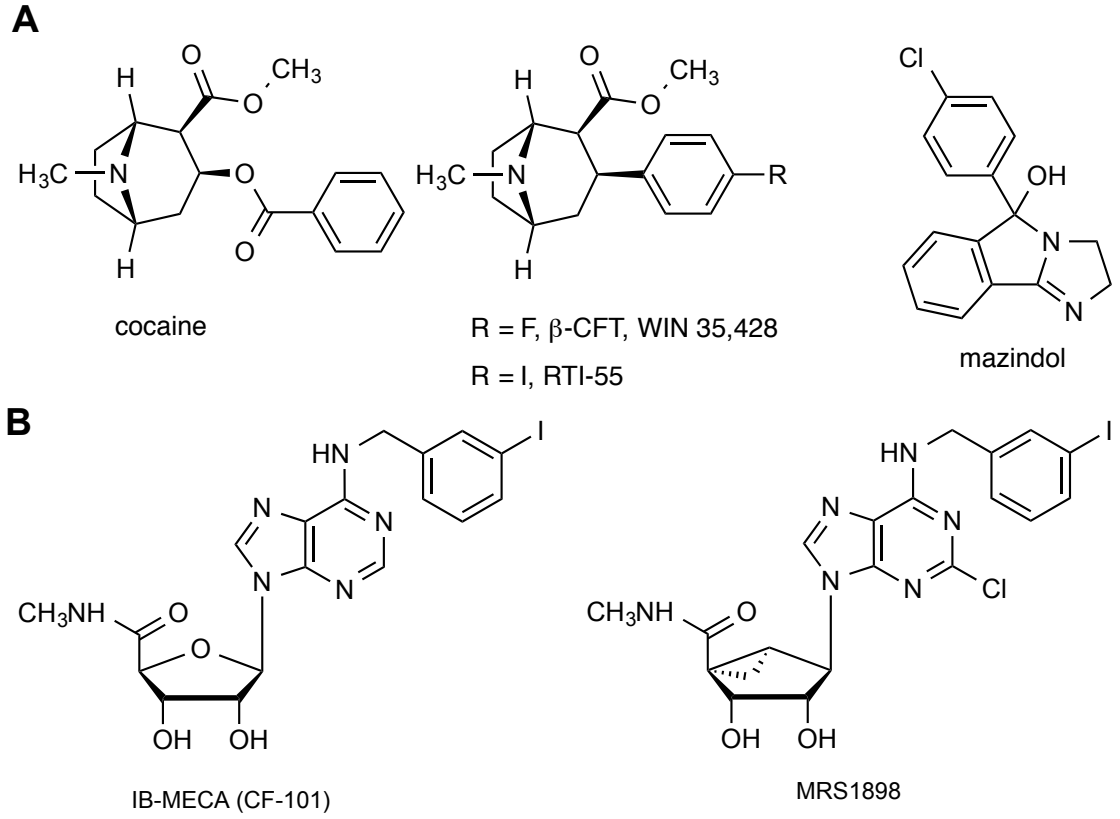
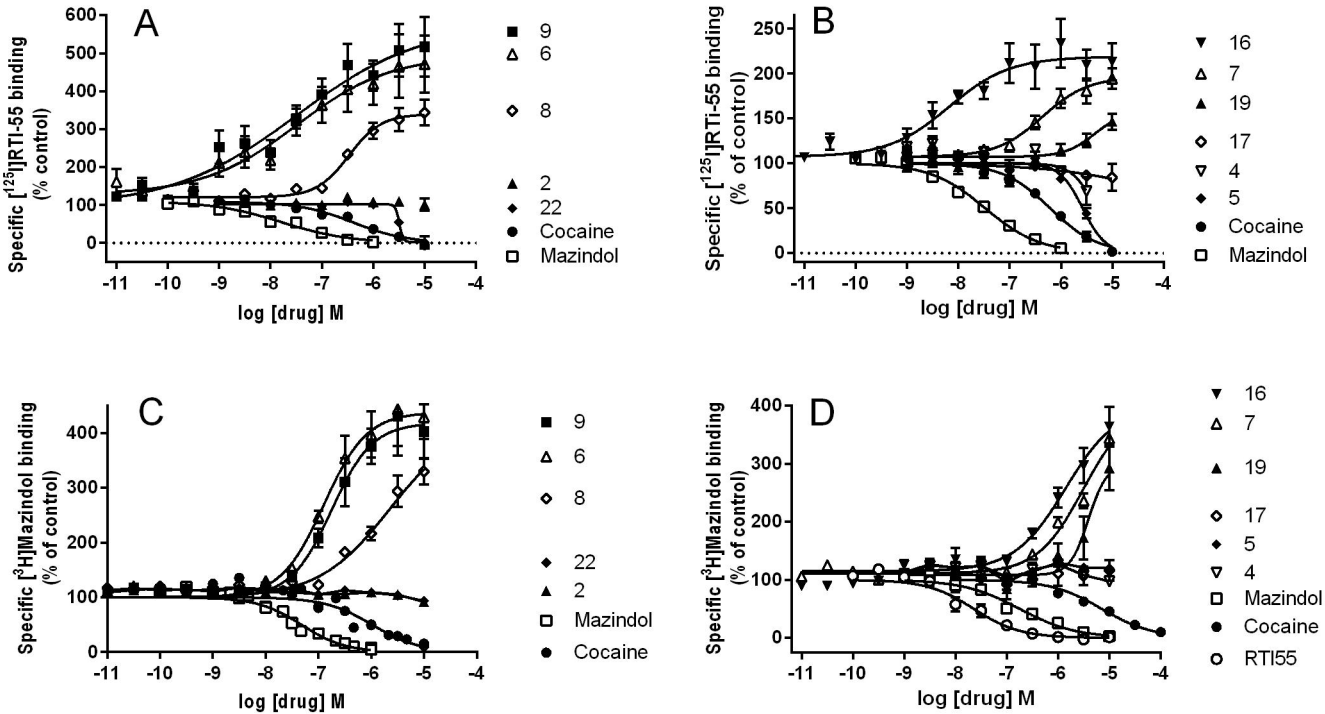
This article has not been copyedited and formatted. The final version may differ from this version.

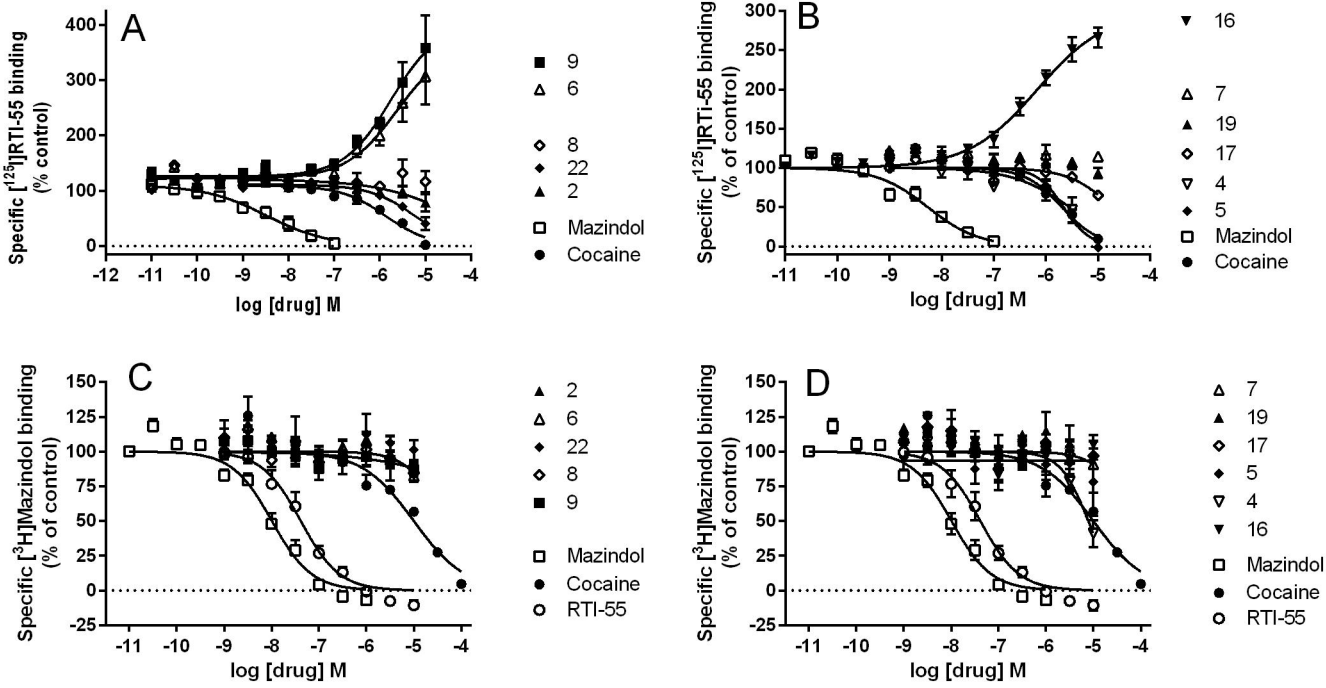
Figure 1



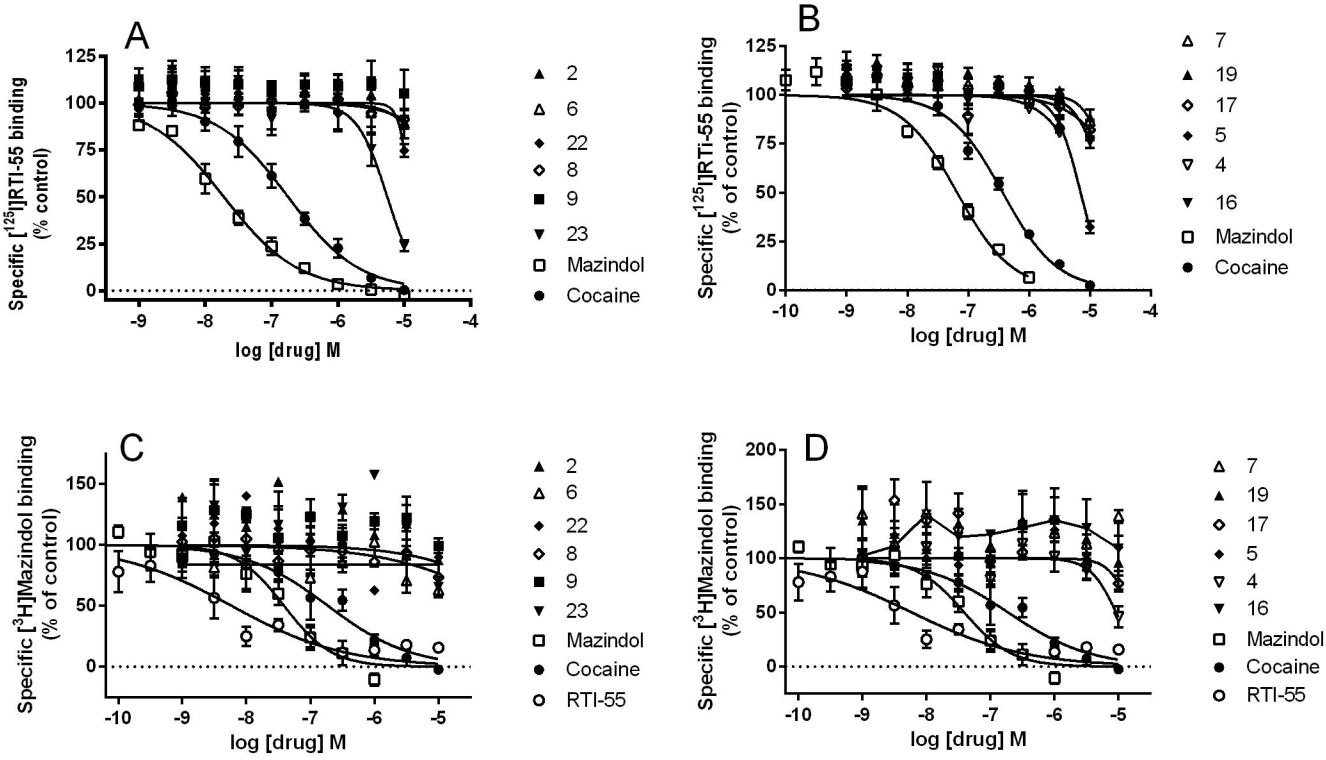
# Figure 2


A
Specific [125I]RTI-55 binding (% control)
log [drug] M
9
6
8
2
22
Cocaine
Mazindol
B
Specific [125I]RTi-55 binding (% of control)
log [drug] M
16
7
19
17
4
5
Cocaine
Mazindol
C
Specific [3H]Mazindol binding (% of control)
log [drug] M
9
6
8
22
2
Mazindol
Cocaine
D
Specific [3H]Mazindol binding (% of control)
log [drug] M
16
7
19
17
5
4
Mazindol
Cocaine
RTI55

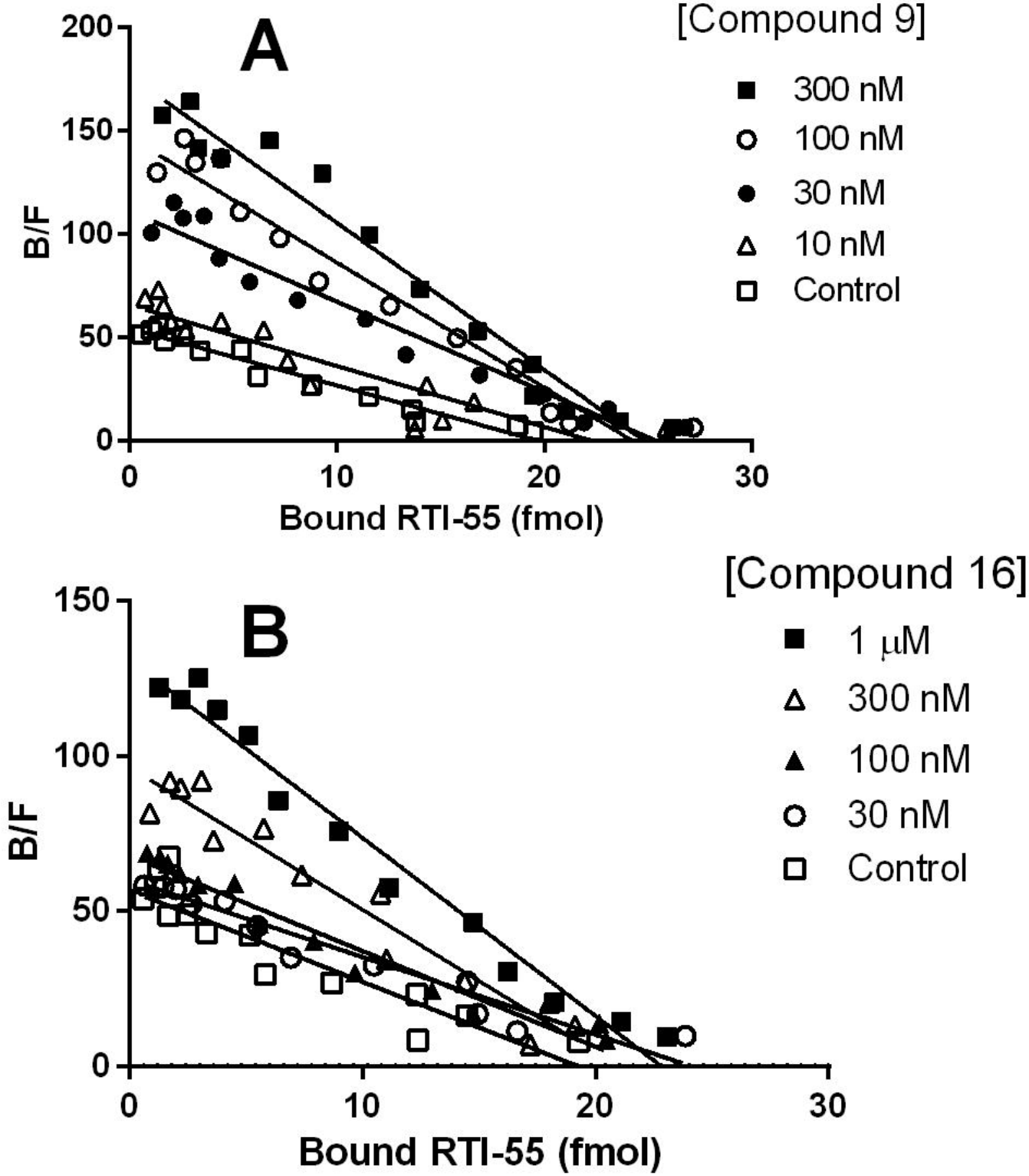
# Figure 3


A
Specific [125I]RTI-55 binding (% control)
log [drug] M
9
6
8
22
2
Mazindol
Cocaine
B
Specific [125I]RTI-55 binding (% of control)
log [drug] M
16
7
19
17
4
5
Mazindol
Cocaine
C
Specific [3H]Mazindol binding (% of control)
log [drug] M
2
6
22
8
9
Mazindol
Cocaine
RTI-55
D
Specific [3H]Mazindol binding (% of control)
log [drug] M
7
19
17
5
4
16
Mazindol
Cocaine
RTI-55

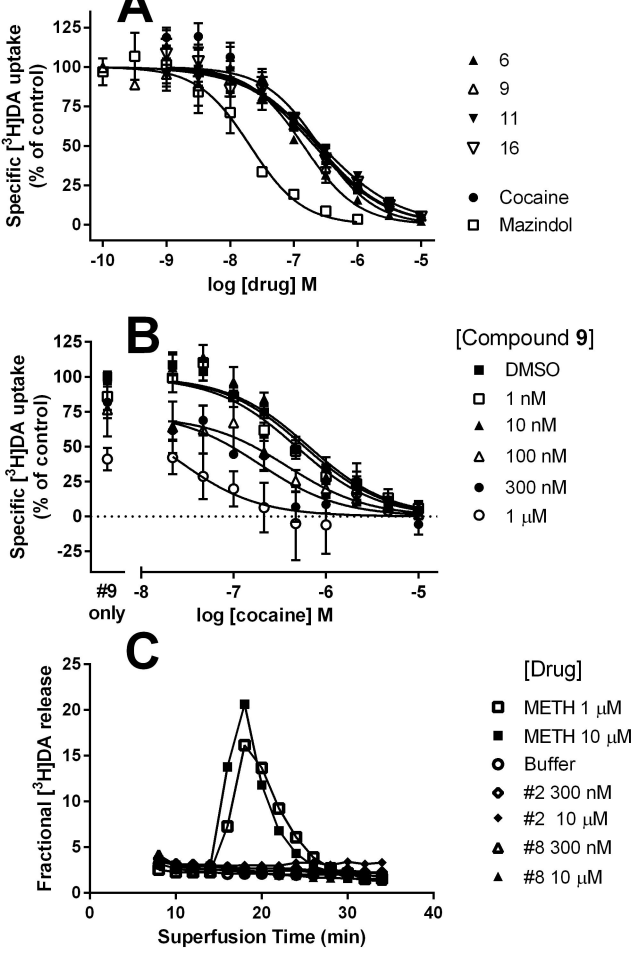
# Figure 4


A
Specific [125I]RTI-55 binding (% control)
log [drug] M
2
6
22
8
9
23
Mazindol
Cocaine
B
Specific [125I]RTI-55 binding (% of control)
log [drug] M
7
19
17
5
4
16
Mazindol
Cocaine
C
Specific [3H]Mazindol binding (% of control)
log [drug] M
2
6
22
8
9
23
Mazindol
Cocaine
RTI-55
D
Specific [3H]Mazindol binding (% of control)
log [drug] M
7
19
17
5
4
16
Mazindol
Cocaine
RTI-55

# Figure 5


A
[Compound 9]
300 nM
100 nM
30 nM
10 nM
Control
B/F
Bound RTI-55 (fmol)
B
[Compound 16]
1 μM
300 nM
100 nM
30 nM
Control
B/F
Bound RTI-55 (fmol)

# Figure 6


A
Specific [3H]DA uptake (% of control)
log [drug] M
6
9
11
16
Cocaine
Mazindol
B
Specific [3H]DA uptake (% of control)
#9 only
log [cocaine] M
[Compound 9]
DMSO
1 nM
10 nM
100 nM
300 nM
1 μM
C
Fractional [3H]DA release
Superfusion Time (min)
[Drug]
METH 1 μM
METH 10 μM
Buffer
#2 300 nM
#2 10 μM
#8 300 nM
#8 10 μM

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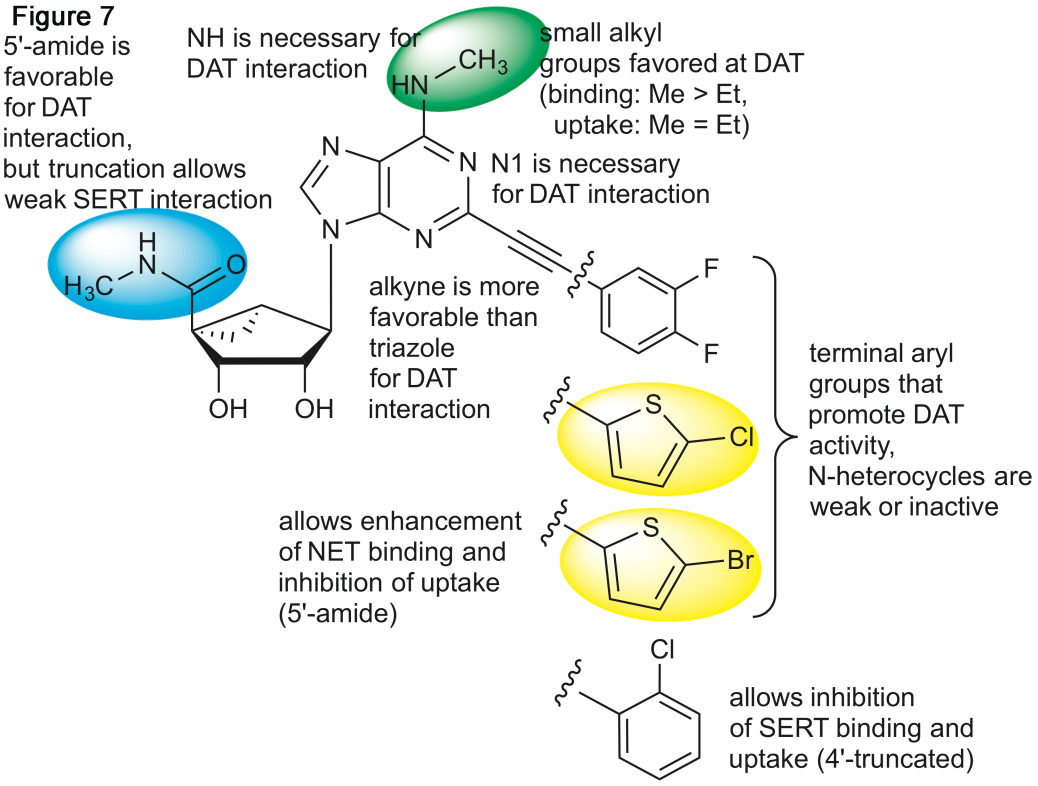


**Figure 7**