Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 3398–3401

A novel $[^{99m}Tc{\equiv}N]^{2+}$ complex of metronidazole xanthate as a potential agent for targeting hypoxia

Madhava B. Mallia,[a] Anupam Mathur,[b] Suresh Subramanian,[a] Sharmila Banerjee,[a] H. D. Sarma[c] and Meera Venkatesh[a,*]

[a]*Radiopharmaceuticals Division, Bhabha Atomic Research Centre, Mumbai 400085, India*
[b]*Medical and Biological Products Program, Board of Radiation and Isotope Technology, Mumbai 400094, India*
[c]*Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Mumbai 400085, India*

Received 10 March 2005; revised 4 May 2005; accepted 9 May 2005
Available online 8 June 2005

Abstract—A xanthate derivative (L) at the pendant hydroxy group of metronidazole, a nitroimidazole known to possess affinity for hypoxic tumors, has been used as the carrier molecule for targeted delivery of the gamma-emitting radioisotope ^{99m}Tc to tumors. The xanthate residues (S_2^-) from two molecules of this ligand (L) were used for chelation with the $[^{99m}TcN]^{2+}$ intermediate to form a square pyramidal and neutral $[^{99m}TcN/L_2]$ complex in >95% yield using a low ligand concentration of 1 mg/mL ($\sim 3 \times 10^{-3}$ M). Biodistribution studies carried out in Swiss mice bearing fibrosarcoma tumor showed selective accumulation of the injected activity in the tumor (1.44 ± 0.26% per gram 1 h pi) with major clearance through hepatobiliary route. The complex showed high tumor/muscle ratio (2.15 and 3.35 at 1 and 3 h post-injection, respectively) and tumor/blood ratio, which were comparable to hypoxia targeting agents ^{99m}Tc-BMS181321 and ^{99m}Tc-BRU59-21 reported earlier.

Hypoxic regions in tumors result when tumor growth exceeds the capacity of accompanying blood vasculature to deliver adequate quantity of oxygen to the growing mass of tumor cells.[1] The resistance of hypoxic cells to conventional radiotherapy and chemotherapy[2,3] is a major obstacle posed in the complete remission of tumors. The inherent drawbacks of invasive techniques to detect hypoxia[4] have provided an impetus for the development of suitable radiopharmaceuticals that can be utilized for imaging tumor hypoxia. Nitroimidazole derivatives have shown a tendency to accumulate in the hypoxic regions of tumors, which provide the attractive possibility of employing these molecules as carriers for targeted delivery of radiation to hypoxic tissues.[5,6] The search for an ideal hypoxia-imaging agent must take into consideration factors such as a simple, cost-effective preparation route, ease of preparation, stability, rapid accumulation in tumors, sufficient retention times therein, and rapid clearance from other tissues to provide better contrast between lesion and background. To circumvent the existing deficiencies in labeling of metronidazole via the $[^{99m}Tc(V){=}O]^{3+}$ route with respect to the requirement of high ligand concentration and low stability of the resultant ^{99m}Tc–metronidazole complex, as have been observed earlier in our group while labeling metronidazole,[7] new methods of ^{99m}Tc labeling that give products of adequate stability are being widely explored. The $[^{99m}Tc{\equiv}N]^{2+}$ intermediate where ^{99m}Tc is in +5 oxidation state is very stable, can be prepared as a precursor, and forms stable complexes, therefore acting as a viable alternative to the isoelectronic conventional $[^{99m}Tc{=}O]^{3+}$ core.[8] It provides an additional advantage of exhibiting high affinity towards chelating ligands containing selected atoms such as sulfur, dithiocarbamates and xanthates.[9] The use of symmetric and asymmetric, monoalkyl and dialkyl dithiocarbamates as potential myocardial agents has been widely documented,[10,11] but in comparison, there are relatively few reports on xanthate derivatives employed as in vivo agents. Metronidazole with a pendant $-CH_2OH$ group acts as a suitable substrate for facile derivatization to the corresponding xanthate in moderate yields. Herein, we report the synthesis of metronidazole xanthate and its ^{99m}Tc labeling using the novel ^{99m}Tc-nitrido core. To the best of our knowledge, this report constitutes the first of its

Keywords: Metronidazole xanthate; Hypoxia imaging; Technetium nitrido complex.
* Corresponding author. Tel.: +91 22 2559 3676; fax: +91 22 2550 5345; e-mail: meerav@apsara.barc.ernet.in

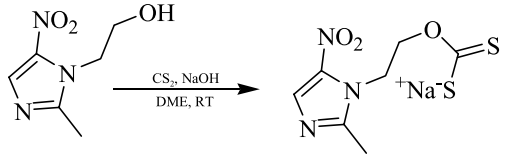
0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.05.030

kind in using the xanthate donor moiety novel nitroimidazole substrate in the preparation of high specific activity complex of ^{99m}Tc in the designing of targeted agents for hypoxia.

The sodium salt of metronidazole xanthate was synthesized by adding carbon disulfide to a well-stirred solution of metronidazole and crushed sodium hydroxide in dimethoxyethane. The reaction is schematically shown in Scheme 1. In a typical procedure, about 0.5 g of metronidazole and 0.17 g (1.5 equiv) of crushed sodium hydroxide were stirred vigorously in dimethoxyethane for 5 min at room temperature. When the solution became clear ~0.2 mL (1.1 equiv) of carbon disulfide was added dropwise. Stirring was continued overnight at room temperature. The yellow precipitate was filtered, washed with dimethoxyethane, and dried (0.25 g, 30%). The product was used as such without further purification. The product was characterized by GC–MS, high-resolution ^{13}C NMR spectroscopy, and C, H, N, S analysis. In GC–MS, though the molecular ion peak was not observed, the M−15 and M−46 peaks corresponding to loss of a methyl and a nitro group, respectively, from the molecular ion peak were observed. The ^{1}H-decoupled ^{13}C NMR of the compound in CD_3OD showed the appearance of a new peak at 170 ppm, indicating the introduction of the CS_2 group further confirming the formation of the product. C, H, N, S: obsd (calcd) 43.96 (44.06), 4.3 (4.52), 5.66 (5.71), 26.45 (26.14).

The $[^{99m}TcN]^{2+}$ intermediate was prepared by using a kit-vial,[12] obtained as a gift from IAEA coordinated research project following an optimized protocol. One millilitre of freshly eluted $^{99m}TcO_4^-$ (15–30 MBq) was added to the kit-vial and the mixture was vortexed for one minute and kept at room temperature for 20 min. The $[^{99m}TcN]^{2+}$ intermediate was characterized by TLC using two solvent systems, viz., ethanol/chloroform/toluene/0.5 M ammonium acetate (6:3:3:0.5 v/v) and saline. In the former solvent system, ~99% of the activity, corresponding to $[^{99m}TcN]^{2+}$ intermediate, was concentrated at the point of spotting, while $^{99m}TcO_4^-$ at $R_f = 0.4–0.6$ accounted for less than 1% of total activity. In the latter solvent system, $[^{99m}TcN]^{2+}$ intermediate species moved with solvent front ($R_f = 0.7–1$) while minimal activity corresponding to reduced technetium remained at the point of spotting.

The parameters such as ligand concentration and time were optimized to obtain maximum yield of complexation. The minimum ligand concentration required for >95% complexation yield was found to be 1.0 mg/mL when incubated for 30 min. This amount was significantly lower than the amount required for the complexation of metronidazole–CAA complex with $[^{99m}TcO]^{3+}$ core reported by our group earlier.[7] The complexation yield declined as the ligand concentration was decreased and it was found to be only 48% at 0.1 mg/mL. An incubation time of 30 min at room temperature was necessary to result in >95% complexation yield. Thereafter, there was no significant improvement in complexation yields with time. This preparation was observed to be stable for over 20 h at room temperature with retention of radiochemical purity of the complex to the extent of ~85%. Stability of the complex in blood serum was studied at 37 °C and the results indicate that the complex exhibits significant stability with retention of radiochemical purity up to an extent of 90% for 3 h. An optimized protocol for obtaining maximum complexation yield therefore involves dissolution of 1 mg (~ 3 mmol) of the ligand in 0.5 mL of saline and subsequent addition to 0.5 mL of the freshly prepared $[^{99m}TcN]^{2+}$ intermediate. The reaction mixture was then vortexed for a minute and incubated at room temperature for 30 min. The ^{99m}TcN complex was characterized by both paper electrophoresis and HPLC. Paper electrophoresis was carried out in 0.05 M phosphate buffer (pH 7.5) for 1 h under a potential gradient of ~10 V/cm. The strip was then dried, cut into 1 cm segments, and the activity associated with each was counted in well-type NaI(Tl) detector. In paper electrophoresis, more than 95% of the activity corresponding to ^{99m}TcN complex was found at the point of spotting, indicating that the complex formed was neutral. Migration of <4% activity toward the anode 4–5 cm from the point of spotting was attributable to the nitrido intermediate in the reaction mixture. Since the complex was found to be neutral from electrophoresis study, the probable structure that could be proposed is $^{99m}TcNL_2$ (L = metronidazole xanthate) using the $[^{99m}TcN]^{2+}$ core as the precursor for the complexation. A similar study reported by Abram et al.[9] involving analogous ^{99m}TcN–xanthate complexes of some simple alcohols has established the structure to be $^{99m}TcNL'_2$ (L′ = xanthate). Studies towards confirmation of the proposed structure in macroscopic amounts will be carried out finally, by preparing the complexes using the long-lived ^{99}Tc.



Scheme 1. Synthesis of metronidazole xanthate.

A dual pump HPLC unit with a C-18 reversed phase column (HiQ Sil (5 μm, 4 × 250 mm)) was used for the characterization of the labeled complex. About 25 μL of the test solution was injected into the C-18 reversed phase column and the elution was monitored by observing the radioactivity profile. The flow rate was maintained at 1 mL/min. 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 HPLC chromatograms of $[^{99m}TcN]^{2+}$ intermediate species and ^{99m}TcN–metronidazole xanthate complex are shown in Figure 1. It was observed that the retention time of $[^{99m}TcN]^{2+}$ intermediate species was 2.9 ± 0.2 min, while that of radiolabeled complex was found to be 10.2 ± 0.1 min. Slight broadening was observed towards the end of the elution of the complex peak, which could be possibly due to the presence of minor amounts of a secondary

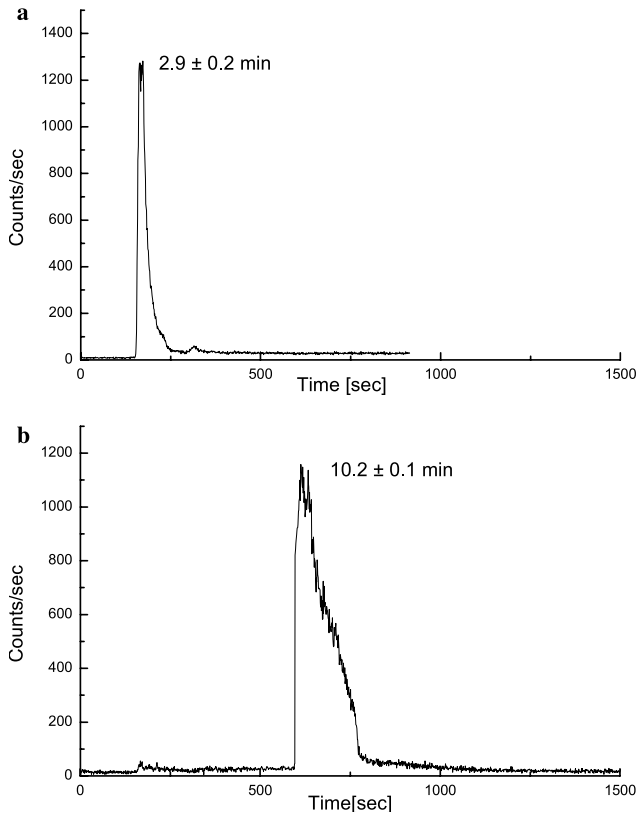


Figure 1. HPLC patterns of (a) $[^{99m}TcN]^{2+}$ intermediate species and (b) ^{99m}TcN–metronidazole xanthate complex.

radiochemical species. However, it was not possible to resolve the peak and estimate the presence of the other species formed if any.

In vitro serum stability studies were performed for the labeled complex using a method adapted from a protocol reported earlier.[13] Stability was assayed on HPLC using same protocol as for chemical characterization studies.

Swiss mice (~25 g body weight) were used for the biodistribution studies. Localized solid tumors were propagated in the animals by transplantation of a fibrosarcoma cell line (barcl-95) into the dorsal region. Around 10^6 cells per animal were injected subcutaneously. The tumor size was allowed to reach approximately 1 cm diameter and then the animals were used for the experiment. All the procedures performed herein were in strict accordance with the national laws pertaining to the conduct of animal experiments.

0.1 mL of the labeled product (~2500 kBq) was injected into the animals via the tail vein. The animals were sacrificed at various time points of 30, 60, and 180 min pi after which the relevant organs and tissues were excised for measurement of associated activity. Radioactivity measurements were carried out in a flat-bed type NaI(Tl) scintillation counter with optimal window for ^{99m}Tc. The accumulated activity was expressed in terms of percentage of total injected dose associated with the specific organ/tissue. The weights of blood, bone, and muscle were extrapolated as 7%, 10%, and 40% of total body weight, respectively.

The results of biodistribution of the labeled preparation in Swiss mice are represented in Table 1. The tumor retains about 1.4% of injected dose per gram and this remains nearly constant over the period of study up to 180 min post-injection. This is appreciably higher than that observed for previously reported nitroimidazole complexes, including ^{99m}Tc-labeled MNZCAA[7,14,15] reported by our group earlier. The striking observation in the present study was the desirable feature of steady retention of the tumor activity throughout the period of study. Although the initial activity level in blood and muscle was high, there was a steady clearance of activity from these tissues with time. Tumor/blood and tumor/muscle ratios of the retained activity improved steadily with time (Table 2) and were observed to be 0.62 and 3.3, respectively, at 180 min post-injection. As seen from Table 3, this is comparable to the results obtained for previously reported agents. Activity from other vital organs also appears to wash out with time. The directional movement of activity from the liver to the intestinal tract indicates that the preparation is excreted predominantly through the hepatobiliary system. A minor portion of the injected dose is also excreted renally, as seen from the activity associated with the kidneys.

To conclude, metronidazole xanthate was synthesized in moderate yields. The xanthate was labeled with the novel $[^{99m}TcN]^{2+}$ intermediate in >95% yield using a substantially low ligand concentration of 1 mg/mL as compared to labeling following the conventional

Table 1. Biodistribution pattern of ^{99m}TcN–metronidazole xanthate ($n = 3$)

Organ	% Injected dose per organ (±1SD)		
	30 min pi[a]	60 min pi	180 min pi
Liver	29.32 ± 0.88	23.53 ± 2.28	18.37 ± 2.33
Int + GB	12.46 ± 0.37	20.19 ± 3.11	32.52 ± 2.17
Kidney	4.25 ± 0.20	3.18 ± 0.74	2.87 ± 0.40
Stomach	1.68 ± 1.42	0.74 ± 0.24	0.71 ± 0.07
Heart	0.38 ± 0.17	0.27 ± 0.09	0.27 ± 0.07
Lungs	1.59 ± 0.69	1.09 ± 0.26	0.75 ± 0.06
Spleen	0.25 ± 0.05	0.34 ± 0.05	0.27 ± 0.00
Bone	2.59 ± 0.53	2.43 ± 0.70	2.27 ± 1.54
Muscle	11.19 ± 1.59	6.43 ± 1.46	4.20 ± 1.79
Blood	11.45 ± 0.89	6.75 ± 0.93	3.95 ± 0.32
Tumor	**0.40 ± 0.17 (1.42 ± 0.21/g)**	**0.43 ± 0.14 (1.44 ± 0.26/g)**	**0.41 ± 0.14 (1.36 ± 0.29/g)**

[a] pi—post-injection.

Table 2. Tumor/blood and tumor/muscle ratios of ^{99m}TcN–metronidazole at various time points

Time post-injection (min)	Tumor:blood	Tumor:muscle
30	0.2	1.19
60	0.36	2.15
180	0.62	3.35

Table 3. Comparison of biological characteristics of ^{99m}TcN–metronidazole derivative with earlier reported agents

Complex	^{99m}Tc-BMS181321	^{99m}Tc-BRU59-21	^{99m}Tc-MNZ-xanthate
Animal	C3H mice	C3H mice	Swiss mice
Tumor type	Fibrosarcoma	Fibrosarcoma	Fibrosarcoma
Time pi (h)	2	2	3
Tumor (%ID/g)	0.55 ± 0.08	0.37 ± 0.14	1.36 ± 0.29
Tumor/blood	0.31	0.86	0.62
Tumor/muscle	2.63	3.84	3.32
Reference	14	15	Present study

$[^{99m}Tc{=}O]^{3+}$ core. Characterization by HPLC showed that the complex was formed as a single species. Biodistribution studies in fibrosarcoma tumor bearing Swiss mice showed considerable retention of activity in tumor even after 3 h post-injection. The in vivo pharmacological behaviour observed with the ^{99m}TcN-labeled metronidazole xanthate complex provides considerable promise toward the development of a hypoxia-imaging agent involving structural variation of metronidazole.

Acknowledgments

The authors are thankful to International Atomic Energy Agency, Vienna, for supplying kit-vials used for the preparation of the Technetium-99m nitride intermediate, and Regional Sophisticated Instrumentation Centre (RSIC), IIT, Mumbai, for carrying out the spectral analyses of the compound.

References and notes

1. Nunn, A.; Linder, K.; Strauss, H. W. *Eur. J. Nucl. Med.* **1995**, *22*, 265.
2. Gary, L. H.; Conger, A. D.; Ebert, M.; Hornsey, S.; Scott, O. C. A. *Br. J. Radiol.* **1953**, *26*, 638.
3. Moulder, J. E.; Rockwell, S. *Cancer Metastasis Rev.* **1987**, *5*, 313.
4. Ballinger, J. R. *Semin. Nucl. Med.* **2001**, *31*, 321.
5. Brown, J. M. *Int. J. Radiat. Oncol. Biol. Phys.* **1989**, *16*, 987.
6. Chapman, J. D. *N. Engl. J. Med.* **1979**, *301*, 1429.
7. Das, T.; Banerjee, S.; Grace Samuel; Sarma, H. D.; Aruna Korde; Meera Venkatesh; Pillai, M. R. A. *Nucl. Med. Biol.* **2003**, *30*, 127.
8. Baldas, J.; Bonnyman, J. *Int. J. Appl. Radiat. Isot.* **1985**, *36*, 133.
9. Abram, S.; Abram, A.; Spies, H.; Munze, R. *J. Radioanal. Nucl. Chem. Art.* **1986**, *102*, 309.
10. Pasqualini, R.; Duatti, A.; Bellande, E.; Comazzi, V.; Brucato, V.; Hoffschir, D.; Fagret, D.; Comet, M. *J. Nucl. Med.* **1994**, *35*, 334.
11. Pasqualini, R.; Duatti, A. *J. Chem. Soc., Chem. Commun.* **1992**, *18*, 1354.
12. Kit-vial compostion: succinic acid dihydrazide (SDH) (5 mg), tin(II)chloride (0.1 mg), 1,2-diaminopropane-*N*,*N*,*N*′,N′-tetraacetic acid (PDTA) (5 mg), sodium dihydrogen phosphate anhyd (0.5 mg), disodium hydrogen phosphate anhyd (5.8 mg).
13. Ma, D.; Lu, F.; Oversheet, T.; Milenic, D. E.; Brechtiel, M. W. *Nucl. Med. Biol.* **2002**, *29*, 91.
14. Ballinger, J. R.; Kee, J. W. M.; Rauth, A. M. *J. Nucl. Med.* **1996**, *37*, 1023.
15. Melo, T.; Duncan, J.; Ballinger, J. R.; Rauth, A. M. *J. Nucl. Med.* **2000**, *41*, 169.