Comparative Evaluation of 4 and 6-Carbon Spacer Conformationally-Flexible Tetrahydroisoquinoliny Benzamide Analogues for Imaging the Sigma-2 Receptor Status of Solid Tumors

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Abstract

Introduction: Nine novel analogues were synthesized including a 6-carbon spacer analogue of **ISO-1 (7)**. They have moderate binding affinity for sigma-2 (σ_2) receptors and high selectivity for σ_2 receptors relative to sigma-1 (σ_1) receptors.

Methods: (^{18}F)**7** was synthesized and evaluated as a candidate ligand for positron emission (PET) imaging of the σ_2 receptor in tumors. Radioligand [^{18}F]**7** was radiolabeled with ^{18}F via displacement of the corresponding mesylate precursor with [^{18}F]fluoride. Cellular uptake study of [^{18}F]**7** was performed in EMT-6 tumor cell and *in vivo* biodistribution study of [^{18}F]**7** carried out in female Balb/c mice bearing EMT-6 tumors.

Results: [^{18}F]**7** had a respectable tumor uptake (1.55 %ID/g at 60 min post-injection) and high tumor/muscle ratios at 60 and 120 min post-injection. MicroPET imaging of [^{18}F]**7** in tumor-bearing mice as above showed significant tumor localization and a high tumor/muscle ratio as well.

Conclusions: These results are similar to or better than [^{18}F]**ISO-1** ([^{18}F]**3**), which indicates that [^{18}F]**7** has potential for imaging the σ_2 receptor status of solid tumors.

Keywords: PET, sigma-2 receptors, tumor imaging, tumor cell proliferation, EMT-6 cells

Introduction

Sigma (σ) receptors represent a class of proteins that were initially thought to be a type of opioid receptor [1]. It has been established that σ receptors are a distinct class of receptors, largely based on in vitro binding and behavioral pharmacology studies [2].

σ receptors are present in many normal tissues including liver, kidney, endocrine glands, and the central nervous system (CNS) [3]. Two subtypes have been pharmacologically established as σ_1 and σ_2 receptors [4, 5]. The σ_1 receptor has been cloned from tissues of guinea pig, rat, mouse, and man [6], and the crystal structure of the protein reveals a trimeric architecture consisting of a single trans-membrane domain in each protomer [7]. The σ_2 receptor has not been cloned yet, but our group has reported that the putative σ_2 receptor binding site resides within the PGRMC1 (progesterone receptor membrane component 1) protein complex [8]. However, recent studies using stable cell lines where the PGRMC1 had been knocked out using either a shRNA or CRISPR vector for the PGRMC1 have shown that there is no decrease in binding of [3 H]DTG binding in PGRMC1 knockout cells [9, 10]. These data indicate that the DTG-sensitive σ_2 receptor binding site is not located within the amino acid sequence of the PGRMC1, and supports the need for further studies in the identification of the σ_2 receptor.

Although the molecular identity of the receptor is subject to debate, it is known that σ_2 receptors are overexpressed in a wide variety of human and murine tumor cell lines and solid tumors such as breast adenocarcinoma, neuroblastoma, leukemia, glioblastoma, lung carcinomas, renal carcinoma, colon carcinoma, sarcoma, urinary bladder tumor, pancreatic cancer, and prostate cancer [11-24]. The density of σ_2 receptors has been reported to be more abundant than σ_1 receptors in a wide panel of tumor cells grown under cell culture conditions [12]. In addition,

the density of σ_2 receptors is found to be 8-10 fold higher in proliferating vs. quiescent mouse mammary adenocarcinoma cells both *in vitro* [24] and *in vivo* [23]. This correlation between σ_2 receptor density and the proliferative status of tumor cells has been reported to be independent of other factors, biological or physiological, such as species, cell types, ploidy, cell-cell contact, nutrient depletion, low pH, altered metabolic states, or tumor size [23, 24]. Our group has also reported the localization of σ_2 receptors in the mitochondria, lysosomes, endoplasmic reticulum, and plasma membrane of breast cancer cells using two-photon and confocal microscopy probes [25].

In addition to being an excellent biomarker of cell proliferation for imaging studies, the σ_2 receptor has also proven to be a promising target for as either a monotherapy for treating cancer, or as a mechanism for delivering a therapeutic cargo to cancer cells [11, 22, 26-29]. Therefore, radiolabeled σ_2 receptor ligands can be used to assess the proliferative status of solid tumors with positron emission tomography (PET) and single photon emission computed tomography (SPECT), as well as serving as a companion diagnostic for identifying tumors that would respond to σ_2 receptor targeting therapeutics [29-33].

Our group has previously radiolabeled several σ_2 receptor ligands having a high affinity for σ_2 receptors and excellent $\sigma_2:\sigma_1$ selectivity ratios as potential PET tracers for clinical research studies. The initial *in vitro* and *in vivo* evaluation of these radiotracers has been reported previously [34-37]. The 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline moiety is an optimal structure to increase binding affinity and selectivity for σ_2 receptors [36, 38-40]. Among these compounds, ^{11}C -radiolabeled benzamide analogues have high binding affinity and selectivity for σ_2 receptors (Figure 1), and a good relationship between tumor uptake and lipophilicity of those compounds has been shown.[38] Although ^{11}C -labeled benzamide analogues have a high

potential for PET imaging of σ_2 receptors (compounds **1** and **2**, Figure 1), we also developed ^{18}F -labeled benzamide analogues (compounds **3** and **4**, Figure 1) due to the short half-life of ^{11}C (20.4 min) [36]. Recently, the first human imaging studies of the σ_2 receptor ligand [^{18}F]**ISO-1** ([^{18}F]**3**) were accomplished and demonstrated the potential utility of this compound in imaging the proliferative status of solid tumors [41]. However, it has been reported that 1,2,3,4-tetrahydroisoquinoline with the flexible 6-carbon spacer, and benzofuran ring as the benzamide moiety (**5**, Figure 1) has higher binding affinities and selectivity for σ_2 receptors than the 4 and 5-carbon spacer group, and the authors also suggested that the corresponding indole and benzothiophene analogs should have improved binding affinities and selectivity for σ_2 receptors [40].

In this study, we further explored this structural optimization strategy by increasing the carbon spacer length from four to six carbons for hydrophobic spacer, and replaced the benzamide ring with either an indole or benzothiophene ring (Figure 2). We also synthesized the 6-carbon spacer analogue of **ISO-1** and evaluated the compounds for their *in vitro* binding properties to the σ receptors. The *in vivo* properties of [^{18}F]**ISO-1** (4-carbon spacer) ([^{18}F]**3**), a well-established σ_2 radioligand, and [^{18}F]**7**, the corresponding [^{18}F]-labeled 6-carbon spacer analogue of **ISO-1**, were also compared.

1. Materials and Methods

1.1. Reagents and Equipment

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Alfa Aesar (Ward

Hill, MA, USA). ^1H NMR spectra were obtained using a Bruker DMX 360 (360 MHz) spectrometer (Rheinstetten, Germany), and chemical shifts (δ) were reported as the ppm downfield of the internal tetramethylsilane. LCMS (Liquid Chromatography Mass Spectroscopy) and HRMS (High Resolution Mass Spectroscopy) were obtained using a Waters microMass zQ and LCT Premier XE LC/MS system (Waters Corporations, Milford, MA, USA). For purification and analysis of radioligands, HPLC analysis was conducted using the Agilent 1100 (Agilent Technologies, Santa Clara, CA, USA) equipped with a semi-preparative column (Agilent SB-C18, 5 μm , 9.4 x 100 mm) or Waters Alliance e2695 HPLC (Waters Corporations, Milford, MA, USA) equipped with an analytical column (Agilent ZORBAX Eclipse XDB-C18, 5 μm , 4.6 x 150 mm). The eluent was monitored simultaneously using UV (281 nm) and NaI(Tl) radioactivity detectors. TLC was performed on Merck F₂₅₄ silica plates and analyzed on a Bioscan Mini-Scan TLC Imaging Scanner (Hopkinson, MA, USA).

[^{18}F]Fluoride was produced by the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ reaction using an IBA Cyclone[®] 18 (Louvain-la-Neuve, Belgium) or JSW (Tokyo, Japan). Radioactivity was measured in a dose calibrator (Capintec, Ramsey, NJ, USA). Binding studies were performed using a Unifilter 96 harvester (Perkin Elmer, Boston, MA), and bound radioactivity was counted on a Microbeta² liquid scintillation counter (Perkin Elmer, Boston, MA) with a counting efficiency of ~45%. All animal experiments were performed under IACUC-approved protocols in compliance with the guidelines for the care and use of research animals established by the University of Pennsylvania.

1.2. Synthesis

1.2.1. 2-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)isoindoline-1,3-dione (**10a**) and 2-(6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)isoindoline-1,3-dione (**10b**)

10a and **10b** were synthesized using a modification of the reported method [42, 43]. 2-(4-bromobutyl)isoindoline-1,3-dione (or 2-(6-bromohexyl)isoindoline-1,3-dione) (15.7 mmol) was added to a solution of 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride (13.1 mmol) and K₂CO₃ (5.40 g, 39.2 mmol) in CH₃CN (37.5 mL), and the mixture stirred at reflux for 20 h. The reaction mixture was cooled to ambient temperature, the CH₃CN removed *in vacuo*, and H₂O (50 mL) was added. The mixture was extracted with EtOAc (2 x 50.0 mL), the combined organic layer was washed with brine (50.0 mL), dried over anhydrous Na₂SO₄ and concentrated under reduced pressure.

Flash column chromatography (15:1 CH₂Cl₂-MeOH) afforded compound **10a** (3.87 g, 75%) as a pale yellow solid. mp 109.3-109.5 °C. ¹H NMR (CDCl₃) δ 7.77 (dq, *J* = 39.6 and 3.60 Hz, 4H), 6.58 (s, 1H), 6.51 (s, 1H), 3.83 (s, 3H), 3.82 (s, 3H), 3.74 (t, *J* = 7.20 Hz, 2H), 3.54 (s, 2H), 2.81 (t, *J* = 5.75 Hz, 2H), 2.70 (t, *J* = 5.40 Hz, 2H), 2.54 (t, *J* = 7.20 Hz, 2H), 1.80-1.76 (m, 2H), 1.66-1.62 (m, 2H). LCMS *m/z* 395.2392 (M+H).

Flash column chromatography (15:1 CH₂Cl₂-MeOH) afforded compound **10b** (4.57 g, 83.0%) as a pale yellow solid. mp 111.8-112.5 °C. ¹H NMR (CDCl₃) δ 7.76 (dq, *J* = 39.6 and 3.6 Hz, 4H), 6.57 (s, 1H), 6.51 (s, 1H), 3.83 (s, 3H), 3.82 (s, 3H), 3.68 (t, *J* = 7.20 Hz, 2H), 3.53 (s, 2H), 2.81 (t, *J* = 5.75 Hz, 2H), 2.70 (d, *J* = 5.40 Hz, 2H), 2.48 (t, *J* = 7.38 Hz, 2H), 1.71-1.65 (m, 2H), 1.59-1.57 (m, 2H), 1.41-1.37 (m, 4H). LCMS *m/z* 423.2752 (M+H).

1.2.2. 4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butan-1-amine (**11a**) and 6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexan-1-amine (**11b**)

11a and **11b** were synthesized using the literature method [42, 43]. Hydrazine monohydrate (10.1 mmol) was added to a solution of compound **10a** (or **10b**) (1.52 mmol) in EtOH (6.00 mL), and stirred at 70 °C for 3 h. The reaction mixture was cooled to ambient temperature and the EtOH removed *in vacuo*. Aqueous NaOH solution (50%) was added until the white solid was fully dissolved, and the mixture extracted with CH₂Cl₂ (4 x 50.0 mL), and dried over anhydrous Na₂SO₄. The solvents were evaporated under reduced pressure to give compound **11a** (or **11b**) which was used without further purification.

11a; a pale yellow viscous oil (374 mg, 93.0%) ¹H NMR (CDCl₃) δ 6.57 (s, 1H), 6.50 (s, 1H), 3.83 (s, 3H), 3.81 (s, 3H), 3.53 (s, 2H), 2.79 (t, *J* = 5.60 Hz, 2H), 2.71-2.66 (m, 4H), 2.50-2.46 (m, 2H), 1.61-1.57 (m, 2H), 1.49-1.47 (m, 2H). LCMS *m/z* 265.2048 (M+H).

11b; a pale yellow viscous oil (647 mg, 88.0%) ¹H NMR (CDCl₃) δ 6.58 (s, 1H), 6.51 (s, 1H), 3.83 (s, 3H), 3.82 (s, 3H), 3.54 (s, 2H), 2.81 (t, *J* = 5.76 Hz, 2H), 2.70-2.67 (m, 4H), 2.50-2.46 (m, 2H), 1.60-1.55 (m, 2H), 1.47-1.44 (m, 2H), 1.38-1.34 (m, 4H). LCMS *m/z* 293.2721 (M+H).

1.2.3. Methyl 2-(2-fluoroethoxy)-5-methylbenzoate (**12**)

2-Fluoroethyl 4-methylbenzenesulfonate (982 mg, 4.50 mmol) was added to a solution of methyl 5-methylsalicylate (500 mg, 3.00 mmol) and K₂CO₃ (892 mg, 6.45 mmol) in CH₃CN (20.0 mL), and the mixture was stirred at reflux for 3h. The reaction mixture was cooled to ambient temperature, the CH₃CN removed *in vacuo*, and H₂O (50.0 mL) was added. After

extraction with EtOAc (2 x 50.0 mL), the combined organic layer was washed with brine (50.0 mL), dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. Flash column chromatography (5:1 hexane-EtOAc) gave compound **12** (628 mg, 98.5%) as a colorless viscous oil. ¹H NMR (CDCl₃) δ 7.60 (s, 1H), 7.26 (d, *J* = 11.5 Hz, 1H), 6.89 (d, *J* = 8.28 Hz, 1H), 4.82 (dt, *J* = 46.1 and 2.70 Hz, 2H), 4.26 (dt, *J* = 26.6 and 4.32 Hz, 2H), 3.88 (s, 3H), 2.31 (s, 3H).

1.2.4. (2-(2-Fluoroethoxy)-5-methylbenzoic acid (**13**))

Solid NaOH (37.9 mg, 0.47 mmol) was added to a solution of compound **12** (100 mg, 0.47 mmol) in MeOH (5.00 mL) and H₂O (0.86 mL), and the mixture was stirred at 70 °C for 3 h. The reaction mixture was cooled, the MeOH removed *in vacuo*, and acidified to pH 2 using 1 N HCl. After extraction with EtOAc (2 x 25.0 mL), the combined organic layer was dried over anhydrous Na₂SO₄. Volatiles were evaporated *in vacuo* to give compound **13** (87.1 mg, 93.3%) as a white solid which was used without further purification. mp 81.3-81.7 °C. ¹H NMR (CDCl₃) δ 10.60 (br, 1H), 8.01 (s, 1H), 7.35 (d, *J* = 10.8 Hz, 1H), 6.93 (d, *J* = 9.36 Hz, 1H), 4.83 (dt, *J* = 47.2 and 4.14 Hz, 2H), 4.45 (dt, *J* = 28.8 and 4.14 Hz, 2H), 2.34 (s, 3H).

1.2.5. *N*-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2-(2-fluoroethoxy)-5-methylbenzamide (**3**) and *N*-(6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)-2-(2-fluoroethoxy)-5-methylbenzamide (**7**)

Compounds **3** and **7** were synthesized using the literature method [36]. DCC (0.15 mmol) and HOBt (0.15 mmol) were added to a solution of compound **11a** (or **11b**) (0.18 mmol) and **13**

(0.15 mmol) in CH₂Cl₂ (2.00 mL). The reaction mixture was stirred at room temperature for 20 h. H₂O (50.0 mL) was added and the mixture was extracted with EtOAc (2 x 30.0 mL). The combined organic layers was washed with brine, and then dried over anhydrous Na₂SO₄ and concentrated *in vacuo*.

Flash column chromatography (15:1 CH₂Cl₂-MeOH) gave compound **3** (49.1 mg, 73.7%) as a white solid. ¹H NMR (CDCl₃) δ 7.99 (d, *J* = 3.60 Hz, 1H), 7.96 (s, 1H), 7.21-7.20 (m, 1H), 6.81 (d, *J* = 5.40 Hz, 1H), 6.57 (s, 1H), 6.49 (s, 1H), 4.85 (t, *J* = 5.40 Hz, 1H), 4.69 (t, *J* = 5.40 Hz, 1H), 4.31 (t, *J* = 5.40 Hz, 1H), 4.22 (t, *J* = 5.40 Hz, 1H), 3.83 (s, 3H), 3.82 (s, 3H), 3.54-3.48 (m, 4H), 2.79 (t, *J* = 5.40 Hz, 3H), 2.69 (t, *J* = 7.20, 3H), 2.53 (t, *J* = 9.00 Hz, 3H), 2.33 (s, 3H), 2.00-1.67 (m, 4H). [36]

Flash column chromatography (15:1 CH₂Cl₂-MeOH) gave compound **7** (61.6 mg, 86.3%) as a white solid. mp 100.2-100.6 °C. ¹H NMR (CDCl₃) δ 8.01 (s, 1H), 7.89 (br, NH), 7.21 (d, *J* = 7.20 Hz, 1H), 6.83 (d, *J* = 7.20 Hz, 1H), 6.59 (s, 1H), 6.52 (s, 1H), 4.79 (dt, *J* = 46.8 and 3.60 Hz, 2H), 4.30 (dt, *J* = 25.2 and 3.60 Hz, 2H), 3.84 (s, 3H), 3.83 (s, 3H), 3.68 (s, 2H), 3.48-3.43 (m, 2H), 2.87 (s, 4H), 2.61 (s, 2H), 2.33 (s, 3H), 1.69-1.59 (m, 4H), 1.48-1.44 (m, 4H). LCMS *m/z* 473.2164 (M+H). HRMS (ESI) (M+H) calcd for C₂₇H₃₈FN₂O₄, 473.2816; found, 473.2831.

1.2.6. Ethyl 5-(2-fluoroethoxy)-1*H*-indole-2-carboxylate (**14**)

2-Fluoroethyl 4-methylbenzenesulfonate (1.28 g, 5.85 mmol) was added to a solution of K₂CO₃ (808 mg, 5.84 mmol) and ethyl 5-hydroxy-1*H*-indole-2-carboxylate (1.28 g, 5.84 mmol) in CH₃CN (90.0 mL), and the mixture was stirred at reflux for 3 h. The reaction mixture was

cooled to ambient temperature, the CH₃CN removed *in vacuo*, and H₂O (50.0 mL) added. After extraction with EtOAc (2 x 50.0 mL), the combined organic layer was washed with H₂O (2 x 50.0 mL), dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. Flash column chromatography (3:1 hexane-EtOAc) afforded compound **14** (1.48 g, 99.0%) as a white oil. ¹H NMR (CDCl₃) δ 7.81 (d, *J* = 8.30 Hz, 2H), 7.34 (d, *J* = 19.8 Hz, 2H), 4.57 (dt, *J* = 45.7 and 4.00 Hz, 2H), 4.27 (dt, *J* = 29.8 and 5.40 Hz, 2H), 4.40 (q, *J* = 7.20 Hz, 2H), 1.41 (t, *J* = 7.20 Hz, 3H).

1.2.7. 5-(2-Fluoroethoxy)-1H-indole-2-carboxylic acid (**15**)

Solid NaOH (160 mg, 4.00 mmol) was added to a solution of **14** (500 mg, 1.99 mmol) in MeOH/H₂O (30.0/5.00 mL), and stirred at 70 °C for 3 h. The reaction mixture was cooled to ambient temperature, the MeOH removed *in vacuo*, and 1 N HCl was added to the reaction mixture until pH ~2. After extraction with CH₂Cl₂ (2 x 50.0 mL), the combined organic layer was washed with H₂O (2 x 50.0 mL), dried over anhydrous Na₂SO₄ and concentrated *in vacuo* to give compound **15** (115 mg, 25.8%) as a white solid. mp 220.5-220.9 °C. ¹H NMR (DMSO-d₆) δ 7.33 (d, *J* = 9.00 Hz, 1H), 7.12 (s, 1H), 6.97 (s, 1H), 6.92 (d, *J* = 9.00 Hz, 1H), 4.74 (dt, *J* = 47.8 and 3.60 Hz, 2H), 4.21 (dt, *J* = 30.2 and 4.00 Hz, 2H).

1.2.8. Methyl 5-methoxybenzo[*b*]thiophene-2-carboxylate (**16**)

Methyl thioglycolate (208 μ L, 2.34 mmol) was dropwise added to a solution of NaH (93.4 mg, 3.89 mmol) in DMSO (9.00 mL), followed by adding 2-fluoro-5-methoxybenzaldehyde (300 mg, 1.95 mmol) quickly. The reaction mixture was stirred at room temperature for 5 min, and

precipitated by pouring into ice cold H₂O. The product was filtered, and washed with H₂O to afford compound **16** (133 mg, 30.0%) as a white solid. mp 109.2-109.3 °C. ¹H NMR (CDCl₃) δ 7.98 (s, 1H), 7.71 (d, *J* = 10.8 Hz, 1H), 7.28 (s, 1H), 7.12 (d, *J* = 7.20 Hz, 1H), 3.94 (s, 3H), 3.88 (s, 3H).

1.2.9. 5-Methoxybenzo[*b*]thiophene-2-carboxylic acid (**17**)

Solid NaOH (42.6 mg, 1.06 mmol) was added to a solution of **16** (120 mg, 0.53 mmol) in MeOH/H₂O (6.00/1.00 mL), and stirred at 70 °C for 3 h. The reaction mixture was cooled to ambient temperature, the MeOH was removed *in vacuo*, and 1 N HCl added to the reaction mixture until pH ~2. After extraction with CH₂Cl₂ (2 x 50.0 mL), the combined organic layer was washed with H₂O (2 x 50.0 mL), dried over anhydrous Na₂SO₄ and concentrated *in vacuo* to give compound **17** (103 mg, 92.8%) as a white solid. mp 180.6-181.3 °C. ¹H NMR (DMSO-*d*₆) δ 13.36 (br, COOH), 7.99 (s, 1H), 7.89 (d, *J* = 7.20 Hz, 1H), 7.50 (s, 1H), 7.13 (d, *J* = 7.20 Hz, 1H), 3.80 (s, 3H).

1.2.10. 5-(2-Fluoroethoxy)-2-nitrobenzaldehyde (**18**)

2-Fluoroethyl 4-methylbenzenesulfonate (1.08 g, 4.98 mmol) was added to a solution of K₂CO₃ (1.65 mg, 12.0 mmol) and 5-hydroxy-2-nitrobenzaldehyde (1.00 g, 5.98 mmol) in CH₃CN (50.0 mL), and the mixture stirred at reflux for 6 hr. The reaction mixture was cooled to ambient temperature, the CH₃CN removed *in vacuo*, and H₂O (100 mL) added. After extraction with EtOAc (2 x 100 mL), the combined organic layer was washed with H₂O (2 x 50.0 mL),

dried over anhydrous Na_2SO_4 and concentrated. Flash column chromatography (2:1 hexane-EtOAc) afforded compound **18** (687 mg, 64.6%) as a pale yellow solid. mp 67.0-67.9 °C. ^1H NMR (CDCl_3) δ 7.98 (s, 1H), 7.71 (d, J = 21.6 Hz, 1H), 7.16 (d, J = 10.8 Hz, 1H), 4.80 (dt, J = 47.5 and 3.60 Hz, 2H), 4.29 (dt, J = 21.6 and 3.60 Hz, 2H), 3.87 (s, 3H).

1.2.11. Methyl 5-(2-fluoroethoxy)benzo[*b*]thiophene-2-carboxylate (**19**)

K_2CO_3 (194 mg, 1.41 mmol) was added to a solution of **18** (300 mg, 1.41 mmol) in DMF (4.50 mL), added methyl thioglycolate (125 μL , 1.41 mmol), and stirred at 80 °C for 20 h. The reaction mixture was cooled to ambient temperature, poured into ice H_2O , extracted with EtOAc (3 x 50 mL), dried over anhydrous Na_2SO_4 , and concentrated *in vacuo*. Flash column chromatography (6:1 hexane-EtOAc) afforded compound **19** (51.3 mg, 14.3%) as a pale yellow solid. mp 124.8-124.9 °C. ^1H NMR (CDCl_3) δ 8.18 (s, 1H), 7.74 (d, J = 10.8 Hz, 1H), 7.30 (s, 1H), 7.16 (d, J = 9.00 Hz, 1 H), 4.79 (dt, J = 47.5 and 4.30 Hz, 2H), 4.28 (dt, J = 27.7 and 3.90 Hz), 3.94 (s, 3H).

1.2.12. 5-(2-Fluoroethoxy)benzo[*b*]thiophene-2-carboxylic acid (**20**)

Solid NaOH (15.8 mg, 0.40 mmol) was added to a solution of **19** (50.0 mg, 0.20 mmol) in MeOH/ H_2O (2.50/0.40 mL), and stirred at 70 °C for 3 h. The reaction mixture was cooled to ambient temperature, MeOH removed *in vacuo*, and 1 N HCl added to the reaction mixture until pH ~2. After extraction with CH_2Cl_2 (2 x 30.0 mL), the combined organic layer was washed with H_2O (2 x 30.0 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give compound

20 (103 mg, 92.8%) as a white solid. mp 192.1-192.6 °C. ¹H NMR (DMSO-d₆) δ 7.98 (s, 1H), 7.93 (d, *J* = 9.00 Hz, 1H), 7.54 (d, *J* = 2.50 Hz, 1H), 7.18 (dd, *J* = 9.00 and 2.50 Hz, 1H), 4.78 (dt, *J* = 47.8 and 3.90 Hz, 2H), 4.30 (dt, *J* = 29.8 and 3.90 Hz, 2H).

1.2.13. *N*-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-5-methoxy-1H-indole-2-carboxamide (**8a**), *N*-(6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)-5-methoxy-1H-indole-2-carboxamide (**8b**), *N*-(4-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-5-(2-fluoroethoxy)-1H-indole-2-carboxamide (**8c**), and *N*-(6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)-5-(2-fluoroethoxy)-1H-indole-2-carboxamide (**8d**).

DCC (0.23 mmol) and HOBt (0.23 mmol) were added to a solution of compound **11a** (or **11b**) (0.23 mmol) and 5-methoxy-1H-indole-2-carboxylic acid (or **15**) (0.23 mmol) in CH₂Cl₂ (5.00 mL). The reaction mixture was stirred at ambient temperature for 20 h. H₂O (50.0 mL) was added to the reaction mixture, and the mixture extracted with EtOAc (2 x 30.0 mL), washed with brine, and the combined organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*.

Flash column chromatography (30:1 CH₂Cl₂-MeOH) gave compound **8a** (89.4 mg, 90.0%) as a white solid. mp 193.2-193.3 °C. ¹H NMR (CDCl₃) δ 9.12 (br, NH), 7.88 (s, 1H), 7.23 (s, 1H), 6.88 (dd, *J* = 3.60 and 3.60 Hz, 1H), 6.67 (d, *J* = 3.60 Hz, 1H), 6.64 (s, 1H), 6.51 (s, 1H), 6.32 (br, NH), 3.85 (s, 3H), 3.82 (s, 3H), 3.70 (s, 2H), 3.53 (q, *J* = 5.70 Hz, 2H), 2.93 (d, *J* = 3.60 Hz, 2H), 2.87 (d, *J* = 7.20 Hz, 2H), 2.69 (t, *J* = 5.40 Hz, 2H), 1.86-1.78 (m, 4H). LCMS *m/z* 438.2298 (M+H). HRMS (ESI) (M+H) calcd for C₂₅H₃₁N₃O₄, 438.2393; found, 438.2383.

Flash column chromatography (30:1 CH₂Cl₂-MeOH) gave compound **8b** (65.1 mg, 68.2%) as a white solid. mp 174.8-175.1 °C. ¹H NMR (CDCl₃) δ 9.27 (br, NH), 7.32 (d, *J* = 3.60 Hz, 1H),

7.05 (s, 1H), 6.95 (d, $J = 3.60$ Hz, 1H), 6.79 (s, 1H), 6.59 (s, 1H), 6.49 (s, 1H), 6.35 (br, NH), 3.87 (s, 6H), 3.84 (s, 3H), 3.63 (s, 2H), 3.49 (q, $J = 7.20$ Hz, 2H), 2.86-2.80 (m, 4H), 2.58 (t, $J = 10.8$ Hz, 2H), 1.67 (t, $J = 7.20$ Hz, 4H), 1.44 (t, $J = 3.60$ Hz, 4H). LCMS m/z 466.2721 (M+H). HRMS (ESI) (M+H) calcd for $C_{27}H_{36}N_3O_4$, 466.2706; found, 466.2686

Flash column chromatography (15:1 CH_2Cl_2 -MeOH) gave compound **8c** (85.0 mg, 80.8%) as a white solid. mp 198.1-198.4 °C. 1H NMR ($CDCl_3$) δ 9.24 (br, NH), 7.98 (s, 1H), 7.27 (d, $J = 3.60$ Hz, 1H), 6.93 (d, $J = 7.20$ Hz, 2H), 6.63 (d, $J = 3.60$ Hz, 2H), 6.51 (s, 1H), 6.24 (br, NH), 4.78 (dt, $J = 46.8$ and 3.60 Hz, 2H), 4.25 (dt, $J = 28.8$ and 3.60 Hz, 2H), 3.85 (s, 3H), 3.79 (s, 3H), 3.68 (s, 2H), 3.55-3.52 (m, 2H), 2.93-2.92 (m, 2H), 2.85-2.84 (m, 2H), 2.67 (t, $J = 7.20$ Hz, 2H), 1.85-1.78 (m, 4H). LCMS m/z 470.2784 (M+H). HRMS (ESI) (M+H) calcd for $C_{26}H_{33}FN_3O_4$, 470.2455; found, 470.2454.

Flash column chromatography (15:1 CH_2Cl_2 -MeOH) gave compound **8d** (54.1 mg, 48.5%) as a white solid. 1H NMR ($CDCl_3$) δ 9.33 (br, NH), 7.33 (d, $J = 10.8$ Hz, 1H), 7.07 (s, 1H), 7.00 (d, $J = 3.60$ Hz, 1H), 6.79 (s, 1H), 6.59 (s, 1H), 6.49 (s, 1H), 6.38 (br, NH), 4.77 (dt, $J = 46.8$ and 3.60 Hz, 2H), 4.24 (dt, $J = 28.8$ and 3.60 Hz, 2H), 3.84 (s, 3H), 3.82 (s, 3H), 3.64 (s, 2H), 3.49 (q, $J = 7.20$ Hz, 2H), 2.86-2.81 (m, 4H), 2.58 (t, $J = 7.20$ Hz, 2H), 1.68-1.65 (m, 4H), 1.45-1.43 (m, 4H). LCMS m/z 498.1904 (M+H). HRMS (ESI) (M+H) calcd for $C_{28}H_{37}FN_3O_4$, 498.2768; found, 498.2766.

1.2.14. N-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-5-methoxybenzo[b]thiophene-2-carboxamide (9a), N-(6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)-5-methoxybenzo[b]thiophene-2-carboxamide (9b), N-(4-(6,7-dimethoxy-3,4-

dihydroisoquinolin-2(1H)-yl)butyl)-5-(2-fluoroethoxy)benzo[b]thiophene-2-carboxamide (**9c**),
and *N-(6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)-5-(2-fluoroethoxy)-*
benzo[b]thiophene-2-carboxamide (**9d**)

DCC (0.19 mmol) and HOBt (0.19 mmol) were added to a solution of compound **11a** (or **11b**) (0.23 mmol) and **17** (or **20**) (0.19 mmol) in CH₂Cl₂ (5.00 mL). The reaction mixture was stirred at ambient temperature for 20 h. H₂O (50.0 mL) was added to the reaction mixture, and the mixture extracted with EtOAc (2 x 30.0 mL), washed with brine, and the combined organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*.

Flash column chromatography (20:1 CH₂Cl₂-MeOH) gave compound **9a** (56.6 mg, 64.8%) as a white solid. mp 122.0-122.4 °C. ¹H NMR (CDCl₃) δ 7.83 (br, NH), 7.60 (d, *J* = 10.8 Hz, 1H), 7.50 (s, 1H), 7.01 (d, *J* = 7.20 Hz, 1H), 6.94 (s, 1H), 6.60 (s, 1H), 6.47 (s, 1H), 3.84 (s, 3H), 3.83 (s, 3H), 3.77 (s, 3H), 3.74 (s, 2H), 3.53 (q, *J* = 7.20 Hz, 2H), 2.93 (s, 4H), 2.74 (t, *J* = 7.20 Hz, 2H), 1.88-1.78 (m, 4H). LCMS *m/z* 455.0548 (M+H). HRMS (ESI) (M+H) calcd for C₂₅H₃₀N₂O₄S, 455.2005; found, 455.1999.

Flash column chromatography (20:1 CH₂Cl₂-MeOH) gave compound **9b** (66.5 mg, 71.7%) as a white solid. mp 57.1-57.3 °C. ¹H NMR (CDCl₃) δ 7.74 (s, 1H), 7.69 (d, *J* = 10.8 Hz, 1H), 7.24 (d, *J* = 3.60 Hz, 1H), 7.07 (dd, *J* = 7.20 and 3.60 Hz, 1H), 6.58 (s, 1H), 6.50 (s, 1H), 6.29 (br, NH), 3.86 (s, 3H), 3.83 (s, 3H), 3.82 (s, 3H), 3.64 (s, 2H), 3.47 (q, *J* = 7.20 Hz, 2H), 2.86-2.81 (m, 4H), 2.58 (t, *J* = 7.20 Hz, 2H), 1.68-1.65 (m, 4H), 1.45-1.43 (m, 4H). LCMS *m/z* 483.2325 (M+H). HRMS (ESI) (M+H) calcd for C₂₇H₃₄N₂O₄S, 483.2318; found, 483.2305.

Flash column chromatography (20:1 CH₂Cl₂-MeOH) gave compound **9c** (35.6 mg, 93.8%) as a white solid. mp 144.5-145.0 °C. ¹H NMR (CDCl₃) δ 8.02 (br, NH), 7.63 (d, *J* = 7.20 Hz, 1H),

7.46 (s, 1H), 7.05 (dd, $J = 7.20$ and 3.60 Hz, 1H), 6.86 (s, 1H), 6.61 (s, 1H), 6.47 (s, 1H), 4.78 (dt, $J = 47.5$ and 3.90 Hz, 2H), 4.23 (dt, $J = 28.4$ and 3.90 Hz, 2H), 3.83 (s, 3H), 3.76 (s, 3H), 3.53 (q, $J = 5.40$ Hz, 2H), 2.94 (s, 4H), 2.75 (s, 2H), 1.90-1.80 (m, 6H). LCMS m/z 487.1054 (M+H). HRMS (ESI) (M+H) calcd for $C_{26}H_{32}FN_2O_4S$, 487.2067; found, 487.2056.

Flash column chromatography (20:1 CH_2Cl_2 -MeOH) gave compound **9d** (25.7 mg, 64.0%) as a white solid. mp 99.7-99.9 °C. 1H NMR ($CDCl_3$) δ 8.02 (br, NH), 7.80 (s, 1H), 7.72 (d, $J = 7.20$ Hz, 1H), 7.27 (s, 1H), 7.11 (dd, $J = 7.20$ and 3.60 Hz, 1H), 6.59 (s, 1H), 6.49 (s, 1H), 4.78 (dt, $J = 47.2$ and 3.90 Hz, 2H), 4.26 (dt, $J = 27.7$ and 3.90 Hz, 2H), 3.83 (s, 3H), 3.82 (s, 3H), 3.74 (s, 2H), 3.47 (q, $J = 7.20$ Hz, 2H), 2.91 (s, 4H), 2.67 (t, $J = 7.20$ Hz, 2H), 1.72-1.66 (m, 4H), 1.47-1.45 (m, 4H). LCMS m/z 515.2862 (M+H). HRMS (ESI) (M+H) calcd for $C_{28}H_{36}FN_2O_4S$, 515.2380; found, 515.2370.

1.2.15. Methyl 2-(2-acetoxyethoxy)-5-methylbenzoate (**21**)

2-Bromoethyl acetate (2.68 g, 16.1 mmol) was added to a solution of methyl 5-methylsalicylate (540 mg, 3.25 mmol) and K_2CO_3 (2.19 g, 15.8 mmol) in CH_3CN (38.0 mL), and stirred at 80 °C for 48 h. The reaction mixture was cooled to ambient temperature, CH_3CN was removed *in vacuo*, and H_2O (50.0 mL) added. After extraction with EtOAc (2 x 50.0 mL), the combined organic layer was washed with brine (50.0 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Flash column chromatography (3:1 hexane-EtOAc) gave compound **21** (235 mg, 28.7%) as a pale yellow liquid. 1H NMR ($CDCl_3$) δ 7.56 (s, 1H), 7.24 (d, $J = 8.64$ Hz, 1H), 6.87 (d, $J = 6.87$ Hz, 1H), 4.44 (t, $J = 3.60$ Hz, 2H), 4.21 (t, $J = 3.60$ Hz, 2H), 3.87 (s, 3H), 2.30 (s, 3H), 2.09 (s, 3H).

1.2.16. 2-(2-Hydroxyethoxy)-5-methylbenzoic acid (22)

Solid NaOH (81.1 mg, 2.03 mmol) was added to a solution of compound **21** (235 mg, 0.93 mmol) in MeOH (10.0 mL) and H₂O (4.70 mL), and the mixture was stirred at 70 °C for 3 h. The reaction mixture was cooled to ambient temperature, the MeOH removed *in vacuo*, and acidified to pH 2 using 1 N HCl. After extraction with EtOAc (2 x 50.0 mL), the combined organic layer was dried over anhydrous Na₂SO₄. The volatiles were removed *in vacuo* to give compound **22** (179 mg, 98%) as a colorless liquid which was used without further purification. ¹H NMR (CDCl₃) δ 7.95 (s, 1H), 7.34 (d, *J* = 7.20 Hz, 1H), 6.95 (d, *J* = 8.28 Hz, 1H), 4.32 (t, *J* = 3.60 Hz, 2H), 4.06 (t, *J* = 3.60 Hz, 2H), 2.34 (s, 3H).

1.2.17. N-(4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)-2-(2-hydroxyethoxy)-5-methylbenzamide (23a) and N-(6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)-2-(2-hydroxyethoxy)-5-methylbenzamide (23b)

DCC (58.9 mg, 0.29 mmol) and HOBt (38.5 mg, 0.29 mmol) were added to a solution of compound **22** (100 mg, 0.34 mmol) and **11a** (or **11b**) (0.29 mmol) in CH₂Cl₂ (3.70 mL). The reaction mixture was stirred at ambient temperature for 20 h. H₂O (50.0 mL) was added to the reaction mixture, and the mixture was extracted with EtOAc (2 x 30 mL), washed with brine, and the combined organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*.

Flash column chromatography (10:1 CH₂Cl₂-MeOH) gave compound **23a** (115.2 mg, 34.5%) as a white solid. ¹H NMR (CDCl₃) δ 8.35 (br, NH), 7.95 (d, *J* = 2.16 Hz, 1H), 7.17 (d, *J* = 3.60 Hz, 1H), 6.82 (d, *J* = 7.20 Hz, 1H), 6.61 (s, 1H), 6.50 (s, 1H), 4.10 (t, *J* = 3.60 Hz, 2H), 3.85 (s, 3H), 3.83 (s, 3H), 3.81 (t, *J* = 3.60 Hz, 2H), 3.71 (s, 2H), 3.58-3.53 (m, 2H), 2.91 (s, 4H), 2.67 (t, *J* = 7.20 Hz, 2H), 2.31 (s, 3H), 1.92-1.84 (m, 2H), 1.76-1.71 (m, 2H)

Flash column chromatography (10:1 CH₂Cl₂-MeOH) gave compound **23b** (49.0 mg, 36.5%) as a white solid. ¹H NMR (CDCl₃) δ 8.26 (br, NH), 7.96 (d, *J* = 2.16 Hz, 1H), 7.18 (d, *J* = 10.4 Hz, 1H), 6.81 (d, *J* = 8.28, 1H), 6.60 (s, 1H), 6.50 (s, 1H), 4.09 (t, *J* = 4.50 Hz, 2H), 3.85 (s, 3H), 3.84 (s, 3H), 3.81 (t, *J* = 4.70 Hz, 2H), 3.74 (s, 2H), 3.50-3.45 (m, 2H), 2.93 (s, 4H), 2.67 (s, 2H), 2.31 (s, 3H), 1.76-1.62 (m, 4H), 1.47-1.45 (m, 4H).

1.2.18. 2-(2-((4-(6,7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)butyl)carbamoyl)-4-methylphenoxy)ethyl methanesulfonate (24a) and 2-(2-((6-(6,7-dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)hexyl)carbamoyl)-4-methylphenoxy)ethyl methanesulfonate (24b)

Et₃N (43.5 μL, 0.31 mmol) was slowly added to a solution of compound **23a** (or **23b**) (0.10 mmol) in CH₂Cl₂ (2.00 mL) at 0 °C. Methanesulfonyl chloride (15.5 μL, 0.20 mmol) was added and the reaction mixture was stirred at ambient temperature for 3 h. The reaction mixture was diluted with CH₂Cl₂ (20.0 mL), washed with H₂O (2 x 30.0 mL), and the combined organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*.

Flash column chromatography (15:1 CH₂Cl₂-MeOH) gave compound **24a** (45.8 mg, 88%) [36] as a pale yellow solid. ¹H NMR (CDCl₃) δ 8.20 (s, 1H), 8.19 (s, 1H), 7.55 (d, *J* = 18.0 Hz,

1H), 7.17 (d, $J = 5.40$ Hz, 1H), 6.95 (s, 1H), 6.88 (s, 1H), 4.96 (t, $J = 3.60$ Hz, 2H), 4.64 (t, $J = 14.4$ Hz, 2H), 4.22 (s, 3H), 4.20 (s, 3H), 4.00-3.80 (m, 4H), 3.40-3.39 (m, 3H), 3.20-3.08 (m, 4H), 3.00-2.80 (m, 2H), 2.71 (s, 3H), 2.10-2.07 (m, 4H).

Flash column chromatography (15:1 CH₂Cl₂-MeOH) gave compound **24b** (30.0 mg, 52.6%) as a pale yellow solid. mp 61.7-62.0 °C. ¹H NMR (CDCl₃) δ 7.99 (d, $J = 6.84$ Hz, 1H), 7.78 (br, NH), 7.22 (d, $J = 7.20$ Hz, 1H), 6.81 (d, $J = 7.20$ Hz, 1H), 6.60 (s, 1H), 6.53 (s, 1H), 4.65 (t, $J = 5.40$ Hz, 2H), 4.35 (t, $J = 5.40$ Hz, 2H), 3.84 (s, 3H), 3.83 (s, 3H), 3.49-3.43 (m, 2H), 3.08 (s, 2H), 2.32 (s, 3H), 1.61-1.44 (m, 14H). LCMS m/z 549.2131 (M+H). HRMS (ESI) (M+H) calcd for C₂₈H₄₁N₂O₇S, 549.2634; found, 549.2620.

1.3. *In vitro* sigma binding assays

The test compounds were dissolved in DMSO or ethanol and then diluted in 50 mM Tris-HCl buffer containing 150 mM NaCl and 100 mM EDTA at pH 7.4 prior to performing the σ_1 and σ_2 receptor binding assays. Briefly, the σ_1 receptor binding assays were conducted in 96-well plates using guinea pig brain membrane homogenates (~300 μ g protein) and 6 nM [³H](+)-pentazocine (~42,000 cpm, Perkin-Elmer, Boston, MA). The total incubation time was 120 min at room temperature. Non-specific binding was determined from samples that contained 10 μ M of cold haloperidol. After 120 min, the reaction was terminated by the addition of 150 μ L of ice-cold wash buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) using a 96 channel transferring pipet (Fisher Scientific, Pittsburgh, PA). The samples were harvested and filtered rapidly through a 96-well fiberglass filter plate (Millipore, Billerica, MA) that had been pre-soaked with 100 μ L of 50 mM Tris-HCl buffer at pH 8.0 for 1 h. Each filter was washed three times with 200 μ L of ice-

cold wash buffer, and the filter was counted. The σ_2 receptor binding assays were conducted using rat liver membrane homogenates (~300 μg protein) and 5 nM [^3H]DTG (~82,000 cpm, Perkin-Elmer, Boston, MA) in the presence of 100 nM (+)-pentazocine to block σ_1 sites. The incubation time was 120 min at room temperature. Non-specific binding was determined from samples that contained 5 μM of cold DTG. All other procedures were identical to those described for the σ_1 receptor binding assay above. Data from the competitive inhibition experiments were modeled using nonlinear regression analysis to determine the concentration that inhibits 50% of the specific binding of the radioligand (IC_{50} value). K_i values were calculated using the reported method [44] and are presented as the mean $\pm$ SEM. For these calculations, we used a K_d value of 7.89 nM for [^3H](+)-pentazocine and guinea pig brain. For [^3H]DTG and rat liver, we used 30.73 nM [38].

1.4. Radiochemical Synthesis of [^{18}F]3 and [^{18}F]7

[^{18}F]Fluoride (1.8 – 2.0 GBq) was trapped on a Sep-pak[®] QMA-bicarb cartridge from the cyclotron, and eluted to the reaction vial using 7.0 mg of Kryptofix 222 and 3.0 mg of K_2CO_3 solution. Three azeotropic distillations were then performed using 0.5-1.0 mL aliquots of anhydrous acetonitrile at 100 $^\circ\text{C}$ (oil bath) under a gentle stream of N_2 . The precursor **24a** (or **24b**) (1.5 mg) in DMSO (200 μL) was added to the resulting $\text{K}[^{18}\text{F}]\text{F}$ in the reaction vial. The reaction mixture was heated at 120 $^\circ\text{C}$ for 15 min (Scheme 4). At the end of the reaction, 1.5 mL of the HPLC mobile phase was added to the reaction vial, after passing through Sep-pak[®] Alumina light cartridge, the crude product was then purified by HPLC using a semi-preparative column eluted with a 45:55 mixture of methanol and 0.1 M ammonium formate (aq) at a flow

rate of 5 mL/min for [^{18}F]**3** or with a 46:54 mixture of methanol and 0.1 M aqueous ammonium formate at a flow rate of 5 mL/min for [^{18}F]**7**. The desired products eluted between 29 and 30 min for [^{18}F]**3** and between 27 and 28 min for [^{18}F]**7**. The collected products were diluted with 30 mL of H_2O , trapped Sep-pak[®] C-18 plus cartridge, and formulated 10% EtOH-saline for the *in vivo* biodistribution study or microPET imaging study. Specific activity was determined by comparing the UV peak area of the desired radioactive peak and the UV peak areas of different concentrations of unlabeled standard **3** or **7** on HPLC. Identification of radioligand [^{18}F]**3** and [^{18}F]**7** was determined by co-injecting the radioligand with the corresponding unlabeled standard into the HPLC system.

*1.5. Cellular uptake and blocking study of [^{18}F]**7** in EMT6 Cells*

EMT6 (mouse mammary carcinoma) cells were cultured at 37 °C under 5% CO_2 in 85% Waymouth's MB 752/1 Medium with 2 mM L-glutamine containing 15% FBS. EMT6 cells (5.0×10^4 cells/well) were plated prior to ligand incubation within a 96 well plate. Radiotracer [^{18}F]**7** (~1,000,000 cpm/50 μL /well) was dissolved in PBS (w/ Ca^{2+} and Mg^{2+}) and incubated with EMT6 cells at 37 °C for 1, 2, 3, 5, 10, 20, 30, 45, 60, 90 and 120 min (n=3). Cells were then washed twice with ice cold PBS (w/ Ca^{2+} and Mg^{2+}), lysed cells using 200 μL of 1 N NaOH, and counted. To investigate the blocking of [^{18}F]**7** uptake by **RHM-4**, EMT6 cells were incubated with [^{18}F]**7** (~1,000,000 cpm /25 μL /well) in the presence of 100 μM **RHM-4** (25 μL in PBS) at 37 °C for same time point as above. Cells were then washed as described above and counted.

1.6. *In vivo* biodistribution studies of [^{18}F]7

EMT6 mammary carcinoma cells were injected into the right flanks of female Balb/C mice. Experiments were conducted when tumors had reached a diameter of between 1.0 and 1.2 cm. Radioligand [^{18}F]7 (1.11 MBq) in 0.15 mL of 10% EtOH-saline was injected into mice *via* a tail vein. At 5, 60, and 120 min postinjection, the mice ($n = 4$) were sacrificed, and blood, heart, muscle, lung, kidney, pancreas, spleen, liver, brain, bone, and tumor removed, weighed and counted. Data are expressed as a percent injected dose per gram of tissue (%ID/g).

1.7. *MicroPET* imaging of [^{18}F]3 and [^{18}F]7

MicroPET imaging was performed on a Philips Mosaic small animal scanner, which has 2 mm spatial resolution, with high sensitivity and transverse imaging field-of-view of 12.8 cm. Two weeks after inoculation with EMT6 mammary carcinoma cells, mice with a tumor size of 1~1.2 cm underwent microPET imaging. Mice were anesthetized with isoflurane, which was maintained *via* nosecone, and placed on a heating pad on the scanner bed. The dynamic images of these mice were acquired for 2 h after injection of [^{18}F]3 (or [^{18}F]7, 10 – 11 MBq) in 0.15 mL of 10% EtOH-saline solution. Elipsoid VOIs were drawn around the tumor (5 x 4 x 4 mm) and muscle (3 x 3 x 4 mm) regions. Biceps brachii muscle (left flank) was used as the reference region. Time activity curves of the tumor regions and the muscle regions were obtained from mean counts/voxel of each VOI by Amide software.

2. Results and discussion

2.1. Chemistry

Compounds **3** and **7** were synthesized from compounds **11** and **13** using a condensation reaction between benzoic acid and amine. Compound **13** was synthesized from methyl 5-methylsalicylate and 2-fluoroethyl 4-methylbenzenesulfonate in 2 steps, and compounds **11a** and **11b** were prepared using phthalimide as a protecting group in 2 steps (Scheme 1). Synthetic pathways of eight novel indole- and benzothiophene-substituted tetrahydroisoquinoline analogues, **8a-8d** and **9a-9d** (Figure 2), are shown in Schemes 2-3. These analogues were synthesized by condensation of **11a** (or **11b**) and the corresponding carboxylic acid, 5-methoxy-1*H*-indole-2-carboxylic acid and **15** for indole-substituted analogues, or **17** and **20** for benzothiophene-substituted analogues in the presence of HOBt and DCC in CH₂Cl₂. Compounds **15**, **17**, and **20** were hydrolyzed from the corresponding carboxylate, **14**, **16**, and **19** in the presence of solid NaOH in MeOH/H₂O. Benzothiophene moieties, **16** and **19**, were cyclized between 2-fluoro-5-methoxybenzaldehyde (or **18**) and methyl thioglycolate as a sulfur source in the presence of NaH in DMSO and DMF, respectively. Compounds **14** and **18** were prepared from ethyl 5-hydroxy-1*H*-indole-2-carboxylate or 5-hydroxy-2-nitrobenzaldehyde with 2-fluoroethyl 4-methylbenzenesulfonate in the presence of K₂CO₃ in high yields (64.6-99%). For the comparison 4 and 6-carbon spacer analogues, [¹⁸F]**3** and [¹⁸F]**7** were radiolabeled from **24a** and **24b** with ¹⁸F, as shown in Scheme 4. Alkylation of the ortho-hydroxyl group of methyl 2-hydroxy-5-methylbenzoate with 1-bromoethyl acetate, followed by hydrolysis of the acetate group produced **22**. Compounds **23a** and **23b** were then converted to the corresponding mesylates, **24a** and **24b**, by treatment with methanesulfonyl chloride in dichloromethane using Et₃N as an acid scavenger (Scheme 4).

2.2. *In vitro* sigma binding assays

The results of the binding assays for compounds **3** and **7**, along with haloperidol and DTG as standard controls are shown in Table 1. Haloperidol (3.18 and 57.9 nM for σ_1 and σ_2 receptors, respectively) and DTG (59.9 and 19.4 nM for σ_1 and σ_2 receptors, respectively) showed similar K_i values to previously reported data. The binding affinities of compound **3** and **7** for the σ_1 receptor were 95.1 and over 1000 nM, and for the σ_2 receptors were 13.3 and 23.1 nM, respectively. The σ_1 : σ_2 ratios of compound **3** and **7** were 7.15 and over 43.3. These results indicate that although compound **7** had a lower affinity for σ_2 receptors versus compound **3**, it had a higher selectivity ratio value for the σ_2 versus σ_1 receptor. K_i values of indole and benzothiophene derivatives, **8a-9d**, for σ_2 receptors were desirable, ranging from 18.2 to 37.9 nM as well as they showed high selective binding to σ_2 receptors compare to σ_1 receptors (σ_2 : σ_1 = 17.4-215.5) (Table 1). In terms of selectivity for σ_2 receptors to σ_1 receptors, 4-carbon spacer derivatives showed higher selectivity than the corresponding 6-carbon spacer derivatives in both analogues.

2.3. Radiochemical synthesis of [^{18}F]**3** and [^{18}F]**7**

[^{18}F]**3** and [^{18}F]**7** were synthesized from mesylate precursors, **24a** and **24b**, with [^{18}F]fluoride/ K_2CO_3 and Kryptofix 222, using DMSO as the solvent (Scheme 4). The following HPLC purification gave [^{18}F]**3** and [^{18}F]**7** in an overall 15-40% decay-corrected radiochemical yield and radiochemical purity higher than 99% with specific activity of 70 and 73 GBq/ μmol . The total synthesis time including HPLC purification was 2 h.

2.4. Cellular uptake and blocking study of [^{18}F]7 in EMT6 Cells

The uptake of [^{18}F]7 in EMT6 cells increased from 18% uptake/100 μg protein at 0 min to 30% uptake/100 μg protein at 20 min; the uptake then remained level from 20 min to 120 min. (25% - 30% uptake/100 μg protein). The uptake of [^{18}F]7 in EMT6 cells was reduced by co-incubation of 100 μM of the σ_2 receptor antagonist, **RHM-4** [36, 45]; that is, the cell uptake in the blocking study ranged from 5 to 10% uptake/100 μg protein, which represents a 70-80% reduction in uptake relative to the control group (Figure 3). This result demonstrated that [^{18}F]7 specifically binds to σ_2 receptors in the EMT6 breast cancer cells.

2.5. In vivo biodistribution of [^{18}F]7 and microPET imaging studies of [^{18}F]3 and [^{18}F]7

The results of the biodistribution studies of [^{18}F]7 in female Balb/c mice bearing EMT-6 tumors are shown in Table 2. [^{18}F]7 displayed a modest tumor uptake 0.52 of the injected dose per gram (%ID/g) at 5 min post-injection and the tumor uptake increased (1.55 %ID/g, respectively) at 1 h post-injection and at 2 h post-injection (1.19 %ID/g) in mice. The tumor uptake of [^{18}F]7 at 1 h and 2 h post-injection is higher than the reported values of [^{18}F]3 (i.e., [^{18}F]ISO-1) at 1h (1.14 %ID/g) and at 2 h (0.64 %ID/g) post-injection [36] in mice. [^{18}F]7 also had good tumor : muscle (left flank) ratios at 1 h (2.92) and 2 h (3.30) post-injection of the radiotracer. Low bone uptake of [^{18}F]7 was observed (Table 2), suggesting that it does not undergo in vivo metabolic defluorination.

MicroPET imaging studies comparing both [^{18}F]3 (i.e., [^{18}F]ISO-1) and [^{18}F]7 were conducted in tumor-bearing mice. The EMT-6 tumors were readily identifiable using both radioligands (Figure 4). [^{18}F]7 showed higher tumor uptake and higher tumor: muscle ratios than [^{18}F]3 in the

images (Figure 5). These results suggest that [^{18}F]**7** may be a suitable PET probe for imaging σ_2 receptor *in vivo* with PET.

3. Conclusions

In this study, we synthesized nine novel σ_2 ligands including a 6-carbon spacer analogue of ISO-1, four indole analogues, and four benzothiophene analogues which have moderate binding affinity and high selectivity for σ_2 versus σ_1 receptors (Table 1). [^{18}F]**7** was also successfully radiolabeled with ^{18}F and evaluated as a potential radiotracer for imaging EMT-6 tumors in female Balb/c mice. Although [^{18}F]**7** has a lower binding affinity for σ_2 receptors than [^{18}F]**3** *in vitro*, it displays a high tumor uptake and suitable tumor: muscle ratios for imaging studies. MicroPET studies demonstrated [^{18}F]**7** had a higher tumor uptake and a higher tumor : muscle ratio than that of [^{18}F]**3**. Therefore, the 6-carbon spacer analogue of **ISO-1** ([^{18}F]**7**) may be a useful probe for detecting solid tumors and imaging their σ_2 receptors status with PET. In contrast to previous suggestions, we observed no difference in σ_2 receptor binding affinity between the 4-carbon and 6-carbon linker within the indole and benzothiophene series.

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5. References

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Table 1. Binding affinity (K_i) of compounds for the σ_1 and σ_2 receptors assayed *in vitro*.

Compound	K_i value (nM)			$C \text{ Log } P^a$ (<i>calculated</i>)
	σ_1	σ_2	$\sigma_1:\sigma_2$ ratio	
Haloperidol	3.18 ± 0.04	57.9 ± 5.25	0.05	
DTG	59.9 ± 1.45	19.4 ± 1.21	3.08	
3 (ISO-1)	95.1 ± 4.40	13.3 ± 1.00	7.15	3.06 ± 0.51
7	>1000	23.1 ± 1.34	>43.3	3.64 ± 0.51
8a	>5000	23.2 ± 5.55	>215.5	3.43 ± 0.87
8b	>2000	33.6 ± 5.29	>59.5	4.19 ± 0.87
8c	>2000	24.0 ± 4.13	>83.3	3.66 ± 0.92
8d	>2000	37.9 ± 1.16	>52.7	4.42 ± 0.92
9a	>2000	25.6 ± 2.21	>78.1	4.57 ± 1.10
9b	>1000	23.5 ± 1.54	>42.5	5.33 ± 1.10
9c	>2000	21.3 ± 1.22	>93.8	4.80 ± 1.14
9d	316 ± 3.5	18.2 ± 1.13	17.4	5.56 ± 1.14

^a *Calculated using ACD log P software, Advanced Chemistry Development*

Table 2. *In vivo* biodistribution of [¹⁸F]7 in female Balb/c mice bearing EMT-6 tumors

	(%ID/g) (n=4)		
	5 min	60 min	120 min
Blood	2.31 ± 0.13	1.65 ± 0.14	1.16 ± 0.06
Heart	3.21 ± 1.55	1.59 ± 0.18	0.85 ± 0.05
Muscle	0.57 ± 0.11	0.53 ± 0.03	0.36 ± 0.07
Lung	9.29 ± 3.25	2.63 ± 0.32	1.46 ± 0.09
Kidney	38.6 ± 6.37	5.59 ± 0.69	3.59 ± 0.37
Pancreas	8.78 ± 1.82	41.0 ± 6.99	25.9 ± 5.43
Spleen	7.13 ± 0.90	3.09 ± 0.35	1.74 ± 0.36
Liver	26.0 ± 3.98	14.5 ± 3.15	6.43 ± 1.28
Brain	0.87 ± 0.14	0.20 ± 0.01	0.12 ± 0.01
Bone	1.65 ± 0.31	0.89 ± 0.16	0.51 ± 0.03
Tumor	0.52 ± 0.14	1.55 ± 0.10	1.19 ± 0.25
T:M ratio	0.92	2.92	3.30

Figure 1. Structures, binding affinities for σ receptors, and $\log P$ of benzamide analogues

Figure 2. Chemical Structures of novel tetrahydroisoquinoline derivatives

Figure 3. Cellular Uptake and Blocking Study of [^{18}F]**7** in EMT-6 Cells; black circle: Cell uptake of [^{18}F]**7**, gray square: Cell uptake of [^{18}F]**7** in the presence of 100 μM **RHM-4**.

Figure 4. MicroPET images of EMT-6 tumors in female Balb/c mice. All microPET images were summed over the 2 h acquisition period after i.v. injection of either [^{18}F]**3** (left panel) and [^{18}F]**7** (right panel).

Figure 5. Time-activity curves and tumor to muscle ratios of [^{18}F]**3** (left panel) and [^{18}F]**7** (right panel) from microPET images studies; left top (tumor and muscle uptake of [^{18}F]**3**), left bottom (tumor/muscle ratio of [^{18}F]**3**, right top (tumor and muscle uptake of [^{18}F]**7**), and right bottom (tumor/muscle ratio of [^{18}F]**7**).

Scheme 1. Reagents and conditions: a) *N*-(bromoalkyl)phthalimide, K_2CO_3 , CH_3CN , 120 °C, 20 h; b) hydrazine monohydrate, EtOH, 70 °C, 3 h; c) 2-fluoroethyl 4-methylbenzenesulfonate, K_2CO_3 , CH_3CN , 120 °C, 3 h; d) NaOH, H_2O , MeOH, 70 °C, 3 h; e) *N,N'*-dicyclohexylcarbodiimide (DCC), hydroxybenzotriazole (HOBt), CH_2Cl_2 , room temperature, 20 h.

Scheme 2. Reagents and conditions: a) 2-fluoroethyl 4-methylbenzenesulfonate, K_2CO_3 , CH_3CN , 120 °C, 3 h; b) NaOH, H_2O , MeOH, 70 °C, 3 h; c) methyl thioglycolate, NaH, DMSO, room temperature, 5 min; d) methyl thioglycolate, K_2CO_3 , DMF, 80 °C, 20 h.

Scheme 3. Reagents and conditions: a) DCC, HOBt, CH_2Cl_2 , room temperature, 20 h.

Scheme 4. Reagents and conditions: a) 2-bromoethyl acetate, K_2CO_3 , CH_3CN , 80 °C, 48 h; b) NaOH, H_2O , MeOH, 70 °C, 3 h; c) DCC, HOBt, CH_2Cl_2 , room temperature, 20 h; d) methanesulfonyl chloride, Et_3N , CH_2Cl_2 , room temperature, 3 h; e) K_{222} , $K^{18}F$, DMSO, 120 °C, 15 min.

1

$\sigma_1 = 10,400 \text{ nM}$
 $\sigma_2 = 13.3 \text{ nM}$
 $\text{Log P} = 2.31$

2 (RHM-1)

$\sigma_1 = 3,078 \text{ nM}$
 $\sigma_2 = 10.3 \text{ nM}$
 $\text{Log P} = 2.84$

3 (ISO-1)

$\sigma_1 = 330 \text{ nM}$
 $\sigma_2 = 7.0 \text{ nM}$
 $\text{Log P} = 3.06$

4 (FE-RHM-4)

$\sigma_1 = 2,150 \text{ nM}$
 $\sigma_2 = 0.26 \text{ nM}$
 $\text{Log P} = 3.46$

5

$\sigma_1 = 780 \text{ nM}$
 $\sigma_2 = 0.88 \text{ nM}$
 $\text{Log P} = 4.47$

6 (RHM-4)

$K_d \text{ for } \sigma_2 = 0.68 \text{ nM}$