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Original article

Structure–activity relationship of trihexyphenidyl analogs with respect to the dopamine transporter in the on going search for a cocaine inhibitor

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Abstract

A series of trihexyphenidyl (THP) analogs were used to search for a derivative that could serve as a cocaine inhibitor. A compound that blocks binding of the cocaine analog carboxyfluorotropane (CFT), allows dopamine uptake and exhibits low side effects could serve as a good candidate for that purpose. All analogs were tested for the extent to which they inhibit CFT binding, dopamine uptake and *n*-methyl scopolamine (NMS) binding. Several structure–function relationships emerged. Methylation/halogenation of THP's benzene ring enhanced the compound's ability to block CFT binding in comparison to its ability to block dopamine uptake (**5a**–**e**). Replacement of the cyclohexyl ring with a benzene ring tended to create compounds that had lower affinities to the dopamine transporter (**7b** compared to THP, **7d** compared to **5h**, **7c** compared to **8c**) and modification of THP's piperidine ring tended to enhance affinity to the dopamine transporter (**5f**–**h**, **8a**, **8c**). One analog (**5f**) that showed little muscarinic activity indicating that it would probably have few side effects was investigated for its effects as an in vivo cocaine inhibitor. However, it showed few antagonistic effects in vivo. Nevertheless, this work greatly elucidates the structure–function relationships required for potential cocaine inhibitors and so lays out promising directions for future research.

Keywords: Trihexyphenidyl; Dopamine; Cocaine; n-methylscopolamine; Transporter; Uptake; Binding

1. Introduction

Cocaine abuse is a major continuing concern in the Western world and there is currently no cocaine antagonist or cocaine substitute available. Ritz et al. [1] examined the correlations between psychostimulant potencies in tests of behavioral reward and their potencies in blocking each of the monoamine transporters at which cocaine displays activity: the dopamine (DAT), serotonin (SERT) and norepinephrine (NET) transporters. The good fit between DAT potencies and behavioral reward described in their work, coupled with data from lesion [2] and dopamine release studies [3–5], suggested that DAT was the principal site for cocaine induced reward [1].

Studies using site directed mutagenesis [6–9], molecular modeling [10], binding, [11,12] and thermodynamic approaches [13] have all suggested that there may be separate or overlapping, but not identical, cocaine and dopamine recognition sites on the DAT. Although the exact relationship of each site with the DAT is not clearly understood, these studies indicate on the possibility to construct a small drug molecule that could impede cocaine recognition but still allow normal dopamine uptake.

Abbreviations: CFT, carboxyfluorotropane; CHO, Chinese hamster ovary; DAT, dopamine transporter; NET, norepinephrine transporter; NMS, *n*-methylscopolamine; SERT, serotonin transporter; THP, trihexyphenidyl.

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In order to characterize the cocaine binding site on the DAT, extensive investigations were conducted to examine the structure–function relationships of a series of cocaine- or carboxyfluorotropane (CFT)-based compounds [14–18]. Those studies focused on compounds that bind better to the DAT than to the SERT or NET [19–21]. They identified, for example, several 3-phenyltropane cocaine analogues that demonstrated a slower onset and longer duration of action than cocaine [22,23] and were proposed for cocaine replacement therapy similar to methadone substitution for heroin addiction [24–27]. They did not, however, report on compound/s that can block cocaine recognition without impairing the DAT's normal dopamine uptake function.

The term cocaine selective inhibitor is used here to describe a compound that block the cocaine site on the DAT better than it block the dopamine site. To find a structure capable of at least partially blocking the binding of cocaine, while allowing for dopamine uptake, the activities of almost 100 non-tropane compounds containing heteroatoms and aromatic features compatible with possible interactions predicted from initial DAT models were screened (reviewed in [27]). Initial screening identified the muscarinic anticholinergic drug THP as providing some selective blockage of cocaine analog recognition, while being less potent in terms of dopamine uptake inhibition [28].

We now report data on the synthesis and structure–function relationships of a series of novel THP congeners. The pharmacological characteristics of these analogs in rat brain membranes and in cells transfected with the dopamine transporter under two different sets of conditions were compared and a similar trend was revealed in all systems. Three main types of modifications of THP's basic structure (Fig. 1) were performed: (1) methylation/halogenation of the benzene ring; (2) replacement of THP's cyclohexyl ring with a benzene ring; and (3) insertions/deletions on THP's piperidine ring. The structure–function activities of THP congeners are described and discussed in detail. The side effects profile of a compound that retains some selective ability to block cocaine while manifesting a much lower potency than THP with respect to blocking muscarinic cholinergic receptors was investigated using a 61-site NovaScreen assay. The implications of the current study for future development of small molecules as drugs targeting the dopamine transporter site is discussed.

2. Chemistry

THP derivatives were prepared by two general methods. As illustrated in Scheme 1, the lithium derivatives of appropriate acetamides (**2**) were treated with the corresponding cyclohexyl substituted phenyl ketone (**3**) to provide the corresponding propionamides (**4**). The commercially unavailable acetamides (**2**) were prepared by acetylation of the appropriate amines (**1**). On reduction with borane dimethylsulfide complex, the propionamides gave the target aminoalcohols (**5**). An alternate route was employed to prepare derivatives **7** and **8a**, **8c** (Scheme 2). The substituted 3-aminopropionic acid ethyl esters (**6**) were prepared by the action of ethyl acrylate on the appropriate amines (**1**), which, on treatment with phenyl magnesium bromide, provided the diphenyl amine alcohols (**7**). Careful reduction of one of the phenyl groups in a hydrogen atmosphere over platinum oxide (IV) gave the target propanolamines (**8a** and **8c**). The various substituents (R_1, R_2, X and Y) on each product are identified in Table 1.

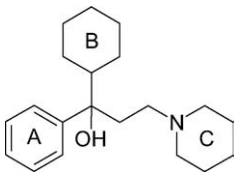
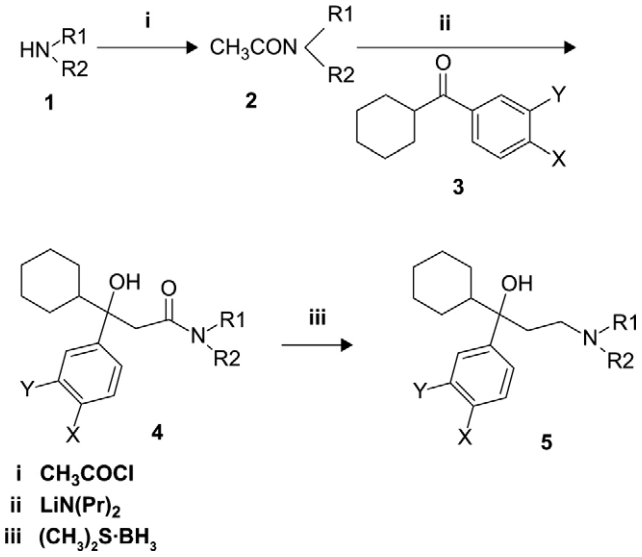
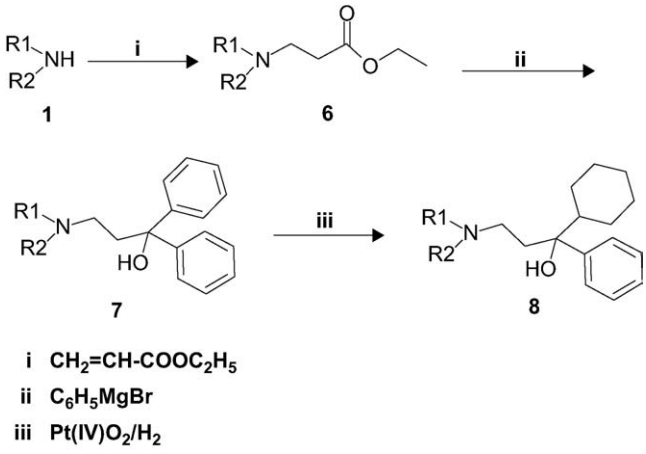


Fig. 1. The structure of THP. A–C refer to the phenylpropane, cyclohexyl and piperidine rings of THP accordingly.

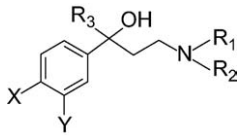


Scheme 1.



Scheme 2.

Table 1
Physical properties of THP analogs



Congener	X (*para*)	Y (*meta*)	R_3	R_1 R_2	Salts	Melting point (°C)
5a	CH_3	H	C_6H_{11}	$–CH_2(CH_2)_3CH_2–$	HCl	> 260
5b	H	CH_3	C_6H_{11}	As above	As above	243–245
5c	Cl	H	C_6H_{11}	As above	As above	> 260
5d	H	Cl	C_6H_{11}	As above	As above	242–243
5e	Cl	Cl	C_6H_{11}	As above	As above	255–256
5f	H	H	C_6H_{11}	$CH_2CH_2C(OH)(C_6H_5)CH_2CH_2$	As above	187–189
5g	H	H	C_6H_{11}	$CH_2CH_2CH(C_6H_5)CH_2CH_2$	As above	190–191
5h	H	H	C_6H_{11}	$CH_2CH_2CH(CH_3)CH_2CH_2$	As above	222–224
5i	H	H	C_6H_{11}	$CH_2CH_2CH_2(C_6H_4)CH_2CH_2$	As above	217–219
7a	H	H	C_6H_5	CH_2CH_3 CH_2CH_3	As above	202–204
7b	H	H	C_6H_5	$CH_2CH_2CH_2CH_2CH_2$	As above	189–195
7c	H	H	C_6H_5	$CH_2CH_2N(CH_3)CH_2CH_2$	2 HCl	235–237
7d	H	H	C_6H_5	$CH_2CH_2CH(CH_3)CH_2CH_2$	HCl	196–197
8a	H	H	C_6H_{11}	CH_2CH_3 CH_2CH_3	HCl	182–184
8c	H	H	C_6H_{11}	$CH_2CH_2N(CH_3)CH_2CH_2$	2 HCl	235–237

3. Results

3.1. Potencies of THP and its analogs in dopamine transporter uptake and binding

Initial results from screening assays demonstrated that THP could block the CFT binding with a significantly greater potency than it exhibited in inhibiting dopamine uptake. Compounds that were closely related structural analogs of THP were then synthesized and tested (Table 1). Many of the previous studies that tested the activity of compounds at the DAT used homogenates of rat brain membranes [29,30,32]. In this study, each compound was tested for its activity at the DAT under three sets of conditions. In condition set I, Chinese hamster ovary (CHO) cells stably transfected with the human DAT were used. An identical buffer composition was used in the [^{3}H]dopamine uptake and [^{3}H]CFT binding experiments, which were repeated three to five times each. Condition set II was similar to condition set I, with minor modifications, and the experiment was conducted only once. In condition set III, homogenated rat brains were used. Different buffers were used in the [^{3}H]dopamine uptake and [^{3}H]CFT binding experiments, which were repeated two to three times each. Condition set I is, therefore, considered the most conservative. Although the specific conditions of each assay influenced the results, they did not usually change the overall tendency. The potencies with which the congeners influenced dopamine uptake and cocaine analog binding differed, with the ratios of K_i for [^{3}H]dopamine uptake to K_i of [^{3}H]CFT binding ranging from 2 to 3 up to more than 20 in DAT-expressing cells. Table 2 shows K_i values for these THP analogs' inhibition of [^{3}H]dopamine uptake and [^{3}H]CFT binding. Inhibition curves for some of the compounds are presented in Fig. 2.

Compounds with methyl- or halogen substituents placed at *meta* or *para* positions on the phenyl ring (Fig. 1) exhibited increased selectivity, inhibiting [^{3}H]CFT binding more than [^{3}H]dopamine uptake. They, thus produced higher values for the ratios of K_i ([^{3}H]dopamine uptake) to K_i ([^{3}H]CFT binding) in DAT-expressing cells. *Para* substituents were generally more selective than *meta* substituents (Table 2, condition sets I and II; **5a** compared to **5b**; **5c** compared to **5d**). Chloride-substituted compounds had higher potencies than methyl-substituted compounds with respect to inhibiting both [^{3}H]dopamine uptake and [^{3}H]CFT binding (Table 2, condition sets I and II; **5c** compared to **5a**; **5d** compared to **5b**). In DAT-expressing cells using condition set I (Table 2), though not in expressing cells using condition set II (Table 2) or rat striatum (Table 2, condition set III), [^{3}H]dopamine uptake was inhibited nearly equipotently by the dicholoro-substituted **5e** and the monochloro-substituted **5d** compounds. The 3′4′ dichloro substitution increased potency with respect to [^{3}H]CFT binding inhibition by about three times, and improved selectivity 22-fold in DAT-expressing cells (Table 2, condition set I). In striatal tissue, halogenation of the phenyl ring also lead to more potent inhibition of [^{3}H]dopamine uptake and [^{3}H]CFT binding than methylation at the same position (Table 2, condition set III; **5c** compared to **5a**; **5d** compared to **5b**). The *meta* substituent was more potent than the *para* substituents (Table 2, condition set III; **5b** compared to **5a**; **5d** compared to **5c**).

Replacement of THP's cyclohexyl ring (Fig. 1) with a phenyl ring usually resulted in reduced affinity for both [^{3}H]dopamine uptake and [^{3}H]CFT binding in DAT-expressing cells. This was true when comparing data from **7b** to THP (Table 2, condition sets I and II), data from **7d** to **5h** (Table 2, condition set I) and data from **7c** to **8c** (Table 2, condition set I), although it did not hold for comparisons of data from **7a** to **8a** (Table 2, condition sets I–III). Substituting an aromatic ring for the cyclohexyl ring also reduced affin-

Table 2
Affinities of THP and related analogs for the human and rat dopamine transporter

Test Compound	K_i (μM) hDAT^{I}			K_i (μM) hDAT^{II}			K_i (μM) rDAT^{III}		
	[^{3}H]dopamine	[^{3}H]CFT 35,428	Ratio	[^{3}H]dopamine	[^{3}H]CFT 35,428	Ratio	[^{3}H]dopamine	[^{3}H]CFT 35,428	Ratio
THP	79.8 ± 4.7	26.9 ± 3.1	2.96	142	21	6.8	26.3 ± 4.6	12.2 ± 1.1	2.1
5a	54.6 ± 2.1	5 ± 1.2	10.85	39.5	6.3	6.3	13.1 ± 1	6.5 ± 0.9	2
5b	18.5 ± 3.2	4.3 ± 0.8	4.33	46	16	2.9	6.8 ± 0.5	4.6 ± 0.3	1.5
5c	16.2 ± 0.3	1.6 ± 0.5	10.15	14.4	1.4	10.4	3.8 ± 1	2.3 ± 0.2	1.65
5d	10.2 ± 2.2	1.5 ± 0.45	6.76	12	2.8	4.3	2.7 ± 0.4	1.5 ± 0.2	1.8
5e	11.6 ± 2.4	0.53 ± 0.12	21.94	4.2	1.3	3.2	0.7 ± 0.1	0.8 ± 0.5	0.875
5f	8.9 ± 1.1	2.2 ± 0.2	4	6.5	0.8	8.1	1.5 ± 0.4	0.9 ± 0.04	1.7
5g	28.5 ± 6.8	3.8 ± 1.1	7.5	8	1.7	4.8	0.8 ± 0.2	0.63 ± 0.03	1.3
5h	40.1 ± 6.1	13.8 ± 3.6	2.89	105	20	5.3	11.8 ± 0.8	9.4 ± 0.8	1.25
5i	384 ± 33.4	48 ± 21.5	8	> 300	> 300	ND	4.4 ± 0.5	6.4 ± 2.6	0.7
7a	32.3 ± 3.3	11.3 ± 1.9	2.86	37	12.5	3	14 ± 0.8	12 ± 2	1.2
7b	139 ± 12.3	40.8 ± 7.8	3.39	> 100	50	ND	33.9 ± 2.2	11.5 ± 1.4	3.1
7c	55.8 ± 4.8	20.9 ± 2.4	2.67	44	23.5	1.9	13.3 ± 2.2	9.8 ± 1.4	1.3
7d	77.2 ± 2.6	23.9 ± 4.2	3.23	105	15	7	17.9 ± 5	6.9 ± 1.6	2.6
8a	42.1 ± 7.9	10.7 ± 2.3	3.93	54	16	3.4	14.2 ± 2.8	13.1 ± 2	1.1
8c	8.9 ± 0.7	2.4 ± 0.1	3.7	11	1.9	5.9	3.8 ± 0.3	3.8 ± 0.7	1

The affinities of the above compounds for the cocaine/CFT site and the dopamine site on the dopamine transporter were determined by inhibition of [^{3}H]CFT binding and [^{3}H]dopamine uptake to CHO cells stably transfected with the human dopamine transporter (condition sets I and II) and on rat striatum (condition set III). Data are presented as the mean ± S.E.M. values for three to five experiments (condition I); one to two experiments (condition II), two to three experiments (condition III); ND = not determined.

ity for [^{3}H]dopamine uptake in rat striatum (Table 2, condition set III; **7d** compared to **5h**; **7c** compared to **8c**).

Five out of the six THP analogs with modifications on the nitrogen-containing (piperidine or piperazine) ring (Fig. 1) demonstrated significantly higher affinities for both [^{3}H]dopamine uptake and [^{3}H]CFT binding sites than did THP itself in DAT-expressing cell systems (Fig. 2 and Table 2, condition sets I and II). Ranking the molecules in decreasing order

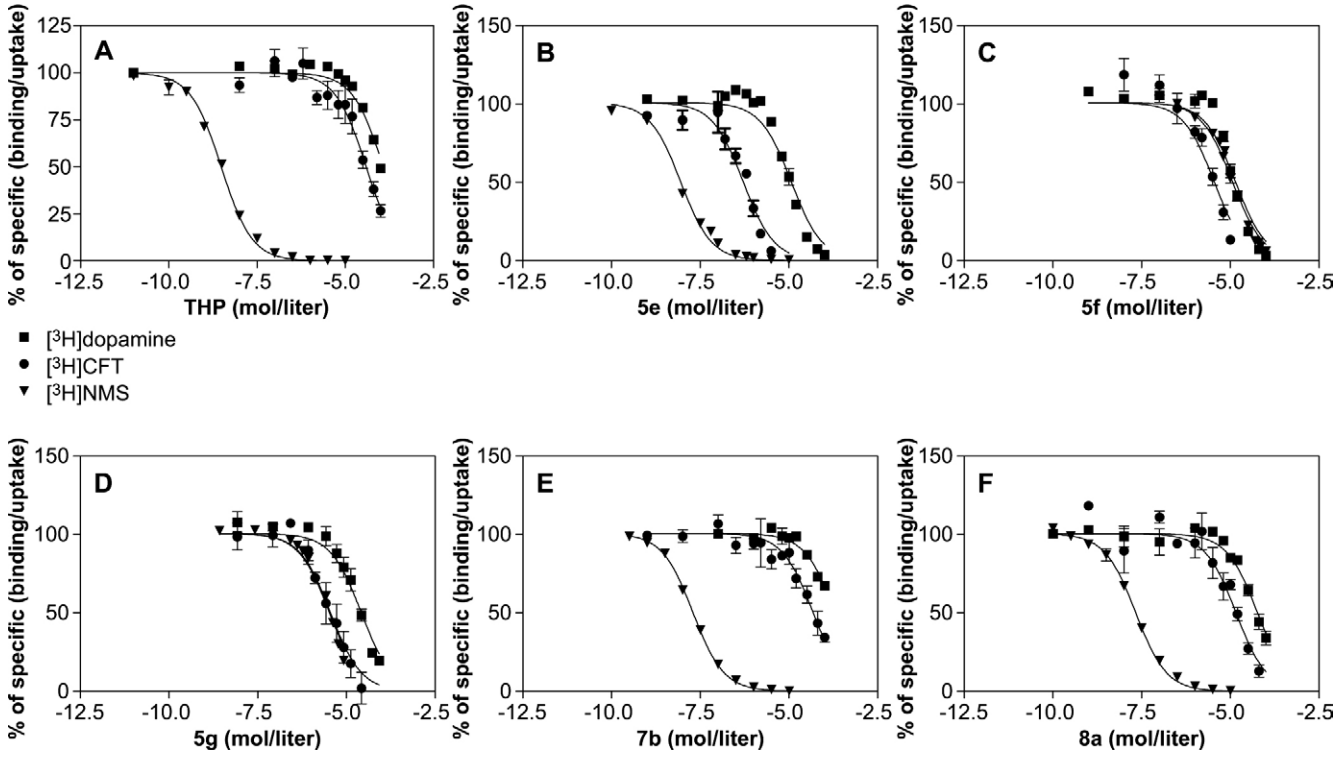


Fig. 2. Representative curves for the inhibition of [^{3}H]dopamine uptake, [^{3}H]CFT binding and [^{3}H]NMS binding by THP and several of its related analogs in CHO cells stably transfected with the human dopamine transporter ([^{3}H]dopamine uptake and [^{3}H]CFT binding) or in rat brain membranes ([^{3}H]NMS binding). (A) THP, (B) Congener **5e**, (C) Congener **5f**, (D) Congener **5g** (E) Congener **7b**, (F) Congener **8a** (see Table 1 and the Appendix for descriptions). Competition studies were conducted with various concentrations of test compound and a fixed concentration of radioligand as indicated in the methods. Data for the graphs were taken from the experiments presented in Table 2 (condition set I) and Table 3.

of potency yields: 4-methylpiperazine (**8c**) ≈ 4-hydroxy-4-phenylpiperadine (**5f**) > 4-phenylpiperadine (**5g**) > 4-methylpiperadine (**5h**) ≈ the diethylamino hydroxyphenylcyclohexylpropane derivative (**8a**). Binding and uptake assays performed under the most conservative condition set revealed ratios of K_i ([^{3}H]dopamine uptake) to K_i ([^{3}H]CFT binding) for that group of compounds ranging between 2.9 and 7.5. Assays in rat striatum also revealed that substituting the piperidine ring with a hydroxyphenyl (**5f**), 4-phenylpiperadine (**5g**), 4-methylpiperidine (**5h**), 3′-diethylamine (**8a**) or 4-methyl piperazine (**8c**) increased a compound's potency in inhibiting [^{3}H]dopamine uptake compared to the parent compounds (Table 2, condition set III).

Congener **5i** inhibited [^{3}H]dopamine uptake only modestly in DAT-expressing cell systems, but inhibited [^{3}H]CFT binding in a complex fashion that made determining meaningful K_i values difficult (Table 2, condition set I). In rat striatal synaptosomes, **5i** inhibited [^{3}H]dopamine uptake with potencies substantially higher than those displayed in the DAT-expressing cell lines (Table 2, condition set III).

3.2. Potencies of THP analogs in inhibiting binding of a muscarinic cholinergic ligand

With the exception of **5f**, THP and its congeners remained substantially more potent in inhibiting [^{3}H]NMS binding to muscarinic cholinergic receptors than they were in inhibiting [^{3}H]CFT binding or [^{3}H]dopamine uptake through the DAT (Table 3). The THP analog series of compounds displayed various potencies at displacing [^{3}H]NMS bound to membranes prepared from rat frontal cortex (Fig. 2 and Table 3). Addition of a second aromatic ring to THP (Fig. 1) substantially reduced affinities for muscarinic receptors (**7a** compared to **8a**; **7b** compared to THP; **7c** compared to **8c**; **7d** compared to **5h**). The phenylpiperidine (**5g**; IC_{50} = 4.2 μM) and the hydroxyphenylpiperidine (**5f**; IC_{50} = 11.2 μM) substitutions were especially effective. Each of the other tested analogs displayed a lower affinity than THP for muscarinic receptors. Potencies varied from 5 nM (Table 3: **5b** or **5d**) to 250 μM (Table 3: **8c**).

Table 3
Affinities of THP and related analogs for the muscarinic receptor

Test compound	[^{3}H]NMS IC_{50} (nM)
THP	3.1 ± 0.09
5a	14.1 ± 2.3
5b	5.9 ± 0.56
5c	19.5 ± 4.1
5d	5.5 ± 0.76
5e	10.4 ± 2.3
5f	11,186.5 ± 817
5g	4,160 ± 196
5h	68.3 ± 4.5
5i	312 ± 68
7a	155 ± 17.5
7b	19.7 ± 1.4
7c	768 ± 94
7d	351 ± 36
8a	21.2 ± 2
8c	254 ± 14

Affinities of THP analogs to the muscarinic cholinergic receptor were determines by inhibition of [^{3}H]NMS binding to rat frontal cortex membranes. Values for each compound are mean ± S.E.M. calculated from three to six independent experiments.

3.3. Potencies of a lead THP analog in inhibiting binding of a number of radioligands

Congener **5f** appeared to provide an interesting potential lead compound for in vivo testing, as it had reduced muscarinic activity and it displayed moderately selective potencies in blocking cocaine analog recognition. To further assess the possible in vivo actions of this structure, **5f** was employed in single concentration (10 μM) tests for inhibition of radioligand binding to 61 diverse brain sites (NovaScreen, Oceanicx Biosystems Inc.). No significant activity was revealed in 53 tests. At that concentration, **5f** inhibited the binding of [^{3}H]LSD, [^{3}H]naloxone, [^{3}H]DTG, [^{3}H]nitrendipine, [^{3}H]batrachotoxin A and [^{125}I]neurokinin A by more than 50%. Follow up assays revealed that **5f** displaced bound sigma-1 receptor ligand [^{3}H]SKF10047 potently (K_i = 106 nM, n = 1) but that its potency to displace the opiate receptor ligand [^{3}H]naloxone was much lower (K_i = 1.4 μM, n = 1).

*3.4. Potencies of **5f** in behavioral assessments of baseline and cocaine induced locomotion and reward*

C57B1/6J male mice were treated with 1 or 4 mg/kg of **5f**, 60 min before they were injected with 30 mg/kg of cocaine. Their locomotive activity was measured for 60 min after cocaine injection. Pretreatment with 1 and 4 mg/kg of **5f** reduced cocaine induced locomotive activity by 11% and 14%, respectively (n = 1, data not shown). In condition place preference (CPP) tests, mice injected with 1 or 2.5 mg/kg of **5f** displayed no significant alteration in the time spent in the chamber paired with this agent (n = 1, data not shown). Mice injected with cocaine alone (no pretreatment) spent significantly more time on the cocaine paired side. Mice pretreated with **5f** before cocaine conditioning spent almost exactly the same time in each chamber as mice pretreated with saline.

4. Discussion

This work has implications for considerations of the structure/activity properties of the dopamine transporter and muscarinic cholinergic receptors. It demonstrates the possibility, and the difficulty, of designing and selecting compounds capable of serving as a new class of DAT blockers.

As mentioned earlier, the vast majority of studies investigating fine interactions of ligands with the DAT have focused on cocaine analogs [19,33–37]. Structure requirements elucidated for those tropane congeners suggested that 2β-carboxy esterification, basic nitrogens capable of protonization at

physiological pH, R-configuration of the tropane and a 3β-aromatic ring at C-3 are required to varying extents [19,29,30,38]. THP's basic structure is substantially different from that of cocaine, though they both possess an aromatic ring and a nitrogen.

The structure–activity relationships of the THP analogs described in detail in this report demonstrated that modifications of THP's phenylpropane group (**5a**–**5e**), cyclohexyl group (**7a**–**7d**) and piperidine group (**5f**–**5i**, **8a**, **8c**) each produced typical pharmacological characteristics. As indicated in Section 3, methylation/halogenation of the phenylpropane ring created compounds with better K_i dopamine uptake/K_i CFT binding selectivity, ranging between 4.33 and 22 (Table 2, condition set I). In agreement with previous studies using cocaine [38] and aryloxantropane analogs [39], chloride substituents (**5c**–**5e**) on the aromatic ring increased affinity for the DAT and followed the same order of $Cl_2 > Cl$. In addition, chloride and methyl substitution on the phenyl ring dramatically enhanced selectivity (Table 2, condition set I). Congener **5e**, which has two chloride substitutions on its phenylpropane ring, displayed the highest affinity for the cocaine/CFT site and the highest selectivity for that site among the THP congeners (Table 2, condition set I; Fig. 2). A compound with this kind of selectivity that does not interfere with other receptors might serve as a cocaine inhibitor.

Replacing the cyclohexyl ring with an aromatic ring (**7a**–**7d**) tended to reduce the compound's affinity for the dopamine transporter as measured by both [^{3}H]dopamine inhibition and by [^{3}H]CFT displacement (Table 2, condition set I). This may be a result of changing the octanol/water partition of the molecule, thus creating a more lipophilic compound with reduced ability for docking at its ligand site. Substitutions on the piperidine ring (**5f**–**5h**, **8a**, **8c**) also influenced both [^{3}H]CFT binding and [^{3}H]dopamine uptake and resulted in compounds that demonstrated higher affinities for those sites. This tendency was robust and appeared in the three sets of conditions tested (Table 2). It may be related to the availability of the nitrogen atom for protonization, which may be required for binding to the transporter.

The cholinergic properties of THP congeners also displayed several structure–function profiles. Compounds that had 4-phenyl (**5g**) or 4-hydroxyphenyl (**5f**) on the piperidine ring showed a markedly reduced affinity to the muscarinic receptor (Table 3).

It was anticipated that congener **5f** would exhibit low side effects due to its reduced affinity for the muscarinic cholinergic receptor (Table 3) and it was, therefore, selected for further measurement. The fact that **5f** exhibited binding affinity to the Sigma-1 receptor is not surprising, as THP and other M_1 selective antagonists demonstrated potent inhibitory activity at that site [40]. Recent studies demonstrated several functional links between cocaine activity and the Sigma-1 receptor [41,42]. If THP and its congeners act as antagonists at that site, it should be interesting to relate their potency at the Sigma-1 receptor to their ability to reduce cocaine's rewarding effects.

A recent study has demonstrated that THP attenuated CPP induced by methamphetamine, but not that induced by cocaine [43]. In the same study, THP failed to attenuate locomotive activity induced by any of the stimulants. It is not clear what mechanism caused the observed attenuation in CPP. It would be interesting to test the in vivo effect of compounds with greater selectivity for each of these drugs. We were not able to test all the described analogs for inhibition of locomotion or other behavioral tests in the current study. In preliminary behavioral studies, congener **5f** demonstrated only a small degree of antagonism towards cocaine activity in vivo. This could be because of this compound's relatively low selectivity, its poor ability to reach the brain or because it is rapidly metabolized. Another possibility is that the dopamine uptake and cocaine binding properties of the DAT can not be pharmacologically separated. Extensive DAT mutagenesis studies, however, indicated that alanine or glycine substitutions of tryptophane [8], proline [9] and phenylalanine [7] can selectively reduce cocaine analog or dopamine affinities. It is conceivable that a compound capable of acting on a certain DAT site without affecting other sites will eventually be designed.

An agent capable of selectively blocking a specific site on the DAT could be beneficial for treating cocaine overdoses, on the one hand, and for preventing neurotoxicities, on the other. Neurotoxicity may be caused by toxins such as MPP^+, which induces Parkinsonism [44]. DAT mutagenesis data suggested that the residues important for MPP^+ uptake are not identical to those important for dopamine uptake by the same transporter [45]. Conceivably, small molecules capable of interfering with the uptake of an MPP^+-like substrate without significantly interfering with dopamine uptake may provide a novel approach to antagonizing neurotoxicities.

In conclusion, we present here results from a structure–function study of a series of novel THP analogs. All the analogs tested demonstrated a better affinity for the cocaine binding site than the dopamine uptake site. We have more specifically defined the characteristics necessary for a drug to be potent at the DAT. One of these analogs, the **5f** congener, had few inihibitory effects in behavioral tests. Unfortunately, other analogs were not tested. As more compounds exhibiting selective DAT inhibition are developed, further structure/activity studies could help to enrich the generalized structure–activity requirements tentatively proposed for selective cocaine antagonism.

5. Experimental

5.1. Chemistry: synthesis of trihexyphenidyl analogs

*5.1.1. Synthesis of substituted phenylcyclohexyl ketones (**3a–e**)*

Cyclohexyl *n*-methyl-*n*-methoxy carboxamide was prepared from cyclohexyl carboxylic acid chloride and *n*-methoxy-*n*-methyl amine. To a solution of this carboxamide (1.71 g., 10 mmol) in ether (20 ml) at –78 °C was added

the appropriately substituted phenylmagnesium bromide (30 ml, 1.0 M in ether solution). The reaction mixture was stirred overnight while allowing the temperature to reach 23–25 °C. After addition of ammonium chloride solution, the ether fraction yielded pure samples of ketones **3a** (100%) and **3b** (100%). The **3c** ketone was purified (yield, 71%) on a silica gel column (2% ethyl acetate in hexane). Ketones **3d** (yield, 31%) and **3e** (yield, 32%) were prepared in a similar manner by the action of cyclohexylmagnesium chloride on the appropriate benzaldehyde. The resulting benzyl alcohols were oxidized with pyridinium dichromate solution to give the corresponding ketones. All ketones gave characteristic IR and NMR spectra and were used without further purification in the next step.

*5.1.2. General procedure for preparation of 3-piperidino-1-substituted propanols (**5a–i**)*

To a freshly prepared and stirred solution of $LiN[CH(CH_3)_2]_2$ (1.1 mmol) in THF (5 ml) at 0 °C was added a solution of the appropriate *n*-acetylpiperidine (**2**) (1 mmol in 4 ml THF). After stirring for 15 min the reaction mixture was cooled to –78 °C and the appropriate ketone (**3**) was added. The reaction mixture was allowed to warm up to –15 °C over a period of 1.5 h and was stirred for 1 h at that temperature. The reaction was quenched with a solution of acetic acid in ether (15%, 10 ml followed by 10 ml water). The aqueous fraction was extracted with ethylacetate. The combined organic fraction was washed with brine and evaporated. The crude amides (**4a–i**) were purified by column chromatography on silica gel (20% ethyl acetate/hexane). The pure amides obtained (yield, 44–82%) were used in the next step.

$BH_3/(CH_3)_2S$ (2.0 M in THF ml/mmol of amide) was added to a solution of the appropriate amide **4** (4 ml/mmol in THF). After stirring at 0 °C for 1 h and at room temperature for 2 h, methanol was added carefully to remove excess borane. The residue obtained on evaporation of the solvent was converted to a hydrogen chloride salt and crystallized from methanol/ether (Scheme 1). The compound's physical properties are listed in the Appendix (see Section 5.1.6).

*5.1.3. Synthesis of ethyl-3-dialkylamino propionates (**6a–d**)*

An equimolar mixture of ethyl acrylate and the appropriate secondary amine (**1**) were heated to 100 °C for 4–8 h. The resulting propionates were purified by distillation and were used in the next step without further purification (Scheme 2).

*5.1.4. Preparation of diphenylpropanols (**7a–d**)*

To a stirred solution of each of **6a–c** (in ether, 5 ml/mmol) at 0 °C was added phenylmagnesium bromide in ether (3 eq./1 eq. of **6**) and the mixture was heated to reflux for 3 h. After quenching the reaction with water at 0 °C the ether layer was washed with brine and dried. The residue obtained on evaporation was purified on a silica gel column. Eluting with a solvent mixture of ethyl acetate and hexane (1:20) gave diphenyl analogs (**7a–d**; Scheme 2) characterized as hydrogen chloride salts. Their physical properties are listed in the Appendix (Section 5.1.6).

*5.1.5. Preparation of 1-cyclohexyl-1-phenylpropanols (**8a and 8c**)*

A solution of the diphenyl propanols (**7a** and **7c**) in acetic acid (10 ml/mmol) was hydrogenated over platinum oxide (IV) catalyst (20 mg/mmol) at atmospheric pressure overnight. The catalyst was removed and the filtrate was rendered basic with dilute ammonium hydroxide solution and was extracted with ether (20 ml/mmol). The ether solution was dried using Na_2SO_4, evaporated and the residue was purified by silica gel column chromatography, and eluted with a solvent mixture of ethyl acetate (5–10%) in hexane. The target compounds (**8a** and **8c**; Scheme 2) were characterized as their hydrogen chlorides. The physical properties are listed in the Appendix (see Section 5.1.6).

5.1.6. Appendix

5a 3-Piperidino-1-cyclohexyl-1-(*p*-tolyl)-1-propanol hydrochloride. $C_{21}H_{33}NO{\cdot}HCl$.

5b 3-Piperidino-1-cyclohexyl-1-(*m*-tolyl)-1-propanol hydrochloride. $C_{21}H_{33}NO{\cdot}HCl$.

5c 3-Piperidino-1-cyclohexyl-1-(4-chlorophenyl)-1-propanol hydrochloride. $C_{20}H_{30}NOCl{\cdot}HCl$.

5d 3-Piperidino-1-cyclohexyl-1-(3-chlorophenyl)-1-propanol hydrochloride. $C_{20}H_{30}NOCl{\cdot}HCl$.

5e 3-Piperidino-1-cyclohexyl-1-(3,4-dichlorophenyl)-1-propanol hydrochloride. $C_{20}H_{30}NOCl_2{\cdot}HCl$.

5f 3-(4-Hydroxy-4-phenyl)piperidino-1-cyclohexyl-1-pheny-l-propanol hydrochloride. $C_{26}H_{35}NO_2{\cdot}HCl$.

5g 3-(4-Phenyl)piperidino-1-cyclohexyl-1-phenyl-1-propanol hydrochloride. $C_{26}H_{35}NO{\cdot}HCl0.5\ H_2O$.

5h 3-(4-Methyl)piperidino-1-cyclohexyl-1-phenyl-1-propanol hydrochloride. $C_{21}H_{32}NO{\cdot}HCl$.

5i 3-(1,2,3,4-Tetrahydroisoquinolino)-1-cyclohexyl-1-phenyl-propan hydrochloride. $C_{24}H_{30}NO{\cdot}HCl$.

7a 3-Diethylamino-1,1-diphenyl-1-propanol hydrochloride. $C_{19}H_{25}NO\ HCl{\cdot}0.25H_2O$.

7b 3-Piperidino-1,1-diphenyl-1-propanol hydrochloride. $C_{20}H_{25}NO\ HCl$.

7c 3-(4-Methyl)piperazeno-1,1-diphenyl-1-propanol hydrochloride. $C_{20}H_{26}NO{\cdot}2HCl$.

7d 3-(4-Methyl)piperidino-1,1-diphenyl-1-propanol hydrochloride. $C_{21}H_{27}NO{\cdot}HCl$.

8a 3-Diethylamino-1-cyclohexyl-1-phenyl-1-propanol hydrochloride. $C_{19}H_{31}NO{\cdot}HCl{\cdot}0.25H_2O$.

8c 3-(4-Methyl)piperazino-1-cyclohexyl-1-phenyl-1-propanol hydrochloride. $C_{20}H_{32}N_2O{\cdot}2HCl$.

5.2. In-vitro assays

5.2.1. [^{3}H]CFT binding and [^{3}H]dopamine uptake

5.2.1.1. Condition set I. CHO cells stably expressing the human DAT in pcDNAI were selected in G418. DAT-expressing subclones were expanded and maintained on F12 medium supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and 200 μg/ml of G418. Cells were

distributed to 24 well plates 2 days prior to harvest. DAT-expressing cells were subjected to radioligand CFT binding and dopamine uptake assays after washing with Krebs–Ringer–Hepes (KRH) buffer (in mM: NaCl 125, NaHepes 25, KCl 4.8, $MgSO_4$ 1.2, glucose 5.6, $CaCl_2$ 1.3, KH_2PO_4 1.2, pH 7.4). For binding, cells were incubated with 2 nM of [^{3}H]CFT (84.5 Ci/mmol; NEN) and the unlabeled compounds for 2 h at 4 °C. For uptake assays, cells were incubated with 1 μM [^{3}H]dopamine (NEN stock diluted to ca. 23 mCi/mmol) and unlabeled compounds for 5 min at 37 °C in KRH supplemented with 10 μM ascorbate. Three washes with KRH terminated both binding and uptake assays. Cells were dissolved in 0.25 ml 1% SDS, and their radioactivity was assessed. 30 μM of (–)cocaine was added to parallel incubations to provide estimates of non-specific binding and uptake.

5.2.1.2. Condition set II. Conditions were identical to those above except that cells were distributed to 96-well plates, 3–4 days prior to the experiment, pre-incubated with unlabeled test compounds for 2 h in cell culture media at 37 °C, washed three times and then incubated with 5 nM [^{3}H]CFT or 100 nM [^{3}H]dopamine and the tested compounds for 10 min at 25 °C. Binding and uptake were terminated by five washes with KRH and radioactivity was assessed. 200 μM (–)cocaine provided estimates of non-specific binding and uptake.

5.2.1.3. Condition set III. Striata were dissected from the brains of 200–250 g male Sprague–Dawley rats. Binding studies were conducted as previously described [29]. Striata were homogenized using a polytron, and membranes washed twice resuspended in 0.32 M sucrose phosphate buffer (pH 7.4) and recentrifuged. Membranes prepared from 1 mg of striatum (wet weight) were incubated with 0.5 nM [^{3}H]CFT in 0.5 ml sucrose phosphate buffer and unlabeled test compound at 4 °C for 2 h. Uptake studies were conducted as previously described [30]. Striatal synaptosomes were prepared, pre-incubated at 30 °C for 10 min with 1 μM pargyline and unlabeled test compounds and incubated for 3 min with 0.5 nM [^{3}H]dopamine in 1 ml of buffer (in mM: NaCl 126, KCl 4.8, $CaCl_2$ 1.3, NaH_2PO_4 16, $MgSO_4$ 1.4, dextrose 11 and ascorbic acid 1.1). Binding and uptake reactions were quenched by rapid filtration onto GF/B filters presoaked in 0.05% polyethyleneimine. Non-specific binding was determined using 30 or 100 μM of (–)cocaine for [^{3}H]CFT binding and [^{3}H]dopamine uptake experiments, respectively.

5.2.2. [^{3}H]n-methyl scopolamine binding

Displacement of [^{3}H]NMS from membranes prepared from rat frontal cortex was determined as described previously [31]. Tissue was homogenized, washed twice in 50 mM Tris–HCl and then membranes were prepared from 0.2 mg of tissue incubated with 0.06 nM of [^{3}H]NMS (NEN, 84 Ci/mmol) and various unlabeled test compounds in 2 ml buffer at 22 °C for 90 min. Reactions were terminated by rapid filtration, and radioactivity was estimated. Parallel incubations containing 1 μM atropine provided estimates of non-specific binding.

5.2.3. NovaScreen

NovaScreen (Hanover, Maryland) uses radioligands selective for 61 neurotransmitters, ion channels, transporters, second messengers, steroids, prostaglandins, growth factors, hormones and enzyme receptor sites. The efficacy of compound **5f** (10 μM) in competing against these radoligands for binding sites on whole rat brain membrane preparations was assessed in duplicate.

5.3. In-vivo assays

5.3.1. Locomotive activity

C57B1/6J male mice obtained from Jackson Laboratories (Bar Harbor, ME, USA), group housed with food and water available ad lib and maintained on a 12 hourly day–night cycle were housed in our facilities for a minimum of 2 weeks prior to testing. Mice were placed in 46 × 25 × 19 ($l \times w \times h$) clear plastic cages and locomotive activity was measured using Columbus Instruments (Columbus, OH, USA) Opto-Varimex activity monitors in sound-attenuating chambers under dim ambient light conditions. Total distance traveled was measured for 60 min for mice injected with 1 or 4 mg/kg of **5f**. Mice were then injected with 30 mg/kg of cocaine HCl and their activity was assessed for an additional 60 min.

5.3.2. Conditioned place preference

CPP was assessed using two compartment chamber with one 18x18x18 compartment with a wire mesh floor and the other with concorb bedding. During two days of twice daily conditioning sessions, C57B1/6J male mice were restricted for 20 min after injection with cocaine or saline, with or without pretreatment with congener **5f** to one side of the apparatus. A single CPP assessment session followed the last conditioning session by 24 h. In this session, mice had access to both compartments and the proportion of the 20 min session spent on each side was recorded using Optivarimax animal activity monitor (Columbus Instruments; Columbus, OH, USA).

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