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Non-amine-based dopamine transporter (reuptake) inhibitors retain properties of amine-based progenitors

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Abstract

Without exception, therapeutic and addictive drugs that produce their primary effects by blocking monoamine transporters in brain contain an amine nitrogen in their structure. This fundamental canon of drug design was based on a prevailing premise that an amine nitrogen is required to mimic the structures of monoamine neurotransmitters and other natural products. Non-amines, a novel class of compounds that contain no amine nitrogen, block monoamine transporters in the nM range and display markedly high selectivity for monoamine transporters, but not for receptors. Non-amines retain the spectrum of biochemical and pharmacological properties characteristic of amine-bearing counterparts. These novel drugs compel a revision of current concepts of drug-monoamine transporter complex formation and open avenues for discovery of a new generation of therapeutic drugs.

Keywords: Dopamine transporter (DAT); Cocaine; Attention deficit hyperactivity disorder (ADHD); Anti-depressant; Serotonin transporter (SERT); Addiction

1. Introduction

Drug-mediated inhibition of dopamine transport elevates extracellular dopamine levels (Hurd et al., 1988), resulting in therapeutic benefit and/or psychostimulant effects. Blockade of the dopamine transporter is considered an important mechanism underlying the therapeutic benefits of anti-hyperactivity (attention deficit hyperactivity disorder or ADHD) medications (e.g. methylphenidate), smoking cessation, and antidepressant medications (e.g. bupropion). The majority of antidepressants, however, block the serotonin and/or norepinephrine transporters. Psychostimulant drugs of abuse also target the dopamine transporter and the prototype, cocaine, blocks the dopamine transporter in brain, a process considered instrumental in producing psychomotor stimulant and reinforcing effects (Reith et al., 1986, Ritz et al., 1987; Bergman et al., 1989; Madras et al., 1989a; Spealman et al., 1989).

The vast majority of therapeutic agents targeted to monoamine transporters and to brain receptors are amine-based. From the time medicinal chemists identified lead compounds from amine-bearing naturally occurring psychoactive drugs or neurotransmitters, they successfully modified the basic structures to optimize affinity and reduce side effects. The fundamental canon of incorporating an amine nitrogen into therapeutic drug structures was also sustained by practical considerations such as water solubility, ease of crystallization and absorption by the intestinal tract. In due course, a model of ligand–receptor interaction postulated the formation of an ionic bond between the amine nitrogen of a ligand and an acidic amino acid residue on the target protein. This model gained momentum with the discovery that ligand binding to the β-adrenergic receptor was mark-

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edly reduced if a highly conserved aspartic acid residue on the receptor was mutated to a neutral amino acid (Strader et al., 1988, 1989). A parallel strategy subsequently was applied to monoamine transporters, by mutating a highly conserved aspartate residue on the dopamine (D79), norepinephrine (D75), or serotonin transporters (D98), to alanine (Kitayama et al., 1992; Barker et al., 1999). These mutant transporters displayed markedly reduced affinities for both their substrates and for transport inhibitors. The diminished transport capacity reinforced the concept that ionic bond formation is as important for monoamine transporters as for receptors.

Empirical evidence to challenge this precept emerged during the design of putative cocaine antagonists, in which the amine nitrogen of phenyltropane analogs of cocaine was replaced with an oxygen or carbon atom (Madras et al., 1996, 2000; Meltzer et al., 1997, 1999, 2000, 2003). We conjectured that substitution of the amine nitrogen by an ether oxygen in the structure of phenyltropanes may attenuate the binding of cocaine, while permitting the transport of dopamine (Madras et al., 1996; Meltzer et al., 1997). With the discovery that oxa analogs of WIN 35,428 (or CFT, 2β-carbomethoxy-3β-4-fluorophenyltropane, Daum et al., 1973) blocked the dopamine transporter with high affinity, the concept that formation of an ionic bond was obligatory for transporter–ligand complex formation became less sustainable (Madras et al., 1996; Meltzer et al., 1997). The oxa analogs of monoamines nevertheless left open the possibility for hydrogen bonding to occur. This premise also became unsustainable, as carba substitutions for the amine nitrogen in tropanes also retained high affinity for the dopamine transporter (Madras et al., 2000; Meltzer et al., 1999). Accordingly, both oxa and carba analogs of phenyltropanes supported the view that neither an ionic bond (formed between the amine nitrogen and an aspartate residue on the transporter), nor a hydrogen bond, were necessary for high affinity binding of compounds to monoamine transporters.

A range of non-amines displayed high affinities in radioreceptor and functional assays but whether these counterintuitive results would generalize to pharmacological properties, behavioral effects, and bioavailability was unknown. This review summarizes recent evidence that non-amines retain a wide spectrum of properties characteristic of monoamine drugs that target monoamine transporters.

2. Materials and methods

2.1. Radioreceptor assays

Brain transporter assays and receptor screens were conducted by methods previously described (Madras et al., 1989b, 1996; Goulet et al., 2001). The binding potencies of non-amines at 30 receptors were screened by the NIMH Psychoactive Drug Screening Program (PDSP) using standard methods. Online protocols are available at http://pdsp.cwru.edu/pdsp.htm.

2.2. Positron Emission Tomography (PET) imaging

Positron Emission Tomography (PET) imaging was conducted in rhesus monkeys (*Macaca mulatta*). Monkeys weighing approximately 7 kg were anesthetized with ketamine/xylazine (15.0 and 1.5 mg/kg) and positioned prone on the imaging bed of a PC 4096 PET camera. A stereotactic head holder was used for head immobilization. Peripheral venous and femoral arterial catheters were inserted for radiopharmaceutical administration and arterial blood sampling. Tropoxene was demethylated in the C-2 position (Fig. 1) and [^{11}C]methyl was inserted by the methyl iodide reaction, followed by injection of ~ 10 mCi of [^{11}C]tropoxene (specific activity >1500 mCi/μmol) through the

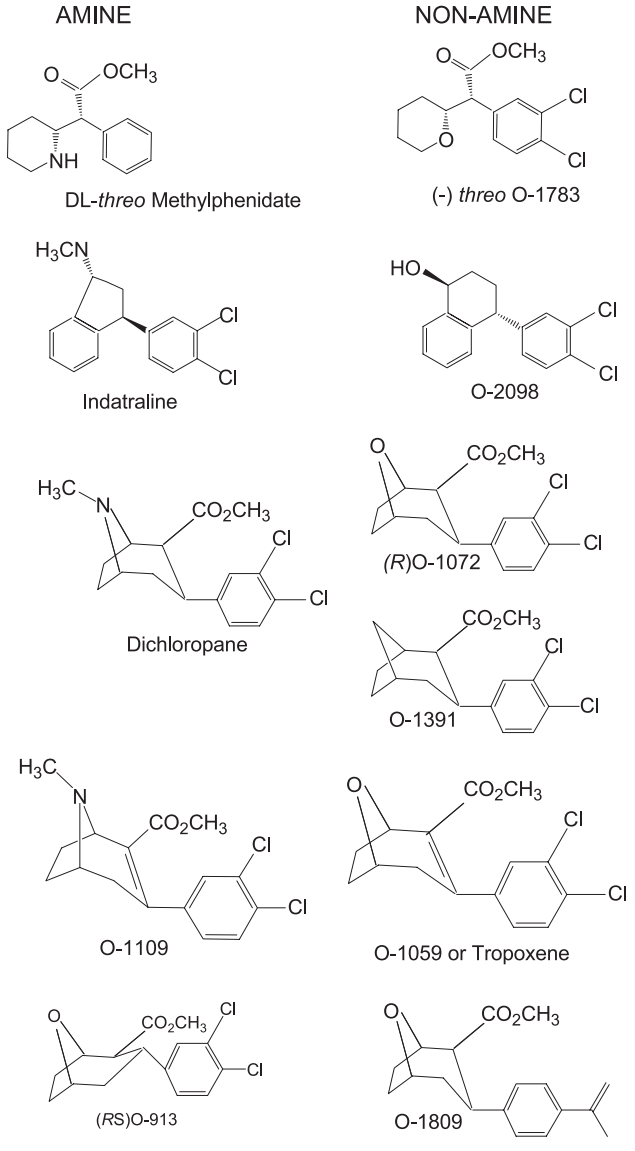


Fig. 1. Structures of novel non-amines.

venous catheter. Sequential images were acquired: 15 s frames for the first 2 min and in 1 min frames for 58 min. In parallel with imaging, arterial blood sampling was performed. At the conclusion of each imaging study, the emission and transmission images were reconstructed using a conventional filtered back-projection algorithm to an in-plane resolution of 6 mm. All projections were corrected for non-uniformity of detector response, dead time, random coincidences and scattered radiation (Fischman et al., 2001).

2.3. Microdialysis

Microdialysis was performed in Male Wistar rats (Charles River, Canada, 270–300 g) were anesthetized with ketamine (66 mg/kg) and pentobarbital (22 mg/kg). Cannulae (22-gauge guide, Plastic Products, Roanoke, VA) were implanted into the left lateral ventricle at a 10° angle with coordinates: AP (anterior-to-posterior plane) 0.8 mm posterior to the bregma, L + 2.2 mm, V 4.1 mm from the surface of the dura. Microdialysis guide cannulae (Carnegie Medicin, Sweden) were implanted on the right side of the striatum (AP 1.6 mm anterior to the Bregma, L 2.3 mm, V 3.9 mm from the surface of the dura). The animals were allowed to recover for a minimum of 3 days before studies were conducted. At the end of the experiments, probe and cannulae placement were verified anatomically by sectioning the frozen brain using a microtome-cryostat. On the day of the experiment, the steel insert from the guide cannula was replaced by a microdialysis probe (2 mm CMA/12, Carnegie Medicin, Sweden) and perfused with artificial cerebrospinal fluid (CSF, NaCl: 145 mM; KCl: 2.7 mM; $CaCl_2 \cdot 2H_2O$: 1.2 mM; $MgCl_2$: 1 mM; Na_2HPO_4: 2 mM; ascorbic acid: 0.2 mM; pH: 7.4) at a rate of 1 μl/min. The perfusion in awake, unrestrained and freely moving animals was continued for 3–4 h until the basal efflux of dopamine and its metabolites were stable for three consecutive measurements. Dialysate was collected over 30 min periods and was injected into a high pressure liquid chromatography (HPLC) system equipped with a CSC-SIL 5 μm 15 × 0.46 cm column and electrochemical detector. The mobile phase consisted of buffer (Na_2PO_4: 25 mM; $NaHPO_4$: 35 mM; citric acid: 28 mM; EDTA: 0.1 mM; lauryl sulfate: 0.076 mM; methanol: 23%; pH: 3.8) at a flow rate of 0.9 ml/min. For intracerebral ventricular injection (i.c.v.), the cannula insert was replaced with an internal cannula attached by PE-50 tubing to a Hamilton syringe. A 170 μg of O-1072 in 4.5 μl dimethyl sulfoxide was perfused slowly over a period of 2 min.

2.4. Drug discrimination

Drug discrimination studies were performed in six adult male squirrel monkeys (*Saimiri sciureus*) during daily experimental session. Animals were maintained at approximately 85% of free-feeding weight (0.74–0.82 kg), by adjusting access to food in the home cage. During experimental sessions, monkeys were seated in Plexiglas chairs enclosed in ventilated, sound-attenuating chambers, provided with white noise to mask extraneous sound. Each monkey was trained to respond differentially on the left and right levers, depending on whether cocaine or saline was injected. Briefly, after injection of cocaine, 10 consecutive responses (fixed ratio 10 or FR 10) on one lever (left for two monkeys; right for the remaining two) produced food, whereas after injection of saline, 10 consecutive responses on the other lever produced food. Responses on the inappropriate lever (e.g. the saline-associated lever when cocaine was injected) reset the FR requirement. Training sessions consisted of a variable number of components ($n = 1–4$) of the FR schedule. Each component ended after the completion of 10 FRs or 10 min, whichever occurred first. A 10-min timeout period, during which the lights were off and responses had no programmed consequences, preceded each component. During most training sessions, saline was injected during timeout periods preceding the first $n - 1$ components, and cocaine was injected before the nth component of the session. Periodically, saline was injected before all four components of a training session to prevent an invariant association between the last component and cocaine. Injections of cocaine (0.3 mg/kg) or saline were made i.m. during the fifth minute of the 10-min timeout periods. Drug discrimination training continued until a criterion of ≥ 90% of responses was made on the injection-appropriate lever for at least five consecutive sessions. Once these criteria were met, drug test sessions were conducted once or twice per week, with training sessions scheduled on intervening days. Test sessions consisted of four FR components, each preceded by a 10-min timeout period. In each component, completion of 10 consecutive responses on either lever produced food. Cocaine, CFT, and the non-amine O-913 were studied using the cumulative dosing procedure as previously described (Spealman et al., 1991). Briefly, incremental doses of the test drug were injected i.m. during the fifth minute of the timeout periods preceding sequential components of the FR schedule, permitting a four-point cumulative dose–effect curve to be determined in a single session. Up to five different doses were studied by administering overlapping ranges of cumulative doses during test sessions on different days. Saline was included as the first injection in at least one test session.

2.5. Animal care and treatment

Animal care and treatment were supervised by veterinarians under the guidelines and in accordance with "Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, National Academy Press, Washington, DC, 1996. An animal care protocol was approved by the Harvard Animal Care Committee, and was in compliance with the Harvard Medical School animal

management program, an institution accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC).

3. Results

3.1. Non-amine and monoamine tropanes: structure–activity relationships

The basic structure of the high affinity phenyltropane analog of cocaine, WIN 35,428 (2β-carbomethoxy-3β-4-fluorophenyltropane), was used as a template for the design of non-amines. Comparisons of amines and non-amines were explored by investigating the influence of substituents on the aromatic ring of tropanes, the 3α vs. 3β placement of the aromatic ring of aryltropanes, replacement of a saturated with a 2,3-unsaturated bond in the tropane skeleton, replacement of an amine nitrogen with an ether oxygen or a methylene.

Initially, the most significant finding was that an amine nitrogen was not essential for formation of drug–transporter complexes and for blockade of monoamine transporters (Table 1, Fig. 1, Madras et al., 1996; Meltzer et al., 1997, 2003). The oxa-based non-amines inhibited radioligand binding to monoamine transporters in a concentration-dependent and saturable manner. Several compounds exhibited low nM affinity for monoamine transporters and were within the same range of potencies of their amine-based progenitors (Table 1). For example, O-1072 (active enantiomer of O-914) bound the dopamine transporter with high affinity (IC_{50}: 3.69 ± 0.39 nM) as well as the serotonin transporter (IC_{50}: 4.66 ± 1.24 nM), O-1914 at the norepinephrine transporter, 27 nM. At the dopamine transporter, O-1072 was more potent than the therapeutic drugs methylphenidate (IC_{50}: 17.2 ± 2.04 nM), cocaine (IC_{50}: 150 ± 18 nM), benztropine (312 ± 1.1 nM, or bupropion (1130 ± 268 nM). At the serotonin transporter, the affinities of O-1072 and O-1809 (IC_{50}: 4.66 and 10.2 nM, respectively) were within the same range as the potent antidepressant drugs fluoxetine and citalopram (IC_{50}: 2.6 and 2.43 nM, respectively).

In contrast to aryltropanes, halogen substituents on the aromatic ring were necessary for high affinity binding (Fig. 1, Table 2, top). The non-halogenated oxa analog of O-1072, O-905 bound the dopamine transporter in the μM range (IC_{50}: 1990 ± 247 nM), whereas the parent 8-aza phenyltropane analog, WIN 35,065-2 (2β-carbomethoxy-3β-fluorophenyltropane), bound with 30-fold higher affinity (IC_{50}: 65.1 ± 11.7 nM). The rank order of potency for the halogenated non-amines, Cl_2>Cl>F>H, corresponded to the rank order observed previously with the parent nitrogen compounds (Table 2, top, Madras et al., 1989b; Meltzer et al., 1993, 1997), although non-amine affinities spanned a wider range of values (3–1990 nM) than in the amine nitrogen-containing series (1–65 nM, Table 2, top). The replacement of an amine nitrogen with an oxygen or carbon atom significantly increased the relative contributions of aryl substituents to binding affinity in this family of compounds.

The potencies of O-913, the 3α epimer of O-914 (a 3β racemic form of O-1072) were similar (Fig. 1, Table 1). In stark contrast, the 3β-epimer of cocaine is 60 times more potent than the α-epimer (Carroll et al., 1992). Within the benztropine series, carbomethoxybenztropine analogs are active only in the 3α-diphenylmethoxy form (Meltzer et al., 1994). Unlike cocaine and benztropine, the

Table 1
Comparison of amine and non-amine affinities that block dopamine and serotonin transporters

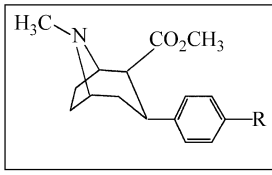
Amine	Dopamine transporter IC_{50} (nM)	Non-amine	Dopamine transporter IC_{50} (nM)
Methylphenidate$_{(2R,R')\text{DL-threo}}$	17 ± 2.04	O-1783$_{(R,R')\text{D-threo}}$[a]	17 ± 1.3
Indatraline (Lu 19-005)	2 ± 0	O-2098	20 ± 3.49
Dicholoropane (O-401)	1.09 ± 0.02	O-913-3α	3.08 ± 0.07
		O-914-3β	3.35 ± 0.55
	–	O-1072-3β(1R)	3.69 ± 0.39
	–	O-1391-3β	13.5 ± 0.9
O-1109	1.16 ± 0.17	Tropoxene (O-1059)	4.59 ± 0.57
WIN 35,428 (CFT)	12 ± 1.2		–
(−)-Cocaine	150 ± 18		
Benztropine	312 ± 1.1		–
Bupropion	1130 ± 268		–
	Serotonin transporter IC_{50} (nM)		Serotonin transporter IC_{50} (nM)
Citalopram	2.43 ± 0.33	O-1072	6.52 ± 2.91
Fluoxetine	2.60 ± 0.15	O-1809	10.2 ± 2.1

Affinities (means ± S.E.M., n = 2–4) for the dopamine transporter ([^{3}H]CFT/WIN 35,428) and serotonin transporter ([^{3}H]citalopram) were measured by radioreceptor assays in striatum of cynomolgus (*Macaca fascicularis*) or rhesus (*M. mulatta*) monkey. Fig. 1 displays structures.

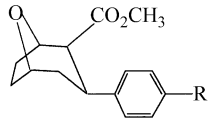
[a] The affinity of the racemic mixture O-1730 (DL-threo), corresponding to methylphenidate: 29.1 ± 5.05 nM.

Table 2
Comparison of non-amine and amine-based tropane affinities

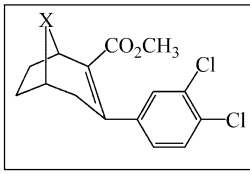
AMINES

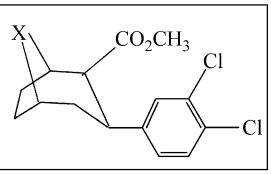


OXA NON-AMINES



Compound	Dopamine transporter (IC_{50}: nM)	R	Compound	Dopamine transporter (IC_{50}: nM)
WIN 35,065-2	65	H	O-904 (±)	4730
WIN 35,428	12	F	O-895 (±)	546
0-371	1.4	Cl	O-916 (±)	10
O-401	1.09	3,4-Cl_2	O-1072 (1R)	3.69





Compound	Dopamine transporter	Serotonin transporter IC_{50}: nM	X	Compound	Dopamine transporter IC_{50}: nM	Serotonin transporter
(1*R*)-O-1109	1.16	867	CH_3N	(1*R*)-O-401	1.09	2.47
O-1014	12.3	1960	O	O-914	3.35	6.52
O-1231	5.5	4580	C	O-1232	11.1	214

Top: Influence of aromatic substituents. Bottom: Influence of aza, oxa, carba substitutions.

3α-epimers of phenyltropane analogs (amine or non-amine) assume a "boat", not a "chair" conformation. This form may allow for potent binding of both the 3α- and 3β-epimers.

Another unanticipated discovery was that replacement of the amine nitrogen with a carba atom retained high affinity binding of non-amines (Fig. 1, Table 2, bottom). In this regard, carba compounds were of similar potency as their aza or oxa progenitors, indicating that even hydrogen bonding was not necessary to confer high affinity transporter binding of this series. The development of 2,3-unsaturated aryl derivatives increased dopamine/serotonin transporter selectivity considerably (Table 2, bottom left). Unsaturated cyclic non-amines that serve as pheromones occur naturally in nature (e.g. dehydro-*exo*-brevicomin, referred to in Madras et al., 1996), and the unsaturated 2,3 enes were intermediates in the synthesis of the saturated phenyltropanes.

To address previous speculation on whether the structure of aryltropanes is essential for non-amines (Madras et al., 1996), we then explored whether the nitrogen could be exchanged for oxygen or methylene in other potent monoamine transport inhibitors and still retain high affinity. Non-amine analogs of methylphenidate and indatraline (Lu 19-005, Arnt et al., 1985) also displayed high affinity for monoamine transporters, indicating that the basic tropane structure is not a prerequisite for high affinity binding of non-amines. Compared with the parent compound methylphenidate, the racemic di-halogenated non-amine analog O-1730 was pf slightly lower potency (IC_{50}: 29.1 ± 5.05 nM), whereas the active enantiomer O-1783 (2R, 2′R) was of similar potency (IC_{50}: 17 ± 1.3 nM vs. methylphenidate: 17.2 ± 2.04 nM, Fig. 1, Table 1). The oxa compound O-2098 was 10-fold less potent than the amine-based progenitor indatraline (Lu 19-005), one of highest affinity compounds at the dopamine transporter (Arnt et al., 1985). Not all non-amines displayed high affinity for the dopamine transporter as 8-oxa derivatives of cocaine are weak ligands for monoamine transporters (Simoni et al., 1999; Kozikowski et al., 1999).

3.2. Affinities of non-amines for receptors

The remarkable selectivity of non-amines for monoamine transporters was highlighted by their low affinities for various cloned and expressed receptors (Table 3). These included human or rodent 5-HT serotonin receptors (5-$HT1_A$, 5-HT_{1D}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, 5-HT_{5a}, 5-HT_6, 5-HT_7 receptors); dopamine receptors (D_1-, D_2-, D_3-, D_4-, D_5-receptors); muscarinic acetylcholine receptors (M_1-, M_2-, M_3-, M_4-, M_5-receptors); α-adrenoreceptors (α-2A, α-2B, α-2C, α-1A, α-1B receptors); β-adrenoreceptors (β-1, β-2); opioid receptors (μ-, κ-, δ-opioid receptors): and H_1 histamine receptors. All receptors were cloned from human

Table 3
Affinities of non-amines for various receptors

Receptor	O-401	O-913	O-1072	O-1391	O-1783	O-1809	O-2098
5-HT$^{a}_{1A}$	6.8	9.9	11	13	18	36	36
5-HT$^{a}_{1Da}$	11	1.4	−2.8	−12	−14	−13	−11
5-HT$^{a}_{1Db}$	34	−37	−26	−7.3	−30	−35	−22
r5-HT$^{a}_{2A}$	−12	−28	12	−8.8	−4.8	−7.2	24
r5-HT$^{a}_{2C}$	5.8	17.8	14	7.3	0	13	25
5-HT$^{a}_{3}$	−11	−13	−17	−1.7	4.1	5.4	11
5-HT$^{a}_{5a}$	5.2	6.6	9.5	−13	−14	−3	−7
5-HT$^{a}_{6}$	35	35	29	14	30	14	18
5-HT7^{a}	13	13	0	−18	−8	−6	−2
D$^{b}_{1}$	−26	0	−21	−^{c}	11	21	16
rD$^{b}_{2}$	4.7	0	6.0	4.8	−2.5	5.0	−5.0
D$^{b}_{3}$	10	14	22	−9	0	−6.3	−2
D$^{b}_{4}$	−1	−4	7	−9	0	−6.3	−2
D$^{b}_{5}$	−3	−2	−^{c}	−18	−8	−6	−2
M$^{d}_{1}$	13	−0.16	16	9.5	7.5	8.0	3.6
M$^{d}_{2}$	8.7	9.4	11	9.7	1.5	−6.7	−4.0
M$^{d}_{3}$	5.4	2.4	8.1	7.6	6.2	5.1	4.9
M$^{d}_{4}$	−5.4	6.3	1	28	8.1	6.0	10
M$^{d}_{5}$	1	19	25	13	9.6	22	16
α $^{e}_{2A}$	4.3	−7.0	−15	1.8	6.0	1	9.6
α-$^{e}_{2B}$	6.1	−3.6	−4.8	−2.8	0	1	−14
α-$^{e}_{2C}$	1.2	−3.5	3.2	1	2.3	5.9	21
α-$^{e}_{1A}$	21	23	4.4	1	3.2	4.9	20
α-$^{e}_{1B}$	−5.5	7.5	−8.0	5.6	−7.7	−3.7	4
rβ-$^{f}_{1}$	−5.3	21	−1.8	−1.9	−5.6	−4.9	−1
rβ-$^{f}_{2}$	0	3.9	0	0	1.5	2.3	2.5
μ-^{g}	5	4	0	13	5	3	23
δ-^{g}	10	17	33	7	3	6	–
κ-^{g}	31	34	23	30	23	–	–
rH$^{h}_{1}$	55	10	16	12	2.6	12	3.4

Data represent the mean % inhibition of binding to each receptor at a compound concentration of 10 μM, with significant inhibition >50%. Stimulation of binding is seen in cases designated as negative inhibition (−) if, on occasion, compounds at high concentrations non-specifically increase binding. All cloned receptors are human receptors unless otherwise specified (r: rodent).

[a] 5-HT: serotonin receptor.

[b] D: dopamine receptor.

[c] A 10 μM of compound were inactive at both the D_1 and D_5 dopamine receptor.

[d] M: muscarinic acetylcholine receptor.

[e] α: α-Adrenoceptor.

[f] β: β-Adrenoceptor.

[g] Opioid receptor.

[h] H1: histamine H1 receptor.

DNA with the exception of those designated "rat" in Table 3). Tested at 10 μM, non-amines displaced less than 50% of the binding of radioligands at these receptors. Non-amines screened included those selective for the dopamine transporter (O-913, O-1072, O-1391), the methylphenidate analog O-1783, the indatraline analog O-2098, a serotonin transporter-selective compound O-1809, and the carba-based non-amine O-1391. Interestingly, dichloropane (O-401) the amine-based progenitor of O-1072 and O-914 also displayed marked selectivity for monoamine transporters and low affinity for biogenic amine receptors. It should be noted that 10 μM levels of O-913, O-1072, and O-1809 displayed 25–40% functional inhibition of the nicotinic cholinergic receptor, as measured by the $^{86}Rb^{+}$ efflux assay.

3.3. Brain distribution of the non-amine [^{3}H] tropoxene: in vitro autoradiography

The distribution of the non-amine [^{3}H]tropoxene in brain (De La Garza et al., 1999) corresponded closely with the distribution of the amine-based selective dopamine transporter probe [^{3}H]WIN 35,428 (CFT). A high and positive correlation was obtained by comparing brain regions that accumulate [^{3}H]WIN 35,428 (CFT, Canfield et al., 1990; Kaufman et al., 1991; Madras et al., 1989a,b), and accumulate the non-amine [^{3}H]tropoxene (Fig. 2). The distribution of [^{3}H]tropoxene also corresponded with the brain distribution of the dopamine transporter previously detected with antibodies (Ciliax et al., 1995; Freed et al., 1995; Nirenberg et al., 1996, 1997a,b) to the dopamine transporter. The high specificity of [^{3}H]tropoxene for the dopamine transporter suggests that, in the absence of an amine nitrogen, compounds targeted to transporters can accumulate selectively in dopamine transporter-rich brain regions.

3.4. Brain distribution of [^{11}C]tropoxene, measured with PET

Based on these findings, we reasoned that other characteristic properties of monoamine transport inhibitors may be retained by non-amines. To affect dopamine transport in vivo, a compound must cross the blood–brain barrier and accumulate in brain regions expressing the dopamine transporter. Although in vitro autoradiography indicated that [^{3}H]tropoxene distributes selectively to dopamine-rich brain areas (De La Garza et al., 1999), this technique does not predict in vivo brain bioavailability or distribution. When converted to a PET imaging ligand, [^{11}C]tropoxene crossed the blood–brain barrier in vivo and accumulated in the dopamine-rich striatum (Fig. 3). The relatively low striatum/cerebellum ratio (2.5) contrasted with >50-fold striatum/cerebellum ratio observed by in vitro autoradiography. A relatively high rate of peripheral metabolism of [^{11}C]tropoxene (not shown) may account for this modest selectivity. Nevertheless, the results indicate that non-amines can access

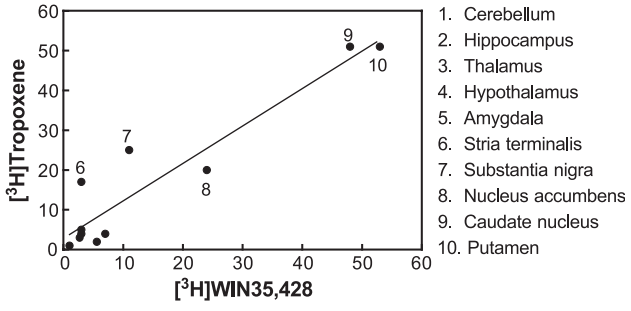


Fig. 2. Comparison of the in vitro autoradiographic distribution of the amine [^{3}H[]WIN 35,428 and of the non-amine [^{3}H]tropoxene. Each brain region is expressed as a ratio of binding site density relative to density in the cerebellum. Data for [^{3}H[]WIN 35,428 (Kaufman et al., 1991) and for [^{3}H]tropoxene (De La Garza et al., 1999) have been reported previously.

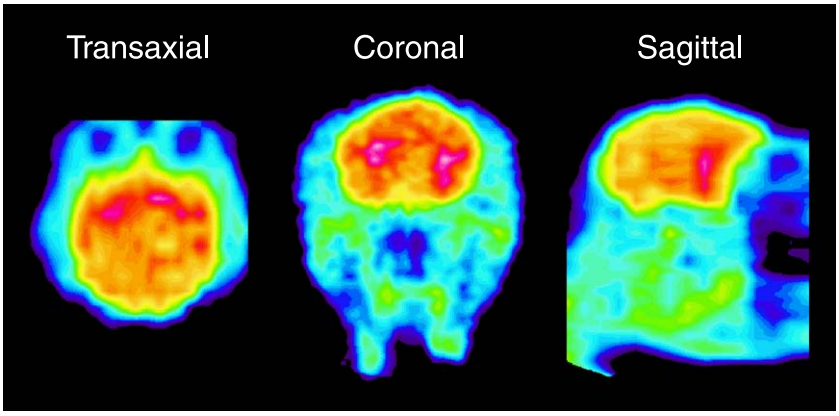


Fig. 3. Brain distribution of the non-amine [^{11}C]tropoxene as measured by positron emission tomography (PET) imaging. The PET ligand [^{11}C]tropoxene was administered i.v. to two rhesus monkeys (*M. mulatta*) and serial PET images were acquired for 90 min. At 30 min, accumulation of [^{11}C]tropoxene was most prominent in monkey striatum. Rapid wash-out from the striatum and other brain regions was apparent and may be attributable to rapid metabolism.

dopamine-rich brain regions in vivo, warranting investigation of their pharmacological properties.

3.5. Non-amine effects on extracellular dopamine levels in rodent striatum

In vivo, monoamine-based dopamine transport inhibitors produce psychomotor stimulation in rodents presumably by increasing extracellular dopamine levels in brain (Hemby et al., 1995; Hurd et al., 1988; Simoni et al., 1999; Tolliver et al., 1999). The effects of non-amines on extracellular dopamine levels were monitored by microdialysis in rodent striatum. Non-amines were administered either by infusion into intracerebral ventricles (i.c.v.) or by intraperitoneal injection (i.p.). Within 3 min of i.c.v. infusion of (*R*)-O-1072 (170 μg), animals became hyperactive and hyperreactive to noise and this behavior lasted for 1 h. Signs of dopaminergic activity were manifest by circling behavior, stereotypies (head nodding and sniffing) and rearing (not shown). At 30 min, extracellular levels of dopamine rose to 193% of control values, fell to 114% at 2 h and displayed a secondary persistent rise to 149% at 3 h (Fig. 4A). A slight increase in dopamine metabolites followed this pattern with maximum levels of homovanillic acid (128%) and dihydroxyphenylacetic acid (107%). Elevated dopamine concentrations cleared gradually and did not return to baseline levels even at 3 h. O-1072 also binds to the serotonin transporter and levels of the serotonin metabolite, 5-hydroxyindoleacetic acids increased to 121% at 3 h. The secondary rise in dopamine levels may reflect release of these lipophilic compounds from depot sites. To test whether peripherally administered non-amines elevated extracellular dopamine levels, (*RS*)-O-913 (3-α epimer of (*R*)-O-1072) was administered i.p. to rodents (0.6 mg/kg). It produced a qualitatively similar increase in extracellular dopamine levels (Fig. 4B). The maximum rise occurred 30 min after the peak levels produced by O-1072 and the increase was 160% of control values. Secondary fluctuations above

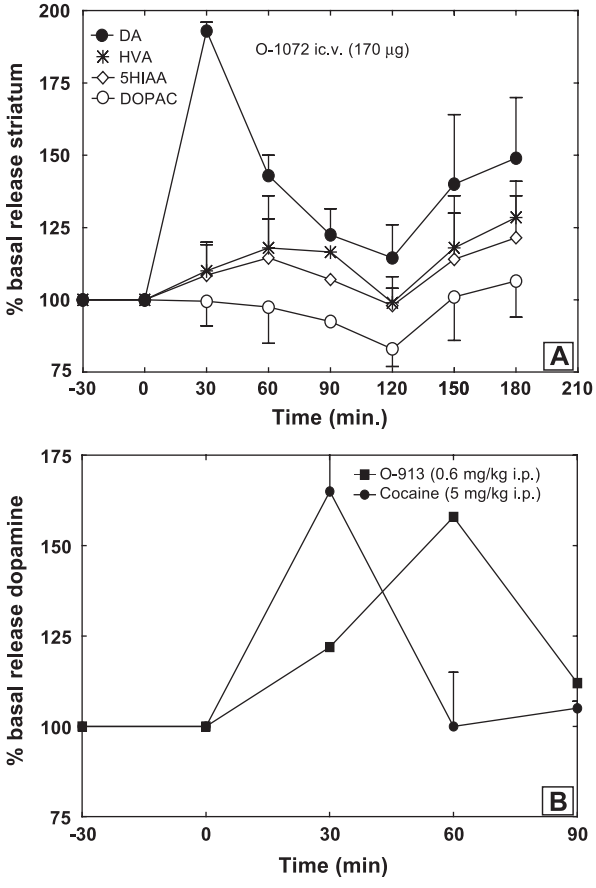


Fig. 4. Effects of non-amines and cocaine on extracellular dopamine levels in rat brain striatum, as measured by microdialysis. (A) Left panel: The non-amine O-1072 was administered to male Wistar rats (170 μg, i.c.v., $n=2$) via a microdialysis probe. O-1072 was perfused via intracerebral ventricular injection over a period of 2 min. Microdialysis samples were taken for 3 h to measure extracellular dopamine, the dopamine metabolites homovanillic acid (HVA), dihydroxyphenylacetic acid (DOPAC) and the serotonin metabolite 5-hydroxyindoleacetic acid (5HIAA) by HPLC. (B) Right panel: The non-amine O-913 (0.6 mg/kg, $n=2$) or cocaine (5 mg/kg; $n=4$) was administered to male Wistar rats intraperitoneally. Each point is the mean of 2–4 experiments.

baseline values occurred several hours later. O-913 (1 mg/kg) was more potent than (−)-cocaine (5 mg/kg), as the maximum rise in extracellular dopamine levels, 130%, was similar for both compounds. Cocaine induced a rapid surge in extracellular dopamine, followed by a precipitous decline below baseline levels. In contrast, O-913 produced peak elevations of dopamine 30 min later than cocaine, indicating different pharmacokinetic properties of the two compounds. As the rapid rise and decline of extracellular dopamine levels may contribute to the reinforcing properties and abuse liability of cocaine, the slower onset and offset effects of O-913, compared with those of cocaine, suggest that O-913 may be less reinforcing than cocaine.

3.6. Subjective effects of a non-amine in a drug discrimination paradigm

Drug discrimination procedures can provide relevant information on brain substrates of specific drugs (Johanson and Fischman, 1989). Cocaine serves as a discriminative stimulus in monkeys, with a major component of the stimulus generally attributable to cocaine-induced blockade of the dopamine transporter (Spealman et al., 1989). Dopamine transport inhibitors typically substitute fully for the discriminative stimulus effects of cocaine, whereas norepinephrine or serotonin transport inhibitors generally fail to engender cocaine-like effects in primates (Spealman et al., 1991; Kleven et al., 1990; Witkin et al., 1991). In our studies, monkeys that were trained to discriminate cocaine from vehicle made an average of $\geq 90\%$ responses on the cocaine-associated lever after injection of ≥ 0.3 mg/kg and $\leq 10\%$ responses on the cocaine-associated lever after injection of saline (Fig. 5). Similarly, both the monoamine-based WIN 35,428 (CFT) and the non-amine O-913 engendered dose-related increases in the proportion of responses on the cocaine-appropriate lever, with O-913 being only slightly less potent than either cocaine or CFT (ED_{50} values: O-913: 0.29 ± 0.20 mg/kg; cocaine: 0.14 ± 0.03 mg/kg; CFT: 0.15 ± 0.02 mg/kg). In comparison with cocaine or CFT, O-913 displayed lower potency as a discriminative stimulus, relative to its affinity at the dopamine transporter in vitro (Table 1), suggesting that bioavailability or delayed onset of the drug may differ compared with monoamine-based dopamine transport inhibitors. Further assessment of O-913 and similar non-amines as candidate cocaine medications and as therapeutics for other indications are warranted.

3.7. Non-amines at the serotonin transporter

Conventional antidepressants universally contain an amine nitrogen in their structures. Again, this precept was driven by an underlying assumption that high affinity drug–serotonin transporter interaction required the formation of an ionic bond between the amine nitrogen and an acidic amino acid on the transporter (Barker et al., 1999).

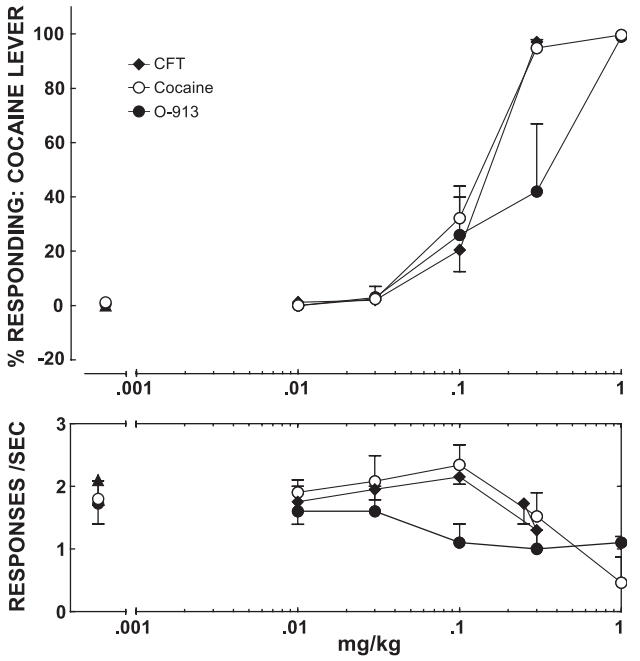


Fig. 5. Effects of the monoamines cocaine and WIN 35,428 (CFT) and the non-amine O-913 in monkeys trained to discriminate cocaine from saline. The *x*-axis represents cumulative dosing on a log scale. Top panel: the *y*-axis represents percent of responses on the cocaine-associated lever. Bottom panel: the *y*-axis represents rates of responding. Each point is the mean of data generated from four to five monkeys.

The feasibility of developing non-amines with high affinity for the serotonin transporter (SERT) was recently demonstrated (Madras et al., 1996; Meltzer et al., 1997; Goulet et al., 2001). Several non-amines blocked [^{3}H]serotonin transport in the low nM range, comparable to potencies of conventional amine antidepressants, imipramine, fluoxetine and amitriptyline (Goulet et al., 1999). Equally interesting was the discovery of a non-amine, O-1809, that displays selectivity for the SERT over the dopamine transporter (Table 1), with the design based on a SERT-selective amine (Blough et al., 1997; Smith, 1998). Structure–activity relationships for monoamines and non-amines at the SERT were parallel. Similar to the trend at the dopamine transporter, substituents on the aromatic ring of non-amines enhanced serotonin transporter affinities by 10- to 1000-fold. The increases were greater than those observed with corresponding monoamines and suggested a possible mechanism for high affinity binding of non-amines to monoamine transporters. Structure–activity studies suggest that ionic bonding and aromatic interactions among cocaine analogs and the dopamine transporter contribute to high affinity binding (Carroll et al., 1992). Conversion of a conserved aspartate residue on the serotonin transporter to a neutral amino acid resulted in a marked loss of ligand recognition and substrate transport (Barker et al., 1999). As phenylalanine, tyrosine and tryptophan can engage in aromatic–aromatic interactions (Burley and Petsko, 1985), aromatic amino acids highly

conserved throughout the superfamily of sodium chloride-dependent transporters may play important functional roles in ligand transporter interaction (Lin et al., 1999). As non-amines cannot engage in ionic bonding, aromatic–aromatic interactions may contribute more significantly to non-amine binding than to conventional monoamine binding (Madras et al., 1996; Meltzer et al., 1997). In a pilot study to test this premise, we identified conserved aromatic amino acids throughout the superfamily of sodium/chloride-dependent transporters and replaced a highly conserved phenylalanine residue with alanine. Non-amines displayed similar affinities for the wild-type SERT and the mutated form F548A but the monoamine imipramine bound to the mutant SERT with 5-fold lower affinity. This residue (F548) on the SERT apparently is not critical for non-amine, but others may be.

4. Discussion

Non-amines display affinities within the range of amine-based therapeutic agents targeted to the dopamine transporter and block dopamine transport. They furthermore distribute in vitro and in vivo to brain regions consistent with the distribution of the dopamine transporter, raise extracellular dopamine levels and engender cocaine-like discriminative stimulus cues in a drug discrimination paradigm (Madras et al., 1996; De La Garza et al., 1999; Meltzer et al., 1997, 1999, 2003). Structural variants also display high affinity and selectivity for the serotonin transporter (Goulet et al., 2001), and highlight the feasibility of developing non-amines that are as potent as conventional anti-depressants for inhibiting serotonin transport. As such, non-amines may provide novel leads for developing improved therapeutic agents for neuropsychiatric disorders (e.g. attention deficit hyperactivity disorder, compulsive nicotine use, depression, anxiety, eating disorders and obsessive-compulsive disorders). As non-amines have different physicochemical properties than their amine-bearing counterparts, they may display pharmacokinetic and pharmacodynamic properties distinct from conventional amine-based therapeutic agents. The lipophilicity of non-amines may confer properties of a natural "depot drug" with prolonged duration of action. The striking transporter selectivities of non-amines and their low affinities for a wide range of receptors also raise the possibility of developing a new class of agents with different therapeutic and side effect profiles than conventional amine-based drugs.

Fundamentally, the high affinity and efficacy of these unique drugs compel the development of new models for transporter–ligand interactions. Mounting evidence suggests that compounds of various chemical classes can bind to differing domains on monoamine transporters (Reith et al., 2001). By virtue of their structure, select non-amines are incapable of engaging in ionic or hydrogen bonding at or near the locus of their amine nitrogen-bearing progenitors. Apparently, the high affinity conferred by formation of an ionic bond with an amine nitrogen can be compensated for in non-amines by aromatic ring substituents. This class of compounds will facilitate the development of more accurate models of drug–transporter complex formation, as borne out by our pilot studies on site-directed mutagenesis.

Amine nitrogen bearing neurotransmitters are the norm in the mammalian nervous system. A "protonatable" or "basic" "protonatable: nitrogen may be advantageous because it confers solubility on a molecule requiring rapid transit across the aqueous synaptic gap and allows for effective formation of an ionic bond. The present report highlights the feasibility of developing non-amines that are as potent as conventional therapeutic drugs for inhibiting the dopamine or serotonin transporter.

This series also raises the question of whether brain or peripheral *receptors* will respond to non-amine nitrogen bearing drugs. Compliance with the premise that receptors respond effectively only to aminergic drugs is evident in virtually all therapeutic drugs targeted to receptors and include anti-psychotics, anti-Parkinsonian drugs, anxiolytics, analgesics, β-adrenergic receptor antagonists, and anti-histaminergics.

Our conjecture that non-amines targeted to receptors also warrant systematic investigation (Madras et al., 1996) is sustained by the mounting list of compounds containing no amine nitrogen, that target receptors. These include the active ingredient of the marijuana plant (Δ^9)-tetrahydrocannabinol, which targets the cannabinoid receptor, the anti-convulsant drug valproic acid, which activates the GABA receptor, and ester and ketone analogs of β-adrenergic amine agonists, which activate only mutant forms of the β-adrenergic receptor, but at high μM concentrations (Strader and Dixon, 1991). In further support of our view that non-amines may effectively activate receptors, the non-amine Salvinorin A was recently reported to be a high affinity and markedly selective κ-opioid receptor agonist (Roth et al., 2002). Parallel to our discovery that the non-amines targeted to monoamines transporters are highly selective and inactive at >30 receptors, Salvinorin A was inactive against a battery of 50 receptors, transporters, and ion channels. Thus the powerful hallucinogenic properties of Salvinorin A could not be accounted for by agonist activity at 5-HT_{2A} serotonin receptors, a principal target of classical hallucinogens. Based on Salvinorin A's psychotomimetic properties at κ-opioid receptors, κ-opioid-selective antagonists may constitute novel psychotherapeutic agents to treat diseases manifested by perceptual distortions (e.g. schizophrenia, dementia, and bipolar disorders). In this regard, another related terpenoid, the non-amine (+)-3-thujone is a modulator of $GABA_A$ receptors, displays anti-nociceptive activity in mice, and is the active ingredient of absinthe (Hold et al., 2000; Rice and Wilson, 1976). Other non-amine receptor activators, including steroid hormones, vertebrate and invertebrate pheromones (e.g. dehydro-*exo*-brevicomin), are abundant in nature.

Non-amines compel a revision of current concepts of the drug binding domains on monoamine transporters. They open new avenues for drug discovery and drug development for transporter proteins. Finally, it is reasonable to speculate on whether mammalian transporters and receptors may respond to, as yet, undiscovered non-amine bearing neurotransmitters or plant products.

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