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ORIGINAL PAPER

Design, synthesis, and evaluation of cyclofenil derivatives for potential SPECT imaging agents

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Abstract To develop technetium- and rhenium-labeled nonsteroidal estrogen imaging agents for estrogen receptor (ER) positive breast tumors, two groups of rhenium and technetium cyclofenil derivatives were synthesized and characterized. The binding affinities of the rhenium complexes for ERs were determined. The tricarbonyl rhenium complex showed the highest binding affinity for ERs (81.2 for ERβ, 16.5 for ERα). Tricarbonyl technetium cyclofenil complexes were obtained in high radiochemical purity and radiochemical yields. The results of studies of their octanol/water partition and in vitro stability are presented. These results demonstrate that these radiolabeled cyclofenil derivatives may be considered as potential breast cancer imaging agents.

Keywords Cyclofenil · Tricarbonyl rhenium · Radiolabeling · Nonsteroidal · Estrogen receptor

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Introduction

Estrogen receptor (ER) is an attractive target in the treatment of breast cancer, in which it has two structurally similar subtypes ERα and ERβ with different biological properties [1, 2]. It is notable that the level of ERβ relative to that of ERα declines with breast cancer progression [3–6]. A number of estradiol derivatives labeled with bromine, iodine, fluorine, rhenium, and technetium have been developed as tumor imaging agents [7–16]. Although most of these agents are based on steroidal estrogens, a few investigations have focused on nonsteroidal estrogens or antiestrogens, including selective ER modulators [17–22].

Cyclofenil and its derivatives have high binding affinity for ER, often comparable to or greater than that of estradiol, and bis(4-hydroxyphenyl)methylidenecyclopentane showed the highest ERβ to ERα ratio [23]. Notably, it has mixed agonist–antagonist activity typical for a nonsteroidal selective ER modulator such as tamoxifen or raloxifene [22, 24]. More recently, ^{18}F-labeled fluorocyclofenil analogues have been synthesized and investigated for subtype-specific imaging of ERα or ERβ by using positron emission tomography (PET). Unfortunately, these ^{18}F-labeled high-affinity ligands for ER failed to show receptor-mediated uptake into the uterus [25]. The corresponding ^{11}C-labeled cyclofenil ester has also been studied. These chemistry results encouraged the further in vivo biological evaluation of ^{11}C-labeled cyclofenil derivatives as new potential PET imaging agents [26].

The PET radionuclides must typically be generated by an on-site cyclotron and used as soon as possible owing to their short half-lives. Single photon emission computed tomography (SPECT) imaging with ^{99m}Tc alleviates some of the problems encountered with PET owing to its convenient 6-h half-life and its wide availability. ^{99m}Tc has

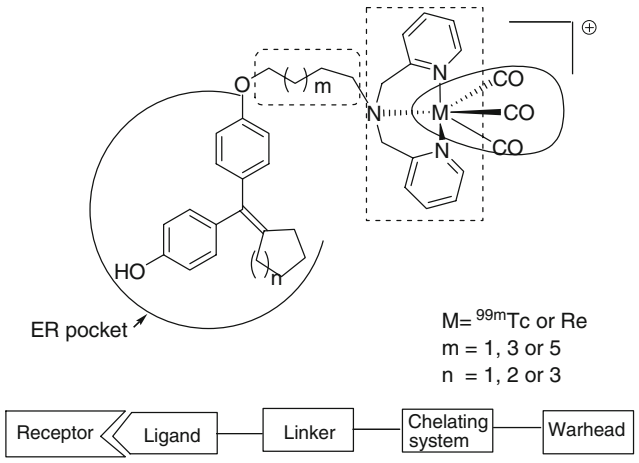


Fig. 1 Structure and proposed mechanism of binding of cyclofenil derivatives with estrogen receptor (*ER*)

emerged as a preeminent radionuclide, and it is used in over 80% of all routine diagnostic nuclear medicine procedures [27].

Structural studies on the ERs have suggested that there is ample unoccupied space within the ligand binding pocket [28]. An important requirement for high affinity for the ERs is that at least one hydroxyl function should remain on the estrogenic nucleus [29–31]. As shown in Fig. 1, a reasonable pharmacophore model has been advanced to guide the design of cyclofenil for a potential SPECT imaging agent [32–34].

Starting from 4, 4′-dihydroxybenzophenone, we prepared a series of cyclofenil derivatives based on a tridentate ligand chelating system by ω-bromoalkylation and subsequent nucleophilic substitution. The corresponding cyclofenil rhenium complexes were synthesized and characterized. These tricarbonyl rhenium complexes showed excellent binding affinities for the ERs when compared with the lead structure. Tricarbonyl technetium cyclofenil complexes were synthesized and preliminarily revaluated with effective specific activities sufficient for in vivo biodistribution studies. All these studies were designed to develop labeled cyclofenil derivatives as new potential SPECT nonsteroidal estrogen radioligands for imaging ER in breast cancer.

Experimental

Materials and methods

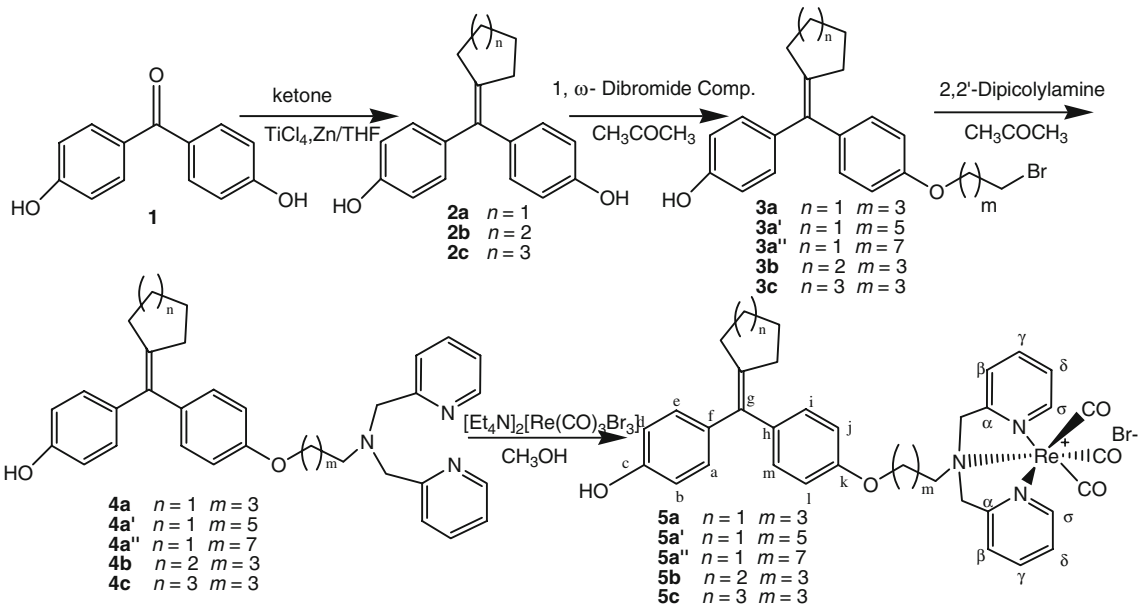
All experiments were performed under the specified temperature conditions. Solvents were distilled from the appropriate drying agents and degassed before use. Melting points were determined using a WRS-IA apparatus and were uncorrected. High-resolution mass spectra were obtained with a Thermo-MAT95XP mass spectrometer under electron impact ionization conditions. NMR spectra were recorded using a Bruker Avance 400 or 500 MHz spectrometer. IR spectra were recorded with an Avataar 370 Fourier transform IR spectrometer (250–4,000 cm^{-1}). The elemental analyses were conducted using an Elementar Analysensysteme (Germany) vario EL III.

Purified full-length human ERα and ERβ were purchased from PanVera/Invitrogen (Carlsbad, CA, USA). [6,7-^{3}H] Estra-1,3,5,(10)-triene-3,17-β-diol ([^{3}H]-E_2), 44.8 Ci/mmol, was from PerkinElmer (Boston, MA, USA). Hydroxyapatite (HAP) was from Aladdin (China). Borosilicate glass tubes were from VWR International (West Chester, PA, USA). The organometallic precursor $[Et_4N]_2[Re(CO)_3Br_3]$ and the radioactive precursor $[^{99m}Tc(H_2O)_3(CO)_3]^+$ were prepared as reported before [35, 36]. Na[$^{99m}TcO_4$] was eluted from a $^{99}Mo/^{99m}Tc$ generator (Shanghai Yuanpu Isotope Technology) using 0.9% saline. High performance liquid chromatography (HPLC) analyses of the rhenium and technetium-99m complexes were performed using a Dionex P680 system equipped with a tunable absorption detector and a PDA-100 photodiode-array detector using a Hypersil BDS C-18 reversed-phase column (5 μm, 250 mm × 4.6 mm). The HPLC solvents were methanol (solvent A) and aqueous triethylammonium phosphate buffer, pH 2.76 (solvent B). The HPLC eluting conditions were as follows: 0–3 min, 15% solvent A; 3–6 min, 15–25% solvent A; 6–9 min, 25–35% solvent A; 9–22 min, 35–98% solvent A; 22–25 min, 98–25% solvent A; 25–30 min, 25–15% solvent A. The flow rate was 1 mL/min.

Chemical synthesis

*Syntheses of compounds **2a**, **2b**, and **2c***

The syntheses of compounds **2a**, **2b**, and **2c** (Scheme 1) were performed according to the following procedure. Cyclofenil and its analogues were prepared via McMurry coupling. The low-valence titanium was formed by reaction of $TiCl_4$ (3.28 g, 17.5 mmol) with zinc power (2.4 g, 36 mmol) in absolute tetrahydrofuran (25 mL) at 100 °C. A solution of **1** (1.00 g, 4.67 mmol) and each ketone (5.0 mmol) dissolved in absolute tetrahydrofuran (15 ml) was injected by syringe, and the reaction mixture was refluxed for 1.5 h. The cooled reaction mixture was slowly poured into a $NaHCO_3$ solution (200 mL) with vigorous stirring. The heterogeneous solution was filtered through Celite. The aqueous layer was extracted with EtOAc (100 mL) three times and finally purified by column chromatography. Under these optimized conditions, all of the reactions gave reasonable yields of 78–85%, which are higher than the yields reported in the literature [23].



Scheme 1 Synthetic strategy for cyclofenil–$Re(CO)_3$ conjugates. *THF* tetrahydrofuran

Analytical data for compound **2a** (a white solid): Yield 81%, white solid, m.p. 193–194 °C; IR (KBr) *v*: 3,280, 2,949, 1,613, 1,592, 1,429, 1,332, 840, 562 cm^{-1}; ^1H NMR (dimethyl-d_6 sulfoxide) δ: 9.25 (s, 2H, –OH), 6.91(d, 4H, aromatic, $J = 8.6$ Hz), 6.66 (d, 4H, aromatic, $J = 8.6$ Hz), 2.28 (t, 4H, C_2–H, $J = 6.8$ Hz), 1.61 (dt, 4H, C_3–H, $^2J_{H–H} = 6.6$ Hz, $^3J_{H–H} = 2.7$ Hz). Analytical data for **2b** and **2c** are given in the electronic supplementary material.

*Syntheses of compounds **3a**, **3a′**, **3a″**, **3b**, and **3c***

Compound **2a**, **2b** or **2c** (2 mmol), and K_2CO_3 were dissolved in 15 mL acetone, then 1, ω-dibromide compound (2.4 mmol) dissolved in another 10 mL acetone was slowly dropped into the mixture. After the reaction mixture had been stirred for 8 h at 46 °C, the reaction was stopped, and the reaction mixture was filtered, evaporated under reduced pressure, and purified by column chromatography.

Analytical data for compound **3a** (straw yellow oil): Yield: 62%; ^1H NMR ($CDCl_3$, 400 MHz) δ: 7.09 (d, $J = 8.1$ Hz, 2H, ArH), 7.05 (d, $J = 8.0$ Hz, 2H, ArH), 6.81 (d, $J = 8.4$ Hz, 2H, ArH), 6.75 (d, $J = 8.1$ Hz, 2H, ArH), 4.83 (s, 1H, –OH), 3.99 (t, $J = 6.6$ Hz, 2H, 1′-CH_2), 3.50 (t, $J = 6.4$ Hz, 2H, 4′-CH_2), 2.39 (s, 4H, 2-CH_2 and 5-CH_2), 2.06–2.10 (m, 2H, 2′-CH_2), 1.94–1.96 (m, 2H, 3′-CH_2), 1.66–1.69 (m, 4H, 3-CH_2 and 4-CH_2); IR (KBr) *v*: 2,949, 2,876, 1,601, 1,507, 1,168, 927, 837, 605, 579 cm^{-1}; electrospray ionization (ESI) high-resolution mass spectrometry (HRMS): calcd. for $C_{22}H_{25}O_2BrK$ $[M + K]^+$ 439.0675, found 439.0684. Analytical data for **3a′**, **3a″**, **3b**, and **3c** are given in the electronic supplementary material.

*Syntheses of compounds **4a**, **4a′**, **4a″**, **4b**, and **4c***

Compounds **3a**, **3a′**, **3a″**, **3b**, and **3c** (1 mmol), K_2CO_3 (280 mg, 2 mmol), and KI (36 mg, 0.2 mmol) were dissolved in 15 mL acetone, then 2,2′-dipicolylamine (300 mg, 1.5 mmol) in acetone (5 mL) was added slowly. After the reaction mixture had been stirred at 44 °C for 18 h, the reaction was stopped, and the reaction mixture was filtered, evaporated under reduced pressure, and purified by column chromatography.

Analytical data for compound **4a** (colorless oil): Yield: 63%; ^1H NMR ($CDCl_3$, 500 MHz) δ: 8.51 (d, $J = 8.6$ Hz, 2H, σ-CH, Py), 7.63 (t, $J = 6.0$ Hz, 2H, γ-CH, Py), 7.54 (d, $J = 7.6$ Hz, 2H, β-CH, Py), 7.14 (t, $J = 6.2$ Hz, 2H, δ-CH, Py), 7.06 (d, $J = 8.4$ Hz, 2H, ArH), 7.04 (d, $J = 8.0$ Hz, 2H, ArH), 6.77 (d, $J = 8.4$ Hz, 2H, ArH), 6.74 (d, $J = 8.4$ Hz, 2H, ArH), 3.86 (s, 4H, –$N(CH_2)_2$), 3.81 (t, $J = 5.7$ Hz, 2H, 1′-CH_2), 2.60 (t, $J = 6.4$ Hz, 2H, 4′-CH_2), 2.38–2.40 (m, 4H, 2-CH_2 and 5-CH_2), 1.60–1.80 (m, 8H); IR (KBr) *v*: 2,432, 1,593, 1,471, 1,364, 1,164, 1,046, 833, 756, 580 cm^{-1}; ESI-HRMS: calcd. for $C_{34}H_{38}N_3O_2$ $[M + H]^+$ 520.2964, found 520.2966. Analytical data for **4a′**, **4a″**, **4b**, and **4c** are given in the electronic supplementary material.

*Syntheses of complexes **5a**, **5a′**, **5a″**, **5b**, and **5c***

Complexes **5a**, **5a′**, **5a″**, **5b**, and **5c** (0.2 mmol) were synthesized according to the following general procedure. $(Et_4N)_2[Re(CO)_3Br_3]$ (180 mg, 0.24 mmol) and ligands **4a**, **4a′**, **4a″**, **4b**, and **4c** were dissolved in ethanol and the mixture was stirred at room temperature for 2 h. The

reaction mixture was evaporated and the residue was purified by flash chromatography. Compounds for bioassay were recrystallized from CH_2Cl_2 and hexane.

Analytical data for compound **5a** (pale-yellow solid): Yield: 85%, melting point more than 200 °C; ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 8.63 (d, $J = 5.4$ Hz, 2H, σ-CH, Py), 7.88 (d, $J = 7.8$ Hz, 2H, γ-CH, Py), 7.74 (t, $J = 7.7$ Hz, 2H, β-CH, Py), 7.17 (t, $J = 6.58$ Hz, 2H, δ-CH, Py), 7.04 (d, $J = 8.6$ Hz, 2H, ArH), 6.97 (d, $J = 7.2$ Hz, 2H, ArH), 6.75–6.83 (m, 4H, ArH), 6.03 (s, 1H, –N(CH_2)$_2$), 5.99 (s, 1H, –N(CH_2)$_2$), 4.35 (s, 1H, –N(CH_2)$_2$), 4.31 (s, 1H, –N(CH_2)$_2$), 4.00 (t, $J = 5.8$ Hz, 2H, 1′-CH_2), 3.84 (t, $J = 8.4$ Hz, 2H, 4′-CH_2), 2.35 (d, $J = 7.2$ Hz, 4H, 2-CH_2 and 5-CH_2), 2.23 (t, $J = 5.9$ Hz, 2H, 3′-CH_2), 1.87 (t, $J = 6.23$ Hz, 2H, 2′-CH_2), 1.60–1.70 (m, 4H, 3-CH_2 and 4-CH_2); ^{13}C NMR (CD_3OD,125 MHz) δ: 197.7 (3C, *fac*-Re$(CO)_3$), 162.6 (2C, Py-αC), 158.9 (1C, Ar-kC), 157.0 (1C, Ar-cC), 153.6 (2C, Py-σC), 142.1 (2C, Py-γC), 138.4 (2C, Ar-iC and Ar-mC), 137.0 (2C, Ar-eC and Ar-aC), 134.4 (2C, Ar-jC and Ar-lC), 131.8 (2C, Ar-dC and Ar-bC), 127.3 (2C, Py-βC), 127.3 (2C, Py-δC), 125.1 (1C, C1), 116.5 (2C, Ar-hC and Ar-fC), 115.7 (1C, gC), 72.3 (1C, C1′), 69.1 (2C, PyCH_2), 69.0 (1C, C4′), 34.4 (2C, C2 and C5), 28.4 (2C, C3 and C4), 28.0 (1C, C2′), 23.9 (1C, C3′); IR (KBr) ν: 2,921, 2,848, 2,026, 1,916, 1,605, 1,503, 1,242, 1,029, 829, 764 cm^{-1}; ESI mass spectrometry (MS): calcd. for $C_{37}H_{37}O_5N_3Re$ [M-Br]$^+$ 789.91, found 790.20; anal. calcd. for [$C_{37}H_{37}N_3O_5Re$]Br·1/2CH_2Cl_2: C 49.37, H 4.20, N 4.61; found C 49.46, H 3.85, N 4.62. Analytical data for **5a′**, **5a″**, **5b**, and **5c** are given in the electronic supplementary material.

Binding affinity assay

The equilibrium and competition binding affinity assays were performed as described by Katzenellenbogen et al. [37, 38] with some modifications [39]. ERα and ERβ were diluted to 2 nM in binding buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 7.4, 150 mM KCl, 1 mM EDTA, 0.1% bovine serum albumin]. [^{3}H]-E_2 was extracted with ethanol and diluted in Tris–HCl to 0.5–30 nM for the saturation binding affinity assay and to 5 nM for the competition binding affinity assay. HAP was used to absorb the receptor–ligand complexes and free ligand was washed away. Each compound (**2a**, **2b**, **2c**, **5a**, **5a′**, **5a″**, **5b**, and **5c**) was dissolved in dimethylformamide and then diluted with binding buffer to obtain concentrations ranging from 0.1 nM to 1 mM. Thirty microliters of the ER solution, [^{3}H]-E_2 solution, estradiol, and each test compound was added to a test tube, followed by binding buffer to a final volume of 300 μL. The mixture was incubated at 25 °C for 2 h. HAP slurry (30% solution) was added and the mixture was vortexed and centrifuged. The supernatant was removed and discarded and the pellet was washed three times with Tris–HCl (0.05 M, pH 7.4). The radioactivity was counted the next day with a scintillation counter (Beckman) with 43% counting efficiency. All numeric data were expressed as the mean of the values ± the standard error of the mean. Graphpad Prism, version 4, was used for statistical analysis.

Evaluation of radioactive cyclofenil–^{99m}Tc$(CO)_3$ conjugates

Preparation of radioactive cyclofenil–^{99m}Tc$(CO)_3$ conjugates

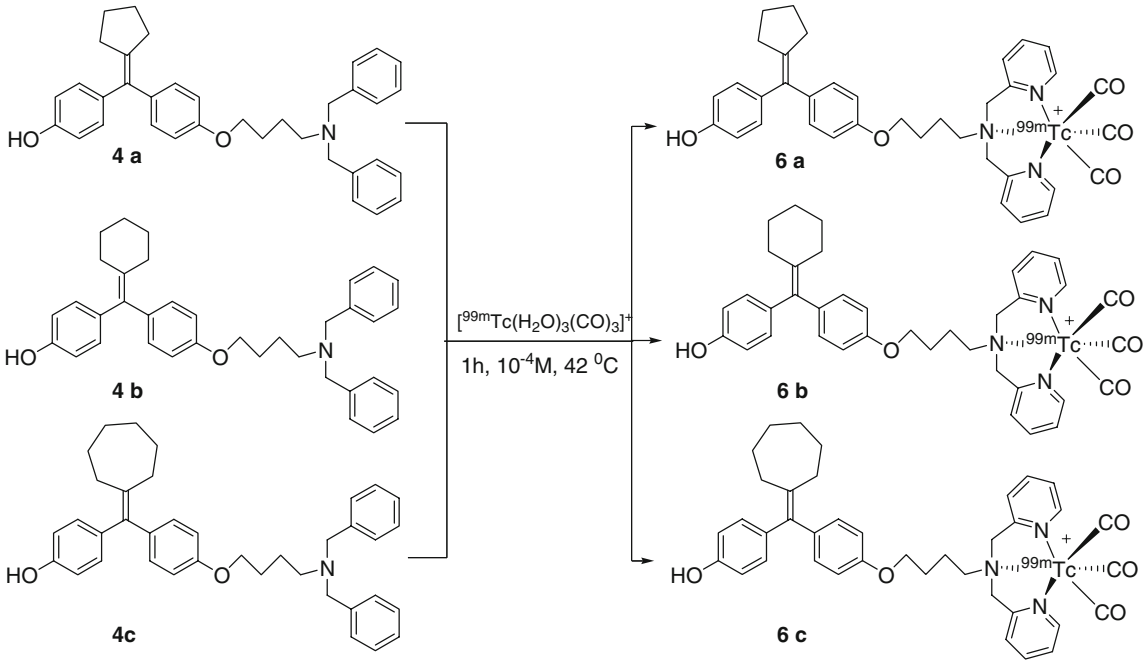
^{99m}Tc$(CO)_3(H_2O)_3^+$ was prepared as reported before [40–42]. In our experiment the radiochemical purity of the [^{99m}Tc$(CO)_3(H_2O)_3$]$^+$ intermediate exceeded 95% as determined by radio-HPLC. To a 5-mL serum vial, 0.2 mL of aqueous [^{99m}Tc$(CO)_3(H_2O)_3$]$^+$ solution was added, followed by 100 μL of **4a**, **4b**, or **4c** (10^{-4} M). The reaction mixture was heated at 42 °C for 60 min (Scheme 2). After the mixture had been cooled to room temperature, the radiotracer was purified by HPLC using conditions identical to those for [^{99m}Tc$(CO)_3(H_2O)_3$]$^+$ analysis.

Octanol/water partition coefficient of cyclofenil–^{99m}Tc$(CO)_3$

The final partition coefficient was expressed as log P. Log P of cyclofenil–^{99m}Tc$(CO)_3$ complexes was determined by measuring the distribution of radioactivity in 1-octanol and phosphate-buffered saline (PBS). A 10-μL sample of cyclofenil–^{99m}Tc$(CO)_3$ in PBS was added to a vial which contained 1 mL of 1-octanol and 1 mL of PBS. After the mixture had been vortexed for 8 min, the vial was centrifuged for 5 min to ensure complete separation of layers. Then, 10 μL of each layer was pipetted into the other test tubes, and log P values were calculated using the formula log P = log(counts in octanol/counts in water) [43].

In vitro stability of cyclofenil–^{99m}Tc$(CO)_3$

The stability of cyclofenil–^{99m}Tc$(CO)_3$ complexes was studied by measuring the radiochemical purity using radio-HPLC at different time intervals after preparation. The complex was added to a test tube with PBS. The mixture was incubated by shaking the test tube at 37 °C in an incubation apparatus. The radiochemical purity was measured at 30 min and at 1, 2, 4, and 6 h by radio-HPLC. The same procedure was applied in an experiment using histidine and cysteine [44].



Scheme 2 Radiolabeling of cyclofenil–$^{99m}Tc(CO)_3$ (**6a**, **6b**, **6c**)

Results and discussion

Design and synthesis of cyclofenil derivatives

Clearly, the hydroxylation pattern is important for receptor binding and in vitro activity [45]. The presence and the nature of the basic amine substituent is critical in determining estrogen antagonist activity [46]. We began with the selective modification of phenolic hydroxyl group. Then dipicolyamin chelators system were introduced for further coordination to *fac*-$[M(CO)_3]^+$ (Scheme 1).

Cyclofenil (**2a**, **2b**, **2c**) contains two phenols, which react with 1,ω-dibromide to form **3a**, **3a′**, **3a″**, **3b**, and **3c**. In the reaction we chose K_2CO_3 as the base and acetone as the solvent. Firstly, all of the starting materials were mixed together at room temperature, then the mixture was stirred at 46 °C for 12 h, the reaction was stopped, and the product (**3a**, **3a′**, **3a″**, **3b**, and **3c**) was obtained with 50–62% isolated yield. With the same procedure a series of cyclofenil derivatives (different lengths of the alkyl chains as well as variation of the ring size in the lead structure) could be easily prepared for further reaction. Their structures were characterized by IR spectroscopy, ^{1}H NMR spectroscopy, and ESI-HRMS.

The tridentate ligands **4a**, **4a′**, **4a″**, **4b**, and **4c** were characterized by IR spectroscopy, ^{1}H NMR spectroscopy, and HRMS. The rhenium complexes **5a**, **5a′**, **5a″**, **5b**, and **5c** were unambiguously characterized by IR spectroscopy, ^{1}H NMR spectroscopy, ^{13}C NMR spectroscopy, ESI–MS, and elemental analysis. The protons of the methylene groups adjacent to the pyridines are equivalent by virtue of their symmetry for ligands **4a**, **4a′**, **4a″**, **4b**, and **4c**. After ligands **4a**, **4a′**, **4a″**, **4b**, and **4c** had coordinated to rhenium, the splitting pattern of these methylene protons became more complicated, resulting in multiplets in the 4.0–6.0-ppm range. The ^{1}H NMR spectra of Py–CH_2– of ligands **4a**, **4a′**, **4a″**, **4b**, and **4c** show a single peak at 3.9 ppm. Rhenium complexes **5a**, **5a′**, **5a″**, **5b**, and **5c** show four single peaks at 6.06, 6.00, 4.35, and 4.00 ppm. The ^{13}C NMR spectra of the five rhenium complexes show chemical shifts of three carbonyl peaks in the range 195.9–197.9 ppm. All the conditions show that the rhenium core lacking electrons makes the entire molecular electric field move towards the rhenium core. So many proton signals show a downfield shift. The IR spectra of complexes **5a**, **5a′**, **5a″**, **5b**, and **5c** exhibit a sharp, strong band in the 2,026–2,030-cm^{-1} range and two broad, intense absorptions at 1,910 and 2,036 cm^{-1}, attributed to ν(C–O) of the *fac*-$Re(CO)_3$ unit [47, 48]. The absorptions are significantly blueshifted compared with the absorptions of the starting material $[Re(CO)_3Br_3]^{2-}$ (1,998, 1,871 cm^{-1}). The ESI–MS of **5a**, **5a′**, **5a″**, **5b**, and **5c** showed cleavage of bromide anion, whereas the corresponding rhenium core is relatively stable. These features indicate the tridentate coordination mode of ligands **4a**, **4a′**, **4a″**, **4b**, and **4c** via the tertiary amine and the two pyridine nitrogens. Elemental analyses of the compounds gave definite ultimate compositions of **5a**, **5a′**, **5a″**, **5b**, and **5c**. Owing to the purity of

Table 1 Relative binding affinities (*RBA*) of cyclofenil and its derivatives at 25 °C

Complexes	RBA[a]		ERβ/ERα[b]
	ERα	ERβ	
2a	17.7 ± 1.8	121.8 ± 4.0	6.9
2b	114.6 ± 5.0	274.8 ± 5.0	2.4
2c	101.3 ± 4.0	339.0 ± 9.0	3.3
5a	13.3 ± 1.2	33.8 ± 1.8	2.5
5a′	9.43 ± 0.8	62.6 ± 2.0	6.7
5a″	7.75 ± 0.6	57.4 ± 2.2	7.4
5b	44.8 ± 1.8	66.7 ± 2.6	1.5
5c	16.5 ± 1.2	81.2 ± 2.4	4.9

The RBA is expressed as the binding affinity relative to that of estradiol (100%)

[a] Determined by a competitive radiometric binding assay with [^{3}H]estradiol, using full-length human estrogen receptor α (ERα) and estrogen receptor β (ERβ). The values are reported as the mean ± range ($n = 2$)

[b] Under these conditions, K_d of estradiol is 0.4 nM for ERα and 1.0 nM for ERβ

the raw products by recrystallization, different numbers of CH_2Cl_2 groups were left on the surface of the crystal.

Binding affinity assay of cyclofenil–Re(CO)$_3$ conjugates

The [^{3}H]-E_2 binding inhibition curves were established for each compound. The relative binding affinity (RBA) was expressed as the binding affinity relative to that of estradiol (100%) [36, 49]. The results are shown in Table 1.

For an ER pharmacophore, the most important requirement for high affinity for the ER is to maintain the integrity of the hydroxyl functions. However, it was not obvious what effect the substituted hydroxyl group had on binding affinity. Since cyclofenil (**2a**, **2b**, and **2c**) shows better binding affinity than estradiol (Table 1), we prepared the first group of cyclofenil–Re(CO)$_3$ conjugates (**5a**, **5a′**, **5a″**, **5b**, and **5c**) to evaluate the RBAs of cyclofenil compounds having different ring size core units with the same substituted hydroxyl group (four carbon alkyl chains). In short, the RBAs range from 13.3 to 44.8 for ERα and from 33.8 to 81.2 for ERβ (Table 1).

As the ring size increased from cyclopentyl (**5a**) to cycloheptyl (**5c**), the binding affinities for ERβ increased, and cycloheptyl (**5c**) showed the highest ERβ affinity. However, the RBAs for ERα did not show this tendency, and **5b** show the highest ERα affinity. All compounds (**5a**, **5a′**, **5a″**, **5b**, and **5c**) show slight selectivity for ERβ from the calculated ERβ to ERα ratio. On this scale, cycloheptyl compound (**5c**) was 4.9-fold ERβ-selective. Examples of the [^{3}H]-E_2 inhibition curves used for the RBA estimations are presented in Figs. 2, S1, and S2. These data suggest that after the modification of hydroxyl group with a carbon side chain, the RBA was slightly reduced (approximately 30% of that of lead structure). However, affinities comparable to the affinity of estradiol are maintained.

To investigate the relation between the lengths of the alkyl chains and ER affinity, further modification of **2a** were present. **2a** has the smallest ring size and it might be more suitable to "enter" the ER pocket (Fig. 1). Rhenium–cyclofenil complexes with a chain of four to eight carbons (**5a**, **5a′**, and **5a″**) were synthesized. In general, the RBAs ranged from 7.7 to 13.3 for ERα and from 33.8 to 62.6 for ERβ (Table 1).

As the number of carbons in the chain increased from four (**5a**) to eight (**5a″**), the binding affinity for ERα gradually decreased, whereas for **5a′** and **5a″** show similar affinity for ERβ, double that of **5a**, greatly improving the selectivity for ERβ. Let us take **5a″** as an example. It is

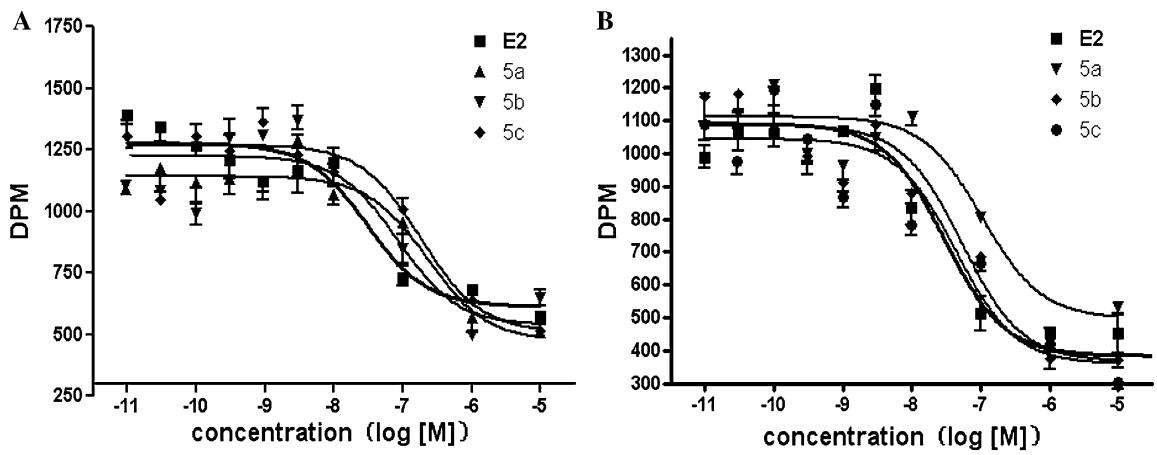


Fig. 2 Determination of specific binding of estradiol (*E2*) and rhenium–cyclofenil (**5a**, **5b**, and **5c**) complexes in purified full-length human ER. *Left* for ERα; *right* for ERβ. Each data point was from the average of three measurements and the *bar* represents the standard deviation

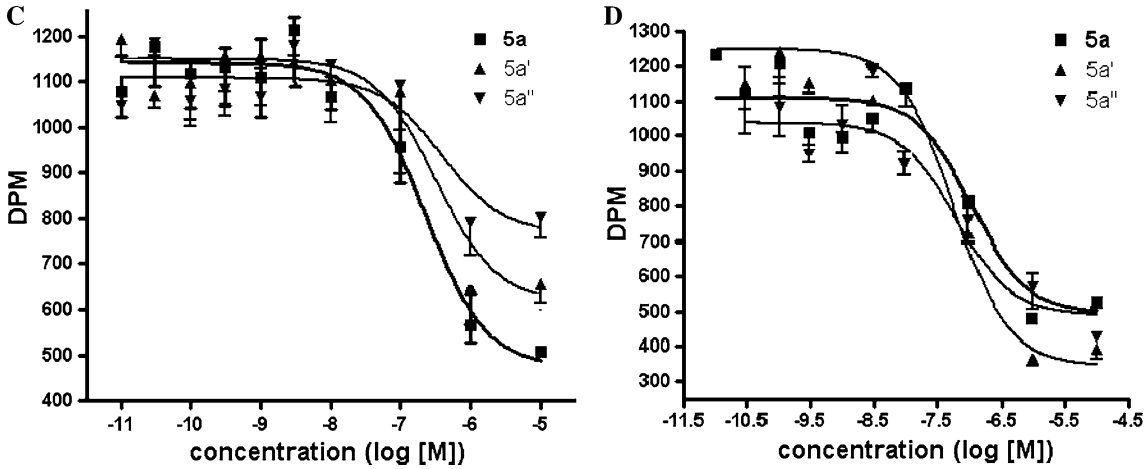


Fig. 3 Determination of specific binding of **5a** and rhenium complexes with different carbon chain lengths (**5a′** and **5a″**) in purified full-length human ER. *Left* for ERα; *right* for ERβ. Each data point came from the average of three measurements and the *bar* represents the standard deviation

7.4-fold ERβ-selective and has highest binding affinity of all the compounds, comparable to that of the original compound (**2a**, ERβ to ERα ratio of 7.7 [23]). Examples of the [^{3}H]-E_2 inhibition curves used for the RBA estimations are presented in Fig. 3. As the carbon chain length increased, the binding affinity of ERβ also increased; however, the situation is opposite that of ERα.

In term of binding affinity, our modification was successful, because we found the cyclofenil–$Re(CO)_3$ compounds **5a′**, **5b**, **5c**, and **5a′** bind to ERs with an affinity comparable to that of estradiol.

Evaluation of cyclofenil–$^{99m}Tc(CO)_3$ conjugates

Preparation of cyclofenil–$^{99m}Tc(CO)_3$

To further study their water solubility and stability, we selected radioactive $^{99m}Tc(CO)_3(H_2O)_3^+$ instead of the stable rhenium complex. In the periodical table of elements, technetium and rhenium are in the same group, and they have extremely similar properties, so **6a**, **6b**, and **6c** show almost the same chemical character as **5a**, **5a′** **5a″**, **5b**, and **5c**, respectively.

The radiolabeling of cyclofenil derivatives is shown in Scheme 2. The radiochemical purity of the $^{99m}Tc(CO)_3(H_2O)_3^+$ intermediate exceeded 95% as determined by radio-HPLC. The intermediate was used directly without further purification. The radiochemical purity of the crude cyclofenil–$^{99m}Tc(CO)_3$ product exceeded 90%.

The retention times were 22.75 min for **4a**, 23.75 min for **4b**, and 23.80 min for **4c** and 23.15 min for **6a**, 24.55 min for **6b**, and 24.24 min for **6c**. The retention times are long enough for the technetium complexes to be separated from unreacted chelate by semipreparative HPLC. Radio-HPLC also readily distinguished cyclofenil–$^{99m}Tc(CO)_3$ (**6a**, **6b**, and **6c**) from $^{99m}Tc(CO)_3(H_2O)_3^+$ and $^{99m}TcO_4^-$, whose retention times were 5.51 and 10.30 min, respectively. The retention times for the cyclofenil–$Re(CO)_3$ analogues (**5a**, **5b**, and **5c**) were 23.12, 24.37, and 24.12 min, respectively (Fig. 4).

Octanol/water partition coefficient of cyclofenil–$^{99m}Tc(CO)_3$

To evaluate the aqueous solubility of the complex, the octanol/water partition coefficient was determined. The values of log $P_{o/w}$ for cyclofenil–$^{99m}Tc(CO)_3$ are shown in Table 2. The octanol/water partition of estradiol was reported as log $P = 3.26$ [13] or log $P = 3.30$ [15]. It is well known that the lipophilicity can affect the tissue permeability properties of a ligand, thus affecting its ability to enter target tissues. By altering the lipophilicity of the cyclofenil derivatives through synthetic modification, one can change the tissue permeability by allowing more or less of the ligand to enter cells in ER-rich target tissues or nontarget tissues. The oil/water partition coefficients of the cyclofenil–$^{99m}Tc(CO)_3$ complexes were up to almost 100 times lower than that of estradiol. According to research by Nayak et al. [50], appropriately reducing radiopharmaceutical lipophilicity can be favorable to target tissue uptake of ER-expressing tumors.

In vitro stability study

The in vitro stability in PBS, histidine, and cysteine was tested, and the results are shown in Fig. S3. The radioactive conjugates for cyclofenil–$^{99m}Tc(CO)_3$ maintain excellent in vitro stability in PBS at 37 °C within 6 h. Decomposition or

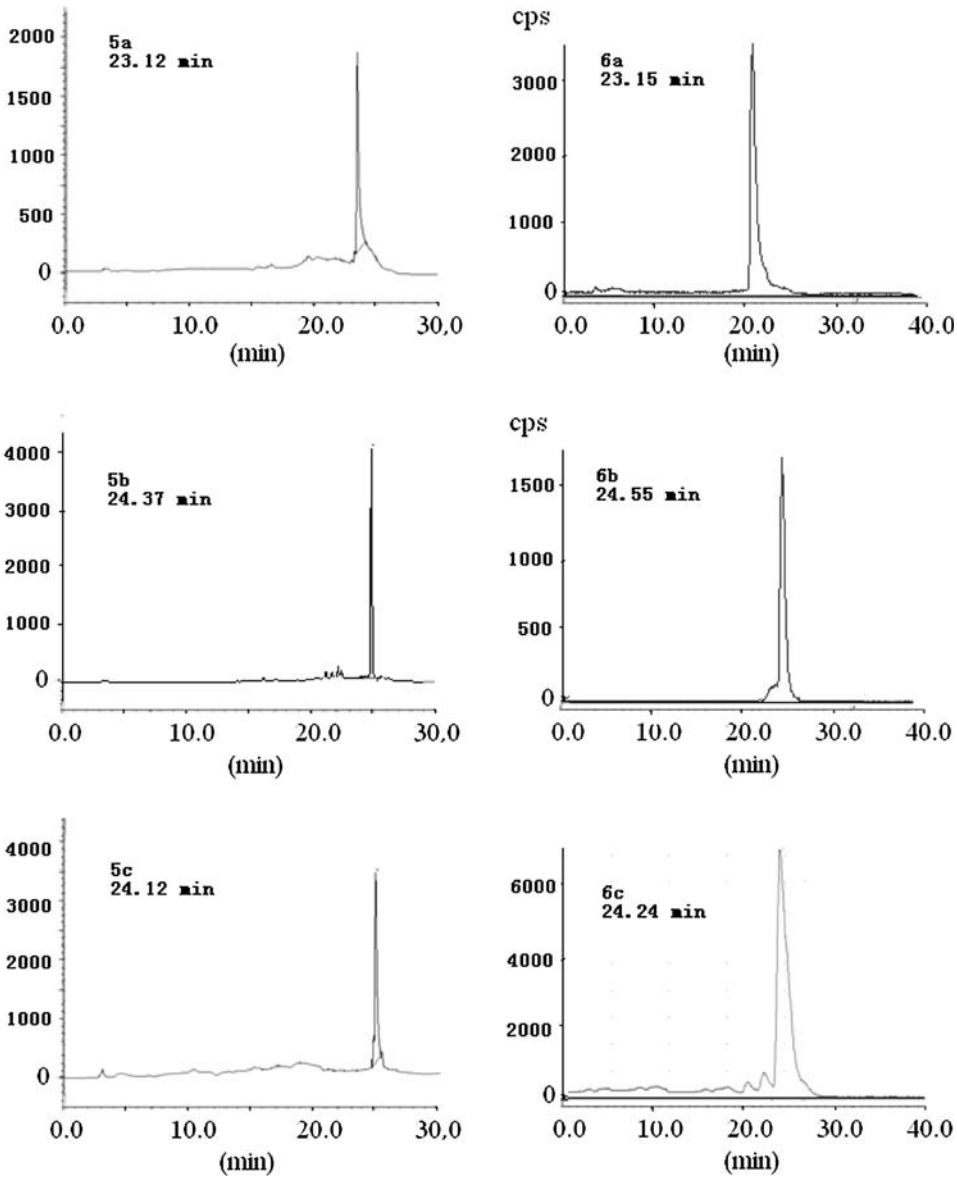


Fig. 4 High performance liquid chromatography analyses of cyclofenil–$Re(CO)_3$ (**5a**, **5b**, and **5c**) and γ-trace of the radioactive cyclofenil–$^{99m}Tc(CO)_3$ conjugates (**6a**, **6b**, and **6c**)

Table 2 Octanol8water partition coefficient of cyclofenil–$^{99m}Tc(CO)_3$ conjugates

Complexes	*P*	Log *P*
6a	9.30 ± 0.94	0.968 ± 0.02
6a′	10.4 ± 0.95	1.02 ± 0.02
6a″	11.0 ± 1.07	1.04 ± 0.03
6b	9.38 ± 0.95	0.972 ± 0.02
6c	9.44 ± 0.95	0.975 ± 0.02

dissociation of the complexes to either $[^{99m}Tc(CO)_3]^+$ or other side products was not observed for all of the complexes under the conditions used. In both histidine and cysteine, more than 90% of the conjugate still maintains the original structure within 6 h. These results indicate that the cyclofenil–$^{99m}Tc(CO)_3$ conjugate has excellent in vitro stability for potential nuclear medical applications.

Conclusion

The strategy for the preparation of a monophenolhydroxyl-substituted 1,1-diarylethylene unit has been developed. A tridentate chelating system was introduced for labeling with *fac*-$[M(CO)_3]^+$ (M is ^{99m}Tc, Re). Rhenium–cyclofenil complexes show binding affinity comparable to that of estradiol in binding to ERs. THe octanol/water partition coefficient and the in vitro stability of ^{99m}Tc–cyclofenil complexes were evaluated. We did not obtain a compound having outstanding affinity preference for either ERα or ERβ. Further work on the optimization of the structures of

the cyclofenil derivatives and other evaluations is currently under way.

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