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Synthesis and biodistribution of fluorine-18 labeled fluorocyclofenils for imaging the estrogen receptor

Jai Woong Seo^a, Dae Yoon Chi^a, Carmen S. Dence^b, Michael J. Welch^b, and John A. Katzenellenbogen^{c,*}

a Department of Chemistry, Inha University, 253 Yonghyundong Namgu, Incheon 402-751, Korea

b Division of Radiological Sciences, Washington University School of Medicine, St. Louis, Missouri 63110, USA

c Department of Chemistry, University of Illinois, Urbana, IL 61801, USA

Abstract

C4-[¹⁸F]Fluorocyclofenil ([¹⁸F]FCF, **6**) and C3-[¹⁸F]fluoroethylcyclofenil ([¹⁸F]FECF, **9**), two high affinity non-steroidal estrogens, were prepared and investigated as potential agents for imaging estrogen receptors (ERs) in breast tumors. Both of these compounds could be prepared conveniently from alkyl methanesulfonate precursors (**5**, **8**) by fluoride displacement reactions, and they were obtained in high radiochemical purity and radiochemical yields, with effective specific activities sufficient for in vivo biodistribution studies. While the biodistribution of [¹⁸F]**6** in immature female rats showed no selective target tissue uptake, the biodistribution of [¹⁸F]**9** showed selective uptake by the uterus, but this uptake could not be blocked by excess estradiol. The poor in vivo biodistribution of these otherwise high affinity ligands is curious, and together with recent results on the biodistribution of other non-steroidal ligands, it suggests that factors other than receptor binding affinity are important for in vivo imaging of estrogen target tissues and ER-positive breast tumors.

Keywords

Cyclofenil; Estrogen receptor; Estrogen receptor imaging; Fluorine-18

1. Introduction

The development of estrogen derivatives for the diagnosis and treatment of breast cancer has been of interest for many years [1–3], and agents for imaging estrogen receptors (ERs) in breast tumors have also been investigated as a means for obtaining—non-invasively—information on the hormonal sensitivity of the cancer and the likelihood that it can be treated successfully by endocrine therapies [4–6]. A number of estradiol derivatives labeled with bromine [7,8], iodine [9], and fluorine [10–12] have been developed as tumor imaging agents for this purpose. While most of these agents have been based on steroidal estrogens, a few investigations were focused on non-steroidal estrogens or antiestrogens including selective estrogen receptor modulators [13–18].

*To whom correspondence should be addressed: Prof. John A. Katzenellenbogen Department of Chemistry, 461 Roger Adams Laboratory, Box 37-5, University of Illinois, 600 S. Mathews Avenue, Urbana, IL 61801. Telephone: +1-217-333-6310, FAX: +1-217-333-7325, e-mail: jkatzene@uiuc.edu

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The discovery that there are two ER subtypes, ER α and ER β , having different tissue distributions, different biological roles, and different activity levels [19,20], has further stimulated the search for ER ligands that might act selectively on these subtypes [20], and has led to the development of the ER α -selective pyrazole **PPT** [21], as well as other furan, and pyrrole derivatives [22], and the ER β -selective diarylpropionitrile **DPN** [23], as well as other cyclofenil, and tetrahydrochrysene derivatives (Figure 1) [24,25]. Also of interest is the development of ER ligands that might be effective in imaging the ERs in a subtype-selective manner [26,27]. This is important because ER α appears to play a pro-proliferative role and ER β an anti-proliferative role in a number of target tissues, including breast cancer [28].

Recently, we investigated the tissue distribution of a fluorine radiolabeled ligand selective for ER α , 4-(3-[18 F]fluoropropyl)-1,3,5-tris-4-(hydroxyphenyl)-1*H*-pyrazole ([18 F]**FPPT**) [26]. Although this compound showed high binding affinity and selectivity for ER α (K_i = 0.4 nM with a 320 fold preference over ER β) and high potency in cell-based assays of ER α transcriptional activity, its uptake in ER α -rich target tissues was minimal [26]. Likewise, a fluorine radiolabeled ligand selective for ER β , 2,3-bis-(4-hydroxyphenyl)-5-[18 F]fluoropentanitrile ([18 F]**FEDPN**), failed to show selective uptake into ER β target tissues such as the ovary, although its ER β binding affinity was more modest than that of estradiol [27].

In our attempts to obtain ER ligands that would be effective and selective for subtype-specific imaging of ER α or ER β using positron emission tomography (PET), we were intrigued by the non-steroidal ER ligand cyclofenil (**1**). This simple molecule binds to ER α and ER β with very high affinity and some ER β selectivity (Figure 1; Table 1) [25], and because of its mixed estrogen agonist/antagonist activity, it has been used as a pharmaceutical agent in humans for ovulation induction properties [29–31]. Moreover, cyclofenil derivatives that have the 1,1-diarylethylene motif connected to a bicyclic or tricyclic core showed 3–5 times higher binding affinities than that of estradiol to both ER subtypes [25,32]. Thus, the cyclofenil-type ligands appeared to be promising starting points for the development of fluorine-18 ligands for PET imaging of ERs.

Recently, we published an extensive structure-activity study of cyclofenil analogues to establish the tolerance of the ERs for cyclofenil ligands having substitution of alkyl, hydroxy, fluoro, and hydroxyalkyl and fluoroalkyl groups on the cyclohexane portion of cyclofenil [32]. Although polar substituents (-OH and -F) caused considerable decrease in binding affinity, we found that fluorine was best tolerated as a direct C4 substituent or as a C3 fluoroethyl group [32]. The structures and relative binding affinities (RBAs) of these fluorocyclofenil analogs to ER α and ER β are given in Figure 1 and Table 1. Although these compounds did not have high ER subtype selectivity, their RBA values were sufficiently high to be of further interest. Herein, we report the syntheses of these two cyclofenil analogues in fluorine-18 labeled form and studies of their biodistribution in immature female rats to evaluate their potential as PET tracers for imaging breast cancer.

2. Experimental

2.1. Materials and methods

Anhydrous THF, Et₂O and CH₂Cl₂ were collected using a solvent dispensing system built by J. C. Meyer based on a design developed by Pangborn et al. [34]. The microwave cavity (model 520A) was obtained from Resonance Instruments Inc. Skokie, IL. ACS grade solvents (Fisher) were used for HPLC purification and analysis. Reaction progress was followed by 0.25 mm silica gel glass TLC plates containing F-254 indicator (Merck). Visualization on TLC was monitored by UV light or phosphomolybdic acid indicator. Radio TLC yield was checked by Bioscan AR-2000 scanner. The chemical shifts were reported in parts per million downfield from the internal tetramethylsilane. Coupling constants were reported in Hertz. 1 H NMR

spectra were obtained on 400 or 500 MHz spectrometers. ^{13}C NMR spectra were acquired at 100 or 125 MHz. Low- and high-resolution electron impact (EI, 70 eV) mass spectra were obtained on a Micromass 70-VSE spectrometer. Some reactions were performed under nitrogen condition if special conditions were not referred. The synthesis of the phenol-protected alcohol precursors **4** and **9** has been described by us previously [32]. Log P values were calculated using ChemDraw (CambridgeSoft Corporation, Cambridge, MA).

2.1.1.4-Methanesulfonyloxy[bis(4-methoxymethoxyphenyl)methylene]

cyclohexane 5—Two-necked round-bottomed flask which contained the phenol-protected alcohol precursor **4** [32] (300 mg, 0.78 mmol) was charged with nitrogen. Freshly distilled dichloromethane (5.0 mL) was added, and reaction was stirred under ice-bath. Methanesulfonic anhydride (204 mg, 1.2 mmol) was added in several portions, followed by the slow addition of triethylamine (240 mg, 2.4 mmol). The reaction mixture was stirred at room temperature about 1 h and quenched by water (1.0 mL) with vigorous stirring. The organic material was extracted with additional dichloromethane (20 mL), which was dried over magnesium sulfate and concentrated under reduced pressure. Flash column chromatography (ethyl acetate/hexane; 3:7) gave **8** (300 mg, 83%) as a white solid: ^1H NMR (500 MHz, CDCl_3) δ 6.93 (d, J = 8.8 Hz, 4H), 6.77 (d, J = 8.8 Hz, 4H), 5.15 (s, 4H), 4.92 (septet, J = 4.0 Hz, 1H), 3.48 (s, 6H), 3.02 (s, 3H), 2.52–2.60 (m, 2H), 2.20 (ddd, J = 14.0, 9.5, 4.0 Hz, 2H), 1.98–2.06 (m, 2H), 1.80–1.88 (m, 4H); ^{13}C NMR (125 MHz, CDCl_3) δ 155.8, 136.2, 135.9, 134.1, 130.6, 115.7, 94.4, 80.3, 56.0, 38.8, 33.6, 28.0; MS (ESI) m/z 463 ($\text{M}^+\text{+H}$, 100). HRMS (EI) m/z calcd for $\text{C}_{24}\text{H}_{30}\text{O}_7\text{S}$ 462.1712, found 462.1705.

2.1.2. 3-(2-Methanesulfonyloxyethyl)[bis(4-methoxymethoxyphenyl)methylene]

cyclohexane 8—Compound **8** was prepared by the same method as compound **5**. Reaction with phenol-protected alcohol **7** [32] (349 mg, 0.82 mmol), methanesulfonic anhydride (215 mg, 1.2 mmol), triethylamine (331 mg, 2.5 mmol) and dichloromethane (10.0 mL) gave **8** (393 mg, 98%) as a clear oil: ^1H NMR (400 MHz, CDCl_3) δ 7.002 (d, J = 8.8 Hz, 2H), 6.999 (d, J = 8.8 Hz, 2H), 6.94 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 5.154 (s, 2H), 5.153 (s, 2H), 4.21–4.23 (m, 2H), 3.479 (s, 3H), 3.477 (s, 3H), 2.88 (s, 3H), 2.60 (bd, J = 10.0 Hz, 1H), 2.53 (bd, J = 13.6 Hz, 1H), 1.75–1.97 (m, 3H), 1.60–1.74 (m, 4H), 1.34–1.47 (m, 1H), 1.12–1.26 (m, 1H); ^{13}C NMR (100 MHz, CDCl_3) δ 155.62, 155.58, 136.7, 136.56, 136.51, 134.5, 130.77, 130.75, 115.7, 115.6, 94.4, 68.1, 56.03, 56.02, 38.0, 37.0, 35.6, 35.5, 32.7, 32.0, 27.0; MS (EI) m/z 490 (M^+ , 100), 211, 141. HRMS (EI) m/z calcd for $\text{C}_{26}\text{H}_{34}\text{O}_7\text{S}$ 490.2025, found 490.2015.

2.2. [^{18}F]Radiolabeling

2.2.1. 4-[^{18}F]Fluoro[bis(4-hydroxyphenyl)methylene]cyclohexane ([^{18}F]FCF, **6)**—Fluorine-18 was obtained from the $^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ reaction on an enriched water target, as previously reported [35]. Radioactivity (85 mCi) of [^{18}F]fluoride was transferred to a Vacutainer[®] which contained Kryptofix-2.2.2[®] (6.8 mg, 18 mol) and K_2CO_3 (1.5 mg, 11 mol). The water was evaporated azeotropically with anhydrous acetonitrile (1.0 mL $\times$ 3) at 100 $^\circ\text{C}$ with a gentle nitrogen gas purge. After the reaction vessel was cooled to room temperature under a continuous flow of argon gas, the activity was dissolved in acetonitrile (300 L) and transferred to a test tube which contained precursor **5** (1.5 mg, 3.2 mol) and a glass bead. The test tube was capped and placed in a microwave reactor. Microwave irradiation (100 W) was performed for 30 sec, at which time the reaction was allowed to cool for 1 min. Irradiation was repeated a total of three times, and the reaction was monitored by radio TLC. The reaction mixture was passed through a silica gel filter (50 mg) and collected. Residual activity in test tube was removed by washing with additional acetonitrile (300 L), and this solution was passed through silica filter. Silica pad was washed again with 300 L of acetonitrile. To remove the MOM (methoxymethyl-) protective groups, 5.0 M HCl (150 L) was added to the combined acetonitrile solution, and the reaction was heated at 100 $^\circ\text{C}$ for 15 min. The solution was

neutralized by 8.4% NaHCO₃ (500 L). The reaction mixture (total volume ~ 1.5 mL) was purified by HPLC purification (column: Alltech altima C18, 250 × 10 mm, 10m, 43% acetonitrile and 57% H₂O, flow rate: 4 mL/min, retention time of radioactivity: 26.0 min). The radiochemical purity of **6** was >95% when rechecked by HPLC (Alltech Altima C18 column; 53 × 7 mm, 45% CH₃CN/55% H₂O, flow rate: 1.0 mL/min). Radiochemical yield (decay corrected EOS) was 42%, and the effective specific activity (determined by comparative radiometric binding assay [36]) was 6,500 Ci/mmol (2.4×10^{14} Bq/mmol).

2.2.2. 3-(2-[¹⁸F]Fluoroethyl)[bis(4-hydroxyphenyl)methylene]cyclohexane ([¹⁸F]FECF, **9)**—Fluorine-18 was obtained by the same method as described in the procedure for the preparation of [¹⁸F]FCF. Radioactivity (35 mCi) was transferred to a Vacutainer® that contained Kryptofix-2.2.2® (6.8 mg, 18 mol) and K₂CO₃ (2.1 mg, 15 mol). H₂O was evaporated azeotropically with acetonitrile (1.0 mL × 3) at 100 °C under a gentle stream of argon gas. After the reaction vessel was cooled to room temperature under a continuous flow of nitrogen gas, the activity was dissolved in acetonitrile (300 L) and transferred to test tube that contained precursor **8** (0.40 mg, 0.82 mol) and a glass bead. The test tube was capped and placed in a microwave reactor. Microwave irradiation (200–250 W) was twice for 20 sec. Reaction was monitored by radio TLC. After addition of 5.0 M HCl (300 L) and capping the test tube tightly, the reaction was heated at 100 °C for 15 min. The cooled solution was neutralized by 8.4% NaHCO₃ solution (500 L), and the reaction mixture was purified by HPLC (column: Alltech altima C18, 250 × 10 mm, 10 L, 50% acetonitrile and 50% H₂O, flow rate: 4 mL/min, retention time of radioactivity: 34.1 min). The radiochemical purity of **9** was 99% when it rechecked by HPLC (Alltech Altima C18 column; 53 × 7 mm, 45% CH₃CN/55% H₂O, flow rate: 2.0 mL/min). Total radiochemical yield (decay corrected EOS) was 39%. The specific activity (determined by comparative radiometric binding assay [36]) was 26 Ci/mmol (9.5×10^{11} Bq/mmol).

2.3. In vivo biodistribution

2.3.1. Biodistribution of 4-[¹⁸F]fluorocyclofenil ([¹⁸F]FCF, **6)**—Animal biodistribution studies were performed at Washington University Medical School under Institutional Animal Care and Use Committee protocols approved at that institution. Animal handling techniques and protocols have been previously described [37]. The activity (241 Ci) of [¹⁸F]FCF was dissolved in 233 L of ethanol and then finally diluted with 1.4 mL of saline (15% ethanol/saline solution). The blocking agent was prepared with 200 Ci of [¹⁸F]FCF and 65 g of estradiol in 65 L of ethanol and diluted up to 1.3 mL with saline (15% ethanol/saline solution). Immature female Sprague-Dawley rats (21 days) used for biodistribution studies weighed from 37 g to 50 g, and radioactivity was injected via tail vein under isoflurane anesthesia. Three sets of animals (n = 4 or 5 per set) were injected with 15 Ci of [¹⁸F]FCF in 100 L volume and sacrificed at the noted time points (1 h, 1 h – blocked study, and 2 h). Target tissue organs (uterus and ovaries) and non-target tissue organs (blood, lung, liver, spleen, kidney, muscle, bone, and thymus) were removed and weighted. Radioactivity was counted in a Beckman Gamma 8000 Well counter, and activities in each tissue were calculated as %ID per gram (Table 3 and 4). For each animal group, the data was averaged and standard deviation calculated.

2.3.2. Biodistribution of 3-[¹⁸F]fluoroethylcyclofenil ([¹⁸F]FECF, **9)**—[¹⁸F]FECF was also prepared by the same procedure as [¹⁸F]FCF except for the activity levels and numbers of animals used. The activity (530 Ci) of [¹⁸F]FECF was diluted with 2.6 mL of saline (15% ethanol/saline solution). The blocking agent was prepared with 435 Ci of [¹⁸F]FECF and 115 g of estradiol in 2.3 mL of saline (15% ethanol/saline solution). Immature female Sprague-Dawley rats (21 days) used for biodistribution studies weighed from 45 g to 55 g. Three sets of animals (n = 4 per set) were injected with 20 Ci of [¹⁸F]FECF in 100 L of solvent.

3. Results

3.1 Synthesis of methanesulfonate precursors (**5** and **8**) for the preparation of the fluorine-substituted cyclofenil derivatives (**6** and **9**)

In our earlier studies directed at optimizing the binding affinity of estrogen receptor ligands, we worked out a convenient approach to various bis(dihydroxyphenyl)methylenebicyclic [25] and cyclofenil derivatives [32], using a McMurry coupling reaction of dihydroxybenzophenone with various commercially available ketones [32]. Thus, to prepare the ^{18}F ($t_{1/2} = 109.7$ min) labeled cyclofenil derivatives, we first synthesized the hydroxyl or hydroxy-alkyl cyclofenil derivatives (**4** and **7**), as previously described [32], and from them prepared the methanesulfonates **5** and **8**, as precursors for fluorine-18 labeling. For these final conversions, the methoxymethyl (MOM) ether proved to be a convenient protecting group for the phenol, because it could be easily removed with acid at the end of the radiochemical synthesis. In fact, the MOM group can sometimes be removed under mild acidic conditions without the need for additional purification of fluorinated intermediate.

The MOM-protected methanesulfonate precursors **5** and **8** were synthesized in quantitative yield from the known primary and secondary hydroxyl compounds **4** and **7** [32] in 83% and 98% yields, respectively, by reaction with methanesulfonic anhydride at room temperature for 1 h (Scheme 1).

3.2 Synthesis of F-18 labeled 4-fluorocyclofenil (FCF, **6**) and 3-fluoroethylcyclofenil (FECF, **9**)

^{18}F Fluorination to produce the target compounds, ^{18}F FCF **6** and ^{18}F FECF **9**, was effected by reaction of the protected methanesulfonates **5** and **8** with ^{18}F fluoride ion, followed by acid exposure. ^{18}F Fluoride ion was converted to the soluble potassium salt with a mixture of potassium carbonate and Kryptofix[®] (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8] hexacosane) and was dried by azeotropic distillation with acetonitrile. The fluoride substitution reaction was effected by microwave heating to reduce the total synthesis time. Under these conditions, the primary mesylate precursor **8** was almost quantitatively converted to methoxymethyl protected C3- ^{18}F fluoroethylcyclofenil (**9**) (89% radiochemical yield, determined by TLC analysis; Table 2). The methoxymethyl protected C4- ^{18}F fluorocyclofenil (**6**), however, was obtained only in lower yield (29% by TLC; Table 2), because the secondary mesylate, directly attached at cyclohexane ring, experiences greater steric crowding and is more sluggish in substitution reactions.

Methoxymethyl protected ^{18}F fluorocyclofenil was purified simply by passage through a short silica gel plug into a vessel where the methoxymethyl protecting groups were removed under acidic conditions at 100 °C, giving C4- ^{18}F fluorocyclofenil (^{18}F FCF, **6**). Purification of ^{18}F FCF was performed by HPLC after neutralization with sodium bicarbonate solution. In case of methoxymethyl protected C3- ^{18}F fluoroethylcyclofenil (^{18}F FECF, **9**), purification of the fluorinated methoxymethyl intermediate was skipped, and deprotection was effected by direct addition of aqueous hydrogen chloride (5.0 M, 0.5 mL) to reaction mixture. This expedited procedure was also applied successfully to the production of ^{18}F FCF.

Fluorine-18 labeled ^{18}F FCF was obtained in a decay-corrected radiochemical yield of 41% and ^{18}F FECF was obtained in 50% yield, in both cases after purification with by HPLC. Total synthesis time was less than 2 h, and radiochemical purities of both labeled fluorocyclofenil derivatives, determined by reversed phase HPLC, were greater than 95%. Curiously, the effective specific activities of ^{18}F FCF and ^{18}F FECF, determined by measuring receptor competing activity in a sample of known activity after complete decay [36], were very different: It was 6,500 Ci/mmol for ^{18}F FCF but only 26 Ci/mmol for ^{18}F FECF. We believe that the

reduced effective specific activity of [^{18}F]FECF was the result of coelution of small amounts of the corresponding elimination product, namely 3-vinyl-cyclofenil, that forms during the fluoride substitution and is difficult to separate from the desired product. Nevertheless, as described below, we do not believe that the low specific activity of [^{18}F]FECF has interfered with the tissue distribution studies.

3.3. In vivo tissue biodistribution

Purified C4-[^{18}F]fluorocyclofenil ([^{18}F]FCF, **6**) and C3-[^{18}F]fluoroethylcyclofenil ([^{18}F]FECF, **9**) were dissolved in 15% ethanolic saline and injected (iv, tail vein) into immature female Sprague Dawley rats (21 days old, $\approx 35\text{--}53\text{ g}$). Doses employed were 15 Ci/animal for [^{18}F]FCF and 20 Ci/animal for [^{18}F]FECF, and the tissue biodistribution of fluorine-18 radioactivity was obtained at 1 h and 2 h after injection (Table 3 and 4). A blocking study was performed with one set of animals by coadministration of 5 g of unlabeled estradiol with the radiopharmaceutical to fully occupy the estrogen receptor with estradiol at the fluorine-18 radiolabeled doses used in these experiments (Table 3 and 4, 1 h block).

Overall, the tissue distribution of both compounds is unremarkable. Low bone activity indicates that metabolic defluorination is not occurring, and low liver and kidney activity levels suggest that the clearance of both compounds is rapid and clean. Curiously, despite its high radiochemical purity ($>95\%$) and very high effective specific activity (6,500 Ci/mmol), the 4-fluorocyclofenil compound, [^{18}F]FCF (**6**), did not show evidence of selective uptake into ER-rich target tissues (uterus and ovary) nor selective uptake blockade in these tissues by coadministration of an excess of unlabeled estradiol (Table 3). The uterus to blood ratios were less than one, and the uterus to muscle ratios never exceeded 1.6; neither value was affected by the estradiol blocking experiment. Perhaps this poor target tissue uptake is a consequence of the modest affinity of this compound for ER α (Table 1), the predominant ER subtype in the uterus. The other, higher affinity fluorine-18 labeled cyclofenil, [^{18}F]FECF (**9**), which is labeled at the end of the 3-ethyl substituent, did exhibit some evidence of selective uptake in the uterus and ovaries; with this compound, the uterus to blood and uterus to muscle ratios were ca. 4–5 at 1 h (Table 4). Curiously, however, coinjection of an excess of unlabeled estradiol was not effective in blocking the target tissue uptake of [^{18}F]FECF (**9**).

While the effective specific activity of [^{18}F]FECF (**9**) (25.7 Ci/mmol) is much lower than that of [^{18}F]FCF (**6**) (6,500 Ci/mmol), we do not believe this difference can account for the limited receptor-mediated uptake of the former compound, based on the following calculations: The 1-hour uterine uptake of [^{18}F]FECF (**9**) is ca. 1% ID/g, which, based on an injected dose of 20 Ci, corresponds to 0.20 Ci/g uterus. At an effective specific activity of 25.7 Ci/mmol (i.e., 25.7 Ci/nmol), this represents 7.8 pmol/g of compound in the uterus. Because the uterus of an immature rat typically weighs ca. 30 mg and contains ca. 1.5 pmol ER, all of which is unoccupied by ligand, uterine ER content of unoccupied receptor is ca. 50 pmol/g. Thus, by this calculation, the administered dose of [^{18}F]FECF (**9**) would occupy only ca. 15% of the available ER sites.

Also relevant to considerations of maximum capacity for uterine uptake are the results from an earlier study [38] in which we did a progressive titration of the uterine uptake of an effective ER imaging agent, 16 α -[^{18}F]fluoroestradiol (FES), in immature rats in vivo. In that study we found that FES uptake saturated at an even higher value, 180 pmol/g uterus. Thus, based on these calculations and our past observations, we believe that the lack of ER-specific uptake of [^{18}F]FECF (**9**) must be accounted for by some unsatisfactory pharmacokinetic or pharmacodynamic behavior of the compound, rather than the low effective specific activity of the material we were able to produce for the tissue uptake study (see Discussion).

4. Discussion

C4-[¹⁸F]Fluorocyclofenil ([¹⁸F]FCF, **6**) and C3-[¹⁸F]fluoroethylcyclofenil ([¹⁸F]FECF, **9**), two high affinity non-steroidal ligands for the estrogen receptor, were synthesized in an efficient manner from mesylate precursors in which the phenols were protected as MOM ethers using microwave-assisted heating. Because the secondary cyclohexylmesylate **5** was less reactive than the primary mesylate **8**, [¹⁸F]fluorination of the former required somewhat more vigorous conditions and proceeded in lower radiochemical yield. The desired compounds, [¹⁸F]**6** and [¹⁸F]**9**, were obtained after phenol deprotection in good radiochemical purity and at adequate effective specific activities, although the effective specific activity of [¹⁸F]**9** is lower than that of [¹⁸F]**6**, most likely because of coelution of the corresponding elimination product, 3-vinyl-cyclofenil, during HPLC purification.

In biodistribution studies of [¹⁸F]**6** and [¹⁸F]**9** performed in immature female rats, the 4-fluoro compound, [¹⁸F]**6**, despite its high effective specific activity, showed no evidence of target tissue-selective uptake or blocking by excess unlabeled estradiol. Curiously, even though the 3-fluoroethyl-cyclofenil compound ([¹⁸F]**9**) had lower effective specific activity, it showed one of the hallmarks of receptor-mediated uptake, namely elevated uterus to blood and uterus to muscle ratios. However, the fact that uterine uptake could not be blocked by excess estradiol indicates that it is not receptor mediated.

It is not clear why these two high affinity ligands for the estrogen receptor fail to show receptor mediated uptake into the uterus. Their binding affinities for the estrogen receptors, ER α and ER β , are of the same order of many other fluorine-18 labeled estrogens that do show ER-mediated uptake in the uterus of immature female rats [10,12]. Their in vivo tissue distribution also did not reveal any obvious unusual pharmacokinetic or metabolism liabilities. Their level of non-specific binding, which generally tracks with compound lipophilicity (as characterized by cLogP values [1]: estradiol (3.8), cyclofenil (5.1), FCF (4.5), FECF (5.4)), is only somewhat greater than that of other ER ligands that show good target tissue selective uptake, such as 16 α -[⁷⁷Br]bromoestradiol (4.3) [7] or 1-[⁷⁷Br]bromopentestrol (5.1) [39].

The most distinguishing feature of these compounds is that they are non-steroidal, whereas most fluorine-18 labeled estrogens studied that have shown clear evidence of ER-mediated uterine uptake have been steroidal [10,12]. Perhaps by following more closely the structure of “nature’s preferred ligand” – namely, a steroid – one can obtain compounds with pharmacokinetic and pharmacodynamic properties that are better suited for selective receptor-mediated uptake and retention, as are required for imaging steroid receptors with PET.

In other studies on the development of agents for imaging estrogen and androgen receptors by PET, we hypothesized that good binding affinity for the plasma steroid carrying protein, sex hormone binding globulin (SHBG), might be a prerequisite for good target tissue uptake and retention [40]. In fact, SHBG has been implicated as a carrier that will facilitate steroid uptake by certain target cells through a membrane receptor for steroid-liganded SHBG [41–43]. SHBG has a strong preference for binding certain androgen and estrogen steroidal ligands; by contrast, non-steroidal ligands, including members of the cyclofenil class, typically have very low SHBG binding affinity. The low binding affinity of FCF and FECF for SHBG is not an adequate explanation for their low uterine uptake in rats, however, because although SHBG is found in primates and certain other species, such as rabbits, it is not found at appreciable levels in immature rat serum. Also, in earlier work, we and others did study the binding and biodistribution of a number of radiofluorine, bromine, and iodine-substituted non-steroidal ligands, those in the hexestrol, norhexestrol, oxohexestrol and 1,1-diarylethylene classes, and these compounds; all of these compounds have low affinity for SHBG, yet they showed clear evidence of ER-mediated uptake in estrogen target tissues [13,39,44]. On the other hand, as

noted in the Introduction, more recently we found that other non-steroidal ER ligands, namely the ER α -selective pyrazole [^{18}F]FPPT [26] and the ER β -selective nitrile [^{18}F]FEDPN [27], showed little if any selective uptake in estrogen target tissues, although it is probably significant that these latter two agents deviated more markedly in structure from that of the more common hexestrol and aryethylene-type non-steroidal estrogens and also that FEDPN has relatively low ER binding affinity.

Thus, we are left without an adequate explanation for the poor target tissue uptake characteristics of these two, high affinity fluorocyclofenil ligands, FCF (**6**) and FECF (**9**), for the estrogen receptor beyond saying that the in vivo behavior of drug-like molecules, such as these, can be very idiosyncratic and that factors other than receptor binding affinity are important for in vivo imaging of estrogen target tissues and ER-positive breast tumors with PET tracers.

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Figure 1.
Cyclofenil and fluorinated analogues

Scheme 1.

Synthesis of C4-[^{18}F]fluorocyclofenil ([^{18}F]FCF, **6**) and C3-[^{18}F]fluoroethylcyclofenil ([^{18}F]FECF, **9**)

Table 1

Binding affinity of cyclofenil (**1**), 4-fluorocyclofenil (FCF, **2**) and 3-fluoroethylcyclofenil (FECF, **3**) to ER α and ER β

Compd. No.	RBA ^a		β/α ratio
	ER α	ER β	
1 (cyclofenil)	120	290	2.4
2 (FCF) [¹⁸ F]FCF 6	27	62	2.3
3 (FECF) [¹⁸ F]FECF 9	120	130	1.1

^aRelative Binding Affinity (RBA) where estradiol is 100%. The K_d value of estradiol is 0.2 nM for ER α and 0.5 nM for ER β . Values represent the average of multiple determinations. Competitive radiometric binding assays were done with purified full length human ER α and ER β (PanVera/Invitrogen Inc), using 10 nM [³H]estradiol as tracer, as previously described [33].

Table 2Conditions and yields of [^{18}F] fluorinations of methanesulfonates 5 and 8

reactant	time	radio TLC yield
5	30 sec $\times$ 3	29 ± 2.0 % (n = 3)
8	20 sec $\times$ 2	89 ± 4.0 % (n = 2)

Table 3Biodistribution of 4- ^{18}F fluorocyclofenil (^{18}F FCF, **6**) in immature SD female rats (%ID/gram)

Tissue	%ID/g		
	1 h control	1 h block	2 h control
Uterus	0.335 $\pm$ 0.235	0.271 $\pm$ 0.217	0.388 $\pm$ 0.266
Ovaries	0.279 $\pm$ 0.298	0.307 $\pm$ 0.198	0.397 $\pm$ 0.242
Blood	0.456 $\pm$ 0.366	0.461 $\pm$ 0.376	0.899 $\pm$ 0.546
Lung	0.372 $\pm$ 0.250	0.381 $\pm$ 0.271	0.536 $\pm$ 0.295
Liver	0.863 $\pm$ 0.524	0.930 $\pm$ 0.697	1.191 $\pm$ 0.270
Spleen	0.118 $\pm$ 0.112	0.205 $\pm$ 0.144	0.278 $\pm$ 0.127
Kidney	0.390 $\pm$ 0.375	0.612 $\pm$ 0.445	0.958 $\pm$ 0.515
Muscle	0.239 $\pm$ 0.151	0.212 $\pm$ 0.145	0.207 $\pm$ 0.074
Fat	0.371 $\pm$ 0.364	0.323 $\pm$ 0.232	0.258 $\pm$ 0.185
Heart	0.249 $\pm$ 0.181	0.245 $\pm$ 0.181	0.346 $\pm$ 0.152
Bone	0.541 $\pm$ 0.332	0.535 $\pm$ 0.316	0.664 $\pm$ 0.210
Thymus	0.275 $\pm$ 0.207	0.220 $\pm$ 0.150	0.147 $\pm$ 0.097
Uterus/blood	0.780 $\pm$ 0.112	0.612 $\pm$ 0.143	0.440 $\pm$ 0.140
Uterus/muscle	1.371 $\pm$ 0.191	1.241 $\pm$ 0.279	1.540 $\pm$ 0.294

Table 4Biodistribution of 3- ^{18}F fluoroethylcyclofenil (^{18}F FECF, **9**) in immature SD female rats (%ID/gram)

Tissue	%ID/g		
	1 h control	1 h block	2 h control
Uterus	0.964 $\pm$ 0.108	0.907 $\pm$ 0.157	0.427 $\pm$ 0.108
Ovaries	0.496 $\pm$ 0.070	0.465 $\pm$ 0.031	0.209 $\pm$ 0.031
Blood	0.219 $\pm$ 0.108	0.168 $\pm$ 0.012	0.159 $\pm$ 0.037
Lung	0.350 $\pm$ 0.084	0.335 $\pm$ 0.046	0.148 $\pm$ 0.038
Liver	1.031 $\pm$ 0.421	0.789 $\pm$ 0.037	0.895 $\pm$ 0.343
Spleen	0.203 $\pm$ 0.039	0.166 $\pm$ 0.021	0.103 $\pm$ 0.032
Kidney	0.459 $\pm$ 0.146	0.400 $\pm$ 0.036	0.293 $\pm$ 0.111
Muscle	0.253 $\pm$ 0.045	0.273 $\pm$ 0.045	0.077 $\pm$ 0.026
Fat	0.597 $\pm$ 0.044	0.561 $\pm$ 0.074	0.149 $\pm$ 0.042
Heart	0.242 $\pm$ 0.063	0.232 $\pm$ 0.030	0.114 $\pm$ 0.028
Bone	0.367 $\pm$ 0.064	0.361 $\pm$ 0.057	0.291 $\pm$ 0.028
Thymus	0.294 $\pm$ 0.035	0.285 $\pm$ 0.045	0.100 $\pm$ 0.026
Uterus/blood	4.949 $\pm$ 1.310	5.402 $\pm$ 0.643	2.710 $\pm$ 0.427
Uterus/muscle	3.861 $\pm$ 0.409	3.338 $\pm$ 0.340	5.769 $\pm$ 1.337