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Conformationally-flexible benzamide analogues as dopamine D_3 and σ_2 receptor ligands

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Abstract—A series of conformationally-flexible analogues was prepared and their affinities for D2-like dopamine (D_2, D_3 and D_4) were determined using in vitro radioligand binding assays. The results of this structure–activity relationship study identified one compound, **15**, that bound with high affinity (K_i value = 2 nM) and moderate selectivity (30-fold) for D_3 compared to D_2 receptors. In addition, this series of compounds were also tested for affinity at σ_1 and σ_2 receptors. We evaluated the affinity of these dopaminergic compounds at sigma receptors because (a) several antipsychotic drugs, which are high affinity antagonists at dopamine $D_{2\text{-like}}$ receptors, also bind to sigma receptors and (b) sigma receptors are expressed ubiquitously and at high levels (picomoles per mg proteins). It was observed that a number of analogues displayed high affinity and excellent selectivity for σ_2 versus σ_1 receptors. Consequently, these novel compounds may be useful for characterizing the functional role of σ_2 receptors and for imaging the σ_2 receptor status of tumors in vivo with PET.

The dopamine receptor subtypes are members of the G protein coupled receptor protein superfamily. Based upon genetic and cDNA cloning studies it is currently thought that there are five functionally active dopamine receptor subtypes expressed in mammalian brain. These five subtypes have been classified into two major classes, the $D_{1\text{-like}}$ and $D_{2\text{-like}}$ receptors. Stimulation of the $D_{1\text{-like}}$ receptor subtypes, which include the D_1 (D_{1a}) and the D_5 (D_{1b}) receptors, results in an activation of adenylyl cyclase and an increase in the production of cAMP. Agonist stimulation of the $D_{2\text{-like}}$ receptors, which consist of the D_2, D_3 and D_4 receptors, results in an inhibition of adenylyl cyclase activity, an increase in the release of arachadonic acid, and an increase in phosphatidylinositol hydrolysis.

Over the past 5 years, there has been interest in developing agents that are antagonists of the dopamine D_3 receptor.[1,2] This interest was largely generated by the hypothesis that dopamine D_3 receptors may play a pivotal role in the development of a number of neurological and neuropsychiatric disorders. Receptor autoradiography studies have shown that both D_2 and D_3 receptors are widely distributed in striatal regions of human[3] and monkey[4] brain. However, the higher ratio of D_3 versus D_2 receptors in limbic structures indicates that the D_3 receptor may play an integral role in the pathological abnormalities that occur in neuropsyciatric disorders. Autoradiography studies have also revealed a decrease of D_3 receptors in the frontal cortex and an increase in expression in the ventral striatum of schizophrenics compared to normal individuals.[5,6] Dopamine D_3 receptors are also believed to play a role in the dyskinesias associated with l-dopa treatment of patients with Parkinson's Disease. For example, chronic treatment of squirrel monkeys with the neurotoxin, 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), which causes a selective destruction of the nigrostriatal dopaminergic system, results in a decline of D_3 receptors in the caudate (motor region) but not the putamen of globus pallidus (limbic regions). However, treatment

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with the drug levodopa led to a restoration of D_3 receptor levels and a reversal of Parkinsonian symptoms in MPTP-treated animals. Finally, the activation of dopamine D_3 receptors in the nucleus accumbens is believed to be involved in the sensitization/rewarding properties of psychostimulants, such as cocaine. Therefore, agents that can block the interaction of psychostimulant-induced increases in synaptic dopamine levels with the D_3 receptor have potential for the pharmacological treatment of cocaine abuse.[1]

A number of conformationally-flexible benzamide analogues displaying a high affinity and selectivity for D_3 versus D_2 receptors have been reported in recent years. Examples of this include NGB 2849, NGB 2904, and the structural congeners **1**–**3** (Fig. 1). A common structural feature in the conformationally-flexible benzamide analogues is the *N*-2,3-dichlorophenylpiperazine ring and the four carbon spacer group separating the benzamide and the basic amino moieties.[7–10]

We have recently reported two different classes of compounds, naphthamide analogues and pyrrole derivatives, displaying a modest affinity and selectivity for D_3 versus D_2 receptors.[11,12] For example, the naphthamide analogues **4** and **5** have a moderate selectivity for D_3 versus D_2 receptors. Unfortunately, these compounds were found to have a relatively high affinity for sigma receptors (Fig. 2). Because sigma receptors are expressed ubiquitously and at high levels (picomoles per mg proteins), the high affinity of these compounds for sigma receptors limits their utility for in vitro and in vivo studies of dopamine D_3 receptors.[11] The pyrrole analogues **6** and **7** have a high affinity and 10-fold selectivity for D_3 versus D_2 receptors. However, unlike the naphthamide analogues, the pyrrole analogues bound with low affinity at sigma receptors.[12] As part of a continuing effort to develop potent and selective D_3 receptor ligands as potential radiotracers for studies of the dopaminergic system with the noninvasive imaging procedure, Positron Emission Tomography (PET), we explored the possibility of preparing hybrid ligand structures of the conformationally-flexible benzamide analogues (Fig. 1) and the naphtamide and pyrrole analogues (Fig. 2). The results of this study led to the identification of a number of compounds possessing a high affinity and moderate selectivity for dopamine D_3 versus D_2 receptors.

In addition, the results of this structure–activity relationship study led to the identification of a number of conformationally-flexible benzamide analogues that had a high affinity and excellent selectivity for σ_2 versus σ_1 receptors. Sigma-2 receptors have been shown to be a potential biomarker for determining the proliferative status of breast tumors.[13–15] Therefore, the results of this study have led to the identification of lead compounds for radiotracer development with two completely different functions: (1) D_3 receptor imaging agents for studying the change in dopamine receptor function in a variety of neurological and neuropsychiatric disorders and, (2) σ_2 selective imaging agents for measuring the proliferative status of breast tumors in vivo with PET.

The strategy chosen for the current study involved the combination of the following structural moieties of the lead compounds: (a) the 2,3-dichlorophenylpiperazine

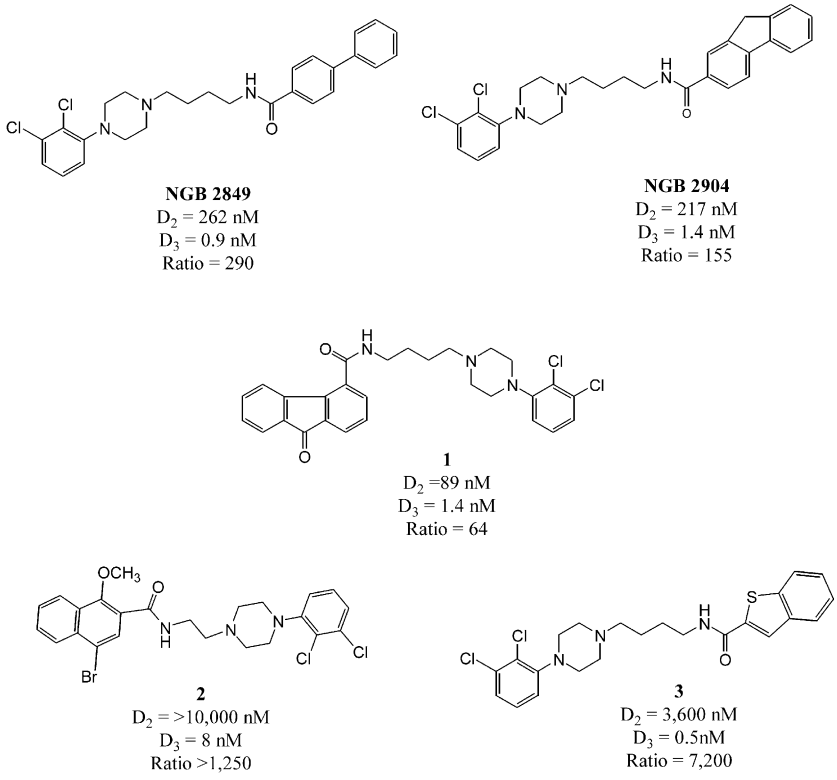


Figure 1.

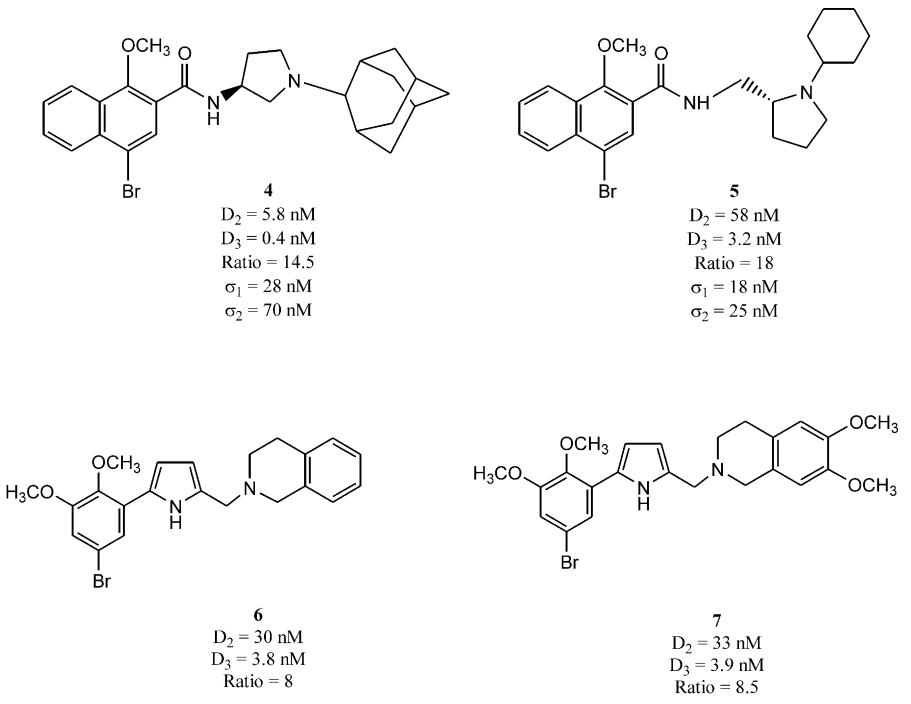


Figure 2.

and the two- and four-carbon spacer groups of the conformationally-flexible benzamide analogues shown in Figure 1; (b) the naphthyl group of naphthamide compounds shown in Figure 2; and, (c) the 2,3-dimethoxyphenyl and tetrahydroisoquinoline moieties of the pyrrole analogues shown in Figure 2.

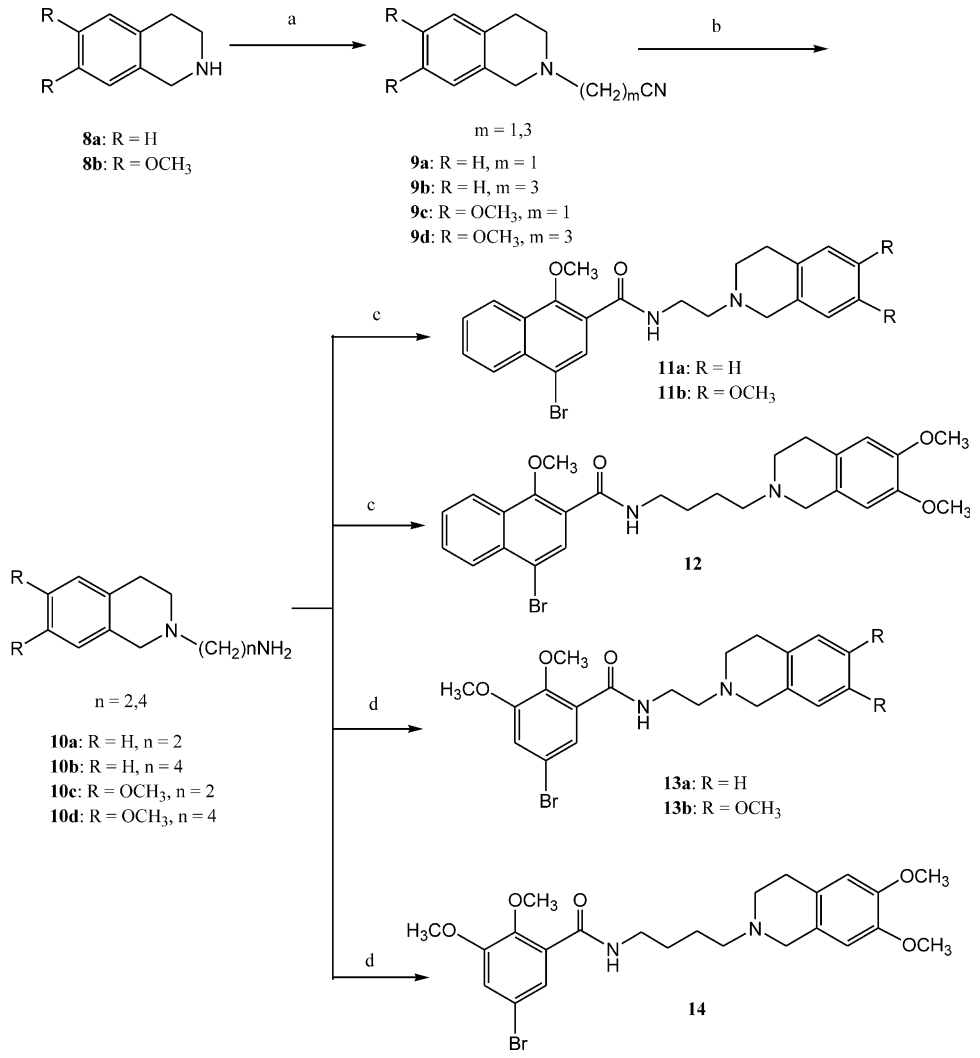
The synthesis of the target tetrahydroisoquinoline analogues is shown in Scheme 1.[16] Reaction of the secondary amine of **8a** and **8b** with either bromoacetonitrile or bromobutyronitrile gave *N*-alkylated products, **9a–d**, in 75–85% yield. Reduction with either lithium aluminum hydride in THF or hydrogenation over palladium on charcoal gave the corresponding amines, **10a–d**, in quantitative yields. Condensation of amines **10a–d** with either 2-methoxy-5-bromonaphthoyl chloride[12] or 5-bromo-2,3-dimethoxybenzoic acid[13] gave the corresponding amide analogues, **11–14**, in excess of 90% yield. Synthesis of the 2,3-dichlorophenylpiperazine benzamide and naphthamide analogues, **15** and **16**, was accomplished using a similar reaction sequence as outlined in Scheme 2.[16]

Table 1. Binding affinities for dopamine D_2/D_3 and sigma σ_1/σ_2 receptors

Compd	K_i (nM)[a]			
	D_2[b]	D_3[c]	σ_1[d]	σ_2[e]
11a	131.6±24.6	81.6±21.28	15.1±1.7	47.7±2.5
11b	240.5±19.4	126.5±42.4	189.1±2.6	21.2±0.1
12	741.0±287.3	106.5±24.3	1,159±7	17.6±0.7
13a	429.7±76.1	17.8±0.5	276.5±35.7	716.5±9.8
13b	714.0±133.7	21.4±2.3	2932±28	16.4±2.0
14	2200±390	627±244	12,900±111	8.2±1.4
15	58.8±13.7	2.1±0.4	809±66	75.0±4.1
16	107.0±19.0	10.2±5.3	751±6	26.4±1.4

[a] Mean±SEM, K_i values were determined by at least three experiments.
[b] K_i values for D_2 receptors were measured on rat $D_{2(long)}$ expressed in Sf9 cells using [^{125}I]IABN as the radioligand.[18]
[c] K_i values for D_3 receptors were measured on rat D_3 expressed in Sf9 cells using [^{125}I]IABN as the radioligand.[18]
[d] K_i values for σ_1 receptors were measured on quinea pig brain membranes using [^{3}H](+)-pentazocine as the radioligand.[17]
[e] K_i values for σ_2 receptors were measured on rat liver membranes using [^{3}H]-DTG as the radioligand in the presence of (+)-pentazocine.[17]

The naphthamide analogue **11a**, which contains a two-carbon spacer group between the amide nitrogen and the basic amino group had a relatively low affinity for dopamine D_3 and D_2 receptors (Table 1). Compound **11a** also had an appreciable affinity for both σ_1 and σ_2 receptors. Introduction of a methoxy groups into the 4- and 5-positions of the tetrahydro-isoquinoline ring resulted in a reduction in affinity for D_2, D_3 and σ_1 receptors, and an increase in affinity for σ_2 receptors. Increasing the length of the spacer group from two carbon units in **11b** to four carbon units (i.e., **12**) resulted in no change in affinity for D_3 and σ_2 receptors, but a dramatic reduction in affinity for D_2 and σ_1 receptors. Replacement of the naphthamide group of **11a** with a 5-bromo-2,3-dimethoxy benzamide group (i.e., **13a**) resulted in an increase in affinity for dopamine D_3 receptors, and a decrease in affinity for D_2, σ_1 and σ_2 receptors. Introduction of the methoxy groups into the 4- and 5-positions of the tetrahydroisoquinoline ring of **13a** to give **13b** resulted in no change in affinity for D_3 receptors and a reduction in affinity for D_2 receptors. However, this change in substitution pattern resulted in

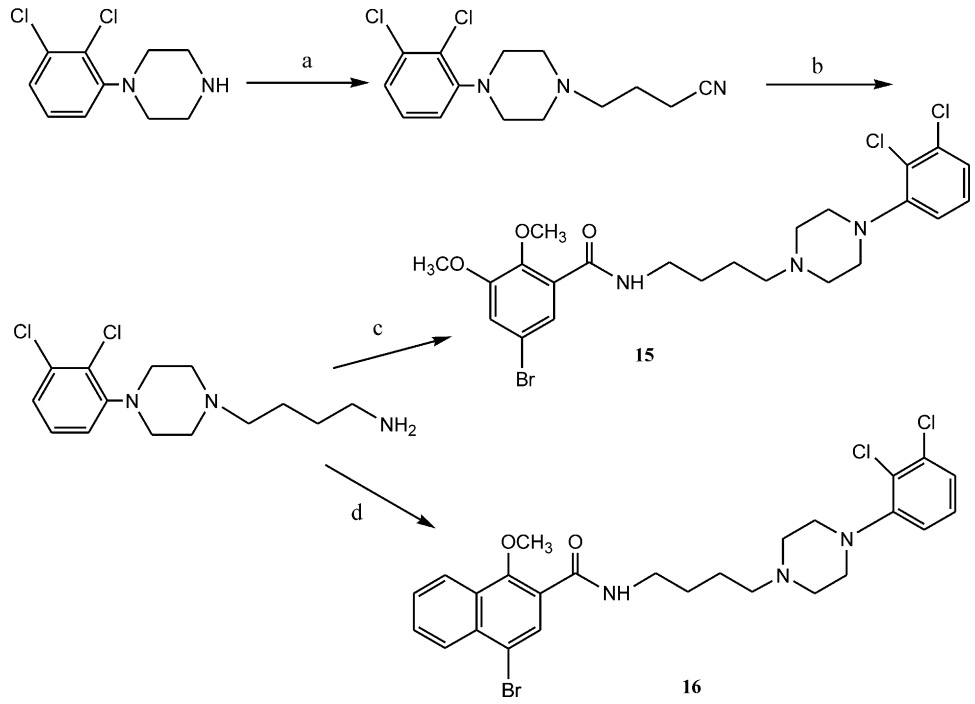


Scheme 1. (a) $BrCH_2CN$ or $BrCH_2CH_2CH_2CN$, Et_3N, CH_2Cl_2, rt; (b) $LiAlH_4$, THF or H_2, Pd(c), ethanol; (c) ref 10; (d) ref 12.

a dramatic decrease in affinity for σ_1 receptors and a large increase in affinity for σ_2 receptors. Increasing the length of the spacer group from two carbons in **13b** to four carbons in **14** resulted in large decrease in affinity for D_2, D_3, and σ_1 receptors and an increase in σ_2 receptor affinity. Compound **14** is unique in that is has over a 1500-fold higher affinity for σ_2 versus σ_1 receptors. Although many different classes of compounds have been shown to bind with high affinity to sigma receptors, most compounds studied to date have either (a) a higher affinity for σ_1 versus σ_2 receptors, or (b) equipotency with respect to binding to σ_1 and σ_2 receptors. Compound **14** is one of the most potent and selective σ_2 receptor ligands reported to date.

Compound **15** was prepared to determine the effect of introducing the 5-bromo-2,3-dimethoxy benzamide moiety into NGB 2904 and its structural congeners (Fig. 1) on D_2 and D_3 receptor affinity. In addition, compound **16** was prepared to assess the effect that increasing the two-carbon spacer group of **2** (Fig. 1) to four carbons would have on dopamine receptor affinity. Compounds **15** and **16** had the highest affinity for D_3 receptors of the analogues prepared in this study, which further emphasizes the importance in the 2,3-dichlorophenyl-piperazine group for binding to dopamine D_3 receptors.

Compounds **17**–**20** were prepared with the goal of increasing the σ_2 receptor affinity and reducing the dopamine D_2 and D_3 receptor affinity of this class of compounds (Scheme 3). The results of our previous structure–activity relationship studies indicated that both the 5-bromo and 3-methoxy groups were important for the binding the binding of pyrrole analogues shown in Figure 2 to dopamine D_2 and D_3 receptors. Therefore, removal of the 3-methoxy group, or replacement of the 5-bromo moiety with a methyl group, was expected to result in a reduction in affinity for D_2 and D_3 receptors. The results of the structure–activity relationship study are shown in Table 2. Compound **14** is included in Table 2 for comparison. Removal of the 3-methoxy group of **13a** to give **17** resulted in a large reduction in affinity for D_2 and D_3 receptors and an increase in affinity for σ_1 and σ_2 receptors. The same change in the structure of **13b** to give compound **18** resulted in a similar effect on dopamine receptor binding. However, there was a marked decrease in affinity of **18** for σ_1 receptors relative to that **13b**. Compound **18** also had a



Scheme 2. (a) $BrCH_2CH_2CH_2CN$, Et_3N, CH_2Cl_2; (b) H_2, Pd(c), ethanol; (c) ref 12; (d) ref 10.

Table 2. Binding affinities of **17**–**20** for dopamine D_2/D_3 and sigma σ_1/σ_2 receptors

Compd	K_i (nM)[a]				
	D_2[b]	D_3[c]	σ_1[d]	σ_2[e]	σ_1/σ_2 ratio
14	2200±390	627±244	12,900±111	8.2±1.4	1573
17	2190±351	310.7±54.4	21.8±5.6	89.4±13.9	0.24
18	3570±796	488.0±70.7	5484±266	12.4±1.8	442
19	2850±316	3760±618	10,412±462	13.3±0.1	783
20	642.0±141.0	313.0±141.0	3078±87	10.3±1.5	300

[a] Mean±SEM, K_i values were determined by at least three experiments.
[b] K_i values for D_2 receptors were measured on rat $D_{2(long)}$ expressed in Sf9 cells using [^{125}I]IABN as the radioligand.
[c] K_i values for D_3 receptors were measured on rat D_3 expressed in Sf9 cells using [^{125}I]IABN as the radioligand.
[d] K_i values for σ_1 receptors were measured on guinea pig brain membranes using [^{3}H](+)-pentazocine as the radioligand.
[e] K_i values for σ_2 receptors were measured on rat liver membranes using [^{3}H]-DTG as the radioligand in the presence of (+)-pentazocine.

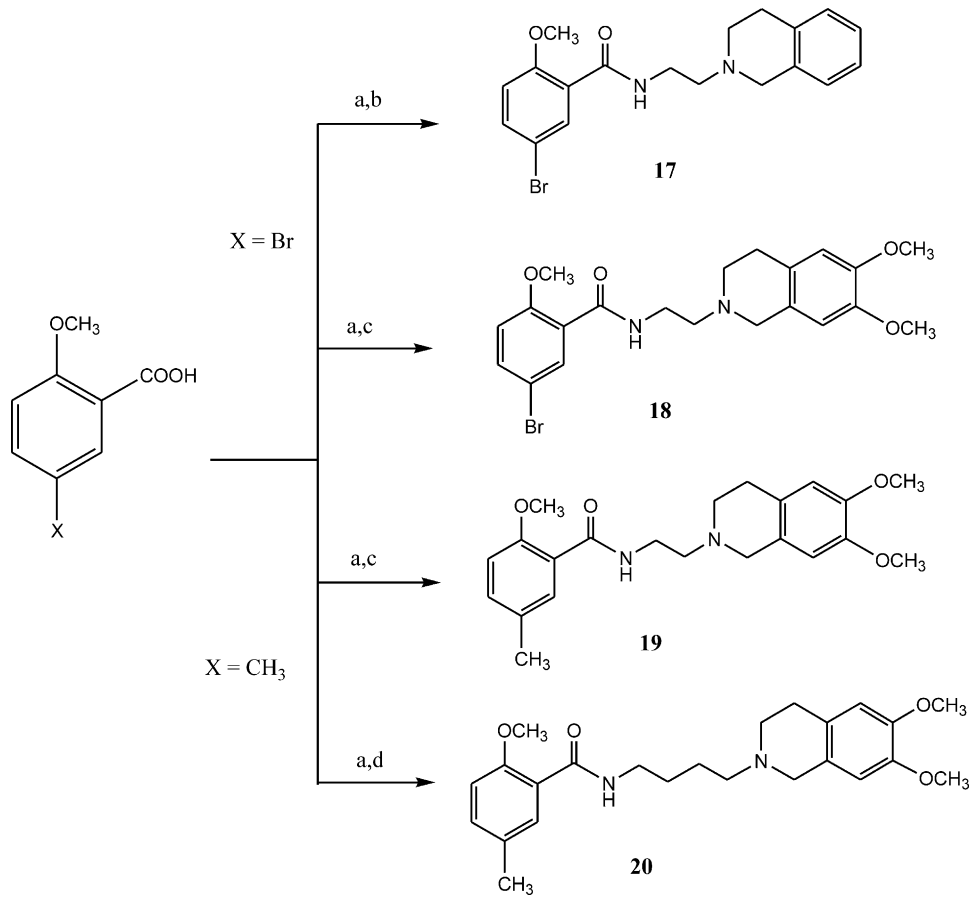
higher affinity for σ_2 receptors than that of **13b**. Replacement of the 5-bromo moiety of **18** with a methyl group (i.e., **19**) resulted in a further reduction in affinity for D_3 and σ_1 receptors, and no change in affinity for D_2 and σ_2 receptors. Increasing the length of the spacer group of **19** from two carbons to four carbons (i.e., **20**) resulted in an increase in affinity for D_2, D_3 and σ_1 receptors, and no change in affinity for σ_2 receptors. The high σ_1/σ_2 selectivity ratio of compounds **14**, **18**, **19**, and **20** indicate that they have the potential to be useful lead compounds for the development of imaging agents for determining the σ_2 receptor status of breast tumors with PET.

Binding assays were also conducted on compounds **13a**, **13b**, **15** and **16** to determine their affinity for dopamine $D_{4.4}$ receptors. The results of this study are shown in Table 3. All four compounds had a lower affinity for $D_{4.4}$ receptors relative to their binding potencies at dopamine D_3 receptors. Compound **15** had the highest D_3 receptor affinity and highest D_2/D_3 and D_4/D_3 selectivity ratios of the four compounds listed in Table 3. The presence of the methoxy groups indicates that a carbon-11 labeled version of **15** can be prepared by

Table 3. In vitro binding data for dopamine D_4 receptors

Compd	K_i (nM)[a]				
	D_2[b]	D_3[c]	D_4[d]	D_2/D_3 ratio[e]	D_4/D_3 ratio[f]
13a	429.7±76.1	17.8±0.5	47.9±11.6	24	2.7
13b	714.0±133.7	21.4±2.3	265.0±60.0	33	12.4
15	58.8±13.7	2.1±0.4	800.0±330.0	28	381
16	107.0±19.0	10.2±5.3	1345±448	10.5	132

[a] Same as Table 1.
[b] Same as Table 1.
[c] Same as Table 1.
[d] K_i for inhibiting the binding of [^{125}I]IABN to human $D_{4.4}$ receptors.
[e] K_i for D_2/K_i for D_3.
[f] K_i for D_4/K_i for D_3.



Scheme 3. (a) $SOCl_2$, benzene, reflux; (b) **10a**, Et_3N, CH_2Cl_2, rt; (c) **10c**, Et_3N, CH_2Cl_2, rt; (d) **10d**, CH_2Cl_2, Et_3N, rt.

alkylation of the des-methyl precursor with ^{11}C-methyl iodide. We are currently exploring [^{11}C]**15** as a potential radiotracer for imaging dopamine D_3 receptors with PET and the relatively low affinity of **15** for σ_1 and σ_2 receptors indicates that there will be minimal in vivo binding to sigma receptors.

In conclusion, we have completed a structure–activity relationship study on a series of benzamides with the goal of identifying a potential radiotracer for imaging dopamine D_3 receptors with PET. The results of this study revealed compound **15** as a candidate for labeling D_3 dopamine receptors in vivo. The low affinity binding of **15** at sigma receptors subtypes servers to minimize sigma receptor interactions as a potential source of nonspecific/background binding. In addition, this study has lead to the identification of a number of novel, σ_2-receptor selective ligands. Based upon those findings we are also exploring the potential of ^{11}C-labeled versions of **14**, **18**, **19** and **20**, and ^{76}Br-labeled versions of **14** and **18**, as potential radiotracers for imaging the σ_2 receptor status of breast tumors.

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16. Data for **11a**. Mp 180–182 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 3.02 (s, 2H), 3.19 (9s, 2H), 3.72–3.74 (m, 3H), 3.94 (s, 3H), 4.21 (s, 3H), 7.16–7.25 (m, 4H), 7.75 (t, J = 8.1 Hz, 1H), 7.81 (t, J = 8.1 Hz, 1H), 8.05 (s, 1H), 8.15 (d, J = 8.3 Hz, 1H), 8.24 (d, J = 8.3 Hz, 1H), 8.77 (t, J = 5.4 Hz, 1H). Calcd: C: 56.72; H: 4.76; N: 5.29. Obsvd: C: 56.50; H: 4.91; N: 5.14.
Data for **11b**. Mp 187–189 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 2.94 (s, 3H), 3.19 (s, 4H), 3.71 (s, 3H), 3.73 (s, 3H), 3.94 (s, 3H), 4.12 (s, 3H), 6.74 (s, 1H), 6.79 (s, 1H), 7.75 (t, J = 7.6 Hz, 1H), 7.82 (t, J = 7.6 Hz, 1H), 8.05 (s, 1H), 8.15 (d, J = 8.3 Hz, 1H), 8.25 (d, J = 8.3 Hz, 1H), 8.76 (s, 1H). Calcd: C: 55.02; H: 4.96; N: 4.75. Obsvd: C: 54.76; H: 5.04; N: 4.65.
Data for **12**. Mp 165–167 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 1.61–1.80 (m, 4H), 2.96 (s, 2H), 3.36–3.39 (m, 5H), 3.71 (s, 3H), 3.73 (s, 3H), 3.95 (s, 3H), 4.19 (s, 3H), 6.77 (s, 1H), 6.80 (s, 1H), 7.75 (t, J = 7.4 Hz, 1H), 7.81 (t, J = 7.4 Hz, 1H), 7.95 (s, 1H), 8.15 (d, J = 8.3 Hz, 1H), 8.25 (d, J = 8.3 Hz, 1H), 8.60 (t, J = 5.6 Hz, 1H). Calcd: C: 56.41; H: 5.39; N: 4.54. Obsvd: C: 56.32; H: 5.45; N: 4.45.
Data for **13a**. Mp 142–144 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 2.99 (s, 2H), 3.10 (s, 2H), 3.61–3.63 (m, 3H), 3.73 (s, 3H), 3.85 (s, 3H), 4.15 (s, 3H), 7.13–7.23 (m, 4H), 7.34 (s, 1H), 7.36 (s, 1H), 8.59 (t, J = 5.0 Hz, 1H). Calcd: C: 51.88; H: 4.95; N: 5.50. Obsvd: C: 51.73; H: 4.95; N: 5.51.
Data for **13b**. Mp 91–93 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 2.92 (s, 2H), 3.13 (s, 2H), 3.59–3.63 (m, 3H), 3.71 (s, 3H), 3.73 (s, 3H), 3.74 (s, 3H), 3.85 (s, 3H), 4.10 (s, 3H), 6.72 (s, 1H), 6.78 (s, 1H), 7.34 (s, 1H), 7.37 (s, 1H), 8.60 (s, 1H). Calcd: C: 50.63; H: 5.13; N: 4.92. Obsvd: C: 50.49; H: 5.30; N: 4.60.
Data for **14**. ^{1}H NMR ($CDCl_3$) δ 1.70–1.79 (m, 4H), 2.59 (s, 2H), 2.73–2.76 (m, 2H), 2.81–2.84 (m, 2H), 3.51–3.52 (m, 2H), 3.58 (s, 2H), 3.85 (s, 3H), 3.86 (s, 3H), 3.88 (s, 3H), 3.90 (s, 3H), 6.52 (s, 1H), 6.60 (s, 1H), 7.12 (d, J = 2.7 Hz, 1H), 7.78 (d, J = 2.7 Hz, 1H), 8.05 (s, 1H). Calcd: C: 52.27; H: 5.57; N: 4.69. Obsvd: C: 52.07; H: 5.40; N: 4.64.
Data for **15**. ^{1}H NMR ($CDCl_3$) δ 1.66 (s, 4H), 2.44–2.66 (m, 6H), 3.06 (s, 2H), 3.17–3.21 (m, 2H), 3.48–3.50 (m, 2H), 3.88 (s, 6H), 6.76–6.96 (m, 2H), 7.12–7.16 (m, 2H), 7.77–7.79 (m, 1H), 7.99 (s, 1H).
Data for **16**. ^{1}H NMR ($CDCl_3$) δ 1.72 (s, 4H), 2.47–2.67 (m, 6H), 3.03 (s, 2H), 3.15–3.20 (m, 2H), 3.54–3.63 (m, 2H), 3.99 (s, 3H), 6.73–6.93 (m, 2H), 7.11–7.17 (m, 1H), 7.60–7.72 (m, 2H), 8.01–8.26 (m, 3H), 8.38 (s, 1H).
Data for **17**. Mp 166–168 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 3.02 (s, 3H), 3.14 (s, 2H), 3.66 (s, 2H), 3.83 (s, 3H), 4.19 (s, 3H), 7.12–7.25 (m, 5H), 7.65–7.67 (m, 1H), 7.86 (s, 1H), 8.56 (t, J = 5.4 Hz, 1H). Calcd: C: 52.62; H: 4.84; N: 5.84. Obsvd: C: 52.38; H: 4.75; N: 5.69.
Data for **18**. Mp 158–160 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 2.93 (s, 2H), 3.16 (s, 2H), 3.60–3.66 (m, 3H), 3.71 (s, 3H), 3.73 (s, 3H), 3.84 (s, 3H), 4.14 (s, 3H), 6.73 (s, 1H), 6.78 (s, 1H), 7.13 (d, J = 8.9 Hz, 1H), 7.66 (d, J = 8.7 Hz, 1H), 7.86 (s, 1H), 8.57 (t, J = 5.4 Hz, 1H). Calcd: C: 51.22; H: 5.05; N: 5.19. Obsvd: C: 51.23; H: 5.07; N: 5.04.
Data for **19**. Mp 160–162 °C (oxalate salt); ^{1}H NMR (DMSO-d_6) δ 2.27 (s, 3H), 2.94 (s, 2H), 3.16 (s, 2H), 3.65–3.66 (m, 3H), 3.72 (s, 3H), 3.73 (s, 3H), 3.82 (s, 3H), 4.14 (s, 3H), 6.73 (s, 1H), 6.79 (s, 1H), 7.03 (d, J = 8.4 Hz, 1H), 7.29 (d, J = 8.4 Hz, 1H), 7.63 (s, 1H), 8.51 (s, 1H). Calcd: C: 60.18; H: 6.42; N: 5.85. Obsvd: C: 60.32; H: 6.39; N: 5.56.
Data for **20**. ^{1}H NMR ($CDCl_3$) δ 1.70–1.79 (m, 4H), 2.32 (s, 3H), 2.59 (s, 2H), 2.73–2.76 (m, 2H), 2.81–2.84 (m, 2H), 3.51–3.52 (m, 2H), 3.58 (s, 2H), 3.81 (s, 3H), 3.83 (s, 3H), 3.89 (s, 3H), 6.50 (s, 1H), 6.58 (s, 1H), 6.86–6.96 (m, 2H), 7.11 (s, 1H), 8.00 (s, 1H). Calcd: C: 62.14; H: 6.82; N: 5.57. Obsvd: C: 62.33; H: 6.60; N: 5.06.
17. **σ Receptor binding assays.** The σ_1 receptor binding assay was conducted using guinea pig brain membrane homogenates (100 μg protein). Membrane homogenates were incubated with 3 nM [^{3}H](+)-pentazocine (31.6 Ci/mmol) in 50 mM Tris–HCl (pH 8.0) at 25 °C for either 120 or 240 min. Test compounds were dissolved in ethanol then diluted in buffer for a total incubation volume of 0.5 mL. Test compounds were added in concentrations ranging from 0.005 to 1000 nM. Assays were terminated by the addition of ice-cold 10 mM Tris–HCl (pH 8.0) followed by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.5% polyethylenimine) using a Brandel cell harvester (Gaithersburg, MD, USA). Filters were washed twice with 5 mL of ice cold buffer. Nonspecific binding was determined in the presence of 10 μM (+)-pentazocine. Liquid scintillation counting was carried out in EcoLite(+) (ICN Radiochemicals; Costa Mesa, CA, USA) using a Beckman LS 6000IC spectrometer with a counting efficiency of 50%.
The σ_2 receptor binding assay was conducted using rat liver membrane homogenates (35 μg of protein). Membrane homogenates were incubated with 3 nM [^{3}H]DTG (38.3 Ci/mmol) in the presence of 100 nM (+)-pentazocine to block σ_1 sites. Incubations were carried out in 50 mM Tris–HCl (pH 8.0) for 120 min at 25 °C in a total incubation volume of 0.5 mL. Test compounds were added in concentrations ranging from 0.005 to 1000 nM. Assays were terminated by the addition of ice-cold 10 mM Tris–HCl (pH 8.0) followed by rapid filtration through Whatman GF/B glass fiber filters (presoaked in 0.5% polyethylenimine) using a Brandel cell harvester (Gaithersburg, MD, USA). Filters were washed twice with 5 mL of ice cold buffer. Nonspecific binding was determined in the presence of 5 μM DTG. Liquid scintillation counting was carried out in EcoLite(+) (ICN Radiochemicals; Costa Mesa, CA, USA) using a Beckman LS 6000IC spectrometer with a counting efficiency of 50%.
The IC_{50} values at sigma sites were generally determined in triplicate from non-linear regression of binding data as analyzed by JMP (SAS Institute; Cary, NC, USA), using eight concentrations of each compound. K_i values were calculated using the method of Cheng–Prusoff[19] and represent mean values ± SEM. All curves were best fit to a one site fit and gave Hill coefficients of 0.8–1.0. The K_d value used for [^{3}H]DTG in rat liver was 17.9 nM and was 4.8 nM for [^{3}H](+)-pentazocine in guinea pig brain.[11,12]
18. **Dopamine receptor binding assays.** A filtration binding assay was used to characterize the binding properties of membrane-associated receptors. For rat $D2_{Long}$, rat D3 receptors expressed in Sf9 cells and human D4 dopamine receptors expressed in HEK 293 cells, tissue homogenates (50 μL) were suspended in 50 mM Tris–HCl/150 mM NaCl/10 mM EDTA buffer, pH 7.5 and incubated with

50 μL of ^{125}I-IABN at 37 °C for 60 min. Nonspecific binding was be defined using 25 μM (+)-butaclamol. For competition experiments the radioligand concentration is generally equal to 0.5 times the K_d value and the concentration of the competitive inhibitor ranges over five orders of magnitude. Binding will be terminated by the addition of cold wash buffer (10 mM Tris–HCl/150 mM NaCl, pH 7.5) and filtration over a glass-fiber filter (Schleicher and Schuell No. 32). Filters will be washed with 10 mL of cold buffer and the radioactivity will be measured using a Packard Cobra gamma counter. Estimates of the equilibrium dissociation constant and maximum number of binding sites are obtained using unweighted nonlinear regression analysis of data modeled according to the equation describing mass action binding.[20] Data from competitive inhibition experiments are modeled using nonlinear regression analysis to determine the concentration of inhibitor that inhibits 50% of the specific binding of the radioligand. Competition curves will be modeled for a single site and the IC_{50} values will be converted to equilibrium dissociation constants (K_i values) using the Cheng–Prusoff[19] correction. Mean K_i values ± SEM are reported for at least three independent experiments.

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