Articles

Novel Azido and Isothiocyanato Analogues of [3-(4-Phenylalkylpiperazin-1-yl)propyl]bis(4-fluorophenyl)amines as Potential Irreversible Ligands for the Dopamine Transporter

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Potential irreversible ligands were prepared, based on a series of 3-(1-piperazinyl)propyl-*N*,*N*-bis(4-fluorophenyl)amines, as molecular probes for the dopamine transporter (DAT). Both azido- and isothiocyanato-substituted phenylalkyl analogues were synthesized and evaluated for displacement of [^{3}H]WIN 35 428 in rat caudate putamen tissue. All of the analogues showed moderate binding potencies at the DAT. The azido analogue, **16b**, was radioiodinated and used to photolabel human DAT-transfected HEK 293 cell membranes. [^{125}I]**16b** irreversibly labeled an ∼80 kDa band corresponding to the DAT detected by sodium dodecyl sulfate−polyacrylamide gel electrophoresis. This radioligand provides a novel addition to the growing arsenal of structurally diverse irreversible ligands that are being used to identify binding domains on the DAT. Characterizing points of attachment of these irreversible probes to the DAT protein will ultimately help elucidate the three-dimensional arrangement of the transmembrane domains, identify individual binding sites of the DAT inhibitors, and direct future drug design.

The development of irreversible ligands for the characterization of drug molecule−receptor protein interactions has been a successful strategy for more than three decades.[1] With the technological advances in molecular pharmacology, the application of these ligands for the identification of binding sites, at the molecular level, has resulted in a resurgence of interest in designing and implementing these highly selective molecular probes.[2]

The dopamine transporter (DAT) is a primary target of psychomotor stimulant drugs of abuse, such as cocaine. Significant and rapid occupation of mesolimbic dopamine transporters that results in the inhibition of dopamine reuptake by cocaine is directly related to its psychostimulant and reinforcing effects.[3,4] Considerable investigation of numerous chemical classes of dopamine uptake inhibitors has provided ligands with both high affinity and selectivity for the DAT.[5] Many of these ligands have provided important molecular tools with which to further elucidate the role of the DAT in cocaine abuse and to provide leads for potential medications for its treatment.[2,5−7] In addition, structure−activity relationships have provided direction toward the design of specific molecular tools such as irreversible ligands for the DAT.

An early report of the photolabel DEEP (**1**, Figure 1), based on the potent dopamine uptake inhibitor GBR 12909, described its covalent attachment to a 58 kDa protein in rat striatum, which was characterized as the DAT.[8] Additional reports of both photolabels and other electrophile-substituted (e.g., NCS) analogues of dopamine uptake inhibitors were subsequently described.[9−13] Initial studies that used epitope-specific immunoprecipitation of proteolytic fragments of the DAT protein showed [^{125}I]DEEP was incorporated near transmembrane (TM) domains 1 and 2, whereas the 3-phenyltropane photolabel [^{125}I]RTI 82 (**2**) was incorporated in the 4−7 TM region of the DAT.[14,15] These studies were the first to provide experimental evidence of differences in the binding domains of these structurally divergent dopamine uptake inhibitors. In a follow-up study and as a result of discovering structure−activity relationship divergence between the two tropane-based classes of dopamine uptake inhibitors (3-aryltropanes vs benztropines), [^{125}I]GA 2−34 (**3**) was discovered to photolabel an 80 kDa protein in rat caudate putamen that was identified as the DAT.[16] Thus, [^{125}I]**3** labeled the 1−2 transmembrane region of the DAT protein, as did the GBR 12909-based photolabel [^{125}I]DEEP. This binding domain for [^{125}I]**3** contrasts to the 4−7 transmembrane region, identified as the binding domain of the tropane-based ligand [^{125}I]RTI-82.[17] Subsequent studies resulted in the design of an N-substituted 3-phenyltropane photolabel, MFZ 2−24 (**4**),[18] which has recently been

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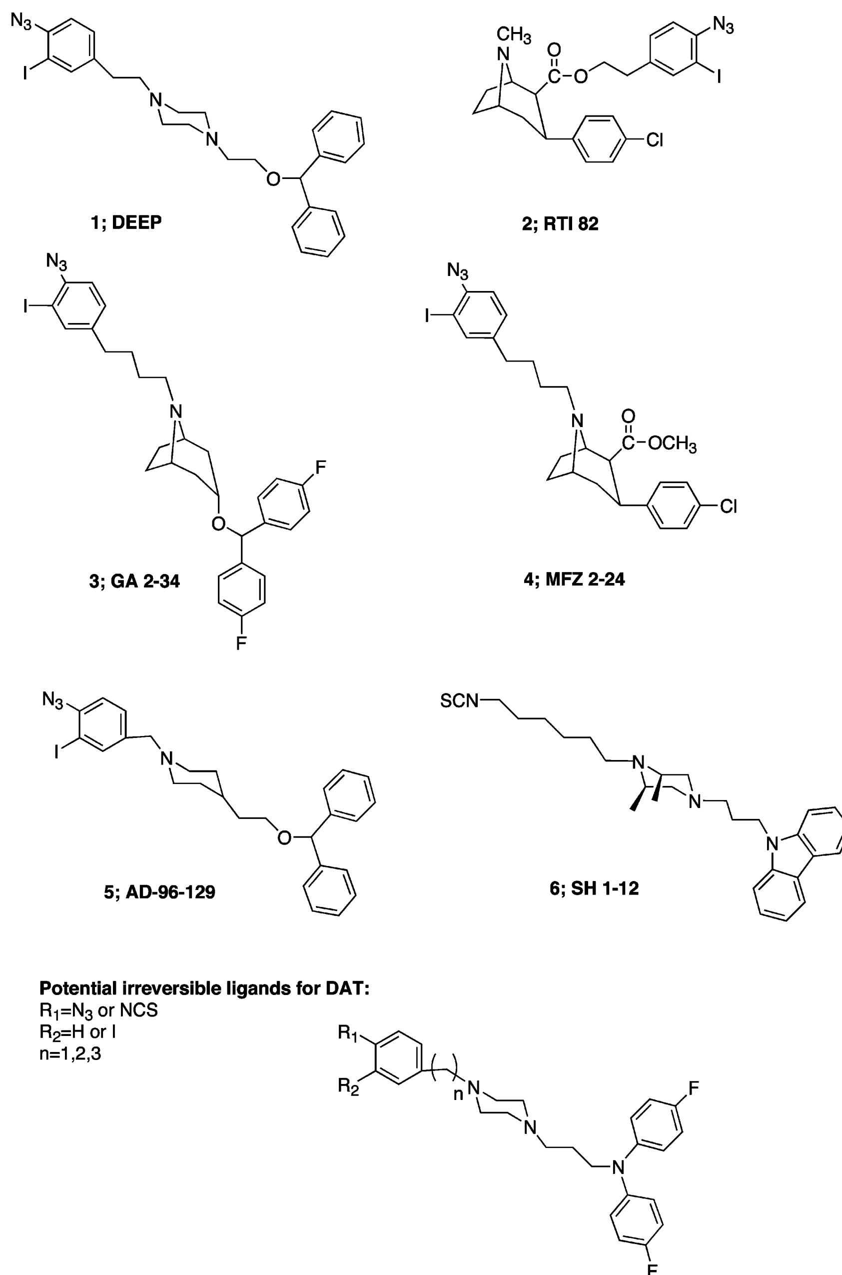


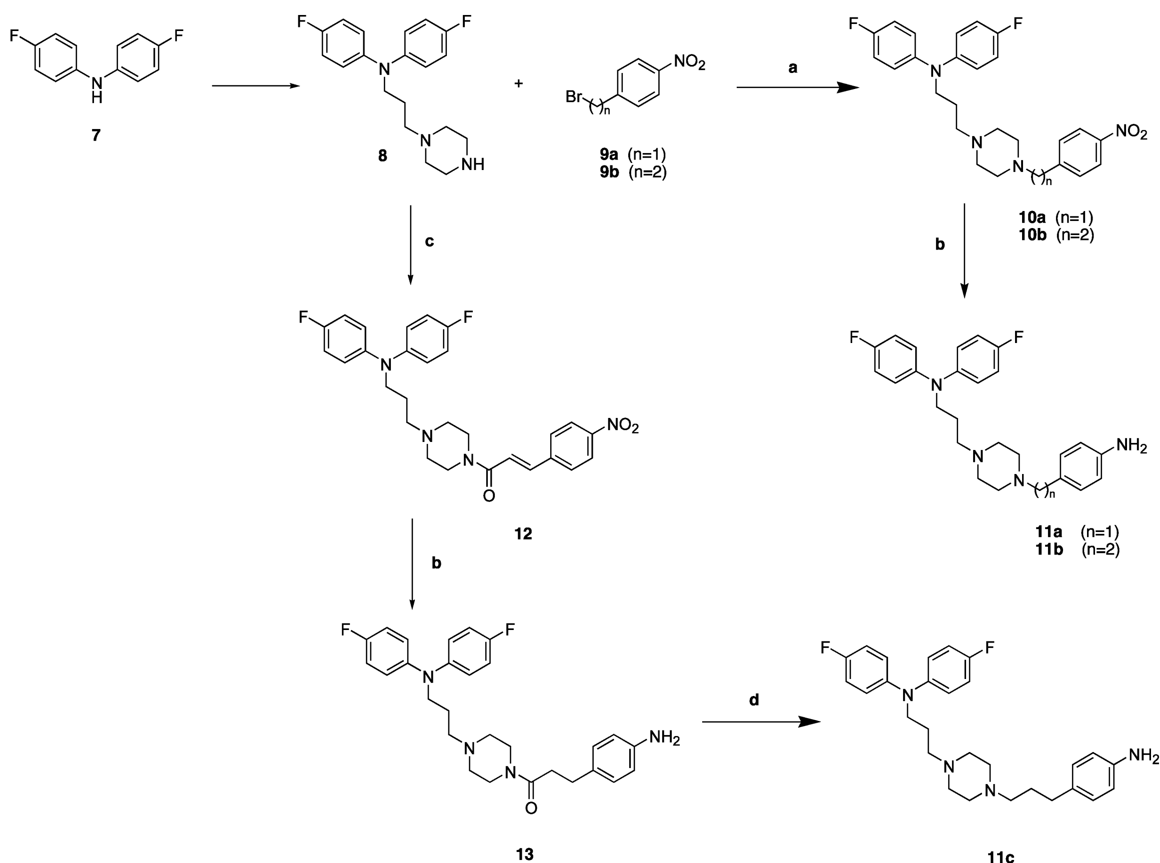
Figure 1. Chemical structures of selected DAT photolabels.

radioiodinated. [^{125}I]**4** is currently being used to identify amino acid residue(s) involved in covalent attachment of this ligand to DAT.

More recently, the dual incorporation of another DAT photoaffinity label, AD-96−129 (**5**) based on a piperidine series of GBR 12909 analogues was reported.[19,20] Proteolysis and immunoprecipitation studies demonstrated that [^{125}I]**5** photolabeled DAT fragments of 45 and 14 kDa, corresponding to those labeled with [^{125}I]**1**, and 32 and 16 kDa fragments, corresponding to those labeled with [^{125}I]**2**.[20,21]

These studies encouraged our interest in another class of dopamine uptake inhibitors, based on rimcazole.[22−24] SAR studies have revealed that the rimcazole analogues show similar trends to those of the GBR 12909 analogues at the DAT. However, divergence in pharmacological and behavioral activity profiles, in animal models of cocaine abuse,[26] prompted us to further investigate binding site interaction at the molecular level. Herein we report the preparation of a series of potential irreversible ligands based on this class of dopamine uptake inhibitors and selection of the most promising analogue for radioiodination and subsequent investigation of the DAT. Previous attempts at an irreversible ligand, based on rimcazole, resulted in SH 1−12 (**6**), which we reported to bind wash-resistantly to the DAT.[22] In that report, addition of an isothiocyanato group extended away from the terminal piperazine nitrogen by an *n*-hexyl chain resulted in the irreversible DAT ligand. Subsequent SAR studies showed that, by replacing the carbazole ring system of rimcazole with a 4,4′-difluorophenylaniline and eliminating the 2,6-dimethyl substitution on the piperazine ring, ligands with higher affinity and selectivity for DAT resulted.[24,25] In addition, substitution of the terminal piperazine ring with alkylaryl groups further improved DAT affinity and provided a template for both isothiocyanato or azido groups, with or without iodo substitution. Hence, a

Scheme 1[a]



[a] Reagents and conditions: (a) K_2CO_3, DMF/H_2O, rt, overnight; (b) H_2 (40 psi), Pd/C (10%), MeOH/EtOAc; (c) (1) 4-nitrocinnamic acid, $SOCl_2$, reflux, 3 h; (2) pentene-stabilized $CHCl_3$, $NaHCO_3$/H_2O, rt, 1 h; (d) AlH_3, THF.

series of potential irreversible ligands based on this design (Figure 1) was prepared.

Chemistry

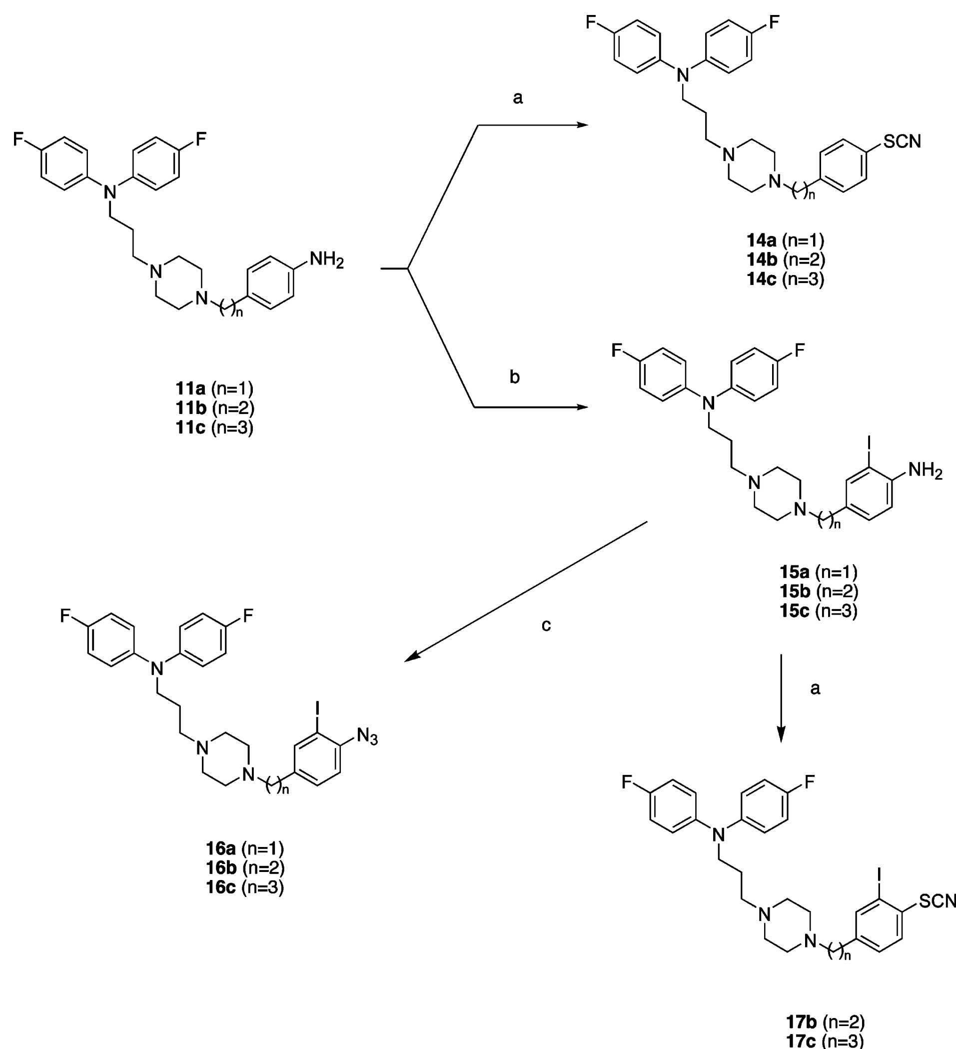
The syntheses of novel analogues **14a**–**c**, **16a**–**c**, and **17b**,**c** are depicted in Schemes 1 and 2. Previously, we reported the synthesis of 4,4-difluorphenylamine (**7**) using literature methods.[25] These methods proved to be unreliable on a larger scale and we report herein a significantly improved procedure using the aryl halide amination catalyst 1,1′-bis(diphenylphosphino)ferrocene (DPPH).[27] Under these conditions, the desired product was obtained in >90% yield and this reaction could be reliably used on a multigram scale. In Scheme 1, compound **8** was prepared as previously reported[25] but with the new method for compound **7**. Coupling of **8** with **9a** followed by catalytic hydrogenation gave the intermediate aniline **11a** in 77% yield. Compound **11b** was prepared in a similar manner with its respective alkylating agent **9b**. Compound **11c** was prepared by the coupling of **8** with 4-nitrocinnamoyl chloride, followed by hydrogenation to **13** and reduction of the amide carbonyl with AlH_3 to give aniline **11c**. In Scheme 2, compounds **11a**–**c** reacted with $CSCl_2$, under classical biphasic conditions, to give products **14a**–**c** in 55–61% yield. Amines **11a**–**c** reacted with ICl in glacial acetic acid to give iodo-substituted products **15a**–**c** in 39–63% yield, which were then treated with $NaNO_2$ followed by NaN_3 to give the azido-iodo products **16a**–**c** in 83–100% yield. Iodo-substituted compounds **15b** and **15c** were treated with $CSCl_2$ to give products **17a** and **17b** in 62% and 67% yield, respectively. All products were purified by flash column chromatography and converted to the oxalate salts.

As shown in Scheme 3, [^{125}I]**16b** was prepared from **11b** in a three-step, one-flask sequence involving electrophilic radioiodination under no-carrier-added conditions, followed by diazotization and substitution of the diazo moiety by azide. Reverse-phase, ion-pair HPLC was used for purification, and [^{125}I]**16b** (t_R = 45.2 min, k' = 77) was well resolved from radioactive and nonradioactive side products that might interfere during photoaffinity labeling trials (Figure 2). Based upon route of synthesis, chromatographic lipophilicity, and previous radiochemical studies of [^{125}I]DEEP[28] and [^{125}I]RTI 82,[29] the major nonradioactive materials are assigned as the corresponding azide (t_R = 18.1 min, k' = 30) and chloroazide (t_R = 31.4 min, k' = 53). In support of this hypothesis, the putative chloroazide was not observed during model studies in the absence of the oxidant chloramine-T. For four consecutive preparations, isolated yields of pure [^{125}I]**16b** ranged from 22% to 30%. Specific radioactivities were within 20% of theoretical and ranged from 1811 to 1942 mCi/μmol.

Results and Discussion

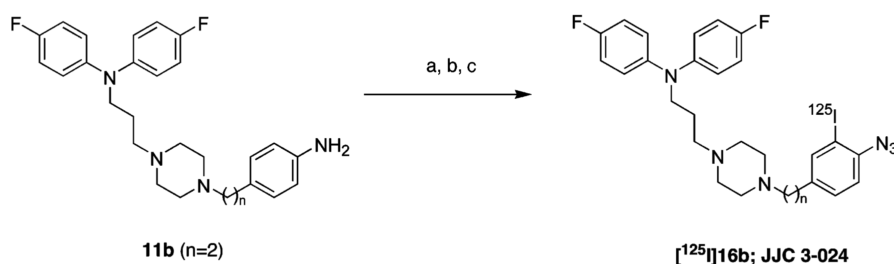
Compounds **14a**–**c**, **16a**–**c**, and **17b**,**c** were evaluated for binding at DAT by displacement of [^{3}H]WIN 35 428 in rat caudate putamen (Table 1). The IC_{50} values show that addition of the 3′-iodo, 4′-azido groups on the alkylphenyl side chains decreased binding affinities as compared to the corresponding unsubstituted arylalkyl

Scheme 2[a]



[a] Reagents and conditions: (a) $CSCl_2$, $NaHCO_3$, $CHCl_3/H_2O$, 0 °C, 3 h; (b) ICl, HOAc (glacial), rt, 3 h; (c) (1) $NaNO_2$, HOAc/H_2O (1:1), 0 °C, 30 min; (2) NaN_3, 0 °C, 30 min.

Scheme 3[a]

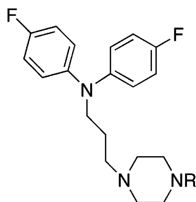


[a] Reagents and conditions: (a) $[^{125}I]NaI$, chloramine-T, NaOAc (0.3 M, pH 5.0)/MeOH, rt, 30 min; (b) HOAc (3.0 M), $NaNO_2$, (0.5 M), −10 °C; (c) NaN_3, rt, 20 min, $Na_2S_2O_5$ (50 mM).

substituted analogues, for example, **18** vs **16a** and **19** vs **16c**.[25] This is consistent with other series of compounds such as the photolabel based on *N*-phenyl-*n*-butyl-4′,4″-difluorobenztropine, **2**,[16] and the piperidine-based photolabel **3**,[19] and may reflect steric bulk and/or electronic properties of these substituents that are not as well tolerated at the DAT. There was not a significant difference in binding affinities between the *N*-benzyl, *N*-ethylphenyl, and *N*-propylphenyl analogues in either the azido- or isothiocyanato-substituted groups.

One observation in this series was that the isothiocyanato analogues consistently showed a 2.5−3-fold higher binding potency for DAT than their corresponding iodo-azido analogues (e.g., **14a** vs **16a**, **14b** vs **16b**, and **14c** vs **16c**). This increase in potency could not be attributed to the detrimental effect of the 3′-iodo group in the azides, as when the 3′-iodo group was added to the isothiocyanates binding affinities were not reduced (e.g., **17b** and **17c**). The binding assay procedure did not include photoactivation, so presumably the azides are not forming a reactive electrophile to covalently attach to an amino acid residue on or near the binding site. However, the isothiocyanates need no photoactivation and are fully reactive as electrophiles under the

Table 1. Binding Data at DAT for Azido and Isothiocyanato Analogues of 3-(4-[3-Phenylalkyl]-1-piperazinyl)Propyl-*N*,*N*-bis(4-fluorophenyl)amines



compd	R	DAT, [^{3}H]WIN 35 428 IC_{50} ± SEM[a] (nM)	Hill coefficient
16a	CH_2-3-I, 4-N_3-Ph	242 ± 30	1.84[b] ± 0.40
16b	CH_2CH_2-3-I, 4-N_3-Ph	163 ± 25	1.54[b] ± 0.29
16c	$CH_2CH_2CH_2$-3-I, 4-N_3-Ph	282 ± 28	1.61[b] ± 0.23
14a	CH_2-4-NCS-Ph	75.4 ± 3.5	1.06 ± 0.13
14b	CH_2CH_2-4-NCS-Ph	68.3 ± 6.6	1.22[b] ± 0.14
14c	$CH_2CH_2CH_2$-4-NCS-Ph	99.8 ± 9.3	1.32[b] ± 0.15
17b	CH_2CH_2-3-I, 4-NCS-Ph	66.0 ± 9.2	1.05 ± 0.13
17c	$CH_2CH_2CH_2$-3-I, 4-NCS-Ph	95.5 ± 12	1.70[b] ± 0.25
18[c]	CH_2-Ph	29.7 ±4.1	1.19 ± 0.15
19[c]	$CH_2CH_2CH_2$-Ph	19.5 ± 2.9	1.44[b] ± 0.12
GBR 12909		21.1 ± 2.18	1.63[b] ± 0.16
1; DEEP		5.61 ± 0.82[d]	1.56[b] ± 0.12

[a] The binding methods used are as reported in ref 33 and described in the Experimental Methods section. [b] This coefficient is significantly different from 1.0 ($p < 0.05$). [c] Synthesis and K_i values for these compounds were previously reported in ref 25. [d] Sucrose PO_4 buffer.

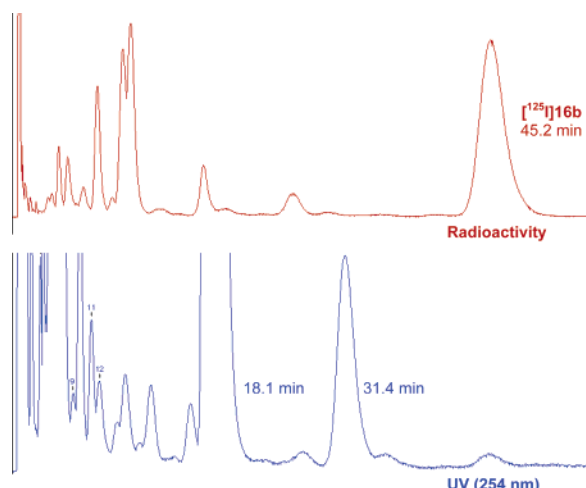


Figure 2. HPLC chromatogram for purification of [^{125}I]**16b**. A 50% incorporation of radioiodine into a peak at 45.2 min, well resolved from side products and appropriate for [^{125}I]**16b**, was observed. The mass of the UV peak associated with the radioactivity was 380 pmol.

conditions of the binding assay. Thus, we speculate that the lower IC_{50} values may be due to covalent attachment to the DAT protein, which results in an apparent higher binding affinity for the isothiocyanates. Indeed, a 3-fold decrease might not be considered very significant when IC_{50} values are compared. However, these small differences have been reported in DAT site-directed mutagenesis studies, wherein a 3-fold change in cocaine's binding affinity has been attributed to a single amino acid residue change that reduces either direct or indirect interaction of the ligand with the mutated protein.[30,31] Others have reported potential covalent interactions with cysteine sulfhydryl groups on the DAT with irreversible and dopamine sparing antagonist.[32] Although the chemical structures of those ligands and these reported herein are significantly different, it suggests that covalent interaction with a cysteine SH on or near the binding domain of these DAT ligands is certainly possible. Additional characterization of these covalent interactions is ongoing.

In general, Hill coefficients of these ligands are >1, suggesting that the actions of these drugs at the site labeled by [^{3}H]WIN 35 428 does not strictly follow the law of mass action. As these compounds are potentially irreversible, covalent attachment and lack of equilibria in binding might account for these values. In fact, Dutta et al.[21] reported that the DAT photolabel **5** had a Hill coefficient of 2. Subsequent studies using immunoprecipitation and protease cleavage of the photolabeled DAT showed dual incorporation of this ligand, suggesting attachment to two separate or overlapping sites on DAT. Further discussion of Hill coefficients of >1 for potential irreversible ligands, based on GBR 12935, has been reported.[10]

On the basis of these studies and our interest in further characterizing the binding domains of irreversible DAT ligands with structural diversity,[17] we selected the azido analogue **16b** to radioiodinate for further investigation. Using procedures similar to those employed for [^{125}I]DEEP,[28] [^{125}I]RTI 82,[29] [^{125}I]**3**,[16] and [^{125}I]-**5**,[20] we prepared [^{125}I]**16b** in moderate yield, with high purity and high specific radioactivity. The radioligand was formulated as a concentrated ethanol solution buffered with pH 7.4 Tris-HCl and was stable when stored at −20 °C in the dark.

Membranes were prepared as described in the Experimental Methods section from HEK-293 cells stably transfected with the human DAT engineered with a 6×-histidine and Flag epitope tags on the N-terminus (F6×H-hDAT). Photolabeling with [^{125}I]**16b** was performed and the solubilized [^{125}I]**16b**-labeled protein was applied directly to SDS−7.5% PAGE. A Western blot of the gel, probed with antibody directed to the Flag epitope incorporated into the hDAT, shows the F6×H-hDAT at approximately 80 kDa (Figure 3). A 24 h exposure of film overlaid onto the developed Western blot generated an autoradiograph in which a dominant band was also observed at approximately 80 kDa (Figure 3), supporting the interpretation that [^{125}I]**16b**

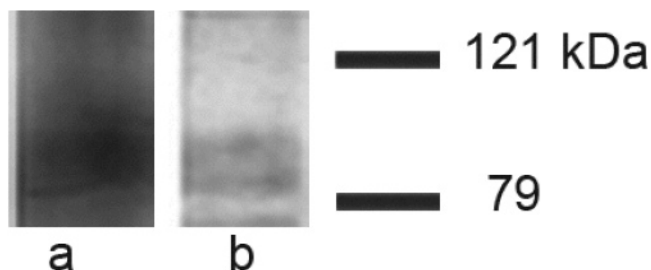


Figure 3. Autoradiograph of a western blot of [^{125}I]**16b**-labeled hDAT. Solubilized N3×Flag6×his-hDAT membranes labeled with [^{125}I]**16b** were separated on a 7.5% Tris glycine minigel and transferred onto a PVDF membrane, as described in the Experimental Methods section. On the right (b) is the Western blot after being probed with the anti-Flag antibody and visualized. A major band is observed at approximately 80 kDa. On the left (a) is an autoradiograph of the western blot. The radioactive signal obtained from [^{125}I]**16b** on the film aligns with the 80 kDa signal obtained from hDAT on the western blot.

labels hDAT. The theoretical weight of the unglycosylated form of the histidine-tagged hDAT is 76 kDa. The 80 kDa band is believed to be the mature glycosylated Flag/histidine-tagged DAT. The immunoblot signal obtained is consistent with western blot analysis routinely performed on hDAT.

Under the assay conditions of this procedure, if covalent attachment of [^{125}I]**16b** had not occurred, the radiolabeled band would not be present. Solubilization of the proteins from the membrane would free any reversibly bound ligand and any other noncovalently bound ligand in the membrane. The protein is also denatured on the gel and any reversibly bound ligand, if not already washed out in solubilizing the protein, would migrate to the bottom of the gel. Together these experiments demonstrate that [^{125}I]**16b** photolabels the DAT protein and can now be used for additional investigation of its binding domain(s).

Summary

Significant indirect support for differing binding domains on the DAT of structurally diverse DAT inhibitors has provided a direction toward the design of novel ligands that may have different pharmacological profiles from cocaine.[2] One approach to further characterizing the DAT inhibitor binding sites is to prepare ligands that can potentially form a covalent linkage with an amino acid residue to ultimately identify points of attachment on the DAT protein. To this end, photoaffinity ligands based on several classes of DAT inhibitors have been designed and have demonstrated early success.[1,17,20] The DAT inhibitors, based on rimcazole, have provided a pharmacologically distinct class of ligands that do not demonstrate a cocaine-like behavioral profile.[26] SAR comparisons to other DAT inhibitors have shown distinctions that might be explained by a differing binding mode that could be exploited toward medication development.[23−25] Thus, we reasoned that further characterization of the binding domain on the DAT, by this class of compounds, might be achieved by use of irreversible ligands, and hence a series of eight 4′-isothiocyanato-, 3′-iodo-4′-isothiocyanato-, and 3′-iodo-4′-azido-substituted phenylalkylpiperazines were prepared. DAT binding data were obtained for these compounds in rat caudate putamen by displacement of [^{3}H]WIN 35 428. Reduction in binding potency of ~10-fold for the iodo-azido analogues, as compared to their unsubstituted parent compounds, was observed. However, this reduction in potency was reduced to ~3-fold for the analogous isothiocyanates. As photolabeling experiments are still preferable for further characterization of DAT binding domains, using immunoprecipitation and proteolytic techniques, compound **16b** was selected for radioiodination. [^{125}I]**16b** labeled an ~80 kDa peptide in rat striatum that has been identified as the DAT. Further investigation by use of this and other irreversible probes will ultimately provide structural characterization of the DAT that has remained elusive due in part to the lack of X-ray data on the crystalline form of the functional DAT protein.

Experimental Methods

All melting points were determined on a Thomas-Hoover melting point apparatus and are uncorrected. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian AS400 instrument. Samples were dissolved in an appropriate deuterated solvent ($CDCl_3$ or D_2O). Proton and carbon chemical shifts are reported as parts per million (δ) relative to tetramethylsilane (Me_4Si; 0.00 ppm), which was used as an internal standard. Mass spectra were recorded on a Hewlett-Packard (Palo Alto, CA) HP 6890 series. Infrared spectra were recorded as a neat film on KBr plates with a Perkin-Elmer RX FT-IR. Microanalyses were performed by Atlantic Microlab, Inc. (Norcross, GA) and agree within ±0.4% of calculated values. All flash column chromatography was performed with flash-grade silica gel (Aldrich, 230−400 mesh, 60 Å). All chemicals and reagents were purchased from Aldrich Chemical Co. or Lancaster Synthesis, Inc. unless otherwise indicated and were used without further purification. Radioactivity was measured with a dose calibrator (Capintec CRC-15W) employing similar counting geometries, coupled with attenuation correction factors as necessary, for each reading.

Chemistry: 4,4′-Difluorophenylamine (7). 4-Fluorophenylamine (278 mg, 2.5 mmol), DPPF (166 mg, 0.3 mmol), (DPPF)$PCl_2\cdot CH_2Cl_2$ (81.7 mg, 0.01 mmol), and sodium *tert*-butoxide (240 mg, 2.5 mmol) were added to 1-fluoro-4-iodobenzene (444 mg, 2 mmol) in dried tetrahydrofuran (THF) (4.5 mL) in a pressure tube under argon.[27] The reaction mixture was heated at 100 °C for 3 h and then cooled to room temperature. HCl (1 M; 4 mL) was added; the mixture was basified with $NaHCO_3$ to pH 9 and extracted with EtOAc (3 × 15 mL); and the combined organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (10:1 to 4:1 hexane/EtOAc) to give the product (380 mg, 93%). MS (EI) 205 (M).

3-[4-(4-Nitrobenzyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (10a). To the mixture of DMF (20 mL) and H_2O (1 mL) was added compound **8**,[25] 4-nitrobenzylbromide (432 mg, 2 mmol), and K_2CO_3 (1.14 g, 8.26 mmol). The mixture was stirred overnight at room temperature and filtered. The filtrate was concentrated in vacuo. The residue was diluted with H_2O (20 mL) and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layer was dried over $MgSO_4$ and evaporated to give the crude product, which was purified by flash column chromatography (2% CH_2Cl_2/MeOH/NH_4OH) to give the pure product (0.83 g, 89%) as an oil. ^{1}H NMR δ 1.74−1.79 (2H, m, J = 7.24 Hz, CH_2), 2.35−2.47 (10H, m), 3.59 (2H, s, N-CH_2-aryl), 3.64−3.69 [2H, t, J = 7.25 Hz, CH_2N-$(PhF)_2$], 6.88−6.98 (10H, m, aryl-Hs), 7.49−7.52 (2H, d, J = 8.75 Hz, aryl-Hs).

3-[4-(4-Aminobenzyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (11a). Compound **10a** (1.25 g, 2.68 mmol) was dissolved in a mixture of MeOH (30 mL) and EtOAc (30 mL), and a catalytic amount of Pd/C (10%) was added. The mixture was placed on a Parr hydrogenator at 40 psi for 0.5 h and then filtered over Celite. The filtrate was concentrated.

The residue was purified by flash column chromatography (2% CH_2Cl_2/MeOH/NH_4OH) to give the pure product (0.90 g, 86%) as a yellow oil. ^1H NMR δ 1.67–1.80 (2H, m, CH_2), 2.23–2.44 (10H, m), 3.40 (2H, s, N-CH_2-aryl), 3.62–3.67 [4H, m, CH_2N-$(PhF)_2$, aryl-NH_2], 6.63–6.65 (2H, m, aryl-Hs), 6.87–6.97 (8H, m, aryl-Hs), 7.07–7.10 (2H, m, aryl-Hs).

3-[4-(4-Aminophenylethyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (11b) was prepared as described for **11a** from **8** (660 mg, 2.0 mmol) by use of 2-(4-nitrophenyl)-ethyl bromide (552 mg, 2.4 mmol). After coupling and hydrogenation, the product (820 mg, 91%) was obtained as an oil. ^1H NMR δ 1.73–1.88 (2H, m, CH_2), 2.35–2.72 (14H, m), 3.64–3.69 [2H, t, $CH_2N(PhF)_2$], 6.60–6.65 (2H, m, aryl-Hs), 6.88–7.00 (10H, m, aryl-Hs).

3-[4-(4-Aminophenylpropyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (11c). A solution of 4-nitrocinnamic acid (676 mg, 3.5 mmol) in $SOCl_2$ (3.5 mL) was stirred at reflux for 3 h. The excess thionyl chloride was removed in vacuo. The pale yellow acid chloride was taken up in 1 mL of pentene-stabilized $CHCl_3$ and added dropwise to the two-phase reaction mixture of **8** (1.16 g, 3.5 mmol), H_2O (17.5 mL), pentene-stablized $CHCl_3$ (56 mL), and $NaHCO_3$ (1.75 g, 21 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature, stirred for 1 h, washed with H_2O (50 mL), and extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layer was dried over $MgSO_4$ and concentrated to give an orange solid (1.73 g, 98%). The intermediate amide (1.1 g, 68%) was prepared as described for **11a** (the reaction time was 7 h) and then reduced by AlH_3 in THF to give the product **11c** as an orange oil (652 mg, 61%). ^1H NMR δ 1.71–1.88 (4H, m), 2.32–2.82 (14H, m), 3.63–3.68 [2H, t, J = 7.30 Hz, CH_2N-$(PhF)_2$], 6.60–6.64 (2H, m, aryl-Hs), 6.87–6.98 (10H, m, aryl-Hs).

3-[4-(4-Isothiocyanatobenzyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (14a). Compound **11a** (240 mg, 0.55 mmol) was dissolved in a mixture of pentene-stabilized $CHCl_3$ (12 mL), H_2O (6 mL), and $NaHCO_3$ (184 mg). To the vigorously stirring mixture was added freshly distilled thiophosgene (54.6 l, 0.71 mmol) at 0 °C, and stirring was continued for 3 h. The two layers were separated, and the aqueous phase was extracted with CH_2Cl_2 (3 × 10 mL). The combined organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (1% CH_2Cl_2/MeOH/NH_4OH) to give the pure product (160 mg, 61%), which was converted to the oxalate salt and recrystallized from MeOH, mp 192–194 C. ^1H NMR δ 1.74–1.80 (2H, m, CH_2), 2.34–2.53 (10H, m), 3.48 (2H, s, N-CH_2-aryl), 3.63–3.68 [2H, t, J = 7.17 Hz, $CH_2N(PhF)_2$], 6.88–7.32 (12H, m, aryl-Hs); ^{13}C NMR δ 25.1, 51.0, 53.5, 53.6, 55.9, 62.7, 116.1, 116.4, 122.6, 122.7, 125.9, 130.3, 130.5, 135.4, 138.3, 144.9, 156.7, 159.9, 167.5; Anal. ($C_{27}H_{28}F_2N_4S2C_2H_2O_4$), C, H, N.

3-[4-(4-Isothiocyanatophenylethyl)-1-piperazinyl]-propyl-*N*,*N*-bis(4-fluorophenyl)amine (14b) was prepared as described for **14a** from **11b** (100 mg, 0.22 mmol) to give the product (60 mg, 55%), which was converted to the oxalate salt and recrystallized from MeOH, mp 198–199 °C. ^1H NMR δ 1.73–1.82 (2H, m, CH_2), 2.35–2.60 (12H, m), 2.76–2.81 (2H, m), 3.64–3.69 [2H, t, J = 7.21 Hz, $CH_2N(PhF)_2$], 6.90–6.97 (8H, m, aryl-Hs), 7.15–7.17 (4H, m, aryl-Hs); ^{13}C NMR δ 25.1, 33.6, 51.0, 53.6, 55.9, 60.4, 116.1, 116.4, 122.6, 122.7, 126.1, 129.5, 130.2, 135.2, 140.4, 144.9, 145.0, 156.7, 159.9, 167.1, 173.8; Anal. ($C_{28}H_{30}F_2N_4S\cdot 2C_2H_2O_4$) C, H, N.

3-[4-(4-Isothiocyanatophenylpropyl)-1-piperazinyl]-propyl-*N*,*N*-bis(4-fluorophenyl)amine (14c) was prepared as described for **14a** from **11c** (120 mg, 0.26 mmol) to give the product (80 mg, 61%), which was converted to the oxalate salt and recrystallized from MeOH, mp 191 °C (decomp); ^1H NMR δ 1.72–1.84 (4H, m), 2.31–2.65 (14H, m), 3.63–3.68 [2H, t, J = 7.20 Hz, $CH_2N(PhF)_2$], 6.88–7.05 (9H, m, aryl-Hs), 6.90–6.97 (8H, m, aryl-Hs), 7.14–7.18 (4H, m, aryl-Hs); ^{13}C NMR δ 25.1, 28.7, 33.7, 51.0, 53.6, 55.9, 58.1, 116.1, 116.4, 122.6, 122.7, 126.0, 129.2, 129.9, 142.2, 144.9, 145.0, 156.7, 159.9, 167.9; Anal. ($C_{29}H_{32}F_2N_4S\cdot 2C_2H_2O_4$) C, H, N.

3-[4-(3-Iodo-4-aminobenzyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (15a). ICl (267 mg, 1.64 mmol) in glacial acetic acid (5.6 mL) was added very slowly (over 3 h) to **11a** (650 mg, 1.49 mmol) in glacial acetic acid (34.6 mL) at room temperature. Acetic acid was then removed in vacuo. The residue was diluted with water (20 mL) and then basified with NH_4OH and extracted with CH_2Cl_2 (3 × 15 mL). The combined organic layer was dried over $MgSO_4$ and concentrated. The crude product was purified by flash column chromatography (1% CH_2Cl_2/MeOH/ NH_4OH) to give the pure product (0.53 g, 63%) as a brown oil.

3-[4-(3-Iodo-4-aminophenylethyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (15b) was prepared as described for **15a** from **11b** (650 mg, 1.44 mmol) to give the product (440 mg, 53%) as a brown oil.

3-[4-(3-Iodo-4-aminophenylpropyl)-1-piperazinyl]-propyl-*N*,*N*-bis(4-fluorophenyl)amine (15c) was prepared as described for **15a** from **11c** (600 mg, 1.29 mmol) to give the product (300 mg, 39%) as a brown oil.

3-[4-(3-Iodo-4-azidobenzyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (16a). Compound **15a** (258 mg, 0.46 mmol) was dissolved in glacial acetic acid (1.5 mL) and H_2O (1.5 mL). To the solution was added $NaNO_2$ (44.6 mg, 0.65 mmol). The mixture was stirred at 0 °C for 30 min. Then NaN_3 (43.7 mg, 0.67 mmol) was added and the mixture was stirred at 0 °C for another 30 min. The reaction mixture was diluted with water (10 mL), basified with $NaHCO_3$ to pH 9, and extracted with CH_2Cl_2 (3 × 7 mL). The combined organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography (2% CH_2Cl_2/MeOH/NH_4OH) to give the pure product (270 mg, 100%), which was converted to the oxalate salt and recrystallized from MeOH, mp 211 °C. ^1H NMR δ 1.74–1.79 (2H, m, J = 7.30 Hz, CH_2), 2.34–2.44 (10H, m), 3.43 (2H, s, N-CH_2-aryl), 3.63–3.68 [2H, t, J = 7.18 Hz, $CH_2N(PhF)_2$], 6.90–7.09 (9H, m, aryl-Hs), 7.26–7.35 (1H, m, aryl-H), 7.75 (1H, m, aryl-H); ^{13}C NMR δ 25.1, 51.0, 53.4, 53.5, 55.9, 61.9, 88.0, 116.1, 116.4, 118.5, 122.6, 122.7, 130.6, 137.3, 140.7, 140.8, 145.0, 159.9, 168.2; Anal. [$C_{26}H_{27}F_2IN_6\cdot 2C_2H_2O_4\cdot 0.5$(2-PrOH)] C, H, N.

3-[4-(3-Iodo-4-azidophenylethyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (16b) was prepared as described for **16a** from **15b** (220 mg, 0.381 mmol) to get the product (190 mg, 83%), which was converted to the oxalate salt and recrystallized from MeOH, mp 217 °C (decomp). ^1H NMR δ 1.73–1.82 (2H, m, CH_2), 2.35–2.58 (12H, m), 2.75–2.76 (2H, m), 3.64–3.69 [2H, t, J = 7.25 Hz, $CH_2N(PhF)_2$], 6.88–7.06 (9H, m, aryl-Hs), 7.21–7.24 (1H, m, aryl-H), 7.63–7.64 (1H, m, aryl-H); ^{13}C NMR δ 25.1, 30.1, 32.8, 51.0, 53.6, 55.9, 60.3, 88.0, 116.1, 116.4, 118.6, 122.6, 122.7, 130.3, 139.3, 139.9, 140.4, 144.9, 145.0, 156.7, 159.9; Anal. [$C_{27}H_{29}F_2IN_6\cdot 2C_2H_2O_4\cdot 0.5$(2-PrOH)] C, H, N.

3-[4-(3-Iodo-4-azidophenylpropyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (16c) was prepared as described for **16a** from **15c** (130 mg, 0.22 mmol) to give the product (120 mg, 90%), which was converted to the oxalate salt and recrystallized from MeOH, mp 212 °C (decomp). ^1H NMR δ 1.75–1.82 (4H, m), 2.30–2.60 (14H, m), 3.64–3.68 [2H, t, J = 7.23 Hz, $CH_2N(PhF)_2$], 6.88–7.05 (9H, m, aryl-Hs), 7.18–7.21 (1H, m, aryl-H), 7.62–7.63 (1H, m, aryl-H); ^{13}C NMR δ 23.8, 25.1, 26.8, 28.7, 30.1, 32.6, 32.8, 51.0, 53.6, 55.9, 57.9, 88.1, 116.1, 116.4, 118.6, 122.6, 122.7, 125.4, 130.0, 135.6, 139.6, 140.2, 141.1, 144.9, 145.0, 156.7, 159.9; Anal. [$C_{28}H_{31}F_2IN_6\cdot 2C_2H_2O_4\cdot 0.5$(2-PrOH)] C, H, N.

3-[4-(3-Iodo-4-(Isothiocyanato)phenylethyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (17b) was prepared as described for **14a** from **15b** (210 mg, 0.35 mmol) to give the product (150 mg, 67%), which was converted to the oxalate salt and recrystallized from MeOH, mp 206 °C (decomp). ^1H NMR δ 1.73–1.82 (2H, m, CH_2), 2.34–2.58 (12H, m), 2.70–2.81 (2H, m), 3.64–3.69 [2H, t, J = 7.15 Hz, CH_2N-$(PhF)_2$], 6.91–6.98 (8H, m, aryl-Hs), 7.16 (2H, m, aryl-Hs), 7.66 (1H, m, aryl-H); ^{13}C NMR δ 25.1, 33.0, 51.0, 53.6, 55.9, 60.0, 94.4, 116.1, 116.4, 122.6, 122.7, 127.0, 130.0, 133.3, 139.9,

141.8, 144.9, 145.0, 156.7, 159.9, 167.9; Anal. ($C_{28}H_{29}F_2IN_4S \cdot 2C_2H_2O_4 \cdot CH_3COCH_3$) C, H, N.

3-[4-(3-Iodo-4-(Isothiocyanato)phenylpropyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine (17c) was prepared as described for **14a** from **15c** (120 mg, 0.20 mmol) to give the product (80 mg, 62%), which was converted to the oxalate salt and recrystallized from MeOH, mp 215 °C (decomp). ^{1}H NMR δ 1.73−1.79 (4H, m), 2.29−2.60 (14H, m), 3.63−3.68 [2H, t, J = 7.50 Hz, $CH_2N(PhF)_2$], 6.91−6.14 (9H, m, aryl-Hs), 7.16−7.23 (1H, m, aryl-H), 7.62−7.64 (1H, m, aryl-H); ^{13}C NMR δ 25.1, 28.5, 30.1, 33.1, 51.0, 53.6, 55.9, 57.8, 94.4, 116.1, 116.4, 122.6, 122.7, 127.0, 129.7, 133.0, 139.7, 143.6, 144.9, 145.0, 156.7, 159.9, 167.9; Anal. [$C_{29}H_{31}F_2IN_4S \cdot 2C_2H_2O_4 \cdot 0.5$(2-PrOH)] C, H, N.

[^{125}I]-3-[4-(3-Iodo-4-azidophenylethyl)-1-piperazinyl]propyl-*N*,*N*-bis(4-fluorophenyl)amine ([^{125}I]**16b**) was prepared by treating aniline **11b** (75 μL, 3.0 mM) in aqueous NaOAc buffer (pH 5.0; 0.3 M) containing MeOH (50% v/v) at ambient temperature with no-carrier-added [^{125}I]NaI (20 μL, 2.21 mCi; ca. 1.0 nmol) followed by *N*-chloro-4-toluenesulfonamide (chloramine-T) trihydrate (15 μL, 3.5 mM) for 30 min. The mixture was chilled at −10 °C, treated with cold HOAc (50 μL, 3.0 M) followed by $NaNO_2$ (25 μL, 0.5 M), and allowed to stand for 15 min. Sodium azide (25 μL, 0.5 M) was added, and the mixture was allowed to warm to ambient temperature over 20 min. The reaction was quenched with $Na_2S_2O_5$ (5 μL, 50 mM), and taken up in a syringe along with rinses (2 × 100 μL) of the vessel with the ternary HPLC mobile phase: MeOH (22.5%), CH_3CN (22.5%), and an aqueous solution (55%) of Et_3N (2.1% v/v) and HOAc (2.8% v/v). The Waters HPLC system was equipped with a UV absorbance detector (254 nm), a flow-through radioactivity detector, and a Waters C-18 Nova-Pak column (radial compression module, 8 × 100 mm, 6 μm). By use of a flow rate of 5 mL/min, radioactive material (t_R = 45.2 min, k' = 77) that corresponded to **16b** was resolved from both nonradioactive and radioactive side products. The [^{125}I]-**16b** was collected (25 mL), diluted with an equal volume of distilled water, and passed through an activated (EtOH/water) solid-phase extraction cartridge (Waters Sep-Pak Light *t*-C-18) that was flushed with water (2.5 mL), to remove residual salts, and then with air. Elution with EtOH (1.0 mL) gave [^{125}I]-**16b** (0.66 mCi; 30%) as a concentrated solution. This material coeluted with **16b** (t_R = 12.8 min, k' = 17), and displayed ≥99% radiochemical purity by HPLC [4 mL/min; 45% aqueous phase as above, 55% MeOH/CH_3CN (1:1)]. Overall recovery of radioligand in the concentration step was 98%. The specific radioactivity of [^{125}I]**16b** was calculated as 1942 mCi/μmol by use of HPLC to determine the mass associated with the UV absorbance peak area of the carrier in a sample of known radioactivity. The UV response for nonradioactive **16b** was linear (r^2 = 1.0) by HPLC for a six-point standard curve over the region of interest (30−450 pmol). Formulations were supplemented (1% v/v) with Tris-HCl buffer (5 mM, pH 7.4) prior to storage at −20 °C in the dark.

DAT Binding Assay in Rat Caudate Putamen. Brains from male Sprague-Dawley rats weighing 200−225 g (Taconic Labs) were removed, and the striatum was dissected and placed on ice. Membranes were prepared by homogenizing tissues in 20 volumes (w/v) of ice-cold modified Krebs-HEPES buffer (15 mM HEPES, 127 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.3 mM NaH_2PO_4, and 10 mM D-glucose, pH adjusted to 7.4) using a Brinkman Polytron (setting 6 for 20 s) and centrifuged at 20000*g* for 10 min at 4 °C. The resulting pellet was resuspended in buffer, recentrifuged, and resuspended in buffer to a concentration of 25 mg/mL. Ligand binding experiments were conducted in assay tubes containing 0.5 mL of modified Krebs-HEPES buffer for 60 min on ice. Each tube contained 1.5 nM [^{3}H]WIN 35 428 (specific activity 84 Ci/mmol) and 2.5 mg striatal tissue (original wet weight). Nonspecific binding was determined by use of 0.1 mM cocaine hydrochloride. For determination of binding affinity, triplicate samples of membranes were preincubated for 5 min in the presence or absence of the compound being tested. Incubations were terminated by rapid filtration through Whatman GF/B filters, presoaked in 0.1% BSA, by use of a Brandel R48 filtering manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed twice with 5 mL of cold buffer and transferred to scintillation vials. Beckman Ready Safe (3.0 mL) was added and the vials were counted the next day in a Beckman 6000 liquid scintillation counter (Beckman Coulter Instruments, Fullerton, CA). Data were analyzed by use of GraphPad Prism software (San Diego, CA).

Labeling hDAT. HEK 293 cells stably expressing DAT were obtained as previously described.[34] HEK-DAT cells were subsequently modified with an N-terminal 3× Flag and an N-terminal 6× histidine affinity tag.[35] Cells were washed with Krebs-Ringer-HEPES (KRH) buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 10 mM glucose, pH 7.4), followed by lysis in 2 mM HEPES and 1 mM EDTA for 10 min at 4 °C. Lysed cells were scraped and centrifuged (31000*g*, 4 °C, 20 min). Pellets and membranes were resuspended twice in incubation buffer (100 mM NaCl and 50 mM Tris base, pH 7.0), sonicated (Heat Systems-Ultrasonics, model W-185F) for 10 s, and centrifuged as above. Each pellet was then resuspended by sonicating in 150 mL of incubation buffer, to which [^{125}I]**16b** was added to a final concentration of 30 nM, and the mixture was incubated for 1 h on ice. The membrane/ligand suspension was transferred to a 60 mm × 15 mm culture dish (Corning) and irradiated for 45 s three times with a shortwave (254 nm) UVG-11 Mineralight lamp (UVP). To wash away unreacted [^{125}I]**16b**, the preparations were diluted 1:2 in incubation buffer and centrifuged (20000*g*, 4 °C, 20 min). Pellets were resuspended in 1 mL of incubation buffer and centrifuged again. The resulting pellets were brought up in 400 μL of Flag solubilization buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl, and 50 mM Tris-HCl, pH 7.4) and thoroughly resuspended by repeatedly drawing the mixture through a pipet tip. Solubilization was carried out overnight at 4 °C. The preparation was then centrifuged for 1 h at 31000*g*, 4 °C. The supernatant, containing the solubilized radiolabeled membrane proteins, was removed and saved at 4 °C for SDS−PAGE.

Gel Electrophoresis. Precast Tris-HC1 minigels, 7.5% acrylamide (10 cm × 10 cm × 0.8 mm), were from Bio-Rad. Samples were prepared by addition of 5 volumes of samples to 1 volume of SDS−PAGE sample buffer (20% glycerol, 1% SDS, and 50 mM Tris, pH 6.8, with bromphenol blue). ProSieve Color Protein Markers (Cambrex) were used for calibration. Gels were run at 200 V for 30−35 min, on a Bio-Rad Protean II apparatus with a Bio-Rad PowerPac 1000. The mini gel was transferred onto a poly(vinylidene difluoride) (PVDF) membrane and probed with primary antibody directed to the Flag epitope (Sigma).

Immunoblotting. Precast gels were soaked in transfer buffer consisting of 39 mM glycine, 40 mM Tris, and 10% MeOH for 15 min. A Fisher Scientific or Bio-Rad semidry blotting apparatus was used for electrotransfer of proteins to PVDF membranes. Whatman 3 M filter paper, 2 × 6 layers, was soaked with transfer buffer, as was the PVDF membrane following wetting with methanol. Electrotransfer was carried out for 90 min at 60 mA constant current per gel. After transfer is complete, the PVDF membrane was allowed to air-dry. Methanol was used to rehydrate the PVDF membrane. The membranes were washed twice for 5 min with TBS buffer (150 mM NaCl and 10 mM Tris, pH 7.6). The membranes are then blocked with blocking buffer (5% nonfat dry milk, 0.1% Tween-20, 150 mM NaCl, and 10 mM Tris, pH 7.6) for 1 h, with shaking, at room temperature. The blocked membrane was then washed three times for 10 min with TTBS (0.1% Tween-20, 150 mM NaCl, and 10 mM Tris, pH 7.6). Primary antibody directed against Flag epitope was used according to the manufacturer's suggestion. The antibody was prepared in blocking buffer, applied to the membrane, and incubated overnight at 4 °C on a shaker. Next, the membranes were washed three times for 10 min each with TTBS at room temperature. A secondary antibody directed toward the primary antibody containing a phosphatase-conjugated IgG (Pierce) was diluted 1:10 000 in blocking buffer and applied

to the membrane for 1 h with shaking at room temperature. The membrane was then washed again three times, for 10 min, with TTBS. The membranes were developed with nitro blue tetrazolium/3-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP; Pierce). After application, the membranes were visually monitored for development and stopped by rinsing the membranes several times with distilled water.

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Supporting Information Available: Results from elemental analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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