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Comparing hypoxia-targeting potential of $^{99m}Tc(CO)_3$-labeled 2-nitro and 4-nitroimidazole

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Non-invasive determination of hypoxia is an important problem in clinical nuclear medicine. Although ^{18}F-fluoromisonidazole is used clinically for hypoxia determination, the short half-life ($t_{1/2}$ = 109.77 min), high cost and less availability of cyclotrone-produced ^{18}F make ^{99m}Tc ($t_{1/2}$ = 6 h)-based agents more desirable. With this aim, $^{99m}Tc(CO)_3$-labeled iminodiacetic acid derivatives of 2- and 4-nitroimidazole are investigated for their ability to target hypoxic tumors. A bifunctional chelating agent, *N, N*-bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine, was synthesized in the first step, which was then conjugated to the nitroimidazoles in the second step. The tert-butyl ester derivatives formed were hydrolyzed to obtain the iminodiacetic acid derivatives of corresponding nitroimidazoles. The radiolabeling of the iminodiacetic acid derivatives with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core was carried out following a reported protocol. Biodistribution of the prepared complexes was carried out in Swiss mice bearing fibrosarcoma tumor. The 2-nitroimidazole complex showed a steady retention in activity (~1.5% injected dose per gram [%ID/g]) in tumor throughout the period of study (3 h) indicating that it may be localized in the hypoxic cells. The 4-nitroimidazole counterpart, however, showed an initial uptake of ~4.6%ID/g at 30 min post injection, which was then observed to wash out rapidly.

Keywords: hypoxia; nitroimidazole; technetium-99m tricarbonyl core; fibrosarcoma; iminodiacetic acid

Introduction

In connection with our current interest of developing suitable radiolabeled agents for targeting hypoxic tumors, it was felt pertinent to explore the potential of two differently substituted nitroimidazoles complexes. Hypoxia plays a major role in contributing toward the resistance of tumors to both radiation therapy and chemotherapy.[1,2] Inherent drawbacks in the invasive methods available today for measuring hypoxia have provided the impetus for the development of non-invasive modalities for the purpose. Currently, ^{18}F-labeled fluoromisonidazole is the standard used for the detection of hypoxia. However, limited availability and high cost of the cyclotron-produced ^{18}F, ^{99m}Tc-based agents could serve as more desirable alternatives. A majority of nitroaromatics studied for targeting hypoxia are based on nitroimidazoles.[3–10] Among the nitroimidazole derivatives, the 2-nitroimidazole derivative is by far the most studied agent. However, 4-nitro and 5-nitro derivatives are also reported. Lipophilicity and the redox potential are the two major factors governing the uptake and retention of the molecule in hypoxic cells. Lipophilicity is primarily responsible for the uptake in tumor, whereas the redox potential determines the efficiency with which the agent gets reduced and is retained in the hypoxic cells. A fine balance between the two factors is sought, which will eventually lead to an ideal hypoxia-targeting agent. Although broad guidelines are available regarding the lipophilicity and the redox potential that a molecule should have to show hypoxia-targeting property, the actual *in vivo* behavior of the molecule may depend on other factors such as the structure of the molecule. W. Peter *et al*. had earlier reported one electron reduction potentials of different nitroimidazole derivatives in aqueous solution at pH 7.[3] The estimated values are in the range −243 millivolt (mV) to −398 mV for differently substituted 2-nitroimidazole derivatives. The values are between −400 and −500 mV for substituted 5-nitroimidazoles and for 4-nitroimidazoles the values are even less. The above data suggest that 2-nitroimidazoles are the most promising candidates as hypoxia-targeting agents. However, Linder *et al*. showed that ^{99m}Tc–BATO complex containing 4-nitroimidazole has the potential for selective retention in hypoxic tissue,[4] and Chu *et al*. have reported a bidentate hydroxyiminoamide derivative of 4-nitroimidazole labeled with $[^{99m}Tc=O]^{3+}$ core, which showed accumulation and slow clearance from tumor.[10] These observations highlight the importance of synthesis and *in vivo* testing of different derivatives of nitroimidazoles irrespective of the

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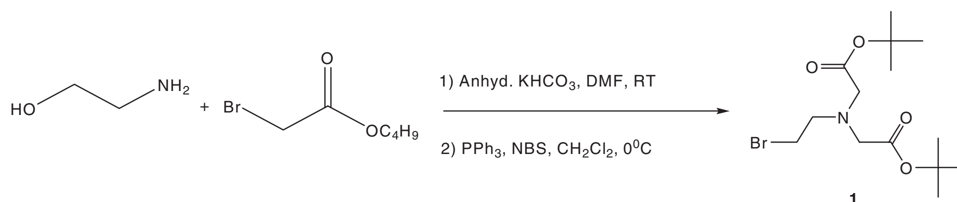

Scheme 1. Synthesis of *N,N*-bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine.

position of the nitro substituent. Generally, labeling of molecules via technetium oxo core requires high ligand concentration to achieve satisfactory labeling yield. The introduction of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core[11] opened up the possibility of labeling molecules using low ligand concentration to yield complexes with high stability. The three substitutionally labile water molecules in $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core facilitate the formation of stable complexes with tridentate ligands such as iminodiacetic acid, diethylene triamine, histidine, etc.[12] and hence target-specific molecules of interest are often modified to possess one of these ligating groups. In the present study, 2-nitro and 4-nitroimidazoles were synthetically modified to iminodiacetic acid derivatives to render them suitable for complexation with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core. Toward this, a bifunctional chelating agent (BFCA) with a pendant bromo substituent was synthesized and used for alkylation of the –NH– of the imidazole ring. The complexes were prepared, characterized and their *in vivo* behaviour as markers for tumor hypoxia were evaluated in the animal model.

Results and discussions

Synthesis

The iminodiacetic acid derivatives of both 2- and 4-nitroimidazole were synthesized in two steps. In the first step, *N, N*-bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine (**1**) was synthesized, following a reported procedure,[13] by reacting ethanolamine and tert-butylbromoacetate in the presence of potassium bicarbonate ($KHCO_3$) in dimethyl formamide (DMF) (Scheme 1). The crude product, *N, N*-bis[(tert-butoxycarbonyl)-methyl]-2-hydroxyethylamine, was then treated with trisphenyl phosphine (PPh_3) and *N*-bromosuccinamide (NBS) in dichloromethane to yield the target compound in 80% yield. This bifunctional chelator was used in the subsequent step for *N*-alkylation of the ring –NH– of the 2- and 4-nitroimidazole to yield *N, N*-bis[(tert-butoxycarbonyl)methyl]-2-(2-nitroimidazolyl)ethylamine and *N, N*-bis[(tert-butoxycarbonyl)methyl]-2-(4-nitroimidazolyl)ethylamine, respectively. The *N*-alkylation was carried out using potassium carbonate as the base in acetonitrile solvent under reflux conditions. During *N*-alkylation of 4-nitroimidazole, two products were observed by thin layer chromatography (TLC) carried out in diethyl ether. Separation of the two products and their analysis by ^{1}H-NMR showed that one was the expected 4-nitroimidazole derivative and the other was the isomeric 5-nitroimidazole derivative. The former was then separated by column chromatography over silica gel using diethyl ether as the solvent. Although the overall yield of 4-nitroimidazole tert-butyl ester derivative was 65%, the 2-nitro imidazole ester derivative could be synthesized in 85% yield. The tert-butyl ester derivatives were then hydrolyzed using 6 N HCl in near-quantitative yield to obtain the desired products **2** and **3**, respectively (Scheme 2).

A reported procedure was followed for the preparation of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core,[11] as mentioned in the Experimental section. For the preparation of the complex, about 1–2 mg of the corresponding ligand in 0.5 mL of phosphate buffer was mixed with 0.5 mL of freshly prepared $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core. The mixture was vortexed for 1 min and then incubated at 80°C for 30 min. The three labile water molecules are replaced by the iminodiacetic acid group resulting in a complex with overall unit negative charge. The probable structure of the complex is shown in Scheme 3. Allali *et al.*[14] had earlier shown, through ^{1}H-NMR and IR, the tridentate coordination of the metal-tricarbonyl core (metal = Tc, Re) via iminodiacetic acid-functionalized molecule. The two complexes reported herein were therefore presumed to have a similar structure. It is pertinent to note that although the overall negative charge on the complexes reported herein may not be a desirable feature as it may hinder/reduce its diffusion across the cell membrane, it may facilitate faster clearance of activity from the body, which is also an important attribute of an imaging agent. Characterization of the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core and the final complex was carried out using a dual pump high-performance liquid chromatography (HPLC) unit coupled with a radioactivity detector. The elution profiles of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core, 2-nitroimidazole complex and 4-nitroimidazole complex are shown in Figure 1(a), (b) and (c), respectively. It can be seen that $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core appeared as a broad peak at 17.42 $\pm$ 0.2 min. The 2-nitroimidazole complex was eluted out at 11.32 $\pm$ 0.2 min and the 4-nitroimidazole complex at 15.27 $\pm$ 0.1 min. *In vitro* serum stability studies were performed for the labeled complex using a method adapted from an earlier reported protocol.[15] About 50 μL of the labeled compound was added to 0.5 mL of human serum and this mixture was incubated at 37°C for 3 h. Aliquots were drawn at 1, 2 and 3 h and analyzed by HPLC to assess stability of the complex in the serum. The elution profiles of the complexes were unaltered indicating that the complexes are not degraded in serum during the period of the study. Lipophilicity of the complex was assessed by determining Log $P_{o/w}$ following a multiple extraction procedure reported by Troutner *et al.*[16] The Log $P_{o/w}$ value of the 4-nitroimidazole–$^{99m}Tc(CO)_3$ complex was found to be -0.86 and that of the 2-nitroimidazole–$^{99m}Tc(CO)_3$ complex was found to be -0.87. Despite showing nearly identical Log $P_{o/w}$ values, it can be seen that there is significant difference in the retention times of the two complexes in the HPLC profile. However, this apparent anomaly that could not be explained has been observed earlier by us as well as reported by Schibli *et al.*[12]

Biodistribution

Swiss mice ($\sim$25 g body weight) were used to develop *in vivo* tumor models for the biodistribution studies. Solid tumors were

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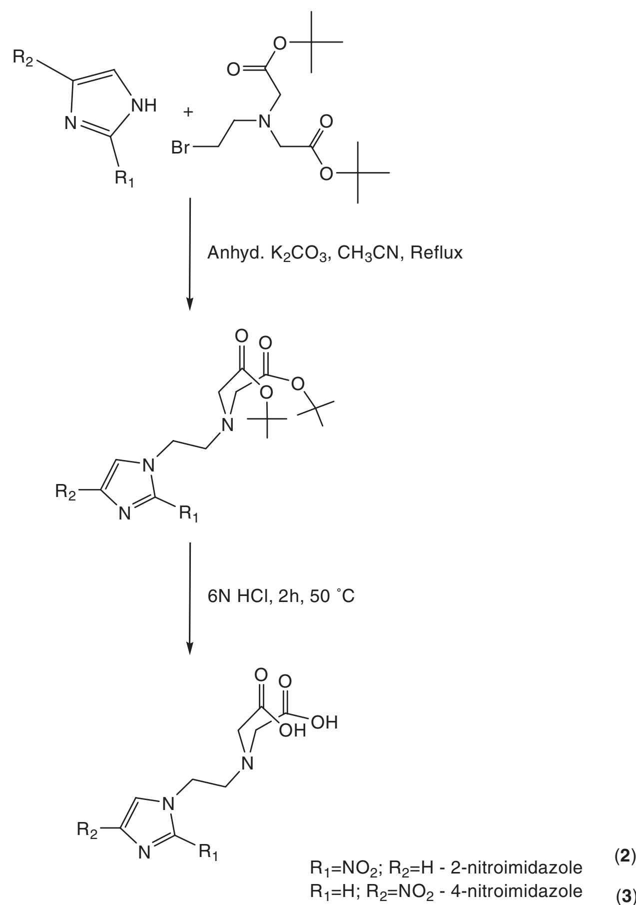
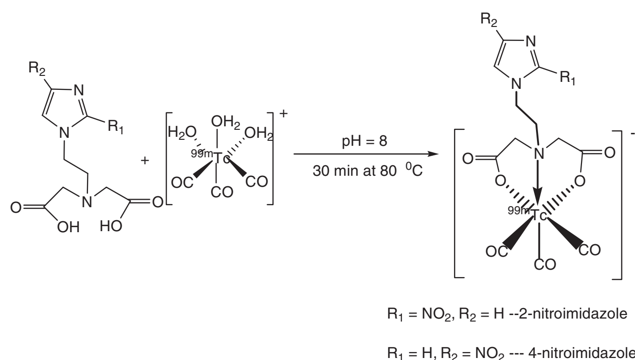


Scheme 2. Synthesis of nitroimidazole derivative.



Scheme 3. Preparation of nitroimidazole-$^{99m}Tc(CO)_3$ complex.

propagated in the animals by subcutaneous administration of a fibrosarcoma cell line ($\sim 10^6$ cells per animal) into the dorsal region. The tumor size was allowed to reach approximately 10 mm diameter after which the animals were used for the experiment. For the *in vivo* distribution studies, about 0.1 mL of the labeled product ($\sim 100\,\mu Ci$) per animal was administered intravenously. The animals were sacrificed at various time points (30, 60 and 180 min p.i.) after which the relevant organs and tissue were excised for measurement of associated activity. Radioactivity measurements were carried out in a flat-bed-type NaI(Tl) scintillation counter with an optimal energy window for ^{99m}Tc. The accumulated activity was expressed in terms of the percentage of the total injected dose associated with the specific organ/tissue per gram. The results of the biodistribution study were shown in Tables 1 and 2. Both the complexes under investigation showed uptake in tumor despite the presence of a negative charge on them. The proposed mechanism of retention of nitroimidazoles in hypoxic cells is attributed to their stepwise reductions, catalyzed by various one-electron reductases, followed by ring fragmentation forming reactive radicals, which then bind to the cellular components.[17] The first reduction step is reversible under well-oxygenated conditions and, hence, nitroimidazoles do not accumulate in normoxic cells. In the present study, the 2-nitroimidazole complex showed a steady retention of activity in the tumor throughout the period of study. This can be attributed to efficient reduction of the

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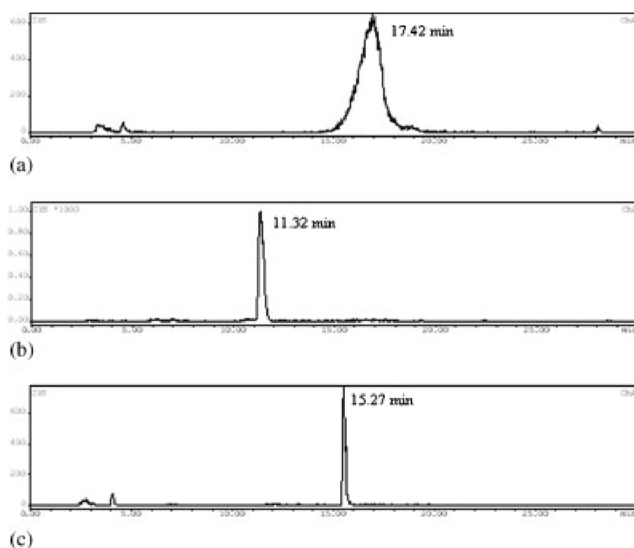

Figure 1. HPLC elution profile of (a) $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core, (b) 2-nitroimidazole–$^{99m}Tc(CO)_3$ complex and (c) 4-nitroimidazole–$^{99m}Tc(CO)_3$ complex.

complex in hypoxic cells. On the contrary, a steady retention of activity was not observed with 4-nitroimidazole complex, despite its high initial uptake. This indicates that the 4-nitroimidazole derivative may be less efficient in being reduced within the time frame that it spends inside the tumor, before being washed out. It is also interesting to observe that after 1 h p.i., the retention of the 4-nitroimidazole complex is similar to that of the 2-nitroimidazole complex, though not identical. However, on careful observation it is evident that the rate of washout of the two complexes from the tumor is not similar, the 4-nitroimidazole complex being cleared at a faster rate than the 2-nitroimidazole complex. Although the present studies carried out for a limited time (3 h max.) do not adequately indicate the clear superiority of one complex over the other, there are possible indications to demonstrate the favorable features of the 2-nitroimidazole complex as a hypoxia-imaging agent in comparison with that of the 4-nitroimidazole complex. The biodistribution results also showed that major clearance of the injected activity was through the hepatobiliary route. The clearance of activity from blood was slow with both the complexes. This affected the tumor to blood ratio, which was found to be lower than one throughout the period of study. Although the tumor to muscle ratio of 2-nitroimidazole complex showed improvement with time, that in case of the 4-nitroimidazole was found to decrease with time.

Experimental

Synthesis

General

2-nitroimidazole, 4-nitroimidazole, 2-aminoethanol, tert-butylbromoacetate, NBS, triphenylphosphine (PPh_3) and carbon monoxide gas, used for the preparation of the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core, were purchased from M/s. Aldrich, U.S.A. Potassium carbonate and potassium bicarbonate were procured from M/s. Fluka, Germany. All solvents used for syntheses were dried as per the general procedure. All reagents were of analytical grade. Sodium pertechnetate was eluted using normal saline from a $^{99}Mo–^{99m}Tc$ column generator. Silica gel plates (silica gel 60 F_{254}) were obtained from M/s. Merck, U.S.A. The HPLC analyses were carried out on a JASCO PU 2080 Plus dual pump HPLC system, Japan, with a JASCO 2075 Plus tunable absorption detector and a radiometric detector system, using a C18 reversed phase HiQ Sil (5 μm, 4 × 250 mm) column. ^1H-NMR spectra were recorded on a 300 MHz Varian VXR 300S spectrophotometer, U.S.A. IR was recorded on a JASCO FT/IR-420 spectrophotometer, Japan. IR of the liquid samples are recorded neat as a thin film over the NaCl/KBr window. For solid samples KBr pellets are used.

Synthesis of N, N*-bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine (**1**)*

To tert-butylbromoacetate (3.3 g, 17.3 mmol) dissolved in 15 mL of DMF, powdered $KHCO_3$ (1.92 g, 19.2 mmol) was added. The suspension was cooled to 0°C, and ethanolamine (0.46 mL, 7.5 mmol) was added dropwise over a period of 5 min. The reaction mixture was stirred at 0°C for 30 min and then at room temperature for 24 h. The semisolid residue obtained after removing the solvent was dissolved in $NaHCO_3$ solution and extracted with ether. The ether layer was then washed with brine, dried and evaporated to give the crude product (~1.8 g, 83%) as oil. To the crude dialkylated product dissolved in 20 mL of dichloromethane, Ph_3P (2.2 g, 8.45 mmol) was added. The solution was cooled to 0°C, and solid NBS (1.5 g, 8.45 mmol) was added over a period of 5 min. The reaction was continued for a period of 2 h after which, the solvent was removed to yield a semisolid residue. The semisolid residue was triturated with ether and the precipitate was filtered off. The ether layer was concentrated and passed through a small column of silica eluting with ether. Evaporation of ether yielded a yellow oil, which was then purified by silica gel chromatography using 5:1 hexane/ether mixture as an eluent to get the target compound (1.7 g, 80%); R_f 0.28 (5:1 hexane/ether); ^1H-NMR ($CDCl_3$, ppm) 1.46 (s, 18H, tert-butyl $C\underline{H}_3$); 3.1 (t, 2H, $J = 7.5$ Hz, $BrCH_2C\underline{H}_2N$); 3.4 (t, 2H, $J = 7.5$ Hz, $BrC\underline{H}_2CH_2N$); 3.5 [s, 4H, (tert-butoxycarbonyl–$C\underline{H}_2)_2N$]. IR (Neat, cm^{-1}) 2985 (m); 2943 (m); 1747 (vs); 1451 (m); 1379 (m); 1254 (s); 1190 (vs); 1145 (w). 1030 (s); 993 (w); 918 (w); 864 (w); 741 (w); 659 (w).

*Synthesis of iminodiacetic acid derivative of 2-nitroimidazole (**2**)*

To 2-nitroimidazole (100 mg, 0.88 mmol) in 15 mL of acetonitrile, anhydrous K_2CO_3 (2 eq) was added and the suspension was stirred for 15 min. To this mixture *N*, *N*-bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine (**1**) (311 mg, 0.88 mmol) was added and the reaction mixture refluxed for 12 h. The progress of the reaction was monitored by TLC. After removing the solvent, the residue was dissolved in 20 mL water and extracted with two 15 mL portions of chloroform. The combined chloroform layer was washed with brine and dried. The pure product, *N*, *N*-bis[(tert-butoxycarbonyl)methyl]-2-(2-nitroimidazolyl)ethylamine, was then obtained by silica gel chromatography, using ethyl acetate as the eluant (287 mg, 85%). R_f 0.88 (ethyl acetate); ^1H-NMR ($CDCl_3$, δ ppm) 1.45 (s, 18H, tert-butyl $C\underline{H}_3$); 3.16 (t, 2H, $J = 6$ Hz, 2-nitroimidazole–$CH_2C\underline{H}_2N$); 3.37 [s, 4H, (tert-butoxycarbonyl–$C\underline{H}_2)_2N$]; 4.55 (t, 2H, $J = 6$ Hz, 2-nitroimidazole–$C\underline{H}_2CH_2N$); 7.12 (d, 1H, $J = 1.2$ Hz, nitroimidazole C2-H); 7.46 (d, 2H, $J = 1.2$ Hz, nitroimidazole C4-H). IR (Neat, cm^{-1}) 3126 (m); 2978 (m); 2930 (m); 1740 (vs); 1540 (s); 1487 (m); 1343 (m); 1216 (m); 1150 (vs);

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Table 1. Distribution of 2-nitroimidazole–$^{99m}Tc(CO)_3$ complex

Organ	% Injected dose per gram (± SD) ($n=3$)		
	30 min p.i.	60 min p.i.	180 min p.i.
Liver	8.22 (0.92)	11.45 (2.31)	15.14 (1.25)
Intestine+gall bladder	7.88 (1.97)	17.71 (4.28)	24.13 (1.56)
Kidney	4.57 (0.36)	5.73 (0.60)	5.05 (0.42)
Heart	1.25 (0.17)	1.42 (0.11)	1.04 (0.13)
Lungs	3.72 (0.26)	4.98 (0.70)	3.37 (0.26)
Muscle	0.61 (0.03)	0.80 (0.15)	0.44 (0.09)
Blood	3.71 (0.55)	3.61 (0.36)	2.89 (0.37)
Tumor	1.36 (0.09)	1.77 (0.15)	1.60 (0.32)
Tumor/blood	0.37	0.49	0.55
Tumor/muscle	2.23	2.38	3.64

Table 2. Distribution of 4-nitroimidazole–$^{99m}Tc(CO)_3$ complex

	% Injected dose per gram (±SD) ($n=3$)		
	30 min p.i.	60 min p.i.	180 min p.i.
Liver	13.32 (1.45)	11.76 (0.95)	9.24 (1.25)
Intestine+gall bladder	20.45 (1.77)	11.43 (0.28)	11.89 (0.61)
Kidney	6.80 (0.29)	6.62 (0.94)	3.90 (0.14)
Heart	2.07 (0.35)	1.47 (0.20)	0.59 (0.05)
Lungs	3.94 (0.57)	3.42 (0.30)	2.41 (0.78)
Muscle	0.44 (0.03)	0.55 (0.05)	0.51 (0.18)
Blood	3.87 (0.52)	3.33 (0.48)	1.66 (0.05)
Tumor	4.69 (0.27)	1.93 (0.31)	1.25 (0.03)
Tumor/blood	1.23	0.58	0.76
Tumor/muscle	10.77	3.50	2.61

1077 (m); 982 (m); 938 (w); 850 (m); 823 (m). The purified ester was dissolved in 1 mL of methanol and to this solution 7 mL of 6 N HCl was added. The reaction was stirred at 50°C for 2 h. The solvent was removed under vacuum to yield the iminodiacetic acid derivative **2** as a white powder (193 mg, 95%). ^{1}H-NMR (D_2O, ppm) 3.51 (t, 2H, $J=5.1$ Hz, nitroimidazole–$CH_2\underline{CH_2}N$); 3.81 (s, 4H, $[(HO_2C–\underline{CH_2})_2N–]$); 4.43 (t, 2H, $J=5.1$ Hz, nitroimidazole–$\underline{CH_2}CH_2N$); 7.34 (d, 2H, $J=1.2$ Hz, nitroimidazole C5-H); 7.50 (d, 2H, $J=1.2$ Hz, nitroimidazole C4-H). IR (KBr, cm^{-1}) 3136 (m); 2970 (m); 2847 (m); 1760 (vs); 1724 (s); 1559 (m); 1499 (m); 1392 (m); 1350 (m); 1230 (m); 1198 (m); 1138 (s); 1006 (w); 860 (m); 827 (m); 751 (w); 653 (m).

*Synthesis of iminodiacetic acid derivative of 4-nitroimidazole (**3**)*

To 4-nitroimidazole (100 mg, 0.88 mmol) in 15 mL of acetonitrile, anhydrous K_2CO_3 (2 eq) was added and the suspension was stirred for 15 min. To this, *N*, *N*-bis[(tert-butoxycarbonyl)methyl]-2-bromoethylamine] (311 mg, 0.88 mmol) was added and the reaction mixture was refluxed for 12 h. Upon completion of the reaction, the target compound was recovered by following the same work up procedure as described above. The TLC of the reaction mixture in ether showed two spots, corresponding to the formation of two products, which were separated by silica gel column chromatography using ether as an eluent. The ^{1}H-NMR of the two compounds revealed that one was the expected 4-nitroimidazole ester derivative and the other was the 5-nitroimidazole ester derivative. The target compound *N*, *N*-bis[(tert-butoxycarbonyl)methyl]-2-(4-nitroimidazolyl)ethylamine was obtained in 65% (219 mg) overall yield. R_f 0.85 (ethyl acetate); ^{1}H-NMR ($CDCl_3$, ppm) 1.46 (s, 18H, tert-butyl $\underline{CH_3}$); 3.13 (t, 2H, $J=6$ Hz, 2-nitroimidazole–$CH_2\underline{CH_2}N$); 3.35 [s, 4H, (tert-butoxycarbonyl–$\underline{CH_2})_2N$]; 4.11 (t, 2H, $J=6$ Hz, 2-nitroimidazole–$\underline{CH_2}CH_2N$); 7.62 (d, 1H, $J=1.5$ Hz, nitroimidazole C2-H); 8.05 (d, 2H, $J=1.5$ Hz, nitroimidazole C4-H). IR (Neat, cm^{-1}) 3133 (m); 2983 (m); 2931 (m); 2954 (m); 1738 (vs); 1537 (s); 1497 (m); 1336 (m); 1229 (m); 1147 (vs); 1070 (m); 987 (m); 938 (w); 857 (m); 825 (m); 767 (m). The purified ester was dissolved in 1 mL of methanol and to this 7 mL of 6 N HCl was added. The reaction was kept stirring at 50°C for 2 h. The solvent was removed under vacuum to yield the iminodiacetic acid derivative **3** as a white powder (147 mg, 95%). ^{1}H-NMR (D_2O, ppm) 3.71 (t, 2H, $J=6.6$ Hz, nitroimidazole–$CH_2\underline{CH_2}N$); 3.99 (s, 4H, $[(HO_2C–\underline{CH_2})_2N–]$); 4.48 (t, 2H, $J=6.6$ Hz, nitroimidazole–$\underline{CH_2}CH_2N$); 7.72 (d, 2H, $J=1.2$ Hz, nitroimidazole C5-H); 8.16 (d, 2H, $J=1.2$ Hz, nitroimidazole C4-H). IR (KBr, cm^{-1}) 3130 (m); 2974 (m); 2847 (m); 1768 (vs); 1724 (s); 1559 (m); 1499 (m); 1392 (m); 1350 (m); 1230 (m); 1185 (m); 1143 (s); 1017 (w); 863 (m); 825 (m); 760 (w).

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Radiolabeling

Preparation of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core

A typical procedure involves dissolution of $NaBH_4$ (~5.5 mg), Na_2CO_3 (~4 mg) and Na/K tartrate (~15 mg) in 0.5 mL double-distilled water in a 10 mL glass vial. The vial was sealed and carbon monoxide gas was purged through the solution for 10 min. After the addition of 1 mL of the generator eluate containing 37–74 MBq of $^{99m}TcO_4^-$, the vial was heated at 80°C for 20 min. After cooling the vial and re-equilibrating with atmosphere, the pH of the reaction mixture was adjusted to 7 using a 1:3 mixture of 0.5 M phosphate buffer (pH 7.5):1 M HCl. The $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core thus prepared was characterized by HPLC.

Preparation of $^{99m}Tc(CO)_3$-complex

Under optimized conditions, 0.5 mL of freshly prepared $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor was added to 1–2 mg of the ligand in 0.5 mL of the phosphate buffer at pH 7.4. The mixture was vortexed for a minute and then incubated at 80°C for 30 min. The complex thus formed was characterized by HPLC.

Quality control

HPLC

The radiochemical purity of the prepared $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core as well as the complex was assessed by HPLC using a C18 reversed phase column. Water (A) and acetonitrile (B) mixtures with 0.1% trifluoroacetic acid were used as the mobile phase and the following gradient elution technique was adopted for the separation (0 min 90% A, 28 min 10% A, 30 min 10% A). The flow rate was maintained at 1 mL/min. About 25 μL of the test solution was injected into the column and the elution was monitored by observing the radioactivity profile.

Partition coefficient (Log $P_{o/w}$)

About 100 μL of the labeled compound was mixed with 0.9 mL of water and 1 mL of octanol on a vortex mixer for about 1 min. The two phases were then allowed to separate. About 0.8 mL of the octanol layer was withdrawn and an equal volume of fresh saline was added. The mixture was re-vortexed for a minute and then the two layers were allowed to separate. Equal aliquots of the two layers were withdrawn and measured for the radioactivity. The readings thus obtained were used to calculate the Log $P_{o/w}$ value of the complex.

Serum stability studies

A volume of 50 μL of the labeled compound was added to 0.5 mL of human serum and this mixture was incubated at 37°C for 3 h. About 100 μL aliquot was drawn at 1, 2 and 3 h into separate centrifuge tubes and an equal volume of ethanol was added to precipitate the serum proteins. The mixture was centrifuged and the supernatant was analyzed by HPLC to assess the stability of the complex in the serum.

Biodistribution studies

Swiss mice were used for an *in vivo* distribution study of the labeled complex. *In vivo* tumor models were developed by subcutaneous administration of murine fibrosarcoma cell line (~10^6 cells/animal) on the dorsal region. The tumors were allowed to grow till they reached a size of approximately 1 cm in diameter and then the animals were used for the biodistribution study. The radioactive preparation (~37 MBq per animal) was injected intravenously into the tumor-bearing mice via the tail vein. Individual sets of animals ($n=3$) were utilized for studying the biodistribution at different time points (30, 60 and 180 min). At the end of the various time periods, the animals in the respective sets were immediately sacrificed and the relevant organs excised for measurement of retained activity. The activity associated with each organ was then measured in a flat-bed-type NaI(Tl) counter with a suitable energy window for ^{99m}Tc. All procedures performed herein were in strict compliance with the national laws of governing the conduct of animal experiments.

Conclusions

The potential of 2-nitroimidazole and 4-nitroimidazole in targeting tumor hypoxia was explored in tumor-bearing animal models. Towards this, the iminodiacetic acid derivatives were synthesized in excellent yield using the BFCA approach for subsequent radiolabeling with the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core in over 95% yield, under mild conditions and low ligand concentration. *In vivo* distribution studies in Swiss mice showed a steady retention of activity in the case of 2-nitroimidazole derivatives, whereas the 4-nitroimidazole derivative showed a high initial uptake but rapid washout from the tumor. The study, carried out for a limited time, demonstrated a better potential of the 2-nitroimidazole derivative over the 4-nitroimidazole derivative, as a tumor-targeting agent.

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