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Evolution of a Compact Photoprobe for the Dopamine Transporter Based on (±)-*threo*-Methylphenidate

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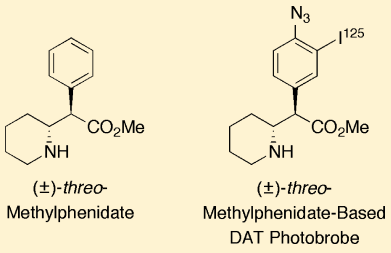
Supporting Information

ABSTRACT: The development of photoaffinity ligands for determining covalent points of attachment to the dopamine transporter (DAT) has predominantly focused on tropane-based compounds bearing variable-length linkers between the photoreactive group and the inhibitor pharmacophore. To expand the array of photoprobes useful for mapping inhibitor-binding pockets within the DAT, a compact nontropane ligand was synthesized featuring a photoreactive azide and iodine tag directly attached to the aromatic ring of (±)-*threo*-methylphenidate. (±)-*threo*-4-Azido-3-iodomethylphenidate [(±)-**6**; K_i = 4.0 ± 0.8 nM] displayed high affinity for hDAT. Moreover, a radioiodinated analogue of (±)-**6** demonstrated covalent ligation to the DAT in cultured cells and rat striatal membranes, thus suggesting the potential utility of this photoprobe in DAT structure−function studies.



KEYWORDS: *methylphenidate, photoaffinity labeling, dopamine transporter, cocaine*

There is sufficient evidence from structure−activity relationship (SAR) studies and site-directed mutagenesis experiments indicating that structurally disparate dopamine transporter (DAT) inhibitors bind to different conformations or domains within the DAT.[1] As a result, the development of irreversible molecular probes, such as affinity labels and photoaffinity ligands, remains an important research objective to understand the binding sites and conformational preferences of DAT inhibitors at the molecular level. To date, the chemical development of DAT molecular probes has predominantly focused on phenyl- and benztropine-based tropane compounds, along with their conformationally flexible piperazine and piperidine analogues, whereas nontropane probes have received significantly less attention.[2−4] On the basis of contrasting SAR between tropane and nontropane DAT inhibitors, these ligand classes may be binding to different specific DAT sites or conformations, which may explain and even dictate their divergent subjective effects in psychostimulant abuse animal models.[5−8] In particular, the effect on DAT binding affinity by the substituents on the aromatic ring of (±)-*threo*-methylphenidate [(±)-**1**, Figure 1], a therapeutic nontropane DAT inhibitor with low abuse potential, appears to be most similar to the so-called "WIN" tropane compounds, wherein the aromatic phenyl ring is directly attached to the 3-position of the tropane ring system.[9] In contrast to highly abused cocaine where the aromatic ring is attached to the tropane system via a 3β-ester,

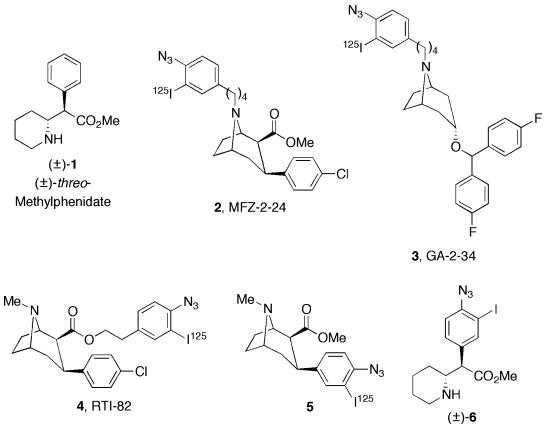


Figure 1. Structural comparison of tropane-based DAT photoaffinity ligands vs target methylphenidate probe (±)-**6**.

the effects of aromatic substituents on DAT affinity are quite different. This difference has been attributed to the greater distance between the tropane nitrogen and the aromatic ring of cocaine.[10]

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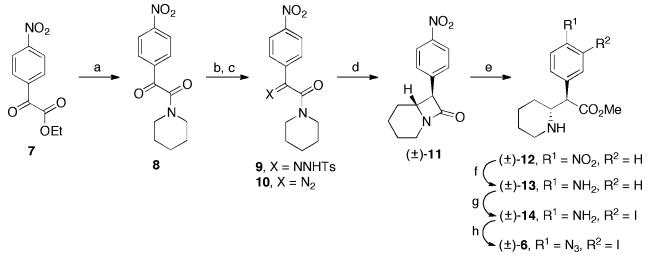
The traditional design of DAT photoprobes features incorporation of a 3′-I, 4′-N_3-phenyl substituent attached to an inhibitor scaffold by means of a variable-length linker (Figure 1). For example, [^{125}I]-MFZ-2−24 (**2**) has been prepared and found to covalently attach to transmembrane domain 1 (TM1) of the DAT,[11] while similar results have also been achieved with benztropine probe [^{125}I]-GA-2−34 (**3**).[12] However, phenyltropane-based probe [^{125}I]-RTI-82 (**4**) covalently ligates to TM6 of the DAT.[13] Multiple studies suggest not all tropane-based inhibitors bind to the same conformation or binding site within the DAT and that covalent conjugation to the DAT protein can occur in different TM regions depending on the position of the photoreactive group within the probe. In this regard, photoprobes **2**−**4** may be viewed as potentially possessing an inherent disadvantage when trying to definitively map the amino acids of the inhibitor-binding pocket or optimally model the photoprobe−DAT complex via the binding ensemble profiling with (f)photoaffinity labeling (BEProFL) approach.[14] That is, the covalent point of probe attachment to the DAT, which is dictated by the location of the photoreactive azide group, is somewhat removed from the inhibitor scaffold by means of a conformationally flexible, variable-length methylene linker. In particular, molecular modeling studies of photoprobe **2** indicate distances of 10.5 and 15.5 Å between the azide and the pharmacophore tropane nitrogen and 3β-phenyl ring.[11] Given that the azide is not directly appended to the tropane pharmacophore for probes **2**−**4**, adduction may occur at a residue near, but not at, a direct inhibitor contact point, thus representing a significant limitation when trying to delineate the discrete molecular interactions between the probe and the DAT protein. Toward potentially addressing this point, 3-(4′-azido-3′-iodo-phenyl)-8-methyl-8-aza-bicyclo[3.2.1]octane-2-carboxylic acid methyl ester (**5**) has been synthesized and displays high affinity, wash-resistant binding to the DAT[15] but fails to label the DAT in subsequent immunoprecipitation and proteolysis experiments (Vaughan et al., unpublished observations). These results, coupled with those from previous DAT photolabeling experiments, have collectively resulted in a hypothesis wherein the azide must be some distance away from the tropane pharmacophore to covalently attach to the DAT protein near, but not within, the ligand-binding domain.[16]

Given our interest in developing nontropane-based DAT molecular probes related to psychostimulant abuse,[2−4] we decided to revisit the possibility that an inhibitor bearing a photoreactive azide directly within its chemical scaffold (i.e., involving no chemical linkers) could covalently label the DAT protein. In particular, we focused on the well-known attention deficit hyperactivity disorder (ADHD) drug (±)-*threo*-methylphenidate [(±)-**1**], analogues of which have received significant attention as potential cocaine abuse therapeutics.[17,18] Previous SAR studies[19] indicated the 3′ and 4′ positions of the aromatic ring within (±)-**1** could potentially be modified to include the 3′-I, 4′-N_3 motif without adversely affecting DAT binding affinity (vide infra). With this in mind, compound (±)-**6** was designed and envisioned as a compact DAT photoprobe bearing no linker functionality. Such a photoprobe is expected to covalently attach to an amino acid residue directly within the methylphenidate-binding pocket of the DAT and also result in a more conformationally restricted photoprobe-protein complex in 3D hDAT molecular modeling studies.

The synthetic strategy toward target photoprobe (±)-**6** is depicted in Scheme 1 and follows methodology initially

Scheme 1. Synthesis of Target Methylphenidate Photoprobe (±)-6[a]



[a]Reagents and conditions: (a) Piperidine, EtOH, 68 °C. (b) $TsNHNH_2$, H_2SO_4, EtOH, reflux. (c) Aliquat 336, 50% aq. NaOH, toluene, reflux. (d) Toulene, reflux and then Et_2O recrystallization. (e) HCl, MeOH, reflux. (f) $SnCl_2$, MeOH, room temperature. (g) lCl, AcOH, room temperature. (h) $NaNO_2$, HCl, H_2O, 0 °C and then NaN_3, 0 °C.

developed by Axten et al.[20] and later modified by Gutman et al.[21] Ketoamide **8** was prepared in 91% yield from commercially available ester **7** by treatment with piperidine in ethanol. Tosylhydrazone **9** was then generated in 59% yield by allowing the ketoamide to react with *p*-toluenesulfonyl hydrazide in acidic ethanol under reflux. Next, utilization of Aliquat 336 as a phase transfer catalyst in refluxing toluene under basic conditions provided α-diazo amide **10** in 95% yield, which underwent thermal cyclization to provide a diastereomeric mixture of racemic *threo*- and *erythro*-β-lactams. Subsequent recrystallization provided diastereomerically pure racemic *threo*-β-lactam (±)-**11** as the major product in moderate yield (53% yield from **10**). In turn, this *threo*-β-lactam was converted to (±)-*threo*-4-nitromethylphenidate [(±)-**12**] in quantitative yield by ring opening with methanol under acidic conditions. It should be noted that a shorter synthesis of (±)-**12** has been previously reported;[9] however, this route generates the 3-nitro isomer as a difficult impurity to remove and utilizes controlled, expensive (±)-**1** as a starting material. The synthesis in Scheme 1 definitively sets the position of the nitro group via starting material **7**. Nitro reduction then provided aniline (±)-**13** in moderate yield (61%), followed by subsequent synthesis of target probe (±)-**6** in 34% yield via iodination and azidization using previously reported procedures.[22] All (±)-*threo*-methylphenidate analogues were determined to be ≥95% diastereomerically pure by ^{1}H NMR upon comparison to the known data for enantiomerically pure *threo*- and *erythro*-methylphenidate and their aromatic ring-substituted derivatives.[23]

With methylphenidate derivatives (±)-**12**−(±)-**14** and target photoprobe (±)-**6** in hand, ligand affinities (K_i values) were determined for inhibition of [^{3}H]-WIN-35,428 (a cocaine analogue) binding to hDAT in N2A neuroblastoma cells (Table 1). Racemic *threo*-methylphenidate [(±)-**1**] was also synthesized[21] and pharmacologically evaluated for comparison to the novel compounds. Analogous to previous reports,[19] substituting the 4-position of (±)-*threo*-methylphenidate with a nitro group resulted in an ∼4.8-fold loss in hDAT affinity, whereas NH_2 substitution at this position increased affinity ∼2.5-fold. The addition of a 3-I group to aniline derivative (±)-**13** resulted in only a slight decrease (∼1.4-fold) in binding affinity for hDAT. However, replacing the aniline of compound (±)-**14** with a photoreactive azide group, resulting in target probe (±)-**6**, gave an ∼2.3-fold increase in hDAT affinity. In particular, our design of target photoprobe (±)-**6** stemmed from known methylphenidate analog (±)-**17**,[19] which features hydrophobic

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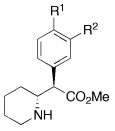
Table 1. Inhibition of [^{3}H]-WIN-35,428 Binding by Methylphenidate Compounds at hDAT N2A Neuroblastoma Cells



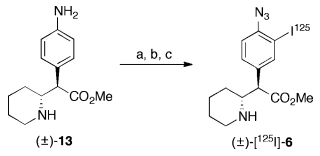
compd no.	R^1	R^2	[^{3}H]-WIN binding inhibition K_i[a] (nM)
(±)-**1**	H	H	16 ± 2.8
(±)-**6**	N_3	I	4.0 ± 0.8
(±)-**12**	NO_2	H	77 ± 14
(±)-**13**	NH_2	H	6.3 ± 2.0
(±)-**14**	NH_2	I	9.1 ± 1.5
(±)-**15**	N_3	H	35 ± 5.4
(±)-**16**	H	I	4.5 ± 1.1
(±)-**17**	Cl	Cl	1.8 ± 0.4

[a]Each K_i value represents data from at least three independent experiments with each data point on the curve performed in duplicate.

chlorine atoms at positions 3′ and 4′ of the aromatic ring. Consistent with a previous report,[19] compound (±)-**17** displayed high DAT affinity in our hands (K_i = 1.8 ± 0.4 nM). In turn, we hypothesized that replacement of the hydrophobic chlorine atoms in (±)-**17** with hydrophobic iodine and azide groups would result a photoprobe with retained DAT affinity. Our results indicate that (±)-**6** (K_i = 4.0 ± 0.8 nM) displays 4-fold higher hDAT affinity than (±)-*threo*-methylphenidate [(±)-**1**], thus representing one of the highest affinity DAT photoprobes synthesized to date. In particular, an ∼91-fold improvement in hDAT affinity is seen relative to our previously reported best methylphenidate photoprobe, (±)-*threo*-*N*-(*p*-azido-benzyl)-4-iodomethylphenidate (K_i = 363 ± 28 nM), which features the photoreactive azide appended to the pharmacophore via an *N*-benzyl linker.[3] Additionally, methylphenidate compounds bearing a 3-iodo group possess high hDAT affinity (K_i <10 nM); adding this functionality to (±)-*threo*-4-azidomethylphenidate [(±)-**15**] increased DAT affinity ∼8.8-fold.

Wash-resistant binding experiments involving nonradioactive azido compounds frequently give false positives in assessment of covalent attachment.[24] As a result, a one-flask synthesis of (±)-[^{125}I]-**6** (Scheme 2) was performed utilizing previously

Scheme 2. Synthesis of Photoprobe (±)-[^{125}I]-6[a]



[a]Reagents and conditions: (a) [^{125}I]-NaI, Chloramine-T, NaOAc (0.2 M, pH 4.0)/MeOH, rt, 30 min. (b) AcOH (3.0 M), $NaNO_2$ (0.5 M), −5 °C. (c) NaN_3 (0.5 M), rt, 15 min, $Na_2S_2O_5$ (50 mM).

described methods.[25] Briefly, electrophilic radioiodination of (±)-**13** with [^{125}I]-NaI (1.49 mCi) under no-carrier-added conditions using Chloramine-T as the oxidant was followed by diazotization and subsequent treatment with sodium azide. Reversed-phase HPLC then provided (±)-[^{125}I]-**6** in 54% isolated yield with high purity (98%) and high specific activity (1986 mCi/μmol). Preparative HPLC confirmed that (±)-[^{125}I]-**6** was resolved from radioactive and nonradioactive side products, and an analytical HPLC trace showed coelution of purified (±)-[^{125}I]-**6** with a fully characterized sample of nonradioactive (±)-**6** (Supporting Information). Compound (±)-[^{125}I]-**6** proved stable and showed 92% radiochemical purity after 3.5 months of storage at −20 °C in the dark.

With (±)-[^{125}I]-**6** in hand, we next turned our attention to preliminary photoaffinity labeling of DAT using previously described procedures.[26] Briefly, rat striatal membranes and $LLCPK_1$ cells expressing 6Xhis-hDAT were photolabeled with 10 nM (±)-[^{125}I]-**6** in the absence or presence of 10 μM (−)-cocaine or D-(+)-methylphenidate. The membranes and cells were then detergent-solubilized, and the lysates were immunoprecipitated with DAT antibody and analyzed by SDS-PAGE/autoradiography. Labeled proteins of ∼80 kDa were obtained from both rat striatal tissue (Supporting Information) and $LLCPK_1$ hDAT cells (Figure 2), demonstrating the

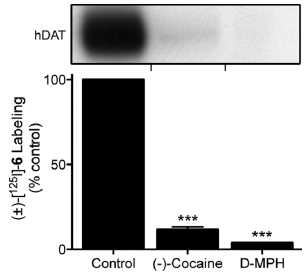


Figure 2. Photoaffinity labeling of DAT with (±)-[^{125}I]-**6**. hDAT $LLCPK_1$ cells were photolabeled with 10 nM (±)-[^{125}I]-**6** in the absence or presence of 10 μM (−)-cocaine or D-methylphenidate (D-MPH). Cells were solubilized and DATs were immunoprecipitated followed by analysis by SDS-PAGE and autoradiography. The relevant portion of a representative autoradiograph is pictured followed by a histogram that quantitates relative band intensities. Means ± SEs of three independent experiments are shown; ***, $p < 0.001$ vs control.

incorporation of (±)-[^{125}I]-**6** into the DAT. Incorporation of the photoprobe was >90% blocked by either (−)-cocaine or D-(+)-methylphenidate, demonstrating the appropriate pharmacological specificity of (±)-[^{125}I]-**6** attachment to the DAT. Similar to results previously reported for tropane, GBR, and benztropine DAT photoaffinity ligands,[27,28] analysis of total cell lysates showed that several proteins undergo adduction with (±)-[^{125}I]-**6** (not shown). However, these do not represent the DAT because they do not immunoprecipitate with DAT antibody as shown for the protein in Figure 2.

In summary, we have designed, synthesized, and pharmacologically evaluated a compact, novel photoaffinity ligand based

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on the well-known ADHD drug (±)-*threo*-methylphenidate. In contrast to tropane-based probes **2**–**4** and our previously reported methylphenidate-based photoprobes,[3] compound (±)-**6** is a nontropane featuring direct attachment of a photoreactive azide within the inhibitor pharmacophore. This photoprobe is expected to covalently attach to an amino acid residue directly within the methylphenidate-binding pocket of the DAT and also result in a more conformationally restricted photoprobe–protein complex in 3-D hDAT molecular modeling studies. As a result, photoprobe (±)-**6** represents an important contribution to the growing arsenal of ligands useful for characterizing DAT function and 3D structure. There is evidence that structurally diverse DAT inhibitors bind to nonidentical DAT sites or conformations,[1,5−8] suggesting that novel irreversible ligands based on methylphenidate may yield new monoamine transporter structure–function information. Additionally, we found that ligand (±)-**6** binds with high affinity to hDAT and [^{125}I]-labeled (±)-**6** binds covalently to rDAT and hDAT expressed in cultured cells. Because the (*R*,*R*)-(+)-enantiomer of *threo*-methylphenidate has been found to be the more biologically active compound,[29] future directions include resolving (±)-**6** with the aim of obtaining a more specific and improved DAT photoaffinity probe. Also, additional DAT irreversible probes based on methylphenidate will be designed, synthesized, and pharmacologically characterized. Their binding site and ligand orientation prediction via docking within 3D DAT homology models[30] and detailed elucidation of DAT binding domains for comparison to established tropane-based probes will be investigated in due course.

■ ASSOCIATED CONTENT

Supporting Information

Experimental section. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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