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Synthesis and evaluation of a new ^{99m}Tc(I)-tricarbonyl complex bearing the 5-nitroimidazol-1-yl moiety as potential hypoxia imaging agent

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The objective of this work was to develop a novel ^{99m}Tc complex bearing the 5-nitroimidazol-1-yl moiety with recognised selectivity towards hypoxic tissue, as a potential radiopharmaceutical for imaging tumour hypoxia.

The new metronidazole derivative (2-amine-3-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethylthio]propanoic acid) (L) containing adequate groups to coordinate technetium through the formation of a Tc(I)-tricarbonyl complex was synthesised with adequate yield (33%) and characterised by spectroscopy.

Labelling was performed by substitution of three labile water molecules of the technetium tricarbonyl precursor, *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ with the ligand. A radiochemical purity higher than 90% was achieved and remained unchanged for more than 4 h. The complex has a high stability in plasma, a moderate plasma protein binding and a moderate hydrophilicity.

***In vitro* cell uptake studies showed a ratio between the activity taken up by cells in hypoxia/normoxia of 1.6 ± 0.4 ($p < 0.5$).**

Biodistribution in normal mice showed rapid depuration and low uptake in all organs and tissues except liver. Biodistribution in mice bearing induced tumours showed a low tumour uptake, but tumour/muscle ratio was favourable thanks to depuration. Comparison with biological results of other metronidazole derivatives clearly shows that modifications of the chelator are very important and contribute to improve the biological behaviour.

Keywords: Tc-tricarbonyl complexes; 5-nitroimidazole derivatives; hypoxia imaging agent

Introduction

Tumour perfusion and oxygenation status have been recognised as important factors that may cause poor treatment outcome after radio and chemotherapy.[1,2]

Bioreductive compounds, which are selectively reduced in hypoxic tissue to reactive intermediates that bind to intracellular molecules, have been used for the development of potential radiopharmaceuticals for targeting hypoxic tumours. The nitroimidazol-1-yl moiety is one of the preferred pharmacophores,[3–8] but other functional groups as nitroaromatics[9,10] and *N*-oxides[11] have also been studied. 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 (Figure 1), which has demonstrated high affinity for hypoxic tumours *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 to enable coordination of ^{99m}Tc without losing the biological activity. As part of our ongoing research on potential radiopharmaceuticals for imaging tumour hypoxia,[9–11,16–19] we have designed and synthesised the novel metronidazole derivative 2-amine-3-[2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)ethylthio] propanoic acid (**L**) (Figure 1). Chemical modifications were performed in order to introduce a cysteine-like donor-atom set (carboxylate, amine and thiol group), which acts as tridentate ligand towards the Tc(I)- tricarbonyl core.

In order to assess the potentiality of the ^{99m}Tc-complex as hypoxia-targeting radiopharmaceutical, we have studied its main physicochemical and biological properties both *in vitro* and *in vivo*.

Materials and methods

General

All laboratory chemicals were reagent grade and were used without further purification. Solvents for chromatographic analyses were

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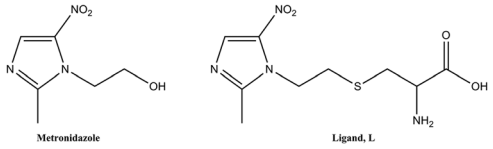


Figure 1. Metronidazole and final ligand **L** structures.

HPLC grade. [^{99m}Tc]$NaTcO_4$ was obtained from a commercial generator (Tecnonuclear S.A., Argentina). Carbonyl labelling agent Isolink was provided by Covidien (Dublin, Ireland). NMR spectra were obtained in the indicated deuterated solvent using a Bruker Avance DPX 400 Spectrometer (Billerica, MA, USA). Chemical shifts are reported as δ values (parts per million) relative to residual protons of deuterated solvent. Coupling constants are reported in hertz (Hz). The multiplicity is defined by s (singlet), t (triplet) or m (multiplet). 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, St. Louis, MO, USA). For column chromatography, we used silica gel (60–230 mesh, Merck (Merck & Co., Inc., NJ, USA)). Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100 Staffordhire, UK.) and were uncorrected. The mass spectra were conducted with a mass spectrometer, Hewlett Packard 5973 MSD or MICROMASS (Triple Quattro Palo Alto, CA, USA.), using electron impact or electrospray (ESI), respectively. HPLC analysis was carried out on an LC-10 AS Shimadzu Liquid Chromatography System using a reverse phase column Phenomenex Luna 5 μm, C18 column (4.6 × 150 mm). Elution was performed with a binary gradient system at 1.0 mL/min flow rate using triethylamine-phosphate buffer pH 2.5 as mobile phase A and methanol as mobile phase B; the elution profile was as follows: 0–3 min 100 % A; 3–6 min linear gradient to 25% B; 6–9 min linear gradient to 34% B; 9–20 min linear gradient to 100% B; 20–27 min 100% B; 27–30 min linear gradient 0% B. Detection was accomplished either with a photodiode array detector (SPD-M10A, Shimadzu (Shimadzu Corporation, Kyoto, Japan)) that recorded UV-vis spectra on flux or with a '3 × 3' NaI (Tl) crystal scintillation detector. Activity measurements were performed either in a Dose Calibrator, Capintec CRC- 5R (Ramsey, NJ, USA) or in a scintillation counter, '3 × 3' NaI (Tl) crystal detector associated to an ORTEC (Atlanta, USA) monochannel analyser.

Synthesis

*Synthesis of 1-(2-iodoethyl)-2-methyl-5-nitro-1H-imidazole (**1**)*

Synthesis was performed according to a previously described method.[20] Triphenylphosphine (0.896 g, 3.42 mmoles) and imidazole (0.233 g, 3.42 mmoles) were dissolved in dry dichloromethane (10 mL). Iodine (0.869 g, 3.42 mmoles) was slowly added to the solution. After incubation at room temperature during 5 min, a solution of metronidazole (0.4 g, 2.34 mmoles) in dichloromethane (1.0 mL) was added to the mixture. Reaction was controlled by TLC until total consumption of metronidazole (approximately 24 h). The solvent was evaporated under vacuum. The residue was purified by column chromatography (SiO_2, dichloromethane/methanol 85:15). Brown solid (47%); mp = 78 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.60 (s, 3H), 3.48 (t, 2H, J = 12.0 Hz); 4.65 (t, 2H, J = 12.0 Hz), 8.00 (s, 1H). IR (KBr) ν (cm^{-1}): 1355, 1368, 1545, 505.

*Synthesis of 2-amine-3-[2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethylthio] propanoic acid (**L**)*

Synthesis was performed according to a previously described method.[21] A solution of the intermediate compound **1** (0.2 g, 0.71 mmoles) dissolved in THF (10 mL) was added to a solution of cysteine hydrochloride (0.125 g, 0.71 mmoles) dissolved in triethylamine (0.10 mL). Progress of the reaction was monitored by TLC until the total consumption of the intermediate compound (approximately 24 h). A white solid corresponding to the impurity triethylamine hydrochloride was removed by filtration. The solvent was evaporated under vacuum to yield the desired product. White solid (70%); mp = 90–94 °C. ^{1}H NMR ($CDCl_3$) δ (ppm): 1.33(m, 2H), 2.57 (s, 3H), 3.27 (m, 1H), 3.57 (t, 2H, J = 14.2 Hz), 4.46 (t, 2H, J = 14.2 Hz), 7.98 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.9 (CH_3), 36.5 (CH_2), 46.0 (CH_2), 51.4 (CH_2), 62.4 (CH), 129.0 (CNO_2), 133.9 (CH), 151.7 (C), 182 (COOH). IR (KBr) ν (cm^{-1}): 1386, 1528, 1732, 3622. Mass spectra (electron impact, 70 eV), *m/z*: 275 (M^+ + H); 244 (M^+ − NO); 199 (M − NO − CO_2H).

Radiolabelling

Preparation of fac-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ complex using a kit formulation

The ^{99m}Tc-sodium pertechnetate (185–1850 MBq, 1 mL) was added to an Isolink kit (Covidien, Dublín, Ireland), and the mixture was incubated at 100 °C for 30 min. After cooling, pH was adjusted to 7.0 with 1N HCl solution. Complex formation was monitored by HPLC analysis as indicated in the general experimental section. Retention time was 4.0 min. Radiochemical purity was >90%.

Preparation of fac-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ complex using CO(g)

The ^{99m}Tc-precursor complex was prepared according to a previously described method[22] as follows: Na/K tartrate (20.0 mg), Na_2CO_3 (4.0 mg) and $NaBH_4$ (7.0 mg) were placed in a vial. The vial was sealed and flushed with carbon monoxide for 30 min. ^{99m}Tc-sodium pertechnetate (185–1850 MBq, 1 mL) was added, and the mixture was incubated at 75 °C for 30 min. After cooling, pH was adjusted to 7.0 with 1N HCl solution. The complex formation was monitored by HPLC analysis as indicated in general experimental section. Retention time was 4.0 min. Radiochemical purity was >90%.

*Substitution with **L***

Neutralised *fac*-[^{99m}Tc(OH$_2$)$_3$(CO)$_3$]$^+$ precursor (100–200 μL, 185–1850 MBq) was incubated with ligand **L** (4 mg, 1×10^{-5} mol) for 30 min at 70 °C. Complex formation was monitored by HPLC analysis. Retention time was 14.0 min. Radiochemical purity was >90%.

Physicochemical evaluation

Stability in labelling milieu

^{99m}Tc-complex was incubated in the labelling milieu at room temperature, and the radiochemical purity was assessed by HPLC using the chromatographic conditions described in general experimental section for up to 4 h after labelling.

Stability in plasma

^{99m}Tc-complex (100 μL) was incubated in human plasma (900 μL) at 37 °C for up to 4 h. After 30, 60 and 120-min incubation, samples (200 μL) were extracted and proteins were precipitated

with ethanol (200 μL), centrifuged (12000 rpm, 5 min) and the supernatants analysed by HPLC using chromatographic conditions described in general experimental section.

Protein binding studies

^{99m}Tc-complex (25 μL) was 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 (GE Healthcare Buckinghamshire, UK.), which have been pre-spun at 2000 g for 1 min. Columns were centrifuged again at 2000 g for 2 min, and the collected eluate and the column were counted in a NaI(Tl)-scintillation counter. Protein bound tracer was calculated as the percentage of activity eluted from the column.

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 cpm/mL of 1-octanol layer divided by that of the buffer. Lipophilicity was expressed as log P.

Biological evaluation

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% foetal bovine serum (Gibco), penicillin 100 U/mL (Sigma) and 100 μg/mL 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. Afterwards, the flasks were pre-incubated in a chamber gassed with N_2 for 1 h to remove oxygen from the milieu, and afterwards ^{99m}Tc-complex 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) to use as control. After incubation, time-elapsed culture milieu was removed, cells were washed with PBS and treated with Trypsin-EDTA (Sigma). Finally, activity in the supernatant and the cells were measured in a solid scintillation counter, and results were expressed as the ratio between the percentage of activity taken up by cells incubated in nitrogen (hypoxia) and incubated in normal conditions (normoxia).

Animal studies

All animal studies were approved by the Ethics Committee of the Faculty of Chemistry from Uruguay.

Biodistribution in normal mice

CD1 mice (female mice, 25–30 g, three animals per group) were injected via a lateral tail vein with ^{99m}Tc-**L** (0.1 mL, 0.037–0.37 MBq). At different intervals after injection, animals were sacrificed by neck dislocation. Whole organs and samples of blood and muscle were collected, weighed and assayed for radioactivity. The 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 % dose/organ. % Dose in blood and in muscle were calculated considering the whole blood as 7% and the whole muscle as 43% of the body weight, respectively

Biodistribution in animals bearing induced tumours

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/six mice (8–10 weeks old). About 20–30 days later, the animals developed palpable tumour nodules ($1.5 \times 0.5 \times 0.5$ cm) and were used for biodistribution studies.

Three animals per group were injected via a lateral tail with ^{99m}Tc-**L** (0.1 mL, 0.037–0.37 MBq). At different intervals after injection, the animals were sacrificed by neck dislocation. Whole tumour and samples of blood and muscle were collected, weighed and assayed for radioactivity. Results were expressed as % dose/organ and % dose/g tissue.

Results and discussion

Synthesis of the ligand

Design of the ligand consisted in the introduction to metronidazole of a high-affinity tridentate chelator system to form a stable neutral complex with the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ synthon. For this purpose, we have prepared a cysteine-derivative ligand that bears an NSO type-chelator unit, similar to the homocysteine-derivative developed by Karagiorgou *et al.*,[23] in which the donor-atom system consists on an *N*-primary amine, an *S*-thioether and an *O*-carboxylate.

Synthesis of the ligand was achieved by a two-step reaction using metronidazole as starting reagent (Figure 2). In the first step, the intermediate compound **1** was obtained by substitution of the hydroxyl group of metronidazole by an iodine moiety. In the second step, attachment to a cysteine unit was performed in an organic solvent and alkaline medium. Intermediate and final products were purified and characterised by common spectroscopic techniques (NMR, IR) and mass spectrometry.

Radiolabelling

Synthesis of the ^{99m}Tc-tricarbonyl complex was achieved by a two-step synthetic route (Figure 3). The first step consisted in the preparation of the precursor, *fac*-$[^{99m}Tc(CO)_3(OH_2)_3]^+$, either using sodium pertechnetate eluted from a ^{99}Mo/^{99m}Tc generator as starting material and sodium borohydride as reducing agent

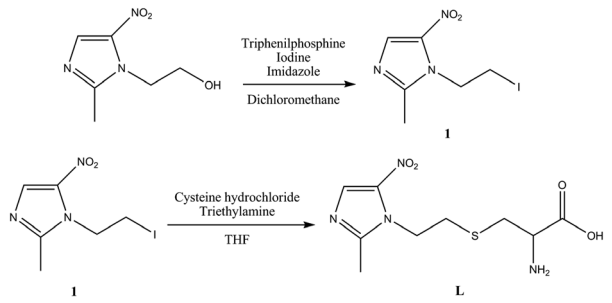


Figure 2. Synthesis of the ligand **L**.

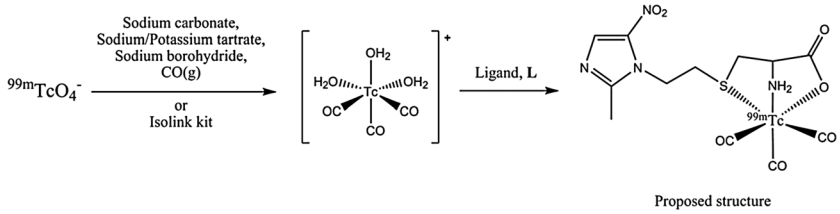


Figure 3. Radiolabelling route.

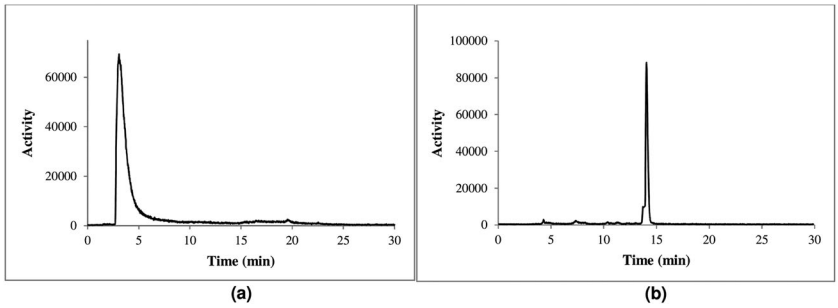


Figure 4. Typical chromatograms for *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)]+ (a) and [^{99m}Tc(CO)$_3$(**L**)] (b) (RP-HPLC).

in the presence of CO(g) or, as an alternative, the Isolink kit. The second step involved the substitution of the precursor by incubation with the ligand, **L**. It is well known that *S*-alkylated cysteine derivatives bind efficiently with the tricarbonyl core via its NSO donor-atom set to provide stable and neutral complexes. Coordination affords two five-membered and one six-membered ring providing thermodynamically stable hexacoordinated complexes[23–26] . The proposed structure for the final complex is shown in Figure 3.

Radiochemical purities of precursor *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ and the final complex [(^{99m}Tc(CO)$_3$(**L**)] were evaluated by reverse phase HPLC (RP-HPLC, Figure 4). The precursor was obtained with high radiochemical purity (>90%). Substitution yielded a single radiolabelled species with retention time of 14 min (Figure 4b) and radiochemical purity above 90%.

Physicochemical evaluation

In order to study the potentiality of the ^{99m}Tc-complex as radiopharmaceutical, some relevant physicochemical properties were studied (i.e. stability, lipophilicity and protein binding).

Stability

The final complex was stable for at least 4 h in labelling milieu at room temperature. Besides, stability after incubation with human plasma for 4 h at 37 °C was also studied, and radiochemical purity after incubation was found to be 91.0 ± 2.0%.

Protein binding studies

Binding to plasma protein was studied using size exclusion chromatography. Ideally, low protein binding is required in order

Table 1. Biodistribution results of [^{99m}Tc(CO)$_3$(**L**)] in normal mice. Each value represents the mean percentage (n = 3) of the injected dose/organ ± standard deviation of the mean

	% Injected dose/organ		
Organ	0.5 h	2.0 h	4.0 h
Blood	8.20 ± 0.98	2.07 ± 0.03	2.00 ± 0.18
Liver	35.5 ± 4.1	19.7 ± 2.3	21.9 ± 4.3
Heart	0.40 ± 0.09	0.09 ± 0.03	0.09 ± 0.08
Lung	0.61 ± 0.09	0.16 ± 0.03	0.13 ± 0.09
Spleen	0.54 ± 0.31	0.08 ± 0.04	0.15 ± 0.03
Kidneys	11.8 ± 3.0	5.42 ± 1.06	5.16 ± 0.44
Thyroid	0.13 ± 0.05	0.02 ± 0.01	0.02 ± 0.01
Muscle	5.4 ± 2.7	2.26 ± 0.80	1.80 ± 0.28
Gallbladder	1.40 ± 0.09	0.97 ± 0.62	0.66 ± 0.13
Stomach	1.68 ± 0.58	0.61 ± 0.12	0.39 ± 0.21
Intestines	23.0 ± 6.0	27.6 ± 2.7	32.1 ± 7.8
Urine + bladder	26.7 ± 5.4	39.9 ± 6.8	35.0 ± 5.3

Table 2. Biodistribution results of [^{99m}Tc(CO)$_3$(**L**)] in C57BL/ six mice bearing induced Lewis murine lung carcinoma. Each value represents the mean percentage (n = 3) of the injected dose/organ ± standard deviation of the mean

	% Injected dose/organ		
Organ	0.5 h	2.0 h	4.0 h
Blood	7.2 ± 1.5	2.53 ± 0.12	1.85 ± 0.32
Liver	38.1 ± 6.5	22.3 ± 1.1	20.2 ± 3.4
Heart	0.32 ± 0.12	0.45 ± 0.17	0.12 ± 0.09
Lung	0.48 ± 0.05	0.09 ± 0.02	0.08 ± 0.05
Spleen	0.71 ± 0.12	0.14 ± 0.08	0.12 ± 0.06
Kidneys	12.9 ± 3.9	6.8 ± 2.1	5.92 ± 0.23
Thyroid	0.09 ± 0.03	0.02 ± 0.01	0.02 ± 0.01
Muscle	4.1 ± 3.2	3.26 ± 0.92	1.92 ± 0.27
Gallbladder	1.98 ± 0.17	0.83 ± 0.13	0.43 ± 0.21
Stomach	3.45 ± 0.89	0.92 ± 0.32	0.14 ± 0.12
Intestines	25.8 ± 8.0	28.5 ± 5.1	30.2 ± 9.2
Urine + bladder	22.5 ± 8.2	34.3 ± 4.3	38.0 ± 8.1

Table 3. Uptake in tumour of [^{99m}Tc(CO)$_3$(**L**)] in C57BL/6 mice bearing induced Lewis murine lung carcinoma. Each value represents the mean percentage ($n = 3$) of the injected dose/g of tissue ± standard deviation of the mean

Organ or ratio	% Injected dose/g			
	0.5 h	1.0 h	2.0 h	4.0 h
Tumour	1.3 ± 0.4	0.5 ± 0.1	0.31 ± 0.03	0.4 ± 0.1
Blood	5.7 ± 0.9	2.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.2
Muscle	1.0 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.20 ± 0.03
Tumour/muscle	1.3 ± 0.5	1.7 ± 0.2	1.2 ± 0.1	2.0 ± 0.1
Tumour/blood	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.3 ± 0.2

to ensure adequate pharmacokinetics of the potential radiopharmaceuticals. Additionally, only the unbound fraction of the radiotracer will penetrate cells and other biological membranes.[27] A relatively low protein binding of 15.0 ± 5.0% was obtained for [^{99m}Tc(CO)$_3$(**L**)], correlating with the high *in vitro* stability and low lipophilicity of this complex. Because protein binding remained constant during all the studied period, the reported value corresponds to the mean of all observed values between 30 and 120 min.

Lipophilicity

The partition coefficient between 1-octanol and phosphate buffer (pH 7.4) was measured in order to assess lipophilicity of the ^{99m}Tc complex. A log P of −0.75 ± 0.08 was found. This value is in accordance with the structure of the ligand, because incorporation of the cysteine unit increases the hydrophilicity of the ligand.

Biological evaluation

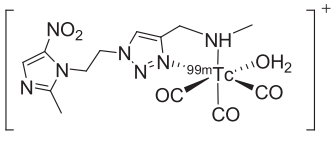
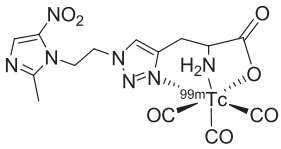
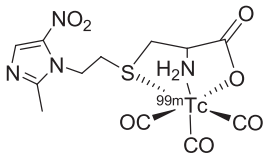
Cell uptake studies

In vitro uptake of [^{99m}Tc(CO)$_3$(**L**)] both in normoxia and 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 (hypoxic conditions). According to literature, oxygen concentration ˂1000 ppm is considered hypoxic condition.[28,3] After 60 min equilibration period, the radiolabelled compound was added and incubated with the cells for other 60 min. HCT-15 cells maintain >90% of viability for at least 90 min under the hypoxic conditions of this assay, as demonstrated by the propidium iodide viability test.[29] Finally, cells were separated from supernatant and activity measured in order to compare the percentage taken up in normoxic and hypoxic conditions. This study has been previously validated by our group and used successfully in the evaluation of other [^{99m}Tc]-complexes.[17–19] Results for [^{99m}Tc(CO)$_3$(**L**)] showed that the ratio between hypoxic and normoxic uptake was 1.6 ± 0.4. This result is statistically significant (p ˂ 0.05) and confirms selectivity towards hypoxia conditions.

Biodistribution in normal mice

The *in vivo* behaviour was evaluated by biodistribution studies in normal mice at 0.5, 2 and 4 h post-injection. Table 1 shows the

Table 4. Some physicochemical and biological properties of our developed ^{99m}Tc(I)-tricarbonyl complexes

Complex	Physicochemical properties			Activity in[a]		
	Stability in plasma (%)[a]	log P	Protein binding	Blood	Liver	Kidneys
 **A**	85	−0.82 ± 0.02	51.5 ± 0.9%	4.7 ± 1.3	17.0 ± 0.6	4.9 ± 0.9
 **B**	92	−0.44 ± 0.04	13.0 ± 3.0%	0.4 ± 0.2	1.8 ± 0.6	0.22 ± 0.04
	91	−0.75 ± 0.08	15.0 ± 5.0%	2.07 ± 0.03	19.7 ± 2.3	5.42 ± 1.06

[a]Percentage of injected dose in each organ after 2 h of complex injection.

results expressed as percentage dose per organ in the most significant organs as a function of time. [^{99m}Tc(CO)$_3$(**L**)] showed considerable blood uptake at 30 min post-injection but clearance was high after 1 h. Lung and muscle activities are low, and moderate liver uptake, which decreases during time was also observed. Excretion occurred through both the urinary and hepatobiliary tracts. Thyroid and stomach activities were very low, indicating minimal *in vivo* reoxidation. Uptake in other organs was negligible.

Biodistribution in animals bearing induced tumours

In order to assess the potentiality of our approach for the design of potential radiopharmaceuticals for nuclear oncology, evaluation of [^{99m}Tc(CO)$_3$(**L**)] in C57BL/6 mice bearing tumours induced by inoculation of 3LL Lewis murine lung carcinoma cells was performed. Cells were inoculated subcutaneously in the right limb, and biodistribution studies were performed 20–30 days after inoculation, when tumours have adequate size. This animal model was selected because histopathologic studies performed previously by our group demonstrated high degree of hypoxia within the tumours.[17] Biodistribution results (Table 2) are in full agreement to those obtained in normal CD1 mice.

Table 3 summarises the *in vivo* tumour uptake (expressed as percentage dose per gramme) as well as the tumour/blood and tumour/muscle ratios. [^{99m}Tc(CO)$_3$(**L**)] showed relatively good initial tumour uptake (1.3 ± 0.4% dose g^{-1} at 0.5 h post-injection) although approximately 50% of the activity was cleared from tumour after 1 h. Uptake remained constant from 1 to 4 h post-injection. Muscle clearance is fast, leading to a favourable tumour/muscle ratio at 4 h post-injection (2.0 ± 0.1% dose g^{-1}). Statistical analysis demonstrated that uptake in tumour was significantly higher in comparison with muscle ($p = 0.05$) at this time point.

Comparison with other ^{99m}Tc-labelled metronidazole derivatives previously described by our group[16–18] is interesting. In spite of having exactly the same pharmacophore, the physicochemical and biological behaviour showed significant differences according to the selected labelling methods. Even if the labelling method is the same, difference in the donor atoms set used for coordination seems to play a significant role. Understanding these differences is a key point for tailoring the biological behaviour of novel compounds.

The new ^{99m}Tc(I)-tricarbonyl complex presented in this paper displays some similar physicochemical properties (stability in the presence of plasmatic proteins and lipophilicity) to those of other tricarbonyl complexes developed by our group (Table 4).[18] However, its protein binding is significantly lower than that of complex **A** (Table 4), formed by a bidentate ligand and having a molecule of water in the coordination sphere. According to the data shown in Table 3, the blood activities seem to be related to complexes lipophilicities. These results contradict previous description[30] and suggest that the lipophilicities, rather than the ligand denticity, seem to better explain the biodistribution in certain organs, such as liver and kidneys (Table 3), because the most hydrophilic complexes exhibit the highest uptakes in these organs. Comparison between complexes **B** and **A** and the new complex support this hypothesis.

Conclusions

Metronidazole, a nitroimidazole, which has demonstrated high affinity for hypoxic tumours *in vitro* and *in vivo*, has been chemically modified in order to prepare a new derivative, **L**, bearing an cysteine-like chelating unit, suitable to coordinate ^{99m}Tc through a Tc(I)-tricarbonyl complex. Labelling with high radiochemical purity was successfully achieved, and the complex was stable both in labelling milieu and in human plasma. Lipophilicity and protein binding value were low. Biodistribution in normal mice was favourable, with rapid depuration and low uptake in all organs and tissues except liver. Although cell studies suggest a preferential uptake by hypoxic tissue, biodistribution in mice bearing hypoxic tumours showed low uptake probably because of hydrophilicity. However, tumour/muscle ratio was favourable thanks to depuration from soft tissues. Comparison with other ^{99m}Tc tricarbonyl metronidazole derivatives developed by our group leads to the conclusion that not only ligand denticity but also lipophilicity plays a key role in the biological behaviour.

Although selective cell uptake in hypoxic conditions was observed and overall biodistribution in normal mice was favourable, low uptake and scarce retention in tumour are drawbacks of this compound as potential hypoxia imaging agent in tumours.

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Conflict of Interest

The authors did not report any conflict of interest.

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