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Synthesis and evaluation of ^{18}F -labeled PPAR γ antagonists

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Abstract

Introduction—Peroxisome proliferator activated-receptor gamma (PPAR γ) transcriptionally modulates fat metabolism and also plays a role in pathological conditions such as cancer, neurodegenerative disease, and inflammation. PPAR γ imaging agents are potential tools for investigating these diseases.

Methods—Four analogs of GW9662, a PPAR γ antagonist, with different fluorine-containing substituents at the *para*-position of the aniline ring were synthesized and evaluated using two different receptor binding assays for measuring PPAR γ affinity. MicroPET imaging studies were performed in a transgenic mouse model having a heart specific overexpression of PPAR γ .

Results—All four analogs were found to have binding affinities that were comparable to or better than the reference antagonist, GW9662, using a scintillation proximity assay. However, only the chloro-based analogs (compounds **3** and **4**) had activity in a whole cell assay measuring activation of the PPAR γ / RXR receptor complex. MicroPET imaging studies in a MHC-PPAR γ transgenic mouse model showed high uptake and PPAR γ specific binding for the irreversible antagonist [^{18}F]**3**, whereas the corresponding reversible methoxy analog ([^{18}F]**5**) displayed only nonspecific uptake in heart.

Conclusions—The results of this preliminary study show that the irreversible antagonist [^{18}F]**3** may represent a novel strategy for imaging PPAR γ in vivo with PET. **1.**

Keywords

PPAR γ ; peroxisome proliferator-activated receptor- γ ; antagonist; imaging agent

1. INTRODUCTION

Peroxisome proliferator activated-receptor gamma (PPAR γ) binds to peroxisome receptor response elements (PPRE) with its heterodimeric partner, retinoid X receptor (RXR); the complex initiates gene remodeling and regulates downstream gene expression [1, 2]. The most widely studied function of PPAR γ is the regulation of fat metabolism and storage. Because PPAR γ ligands reduce free fatty acids in blood, agonists of this receptor have been used as insulin sensitizers to treat type II diabetes. In addition, PPAR γ has been shown to

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play a role in various diseases, such as cancer, neurodegenerative disease, and inflammation. PPAR γ agonists have been used to treat these diseases with variable results [3–7]. Methods that can measure not only the level of PPAR γ but also ligand binding ability would provide a more accurate prediction of the outcome of PPAR γ agonist treatment. A noninvasive imaging modality, such as PET, could be used to monitor receptor expression over time and to identify patients that would most likely benefit from PPAR γ agonist treatments.

PPAR γ antagonists, such as GW9662 and T0070907, have also been reported [8, 9]. GW9662, 2-chloro-5-nitro-*N*-phenyl-benzamide, was characterized with both a competitive binding assay and a whole-cell assay. This compound showed nanomolar binding affinity toward PPAR γ and the subtype selectivity was 10- and 600-fold lower against PPAR α and PPAR β/δ , respectively. Interestingly, pretreatment of PPARs with GW9662 resulted in a loss in binding of [^3H]rosiglitazone in a scintillation proximity assay (SPA) [8]. This loss of binding was shown to be caused by the formation of a covalent bond between GW9662 and Cys²⁸⁵ of PPAR γ . Consistent with this hypothesis, the same phenomenon was not observed when rosiglitazone was used for pretreatment. The antagonistic effect of GW9662 was confirmed by its inhibition of adipocyte differentiation by stimulation with troglitazone, a widely used PPAR γ agonist [8].

Another antagonist, T0070907, a pyridine analog of GW9662 (Figure 1), is a subtype specific ligand with an IC₅₀ value in the nanomolar range [9]. Its antagonist properties at PPAR γ were additionally examined in the inhibition of adipocyte differentiation seen in 3T3-L1 cells stimulated with dexamethasone, 3-isobutyl-1-methylxanthine, and insulin. The same covalent bond formation was confirmed by analyzing the protein/ligand fragments with mass spectrometry [9].

Previous PET imaging studies have involved three PPAR γ agonists, SB213068, GI262570, and GW7845, radiolabeled with either carbon-11 or fluorine-18 [10–13]. Both [^{18}F]SB213068 and [^{11}C]GW7845 showed moderate radioactivity uptake in target tissues. However, the radiotracer uptake was demonstrated to not be receptor-specific. This might be due to the modest subtype specificity of these ligands (PPAR γ /PPAR α $\approx$ 8). Analogs of another agonist, GI262570 (Farglitazar), have been radiolabeled with fluorine-18 [10, 11]. These fluorine-18 labeled radiotracers had good potential for PPAR γ imaging because of their high subtype specificity (PPAR γ /PPAR α $>$ 100). However, despite high affinity and good metabolic stability, in vivo studies demonstrated poor uptake in target tissues, which was attributed to their high lipophilicity [14].

The first radiolabeled antagonist of the PPAR γ receptor, [^{76}Br]2-bromo-5-nitro-*N*-phenyl-benzamide, was synthesized and evaluated for its in vivo efficacy by our research group [15]. The initial biodistribution study of [^{76}Br]**1** indicated that there was a high tumor uptake of radioactivity (4–5 %ID/g uptake within 2 h) in nude mice implanted with MDA-MB435, a human melanoma shown to have a high expression of PPAR γ receptors [16]. However, metabolite analysis suggested that the radiotracer was rapidly degraded in whole blood to [^{18}F]bromide, thus [^{76}Br]**1** is not a viable candidate for imaging PPAR γ in vivo.

In an effort to identify radiolabeled PPAR γ antagonists which can be labeled with fluorine-18, we chose to explore GW9662 as a lead compound radiotracer development. Two patents exploring the structure-activity relationships of GW9662 suggested that there is a high degree of bulk tolerance in the *para*-position of the aniline ring [17, 18]. Our first attempt involved substituting the *para* position of GW9662 with a methoxy group (compound **2** in Figure 1) to facilitate radiolabeling with carbon-11. This compound was found to have a similar potency for inhibiting PPAR γ ; [^{11}C]**2** also degraded slower than [^{76}Br]**1** in both rat plasma and whole blood samples. However, [^{11}C]**2** suffered from a low

radiolabeling yield, poor chemical purity, and poor specific activity in our hands. On the other hand, these studies did suggest that it should be possible to prepare an ^{18}F -labeled analog of GW9662 assuming the radiolabeling conditions could be established to produce a tracer in high yield, high chemical purity, and suitable specific activity for conducting imaging studies of the PPAR γ .

In the current study, we describe the synthesis, in vitro characterization, and in vivo evaluation of four potential radiotracers for imaging PPAR γ in vivo (Figure 1). Two of the compounds possess a chloro group, (**3** and **4**), and were shown to be the irreversible PPAR γ antagonists; two additional reversible compounds were prepared where a methoxy group replaced the chloro group (**5** and **6**). All four compounds were screened for ligand binding affinities and showed good binding affinity in the protein-only assay. However, only **3** and **4** were found to be active in a whole cell assay for measuring PPAR γ antagonist activity (Table 1). [^{18}F]**3** and [^{18}F]**5** were synthesized and microPET imaging studies were conducted in a transgenic mouse model in which PPAR γ is overexpressed in heart. [^{18}F]**3** showed a high target/background ratio, which was blocked GW9662 suggesting that the tissue uptake is receptor specific. Although further studies are needed, [^{18}F]**3**, an irreversible antagonist of PPAR γ , represents an alternative strategy for imaging PPAR γ in vivo with PET versus the radiolabeled agonists reported previously.

2. MATERIALS AND METHODS

2.1 Chemistry

2.1.1 General—All chemicals were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI) and used without further purification unless otherwise stated. Melting points were determined using MEL-TEMP 3.0 apparatus and are uncorrected. ^1H NMR spectra were recorded at 300 MHz on a Varian Mercury-VX spectrometer. All chemical shift values are reported in ppm downfield from tetramethylsilane (TMS) or, when chloroform-*d* was used as solvent, the solvent peak at δ 7.25 ppm was utilized as an internal standard. All coupling constants (*J*) are given in Hz. Splitting patterns are described as follows: s, singlet; d, doublet; t, triplet; m, multiplet. High-resolution mass spectrometric analyses were done at the Washington University Center for Biomedical and Bioorganic Mass Spectrometry. The analyses were performed on Hewlett Packard 5988A GC/MS mass spectrometers or Agilent 5973 GC/MS mass spectrometers with positive ion electrospray ionization (EI-MS).

2.1.2. Synthesis of Anilines **9** and **10**

4-(2-Fluoroethyl)aniline (9**):** In a solution of (2-(4-nitrophenyl)ethanol (1g, 6.0 mmol) in dichloromethane (10 mL), diethylaminosulfur trifluoride (DAST) (1 mL, 7.6 mmol) was added in drop-wise fashion and the reaction mixture became brown in color. The reaction was allowed to sit for 60–120 min at -80°C and was then quenched with saturated sodium bicarbonate solution (1 $\times$ 20 mL) and the aqueous solution was extracted with ethyl acetate (3 $\times$ 20 mL). It was then washed with water (1 $\times$ 20 mL) and brine (1 $\times$ 20 mL) and dried over Na_2SO_4 . Purification was performed with silica gel column chromatography using 70% hexane and 30% ethyl acetate to give 1-(2-fluoroethyl)-4-nitrobenzene **7** (0.49g, 3.6 mmol, 60%), which was then dissolved in 20 mL of ethanol.

Using a high-pressured Parr Shaker hydrogenator (Parr Instrument Company, Moline, IL) in the presence of 2–3 equivalents of Pd/C catalyst (1g, 10 mmol), **7** was reduced to the amine. The catalyst was removed by filtration and the product was purified by silica gel column chromatography with a solution of 50% hexane and 50% ethyl acetate to give **9** (0.43g, 95%

yield). ¹H-NMR (300 MHz, CDCl₃, δ): 2.918 (dt, J=1 Hz, J=1 Hz, 2H), 4.574 (dt, J=2 Hz, J=1 Hz, 2H), 6.652 (d, J=3 Hz, ArH, 2H), 7.031 (d, J= 3 Hz, ArH, 2H).

4-(2-Fluoroethoxy)aniline (10): In a solution of (2-(4-nitro-phenoxy)ethanol (1g, 5.5 mmol) in dichloromethane (10 mL), DAST (1 mL, 7.6 mmol) was added in drop-wise fashion and the reaction mixture started to become brown in color. The reaction was allowed to sit for 60–120 min at –80 °C. Saturated sodium bicarbonate solution (1×20 mL) was added to quench the reaction. The aqueous solution was extracted with ethyl acetate (3×20 mL). It was then washed with water (1×20 mL) and brine (1×20 mL) and dried over Na₂SO₄. Purification was performed with silica gel column chromatography with 75% hexane and 25% ethyl acetate to give 1-(2-fluoroethoxy)-4-nitrobenzene **8** (0.51g, 3.3 mmol, 60%), which was then dissolved in 20 mL of ethanol.

Using a high-pressured Parr Shaker hydrogenator (Parr Instrument Company, Moline, IL) in the presence of 2–3 equivalents of catalyst, Pd/C (1g, 10 mmol), **8** was reduced to the corresponding amine. The catalyst was removed by filtration and the product was purified by silica gel column chromatography with a solution of 50% hexane and 50% ethyl acetate to give **10** (0.49g, 95%). ¹H-NMR (300 MHz, CDCl₃, δ): 4.141 (dt, J=1 Hz, J=1 Hz, 2H), 4.715 (dt, J=2 Hz, J=1 Hz, 2H), 6.650 (d, J=2 Hz, ArH, 2H), 6.771 (d, J= 2 Hz, ArH, 2H).

2.1.3 Synthesis of PPAR γ Ligands 3 – 6

2-Chloro-N-[4-(2-fluoroethyl)phenyl]-5-nitrobenzamide (3): 2-Chloro-5-nitrobenzoic acid (1g, 5.0 mmol), dissolved in dry dichloromethane (10 ml), was placed on ice and under nitrogen gas. Slowly, 1.5 equivalents of triphosgene (2.2g, 7.5 mmol) were added to the solution. The solution turned clear upon the slow addition of 5 equivalents of *N,N*-diisopropylethylamine (DIEA) (4.35 mL, 25 mmol). The amine **9** (0.68g, 5 mmol) was dissolved in 5 mL of dry dichloromethane and slowly added into the reaction mixture. The reaction was slowly warmed to room temperature and allowed to stir at ambient temperature for 14 h. The reaction mixture was washed with saturated sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL). The organic layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. Purification was performed with a silica column with a mixture of 60% hexane and 40% ethyl acetate and the product was recrystallized from methanol to give **3** as a white solid, mp 149.8–151.1 °C (0.64g, 40%). ¹H-NMR (300 MHz, CDCl₃, δ): 2.934 (t, J=1 Hz, 1H), 3.017 (t, J=1 Hz, 1H), 4.565 (t, J=1 Hz, 1H), 4.723 (t, J=1 Hz, 1H), 7.282 (d, J=2.8 Hz, ArH, 2H), 7.643 (d, J= 3 Hz, ArH, 2H), 7.895 (d, J= 3Hz, ArH, 1H) 8.357 (dd, J=3 Hz, J=1 Hz, ArH, 1H), 8.471 (d, J=1 Hz, ArH, 1H), 10.690 (s, NH, 1H); HRMS: calculated for C₁₅H₁₂ClFN₂O₃: 322.0520, found: 322.0532.

2-Chloro-N-[4-(2-fluoroethoxy)phenyl]-5-nitrobenzamide (4): 2-Chloro-5-nitrobenzoic acid (1g, 5.0 mmol), dissolved in dry dichloromethane (10ml), was placed on ice under nitrogen gas. Slowly, 1.5 equivalents of triphosgene (2.2g, 7.5 mmol) were added to the solution. The solution turned clear upon the slow addition of 5 equivalents of DIEA (4.35 mL, 25 mmol). Amine **10** (0.78g, 5 mmol) was dissolved in 5 mL of dry dichloromethane and slowly added into the reaction mixture. The ice bath was removed and the reaction mixture was stirred at ambient temperature for 14 h. The crude reaction mixture was washed with saturated sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. Purification was performed with a silica column with a mixture of 50% hexane and 50% ethyl acetate and the compound was recrystallized from methanol to give **4** as a pale yellow solid, mp 205.4–206.4 °C (0.59g, 35%). ¹H-NMR (300 MHz, CDCl₃, δ): 4.19 (t, J=1 Hz, 1H), 4.30 (t, J=1 Hz, 1H), 4.69 (t, J=1 Hz, 1H), 4.85 (t, J=1 Hz, 1H), 7.01 (d, J=3 Hz, ArH, 2H), 7.637 (d, J=

3 Hz, ArH, 2H), 7.898 (d, J= 3 Hz, ArH, 1H) 8.35 (dd, J=3 Hz, J=1 Hz, ArH, 1H), 8.464 (d, J=1 Hz, ArH, 1H), 10.608 (s, NH, 1H); HRMS: calcd for C₁₅H₁₂ClFN₂O₄: 338.0470, found: 338.0475.

N-[4-(2-Fluoroethyl)phenyl]-2-methoxy-5-nitrobenzamide (5): 2-Methoxy-5-nitrobenzoic acid (1g, 5.1 mmol), dissolved in dry dichloromethane (10 ml), was placed on ice and under nitrogen gas. Slowly, 1.5 equivalents of triphosgene (2.2g, 7.5 mmol) were added to the solution. The solution turned clear upon the slow addition of 5 equivalents of DIEA (4.35 mL, 25 mmol). Amine **9** (0.68g, 5 mmol) was dissolved in 5 mL of dry dichloromethane and slowly added into the reaction mixture. The ice bath was removed and the reaction mixture was stirred at ambient temperature for 14 h. The crude reaction mixture was washed with saturated sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. Purification was performed with a silica column with a mixture of 50% hexane and 50% ethyl acetate and the compound was recrystallized from methanol to give **5** as a white solid, mp 183.4–184.6 °C (0.49g, 30%). ¹H-NMR (300 MHz, d₆-DMSO, δ): 2.89 (t, J=1 Hz, 1H), 2.97 (t, J=1 Hz, 1H), 3.997 (s, 3H), 4.52 (t, J=1 Hz, 1H), 4.68 (t, J=1 Hz, 1H), 7.227 (d, J=1.9 Hz, ArH, 2H), 7.38 (q, J= 0.5 Hz, ArH, 1H), 7.623 (dd, J= 2.7Hz, J=1 Hz, ArH, 2H) 8.357 (m, J=1 Hz, ArH, 2H), 10.261 (s, NH, 1H); HRMS: calculated for C₁₆H₁₅FN₂O₄: 318.1016, found: 318.1001.

N-[4-(2-Fluoroethoxy)phenyl]-2-methoxy-5-nitrobenzamide (6): 2-Methoxy-5-nitrobenzoic acid (1g, 5.1 mmol), dissolved in dry dichloromethane (10 ml), was placed on ice under nitrogen gas. Slowly, 1.5 equivalents of triphosgene (2.2g, 7.5 mmol) were added to the solution. The solution turned clear upon the slow addition of 5 equivalents of DIEA (4.35 mL, 25 mmol). Amine **10** (0.78g, 5 mmol) was dissolved in 5 mL of dry dichloromethane and slowly added into the reaction mixture. The ice bath was removed and the reaction mixture was stirred at ambient temperature for 14 h. The crude reaction mixture was washed with saturated sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. Purification was performed with a silica column with a mixture of 50% hexane and 50% ethyl acetate and the compound was recrystallized from methanol to give **6** as a light yellow solid, mp 197.6–198.8 °C (0.67g, 40%). ¹H-NMR (300 MHz, d₆-DMSO, δ): 3.997 (s, 3H), 4.15 (t, J=1 Hz, 1H), 4.25 (t, J=1 Hz, 1H), 4.64 (m, J=1 Hz, 1H), 4.79 (m, J=1 Hz, 1H), 6.95 (dd, J=1.9 Hz, J=1 Hz, ArH, 2H), 7.38 (dd, J= 2.5 Hz, J=1 Hz, ArH, 1H), 7.62 (dd, J= 2.5Hz, J=1 Hz, ArH, 2H) 8.350–8.385 (dd, J=3 Hz, J=1 Hz, ArH, 2H), 10.173 (s, NH, 1H); HRMS: calculated for C₁₆H₁₅FN₂O₅: 334.0965, found: 334.0951.

2.1.4 Synthesis of Radiolabeling Precursor

2-Chloro-N-(4-(2-hydroxyethyl)phenyl)-5-nitrobenzamide (11): 2-Chloro-5-nitrobenzoic acid (1g, 5.0 mmol), dissolved in 10 mL of dry dichloromethane, was placed on ice under nitrogen gas. Slowly, 1.5 equivalents of triphosgene (2.2g, 7.5 mmol) were added to the solution. The solution turned clear upon the addition of 5 equivalents of DIEA (4.35 mL, 25 mmol). 2-(4-Aminophenyl)ethanol (0.68g, 5 mmol) was dissolved in 5 mL of dry dichloromethane and slowly added into the reaction mixture. The reaction was slowly warmed to room temperature and reacted overnight. The mixture was washed with saturated sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL). Silica column chromatography with a solvent mixture of 50% ethyl acetate and 50% hexane was used for further compound purification. The process afforded **11** as a white solid (0.64g, 2 mmol, 40%). ¹H-NMR (300 MHz, CDCl₃, δ): 2.483 (t, J=1 Hz, 2H), 3.555 (t, J=1 Hz, 2H), 4.620 (t, J=1 Hz, 1H), 7.181 (d, J=2 Hz, ArH, 2H), 7.563 (d, J= 3 Hz, ArH, 2H), 7.855 (d, J=3Hz, ArH, 1H), 8.306 (dd, J=3 Hz, J=1 Hz, ArH, 1H), 8.413 (d, J=1 Hz, ArH, 1H), 10.610

(s, NH, 1H); HRMS: calculated for $C_{15}H_{13}ClN_2O_4$: 320.0564, found: 320.0551 and 322.0539.

N-(4-(2-Hydroxyethyl)phenyl)-2-methoxy-5-nitrobenzamide (12): 2-Methoxy-5-nitrobenzoic acid (1g, 5.1 mmol), dissolved in 10 mL of dry dichloromethane, was placed on ice under nitrogen gas. Slowly, 1.5 equivalents of triphosgene (2.2g, 7.5 mmol) were added to the solution. The solution turned clear upon the addition of 5 equivalents of diisopropyl ethylamine (DIEA) (4.35 mL, 25 mmol). 2-(4-Aminophenyl)ethanol (0.68g, 5 mmol) was dissolved in 5 mL of dry dichloromethane and slowly added into the reaction mixture. The ice bath was removed and the reaction mixture was stirred at ambient temperature for 14 h. The reaction mixture was washed with saturated sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The product was purified with silica gel column chromatography using a mixture of 50% hexane and 50% ethyl acetate to give **12** as a white solid (0.48g, 1.52 mmol, 30%). 1H -NMR (300 MHz, D-DMSO, δ): 2.79 (t, J=1 Hz, 2H), 3.67 (t, J=1 Hz, 2H), 3.98 (s, 3H), 7.23 (d, J=2 Hz, ArH, 2H), 7.38 (q, J= 0.5 Hz, ArH, 1H), 7.62 (dd, J= 2.7Hz, J=1 Hz, ArH, 2H), 8.36 (m, J=1 Hz, ArH, 2H), 8.48 (m, J=1 Hz, ArH, 2H), 10.261 (s, NH, 1H); HRMS: calculated for $C_{16}H_{16}N_2O_5$: 316.1059, found: 316.1058.

4-(2-Chloro-5-nitrobenzamido)phenethyl methanesulfonate (13): Compound **11** (0.641 g, 2 mmol) was dissolved in 10 mL of dichloromethane and 3 equivalents of methanesulfonyl chloride (4.3 mL, 6 mmol) were slowly added at 0 °C. The ice bath was removed and the reaction mixture was stirred at ambient temperature for 14 h. The reaction mixture was washed with sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude product was purified by silica column chromatography with a solvent mixture of 60% ethyl acetate and 40% hexane. Recrystallization from methanol gave **13** as a white solid (0.44g, 55% yield). 1H -NMR (300 MHz, $CDCl_3$, δ): 3.110 (s, 3H), 2.940 (t, J=2 Hz, 1H), 4.361 (t, J=2 Hz, 1H), 7.267 (d, J=2.8 Hz, ArH, 2H), 7.640 (d, J= 1 Hz, ArH, 2H), 7.860 (d, J= 3Hz, ArH, 1H), 8.302–8.8341 (dd, J=3 Hz, J=1 Hz, ArH, 1H), 8.432 (d, J=1 Hz, ArH, 1H), 10.679 (s, NH, 1H).

4-(2-Methoxy-5-nitrobenzamido)phenethyl methanesulfonate (14): Compound **12** (0.485 g, 1.5 mmol) was dissolved in 10 mL of dichloromethane and 3 equivalents of methanesulfonyl chloride (2.9 mL, 4.6 mmol) were slowly added at 0 °C. The ice bath was removed and the reaction mixture was stirred at ambient temperature for 14 h. The reaction mixture was washed with sodium bicarbonate solution (1×20 mL), water (1×20 mL), and brine (1×20 mL), dried over anhydrous sodium sulfate and concentrated *in vacuo*. The crude product was purified by silica column chromatography with a solvent mixture of 60% ethyl acetate and 40% hexane. Recrystallization from methanol gave **14** as a white solid (0.24g, 40% yield). 1H -NMR (300 MHz, D-DMSO, δ): 2.903 (s, 3H), 3.049 (t, J=2.3, 2H), 4.411 (s, J=2.3, 2H), 7.158 (d, J=3 Hz, ArH, 1H), 7.270–7.284 (m, ArH, 4H), 8.380 (dd, J= 3.1Hz, J=1 Hz, ArH, 2H), 9.177 (d, J=1 Hz, ArH, 1H), 9.500 (s, NH, 1H).

2.2 Radiochemistry

No-carrier-added [^{18}F]Fluoride was produced in Washington University by the $^{18}O(p,n)^{18}F$ reaction through proton irradiation of enriched (95%) ^{18}O water using RDS Eclipse cyclotron. Radioactivity (100 – 150 mCi) was trapped on an anion exchange resin and eluted with 0.5 mL aqueous potassium carbonate (0.02 M) into the reaction vial. Screw-cap test tubes used for drying fluoride and radiolabeling were purchased from Fisher Scientific (Pyrex No. 9825). C18 Sep-Pak cartridges were purchased from Waters Corporation (Milford, MA). For the TLC analyses, EM Science Silica Gel 60 F₂₅₄ TLC plates were

purchased from Fisher Scientific (Pittsburgh, PA). Radio-TLC was accomplished using a Bioscan 200 imaging scanner (Bioscan, Inc., Washington, DC). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA).

Synthesis of [^{18}F]3—Before the reaction, the radioactivity was dried under nitrogen gas and heated at 110 °C in the presence of potassium carbonate (2 mg) and Kryptofix-2,2,2 (6–7 mg). Precursor **13** (0.5–1 mg) was dissolved in DMSO (0.5 mL) and transferred into a screw-cap reaction vial containing pre-dried [^{18}F]fluoride. The reaction mixture was heated at 110 °C for 5 min. Upon completion, the reaction was quenched by adding 5 mL of water and pre-purified with solid phase extraction (C-18 Sep-Pak). The crude mixture was purified with a HPLC system with a mobile phase containing 50% acetonitrile and 50% 0.1 M formate buffer. The retention times for the precursor **13** and labeled compound [^{18}F]3 were 13 min and 18 min, respectively. The radiotracer was concentrated via solid phase extraction (C-18 Sep-Pak) and eluted with ethanol. Final samples were prepared in 10% ethanol/saline solution for subsequent studies. The decay-corrected yield was 10 – 20% and the specific activity was 1201 ± 895 mCi/ μmol decay corrected to EOB.

Synthesis of [^{18}F]5—Before the reaction, the radioactivity was dried under nitrogen gas and heated at 110 °C in the presence of potassium carbonate (2 mg) and Kryptofix-2,2,2 (6–7 mg). Precursor **14** (0.5–1 mg) was dissolved in DMSO (0.5 mL) and transferred into a screw-cap reaction vial containing pre-dried [^{18}F]Fluoride. The reaction mixture was heated at 110 °C for 10 min. Upon completion, the reaction was quenched by adding 5 mL of water and pre-purified with solid phase extraction (C-18 Sep-Pak). The crude mixture was purified with a HPLC system with a mobile phase containing 50% acetonitrile and 50% 0.1M formate buffer. The retention times for the precursor **14** and labeled compound [^{18}F]5 were 18 min and 25 min, respectively.. The radiotracer was concentrated via solid phase extraction (C-18 Sep-Pak) and eluted with ethanol. Final samples were prepared in 10% ethanol/saline solution for subsequent studies. The decay-corrected yield was ~50–70% and the specific activity was 764 ± 282 mCi/ μmol decay corrected to EOB.

2.3 In vitro Evaluation

2.3.1 Ligand Screening Using a Scintillation Proximity Assay—The ligand binding affinities were measured by utilizing a modification of the scintillation proximity assay (SPA) developed by Sun *et al.* [19]. Briefly, [^3H]rosiglitazone (American Radiolabeled Compounds, St. Louis, MO) with or without test ligand, and histidine-tagged PPAR γ ligand binding domain (His-tagged PPAR γ LBD; Invitrogen, Calsbad, CA), were incubated together for 2 h. Concurrently, copper-chelated and scintillant-coated SPA beads (GE Healthcare Bio-Sciences Corp. Piscataway, NJ) were diluted to appropriate concentrations based on the manufacturer's protocol, then aliquoted into a clear-bottom, white 96-well assay plate (Corning Inc, Corning, NY.). The plate was gently agitated to prevent bead aggregation. The [^3H]rosiglitazone and PPAR γ ligand binding domain/ligand mixture were then gently introduced to the SPA beads in the 96-well plate and incubated at room temperature for an additional 2 h. At the end of the incubation, the plate was read by a Packard TopCount microplate β -counter (Perkin-Elmer Inc., Waltham, MA). This data was exported into a Microsoft Excel file and the IC_{50} values were calculated with Prism GraphPad 4.0 (San Diego, CA), using a one-site competition function under the non-linear regression type analysis. The binding affinities are also expressed in relative binding affinity (RBA) due to the high variability of the assay. The definition of RBA is as follows: $\text{RBA} = (\text{IC}_{50} \text{ reference compound} / \text{IC}_{50} \text{ test compound}) \times 100$.

2.3.2 Ligand Screening Using a Whole-cell Functional Assay—The antagonistic properties of compounds **3 – 6** were confirmed by in vitro ligand binding experiments in a whole-cell system. This method employed a white-bottom 96-well tissue culture plate (Corning Inc, Corning, NY), Renilla luciferase for transfection control, and Lipofetamine (Invitrogen, Carlsbad, CA) as the transfection reagent. The amount of DNA used for transfection (per triplicate) was as follows: PPRE-TK-luciferase (1μg), pEF-BOS-PPAR γ ₂ (0.125μg), pMT-RXR (0.035μg), and Renilla luciferase (0.05μg). After the cells were transfected for 24 h, they were washed with PBS three times prior to the addition of the ligands. In each well, 250 μM of GW1929 was added to increase the basal PPAR γ expression. GW9662, an antagonist, was used in each plate as a reference compound. Test compounds, at a concentration of 2, 10, 20, 50, 100 and 200 nM, were added to corresponding wells for 24 h. If the compound behaved as an antagonist, it competed with GW1929 and inhibited the transcription of tyrosine kinase, hence firefly luciferase. The detection of firefly and Renilla luciferase was performed with Dual-Luciferase™ Reporter Assay (Promega Corporation, Madison WI) following the manufacturer's protocol. A 96-well plate reader, Chameleon Multilabel Microplate Reader (Bioscan, Washington DC), was used to measure the levels of luciferase in each well. The data was exported into a Microsoft Excel file and the IC₅₀ values were calculated with Prism GraphPad 4.0 (San Diego, CA), using a one-site competition function under the non-linear regression type analysis. The binding affinities are also expressed in relative binding affinity (RBA) due to the high variability of the assay. The definition of RBA is as followed: $RBA = (IC_{50} \text{ reference compound} / IC_{50} \text{ test compound}) \times 100$.

2.4. In vivo Evaluation

2.4.1. General—All animal experiments were conducted in compliance with the NIH Guidelines for the Care and Use of Research Animals under IACUC approved protocols reviewed by the Washington University Medical School Animal Studies Committee. PET imaging studies were performed with mature female MHC-PPAR γ transgenic mice and age-matched wild-type controls. The MHC-PPAR γ mice were a generous gift from Dr. Daniel Kelly. These transgenic mice overexpress PPAR γ 1 in the heart via the cardiac α -myosin heavy chain (α -MHC) promoter [20]. The tissue-specificity was confirmed with RT-PCR of RNA from fat, liver, heart, and muscle, using primer A: 5' end specific to α -MHC promoter exon 2 and 3' end specific to PPAR γ 1 cDNA nucleotides, when the mice were three months of age. The PPAR γ protein expression level in the tested transgenic mice was 2-fold higher than that in the controls. In addition to the microPET imaging study, a biodistribution study was carried out in male Zucker fa/fa rats. Oana et. al. examined the levels of PPAR expression of obese (fa/fa) and lean Zucker rats and found high expressions of PPAR γ in both the white and brown adipose tissues in both species of rats [21]. The biodistribution study evaluated the Zucker fa/fa rats as another potential pre-clinical model for PPAR γ imaging agents..

2.4.2. microPET Imaging of Transgenic Mice—Mice were anesthetized with 1–2% isoflurane/oxygen mixture and were kept under anesthesia throughout the entire study. After the anesthetized mice were securely set in the supine position in a custom cradle and placed on the microPET scanner, the radiotracer was injected via the tail vein. Two mice were imaged side by side, at a single bed position. A fiducial marker was attached on the bed to facilitate the microPET/microCT coregistration. Each pair of wild-type control (C) and transgenic (Tg) mice were injected with radioactivity (120–150 μCi/100μL of [¹⁸F]**3** or [¹⁸F]**5**) and imaged using a 1 h dynamic scan acquisition. Four mice were imaged for each radiotracer.

All imaging studies were done with a Concord microPET-Focus-F120 or a microPET-Focus-F220 system (Siemens CTI Microsystems, Knoxville, TN USA) [22]. A scanning session was also done with a microCAT-II camera (Imtek Inc., Knoxville, TN) for image co-registration. This was accomplished by using a landmark registration technique and AMIRA image display software (AMIRA; TGS Inc., San Diego, CA).

For quantitative analysis, all volumes of interest (VOIs) were drawn manually by a single individual to minimize operator variability. A second set of VOIs were generated by a second independent individual to validate the VOIs. The drawings were based on visible anatomical structures such as non-target background organs (brain and liver) or tissues of interest (heart) using ASIPro software (Concorde, Knoxville, TN USA). VOIs from all images from the same group were averaged and presented as a single value. Blocking studies were conducted by treatment of the transgenic and control mice with GW9662 (10 mg/kg, i.p.) 15 min prior to the injection of radiotracer.

2.4.3. Biodistribution in Zucker Rats—Mature male obese Zucker (*fa/fa*) rats (Charles River Laboratory, Wilmington, MA), > 14 weeks of age and weighing 450–650 g, were used to evaluate the uptake of [¹⁸F]3. Due to the limited number of 14 week old Zucker (*fa/fa*) rats available and based on the results of the microPET imaging study, the 60 min post injection time point was selected for the biodistribution evaluation. Rats were anesthetized with 2–3% isoflurane/oxygen and approximately 30 μ Ci/100 μ L of [¹⁸F]3 was administered i.v. Rats (n=4) were again anesthetized and euthanized 60 min post injection. Samples of blood, lung, liver kidney, muscle, fat, brown fat, heart, brain, and bone were collected. All samples were counted on a Beckman Gamma 8000 well counter with a standard dilution of the injectate. Tissues were weighed and the percentage of the injected dose per gram of tissue (%ID/g) was calculated.

3. RESULTS

3.1 Chemistry

The synthesis of the target compounds is shown in Scheme 1. The 4-nitrobenzene derivatives, 2-(4-aminophenoxy)ethanol and 2-(4-aminophenyl)alcohol, were fluorinated with DAST to produce the corresponding fluorinated analogs, **7** and **8**. Catalytic hydrogenation using a high-pressure hydrogenator in the presence of Pd/C gave the corresponding amines **9** and **10**. A coupling method using triphosgene and diisopropylethylamine gave benzamides **3** and **4**. Amines **9** and **10** were also coupled with 2-methoxy-5-nitrobenzoic acid for the syntheses of the methoxy analogs **5** and **6**. The benzamides were purified with silica gel chromatography and their structures were confirmed by both ¹H-NMR and high resolution EI-MS prior to being screened for PPAR γ affinity. As shown in Scheme 2, benzamides **11** and **12** were synthesized from 2-(4-aminophenyl)alcohol and the corresponding benzoic acid, which were converted to the corresponding mesylate precursors for radiolabeling, **13** and **14**.

3.2. In vitro Ligand Screening

All compounds were screened for their PPAR γ binding affinities using both an SPA technique and a whole-cell assay. In this assay, the amount of radiolabeled compound ([³H]rosiglitazone) bound to the protein (histidine-tagged PPAR γ ligand binding domain) is directly proportional to the light emitted from the copper-chelated and scintillant-coated SPA beads. When a test ligand competes with the binding, the amount of emitted light is reduced relative to the blank and produces a weaker signal. In addition, only the histidine-tagged peptide bound to the SPA beads via the copper-chelate coating generate a signal; unbound peptide in the solution will not produce a light signal.

Compounds **3** – **6** all exhibited better IC₅₀ values (ranged from 30–50 nM, obtained from protein-only competitive assay) than the standard PPAR γ antagonist, GW9662. The IC₅₀ (145 nM) value for GW9662 from our scintillation proximity assay was lower than the published value for this compound (5 nM) [8]. This difference in IC₅₀ values can be attributed to the different protocols used in the binding assays. Screening was also conducted in a whole cell assay described previously [15] and confirmed that the compounds are antagonists. The IC₅₀ values from the whole cell assay for compounds **3** and **4** were similar to the values obtained from the SPA method. However, compounds **5** and **6** were found to be inactive in the whole cell assay (IC₅₀ > 2000 nM), which was an unexpected finding. These data suggest that **5** and **6** are not able to cross the cell membrane and inhibit intracellular PPAR γ . An alternative explanation is that the methoxy-containing analogs, which are expected to be reversible inhibitors of PPAR γ , are not able to compete as well as the irreversible inhibitors **3** and **4** in blocking PPAR γ agonist-induced luciferase activity in the whole cell assay. Screening for PPAR specificity was also done using a whole cell assay for measuring PPAR α activity. The results of this study indicated compounds **3** and **4** are inactive at PPAR α since their IC₅₀ values were >2,000 nM (data not shown).

Based on the results of the in vitro binding data, compound **3** was chosen for radiolabeling and in vivo evaluation. In addition, [¹⁸F]**5**, the methoxy-based analog of [¹⁸F]**3**, was synthesized and evaluated in vivo in order to compare the properties of radiotracers which bind irreversibly versus reversibly to PPAR γ .

3.3. Radiolabeling

Both conventional and microwave heating were attempted to facilitate the nucleophilic substitution of [¹⁸F]fluoride into precursors **13** and **14**. However, microwave irradiation did not yield the desired compounds, [¹⁸F]**3** and [¹⁸F]**5**. The optimal conditions for the radiolabeling procedure (Scheme 3) turned out to be thermal incorporation at 110 °C for 10 min. The radiolabeling yield of [¹⁸F]**5** was consistently higher (50–70%) than that observed for [¹⁸F]**3**. The specific activities were determined by analytical HPLC with the following results: [¹⁸F]**3**: 1201 ± 895 mCi/μmol; [¹⁸F]**5**: 764 ± 282 mCi/μmol.

3.4. MicroPET Study

MicroPET imaging studies were conducted in PPAR γ overexpressing transgenic mice (MHC-PPAR γ 1). These transgenic mice overexpress PPAR γ 1 in the heart via the cardiac α -myosin heavy chain (α -MHC) promoter [20]. The PPAR γ protein expression level in the transgenic mice is 2-fold higher when compared with the wild-type animals. Each pair of wild-type and transgenic mice had dynamic microPET scans for 60 min followed by a microCT scan for co-registration purposes. Scans were conducted under inhalation anesthesia using a 1–2% isoflurane/oxygen mixture. The data was analyzed with ASIPro software (Concorde, Knoxville, TN). A non-target reference organ, brain, was chosen for comparison with the uptake of each radiotracer in the heart.

Figure 2 summarizes the time activity curves (TACs) of [¹⁸F]**3**. The uptake of [¹⁸F]**3** in the heart of the transgenic animals was higher than that of the control (wild type) animals (Figure 2 A). A plot of the ratio of the uptake of [¹⁸F]**3** in transgenic (Tg) versus control (C) heart indicated a gradual increase over time reaching a plateau value of ~4 at 30 min post-i.v. injection of the radiotracer (120–150 μCi). There was no significant uptake or accumulation of [¹⁸F]**3** in brain of either transgenic or control mice which was expected since there is no expression of PPAR γ in the CNS. Pretreatment of the transgenic animals with GW9662 (10 mg/kg i.p. 15 min prior to injection of the radiotracer) reduced the uptake of [¹⁸F]**3** in heart significantly to levels similar to the heart uptake in control mice, which indicates that the uptake of [¹⁸F]**3** in transgenic heart is receptor specific (Figure 2 B).

Figure 2 C shows the uptake of [^{18}F]5 in transgenic and wild type mice. Although there was higher uptake of [^{18}F]5 in transgenic versus control heart, the Tg : C ratios of [^{18}F]5 were much lower than that of [^{18}F]3 (Tg : C ratio ~1.1). Furthermore, blocking studies with GW9662 had no effect on the Tg : C ratio [^{18}F]5, indicating nonspecific uptake of the radiotracer in the heart of the transgenic animals (Figure 2 D). It is of interest to note that compound 3 displayed nM affinity in both the SPA and whole cell binding assays, whereas compound 5 had nM affinity in the SPA assay and was inactive in the whole-cell assay for measuring PPAR γ activity. These data suggest that the in vivo behavior of the radiotracers was more consistent with the results of the whole cell assay than the SPA binding assay..

3.5. Biodistribution Study

The biodistribution of [^{18}F]3 was evaluated in 14 week-old male Zucker fa/fa rats 60 min post-injection. (Table 2) Although the absolute tracer accumulation of [^{18}F]3 in fat was low, levels in fat and brown fat were comparable which is consistent with the reported high expression of PPAR γ in both the white and brown adipose tissues of these rats [21]. Oana et al also reported low expression of PPAR γ in muscle. Muscle accumulation (0.012 ± 0.002 %ID/g) was lower than any other observed tissue, and the ratio of activity in fat: muscle at 60 min post-injection in the Zucker rats was ~2.9, which suggests that [^{18}F]3 was retained in adipose tissue and washed out of muscle. Liver activity (0.087 ± 0.104 %ID/g) represented less than 3% of the total injected dose. Bone accumulation was also modest (~10% of the total injected dose, calculations not shown), which suggests that metabolic defluorination was not significant. Because tracer accumulation was low in all organs including the target organ (fat/brown fat) of the Zucker fa/fa rats, other animal models may be more useful for future studies.

4. DISCUSSION

PPAR γ agonists have been widely used as therapeutic agents in treating patients with diabetes and other ailments. Despite the utility of its agonists, the roles this receptor plays in various diseases are complex and further investigation will require both agonist and antagonist ligands. A PPAR γ imaging agent would provide additional insight into the study of this receptor.

Earlier studies on the development of radiotracers for imaging this receptor focused on three PPAR γ agonists, SB213068, GI262570, and GW7845, which were radiolabeled with either carbon-11 or fluorine-18 [10–13]. Both [^{18}F]SB213068 and [^{11}C]GW7845 had moderate radiotracer uptake in the target tissue when injected into tumor bearing rodents. The co-administration of cold ligands with these two PET radiotracers did not hinder the radioactivity uptake of either radiotracer, thus failing to demonstrate the specificity. ^{18}F -labeled analogs of GI262570 (Farglitazar) [10, 11] exhibited good potential for PPAR γ imaging because of its high subtype specificity (PPAR γ /PPAR α : > 100). However, recent tissue distribution studies of two fluorine-18 analogs of GI262570 in normal female rats did not demonstrate increased tracer uptake in target tissues, and blocking studies showed little in vivo selectivity. Metabolic stability studies and the low fluoride incorporation into the bone suggested that the compounds were stable [14], and rapid metabolism was not responsible for the low uptake of radiotracers in the target tissue.

In a previous study, we reported the first radiolabeled *antagonist* of the PPAR γ receptor, [^{76}Br]2-bromo-5-nitro-*N*-phenyl-benzamide ([^{76}Br]1) [15]. However, the rapid debromination of [^{76}Br]1 in whole blood indicated that it was not a useful radiotracer for imaging PPAR γ in vivo. In contrast, [^{11}C]2, which has a chlorine atom in the benzamide ring versus a bromine atom in [^{76}Br]1, was found to have greater in vitro stability. After incubation in heparinized rat blood, [^{11}C]2 remained 52% and 30% intact at 5 and 30 min

respectively, while [^{76}Br]**1** was only 15% and 10% intact (unpublished data). However, the low radiochemical yield and poor effective specific activity prevented the *in vivo* evaluation of [^{11}C]**2**.

In the current study, we synthesized analogs of GW9662 in which the *para*-position of the aniline aromatic ring was substituted with either a 2-fluoroethyl (**3**) or 2-fluoroethoxy (**4**) group to facilitate radiolabeling with fluorine-18. Previous structure-activity relationship studies indicated that there was good bulk tolerance at the *para*-position of the aniline ring of GW9662. In addition, analogs **5** and **6** were made in which the 5-chloro group of GW9662 was also replaced with a methoxy group in order to prepare a radiotracer which is expected to bind reversibly to PPAR γ . Reversible binding kinetics is predicted for compounds **5** and **6** since the methoxy group is a poor leaving group and is not expected to react with the thiol group of Cys²⁸⁵ in the binding ligand binding domain of PPAR γ .

Two different receptor binding assays were used in measuring the affinity of our analogs of GW9662 for binding to PPAR γ . The first assay consisted of a scintillation proximity assay (SPA) method using histidine-tagged PPAR γ ligand binding domain. This assay measures the ability of a competing ligand to block the binding of [^3H]rosiglitazone, a PPAR γ agonist to the ligand binding domain. In this regard, this method is a rapid throughput assay for measuring the affinity of a ligand for binding to PPAR γ . In this assay, all four analogs displayed an affinity for inhibiting [^3H]rosiglitazone binding to the PPAR γ ligand binding domain in the range of 30 – 50 nM. The second assay consisted of a whole cell assay in which three DNA constructs, PPRE-TK-luciferase, pEF-BOS-PPAR γ 2, and pMT-RXR were transiently transfected into HepG2 cells. This assay measures the ability of ligands to block the action of a PPAR γ agonist, GW1929, to induce the transcription of luciferase through its activation of the heterodimeric receptor complex, PPAR γ /RXR. The results of the whole cell binding assay indicate that compounds **3** and **4**, which are expected to be irreversible inhibitors of PPAR γ in a manner analogous to GW9662, have IC₅₀ values for inhibiting GW1929-mediated expression of firefly luciferase of 20 nM for **3** and 60 nM for **4**, which is similar to the results obtained from the SPA method. In contrast, the two methoxy-containing analogs **5** and **6** were found to be inactive in the whole cell assay, each having IC₅₀ values >2,000 nM. The reasons for this discrepancy between the SPA method and the whole cell assay are not clear and may be due to either the poor cell uptake of **5** and **6** into HepG2 cells, or the high k_{off} rate of a reversibly-binding ligand versus a ligand which irreversibly alkylates PPAR γ by forming a covalent bond with Cys²⁸⁵. It is of interest to note that the results of the microPET imaging studies in transgenic mice having an overexpression of PPAR γ in heart are consistent with results of the whole cell assay versus the SPA assay since [^{18}F]**3**, which had nM potency in the whole cell assay, labeled PPAR γ *in vivo* whereas [^{18}F]**5**, which was inactive in the whole cell assay, did not display specific binding to PPAR γ in the transgenic animals. The results of the microPET imaging studies indicate that [^{18}F]**3** is a potential radiotracer for imaging PPAR γ receptors *in vivo* with PET.

In summary, four putative ligands for imaging PPAR γ were synthesized and evaluated for *in vitro* binding affinity in a receptor binding and whole cell assay. Based on the results of the *in vitro* assays, two analogs were chosen for evaluation in a transgenic mouse model having an overexpression of PPAR γ in heart. Although additional studies are needed, the microPET imaging results indicate that the irreversible antagonist [^{18}F]**3** is a promising radioligand for imaging PPAR γ *in vivo*.

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Figure 1.
Structures of the PPAR γ antagonists GW9662, T007907, and PET radiotracers [⁷⁶Br]**1** and [¹¹C]**2**.

Figure 2.

MicroPET heart time activity curves (TACs) and Tg : control ratios for heart and brain of $[^{18}\text{F}]\mathbf{3}$ and $[^{18}\text{F}]\mathbf{5}$ from 60 min scans under isoflurane anesthesia after injection of 120–150 μCi of radiotracer in MHC-PPAR γ 1 and wild-type mice: (A) TACs for $[^{18}\text{F}]\mathbf{3}$; (B) Tg : C ratios of $[^{18}\text{F}]\mathbf{3}$; (A) TACs for $[^{18}\text{F}]\mathbf{5}$; (B) Tg : C ratios of $[^{18}\text{F}]\mathbf{5}$; (n=2 for each set). (B) and (D) also show Transgenic (Tg) : control (C) ratios for heart under no-carrier-added conditions and blocking studies with GW9662 (10 mg/kg, i.p.)

Scheme 1.

Reagents and Conditions: (a) DAST, CH_2Cl_2 , -80°C ; (b) Pd/C, H_2 , EtOH, rt; (c) 2-chloro-5-nitrobenzoic acid, CH_2Cl_2 , 0°C , triphosgene, DIEA; (d) 2-methoxy-5-nitrobenzoic acid, CH_2Cl_2 , 0°C , triphosgene, DIEA.

Scheme 2.

Reagents and Conditions: (a) 2-chloro-5-nitrobenzoic acid, CH₂Cl₂, 0 °C, triphosgene, DIEA; (b) 2-methoxy-5-nitrobenzoic acid, CH₂Cl₂, 0 °C, triphosgene, DIEA; (c) 2 MsCl, CH₂Cl₂, 4 °C.

Scheme 3.

Table 1

In vitro binding data from the SPA and whole cell assays.

Compound	IC ₅₀ SPA [nM]	IC ₅₀ Whole Cell Assay [nM]
3	30.1 ± 5.8	19.9 ± 6.6
4	47.5 ± 7.6	63.1 ± 21.6
5	46.6 ± 0.7	>2,000
6	53.6 ± 6.2	>2,000
GW9662	144.6 ± 7.5	169.0 ± 49.0

Table 2Biodistribution in male Zucker (*fa/fa*) rats (%ID/g).

Organ/Time point	60 min
Blood	0.029 ± 0.004
Lung	0.052 ± 0.010
Liver	0.087 ± 0.022
Kidney	0.277 ± 0.104
Muscle	0.012 ± 0.002
Fat	0.036 ± 0.025
Brown Fat	0.038 ± 0.017
Heart	0.015 ± 0.002
Brain	0.113 ± 0.009
Bone	0.166 ± 0.016