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Evaluation of $^{99m}Tc(CO)_3$ complex of 2-methyl-5-nitroimidazole as an agent for targeting tumor hypoxia

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Abstract—An iminodiacetic acid (IDA) derivative of 2-methyl-5-nitroimidazole was synthesized as a carrier molecule for radiolabeling with the gamma emitting radioisotope, ^{99m}Tc, for imaging hypoxic regions of tumors. The ligand was synthesized in excellent yield and labeled using freshly prepared $[^{99m}Tc(CO)_3(H_2O)_3]^+$ intermediate. A complexation yield of over 95% could be achieved under mild conditions using a ligand concentration of 1 mg/mL [$\sim 3 \times 10^{-3}$ M]. The complex was characterized by HPLC and its stability in human serum was studied. Biodistribution studies performed in Swiss mice bearing fibrosarcoma tumor showed maximum accumulation in the tumor to the extent of ~0.52 %ID/g at 30 min post-injection (pi). The major clearance of the complex was through the hepatobiliary route. The complex showed tumor/muscle ratio of 1.75 at 30 min pi, which significantly increased to 17 at 180 min pi. However, the tumor/blood ratio was below one throughout the period of study, which could be due to slow clearance of the complex from blood.

1. Introduction

The inability of surrounding vasculature in keeping pace with the tumor growth is a major factor leading to the development of hypoxic regions in tumors.[1] Resistance of hypoxic tissues toward radiation damage is well documented and therefore, the knowledge of the extent of hypoxia in tumor tissue could be exploited in planning an effective treatment strategy. The inherent drawbacks in current available invasive techniques to gauge hypoxia[2] provided the impetus to explore the possibilities of developing new agents. Nitroimidazoles, since long, are known for their ability to accumulate in hypoxic regions. When inside the hypoxic cell, nitroimidazoles undergo a series of enzymatic reductions, mediated by nitroreductase enzymes, followed by ring fragmentation, leading to the formation of reactive radicals, which then irreversibly bind to the cellular components.[3] In normoxic cells, the presence of oxygen prevents the enzymatic reduction of nitroimidazole, and hence no binding occurs. Therefore, nitroimidazoles could be used as vectors for the development of imaging agents for in vivo assessment of tumor hypoxia. While 2-nitroimidazoles[2–5] are more extensively studied for use in imaging hypoxia, 5-nitroimidazoles[6–9] and 4-nitroimidazoles[10,11] have also been reported in designing of similar agents. Apart from being cost effective, the desirable characteristics of an ideal hypoxia imaging agent include, ease of preparation, high stability, rapid accumulation with adequate retention in hypoxic tumor, and rapid clearance from other vital organs and tissues in order to provide a better contrast between lesion and background. In connection with the ongoing research of our group to develop a nitroimidazole based agent to non-invasively probe hypoxia, we have earlier reported a xanthate derivative[9] of metronidazole labeled with ^{99m}TcN core, which showed surprisingly steady retention in fibrosarcoma tumor (~1.4%ID/g up to 3 h pi). However, possibly due to the neutral and lipophilic nature of the complex, the undesirable characteristics like high liver uptake and slow clearance from liver and blood could not be circumvented. Toward efforts to strike an optimal balance between the much-required lipophilicity of the nitroimidazole complex albeit not simultaneously increase its uptake in the liver, the challenging task of envisaging a ^{99m}Tc-complex with a resultant charge

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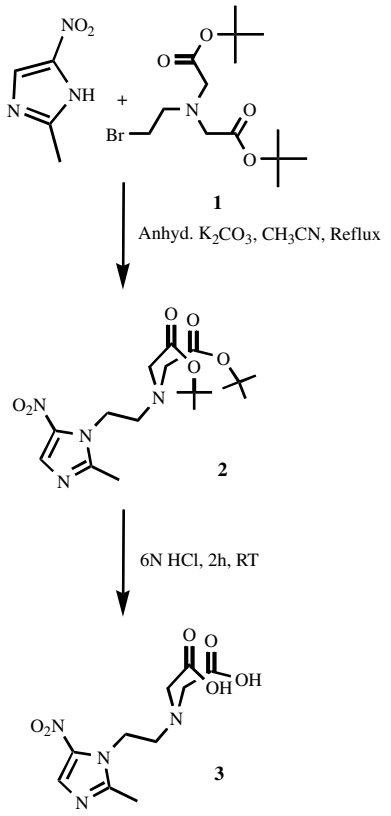
was felt pertinent. Commonly observed complexes with ^{99m}Tc(V) oxo core have a disadvantage of low specific activity, since higher ligand concentration is required to stabilize the +5 oxidation state. Advent of novel cores of technetium has opened fresh avenues for the preparation of stable complexes with high specific activity.[12,13] The *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ core, as demonstrated by Alberto et al.[14] can be prepared as a versatile precursor for reacting with a host of bi- and tridentate ligands. The intermediate is less sensitive to oxidation and forms complexes with ligands at low ligand concentration. The three substitutionally labile water molecules facilitate the formation of stable complexes with tridentate ligands such as iminodiacetic acid, diethylene triamine, histidine, etc. and hence the target-specific molecule of interest is often modified to possess one of these ligating groups. In the present study, iminodiacetic acid derivative of 2-methyl-5-nitroimidazole was the choice. The nitroimidazole was derivatized to the iminodiacetate and labeled with [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ core under mild conditions. The preparation was then evaluated in Swiss mice bearing fibrosarcoma tumor.

2. Results and discussion

2.1. Synthesis

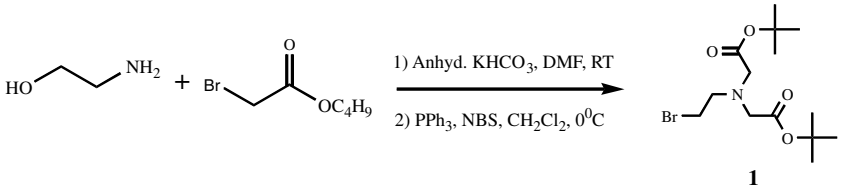
The synthesis of the iminodiacetic acid derivative of 2-methyl-5-nitroimidazole was carried out in two steps. In the first step, *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-bromoethylamine was synthesized, following a reported procedure,[15] (Scheme 1) by reacting ethanolamine and *tert*-butylbromoacetate in presence of $KHCO_3$ in DMF. The crude product, *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-hydroxyethylamine], was then treated with PPh_3 and *N*-bromosuccinimide in dichloromethane to yield the target compound (**1**). This intermediate, a bifunctional chelator, in the subsequent step is used for N-alkylation of the –NH– of the 2-methyl-5-nitroimidazole to yield *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-(2-methyl-5-nitroimidazolyl)ethylamine] **(2)**. The resultant ester was then hydrolyzed using 6 N HCl in near quantitative yield to obtain the desired product (**3**) (Scheme 2). The hydrolysis of the ester can be confirmed by the disappearance of the singlet at δ 1.43 integrating for 18 protons, which was representative of the *tert*-butyl group, in the ^{1}H NMR spectra of **3** and also the molecular ion peak observed at *m*/*z* 286.9, in the mass spectrum of **3**.

Prior to complexation, the [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ core was prepared as described in the experimental section. The core could be prepared in >97% yield, as determined by



Scheme 2. Synthesis of IDA-derivative of 2-methyl-5-nitroimidazole.

C18 reversed phase HPLC. The carbonyl peak appears as a characteristic broad peak at 12.08 ± 0.1 min (Fig. 1a). The nitroimidazole complex could be prepared by incubating **3** with [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ core for 30 min at room temperature. In the HPLC chromatogram Figure 1b, the sharp peak representing the complex appeared at 10.50 ± 0.2 min. The peak area calculations showed that the complex was formed in over 96% yield. Roger Schibli et al.[16] had earlier reported the preparation and characterization of a variety of technetium carbonyl complexes of tridentate and bidentate ligands, including iminodiacetic acid. It was shown that the technetium carbonyl complex formed with iminodiacetic acid bears a resultant negative charge. Therefore, it can be presumed that the tricarbonyl complex formed with iminodiacetic acid derivative of 2-methyl-5-nitroimidazole would also be similar and have a resultant negative charge. Lipophilicity of the radiolabeled complex was determined by distribution in octanol and water as explained in the experimental section. The log *P* value of the complex was found to be −0.82 reflecting its low lipophilicity. In vitro serum stability of the complex was studied by incubation of the complex in human serum at 37 °C for 3 h.



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

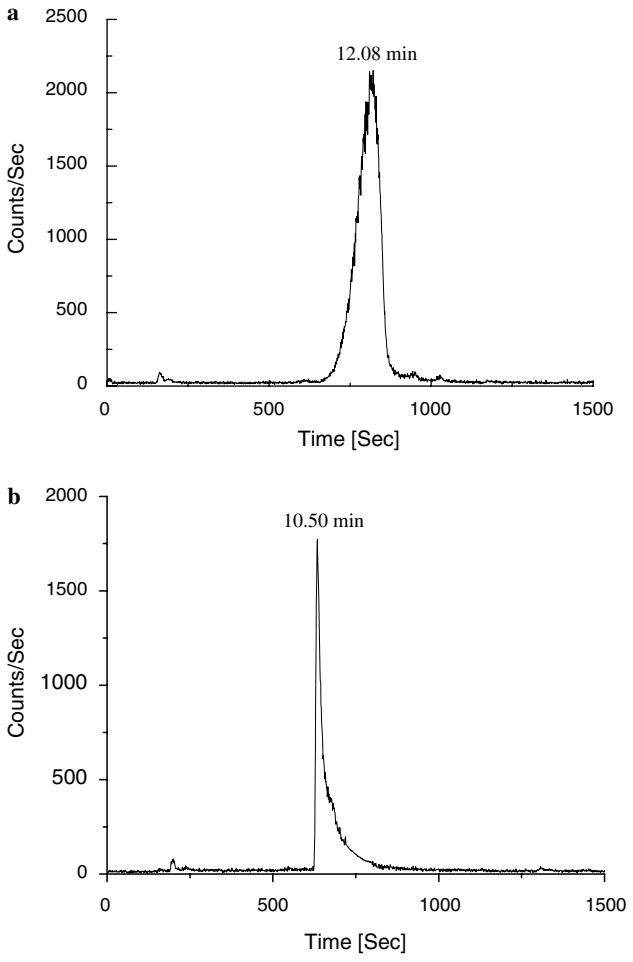


Figure 1. HPLC pattern of (a) $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core and (b) $^{99m}Tc(CO)_3$-complex.

Serum stability of the complex was ascertained by HPLC at 1 and 3 h, wherein a single sharp peak with retention time of 10 min was observed.

2.2. Biodistribution studies

Biodistribution studies were carried out in Swiss mice bearing fibrosarcoma tumor. A maximum tumor uptake of 0.52%ID/g was observed at 30 min pi, which reduced to 0.34%ID/g at 180 min pi (Table 1). The injected activity was found to clear mainly through the hepatobiliary route. About 10–14%ID of activity was found to be associated with urine at each time point. The clearance of activity from liver was fast as can be observed from the accumulation of large amount of activity in intestine as early as 30 min pi. It can be recalled here that the log *P* value of ^{99m}TcN-metronidazole xanthate[9] was over 1.5 as compared to the value of −0.82 for the present complex. Therefore, the lower liver uptake and faster washout could be possibly attributed to the reduced lipophilicity. The tumor/muscle ratio was found to be 1.7 at 30 min pi, which then increased to 17 at 180 min pi. The tumor/blood ratio was below 1 throughout the period of study. This may be due to slow clearance of activity from blood. However, the level of blood binding was significantly lower as compared to the erstwhile reported ^{99m}TcN-metronidazole xanthate.[9]

3. Conclusion

The IDA-derivative of 2-methyl-5-nitroimidazole was synthesized in excellent yield and successfully labeled with the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor under mild conditions. Over 96% complexation yield could be achieved with a low ligand concentration of ~1 mg/mL. The biodistribution in Swiss mice bearing fibrosarcoma tumor showed significant tumor uptake with high tumor/muscle ratio. The uptake in liver was lower and clearance faster than observed with similar ^{99m}Tc-nitroimidazole complexes. There was no significant uptake in other vital organs.

4. Experimental

2-Methyl-5-nitroimidazole, 2-aminoethanol, *tert*-butylbromoacetate, *N*-bromosuccinamide (NBS), and triphenylphosphine were purchased from Aldrich, USA. Potassium carbonate and potassium bicarbonate were procured from Fluka, Germany. Carbon monoxide gas used for the preparation of ^{99m}Tc carbonyl core was also obtained from Aldrich, USA. All the solvents used for synthesis were dried as per the reported 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 Merck, USA. Chromatogram of the prepared complexes was obtained on a JASCO PU

Table 1. Distribution of $^{99m}Tc(CO)_3$-complex in Swiss mice bearing fibrosarcoma tumor ($n = 3$)

Organ	% Injected dose per gram (±SD)		
	30 min pi	60 min pi	180 min pi
Liver	7.7 (0.76)	8.3 (0.4)	8.74 (0.02)
Int + GB	25.01 (5.49)	27.73 (2.96)	26.84 (3.26)
Kidney	3.65 (0.90)	4.15 (0.56)	2.45 (0.39)
Heart	0.78 (0.61)	1.98 (0.96)	1.29 (0.62)
Lungs	1.4 (0.89)	2.56 (0.66)	2.96 (0.72)
Muscle	0.31 (0.12)	0.14 (0.01)	0.02 (0.01)
Blood	1.96 (0.71)	1.36 (0.26)	1.04 (0.07)
Tumor	0.52 (0.1)	0.50 (0.02)	0.34 (0.04)
Tumor/blood	0.26	0.37	0.33
Tumor/muscle	1.7	3.6	17

Int + GB, intestine and gall bladder.

1580 dual pump HPLC system, Japan, with a JASCO 1575 tunable absorption detector and a radiometric detector system, using a C18 reversed phase HiQ Sil (5 μm, 4 × 250 mm) column. The solvents used for HPLC were filtered through Millipore filter paper and contained 0.1% trifluoroacetic acid. ^{1}H NMR spectra were recorded on a 300 MHz Varian VXR 300S spectrophotometer, USA. The mass spectra were recorded on a QTOF Micromass instrument using electron spray ionization (ESI) in positive mode. Elemental analysis was performed on C, H, N, S elemental analyzer, Thermofinnigan, Flash EA 1112 series.

4.1. Synthesis

4.1.1. Synthesis of *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-bromoethylamine (1). To *tert*-butylbromoacetate (3.3 g, 17.3 mmol) dissolved in 15 mL DMF was added powdered $KHCO_3$ (1.92 g, 19.2 mmol). The suspension was cooled to 0 °C, and ethanolamine (0.46 mL, 7.5 mmol) was added dropwise over a period of five minutes. The reaction mixture was stirred at 0 °C for 30 min and then for 24 h at room temperature. 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 resulting solid was separated. The ether layer was concentrated and passed through a small column of silica using ether as the eluant. Evaporation of ether yielded a yellow oil, which was then purified by silica gel chromatography (1.7 g, 80%); R_f 0.28 (5/1 hexane/ether); ^{1}H NMR ($CDCl_3$, δ ppm) 1.46 (s, 18H, *tert*-butyl CH_3); 3.1 (t, 2H, J = 7.5 Hz, BrCH_2CH_2N); 3.4 (t, 2H, J = 7.5 Hz, BrCH_2CH_2N); 3.5 [s, 4H, (*tert*-butoxycarbonyl-CH_2)$_2$N]. C, H, N: Observed (calcd) 47.81 (47.73), 7.97 (7.44), 3.96 (3.98).

4.1.2. Synthesis of iminodiacetic acid derivative of 2-methyl-5-nitroimidazole (3). To 2-methyl-5-nitroimidazole (100 mg, 0.79 mmol) in 15 mL of acetonitrile was added anhydrous K_2CO_3 and the suspension stirred for 15 min. *N*,*N*-Bis[(*tert*-butoxycarbonyl)methyl]-2-bromoethylamine] (277 mg, 0.79 mmol) was added and the reaction mixture refluxed for 12 h following which it was cooled and filtered free of potassium carbonate. The pure compound, *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-2-(2-methyl-5-nitroimidazolyl)ethylamine (**2**), was then obtained by silica gel chromatography using chloroform as the eluant (267 mg, 85%). ^{1}H NMR ($CDCl_3$, δ ppm) 1.43 (s, 18H, [($H_9C_4O_2C$–CH_2)$_2$N–]); 2.43 (s, 3H, 5-nitroimidazole-CH_3); 3.04 (t, 2H, J = 6 Hz, nitroimidazole-CH_2CH_2N); 3.35 (s, 4H, [($H_9C_4O_2C$–CH_2)$_2$N–]); 4.06 (t, 2H, J = 6 Hz, nitroimidazole-CH_2CH_2N); 7.96 (s, 1H, imidazole H). C, H, N: Observed (calcd) 54.29 (54.26); 8.11 (7.59); 14.15 (14.06). The purified ester **2** was then dissolved in 1 mL of methanol and 7 mL of 6 N HCl. The reaction mixture was then stirred at room temperature for 2 h, after which the solvent was removed under vacuum to yield the iminodiacetic acid derivative **3** (183 mg, 95%); MS (ESI) *m*/*z*: 286.9 (M^++1); ^{1}H NMR (D_2O, δ ppm) 2.3 (s, 3H, 5-nitroimidazole-CH_3); 3.6 (t, 2H, J = 7.2 Hz, nitroimidazole-CH_2CH_2N); 4.1 (s, 4H, [(HO_2C–CH_2)$_2$N–]); 4.4 (t, 2H, J = 7.2 Hz, nitroimidazole-CH_2CH_2N); 8.06 (s, 1H, imidazole H). C, H, N: Observed (calcd) 41.91 (41.96); 5.21 (4.93); 19.63 (19.57).

4.2. Radiolabeling

4.2.1. 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 glass serum vial. The vial was sealed and carbon monoxide gas was purged through the solution for 5 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 of the vial and re-equilibration with atmospheric pressure, pH of the reaction mixture was adjusted to 7 using a mixture of 0.5 M phosphate buffer (pH 7.5):1 M HCl (1:3). The intermediate thus prepared was characterized by HPLC.

4.2.2. Labeling of 3 with $[^{99m}Tc(CO)_3(H_2O)_3]^+$. Under optimized conditions, 0.5 mL of freshly prepared $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor was added to 0.5 mL of double distilled water containing 0.5–1 mg of the ligand **3**. The pH of the mixture was maintained between 7 and 8. The mixture was vortexed for a minute and then incubated at room temperature for 30 min. The complex was characterized by HPLC.

4.3. Quality control techniques

4.3.1. Serum stability studies. Fifty microliters of the labeled compound was added to 0.5 mL of human serum and this mixture was incubated at 37 °C for up to 3 h. Aliquots were taken at 1 and 3 h, and analyzed by HPLC to assess the stability of the complex in serum.

4.3.2. 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 acetonitrile/water, binary solvent system, and a gradient elution program (Solvent A–acetonitrile, Solvent B–water; 0 min–10% A, 28 min–90% A, 30 min–90% A). The flow rate was maintained at 1 mL/min.

4.3.3. Partition coefficient. About 100 μL of the labeled compound was mixed with 0.9 mL water and 1 mL of octanol on a vortex mixer for about 1 min. The two phases were allowed to separate. Equal aliquots of the organic and aqueous layers were withdrawn and measured for radioactivity to determine the partition coefficient.

4.4. Biodistribution studies

Swiss mice were used to perform the in vivo uptake as well as the distribution study of the labeled complex.

The mice were injected subcutaneously on the dorsal region with murine fibrosarcoma cell line ($\sim 10^6$ cells/animal). The tumors were allowed to develop until they reached approximately 1 cm in diameter and then the animals were used for biodistribution study.

In the study, the tumor bearing mice were administered intravenous injection of ~3.7 MBq (~100 μCi) of the radioactive preparation via tail vein. Different sets (3 animals per set) were kept for different time points (30, 60, and 180 min). After the respective incubation periods, the animals were 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 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.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.08.011.

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