Supporting Information.

**Novel Cyclopentadienyl Tricarbonyl $^{99m}$Tc Complexes Containing 1-Piperonylpiperazine Moiety: Potential Imaging Probes for Sigma-1 Receptors**

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3. HPLC analysis of lipophilicity of rhenium complexes 10a and 16a.
1. General information and some parts of evaluation of the radiotracers in the Experimental Section.

General information

All reagents and chemicals were purchased from commercial source and used without further purification unless otherwise stated. $^{99m}$Tc-pertechnetate was eluted from a commercial $^{99}$Mo–$^{99m}$Tc generator which was obtained from Beijing Atomic High-Tech Co.. Haloperidol was obtained from Sigma-Aldrich Co. Ltd. (Beijing, China). 1,3-Di- $o$-tolyl-guanidine (DTG) was purchased from Heowns Biochem LLC. (Tianjin, China). 1-(3,4-Dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine (SA4503) was synthesized according to the method in the literature. Reactions were monitored by thin layer chromatography (TLC) (TLC silica gel 60 F$_{254}$ plates, E. Merck). Flash column chromatography was conducted on silica gel (45–75 µm). The mobile phase was reported in the experimental procedure. $^1$H NMR spectra were recorded on a Bruker Avance III (400 MHz) NMR spectrometer. Chemical shift (δ) are reported in ppm downfield from tetramethylsilane and coupling constants (J) are reported in Hertz (Hz). $^{13}$C NMR spectra were recorded on a Bruker Avance III (100 MHz) NMR spectrometer. Mass spectrometry (MS) spectra were obtained by Quattro micro API ESI/MS (Waters, USA). The purity of rhenium compounds and ferrocene precursors were analyzed by high performance liquid chromatography (HPLC). All final complexes were tested with a purity of > 95%.

HPLC separations were performed on a Shimadzu SCL-20 AVP HPLC system (SHIMADZU Corporation, Japan) equipped with a SPD-M20AUV-VIS detector operating at a wavelength of 254 nm, and a Bioscan Flow Count 3200 Nal/PMT γ-radiation scintillation detector. HPLC analyses were performed on a Waters 600 system.
(Waters, corporation, USA) equipped with Waters 2489 UV-VIS detector, and Raytest Gabi NaI (Tl) scintillation detector (Raytest, Germany).

Samples were analyzed and separated on a Agela Venusil MP C18 column (250 mm × 4.6 mm, 5 μm) using acetonitrile with 0.1% trifluoroacetic acid (TFA) and water with 0.1% TFA as mobile phase at a flow rate of 1 mL/min.

Male ICR mice (4–5 weeks, 22–25 g) were purchased from Peking University Health Science Center. All procedures of the animal experiments were performed in compliance with relevant laws and institutional guidelines. All of the animal experiments were approved by the Institutional Animal Care and Use Committee of Beijing Normal University.

**In vitro radioligand competition studies**

**σ Receptor Binding Assays.** All the procedures for the radioligand competition studies were previously described. Briefly, the σ₁ receptor assay was conducted with rat brain membrane homogenate and the σ₁ specific radioligand (+)-[³H]pentazocine. The σ₂ receptor affinity was performed using rat liver membrane homogenate with the radioligand [³H]DTG in the presence of 10 μM dextrallorphan for the selective masking of σ₁ receptor binding sites. Nonspecific binding was determined with 10 μM haloperidol. \( K_i \) values were calculated according to the Cheng-Prusoff equation and represent means ± standard deviation (SD) from at least two independent experiments, each performed in triplicate.

**VAChT Binding Assays.** Radioligand competition binding assays were performed using membrane homogenates obtained from PC12 cells stably transfected with rat
vesicular acetylcholine transporter (VACChT, obtained from Ali Roghani, Department of Pharmacology and Neuroscience, Texas Tech University Health Sciences Center, Lubbock, USA) and (-)[3H]vesamicol (1.1–1.2 nM working concentration). Assays were performed with compound 10a or 10b (10^{-11}–10^{-6} mol/L) in 50 mM Tris-HCl, pH 7.4, by incubation at room temperature for 120 min. Nonspecific binding was measured in the presence of 10 µM (-)-vesamicol. K_i values were calculated according to the Cheng-Prusoff equation and represent means ± SD from two independent experiments, each performed in triplicate.

**Radiochemistry**

The $^{99m}$Tc-pertechnetate was eluted from a commercial $^{99}$Mo–$^{99m}$Tc generator with saline. The $^{99m}$Tc-pertechnetate in saline (13 mCi, 0.5–1.0 mL) was added in a 10-mL vial containing ferrocene precursor 20 (1.0 mg), Mn(CO)$_5$Br (3.0 mg) and dimethylformamide (DMF) (0.6 mL). The vial was then sealed with an aluminum-capped rubber stopper. For safety, the pressure of gas was balanced through a syringe inserted in the rubber stopper. The reaction mixture was heated at 140 °C for 1 h. After cooling to room temperature, the crude product was poured over 3 mL H$_2$O, extracted by CH$_2$Cl$_2$, and then passed through 0.22 µm hydrophobic membranes (about 7.4 mCi remaining). The residue was purified by HPLC using 35% acetonitrile (with 0.1% TFA) and 65% H$_2$O (with 0.1% TFA) as the mobile phase (Agela Venusil MP C18 column, 250 mm × 4.6 mm, 5 µm, 1 mL/min). The product of $[^{99m}$Tc]23 accounts for 80–90% of all radioactivities. And the calculated radiochemical yield was about 50–60% (n > 3, decay-corrected). To identify the radioactive product, stable rhenium compound 10a was
co-injected and co-eluted with $[^{99m}\text{Tc}]{\text{23}}$ (Agela Venusil MP C18 column, 250 mm × 4.6 mm, 5 μm, 1 mL/min; eluent: 40% acetonitrile with 0.1% TFA).

For radiolabeling of $[^{99m}\text{Tc}]{\text{24}}$, precursor 21 (1.0 mg) and $[^{99m}\text{Tc}]$-pertechnetate in saline (11.5 mCi, 0.5–1.0 mL) were added. The reaction mixture was heated at 140 °C for 1 h. After cooling to room temperature, the crude product was poured over 3 mL H$_2$O, extracted by CH$_2$Cl$_2$, and then passed through 0.22 μm hydrophobic membranes (about 6.8 mCi remaining). The residue was purified by HPLC using 30% acetonitrile (with 0.1% TFA) and 70% H$_2$O (with 0.1% TFA) as the mobile phase (Agela Venusil MP C18 column, 250 mm × 4.6 mm, 5 μm, 1 mL/min). The product of $[^{99m}\text{Tc}]{\text{24}}$ accounts for 80–90% of all radioactivities. And the calculated radiochemical yield was about 50–60% (n = 3, decay-corrected). To identify the radioactive product, stable rhenium compound 10b was co-injected and co-eluted with $[^{99m}\text{Tc}]{\text{24}}$ (eluent: 35% acetonitrile with 0.1% TFA).

For radiolabeling of $[^{99m}\text{Tc}]{\text{25}}$, precursor 22 (1.0 mg) and $[^{99m}\text{Tc}]$-pertechnetate in saline (5.3 mCi, 0.5–1.0 mL) were added. The reaction mixture was heated at 160 °C for 1 h. After cooling to room temperature, the crude product was poured over 3 mL H$_2$O, extracted by CH$_2$Cl$_2$, and then passed through 0.22 μm hydrophobic membranes (about 3.4 mCi remaining). The residue was purified by HPLC using 40% acetonitrile (with 0.1% TFA) and 60% H$_2$O (with 0.1% TFA) as the mobile phase (Agela Venusil MP C18 column, 250 mm × 4.6 mm, 5 μm, 1 mL/min). The product of $[^{99m}\text{Tc}]{\text{25}}$ accounts for 75–85% of all radioactivities. And the calculated radiochemical yield was about 50–60% (n = 3, decay-corrected). To identify the radioactive product, stable rhenium compound 16a was co-injected and co-eluted with $[^{99m}\text{Tc}]{\text{25}}$ (eluent: 35% acetonitrile with 0.1%
Measurement of log $D$ values

The log $D$ values of $[^{99m}\text{Tc}]^{23-25}$ were determined by measuring the distribution of the radiotracer between 1-octanol and 0.05 mol·L$^{-1}$ sodium phosphate buffer at pH 7.4. The two phases were pre-saturated with each other. 1-Octanol (3 mL) and phosphate buffer (3 mL) were pipetted into a 15 mL plastic centrifuge tube, and 20 µL HPLC-purified radiotracer (370 kBq) in saline was added. The tube was vortexed for 3 min followed by centrifugation for 5 min (3500 rpm, AnkeTDL80-2B, China). Approximately 50 μL of the 1-octanol layer was weighed. The 1-octanol layer was removed, and approximately 500 μL of the buffer layer was weighed. The activity in both tubes was measured in an automatic γ-counter (Wallac 1470 Wizard, USA). Accurate volumes of each counted phase were determined by weight and known densities. The distribution coefficient was determined by calculating the ratio of cpm/mL in the 1-octanol layer to that in the buffer layer and expressed as log $D$. Samples from the 1-octanol layer were redistributed until a consistent distribution of coefficient values was obtained. The measurement was performed in triplicate and repeated three times.

Biodistribution studies in mice and blocking studies.

All animal experiments in ICR mice (n = 5, 4–5 weeks, 22–25 g) were performed in compliance with the national laws related to the care and experiments on laboratory animals. For the biodistribution studies, HPLC-purified $[^{99m}\text{Tc}]^{23}$ or $[^{99m}\text{Tc}]^{24}$ (370 kBq in 0.1 mL saline) was injected via the tail vein. Mice were sacrificed by decapitation at 2,
15, 30, 60, 120, and 240 min. Samples of the blood, brain, heart, liver, spleen, lung, kidneys, muscle, and thyroid were removed, weighed and counted in an automatic γ-counter (Wallac 1470 Wizard, USA). The results were expressed in terms of the percentage of injected dose per gram (%ID/g) of blood or tissue.

For blocking studies, mice were injected via the tail vein with haloperidol (0.1 mL, 1.0 mg/kg) 5 min prior to radiotracer injection. All mice were sacrificed at 60 or 120 min postinjection. The blood and organs were isolated and analyzed as described above for the biodistribution study. Significant differences between control and test groups were determined by Student’s t test (independent, two-tailed). The criterion for significance was $p\leq0.05$.

**In Vivo Radiometabolic Stability of [$^{99m}$Tc]23.**

The *in vivo* metabolism of [$^{99m}$Tc]23 was studied in male ICR mice. The mice were intravenously administered a saline solution of [$^{99m}$Tc]23 (33 MBq, 0.15 mL) via the tail vein and sacrificed by decapitation at 15 min after radiotracer injection. The blood, brain and liver were collected. The brain and liver were washed with saline and homogenized for 5 min with a LabGEN 7 homogenizer in 2 mL ice-cold acetonitrile. Protein precipitation was achieved with ice-cold acetonitrile. The mixture was vortexed and centrifuged at 14,000 rpm for 5 min (Eppendorf Centrifuge 5418). Acetonitrile extracts were collected and passed through a 0.22 μm organic Millipore filter. The filtrates were concentrated to 0.1 mL under a stream of nitrogen gas and then injected into the radio-HPLC system for analysis (Waters 600 system, Agela Venusil MP C18 column, 250 mm × 4.6 mm, 5 μm, eluent 35% acetonitrile (0.1% TFA) and 65% water (0.1% TFA),
Effect of P-gp on the brain uptake of $[^{99m}\text{Tc}]23$ in mice.

For the investigation of the effect of permeability-glycoprotein (P-gp) on the brain uptake of $[^{99m}\text{Tc}]23$, mice were injected via the tail vein with either saline (0.1 mL) or cyclosporine A (0.1 mL, 50.0 mg/kg) 60 min prior to $[^{99m}\text{Tc}]23$ injection (370 kBq in 0.1 mL saline). All mice were sacrificed at 2 min postinjection. The blood and brain were isolated and analyzed as described above for the biodistribution study. Significant differences between control and test groups were determined by Student’s $t$ test (independent, two-tailed). The criterion for significance was $p \leq 0.05$. 
2. **Purity of key target compounds.**

UV-wavelength: 254 nm

<table>
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<th>Compd</th>
<th>Flow rate (mL/min)</th>
<th>Mobile phase (acetonitrile (0.1% TFA) : water (0.1% TFA))</th>
<th>Column (AgelaVenusil MP C18, 5 μm)</th>
<th>Retention time (t&lt;sub&gt;R&lt;/sub&gt;, min)</th>
<th>Purity (%)</th>
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Figure S1. HPLC profiles of 10a−10c, 16a−16c, 20−22.
3. HPLC analysis of lipophilicity of rhenium complexes 10a and 16a.

Figure S2. HPLC profile of 10a and 16a.
4. HPLC coinjection profiles of $[^{99}\text{mTc}]23$ and 10a, $[^{99}\text{mTc}]24$ and 10b, $[^{99}\text{mTc}]25$ and 16a.

Figure S3. HPLC co-elution profiles (A) $[^{99}\text{mTc}]23$ and 10a, $[^{99}\text{mTc}]23$ $t_R = 7.13$ min, 10a $t_R = 6.82$ min (40% acetonitrile with 0.1% TFA); (B) $[^{99}\text{mTc}]24$ and 10b, $[^{99}\text{mTc}]24$ $t_R = 12.88$ min, 10b $t_R = 11.76$ min (35% acetonitrile with 0.1% TFA); (C) $[^{99}\text{mTc}]25$ and 16a, $[^{99}\text{mTc}]25$ $t_R = 8.73$ min, 16a $t_R = 8.48$ min (35% acetonitrile with 0.1% TFA).
References: