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Synthesis, in vitro and in vivo characterization of novel ^{99m}Tc-‘4+1’-labeled 5-nitroimidazole derivatives as potential agents for imaging hypoxia

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Abstract

The evaluation of oxygenation status of solid tumors is an important field of radiopharmaceutical research. With the aim to develop new potential ^{99m}Tc-radiopharmaceuticals for imaging hypoxia, we have synthesized two novel isocyanide derivatives of metronidazole, which has demonstrated high affinity for hypoxic tumors in vitro and in vivo.

Methods: Metronidazole derivatives 4-isocyano-*N*-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl]butanamide (M1) and 1-(4-isocyanobutanoyl)-4-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl]piperazine (M2) were synthesized, and labeling was performed through preparation of their corresponding ^{99m}Tc-(4+1) complexes, ^{99m}Tc-NS$_3$M1 and ^{99m}Tc-NS$_3$M2. The structure of the technetium complexes was corroborated by preparation and characterization of the corresponding rhenium complexes. We have studied the main physicochemical properties (stability, lipophilicity and plasma protein binding). Biological behavior in HCT-15 cells both in oxia and in hypoxia was assessed. Biodistribution in normal mice and in animals bearing induced 3LL Lewis murine lung carcinoma was also studied.

Results: Metronidazole derivatives were successfully synthesized. Labeling with high radiochemical purity was achieved for both ligands. ^{99m}Tc complexes were stable in labeling milieu and human plasma. However, presence of the piperazine linker in M2 resulted in higher lipophilicity and protein binding. Although cell uptake in hypoxic conditions was observed for both radiotracers, ^{99m}Tc-NS$_3$M2 biodistribution was considered unsuitable for a potential radiopharmaceutical due to high liver uptake and poor blood clearance. However, ^{99m}Tc-NS$_3$M1 demonstrated a very favorable in vivo profile both in normal mice and in mice bearing induced tumors.

Conclusion: Selective uptake and retention in tumor together with favorable tumor/muscle ratio make ^{99m}Tc-NS$_3$M1 a promising candidate for further evaluation as potential hypoxia imaging agent in tumors.

Keywords: Technetium; 5-nitroimidazole; Hypoxia; 4+1 complexes; Imaging agent

1. Introduction

The evaluation of oxygenation status of solid tumors is an important field of radiopharmaceutical research, since low oxygen pressure and diffusion limitation of hypoxic regions in tumors are major causes of resistance to conventional radiotherapy and chemotherapy [1,2]. Bioreductive compounds, which are selectively reduced in hypoxic tissue to reactive intermediates that bind to intracellular molecules,

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have been used for the development of potential radiopharmaceuticals for targeting hypoxic tumors. Nitroimidazole is one of the preferred pharmacophores [3-8], but other functional groups such as nitroaromatics [9,10] and *N*-oxides [11] have been studied also. However, properties of the previously studied technetium complexes are not yet ideal and new potentially active compounds are still being developed.

The 5-nitroimidazole metronidazole (Fig. 1), which has demonstrated high affinity for hypoxic tumors in vitro and in vivo, is an adequate starting material for the preparation of ^{99m}Tc radiopharmaceuticals [12-15]. Metronidazole should be attached to adequate chelating groups that enable the coordination of ^{99m}Tc without losing the specific recognition by target sites in vivo. We have selected the ^{99m}Tc-(4+1) core for this purpose because this labeling approach yields high specific activity tracers while preserving the biological activity of the ligand. The 4+1 mixed ligand complexes proposed by us are oxo-free complexes with the metal at oxidation state +3, formed by combination of the tripodal chelator 2,2′,2″-nitrilotris(ethanethiol) and a monodentate isocyanide. The non-polar isocyanide building block is stable against ligand exchange in vivo and provides ^{99m}Tc complexes with high in vitro stability both in aqueous solution and in plasma [16-19]. It has been successfully employed in the preparation of potential radiopharmaceuticals for imaging sympathetic nervous system receptors, myocardial metabolism, tumor angiogenesis, etc. [20-22].

With the aim to develop new potential ^{99m}Tc-radiopharmaceuticals for imaging hypoxia, we have synthesized two novel isocyanide derivatives of metronidazole, 4-isocyano-*N*-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl]butanamide (M1) and 1-(4-isocyanobutanoyl)-4-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethyl]piperazine (M2) (Fig. 1), and prepared their corresponding ^{99m}Tc-(4+1) complexes. The structure of the technetium complexes was corroborated by preparation and characterization of the corresponding rhenium complexes. In order to assess their potential as hypoxia-targeting radiopharmaceuticals, we have studied the main physicochemical and biological properties both in vitro and in vivo.

2. Materials and methods

2.1. General

All laboratory chemicals were reagent grade and were used without further purification. Intermediates **1**–**3** were prepared according to procedures previously described [18,19,23]. Solvents for chromatographic analysis were HPLC grade. [^{99m}Tc]$NaTcO_4$ was obtained from a commercial generator (Tecnonuclear, Argentina). NMR spectra were obtained at 400 MHz in the indicated deuterated solvent (Bruker DPX 400 spectrometer). Chemical shifts are reported as δ values (parts per million) relative to the residual protons of the deuterated solvent. Coupling constants are reported in Hertz. The multiplicity is defined by s (singlet), t (triplet) or m (multiplet). The mass spectra (MS) were conducted with a mass spectrometer, Hewlett Packard 5973 MSD or MICROMASS (Triple Quattro), using electron impact (EI) or electrospray (ESI), respectively. IR spectra were obtained in the range 4000–200 cm^{-1} in KBr pellets at 1% in a Bomen MB-102 FT-IR spectrometer. Thin-layer chromatography (TLC) was carried out on precoated plates of silica gel 60 F254. Flash chromatography was performed using silica gel 60 (230–400 mesh, Sigma-Aldrich). For column chromatography, we used silica gel (Merck, 60–230

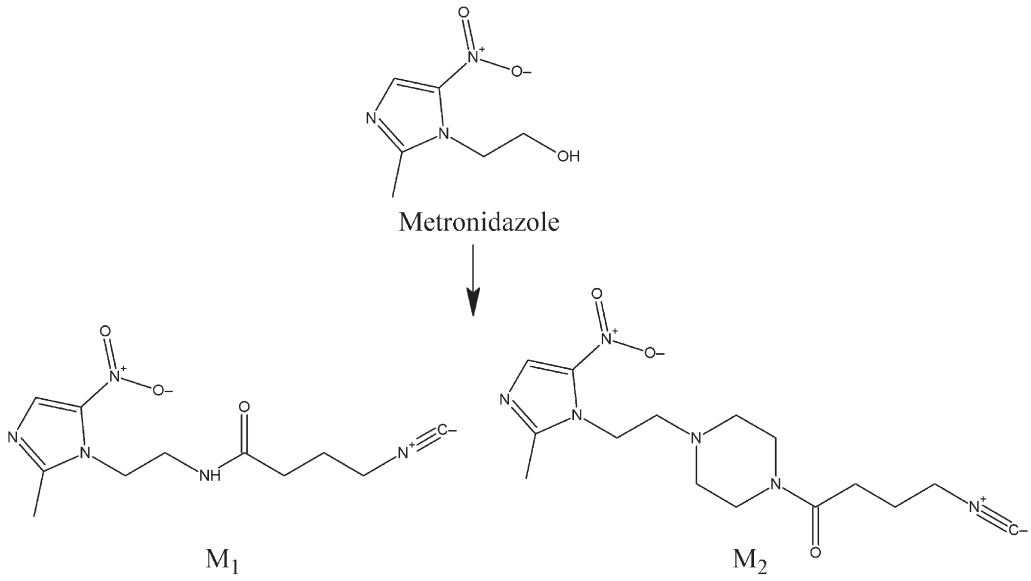

Fig. 1. Structure of metronidazole and isocyanide derivatives used in this study.

mesh) or neutral alumina (Merck, 70–230 mesh). Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and were uncorrected. HPLC analysis was developed on a LC-10 AS Shimadzu Liquid Chromatography System using a reversed-phase column [μ-Bondapack, Waters, 5 μm, C18 column (4.69×300 mm)]. Elution was performed with a binary gradient system at 1.0 ml min^{-1} flow rate using trifluoroacetic acid 0.1% in water as mobile phase (A) and trifluoroacetic acid 0.1% in acetonitrile as mobile phase (B). The elution profile was as follows: t=0, % A=100, % B=0; from 0 to 10 min, linear gradient up to 100% B; from t=10 min to t=20 min, % A=0, % B=100. Detection was accomplished either with a photodiode array detector (SPD-M10A, Shimadzu) that recorded UV–Vis spectra on flux or with a 3×3-in. NaI (Tl) crystal scintillation detector. Activity measurements were performed either with a dose calibrator (Capintec CRC- 5R) or with a scintillation counter (3×3-in. NaI (Tl) crystal detector) attached to an ORTEC monochannel analyzer.

2.2. Synthesis

2.2.1. 4-Isocyano-N-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyl]butanamide (M1)

A mixture of 2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethylamine (**1**, 150 mg, 0.88 mmol), *N*-(4-isocyanobutanoyloxy) succinimide (**2**, 124 mg, 0.59 mmol) and triethylamine (one drop) in dry THF (20 ml) was stirred at room temperature until total consumption of amine **1** (approximately 12 h). The solvent was evaporated under vacuum. The residue was purified by column chromatography [Al_2O_3, hexane/ethyl acetate (3:7)]. Yellowish solid (42 %); mp=88–89°C. ^{1}H NMR (acetone-d_6) δ (ppm): 1.91 (m, 2H), 2.07 (m, 2H), 2.49 (s, 3H), 3.52 (m, 2H), 3.64 (t, 2H, J=6.0), 4.49 (t, 2H, J=6.0), 7.89 (s, 1H). ^{13}C NMR (CD_3OD) δ (ppm): 13.4, 24.8, 31.5, 38.3, 40.6, 45.5, 132.6, 138.9, 151.2, 157.7 (NC), 171.5. IR (KBr) ν (cm^{-1}): 1382, 1547, 1658, 2151 (NC), 3333. MS (EI, 70 eV) *m/z*: 238 ($M^{+\cdot}$-HCN), 222, 206, 191.

2.2.2. 1-(4-Isocyanobutanoyl)-4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethyl]piperazine (M2)

A mixture of 4-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl) ethyl]piperazine (**3**, 10 mg, 0.42 mmol), **2** (132 mg, 0.50 mmol) and triethylamine (one drop) in dry THF (20 ml) was stirred at room temperature until total consumption of amine **1** (approximately 12 h). The solvent was evaporated under vacuum. The residue was purified by column chromatography [Al_2O_3, hexane/ethyl acetate (4:6)]. Dark oil (72 %). ^{1}H NMR (acetone-d_6) δ (ppm): 1.92 (m, 2H), 2.08 (m, 2H), 2.32 (s, 3H), 2.62 (t, 4H, J=5.0), 3.50 (t, 4H, J=5.0), 3.64 (t, 2H, J=6.0), 4.49 (t, 2H, J=6.0), 7.89 (s, 1H). ^{13}C NMR (CD_3OD) δ (ppm): 13.4, 24.8, 31.5, 38.3, 40.6, 45.5, 49.4, 55.1, 132.6, 138.9, 151.2, 157.7 (NC), 171.5. IR (KBr) ν (cm^{-1}): 1384, 1534, 1658, 2151 (NC), 3408. MS (EI, 70 eV) *m/z*: 289 ($M^{+\cdot}$-HNO_2), 248, 219, 191.

2.3. Radiolabeling

2.3.1. Preparation of ^{99m}Tc-EDTA precursor

The ^{99m}Tc-EDTA precursor was prepared by adding $^{99m}TcO_4^-$ (300 MBq, 1 ml) to a vial containing 5 mg EDTA, 5 mg mannitol and 0.1 mg $SnCl_2$. After 5 min, the radiochemical purity was checked by TLC in silica gel using acetone and water as mobile phase.

2.3.2. Substitution by M1 and M2

0.2 mg of the selected Mn ligand (M1=7.5×10^{-7} mol; M2=6.0×10^{-7} mol) and 1 mg of 2-[bis-(2-mercaptoethyl)] aminoethanethiol (NS3) were mixed with 200 μl of the ^{99m}Tc-EDTA precursor and incubated at 70°C for 30 min. Radiochemical purity was assessed by HPLC using the chromatographic condition described in Section 2.1.

2.4. Physicochemical evaluation

2.4.1. Stability in labeling milieu

Complexes ^{99m}Tc-NS_3M1 and ^{99m}Tc-NS_3M2 were incubated in the labeling milieu at room temperature, and the radiochemical purity was assessed by HPLC using the chromatographic condition described in Section 2.1 for up to 4 h after labeling.

2.4.2. Protein binding studies

Complexes ^{99m}Tc-NS_3M1 and ^{99m}Tc-NS_3M2 (25 μl) were incubated with human plasma (475 μl) at 37°C for up to 120 min. At 30 and 120 min, aliquots (50 μl) were added to Microspin G-50 columns (Pharmacia Biotech), which had been pre-spun at 2000×g for 1 min. Columns were centrifuged again at 2000×g for 2 min, and the collected elute and the column were counted in a NaI-scintillation counter. Protein bound tracer was calculated as the percentage of activity eluted from the column.

2.4.3. Lipophilicity

Lipophilicity was studied through the apparent partition coefficient between 1-octanol and phosphate buffer (0.125 M, pH 7.4). In a centrifuge tube, containing 2 ml of each phase, 0.1 ml of the ^{99m}Tc complex solution was added, and the mixture was shacked on a Vortex mixer and finally centrifuged at 5000 rpm for 5 min. Three samples (0.2 ml each) from each layer were counted in a gamma counter. The partition coefficient was calculated as the mean value of each counts per minute per milliliter of 1-octanol layer divided by that of the buffer. Lipophilicity was expressed as log P.

2.5. Preparation of rhenium analogs

The complexes of Re (III) 4+1 were synthesized by substitution using [Re(NS_3)(PMe_2Ph)] as precursor [19,24]. A solution of ligand M1 or M2 (16 and 20 mg, respectively, 0.06 mmol) in chloroform (3 ml) was added to a solution of [Re(NS_3)(PMe_2Ph)] (0.06 mmol) in methylene dichloride (3 ml). The mixture was stirred at room temperature for 1 h, the solvent was evaporated under

reduced pressure and the residue washed with diethyl ether. The desired product was purified by HPLC under the conditions mentioned in Section 2.1.

$ReNS_3M1$. MS (ESI) *m/z*: 647 ($M^{+\cdot}$+H, 8 %), 645 (4 %). Calculated for $C_{17}H_{27}N_6O_3ReS_3$: C=31.6%; H=4.2%; N=13.0%; S=14.9%. Found: C=32.4%; H=3.3%; N=12.9%; S=15.1%.

$ReNS_3M2$. MS (ESI) *m/z*: 716 ($M^{+\cdot}$+H, 50 %), 714 (25 %). Calculated for $C_{21}H_{34}N_7O_3ReS_3$: C=35.3%; H=4.8%; N=13.7%; S=13.4%. Found: C=36.3%; H=5.0%; N=13.9%; S=13.2%.

2.6. Biological evaluation

2.6.1. Cell uptake studies

The cell culture studies were performed using the adherent cell line HCT-15 (CCL-255TM ATCC) corresponding to human adenocarcinoma. Cells were cultured in RPMI-1640 (R6504 Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco), penicillin 100 U ml^{-1} (Sigma) and 100 μg ml^{-1} streptomycin (Sigma) in T75 tissue culture flasks (Nunc, Denmark) at 37°C and 5% CO_2 until approximately 7.5×10^6 cells were obtained. Then, the flasks were preincubated in a chamber gassed with N_2 for 1 h to remove oxygen from the milieu and afterwards radiopharmaceutical was added and incubated for additional predefined incubation times (60–120 min). The same procedure was repeated in normal culture conditions (37°C and 5% CO_2) for use as control. After the incubation time elapsed, the culture milieu was removed and cells were washed with PBS and treated with Trypsin-EDTA (Sigma). Finally, activity in the supernatant and the cells was measured in a solid scintillation counter and the results were expressed as the ratio between the percentage of activity taken up by cells incubated in nitrogen (hypoxia) and those incubated in normal conditions (oxia).

2.6.2. Animal studies

All animal studies were approved by the Ethics Committee of the Facultad de Química, UdelaR, Montevideo, Uruguay.

2.6.2.1. Biodistribution in normal mice. CD1 mice (female, 25–30 g, three animals per group) were injected via a lateral tail vein with ^{99m}Tc-labeled derivatives of metronidazole (0.1 ml, 0.037–0.37 MBq). At different intervals after injection, the animals were sacrificed by neck dislocation. Whole organs and samples of blood and muscle were collected, weighed and assayed for radioactivity. Total urine volume was collected during the biodistribution period and also removed from bladder after sacrifice. The bladder, urine and intestines were not weighed. Corrections by different sample geometry were applied when necessary. Results were expressed as percent dose per organ.

2.6.2.2. Biodistribution in animals bearing induced tumors. A culture of 3LL Lewis murine lung carcinoma cells was expanded and treated with trypsin previous to inoculation. A cell suspension in PBS containing 3×10^6 cells was prepared and injected subcutaneously in the right limb of C57BL/6 mice (8–10 weeks old). Twenty to 30 days later, the animals developed palpable tumor nodules (1.5×0.5×0.5 cm) and were used for biodistribution studies.

Three animals per group were injected via a lateral tail vein with a ^{99m}Tc compound (0.1 ml, 0.037–0.37 MBq). At different intervals after injection, the animals were sacrificed by neck dislocation. Whole tumor and samples of blood and muscle were collected, weighed and assayed for radioactivity. Results were expressed as percent dose per gram of tissue.

3. Results and discussion

3.1. Synthesis of ligands

Synthesis of ligands M1 and M2 was achieved by reaction of the corresponding amino derivatives of metronidazole (**1** and **3** [23]) with the succinimide derivative of 4-isocyanobutanoic acid, **2**, as shown in Fig. 2. We selected the amide linker due to its well-known stability in biological milieu. These procedures yielded the desired products in excellent yield after chromatographic isolation. Structural characterization was performed by NMR (^{1}H and ^{13}C), IR and mass spectrometry. Results were consistent with proposed structures.

3.2. Radiolabeling

Labeling through formation of '4+1' Tc complexes was performed by substitution using ^{99m}Tc-EDTA/mannitol as precursor. This precursor was obtained by direct reduction of pertechnetate using stannous chloride. Radiochemical purity of the labeled precursor was evaluated by classical chromatographic procedures and found to be higher than 90%. Substitution was achieved by the simultaneous reaction of M1 or M2 and the tetradentate tripodal NS_3 coligand 2-[bis-(2-mercaptoethyl)]aminoethanethiol. HPLC analysis of the labeling mixture revealed the formation of a main product with a retention time of 14.3 and 17.5 min for ^{99m}Tc-NS_3M1 and ^{99m}Tc-NS_3M2, respectively (Fig. 3), and a radiochemical purity above 90% after HPLC purification. The radioactivity recovery from the column was monitored by means of an online solid scintillation detector coupled to the HPLC system and found to be quantitative.

3.3. Physicochemical evaluation

3.3.1. Stability studies

The complexes were stable for at least 6 h after isolation. On the other hand, stability after incubation in human plasma for 4 h at 37°C was also studied and found to be 95±4% and 85±2%, respectively.

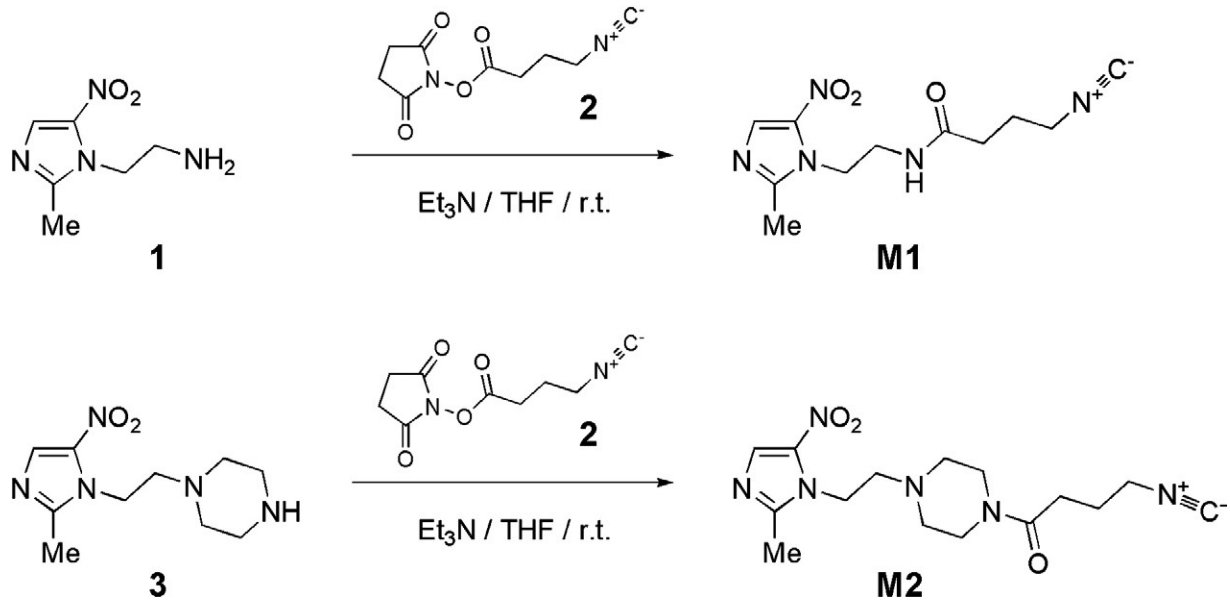

Fig. 2. Synthesis of ligands M1 and M2.

3.3.2. Lipophilicity

The partition coefficient between 1-octanol and phosphate buffer (pH 7.4) of the technetium complexes was measured in order to assess their lipophilicity. A log*P* of 0.7±0.1 and 1.2±0.3 for M1 and M2 complexes, respectively, was found. Values are in accordance with the structure of the ligands, since incorporation of the piperazine linker increases significantly the lipophilicity as was previously observed [23].

3.3.3. Protein binding

Binding to plasma protein was studied using size exclusion chromatography. Ideally, low protein binding is required in order to ensure adequate pharmacokinetics of the potential radiopharmaceuticals. Additionally, only the unbound fraction of the radiotracer will penetrate cells and other biological membranes [25]. A relatively low protein binding of 12±1% was obtained for ^{99m}Tc-NS$_3$M1, correlating with the high in vitro stability and low lipophilicity of this complex. On the other hand, protein binding of ^{99m}Tc-NS$_3$M2 was extremely high, 69±3%. This result might be partially explained by the higher lipophilicity of this complex but does not correlate with the high stability observed during in vitro experiments. However, substitution of M2 ligand in the metal coordination sphere by other potential electron donor groups of the proteins could be considered as a possible cause of this result, since stability studies in human plasma include precipitation of proteins and therefore only evaluate the HPLC profile of the unbound fraction.

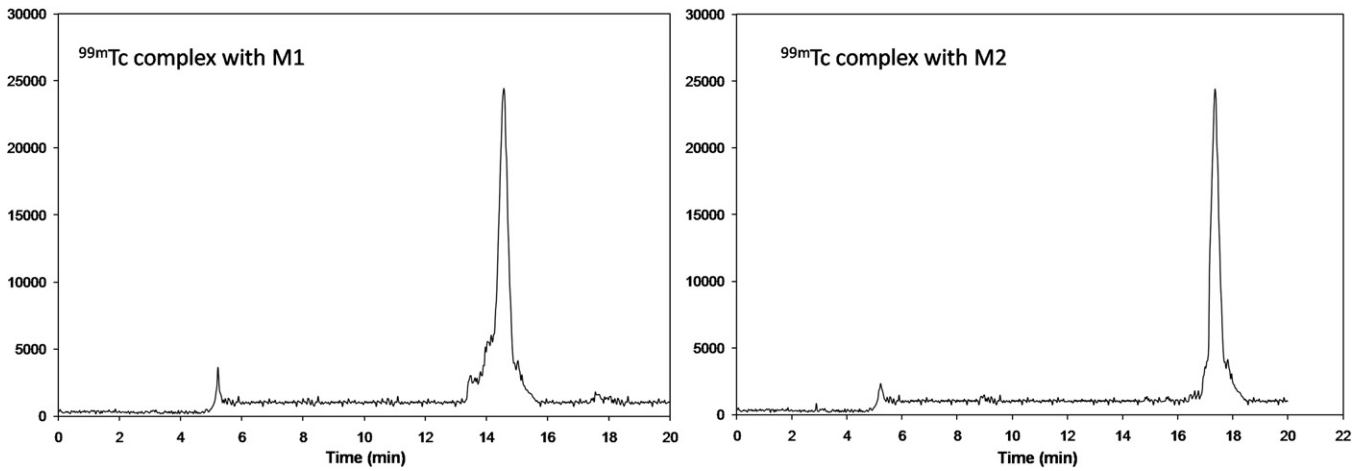

Fig. 3. Reversed-phase HPLC profiles of ^{99m}Tc complex with M1 and M2.

3.4. Preparation of rhenium analogues

The structure of ^{99m}Tc complexes was corroborated using stable rhenium as a surrogate for technetium. Rhenium, as a technetium third-row congener, exhibits many of the chemical properties of Tc. Furthermore, Re and Tc complexes with the same ligand have essentially identical coordination parameters since the ionic radii of both metals are about the same, due to the lanthanide contraction [19]. The rhenium complexes were prepared by substitution of M1 or M2 on the rhenium precursor [Re(NS_3)(PMe_2Ph)] as shown in Fig. 4. HPLC analysis of the reaction mixture for both ligands showed a main peak with the same retention time of the corresponding technetium complexes (Fig. 3). These peaks were isolated by HPLC and analyzed by mass spectrometry and elemental microanalysis. Elemental microanalysis results for C, H, N and S were consistent with the proposed structures. Mass spectra demonstrated the presence of the *m/z* fragments corresponding to the expected molecular ions with the isotopic distribution of the two rhenium isotopes, thus confirming the structures schematized in Fig. 4.

3.5. Biological evaluation

3.5.1. Cell uptake studies

In vitro uptake of ^{99m}Tc-NS_3M1 and ^{99m}Tc-NS_3M2 both in oxia and in hypoxia was evaluated using human colon adenocarcinoma HCT-15 cells in culture. Cells were incubated at 37°C under an atmosphere of 95% air plus 5% carbon dioxide (aerobic exposure) or 95% nitrogen plus 5% carbon dioxide according to literature oxygen concentration where <1000 ppm is considered a hypoxic condition [4,26]. After a 60-min equilibration period, the radiolabeled compounds were added and incubated with the cells for another 60 min. It has been shown by means of propidium iodide viability test [27] that HCT-15 cells maintain >90% of viability for at least 90 min under the hypoxic conditions of this assay. Finally, cells were separated from supernatant and activity measured in order to compare the percentage taken up in oxic and hypoxic conditions. Both complexes showed preferential uptake in hypoxia, as shown in Table 1.

Table 1
Uptake of ^{99m}TcNS$_3$M1 and ^{99m}TcNS$_3$M2 both in oxia and in hypoxia

Complex	Uptake hypoxia/oxia (n=3)
^{99m}TcNS3M1	1.5±0.2
^{99m}TcNS3M1	1.4±0.3

Values are shown as mean±S.D.

3.5.2. Biodistribution in normal mice

The in vivo behavior was evaluated by biodistribution studies in normal mice at 2 and 4 h postinjection. Table 2 shows the results expressed as percent dose per organ in the most significant organs as a function of time. Results were in full agreement with the physicochemical properties of each ^{99m}Tc complexes. ^{99m}Tc-NS_3M1 showed low blood, lung and liver uptake. Excretion occurred through both the urinary and hepatobiliary tract as expected for a compound with intermediate lipophilicity. Thyroid and stomach activities were very low, indicating minimal in vivo reoxidation. Uptake in other organs was negligible. On the other hand, ^{99m}Tc-NS_3M2 exhibited a very high liver activity (50.0±1.9 and 54±2% of the injected dose at 2 and 4 h postinjection), probably due to the high protein binding capability. Excretion was very low within the studied period. This in vivo profile was not adequate for a potential radiopharmaceutical.

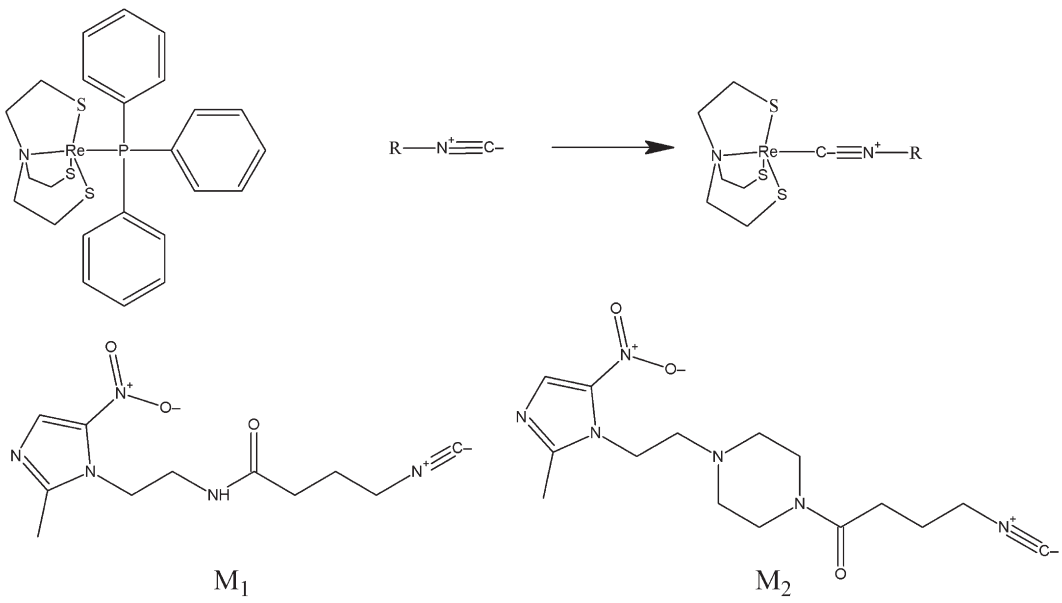

Fig. 4. Synthesis of ReNS$_3$M1 or M2.

Table 2
% Dose organ^{-1} in the most significant organs as a function of time

Organ	^{99m}Tc-NS3M1 % Dose organ^{-1} (n=3)		^{99m}Tc-NS3M2 % Dose organ^{-1} (n=3)	
	2 h	4 h	2 h	4 h
Blood	2.24±0.66	1.31±0.54	3.57±0.56	1.31±0.54
Liver	5.03±0.90	9.26±1.61	50.00±1.91	54.00±1.61
Heart	0.22±0.12	0.09±0.03	0.10±0.01	0.13±0.05
Lung	0.34±0.08	0.28±0.09	0.37±0.04	0.48±0.02
Spleen	0.26±0.11	0.12±0.02	0.11±0.08	0.22±0.04
Kidney	3.08±1.05	6.27±2.27	4.76±0.61	6.57±2.81
Thyroid	0.00±0.00	0.01±0.01	0.03±0.01	0.03±0.01
Muscle	2.30±0.66	3.11±1.98	1.75±0.72	3.21±1.58
Gallbladder	3.12±1.23	2.43±0.86	1.12±0.18	1.61±0.47
Stomach	0.80±0.44	0.61±0.15	3.36±1.02	2.57±1.24
Intestine	32.26±3.92	29.88±3.20	0.82±4.34	0.56±0.11
Bladder+urine	37.76±3.55	35.69±3.93	9.00±3.92	10.00±2.69

Values are shown as mean±S.D.

3.5.3. Biodistribution in animals bearing induced tumors

In order to assess the potentiality of our approach for the design of potential radiopharmaceuticals for nuclear oncology, evaluation of ^{99m}Tc-NS_3M1 in C57BL/6 mice bearing tumors induced by inoculation of 3LL Lewis murine lung carcinoma cells was performed. ^{99m}Tc-NS_3M2 was not tested due to the unfavorable in vivo profile demonstrated in experiments with normal animals. Cells were inoculated subcutaneously in the right limb, and biodistribution studies were performed 20 to 30 days after inoculation, when tumors have adequate size. This animal model was selected because histopathologic studies demonstrated high degree of hypoxia within the tumors (Fig. 5).

Table 3 summarizes the in vivo tumor uptake (expressed as percent dose per gram) as well as the tumor/blood and tumor/muscle ratios. ^{99m}Tc-NS_3M1 showed relatively high initial tumor uptake (2.5±0.3 % dose g^{-1} at 0.5 h postinjection) and significant retention until 4 h (1.2±0.2 % dose g^{-1} at 2 h postinjection). Soft tissue clearance is fast, and this was reflected in tumor/muscle ratios, which were very favorable in the studied period (2.8±0.4 at 0.5 h to 2.0±0.4 at 4 h). Statistical analysis demonstrated that uptake in tumor was significantly higher in comparison to muscle (P=.05) at all time points.

Table 3
Tumor uptake (expressed as % dose g^{-1}) ^{99m}TcNS$_3$M1

	% Injected dose g^{-1} (n=3)			
	30 min	1 h	2 h	4 h
% Tumour g^{-1}	2.5±0.3	2.2±0.5	1.9±0.4	1.2±0.2
% Blood g^{-1}	4.1±0.4	3.8±1.0	1.9±0.3	1.2±0.6
% Muscle g^{-1}	0.9±0.1	0.9±0.6	0.8±0.1	0.6±0.2
Tumour/muscle ratio	2.8±0.4	2.4±0.9	2.4±0.5	2.0±0.4
Tumour/blood ratio	0.6±0.3	0.6±0.2	1.0±0.4	1.0±0.4

Values are shown as mean±S.D.

Although the uptakes of various ^{99m}Tc-nitroimidazole complexes in tumor (target to non-target ratio) have been reported earlier, their comparison was difficult due to both the heterogeneity in animal models used and the nature of the tumors. However, analysis of previously reported data showed that tumor uptake of ^{99m}Tc-NS_3M1 was in the same order as or even higher than those of two well-known agents, ^{99m}Tc-BMS181321 [28] and ^{99m}Tc-BRU59-21 [29], and also of other reported ^{99m}Tc-labelled metronidazole derivatives [14,15]. Blood and liver clearance of our compound were also favorable. Consequently, ^{99m}Tc-NS_3M1 showed promising biological behavior as potential agent for hypoxia imaging in tumors.

4. Conclusion

Metronidazole, a nitroimidazole which has demonstrated high affinity for hypoxic tumors in vitro and in vivo, has been chemically modified in order to prepare two new derivatives, M1 and M2, bearing an isocyanide moiety, suitable to coordinate to ^{99m}Tc through a 4+1 Tc(III)-complex strategy. Labeling with high radiochemical purity was successfully achieved for both complexes. Both ^{99m}Tc complexes were stable both in labeling milieu and in human

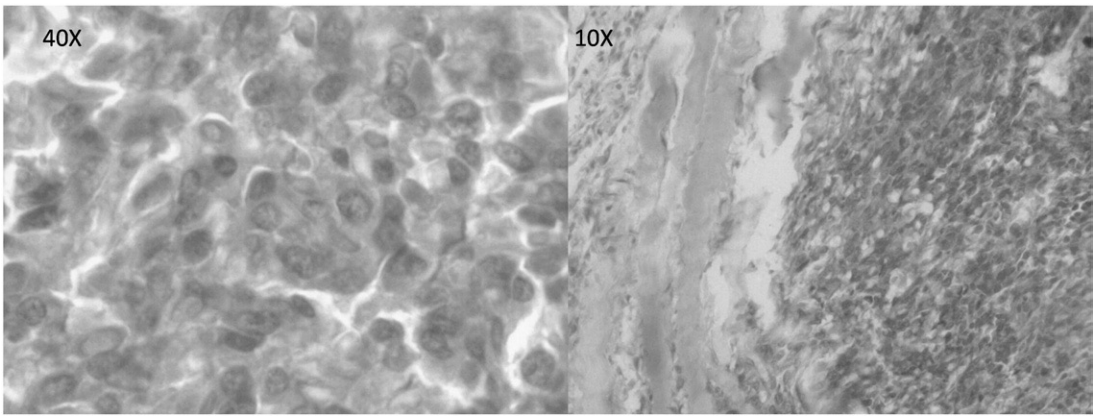

Fig. 5. Histopathologic studies of tumor model induced by 3LL cells.

plasma. However, presence of the piperazine linker in M2 resulted in higher lipophilicity and protein binding. Although cell uptake in hypoxic conditions was observed for both radiotracers ^{99m}Tc-NS_3M2 biodistribution was considered unsuitable for a potential radiopharmaceutical due to its high liver uptake and poor blood clearance. On the other hand, ^{99m}Tc-NS_3M1 demonstrated a very favorable in vivo profile both in normal mice and in mice bearing induced tumors. Selective uptake and retention in tumor together with favorable tumor/muscle ratio makes this compound a promising candidate for further evaluation as potential hypoxia imaging agent in tumors.

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