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Research Article

Preparation and biological evaluation of ^{99m}TcN-4-(cyclohexylpiperazin-1-yl)-dithioformate as a potential sigma receptor imaging agent

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Abstract: The goal of this study is to develop a novel ^{99m}Tc-labeled σ receptor imaging agent. Potassium 4-(cyclohexylpiperazin-1-yl)-dithioformate, **2**, and the corresponding rhenium complex, **ReN-2**, were synthesized and characterized. **ReN-2** possessed moderate affinity toward σ_1 ($K_i = 1.94 \pm 0.60$ μmol/L) and σ_2 ($K_i = 2.83 \pm 1.39$ μmol/L) receptors. The radiolabeled complex **^{99m}TcN-2** was prepared in high yield (> 95%) through the $[^{99m}TcN]^{2+}_{int}$ precursor and characterized by HPLC. **^{99m}TcN-2** was found to be a lipophilic and neutral complex with good stability. The biodistribution in tumor-bearing mice showed that **^{99m}TcN-2** had good tumor uptake (2.12 ± 0.01 %ID/g at 2 h p.i.) and moderate brain uptake (0.27 ± 0.05 %ID/g at 2 h p.i.). After blocking with haloperidol, the uptakes by tumor and brain were lower than control. The results indicated that the complex has specific binding to the σ receptors *in vivo*. Further structural modifications of this complex are needed to obtain ^{99m}Tc-based σ receptor imaging agents with high affinity and subtype selectivity. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: technetium-99m; sigma receptor; tumor imaging agent; biodistribution

Introduction

Sigma receptors were first described by Martin in 1976 as a subtype of opioid receptors.[1,2] However, subsequent studies revealed that sigma receptors are a distinct class, divided into at least two subtypes termed σ_1 and σ_2.[2,3] They are localized in the cell cytoplasm of brain, internal organs, endocrine, immune and reproductive tissues. Sigma receptors are thought to be involved in several psychiatric and neurological disorders involving the central nervous system, such as schizophrenia, movement disorders, depression, anxiety, drug abuse and pain.[4,5] Moreover, a number of studies have demonstrated an overexpression of sigma receptors in human tumors, such as melanoma, breast cancer, small cell lung carcinoma and prostate cancer.[6,7] The high expression of sigma receptors in tumors suggested that sigma receptors selective ligands could play an important role as biomarkers of tumor diagnosis by SPECT scintigraphy and PET analysis.[8]

Significant progress has been made in the development of ^{11}C- and ^{18}F- labeled sigma receptors ligands for PET imaging and ^{123}I-labeled sigma receptors ligands for SPECT imaging, such as benzamides,[9–13] disubstituted piperidines[14–18] and piperazines.[19,20] Compared to ^{11}C, ^{18}F and ^{123}I, ^{99m}Tc is the most common radionuclide in routine nuclear medicine due to its almost ideal physical decay properties ($T_{1/2} = 6.02$ h, $E_\gamma = 140$ keV) and the availability through commercial ^{99}Mo/^{99m}Tc generator. Over the past few years, several ^{99m}Tc-based complexes for sigma receptors imaging have been reported, such as [^{99m}Tc]BAT-EN6,[21] ^{99m}Tc-labeled analogue of *O*-(9-benzyl-9-azabicyclo[3.3.1]nonan-3α-yl)-*N*-(2-methoxy-4-methylphenyl)carbamate[22,23] and PPPE-MA-MA'-^{99m}TcO.[24] However, these ^{99m}Tc complexes are far from ideal and cannot be used in the clinic, and the

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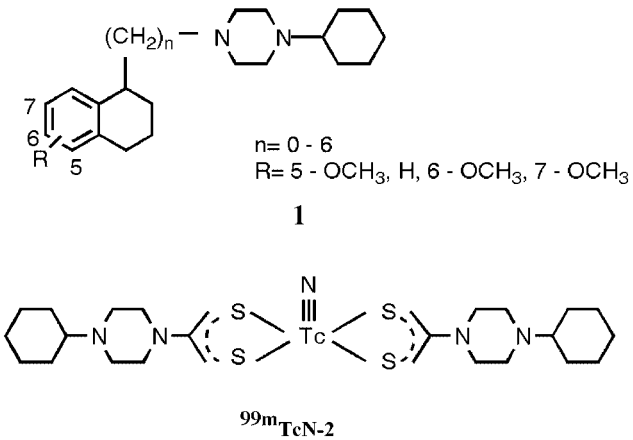


Figure 1 Structures of the lead compound **1** and **^{99m}TcN-2**.

effort to find new ^{99m}Tc-labeled complexes as sigma receptors imaging agents continues. All of the reported ^{99m}Tc-labeled complexes for sigma receptors imaging are based on the N_2S_2 ligand system with the $[^{99m}TcO]^{3+}$ core until now. Recently, several complexes based on the ^{99m}Tc-nitrido core have been proposed to design receptor-specific ^{99m}Tc-tracers.[25] Compared to the ^{99m}Tc-oxo core, ^{99m}Tc-nitrido core is more stable toward oxidation and reduction.

Several 1-cyclohexylpiperazine derivatives have been reported to show high affinities toward σ_1 (K_i = 0.036–13.6 nmol/L) and σ_2 (K_i = 0.34–30.5 nmol/L)[26] (Figure 1). To develop a novel ^{99m}Tc-labeled sigma receptors imaging agent, we designed a ^{99m}Tc-labeled analogue of 1-cyclohexylpiperazine based on the ^{99m}Tc-nitrido core. Reported herein are the syntheses of the ligand and corresponding Re complex, radiolabeling, *in vitro* binding affinity and preliminary *in vivo* evaluation of the ^{99m}Tc-nitrido complex **^{99m}TcN-2** (Figure 1).

Results and discussion

Chemistry

Potassium 4-(cyclohexylpiperazin-1-yl)-dithioformate, **2**, was prepared by the treatment of 1-cyclohexylpiperazine with carbon disulfide in basic condition with 63% yield. The product was characterized by ^{1}H-NMR, GC-MS and elemental analysis.

Since the chemistry of technetium and rhenium is similar, the corresponding rhenium complex was prepared for *in vitro* binding studies. Treatment of **2** with $[NBu_4][ReNCl_4]$ gave the corresponding rhenium complex, **ReN-2** with 66% yield. The complex was characterized by IR, NMR, MALDI-TOF mass and elemental analysis.

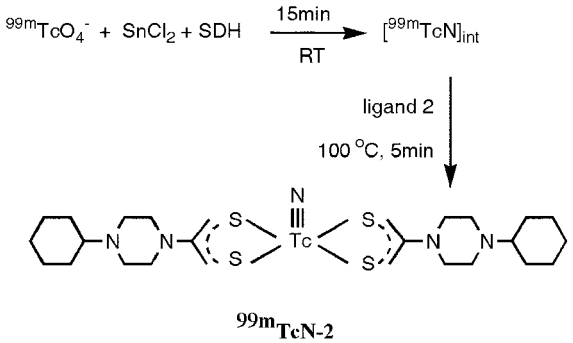
In vitro binding studies

In vitro binding studies were conducted in order to measure the affinity of compound **2** and **ReN-2** for σ_1 and σ_2 receptors using established assays for these receptors. The K_i values of **2** were 0.13 ± 0.04 and 0.91 ± 0.08 μmol/L toward σ_1 and σ_2 receptors, respectively. The K_i values of **ReN-2** were 1.94 ± 0.60 and 2.83 ± 1.39 μmol/L toward σ_1 and σ_2 receptors, respectively. Notably, the derivative still maintained certain affinity for σ_1 and σ_2 receptors although it is much lower than the affinities reported for the lead structures.

Radiochemistry

The complex **^{99m}TcN-2** can be prepared by a two-step procedure, as reported in Scheme 1.The SDH kit vial, containing the following lyophilized formulation: 0.05 mg $SnCl_2 \cdot 2H_2O$, 5.0 mg succinic dihydrazide (SDH) and 5.0 mg propylenediamine tetraacetic acid (PDTA), was used for preparing the $[^{99m}TcN]^{2+}$ intermediate. Quality control of the $[^{99m}TcN]^{2+}$ intermediate was performed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). The final ^{99m}Tc-nitrido complex, **^{99m}TcN-2**, was obtained with high yield (> 95%) by reaction of the ligand, **2**, and $[^{99m}TcN]^{2+}$ intermediate at 100°C for 5 min. **^{99m}TcN-2** was further purified by HPLC to remove the excess ligand, **2**, before the injection of **^{99m}TcN-2** to mice for *in vivo* study.

TLC and HPLC analyses were used to evaluate the radiochemical purity (RCP) and the stability of the complex. TLC was performed on a polyamide film with a mixture of CH_2Cl_2 and CH_3OH ($V/V = 9:1$) as the mobile phase. R_f values for $[^{99m}TcN]^{2+}$ intermediate was 0–0.1 and for **^{99m}TcN-2** was 0.7–1.0. The HPLC chromatogram of **^{99m}TcN-2** is shown in Figure 2. The retention time of **^{99m}TcN-2** was found to



Scheme 1

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be about 32.60 min, while that of $[^{99m}TcN]^{2+}$ intermediate was 3.2 min.

The paper electrophoresis pattern of **^{99m}TcN-2** showed that more than 90% of the activity was maintained at the point of application, indicating neutrality of the complex. The partition coefficient (*P*) of the complex was 31.06 ± 1.78 ($\log P = 1.49$) at pH 7.0, which showed that it was a lipophilic complex.

The *in vitro* stability of the complex was evaluated by measuring the RCP at different time points. The result showed that it was stable over 6 h after diluted with saline. After 4 h of incubation with mouse plasma at 37°C, the RCP was still over 90%, which suggested that the complex **^{99m}TcN-2** possessed high stability.

Biodistribution and blocking studies

Because **ReN-2** has a moderate affinity for the sigma receptor, the biodistribution of **^{99m}TcN-2** was performed in TA2 mice bearing tumor of mammary cancer MA-891. The results are shown in Table 1. The radiotracer excretion from the body occurred mainly via the hepatobiliary system due to the high lipophilicity of the complex. The complex **^{99m}TcN-2** displayed a significant tumor uptake of 2.12 ± 0.01 %ID/g at 2 h p.i. The ratios of tumor/blood and tumor/muscle rose with time were 1.22 and 1.09 at 1 h p.i., 2.92 and 1.93 at 4 h p.i., respectively. It also had a moderate uptake in the brain (0.27 ± 0.05 %ID/g at 2 h p.i.), an organ that is known to possess high densities of σ_1 receptors. The complex displayed an increase in uptake in tumor and brain between 1 and 4 h p.i., which may be due to a slow pharmacokinetics of **^{99m}TcN-2**.

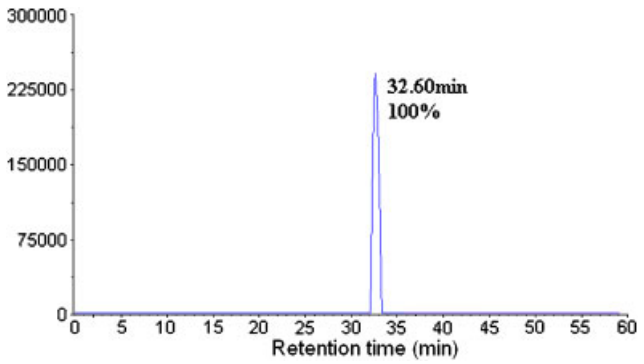

Figure 2 The HPLC chromatogram of **^{99m}TcN-2**. Figure available in colour online at www.interscience.wiley.com

To further characterize the uptake specificity of **^{99m}TcN-2**, haloperidol (1 mg/kg) was co-injected with **^{99m}TcN-2** in tumor-bearing mice. The mice were sacrificed at 2 h p.i. The blocking study resulted in an inhibition of 54.7% binding at 2 h p.i. in the tumor. Similarly, a significant reduction in uptake of radioactivity was also found in brain, liver, lung and muscle (Table 1).

These data provide evidence that the complex **^{99m}TcN-2** binds specifically at sigma receptors *in vivo*. The results also suggested that the MA-891 cell line, a mouse mammary carcinoma line, might be overexpressing sigma receptors. Additional studies are needed to measure the density of sigma receptors in the MA-891 tumors.

Methods and materials

Materials

If not otherwise stated, all chemicals were purchased from Sigma. 1-Cyclohexyl- piperazine was purchased from Fluka (97%). The complex $[NBu_4][ReNCl_4]$ was

Table 1 Biodistribution of ^{99m}TcN-2 in TA2 mice bearing tumor of mammary cancer MA-891 ($n = 3$)

	%ID/g (mean ± SD)			
	1 h	2 h	2 h (blocking)	4 h
Tissue				
Heart	5.38 ± 0.40	3.61 ± 0.50	3.61 ± 0.59	3.06 ± 1.13
Liver	25.63 ± 4.40	31.82 ± 6.10	25.87 ± 2.54	44.10 ± 7.68
Lung	4.34 ± 0.48	3.93 ± 0.28	2.77 ± 0.39	3.32 ± 0.01
Blood	1.37 ± 0.45	0.87 ± 0.04	0.60 ± 0.10	0.79 ± 0.02
Kidney	4.36 ± 0.75	4.08 ± 0.20	3.96 ± 0.36	4.30 ± 0.29
Brain	0.16 ± 0.03	0.27 ± 0.05	0.11 ± 0.03	0.36 ± 0.04
Muscle	1.53 ± 0.05	1.23 ± 0.04	0.96 ± 0.10	1.20 ± 0.14
Bone	1.03 ± 0.33	1.23 ± 0.22	0.77 ± 0.19	1.21 ± 0.24
Spleen	5.36 ± 0.59	4.94 ± 0.65	6.32 ± 1.19	4.72 ± 0.74
Tumor	1.67 ± 0.27	2.12 ± 0.01	0.96 ± 0.21	2.31 ± 0.07
Ratios				
Tumor/blood	1.22	2.44	1.60	2.92
Tumor/muscle	1.09	1.72	1.00	1.93

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prepared according to the literature methods.[27] [^{3}H](+) Pentazocine (1.354×10^{12} Bq/mmol, Perkin–Elmer Life Sciences, Boston, MA, USA) and [^{3}H]DTG (1.147×10^{12} Bq/mmol, NEN Life Science Products, Boston, MA, USA) were applied as σ receptor ligands in the receptor competition binding assays. All other chemicals were of reagent grade and used without further purification. Technetium-99m as sodium pertechnetate ($Na^{99m}TcO_4$) was obtained from commercial $^{99}Mo/^{99m}Tc$ generator (China Institute of Atom Energy) just before use. The SDH kit was provided by courtesy of Beijing Shihong Pharmaceutical Center. Paper electrophoresis experiments were performed on Whatman 3MM chromatography paper (Whatman, England). Polyamide-6 film (SIJIA Co., China) was used for TLC. Reversed-phase high-pressure liquid chromatography (RP-HPLC) experiments were performed on a SHIMAZU system with SCL-10AVP HPLC pump system (SHIMAZU Corporation, Japan) and liquid scintillation analyzer (Packard BioScience Co.,USA), using a C-18 column (4.6×250 mm, 5-μm particle size, Alltech Associates, Inc., USA) and methanol:water mixture as the mobile phase at a flow rate of 1 mL/min. IR spectrum was obtained with an AVATAR 360 FT-IR spectrometer using KBr pellets. NMR spectra were recorded on a Bruker Avance-500 (500 MHz) spectrometers. GC-MS was obtained with a GC 2000/TRACE MS mass spectrometer (Finigan Co., USA), and TOF-MS was obtained with a BIFLEXII MALDI-TOF mass spectrometer (Bruker, USA). Elements analyses were carried out on a Vario EL elemental analyzer (Elementar Co., Germany).

Synthesis of potassium 4-(cyclohexylpiperazin-1-yl)-dithioformate (2)

The dithiocarbamate ligand was prepared by treatment of the amine with carbon disulfide in basic condition. 1-Cyclohexylpiperazine (0.5 g, 3.0 mmol) was dissolved in ethanol (10 mL), and 3.5 mL of a 1 mol/L KOH solution (3.5 mmol) was added. While the solution was stirred in an ice bath at 0°C, 0.2 mL of carbon disulfide was added dropwise. The solution was stirred at room temperature for 3 h. The solvent was removed under vacuum. The solid residue was recrystallized from ethanol to give 0.57 g potassium 4-(cyclohexylpiperazin-1-yl)- dithioformate, **2**, as pale yellow crystals. ^{1}H-NMR (500 MHz, D_2O) δ: 0.98–1.53 and 1.67–1.84 (m, 10H, cyclohexyl (CH_2)$_5$), 2.21 (m, 1H, NCH), 2.57 (m, 4H, N(CH_2)$_2$), 4.26 (m, 4H, (CH_2)$_2$NCS_2). GC-MS: calculated for *m/z* $C_{11}H_{19}N_2S_2$ (M^-): 243, found 243. Anal. (%) calculated for $C_{11}H_{19}N_2S_2K \cdot H_2O$: C 43.94, H 7.06, N 9.32; found C 43.60, H 7.35, N 9.23.

Synthesis of ReN-2

The compound [NBu_4][$ReNCl_4$] (60 mg, 0.1 mmol) was dissolved in acetone (50 mL), and **2** (90 mg, 0.3 mmol) dissolved in methanol (20 mL) was added. The mixture was refluxed for 3 h to give a yellow-brown solution. The solution was filtered and concentrated *in vacuo*. The volume was reduced to 10 mL, and 10 mL of diethylether was added. Upon cooling, the solid was collected, washed with water and recrystallized from CH_2Cl_2–methanol to give the complex **ReN-2**, as tiny pale yellow-brown crystals, yield 66%. IR (cm^{-1},KBr): 1126.4(Re ≡ *N*). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 1.12–1.28 and 1.66–1.69 (m, 10H cyclohexyl (CH_2)$_5$), 2.38 (m, 1H, NCH), 2.74 (m,4H, N(CH_2)$_2$), 3.86–4.13 (m, 4H, (CH_2)$_2$NCS_2). MALDI-TOF-MS: calculated for *m/z* $C_{22}H_{39}N_5S_4Re$ (M + 1): 688.1, found 688.6. Anal. (%) calculated for $C_{22}H_{38}N_5S_4Re$: C 38.45, H 5.59, N 10.19; found C 38.22, H 5.47, N 10.13.

Preparation of ^{99m}TcN-2

The complex **^{99m}TcN-2** was prepared by a two-step procedure. First, 1 mL saline containing $^{99m}TcO_4^-$ (370 MBq) was added into a SDH kit containing 0.05 mg of stannous chloride dihydrate, 5.0 mg of SDH, 5.0 mg of PDTA, then kept for 15 min at room temperature to obtain the $[^{99m}TcN]^{2+}$ intermediate. Second, the ligand **2** (1.0 mg) was added into the $[^{99m}TcN]^{2+}$ intermediate, and the reaction mixture was sealed and heated at 100°C for 5 min. After cooling to room temperature, the reaction mixture was carefully extracted with CH_2Cl_2 (2×1 mL). The organic layer was concentrated *in vacuo* and purified by HPLC using a C-18 column with a mobile phase of water (A): methanol (B) mixture (0–10 min, 70% B, 10–20 min, 70–90% B, 20–60 min, 90% B) at a flow rate of 1 mL/min. The retention time was 32.6 min for ^{99m}TcN-2. The collected HPLC fraction of ^{99m}TcN-2 was evaporated and diluted with ethanol (0.2 mL) and saline (5 mL).

The $[^{99m}TcN]^{2+}$ intermediate and the final complex ^{99m}TcN-2 were identified by TLC and HPLC.

In vitro stability

The *in vitro* stability of **^{99m}TcN-2** was evaluated by monitoring the RCP at different time points. The solution was diluted with 4–5 mL saline and kept at room temperature for 6 h. The RCP was determined by TLC and HPLC chromatography at 30 min, 1, 2, 4 and 6 h.

To 900 μL of fresh mouse plasma, 100 μL of the ^{99m}Tc complex solution was added and incubated at 37°C. At

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30 min, 2 and 4 h, 100 μL aliquots were withdrawn and treated with 200 μL ethanol to precipitate the proteins. Samples were then cooled at 4°C and centrifuged at 3000 rpm. The supernatant was analyzed by TLC.

Paper electrophoresis

Sample of the final complex **^{99m}TcN-2** was spotted at the center of the Whatman 3MM chromatography paper strips (10 cm × 1 cm) which were pre-treated with phosphate buffer (0.05 mol/L, pH = 7.4). The analyses were carried out using phosphate buffer (0.05 mol/L, pH = 7.4) at 150 V for 2 h. The developed paper strips were left to dry, and the distribution of radioactivity on the strip was determined.

Octanol/water partition coefficient

The octanol/water partition coefficient of **^{99m}TcN-2** was measured following 1 min vigorous vortex mixing of 2 mL of octanol, 1.9 mL of phosphate buffer (0.05 mol/L, pH = 7.0), and 100 μL of test solution in a centrifuge tube. The sample was centrifuged at 4000 rpm for 5 min and the counts in 100 μL aliquots of both organic and inorganic layers were determined using a NaI well-type γ-counter. The partition coefficient (*P*) was calculated using the following equation: P = (cpm in octanol −cpm background)/(cpm in water − cpm background). The reported octanol/water partition coefficient represents the mean of three measurements.

Sigma receptor competition binding assay

The affinities of **ReN-2** for sigma receptors were determined in tissues that are enriched in the respective σ receptor subtypes: rat brain homogenates for σ_1 receptors and rat liver homogenates for σ_2 receptors.[28] Use of animals was carried out in accordance with the national laws regulating experiments on animals and followed the principles of laboratory animal care.

Female Sprague–Dawley rats (8 weeks) were anesthetized and sacrificed by decapitation. Membranes of the rat cortex were prepared as described recently.[24] The final membrane fraction was re-suspended in standard buffer and stored at −25°C. Preparation and storage of liver membranes were carried out accordingly.

The *in vitro* affinity of ReN-2 was determined by a method previously described. The membrane homogenates from rat cortices and [^{3}H](+)pentazomicine (0.8 nmol/L, K_D = 6.9 nmol/L[20]) were used for the σ_1 receptors. For measuring the binding for σ_2 receptors, rat liver membrane homogenates and [^{3}H] DTG (0.5 nmol/L, K_D = 29.2 nmol/L[20]) in the presence of 10 μM dextrallorphan ($K_{i,\sigma 1}$ = 125 nmol/L,[29] to mask σ_1 sites) were used. The respective membrane fractions were thawed on ice, diluted with incubation buffer (50 mmol/L Tris–HCl, pH 7.4, 21°C) and re-homogenized by a 27-gauge needle. The protein concentrations were measured by the BCA assay (approx. 170 μg/mL) (Perbio Sciences, Germany). Nonspecific binding was determined in the presence of 10 μmol/L haloperidol. Incubations containing ReN-2 at the indicated concentrations were carried out at 21°C for 120 min. The DMSO content at the highest concentration of the dilution curves did not exceed 1%. Non-labeled haloperidol (to σ_1 receptors: seven concentrations from 0.01 nmol/L to 0.1 μmol/L; to σ_2 receptors: 13 concentrations from 0.1 nmol/L to 1 μmol/L) and DTG (to σ_2 receptors: six concentrations from 0.1 nmol/L to 0.1 μmol/L) were applied in addition to competitive experiments in order to verify the chosen incubation protocol.

All experiments were performed in triplicate. The binding parameter IC_{50} was estimated using iterative non-linear curve fitting. The Cheng–Prusoff equation was applied to calculate K_i values from the estimated IC_{50} values.[30]

Biodistribution and blocking study

The *in vivo* biodistribution study of **^{99m}TcN-2** was carried out in TA2 mice bearing tumor of mammary cancer MA-891. The complex **^{99m}TcN-2** (100 μl, 74 kBq) was injected into the mice via the tail vein. Then, the mice (n = 3) were sacrificed by cervical dislocation at 1, 2 and 4 h post-injection. The organs or tissues of interest were removed, weighted and measured in a well-type NaI(Tl) gamma counter. The percentage of injected dose per gram (%ID/g) for each sample was calculated by comparing its activity with appropriate standard of injected dose (ID), the values are expressed as mean ± SD.

To further determine the receptor-specific uptake of ^{99m}TcN-2, the blocking experiment was performed. Haloperidol (1 mg/kg), a non-selective sigma receptor ligand was co-injected with **^{99m}TcN-2**. The mice (n = 3) were sacrificed at 2 h post-injection. All tissues were treated as described above.

All biodistribution studies were carried out in compliance with the national laws related to the conduct of animal experimentation.

Conclusion

A novel ^{99m}Tc-labeled complex (**^{99m}TcN-2**) and the corresponding rhenium complex have been designed, synthesized and characterized as potential σ receptors ligands. The complex **ReN-2** possesses moderate

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affinity for σ_1 and σ_2 receptors. **^{99m}TcN-2** was prepared in high yield (> 95%) through the $[^{99m}TcN]^{2+}_{int}$ precursor. It was found that **^{99m}TcN-2** was a lipophilic and neutral complex with good stability. *In vivo* biodistribution showed that **^{99m}TcN-2** had good tumor uptake and moderate brain uptake. The result of the blocking study showed a significant reduction in the uptakes by tumor, brain, liver, lung and muscle, which indicated the specific binding of this radiotracer to the σ receptors *in vivo*. **^{99m}TcN-2** may be used as a lead complex for further structural modifications to obtain ^{99m}Tc-based σ receptors imaging agents with high affinity and subtype selectivity.

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