Bioorganic & Medicinal Chemistry 18 (2010) 396–402

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

^{99m}Tc-tricarbonyl labeled agents for cell labeling: Development, biodistribution in normal mice and preliminary in vitro evaluation

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ARTICLE INFO

Article history:
Received 3 August 2009
Revised 23 October 2009
Accepted 24 October 2009
Available online 31 October 2009

Keywords:
Cell labeling
^{99m}Tc(CO)$_3$
Lipophilic hexadecyl derivatives

ABSTRACT

We have developed four ^{99m}Tc(CO)$_3$-labeled lipophilic tracers as potential radiolabeling agents for cells based on a hexadecyl tail. ^{99m}Tc(CO)$_3$-hexadecylamino-*N*,*N*′-diacetic acid (negatively charged), ^{99m}Tc(CO)$_3$-hexadecylamino-*N*-α-picolyl-*N*′-acetic acid (uncharged), ^{99m}Tc(CO)$_3$-*N*,*N*′-dipicolylhexadecylamine (positively charged), ^{99m}Tc(CO)$_3$-*N*-hexadecylaminoethyl-*N*′-aminoethylamine (positively charged) were prepared in a radiolabeling yield: >90%. Preliminary cell uptake studies were performed in mixed blood cells with or without plasma and were compared with ^{99m}Tc-d,l-HMPAO and [^{18}F]FDG. In plasma-free blood cells, maximum uptake (78%) was obtained for ^{99m}Tc(CO)$_3$-*N*-hexadecylaminoethyl-*N*′-aminoethylamine after 60 min incubation (compared to 55% and 23% for ^{99m}Tc-d,l-HMPAO and [^{18}F]FDG, respectively) while in plasma-rich medium, ^{99m}Tc(CO)$_3$-*N*,*N*′-dipicolylhexadecylamine was best bound (54%, similar to the binding of ^{99m}Tc-d,l-HMPAO). Biodistribution in normal mice showed mainly hepatobiliary clearance of the agents and initial high lung uptake. The radiolabeled compounds showed good blood clearance with maximally 7.9% injected dose per gram at 60 min post injection. While the least lipophilic agent (^{99m}Tc(CO)$_3$-*N*,*N*′-dipicolylhexadecylamine, log *P* = 1.3) showed the best cell uptake, there appears to be no direct correlation between lipophilicity and tracer uptake in mixed blood cells. In view of its comparable cell uptake to well known cell labeling agent ^{99m}Tc-d,l-HMPAO, ^{99m}Tc(CO)$_3$-*N*,*N*′-dipicolylhexadecylamine merits further evaluation as a potential cell labeling agent.

1. Introduction

Cell-based therapies hold great promise in the treatment of many human diseases notably in cardiology, neurology and genetically inheritable diseases.[1–4] Tracking of these cells is necessary to evaluate the efficacy of therapy as well as plan subsequent treatment regimens. In cardiology, radiolabeled stem cells have been used on numerous occasions to study the migration, distribution and efficacy of therapy following administration of stem cells.[5–7] In some instances labeled cells have been used to understand the involvement of certain cells or factors in the pathogenesis of diseases. For example, tracking of endothelial progenitor cells labeled with [^{18}F]-fluorodeoxylglucose ([^{18}F]FDG) has been used to study their involvement in vascuologenesis and angiogenesis hence their involvement in tumor growth and development using positron emission tomography (PET).[8] These and many other interesting preclinical studies on cell-based therapies have led to a growing number of early phase human studies to demonstrate the potential usefulness of these cell-based therapies. However, in order for these therapies to finally reach the bedside, questions such as optimal time and route of delivery, their contribution to functional repair and biodistribution need to be answered. Labeling and tracking of cells is therefore an invaluable tool in the evaluation of cell-based therapies.

Indirect radionuclide labeling of cells using reporter genes-reporter probe concept provides one of the most elegant approaches to track in vivo the fate of administered cells. Accumulation of the reporter probe depends on the expression of a reporter gene product which directly correlates with cell viability.[9] However, design of the reporter gene requires extensive molecular biological techniques limiting this application to centers with such facilities. Indirect labeling involving incubation of cells with appropriate radiolabeling agents remains, however, very popular. Many radiopharmaceuticals have been used to radiolabel cells and track their fate in vivo. Agents for positron emission tomography (PET) such as [^{18}F]FDG only permit short tracking of labeled cells due to its short half-life ($t_{1/2}$ = 110 min). Recently, copper-64-pyruvaldehyde-bis(N^4-methylthiosemicarbazone) ([^{64}Cu]PTSM, ^{64}Cu, $t_{1/2}$ = 12.7 h) has been introduced as a potential cell radiolabeling agent even though its potential usefulness remains to be fully evaluated.[10] Technetium-99m labeled d,l-hexamethylpropylene amine oxime (^{99m}Tc-d,l-HMPAO, exametazime) is also a well established single photon emission computed tomography (SPECT) labeling agent.[11]

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doi:10.1016/j.bmc.2009.10.045

Recently, a simple method has been described for labeling stem cells using a lipophilic long-chain ester, hexadecyl-4-[^{18}F]fluorobenzoate ([^{18}F]HFB), which is efficiently and quickly absorbed onto the cellular membranes in a similar fashion as fluorescent dyes used for cell labeling.[12] Absorption onto cell membranes means less toxicity to cell's nuclei resulting from Auger electrons as in the case of indium-111 labeled cells. The short half-life of fluorine-18 means these cells cannot be tracked for a long period in addition to the fact that the use of short-lived cyclotron-based radionuclides is until now limited to centers with an onsite cyclotron. Labeling with technetium-99m ($t_{1/2}$ = 6 h) would be an advantage for longer cell tracking. Technetium-99m tricarbonyl ($^{99m}Tc(CO)_3^+$) labeling of biomolecules has the advantage of ease of preparation, efficient complex formation and inertness of the complex due the d^6 metal core.[13] We herein report the synthesis of lipophilic agents using ligand precursors with a hexadecyl tail and their labeling with $^{99m}Tc(CO)_3^+$ precursor (Fig. 1A–D). We have studied their biodistribution in normal NMRI mice and evaluated their potential for cell labeling in preliminary in vitro experiments using mixed blood cells. Uptake in cells was compared with that of ^{99m}Tc-d,l-HMPAO and [^{18}F]FDG, which are well known cell labeling agents.

2. Results and discussion

2.1. Chemistry and radiolabeling

Synthesis and labeling of hexadecylamino-*N*,*N*′-diacetic acid with $^{99m}Tc(CO)_3^+$ ($^{99m}Tc(CO)_3$-hexadecylamino-*N*,*N*′-diacetic acid, compound **3**) is shown in Scheme 1. Synthesis of hexadecylamino-*N*,*N*′-diacetic acid was accomplished in two steps starting from the alkylation of hexadecylamine with methyl bromoacetate followed by base hydrolysis in an overall yield of 75%. Radiolabeling with the $^{99m}Tc(CO)_3^+$ precursor (pH 7.4) was accomplished by incubating compound **2** with the precursor at 70 °C for 20 min. An overall radiolabeling yield of 97% was obtained resulting in a negatively charged complex (Fig. 1A, compound **3**).

$^{99m}Tc(CO)_3$-hexadecylamino-*N*-α-picolyl-*N*′-acetic acid (compound **8**) was prepared in four steps as shown in Scheme 2 by alkylation of α-picolylamine with methyl bromoacetate followed by monoester hydrolysis and in situ activation of the monomethyl ester (α-picolyl-*N*-methylacetyl-*N*′-acetic acid) with benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) in the presence of hexadecylamine. Hydrolysis yielded the final product hexadecylamino-*N*-α-picolyl-*N*′-acetic acid. Radiolabeling (pH 7.4 at 70 °C for 20 min) yield of 95% was obtained resulting in a neutral complex (Fig. 1B, compound **8**).

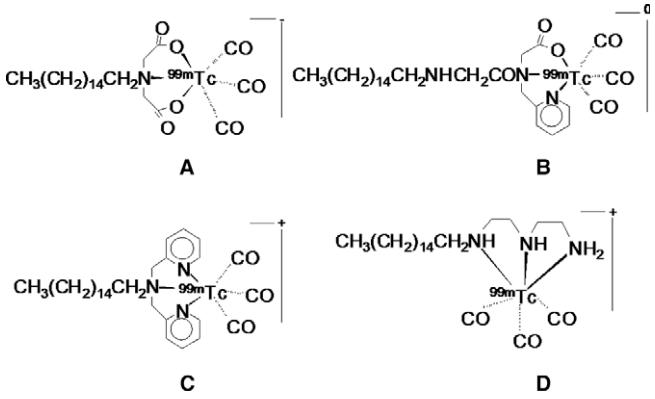
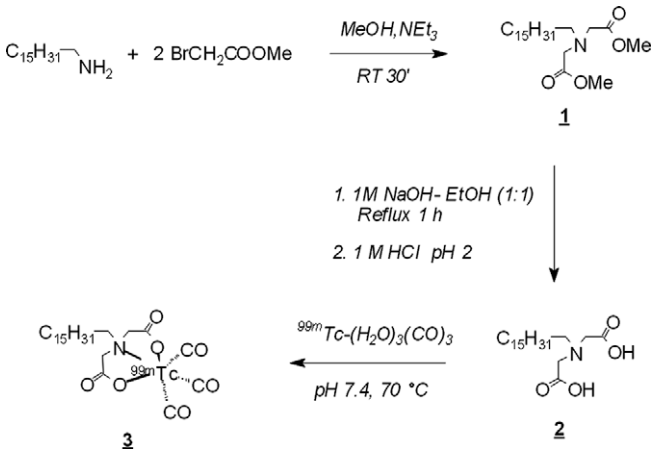
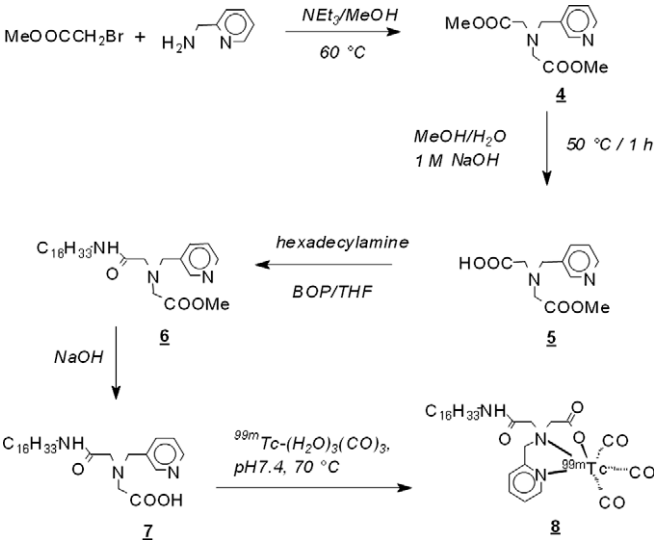


Figure 1. Proposed structure of (A) $^{99m}Tc(CO)_3$-hexadecylamino-*N*,*N*′-diacetic acid (compound **3**), (B) $^{99m}Tc(CO)_3$-hexadecylamino-*N*-α-picolyl-*N*′-acetic acid (compound **8**), (C) $^{99m}Tc(CO)_3$-*N*,*N*′-dipicolylhexadecylamine (compound **10**) and (D) $^{99m}Tc(CO)_3$-*N*-hexadecylaminoethyl-*N*′-aminoethylamine (compound **12**).

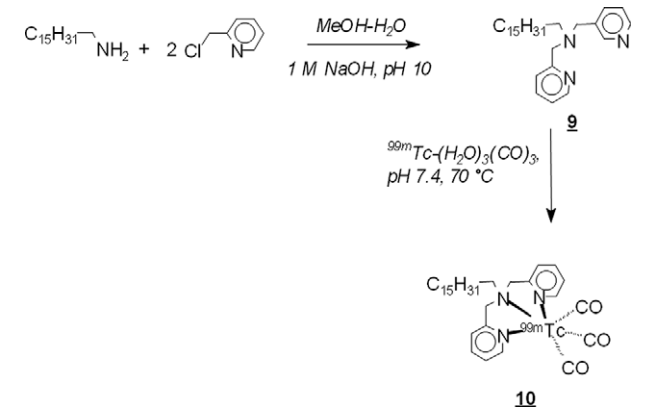


Scheme 1. Reaction scheme for the synthesis of hexadecylamino-*N*,*N*′-diacetic acid and labeling with $^{99m}Tc(CO)_3^+$.

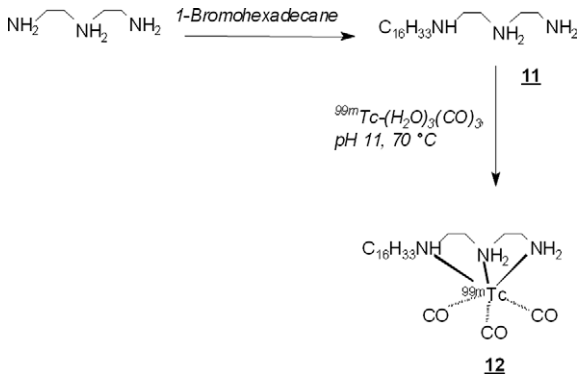


Scheme 2. Reaction scheme for the synthesis of hexadecylamino-*N*-α-picolyl-*N*′-acetic acid and labeling with $^{99m}Tc(CO)_3^+$.

$^{99m}Tc(CO)_3$-*N*,*N*′-dipicolylhexadecylamine (compound **10**) was synthesized as shown in Scheme 3 (overall chemical yield for the precursor was 12%). Radiolabeling (pH 7.4 at 70 °C for 20 min)



Scheme 3. Reaction scheme for the synthesis of *N*,*N*′-dipicolylhexadecylamine and labeling with $^{99m}Tc(CO)_3^+$.



Scheme 4. Reaction scheme for the synthesis of *N*-hexadecylaminoethyl-*N*′-aminoethylamine and labeling with $^{99m}Tc(CO)_3^+$.

yield: ⩾90% was achieved. $^{99m}Tc(CO)_3$-*N*-hexadecylaminoethyl-*N*′-aminoethylamine (compound **12**) was prepared in a one step akylation reaction of diethylenetriamine with 1-bromohexadecane. Radiolabeling (pH 11 at 70 °C for 20 min) yield of 99% was achieved (Scheme 4). Compounds **10** and **12** resulted in positively charged complexes (Fig. 1C and D). The structures of the precursors and final products were confirmed by high resolution mass spectroscopy (HRMS) using different standards and ^{1}HNMR. Stability study of HPLC-purified compounds **3**, **8**, **10** and **12** was examined after reinjection of the compounds on RP-HPLC over a 24 h period at rt. All compounds were >97% stable during this period.

Figure 2A–C shows reversed-phase (RP)-HPLC chromatograms of the crude radiolabeling mixture of compounds **3**, **8**, **10** and **12**, respectively. Compounds **8**, **10**, and **12** have identical retention times (t_R ~10.1 min) on RP-HPLC while compound **3** (t_R ~8 min) was least retained. Lipophilicity of the HPLC-purified compounds was determined by their octanol–buffer partition coefficients.

2.2. Octanol–buffer partition coefficient

Since the labeling of cells with [^{18}F]HFB was hypothesized to be due to absorption of the agent to cell membrane as a result of its lipophilicity, it was necessary in this preliminary study to correlate lipophilicity (log *P*) of the radioligands with their uptake in cells. Octanol–buffer partition coefficient values provide a more reliable measure of the relative lipophilicity. Log *P* values (mean of five determinations ± standard deviation, SD) of the tracers ranged from 1.3 to 2.5. The compounds were ranked according to increasing log *P* values (±standard deviation, SD): Compound **10** (1.3 ± 0.04) < compound **3** (2.17 ± 0.34) < compound **12** (2.22 ± 0.31) < compound **8** (2.50 ± 0.21). The log *P* of [^{18}F]HFB was not determined by Ma et al.[12] but is probably in the 2.5–3 range being a lipophilic ester,[14] while log *P* of ^{99m}Tc-d,l-HMPAO is 1.9.[15]

2.3. Preliminary in vitro cell labeling

Figure 3 shows that all the radiolabeled compounds were well bound in plasma-free mixed blood cells after 60 min. Uptake of the radiolabeled compounds in blood cells after an incubation period of 60 min (Fig. 3) in PBS (plasma-free mixed blood cells) with mixed blood cells was of the order: compound **12** (positively charged, 78%) > compound **8** (uncharged, 77%) > compound **10** (positively charged, 69%) > compound **3** (negatively charged, 55%). Cell uptake of all radiolabeled compounds was better than that of [^{18}F]FDG. When incubated with mixed blood cells containing plasma (Fig. 4), 54% of compound **10** was bound to blood cells (compared to 55% binding of ^{99m}Tc-d,l-HMPAO) while compounds **3** and **8** were more associated with plasma. The amount of the radiolabeled compound bound to blood cells after an incubation period of 60 min (in mixed blood cells containing plasma) was of the order: compound **10** (54%) > compound **12** (46%) > compound **8** (35%) > compound **3** (24%). The order indicates that the positively charged compound **10** had the best uptake in cells while the negatively charged compound **3** was more associated with plasma. High uptake in blood cells in plasma-rich medium means better uptake of the radiolabeled agent in vivo and less transchelation or association with plasma proteins. Although the least lipophilic (compound **10**, log *P* = 1.3) was the most bound to the cells, a clear relationship between lipophilicity and tracer uptake in the cells could not be established. Uptake of compound **10** was similar to ^{99m}Tc-d,l-HMPAO (Fig. 4), a widely used cell labeling compound.

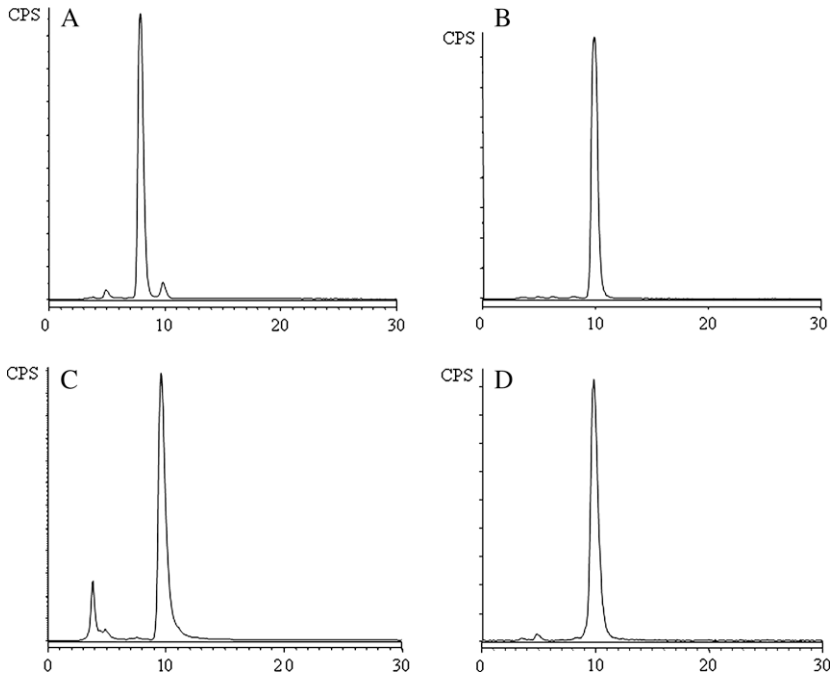


Figure 2. RP-HPLC chromatograms of crude labeling mixture of (A) compounds **3**, (B) compound **8**, (C) compound **10** and (D) compound **12** (see text for HPLC condition).

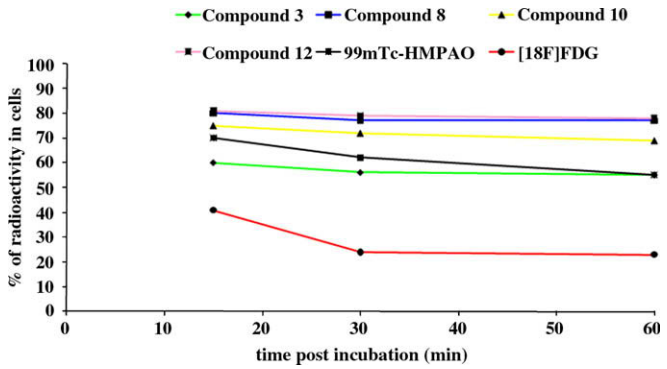


Figure 3. Percentage of tracer uptake after incubation in plasma-free mixed blood cells for 60 min. In plasma-free mixed blood cells, all compounds were bound with a maximum uptake obtained for positively charged compound **12**, (^{99m}Tc-d,l-HMPAO, 55%) while [^{18}F]FDG (23%) were less taken-up.

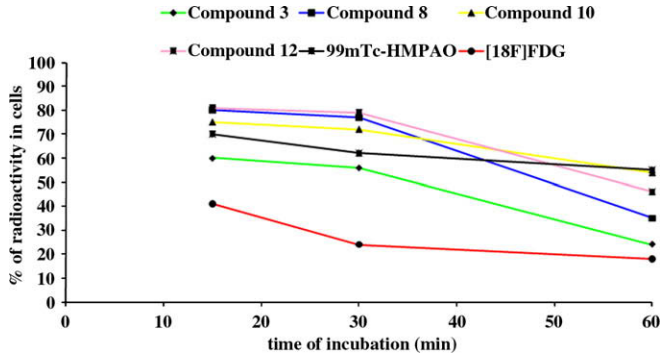


Figure 4. Percentage of tracer uptake after incubation in mixed blood cells with plasma for 60 min. Compound **10** had the highest uptake (54%) while compounds **3** (35%) and **8** (24%) were more associated with plasma. In comparison ^{99m}Tc-d,l-HMPAO and [^{18}F]FDG had 55% and 18% uptake, respectively.

In comparison, [^{18}F]FDG had 23% and 18% uptake in plasma-free and plasma-rich mixed blood cells, respectively. Twenty-five percent of [^{18}F]HFB was bound to mesenchymal stem cells 30 min after incubation with the compound in PBS.[12]

2.4. Biodistribution in normal mice

Biodistribution results in normal NMRI mice of compounds **3**, **8**, **10** or **12** at 10 and 60 min post injection (p.i.) are shown in Table 1 expressed as percentage of injected dose per gram (%I.D./g) of tissue. As was observed after systemic administration of [^{18}F]HFB labeled mesenchymal stem cells,[12] the radiolabeled compounds were excreted mainly via the hepatobiliary pathway. ^{99m}Tc(CO)$_3$-*N*,*N*′-dipicolylhexadecylamine (compound **10**) had the highest liver uptake at 60 min p.i. (38.8%I.D./g). Except for compound **8** which showed fast hepatobiliary clearance (36.1%I.D./g and 6.7%I.D./g at 10 min and 60 min, respectively), all the other agents showed a rather slow hepatobiliary clearance at 10 and 60 min p.i. Lung uptake of all tracers was high. If used to track stem cells targeting the myocardium such a high lung uptake might obscure visualization of the heart at early time points. However, lung uptake decreases rapidly at 60 min p.i. making it favorable for imaging at 60 min p.i. and at later time points. Renal clearance was generally low. Blood clearance was fast with maximally 7.9%I.D./g at 60 min p.i. (compound **12**). Higher blood activity for compounds **10** and **12** reflects the higher affinity for blood cells and plasma proteins, respectively, that was observed in vitro while compounds **3** and **8** were more rapidly cleared. Negligible stomach uptake is an indication of the in vivo stability of the agents.

3. Conclusions

In vitro radiolabeling using mixed blood cells served as a useful tool to evaluate the affinity of these lipophilic agents for blood cells or lipoproteins. If a tracer with high association with plasma protein is used to radiolabel cells, the radiolabel will be rapidly lost in vivo. On the other hand, a tracer with high uptake in blood cells in a plasma-rich blood cell environment has good in vivo stability when used to radiolabel cells. The results show that compound **10** (^{9m}Tc(CO)$_3$-*N*,*N*′-dipicolylhexadecylamine) is potentially the most stable for in vivo tracking of radiolabeled cells: compound **10** > compound **12** > compound **8** > compound **3**. Compound **10** shows more promising results as a potential cell labeling agent. There was no relationship between the lipophilicity and labeling efficiency of the radiolabeled compounds. Compound **10** shall be further evaluated for its ability to label stem cells.

4. Experimental

4.1. Reagents

Hexadecylamine, benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP), diethylenetriamine, picolylchloride and triethylamine were from Sigma Aldrich (Steinheim, Germany); methyl bromoacetate was from Janssen Chimica (Geel, Belgium); 2-chloromethylpyridine, bromohexadecane and all solvents were obtained from Acros Organics (Geel, Belgium) and were used as purchased. Generator eluate containing Na^{99m}TcO$_4$ was obtained from an Ultratechnekow generator (Tyco Healthcare, Petten, The Netherlands). The Isolink kit used for preparing ^{99m}Tc(CO)$_3^+$ was a generous gift from Tyco Healthcare.

4.2. Instrumentations

^{1}H NMR spectra were acquired with a Gemini 300 MHz spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts are reported in parts per million (ppm) relative to TMS ($\delta = 0$). Coupling constants are reported in hertz (Hz). Normal-phase column chromatography was performed using silica gel (silica 63–200, 60 Å, MP Biomedicals, Eschwege, Germany). High-performance liquid chromatography (HPLC) analysis was performed on a LaChrom Elite HPLC system (Hitachi, Darmstadt, Germany) using an XTerra-RP C_{18} column (5 μm, 4.6 mm × 250 mm; Waters, Milford, USA). The compounds were eluted from the column using an isocratic mixture of 0.05 M ammonium acetate buffer pH 6.8/ethanol (25/75 v/v) at a flow rate of 0.9 mL/min over 30 min. The column effluent was monitored using a UV detector set at 254 nm, and the output signal was acquired on a Laura Lite system (Lablogic, Sheffield, UK). For analysis of radiolabeled compounds, the HPLC eluate was led over a 3 in NaI(Tl) scintillation detector connected to a single-channel analyzer. Radioactivity counting for biodistribution studies was done using an automated system with a gamma counter (3 in NaI(Tl) well crystal) coupled to a multichannel analyzer in a sample changer (Wallac, 1480 Wizard 3″, Turku, Finland). The results were corrected for background radiation and physical decay during counting. Accurate mass measurement was performed by co-infusion with a 10 μg/mL solution of a reference as an internal calibration standard on a time-of-flight mass spectrometer (LCT, Micromass, Manchester, UK) equipped with an electrospray ionization (ESI) interface, operated in positive (ES^+) or negative (ES^-) mode. Samples were infused in acetonitrile/water using a Harvard 22 syringe pump (Harvard instruments, Massachusetts, USA).

Table 1
Biodistribution in normal NMRI mice of compounds **3**, **8**, **10** or **12** at 10 and 60 min post injection (p.i.) expressed as % injected dose (I.D.)/gram (standard deviation, $n = 4$ per time point)

Organ	%I.D./g (SD) at 10 min p.i.				%I.D./g (SD) at 60 min p.i.			
	3	**8**	**10**	**12**	**3**	**8**	**10**	**12**
Kidneys	9.5 (1.9)	3.5 (0.7)	2.6 (0.6)	2.5 (0.2)	6.6 (0.9)	6.2 (1.3)	4.5 (2.8)	3.5 (0.3)
Liver	18.8 (1.7)	36.1 (3.0)	36.0 (2.4)	30.5 (3.7)	30.6 (4.4)	6.7 (1.5)	38.8 (3.7)	28.9 (2.3)
Spleen + pancreas	6.6 (0.8)	21.9 (2.8)	17.9 (1.5)	29.3 (16.1)	6.2 (1.1)	3.8 (0.8)	15.4 (2.3)	12.1 (0.6)
Lungs	24.2 (3.1)	43.9 (12)	18.3 (4.6)	11.4 (1.8)	12.0 (2.9)	10.3 (2.4)	11.7 (5.0)	8.5 (0.8)
Heart	5.8 (1.1)	3.8 (0.3)	2.9 (0.8)	2.7 (0.9)	4.3 (0.8)	1.8 (0.3)	2.2 (0.5)	2.7 (0.3)
Intestines + feces	1.8 (0.4)	0.5 (0.1)	0.4 (0.0)	0.3 (0.0)	5.6 (1.1)	8.1 (2.0)	0.8 (0.2)	0.8 (0.1)
Stomach	0.9 (0.4)	0.4 (0.1)	0.6 (0.3)	0.3 (0.1)	1.5 (1.2)	3.6 (1.4)	0.8 (0.2)	0.4 (0.0)
Cerebrum	0.7 (0.2)	0.2 (0.0)	0.1 (0.1)	0.2 (0.0)	0.1 (0.0)	0.3 (0.1)	0.1 (0.0)	0.1 (0.0)
Cerebellum	1.3 (0.3)	0.4 (0.1)	0.3 (0.1)	0.3 (0.0)	0.1 (0.0)	0.5 (0.1)	0.2 (0.0)	0.2 (0.0)
Blood	15.5 (2.1)	7.5 (1.5)	6.9 (1.5)	9.9 (0.9)	1.7 (0.4)	1.3 (0.3)	5.6 (1.3)	7.9 (0.5)

%I.D./g: percentage of injected dose per gram tissue, SD: Standard deviation. Compound **3**: $^{99m}Tc(CO)_3$-hexadecylamino-*N*,*N*′-diacetic acid, compound **8**: $^{99m}Tc(CO)_3$-hexadecylamino-*N*-α-picolyl-*N*′-acetic acid, compound **10**: $^{99m}Tc(CO)_3$-*N*,*N*′-dipicolylhexadecylamine, compound **12**: $^{99m}Tc(CO)_3$-*N*-hexadecylaminoethyl-*N*′-aminoethylamine.

Acquisition and processing of data was done using *Masslynx* software (Micromass, version 3.5). All animal experiments were conducted with the approval of the institutional ethical committee for conduct of experiments on animals.

4.3. Chemistry

4.3.1. Hexadecylamino-*N*,*N*′-dimethyl acetate (1)

Hexadecylamine (238 mg, 0.99 mmol) was dissolved in methanol (50 mL) under N_2. Triethylamine (NEt_3, 0.41 mL, 3 mmol) was added and the mixture was stirred at rt for 30 min. Methyl bromoacetate (0.19 mL, 2 mmol) was added drop wise after which the solution was refluxed for 96 h and then cooled to rt. Water (50 mL) and CH_2Cl_2 (50 mL) were added and the organic layer was separated. Additionally, the aqueous layer was twice washed with CH_2Cl_2 and the combined extracts was dried over $MgSO_4$ and evaporated under reduced pressure. The resulting oil was purified by column chromatography on silica gel with CH_2Cl_2/MeOH (95:5 v/v) as eluting solvent to give a clear oil. Yield: 290 mg (75%). HRMS theoretical $C_{22}H_{44}NO_4$ $[M+H]^+$: 386.5899, found: 386.5889. ^1H NMR ($CDCl_3$): δ (ppm) 0.9 (t, 3H, CH_3–CH_2–CH_2); 1.3 (m, 28H, $(CH_2)_{14}$), 2.3 (q, 2H, CH_2–CH_2–N); 3.5 (s, 4H, OC–CH_2–N–CH_2–CO); 3.7 (s, 6H, COOCH_3).

4.3.2. Hexadecylamino-*N*,*N*′-diacetic acid (2)

Compound **1** was treated with 1 M NaOH (2 mL) and EtOH (2 mL) under reflux for 1 h. After cooling to rt, water (20 mL) was added followed by acidification to pH 2 with 1 N HCl. The precipitate formed was filtered off, washed with water and dried in vacuo at 60 °C to give a white powder. Yield: 270 mg (100%). HRMS theoretical $C_{20}H_{40}NO_4$ $[M+H]^+$ 358.5367, found 358.5314. ^1H NMR (DMSO-d_6): δ (ppm) 0.9 (t, 3H, CH_3–CH_2–CH_2); 1.2 (m, 28H, $(CH_2)_{14}$)); 2.4 (m, 2H, CH_2–CH_2–N); 3.4 (s, 4H, OC–CH_2–N–CH_2–CO).

4.3.3. α-Picolyl-*N*,*N*′-dimethylacetate (4)

Picolylchloride (7.7 mL, 75 mmol) was dissolved in MeOH (100 mL) and NEt_3 (25 mL) was added. Methyl bromoacetate (15 mL, 150 mmol) was added drop wise and the reaction mixture was stirred at 60 °C overnight. After cooling to rt, solution was filtered and the organic solvent was removed in vacuo. The residue was taken up with ethyl acetate, washed with H_2O (3×100 mL), dried over $MgSO_4$ and purified by column chromatography on silica gel with hexane/EtOAc/NEt_3, (30:10:1 v/v) as eluting solvents. Yield: 8.5 g (43%). HRMS theoretical $C_{12}H_{15}N_2O_4$-Na $[M+Na]^+$ 275.10, found 275.32. ^1H NMR ($CDCl_3$): δ (ppm) 3.4 (s, 4H, N–CH_2–COO); 3.6 (s, 6H, COOCH_3); 3.9 (s, 2H, N–CH_2–Pyr); 7.1 (dt, 1H, Pyr*H*); 7.3 (d, 1H, Pyr*H*); 7.6 (dt, 1H, Pyr*H*); 8.5 (d, 1H, Pyr*H*).

4.3.4. α-Picolyl-*N*-methylacetyl-*N*′-acetic acid (5)

Saponification of one methyl ester of compound **4** (2.52 g, 10 mmol) was done in a mixture of 1 M NaOH (10 mL) and MeOH/H_2O (1:1, 20 mL) at 50 °C for 4 h. After cooling to rt, the reaction mixture was evaporated in vacuo with MeOH (4×10 mL). The crude dry reaction mixture was applied on a silica gel column and washed with EtOAc/NEt_3 (95:5 v/v) to remove residual compound **4**. The monomethyl ester (compound **5**) was eluted with EtOAc/MeOH/NEt_3 (10:10:0.5 v/v). Yield: 2.0 g (83%). HRMS theoretical $C_{11}H_{15}N_2O_4$ $[M+H]^+$ 239.2483, found 239.2408. ^1H NMR ($CDCl_3$): δ (ppm) 3.3 (s, 2H, N–CH_2–COOH); 3.4 (s, 2H, N–CH_2–COOCH_3); 3.6 (s, COOCH_3), 3.9 (s, 2H, CH_2–N–CH_2); 7.1 (dt, 1H, Pyr*H*); 7.3 (d, 1H, Pyr*H*); 7.6 (dt, 1H, Pyr*H*); 8.5 (d, 1H, Pyr*H*).

4.3.5. Hexadecylamino-*N*-α-picolyl-*N*′-acetic acid (7)

The monomethyl ester (compound **5**, 0.4 mmol, 95 mg) was dissolved in THF (10 mL). HOBt (0.4 mmol, 54 mg), hexadecylamine (0.52 mmol, 127 mg) and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP, 0.6 mmol, 228 mg) were added, respectively. The reaction mixture was stirred at rt overnight. Analysis by TLC (MeOH/EtOAc/NEt_3, 10:10:0.5 v/v) revealed that the reaction was incomplete. An additional amount of BOP (0.6 mmol, 228 mg) was added and the reaction mixture was further stirred at rt for 5 days. The precipitate was filtered, washed with THF and dried in vacuo. The product was purified by column chromatography on silica gel with CH_2Cl_2/MeOH (97:3 v/v) to give a white solid (compound **6**). Yield: 70 mg (38%). HRMS: theoretical $C_{27}H_{48}N_3O_3$ $[M+H]^+$ 462.6894, found 462.6796. ^1H NMR ($CDCl_3$): δ (ppm) 0.9 (t, 3H, CH_3–CH_2); 1.2 (m, 26H, $(CH_3)_{13}$); 1.5 (m, 2H, CH_2); 3.2 (m, 2H, NH–CH_2); 3.4 (d, 4H $2\times CH_2$–N); 3.7 (s, 3H, COOCH_3); 3.9 (s, 2H, N–CH_2–Pyr); 7.2 (dDD, Pyr*H*); 7.25 (d, Pyr*H*); 7.6 (dt, 1H, Pyr*H*); 8.2 (m, N*H*–CO); 8.6 (d, 1H, Pyr*H*).

Hydrolysis of compound **6** in 1 M NaOH gave compound **7** as a white powder. HRMS $C_{26}H_{46}N_3O_3$ $[M+H]^+$ theoretical 448.6623, found 448.6634.

4.3.6. *N*,*N*′-Dipicolylhexadecylamine (9)

Hexadecylamine (1.15 g, 4.8 mmol) and 2-chloromethylpyridine (1.57 g, 9.6 mmol) were suspended in a mixture of water/MeOH (1:1 v/v, 60 mL). 5 M NaOH was added until pH 10. The pH was kept constant (at 10) by addition of 5 M NaOH. The mixture was stirred overnight at rt, evaporated and suspended in a mixture of water/EtOAc (1:1 v/v). The organic layer was separated, dried over $MgSO_4$ and concentrated in vacuo. The resulting clear oil was purified by column chromatography on silica gel using hexane/EtOAc/NEt_3 (75:20:5 v/v) as eluting solvent. Yield: 270 mg

(12%). HRMS theoretical $C_{28}H_{46}N_3$ $[M+H]^+$ 424.6863, found 424.6848. ^1H NMR ($CDCl_3$): δ (ppm) 0.9 (t, 3H, CH_3–CH_2–CH_2); 1.2 (d, 28H, (CH_2)14); 1.5 (m, 2H, CH_2–CH_2–N); 3.8 (s, 4H, CH_2–N–CH_2); 7.3 (dt, 2H, Pyr*H*); 7.5 (d, 2H, Pyr*H*), 7.6 (dt, 2H, Pyr*H*); 8.5 (dd, 2H, Pyr*H*).

4.3.7. *N*-Hexadecylaminoethyl-*N*′-aminoethylamine (11)

Diethylenetriamine (DETA, 21.6 mL, 200 mmol) and 1-bromohexadecane (15 mL, 50 mmol) were heated at 180 °C during 3.5 h. After cooling, the precipitate was filtered and the filtrate was distilled to remove unreacted DETA (60–100 °C, 0.1 mmHg). The residue containing the final product and some side products was used as such for labeling experiments. HRMS theoretical $C_{20}H_{45}N_3$ $[M+H]^+$ 327.5922, found 327.5931.

4.4. Radiolabeling with technetium-99m

$^{99m}Tc(CO)_3(H_2O)_3^+$ *precursor*. The technetium-99m tricarbonyl precursor was prepared using an Isolink kit by adding 1.1–2.5 GBq of $Na^{99m}TcO_4$ in 1 mL saline to the kit followed by heating at 100 °C for 20 min. After cooling, the solution was neutralized with 1 M HCl and then adjusted to desired pH with 1 M NaOH.

*$^{99m}Tc(CO)_3$-hexadecylamino-N,N′-diacetic acid (compound **3**), $^{99m}Tc(CO)_3$-hexadecylamino-N-α-picolyl-N′-acetic acid (compound **8**), $^{99m}Tc(CO)_3$-N,N′-dipicolylhexadecylamine (compound **10**) and $^{99m}Tc(CO)_3$-N-hexadecylaminoethyl-N′-aminoethylamine (compound **12**)*. To a 10-mL labeling vial was added 1 mg of compounds **2**, **7**, **9** or **11** dissolved in 0.2 mL of ethanol. The vial was purged with nitrogen, and 0.2–1.1 GBq of $^{99m}Tc(CO)_3(H_2O)_3^+$ precursor (0.2–0.5 mL, pH 7.4 or 11 (for compound **11**)) was added. The mixture was heated at 70 °C for 20 min followed by cooling to rt. The crude labeling reaction mixture was analyzed and purified by RP-HPLC as described above.

^{99m}Tc-d,l-HMPAO. d,l-HMPAO was synthesized as described by Troutner and Volkert[16] Radiolabeling of d,l-HMPAO was performed in a 10-mL glass vial containing 1 mg of d,l-HMPAO in a mixture of 100 μL ethanol and 900 μL saline (0.9% NaCl) and consecutively adding 7 μg $SnCl_2.2H_2O$ in 3.5 μL 0.05 M HCl and 400 MBq $Na^{99m}TcO_4$ in 1 mL saline followed by rt incubation[17] [^{18}F]FDG was synthesized at our PET radiochemistry laboratory.

4.5. Octanol–buffer partition coefficient

To 50 μL (74 kBq) of a solution of HPLC-purified radiolabeled compound (**3**, **8**, **10** or **12**) in a test vial was added 2 mL of 1-octanol and 2 mL of 0.025 M phosphate buffer pH 7.4. The vial was vortexed at rt for 2 min and then centrifuged (Centrifuge 4226, Analis, Gent, Belgium) at 1700 g for 10 min. Approximately 50 μL of the 1-octanol phase and 500 μL of the phosphate buffer phase were pipetted and weighed into separate tared test tubes with adequate care to avoid cross contamination between the phases. The volume of fluid pipetted was calculated by dividing the net weight of the fluid by its density. The radioactivity of the test tubes was counted using a 3-inch NaI(Tl) scintillation detector mounted in a sample changer. Corrections were made for background radiation and physical decay during counting. The octanol–buffer partition coefficient *P* was calculated as:

$$P = \frac{cpm/mL\ in\ octanol}{cpm/mL\ in\ buffer} \qquad \text{where cpm} = \text{counts per min}$$

4.6. Preliminary in vitro cell labeling

Preliminary in vitro labeling of tracer was performed in mixed blood cells by incubating the agents in mixed blood cells with or without plasma for 60 min. Blood was collected into a BD vacutainer™ (containing 7.2 mg K_2EDTA; Beckton Dickinson, Franklin Lakes, USA) and subsequently transferred to a 50-mL conical falcon tube. Five-mL blood samples were transferred into 15-mL conical falcon tubes and centrifuged at 3000 rpm (1837 g) for 5 min, to separate plasma. Plasma was carefully transferred into a 15-mL conical falcon tube. To the mixed blood cells (plasma-free) from 5 mL blood was added HPLC-purified compounds **3**, **8**, **10**, **12**, ^{99m}Tc-d,l-HMPAO, or [^{18}F]FDG followed by gentle mixing. After 15 min of incubation at rt in plasma-free blood cells, the cells were centrifuged for 5 min and the activity in the cells and supernatant fractions was counted in a gamma counter. This was immediately followed by twice washing with PBS, pH 7.4. The total time for this uptake phase was 30 min, after which, the cells were fractioned into two to which was added an equal volume of saline or plasma followed by 60 min incubation at rt. Afterwards, the cells were centrifuged for 5 min and the supernatant (saline or plasma) was carefully separated followed by two PBS washes. Radioactivity in the supernatant (saline or plasma) and the cells was counted in a gamma counter. The percentage of radiolabeled compounds bound to the cells was calculated by dividing the counts (decay corrected) in the cells at time (t) by the total counts (cells + supernatant) at time (t) × 100.

4.7. Biodistribution in mice

Mice were anesthetized with isoflurane (2%) in oxygen at a flow rate of 1 L/min and then injected via a tail vein with 74 kBq of HPLC-pure compound **3**, **8**, **10** or **12** in 0.1 mL of saline. They were sacrificed by decapitation under anesthesia at 10 and 60 min p.i. (n = 4 mice per time point). The organs were dissected and weighed in tared tubes and radioactivity in all organs was counted in a gamma counter. Corrections were made for background radiation and physical decay during counting. Activity in the organs was expressed as % I.D./organ and % I.D./g of organ. Activity in blood was calculated on the assumption that blood constitutes 7% of total body weight.

Acknowledgments

The authors would like to thank Peter Vermaelen for invaluable contribution to the study. Financial support for the project was obtained from grants awarded by Geconcerteerde Onderzoeksactie (GOA) of the Flemish Government, FWO-Vlaanderen Grant G. 0257.05 and in part by the EC-FP6-project DiMI, LSHB-CT-2005-512146.

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