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Synthesis and evaluation of 2-, 4-, 5-substituted nitroimidazole-iminodiacetic acid-$^{99m}Tc(CO)_3$ complexes to target hypoxic tumors

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Determination of hypoxia in tumor is an important problem in the clinical management of cancer. Towards this, a series of differently substituted nitroimidazoles, viz. 2-nitro, 4-nitro and 5-nitroimidazole iminodiacetic acid (IDA) derivatives were synthesized and radio-labeled with a $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core. The corresponding $^{185/187}Re(CO)_3$ analogue of 2-nitroimidazole-IDA-$^{99m}Tc(CO)_3$ complex was also prepared and characterized to elucidate the mode of bonding between the ligand and the $M(CO)_3$ core ($M = ^{99m}Tc$, $^{185/187}Re$). All the three nitroimidazole-IDA-$^{99m}Tc(CO)_3$ complexes could be prepared in over 95% yield determined by HPLC. The three complexes were then evaluated in a suitable animal model bearing tumor. Though the *in vivo* accumulation of complexes in hypoxic tissue is governed by factors such as lipophilicity, charge, etc., the variation in accumulation in hypoxic tissue, in the present case, could be explained by considering the reported values of single electron reduction potential of the respective nitroimidazoles. Among the three derivatives studied, the 2-nitroimidazole-IDA-$^{99m}Tc(CO)_3$ complex produced the best result followed by the 5-nitroimidazole complex.

Keywords: hypoxia; tumor; 99m-technetiumtricarbonyl core; 2-nitroimidazole; 4-nitroimidazole; 5-nitroimidazole

Introduction

The negative influence of hypoxia in the chemotherapy and radiotherapy of cancer is well established. The reason often attributed to this phenomenon is 'oxygen effect', wherein the presence of oxygen is required for fixation of radiation-induced damage to DNA.[1] It has been shown that hypoxia can induce gene amplification, chromosomal rearrangements, DNA over replication, etc., the consequence of which is the acquisition of drug resistance.[2] Information about the presence of hypoxia in tumor could possibly help the physicians to modify the treatment strategy to improve the effectiveness of treatment. Although several invasive procedures are available to measure hypoxia in tumors, which are also predictive of response to therapy, none of these are in routine clinical use because of their technical complexity, inconvenience and their inability to enable repetitive measurements. Several times the results are erratic due to heterogeneous nature of hypoxia. Hence, a non-invasive procedure could be of immense clinical utility.

Nitroaromatics, particularly nitroimidazoles, are probably the most widely explored molecules for targeting hypoxic tissues. The nitroimidazole tracers undergo a series of enzymatic one-electron reductions in viable hypoxic cells and its metabolites bind to the cellular components. In normoxic cells, the reduction does not proceed beyond first step and hence there is no accumulation of the tracer.[3] This peculiar property of nitroimidazole is taken advantage in specifically targeting the hypoxic cells.

The ^{18}F-Fluoromisonidazole[4] and ^{123}I-Iodoazomycinarabinoside (IAZA)[5] are two nitroimidazole-based radiotracers being widely evaluated clinically. However, owing to limited availability, high cost of production and short half-life of these cyclotron produced isotopes, a ^{99m}Tc-based agent could be a better alternative. The 2-nitroimidazole derivatives were by far the most widely evaluated for their potential to target hypoxia. This is because, among differently substituted nitroimidazoles, i.e. 2-, 4- and 5-nitroimidazole, 2-nitroimidazole possess the more positive single-electron reduction potential (SERP) value [−250 to −350 mV, with respect to standard hydrogen electrode (SHE)],[6] which facilitates efficient trapping inside the hypoxic cells. The SERP of 4-nitroimidazole range from −500 to −550 mV, whereas, for 5-nitroimidazole the SERP range from −400 to −450 mV with respect to SHE. The exact value of the SERP depends on the substitution in the nitroimidazole ring.

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However, the single electron reduction potential is not the only parameter which decides the uptake and retention of the tracer inside the hypoxic cells. The lipophilicity and the charge of the tracer may also play significant role in determining the usefulness of the agent *in vivo*. While lipophilicity is important for the tracer to cross the cell membrane, charge affects the pharmacokinetics of the tracer. These parameters operate in an intricate manner, which then decides the overall behavior of the agent *in vivo*. This may be the reason for few observations in the literature where even the 4- and 5-nitroimidazole derivatives show encouraging results with respect to targeting hypoxia, although the SERP of 4- and 5-nitroimidazoles are less positive compared to 2-nitroimidazole.[7–9] This fact indicates the significance of carrying out the preliminary evaluation of even 4- and 5-nitroimidazole derivatives to target hypoxia.

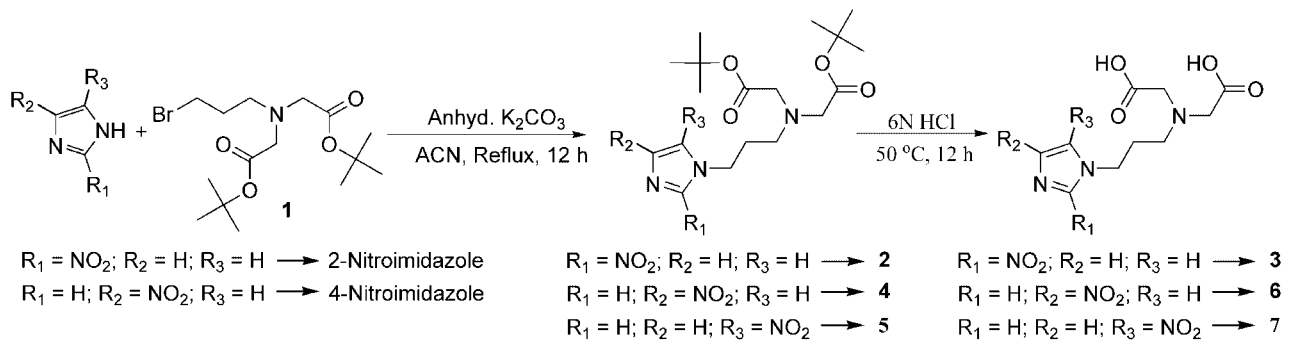
The introduction of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core opened up the possibility of labeling molecules with ^{99m}Tc using low ligand concentration yielding complexes with high *in vivo* stability.[10] The three substitutionally labile water molecules in $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core facilitate the formation of stable complexes with tridentate ligands such as IDA, diethylenetriamine, histidine, etc.[11] and hence target-specific molecules of interest are often modified to possess one of these ligating groups. Towards this, a bifunctional chelating agent (BFCA), with a pendant bromo substituent at one end and an iminodiacetic acid ester at the other, was synthesized and used for the alkylation of the –NH– of the imidazole ring. Hydrolysis of the resultant conjugate generated the required iminodiacetic acid functionality suitable for radiolabeling with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core. In the present work, synthesis, characterization and *in vivo* evaluation of the three $[^{99m}Tc(CO)_3]$-labeled nitroimidazole derivatives viz. 2-nitro, 4-nitro and 5-nitroimidazole are reported.

Results and discussion

The iminodiacetic acid derivatives of differently substituted nitroimidazoles are synthesized via the bi-functional chelator approach as depicted in Scheme 1. The bi-functional chelating agent (BFCA), *N*,*N*-bis[(*tert*-butoxycarbonyl)methyl]-3-bromopropylamine **(1)**, was synthesized as reported earlier.[12] Subsequently, the BFCA was conjugated to the 2-nitroimidazole to obtain corresponding *tert*-butyl ester derivative. Following similar strategy, the 4-nitroimidazole *tert*-butyl ester derivative could be obtained. However, 5-nitroimidazole derivative could not be prepared directly since 5-nitroimidazole is not available commercially. Rao *et al.* had reported the formation of 5-nitroimidazole derivative during base-mediated N-alkylation of 4-nitroimidazole[13] and it was found that the base-catalyzed N-alkylation of 4-nitroimidazole with BFCA resulted in the formation of 5-nitroimidazole *tert*-butyl ester along with the 4-nitroimidazole ester.

The respective purified esters of 2-, 4- and 5-nitroimidazoles were then hydrolyzed with 6 N HCl to obtain the corresponding iminodiacetic acid derivatives in quantitative yields. All the compounds synthesized are characterized by IR, ^{1}H-NMR and elemental analysis. The $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core was prepared using Isolink carbonyl kit vial and subsequently used for the preparation of $^{99m}Tc(CO)_3$ complexes. The procedure followed was briefly described in the experimental section. The prepared complexes were characterized by HPLC. The HPLC elution profile of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core and three complexes were given in Figure 1. It can be observed that $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core appeared as a broad peak at 17.4 min, whereas the 2-, 4- and 5-nitroimidazole complexes eluted as a single sharp peaks at 12.7, 15.6 and 15.7 min respectively. To elucidate the mode of binding of the ligands with the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core, the corresponding $^{185/187}Re(CO)_3$-analogue of 2-nitroimidazole iminodiacetic acid was synthesized as a representative of the iminodiacetic acid series being evaluated in the present study. The $^{185/187}Re(CO)_3$-analogue was prepared following reported procedure (Scheme 2). To confirm the formation of analogous rhenium complex, the HPLC profiles were compared and it could be observed that the elution profile of the 2-nitroimidazole-$^{185/187}Re(CO)_3$ complex matches with that of the 2-nitroimidazole-$^{99m}Tc(CO)_3$ complex prepared in the no-carrier-added (nca) level (Figure 2). The ^{1}H-NMR of the rhenium complex showed two AB quartets, centered at 3.54 δ and 3.78 δ respectively, corresponding to the $-N(CH_AH_BCO-)_2$ proton, which otherwise appeared as a singlet at 3.88 δ in the un-complexed ligand **3**. This change in the ^{1}H-NMR pattern suggested the tridentate coordination of the ligand to the metal core as envisaged in the Re-complex. The Log$P_{o/w}$ of the complex was determined as per reported procedure described earlier.[14] The Log$P_{o/w}$ of the 2-, 4- and 5-nitroimidazole complexes were found to be 0.48, 0.43 and 0.39 respectively. Incubation of the respective complexes in serum did not show any signs of decomposition during the period of study.

The uptake and retention of the three complexes observed in the tumor during the bio-distribution studies is graphically shown in Figure 3. The 2-nitroimidazole complex showed higher uptake than the 4- and 5-nitroimidazole complexes. The tumor uptake observed with 2- and 4-nitroimidazole complex at 30 min post injection (p.i.) may not be strictly due to the complex trapped in the hypoxic cells, since clearance of activity from tumor can be observed till 60 min p.i. Thereafter, clearance of



Scheme 1.

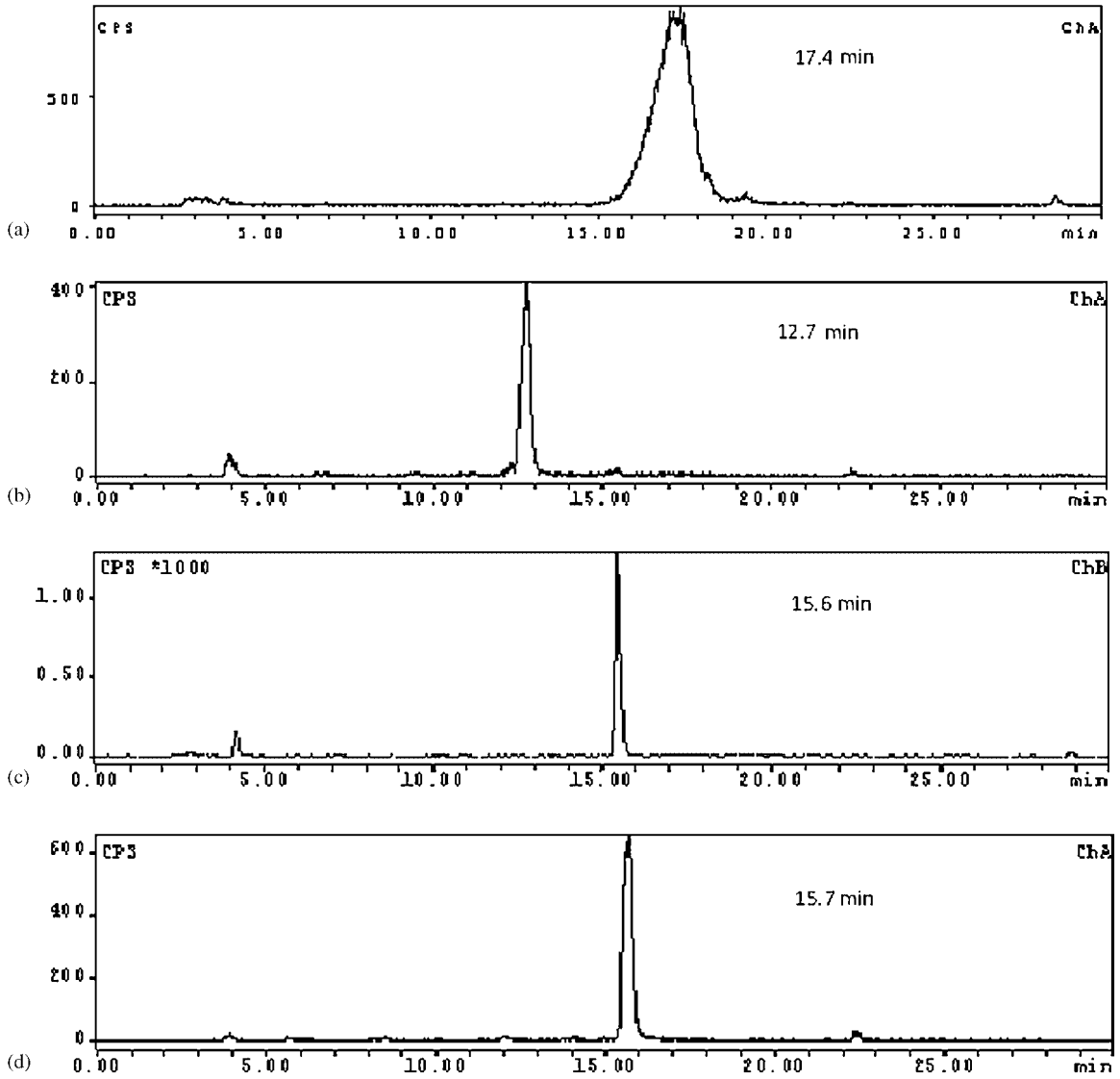
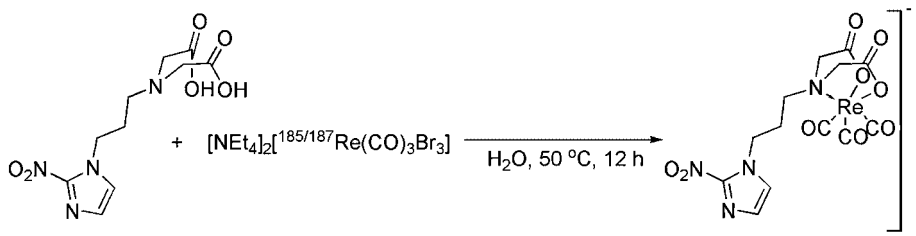


Figure 1. The HPLC elution profiles of: (a) [^{99m}Tc(CO)$_2$] core; (b) 2-nitrroimidazole-IDA-^{99m}Tc(CO), complex; (c) 4-nitroimidazole-IDA-^{99m}Tc(CO)$_2$ complex; and (d) 5-nitroimidazole-IDA-^{99m}Tc(CD)$_3$ complex.



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Scheme 2.

activity from tumor becomes slow for all the complexes, possibly indicating the trapping mechanism being operated. However, the rate of clearance of activity among the complexes was not observed to be the same, with the 2-nitroimidazole complex showing relatively slower clearance compared to the other two nitroimidazole complexes.

Reports available in the literature indicate that the trapping mechanisms of nitroimidazole-based agents are not the same in all cases. Metronidazole was observed to make adduct with DNA, whereas ^{18}F-FMISO adduct with glutathione was detected in cells.[15] Transient trapping of agents has also been suggested where they are not irreversibly bound to the cell components

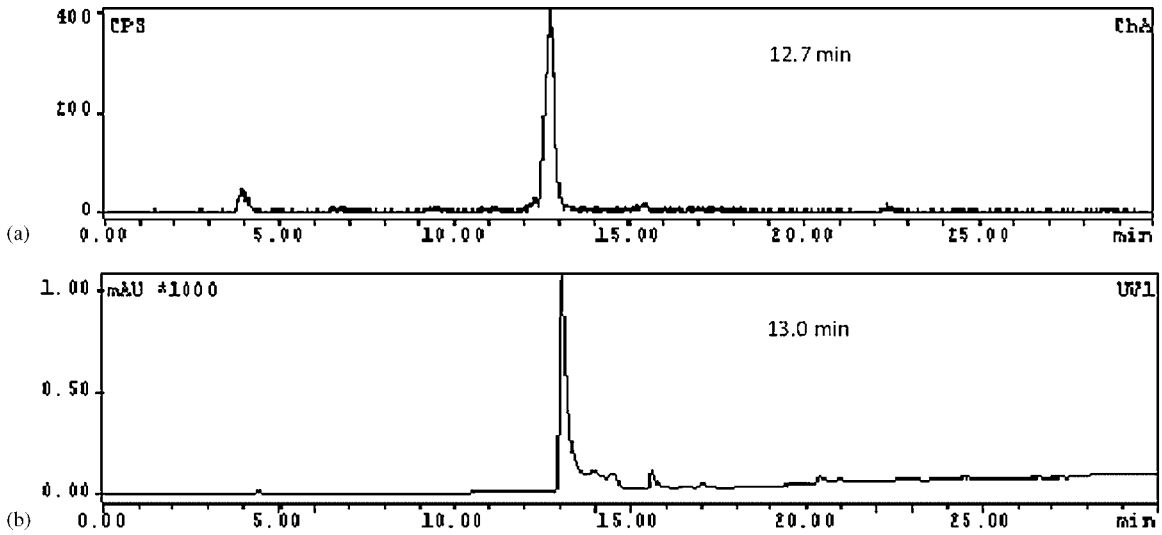

Figure 2. The HPLC elution profile of (a) 2-nitromidazole-IDA-$^{99m}Tc(CO)_3$ complex and (b) 2-introimidazole-IDA-$^{186/187}Re(CO)_3$ complex.

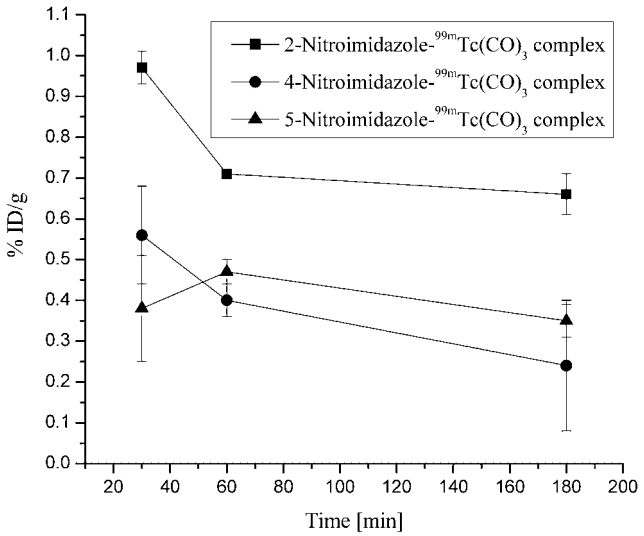

Figure 3. Uptake and retention characteristics of different complexes in tumor with time.

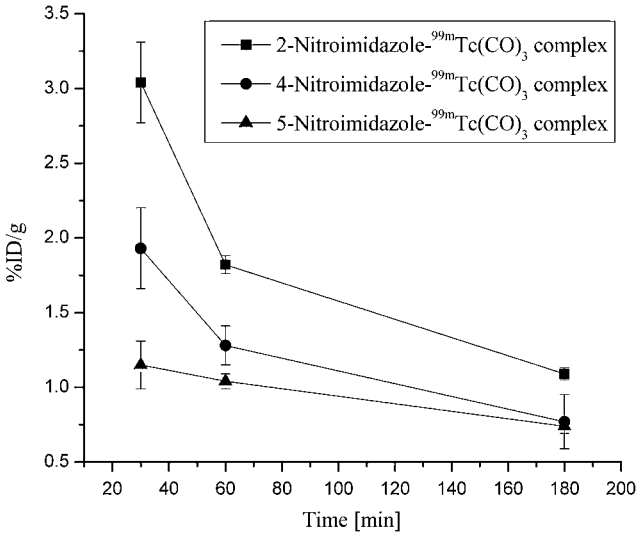

Figure 4. Clearance of blood pool activity of different complexes with time.

but simply metabolized into more hydrophilic species, which clear out gradually from hypoxic cells.

In the present study, since clearance of the activity was observed from muscle, the possibility that the activity observed in tumor could be due to the non-clearance of the diffused unbound complex could be ruled out. Additionally, it is pertinent to note that, in order that the activity associated with the tumor is entirely due to the blood pool unbound activity, the tumor need to have unrealistically high amount of blood in it. Although no attempt has been made to elucidate the trapping mechanism of the complexes under investigation, available evidence indicates that the activity observed in tumor could be due to the transient trapping mechanism operating in hypoxic tissue.

The clearance pattern of the different complexes from blood is shown in Figure 4. The clearance follows expected pattern derived from the Log$P_{o/w}$ of the complexes. The 2-nitroimidazole complex with highest Log$P_{o/w}$ clears relatively slowly compared to the 4- and 5-nitroimidazole complexes. The rate of clearance of the complex from blood decides the average residence time of that complex in cells, irrespective of the oxygenation status of the cell. The complex enters or exits the cells via passive diffusion. Initially, when the concentration of the complex in blood is high, diffusion is from blood to the cells and later, as the concentration of complex in blood falls, the direction of diffusion reverses. So, the faster the clearance of the complex from blood, the earlier will be the reverse diffusion and consequently, lesser will be the time spent by the complex in cells. Thus, in the present case, the residence time of the 2-nitroimidazole complex in cells should be more compared to that of the 4- and 5-nitroimidazole complexes.

Once the complex is inside the hypoxic cell, its single electron reduction potential decides the efficiency with which it gets reduced and trapped. Complexes with more positive reduction potential reduce more readily compared to the complexes with less positive reduction potential. This factor combined with the average time a complex spends inside the cell can possibly

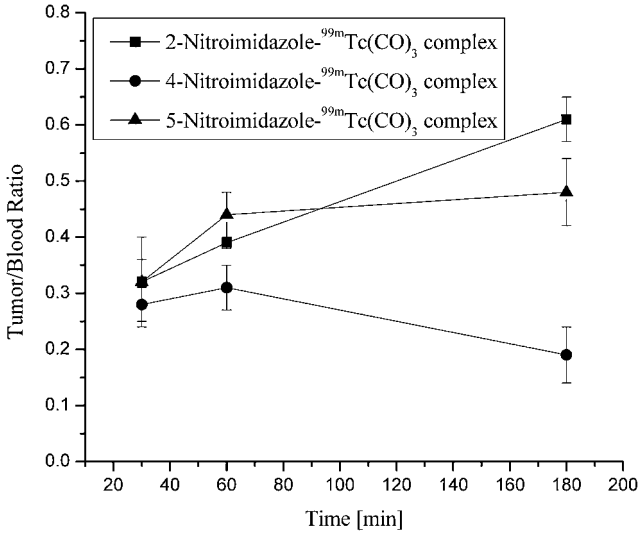

Figure 5. Variation in tumor/blood ratio of different complexes with time.

explain the observed tumor uptake and retention of the complexes under investigation. The 2-nitroimidazole complex, which spends more time in cells, with more positive single-electron reduction potential, shows the highest uptake followed by 5- and 4-nitroimidazole complexes respectively.

The tumor-to-blood ratio of the three complexes at different time points is shown in Figure 5. For 2- and 5-nitroimidazole complex, the tumor-to-blood ratio improved with time, attaining a maximum of 0.61 and 0.48, respectively, at 3 h p.i. In the case of 4-nitroimidazole complex, the tumor-to-blood ratio deteriorated with time. Significantly high tumor-to-muscle ratio was obtained with 5-nitroimidazole complex, whereas a maximum value of 2.4 at 3 h p.i. was obtained for 2-nitroimidazole complex. The activity distribution of the three complexes in other organs is shown in Figure 6. Major clearance of activity was through hepatobiliary route for all the three complexes. Steady increase in the accumulation of activity in liver and intestine could also been seen for all the complexes with time. There were no indications, such as presence of activity in stomach, fast clearance of activity from liver and intestine, etc., which may point to the possibility of hydrolysis of the complexes *in vivo*. Activity accumulated in other vital organs cleared with time.

Experimental

Synthesis

General

2-Nitroimidazole, 4-nitroimidazole and anhydrous potassium carbonate were purchased from Aldrich, U.S.A. All reagents were of analytical grade. Sodium pertechnetate was eluted using normal saline from a ^{99}Mo-^{99m}Tc column generator. The $[^{99m}Tc(CO)_3(H_2O)_3]^+$ core was prepared using Isolink carbonyl kit vial obtained as a gift from Mallinckrodt Medical B. V. Silica gel plates (silica gel 60 F_{254}) were obtained from Merck, India. The HPLC analyses were performed on a JASCO PU 2080 Plus dual pump HPLC system, Japan, with a JASCO 2075 Plus tunable absorption detector and a Gina Star radiometric detector system, using a C-18 reversed phase HiQ Sil (5 µm, 4 × 250 mm) column. IR spectra of the synthesized compounds were recorded on Jasco-FT/IR-420 spectrophotometer, Japan. ^{1}H-NMR spectra were recorded on a 300 MHz Varian VXR 300S spectrophotometer, U.S.A. Elemental analysis was performed on C, H, N, S elemental analyzer, Thermofinnigan, Flash EA 1112 series.

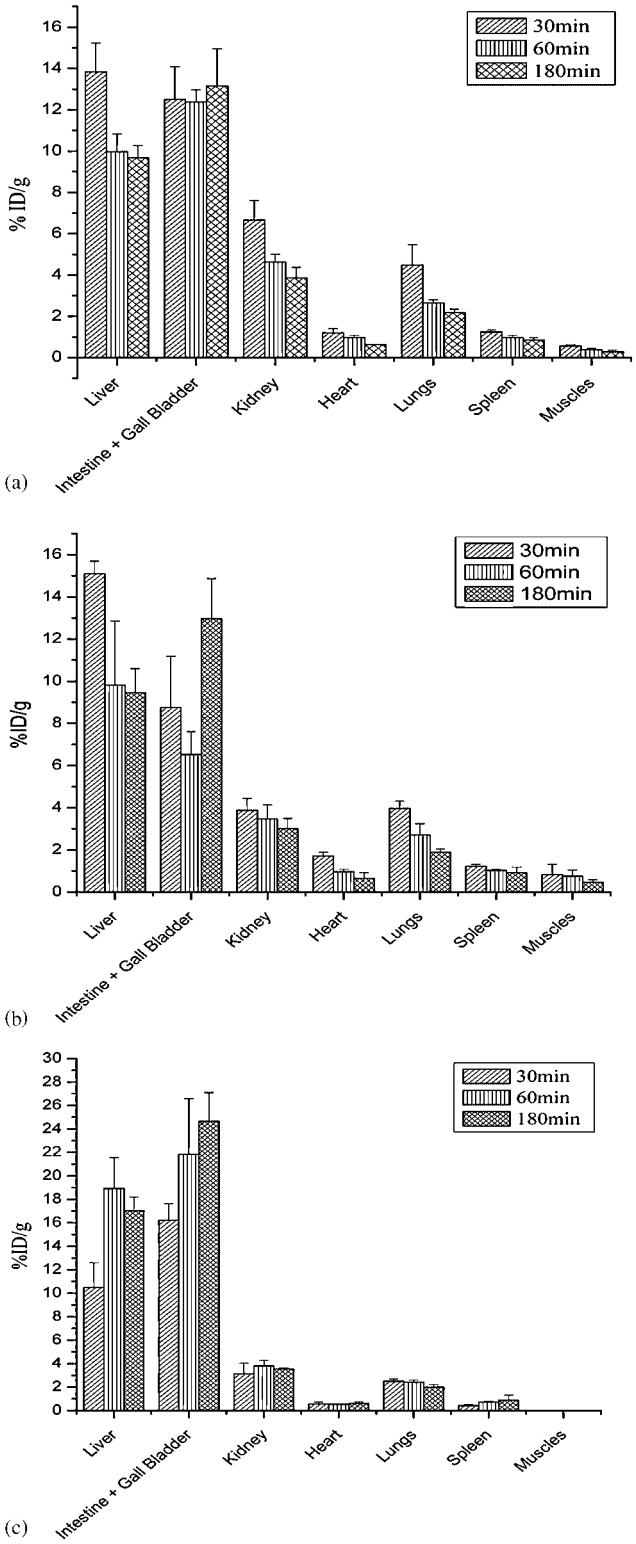

Figure 6. Activity profile of different complexes in various organs: (a) 2-nitroimidazole-IDA-^{99m}Tc(CO)$_3$ complex; (b) 4-nitroimidazole-IDA-^{99m}Tc(CO)$_3$ complex; and (c) 5-nitroimidazole-IDA-^{99m}Tc(CO)$_3$ complex.

General procedure for N-alkylation of different nitroimidazoles

To nitroimidazole (1.1 equiv.) in acetonitrile (15 mL), powdered anhydrous K_2CO_3 (2 equiv.) was added and the suspension stirred for 15 min. To this mixture *N,N*-bis[(*tert*-butoxycarbonyl)-methyl]-3-bromopropylamine (**1**) (1 equiv.) was added and the reaction mixture refluxed with continuous stirring. The progress of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was removed under vacuum and the residue was dissolved in water (15 mL) and extracted twice with chloroform (15 mL × 2). The combined chloroform layer was washed with brine and dried over anhydrous sodium sulphate. The dried chloroform layer was concentrated and purified by silica gel column chromatography.

General procedure for hydrolysis of tert-*butyl esters of different nitroimidazoles*

The *tert*-butyl esters of respective nitroimidazoles (0.25 mmol) was dissolved in 6 N HCl (10 mL) and heated at 50°C for 12 h with continuous stirring. The reaction mixture was then cooled and solvent removed under vacuum. The pure compound could be obtained by re-crystallization from methanol–diethyl ether.

*N,N-Bis[(*tert-*butoxycarbonyl)methyl]-3-bromopropylamine* ***(1)***

The compound N,N-Bis[(*tert*-butoxycarbonyl)methyl]-3-bromopropylamine was synthesized as reported earlier.[12] Analysis for $C_{15}H_{28}BrNO_4$ (Calc) Found: C (49.19) 49.01; H (7.70) 7.96; N (3.82) 3.78.

*N,N-Bis[(*tert-*butoxycarbonyl)methyl]-3-(2-nitroimidazolyl)propylamine (**2**)*

The compound **2** was synthesized following the general procedure for *N*-alkylation using 2-nitroimidazole (169 mg, 1.5 mmol) and N,N-Bis[(*tert*-butoxycarbonyl)methyl]-3-bromopropylamine (**1**) (516 mg, 1.36 mmol). Progress of the reaction was monitored by TLC in ethyl acetate. The pure compound, *N,N*-bis[(*tert*-butoxycarbonyl)methyl]-3-(2-nitroimidazolyl)propylamine (**2**), was then obtained by silica gel chromatography, using chloroform/ethyl acetate (80:20 v/v) mixture as the eluent (512 mg, 94%). R_f (Ethyl acetate) = 0.7; IR (neat, cm^{-1}) 3124 (m); 2977 (m); 2931 (m); 1737 (vs); 1543 (s); 1491 (m); 1336 (m); 1222 (m); 1147 (vs); 1077 (m); 982 (m); 938 (w); 850 (m); 823 (m); 754 (m); 658 (m). ^{1}H-NMR ($CDCl_3$, δ ppm) 1.47 (s, 18H, $-N(CH_2CO_2CC\underline{H}_3)_2$); 1.98 (quintet, 2H, nitroimidazole–$CH_2C\underline{H}_2CH_2N$–); 2.73 (t, 2H, nitroimidazole–$CH_2CH_2C\underline{H}_2N$–); 3.39 (s, 4H, $-N(C\underline{H}_2CO_2CCH_3)_2$); 4.60 (t, 2H, nitroimidazole–$C\underline{H}_2CH_2CH_2N$–); 7.12 (d, 2H, nitroimidazole C4-H,); 7.35 (d, 2H, nitroimidazole C5-H). Analysis for $C_{18}H_{30}N_4O_6$ (Calc) Found: C (54.26) 54.15; H (7.59) 7.65; N (14.06) 14.12.

2-{Carboxymethyl[3-(2-nitroimidazolyl)propyl]amino}acetic acid ***(3)***

The purified ester **2** (100 mg, 0.25 mmol) was hydrolyzed following the general procedure mentioned above. The target compound **3** was obtained as a white powder in quantitative yield (80 mg). 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). ^{1}H-NMR (D_2O, δ ppm) 2.23 (quintet, 2H, nitroimidazole–$CH_2C\underline{H}_2CH_2N$–); 3.32 (t, 2H, nitroimidazole–$CH_2CH_2C\underline{H}_2N$–); 3.88 (s, 4H, $-N(C\underline{H}_2CO_2CCH_3)_2$); 4.21 (t, 2H, nitroimidazole–$C\underline{H}_2CH_2CH_2N$–); 7.35 (d, 2H, nitroimidazole C4-H,); 7.45 (d, 2H, nitroimidazole C5-H). Analysis for $C_{10}H_{14}N_4O_6$ (Calc) Found: C (41.96) 41.68; H (4.93) 5.16; N (19.57) 19.52.

*N,N-Bis[(*tert-*butoxycarbonyl)methyl]-3-(4-nitroimidazolyl)propylamine (**4**) and N,N-Bis[(*tert-*butoxycarbonyl)methyl]-3-(5-nitroimidazolyl)propylamine (**5**)*

The compounds **4** and **5** were synthesized following the general procedure for N-alkylation using 4-nitroimidazole (218 mg, 1.93 mmol) and N,N-Bis[(*tert*-butoxycarbonyl)methyl]-3-bromopropylamine (**1**) (595 mg, 1.6 mmol). Progress of the reaction was monitored by TLC in diethyl ether. The pure compounds, N,N-Bis[(*tert*-butoxycarbonyl)methyl]-3-(4-nitroimidazolyl)propylamine (**4**) and N,N-Bis[(*tert*-butoxycarbonyl)methyl]-3-(5-nitroimidazolyl)propylamine (**5**), were then obtained by silica gel chromatography, using diethyl ether as eluent.

Compound **4**: Yield 65% (420 mg). R_f (Diethyl ether) = 0.52; IR (Neat, cm^{-1}) 3134 (m); 2980 (m); 2929 (m); 1734 (vs); 1540 (s); 1500 (m); 1337 (m); 1225 (m); 1150 (vs); 1071 (m); 988 (m); 940 (w); 854 (m); 823 (m); 761 (m); 659 (m). ^{1}H-NMR ($CDCl_3$, δ ppm) 1.46 (s, 18H, $-N(CH_2CO_2CC\underline{H}_3)_2$); 1.92 (q, 2H, nitroimidazole–$CH_2C\underline{H}_2CH_2N$–); 2.71 (t, 2H, nitroimidazole–$CH_2CH_2C\underline{H}_2N$-); 3.36 (s, 4H, $-N(C\underline{H}_2CO_2CCH_3)_2$); 4.28 (t, 2H, nitroimidazole–$C\underline{H}_2CH_2CH_2N$-); 7.56 (d, 2H, nitroimidazole C2-H,); 7.88 (d, 2H, nitroimidazole C5-H). Analysis for $C_{18}H_{30}N_4O_6$ (Calc) Found: C (54.26) 54.15; H (7.59) 7.68; N (14.06) 14.07.

Compound **5**: Yield 15% (98 mg). R_f (Diethyl ether) = 0.72; IR (Neat, cm^{-1}) 3130 (m); 2980 (m); 2931 (m); 1733 (vs); 1540 (s); 1497 (m); 1336 (m); 1222 (m); 1147 (vs); 1070 (m); 987 (m); 938 (w); 854 (m); 823 (m); 760 (m); 658 (m). ^{1}H-NMR ($CDCl_3$, δ ppm) 1.46 (s, 18H, $-N(CH_2CO_2CC\underline{H}_3)_2$); 1.93 (q, 2H, nitroimidazole–$CH_2C\underline{H}_2CH_2N$–); 2.71 (t, 2H, nitroimidazole–$CH_2CH_2C\underline{H}_2N$–); 3.37 (s, 4H, $-N(C\underline{H}_2CO_2CCH_3)_2$); 4.57 (t, 2H, nitroimidazole–$C\underline{H}_2CH_2CH_2N$–); 7.82 (d, 2H, nitroimidazole C2-H,); 8.0 (d, 2H, nitroimidazole C4-H). Analysis for $C_{18}H_{30}N_4O_6$ (Calc) Found: C (54.26) 54.24; H (7.59) 7.62; N (14.06) 14.17.

2-{Carboxymethyl[3-(4-nitroimidazolyl)propyl]amino}acetic acid ***(6)***

The purified ester **4** (100 mg, 0.25 mmol) was hydrolyzed following the general procedure mentioned above. The target compound **6** was obtained as a white powder in quantitative yield (77 mg). IR (Neat, cm^{-1}) 3132 (m); 2975 (m); 2847 (m); 1761 (vs); 1727 (s); 1559 (m); 1498 (m); 1394 (m); 1350 (m); 1231 (m); 1182 (m); 1140 (s); 1015 (w); 861 (m); 829 (m); 758 (w); 656 (m). ^{1}H-NMR (D_2O, δ ppm) 2.23 (q, 2H, nitroimidazole–$CH_2C\underline{H}_2CH_2N$–); 3.27 (m, 2H, nitroimidazole–$CH_2CH_2C\underline{H}_2N$–); 3.96 (s, 4H, $-N(C\underline{H}_2CO_2CCH_3)_2$); 4.14 (t, 2H, nitroimidazole–$C\underline{H}_2CH_2CH_2N$–); 7.65 (d, 2H, nitroimidazole C2-H,); 8.10 (d, 2H, nitroimidazole C5-H). Analysis for $C_{10}H_{14}N_4O_6$ (Calc) Found: C (41.96) 41.94; H (4.93) 5.09; N (19.57) 19.61.

2-{Carboxymethyl[3-(5-nitroimidazolyl)propyl]amino}acetic acid ***(7)***

The purified ester **5** (100 mg, 0.25 mmol) was hydrolyzed following the general procedure mentioned above. The target compound **7** was obtained as a white powder in quantitative yield (74 mg). IR (Neat, cm^{-1}) 3130 (m); 2974 (m); 2847 (m); 1763 (vs); 1724 (s); 1559 (m); 1499 (m); 1392 (m); 1350 (m); 1230 (m); 1180 (m); 1140 (s); 1014 (w); 861 (m); 827 (m); 758 (w); 654 (m). ^{1}H-NMR (D_2O, δ ppm) 2.27 (q, 2H, nitroimidazole–$CH_2C\underline{H}_2CH_2N$–); 3.42 (m, 2H, nitroimidazole–$CH_2CH_2C\underline{H}_2N$–); 4.04 (s, 4H, $-N(C\underline{H}_2CO_2H)_2$); 4.52 (m, 2H, nitroimidazole–$C\underline{H}_2CH_2CH_2N$–); 8.26 (d, 2H, nitroimidazole C2-H,);

8.46 (d, 2H, nitroimidazole C4-H). Analysis for $C_{10}H_{14}N_4O_6$ (Calc) Found: C (41.96) 42.01; H (4.93) 5.19; N (19.57) 19.58.

*Preparation of 2-nitroimidazole-IDA-$^{185/187}Re(CO)_3$ complex (**8**)*

The rhenium analogue of 2-nitroimidazole-IDA-[$^{99m}Tc(CO)_3$] was prepared by mixing sodium salt of 2-nitroimidazole-IDA derivative (119 mg, 0.41 mmol) with bis(tetraethylammonium)-*fac*-tribromotricarbonylrhenate (330 mg, 1.1 equiv.) in water (5 mL). The compound bis(tetraethylammonium)-*fac*-tribromotricarbonylrhenate was prepared following a procedure reported by Alberto *et al.*[16] The reaction mixture was refluxed for 12 h and then cooled to room temperature. The precipitate formed was removed by filtration and the filtrate upon evaporation gave the target compound **8** as tetraethylammonium salt (118 mg, 52%). IR (KBr, cm^{-1}) 3130 (m); 2984 (m); 2950 (m); 2692 (w); 2016 (vs); 1878 (vs); 1646 (s); 1491 (w); 1395 (m); 1337 (m); 1288 (w); 1183 (w); 1133 (w). ^{1}H-NMR (D_2O, δ ppm) 1.15 (t, 24H, $[N(CH_2\underline{CH_3})_4]_2$; 2.20 (quintet, 2H, nitroimidazole–$CH_2\underline{CH_2}CH_2N$–); 3.14 (q, 16H, $[N(\underline{CH_2}CH_3)_4]_2$; 3.39 (t, 2H, nitroimidazole–$CH_2CH_2\underline{CH_2}N$–); 3.54 (d, 2H, –$NCH_AH_B$ CO–); 3.76 (d, 2H, –NCH_AH_B CO–); 4.13 (t, 2H, nitroimidazole–$\underline{CH_2}CH_2CH_2N$–); 7.23 (s, 1H, nitroimidazole C4-H,); 7.41 (s, 1H, nitroimidazole C5-H).

Radiolabeling

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

Briefly, the procedure involved the addition of 1 mL of freshly eluted $Na^{99m}TcO_4$, from $^{99}Mo/^{99m}TcO_4^-$ alumina column generator, to the Isolink carbonyl kit vial and heating at 95°C for 30 min. After cooling and re-equilibrating to atmospheric pressure, the pH of the reaction mixture was adjusted to 7 using 1:3 mixture of 0.5 M phosphate buffer (pH 7.5) and 1 M HCl.

General procedure for the preparation of nitroimidazole-$^{99m}Tc(CO)_3$ complex

To prepare the complex, freshly prepared [$^{99m}Tc(CO)_3(H_2O)_3]^+$ core (0.5 mL) was added to the nitroimidazole ligand (0.5 mg of **3**, **6** or **7**) in phosphate buffer (0.5 mL) of pH 7.4. The mixture was vortexed for 1 min and then incubated at 80°C for 30 min in a constant temperature water bath. The reaction mixture was then cooled to room temperature and 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 C-18 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). Flow rate was maintained at 1 mL/min. About 25 μL of the test solution was injected into the column and elution was monitored by observing the radioactivity profile.

Partition coefficient ($LogP_{o/w}$)

The labeled compound (0.1 mL) was mixed with water (0.9 mL) and octanol (1 mL) on a vortex mixer for about 1 min and then centrifuged at 3000 rpm for 5 min to effect clear separation of the two layers. The octanol layer (0.8 mL) was withdrawn and equal volume of fresh saline was added. The mixture was vortexed and then centrifuged as described above. Equal aliquots of the two layers were withdrawn and measured for the radioactivity. The readings thus obtained were used to calculate the $LogP_{o/w}$ value of the complex.

In vitro serum stability

The labeled compound (0.05 mL) was added to human serum (0.5 mL) and this mixture was incubated at 37°C for up to 3 h. Aliquots (0.1 mL) were drawn at 1, 2 and 3 h and an equal volume of ethanol was mixed to precipitate the serum proteins. The mixtures were centrifuged and the supernatants were analyzed by HPLC to assess stability of the complex in serum.

In vivo studies

All procedures performed herein were in strict compliance with the national laws governing the conduct of animal experiments. Solid tumor models were developed in Swiss mice by implantation of HSDM1C1 murine fibrosarcoma cells (10^6 cells/100 μl) subcutaneously on the dorsum of every animal. The tumors were allowed to grow till they reached approximately 10 mm in diameter, after which the animals were immediately taken for *in vivo* studies. For the biodistribution study, the radioactive preparation (~37 MBq per animal in 100 μL volume) was administered intravenously through the lateral 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 respective time periods, the animals were sacrificed and the relevant organs excised for the measurement of retained activity. The organs were weighed and the activity associated with each was measured in a flat-bed type NaI(Tl) counter with suitable energy window for ^{99m}Tc (140 keV ± 10%). For the sake of comparison the activity retained in each organ/tissue was expressed as a percent value of the injected dose per gram (% ID/g).

Conclusions

Three differently substituted nitroimidazole–iminodiacetic acid derivatives were synthesized and radiolabeled with [$^{99m}Tc(CO)_3(H_2O)_3]^+$ core in excellent yield. All the complexes are characterized by HPLC and then evaluated in suitable tumor-bearing animal model. The studies carried out for limited time period (3 h) indicated that the 5- and 4-nitroimidazole complexes showed faster clearance from the tumor, limiting their potential to target hypoxia, whereas the 2-nitroimidazole complex cleared slowly possibly indicating its suitability to target hypoxic cells. Further evaluation of this complex at an extended time point is therefore warranted.

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