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Influence of ligand denticity on the properties of novel ^{99m}Tc(I)–carbonyl complexes. Application to the development of radiopharmaceuticals for imaging hypoxic tissue

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ABSTRACT

An important issue in the development of metal-based radiopharmaceuticals is the selection of the labelling strategy in order to couple the metal to the pharmacophore without losing the biological activity. With the aim to evaluate the correlation between ligand denticity and biological behaviour of the corresponding ^{99m}Tc complexes, we designed a tridentate and a bidentate 5-nitroimidazole derivatives suitable for ^{99m}Tc(I) tricarbonyl complexation and with potential use as radiopharmaceuticals towards hypoxic tissue diagnosis. Ligands were synthesized using metronidazol, a pharmaceutical containing the bioreductive pharmacophore as starting material. The chelating units were connected to the pharmacophore using the click reaction of Huisgen. Both ^{99m}Tc complexes were obtained in high yield and were hydrophilic and stable in labelling milieu. The complex obtained from the tridentate ligand exhibited high stability in human plasma, low protein binding and a favourable biodistribution characterized by low blood and liver uptake, fast elimination and negligible uptake in other organs or tissues. Selective uptake and retention in tumour together with favourable tumour/muscle ratio makes this ^{99m}Tc-complex a promising candidate for further evaluation as potential hypoxia imaging agent in tumours. The bidentate ligand, on the other hand, yielded a less stable ^{99m}Tc-complex that experimented hydrolysis in vitro and decomposition in human plasma and showed high protein binding, high blood and liver uptake and moderate excretion. Although selective uptake and retention in tumour was also observed physicochemical and biological behaviour are inadequate for in vivo use, demonstrating that denticity of the ligand is particularly important and that tridentate ligands are preferable in order to prepare ^{99m}Tc-tricarbonyl complexes for Nuclear Medicine imaging.

1. Introduction

Nuclear Medicine techniques based on the administration of small amounts of radioactive compounds, the so-called radiopharmaceuticals, followed by detection of the radiation escaping from the body, provide a non-invasive and accurate method for in vivo imaging highly specific biochemical processes. An important issue in the development of metal-based radiopharmaceuticals is the selection of the labelling strategy in order to couple the metal to the pharmacophore without losing the biological activity. Recently, new ^{99m}Tc-coordinated moieties have been introduced as potentially adequate for labelling small molecules. Alberto et al.[1] have succeeded in the low pressure synthesis of Tc-tricarbonyl complexes, which can be used as precursors to obtain a great diversity of potential radiopharmaceuticals.[2–4] The CO is a tightly bound ligand which stabilizes metals in low oxidation states. In aqueous milieu, the well-defined aqua ion of technetium $[Tc(CO)_3(OH_2)_3]^+$ is formed. This novel type of complexes are at the same time small and very stable, due to the d^6 configuration of the metallic centre, and give the possibility to substitute the three weakly bound water molecules by a great variety of bi and tridentate ligands. Derivatization of different pharmacophores to introduce adequate chelator units (see examples in Fig. 1a)[5–10] could produce a variety of Tc-labelled small molecules. This approach has been applied recently to the labelling of peptides, CNS receptor ligands, myocardial imaging agents, DNA intercalators, among others.[11–15] In these cases different ligand denticity were employed with different success.

Hypoxia in tumours is a pathophysiologic consequence of structurally and functionally disturbed microcirculation and deterioration of diffusion conditions. The evaluation of tumour perfusion and oxygenation status is very important in oncology since tumour hypoxia appears to be strongly associated with tumour propagation, malignant progression, and resistance to therapy and it has

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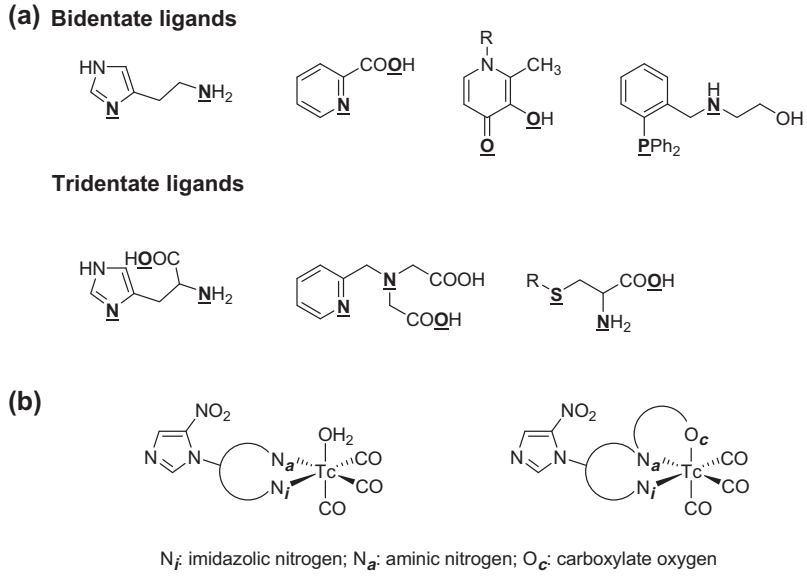


Figure 1. (a) Bidentate and tridentate chelator units used in the preparation of ^{99m}Tc(I) tricarbonyl complexes. (b) General structures of the herein designed ^{99m}Tc-complexes.

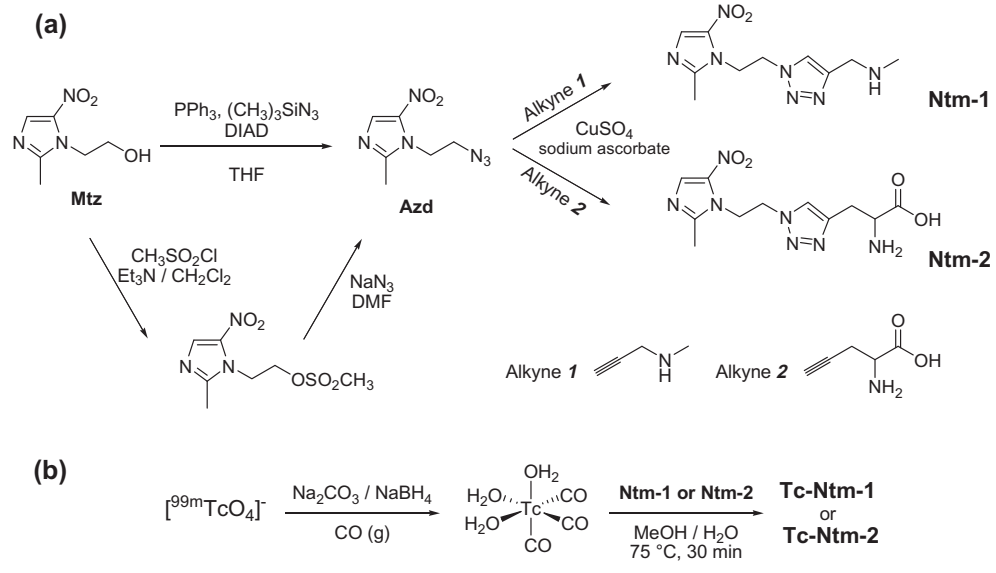
thus become a central issue in tumour physiology and cancer treatment.[16,17] Bioreductive compounds, which are selectively reduced in hypoxic tissue to reactive intermediates that bind to intracellular molecules, have been used for the development of potential radiopharmaceuticals for targeting hypoxic tumours being nitroaryl moiety one of the preferred pharmacophore.[18,19] In this sense, 5-nitroimidazol-1yl moiety[20,21] has recognized bioreductive capacity since in vivo reduction is irreversible in hypoxic tissue resulting in entrapping of metabolites within the cells. Combination of this bioreductive pharmacophore with Tc is useful in the design of potential markers of hypoxic tissue.

With the aim to evaluate the correlation between ligand denticity and biological behaviour of the corresponding ^{99m}Tc complexes, herein we designed 5-nitroimidazole derivatives with different denticity suitable for ^{99m}Tc(I) tricarbonyl complexation (Fig. 1b) and with potential use as radiopharmaceuticals towards hypoxic tissue diagnosis. One of the designed ligands contains an imidazolic nitrogen, from a 1,2,3-triazole system, and an aliphatic amine nitrogen as electron donor atoms for Tc coordination. On the other hand, the second designed ligand has three coordinating groups, an imidazolic nitrogen, an aliphatic amine nitrogen and a carboxylate oxygen, from a carboxylic acid. These ligands were used to prepare the corresponding ^{99m}Tc(I)-tricarbonyl complexes whose structures were proposed from the preparation of the corresponding rhenium complexes. The main physicochemical and biological properties were studied both in vitro and in vivo in order to assess the influence of the denticity of the ligands and to establish their potentiality as hypoxia targeting radiopharmaceuticals.

2. Results and discussion

2.1. Synthesis of the ligands

Two different ligands were designed and synthesized in order to achieve the proposed objectives, that is *N*-methyl-1-[1-(2-(2-methyl-5-nitro-1*H*-imidazole-1-yl)ethyl)-1*H*-1,2,3-triazole-4-yl]methylamine (**Ntm-1**) and 2-amine-3-{1-[2-(2-methyl-5-nitro-1*H*-imidazole-1-yl)ethyl]-1*H*-1,2,3-triazole-4-yl}propanoic acid



Scheme 1. Synthesis of ligands **Ntm-1** and **Ntm-2 (a)** and the corresponding ^{99m}Tc complexes **Tc-Ntm-1** and **Tc-Ntm-2 (b)**.

(**Ntm-2**) (Scheme 1a). For their preparation the key reagent was the azide **Azd** (Scheme 1a) that was obtained from the commercially available antiparasite drug metronizadole (**Mtz**, Scheme 1a). As shown in Scheme 1, in a first stage, the **Mtz** OH-group was transformed into the azide in a single step through a Mitsunobu reaction,[22] employing azidotrimethylsilane as nucleophylic reagent. Alternatively the **Azd** intermediate was also prepared via sulfonate intermediate with similar yield but without chromatographic purification processes (Scheme 1a).[23] Following this step, **Azd** was reacted with the adequate terminal alkyne, *N*-methyl-propargylamine (*alkyne* ***1***) or D,L-propargylglycine (*alkyne* ***2***), to obtain the final ligands **Ntm-1** and **Ntm-2**, respectively. According to the NMR experiments results, ^{1}H NMR[24] and NOE-diff, the Huisgen [4+2] cycloaddition, *click reaction*, occurred regioselectively in presence of Cu(I), produced *in situ* by reduction of Cu(II) with sodium ascorbate, to yield the desired 1,4-disubstituted-1,2,3-triazoles.[25] All the compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR and MS (see Supplementary data). The purity of the ligands was established by HPLC.

2.2. ^{99m}Tc-labelling

Synthesis of ^{99m}Tc-tricarbonyl complexes was achieved by a two-step synthetic route (Scheme 1b). The first stage consisted in the preparation of the precursor, *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$, using sodium pertechnetate eluted from a ^{99}Mo/^{99m}Tc generator as starting material and sodium borohydride as reducing agent. The second stage involved the substitution of the precursor by incubation with the desired ligand, **Ntm-1** or **Ntm-2**, and heating at 75 °C during 30 min. Proposed structures for final complexes, **Tc-Ntm-1** and **Tc-Ntm-2**, are shown in Figure 2 (see Section 2.3.). For the most promising radiopharmaceutical, **Tc-Ntm-2**, a one-pot preparation procedure (Fig. 3) was also assayed. The combination of the chelating unit with the pharmacophore was achieved as a first step by the click reaction and the reaction mixture without any purification was incubated with the neutralized ^{99m}Tc-tricarbonyl precursor to yield the desired technetium complex in high purity. Click chemistry has been proposed as an innovative functionalization strategy for biomolecules in the preparation of potential radiopharmaceuticals because it is efficient, selective and devoid of side reactions. The one-pot procedure represents a remarkable improvement since derivatization of the pharmacophore and labelling is achieved in a very short time and without need of purification of intermediates.

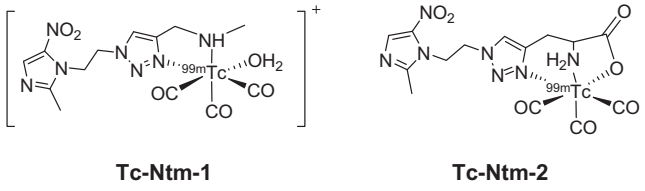


Figure 2. Proposed structures for complexes **Tc-Ntm-1** and **Tc-Ntm-2**.

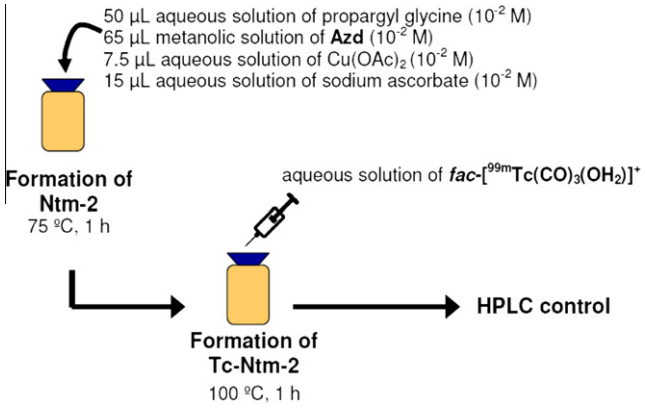


Figure 3. One-pot procedure to prepare **Tc-Ntm-2**.

Radiochemical purities of precursor *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ and final complexes (**Tc-Ntm-1** and **Tc-Ntm-2**) were evaluated by reverse phase HPLC (RP-HPLC, Fig. 4). Analysis of the labelling mixture for **Ntm-2** revealed the presence of a main radio-product with retention time of 18 min (Fig. 4b). On the other hand, for **Ntm-1** labelling the RP-HPLC analysis of the reaction milieu indicated the formation of two Tc-complexes with retention times of 15.0 and 16.0 min (Fig. 4c), ratio 1.0:2.5. Isolation of the main product from the reaction milieu of the **Tc-Ntm-1** synthesis, t_R= 16.0 min, was attempted by HPLC (Fig. 4d). However, HPLC analysis of the isolated peak, during the time, also showed the minor product, t_R= 15.0 min, (Fig. 4e), suggesting that an equilibrium between two ^{99m}Tc-species was established. The chromatogram also showed a small amount of the precursor *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ indicating that technetium complex **Tc-Ntm-1** (Fig. 2), might suffers, in aqueous milieu, transformation to the di-*aquo* minor Tc-complex, t_R = 15.0 min (Fig. 4c), and finally to the precursor, t_R = 3.5 min (Fig. 5a). However another possibility[†] to explain the presence of two Tc-complexes for the bidentate ligand **Ntm-1** is the presence of two configurational isomers (i.e. **A-Tc-Ntm-1** and **B-Tc-Ntm-1**, Fig. 5b). This kind of isomers, diastereomers, could be chromatographically differentiated and could be in equilibrium depending on the pH, as it was recently described.[26] No further studies to explain the presence of two labelled entities in the **Tc-Ntm-1** synthesis were done.

Taking into consideration that during the preparation of **Tc-Ntm-1** the two generated species were in equilibrium (Figs. 4c and 5) and that the main species was converted to the other complex in aqueous solution (Fig. 4e), (and probably also in the biological milieu), we decided to perform all the biological studies using the mixture of both Tc-complex, ratio = 2.5, obtained in the labelling reaction.

2.3. Preparation of rhenium analogues

The structure of ^{99m}Tc-complexes was studied using stable rhenium as a surrogate for technetium. Rhenium, as 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.[27] In our case, the analogue rhenium complexes were prepared by substitution of ligands **Ntm-1** or **Ntm-2** on the rhenium precursor *fac*-[NEt$_4$]$_2$[Re(CO)$_3$Br$_3$] (Scheme 2).[28–30] The HPLC analyses, UV detection, of the reaction mixture for both processes showed main peaks with the same retention time of the corresponding technetium complexes (see Supplementary data). The results of the analysis of the isolated main complexes, **Re-Ntm-1** and **Re-Ntm-2**, were compatible with the proposed structures for the Tc-complexes (Fig. 2) confirming the presence of one ligand molecule per [^{99m}Tc(CO)$_3$]$^+$ core and the presence of one molecule of coordinated water in the case of **Re-Ntm1**.

2.4. Physicochemical studies

In order to study the potentiality of the technetium complexes as radiopharmaceuticals some relevant physicochemical

[†] We would like to thank Referee #1 for his helpful suggestions concerning to this point.

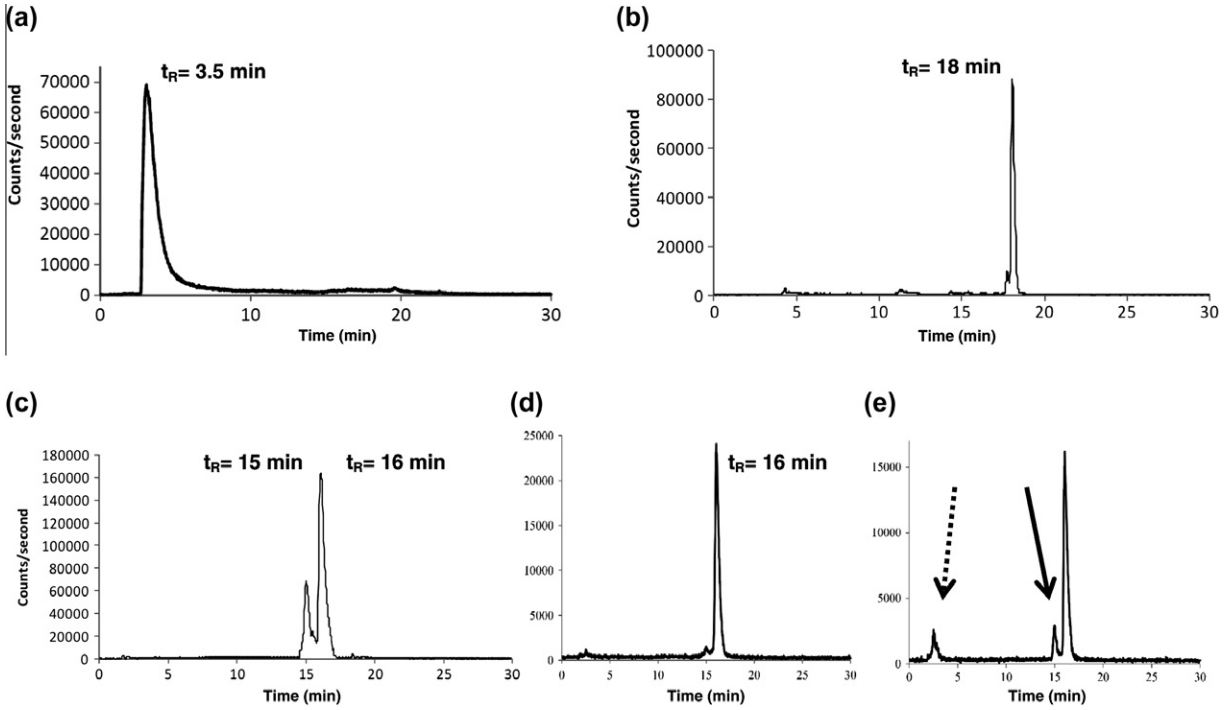


Figure 4. Typical chromatograms for ^{99m}Tc(I)-tricarbonyl complexes (RP-HPLC eluted with phosphate buffer (pH 2.5):MeOH (0 to 100%), coupled to γ-detector). (a) *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$. (b) Labelling mixture of **Tc-Ntm-2**. (c) Labelling mixture of **Tc-Ntm-1**. (d) Isolated main product from the reaction milieu of **Tc-Ntm-1**. (e) Example of stability study by RP-HPLC of radio-product with t_R = 16.0 min. The arrows show the presence of *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ (dotted) and the minor ^{99m}Tc complex with t_R = 15.0 min at 4 h of incubation.

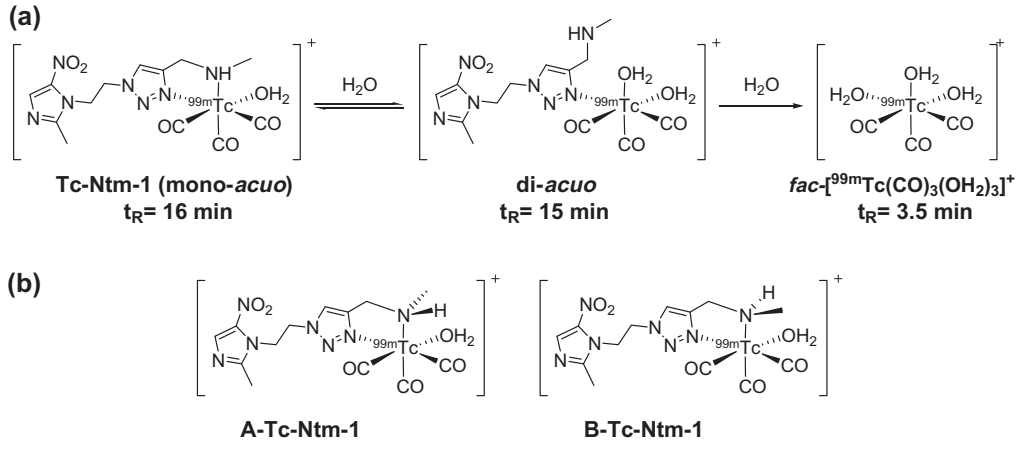
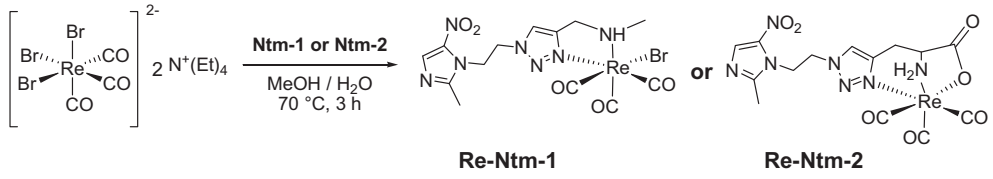


Figure 5. Proposed processes that explain the presence of multiple labelled species in the **Tc-Ntm-1** preparation.



Scheme 2. Synthesis of the Re-complexes analogues, **Re-Ntm-1** and **Re-Ntm-2**.

properties were studied (i.e. stability, lipophilicity, and binding to plasmatic proteins).

2.4.1. Stability

^{99m}Tc-complexes, in the labelling milieu, were incubated at room temperature and the radiochemical purity checked by HPLC analysis for up to 4 h after labelling. The complexes, **Tc-Ntm-1** as the mixture of the two forms and **Tc-Ntm-2**, were stable for at least 4 h in the labelling milieu. On the other hand, stability after incubation in human plasma for 2 h, at 37 °C, was also studied (Table 1). **Tc-Ntm-1** showed a lower stability than **Tc-Ntm-2** in agreement with the bidentate-monodentate behaviour of the ligand, **Ntm-1**,

Table 1
Some physicochemical and biological properties of the developed Tc-complexes

Complex	Physicochemical properties			Activity in[d]		
	Stability in plasma[a]	Log P[b]	Protein binding[c]	Blood	Liver	Kidneys
Tc-Ntm-1[e]	85%	−0.82 ± 0.02	51.5 ± 0.9%	4.7 ± 1.3	17.0 ± 0.6	4.9 ± 0.9
Tc-Ntm-2	92%	−0.44 ± 0.04	13.0 ± 3.0%	0.4 ± 0.2	1.8 ± 0.6	0.22 ± 0.04

[a] Percentage of radioactivity remaining in the main peak after 2 h of incubation in human plasma at 37 °C.
[b] For experimental details see Text.
[c] Percentage of radioactivity bound to plasma proteins after 2 h of incubation.
[d] Percentage of injected dose in each organ after 2 h of complex injection.
[e] Studied as the mixture of complexes.

that entails to a more reactive complex towards plasma nucleophiles.

2.4.2. Lipophilicity

The partition coefficient between 1-octanol and phosphate buffer (0.1 M, pH 7.4) of the technetium complexes was measured in order to assess their lipophilicity (Table 1). The values are in accordance to the proposed structures of the complexes since **Tc-Ntm-1** has a formal positive charge and consequently the highest hydrophilicity.

2.4.3. Protein binding

Binding to plasma protein was studied using size exclusion chromatography (Table 1). 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.[31] A relatively low protein binding was obtained for **Tc-Ntm-2**, correlating with the highest lipophilicity of this complex. On the other hand, protein binding of **Tc-Ntm-1** was considerably high. Once more the substitution of the labile-bound water molecule in the coordination sphere of this complex by other potential electron donor groups from the proteins, that is, imidazole, thiolate, and carboxylate groups, could be considered as a possible cause of this result.

2.5. Biological evaluation

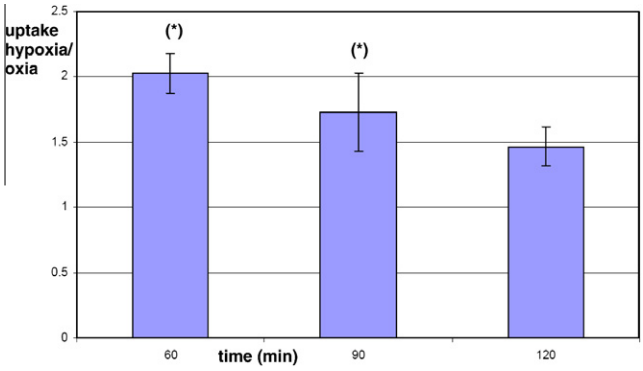
2.5.1. Cell uptake studies

For the most promising radiopharmaceutical, **Tc-Ntm-2**, in vitro uptake both in oxia 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 according to literature oxygen concentration <1000 ppm is considered hypoxic condition.[32,33] After 60 min equilibration period, the radiolabeled compound was added and incubated with the cells for other 60, 90, or 120 min. It has been shown by means of propidium iodide viability test[34] that HCT-15 cells maintain >90% of viability for at least 90 min under the hypoxic conditions of this assay (data not shown). Finally, cells were separated from supernatant and radioactivity measured in order to compare the percentage taken up in oxic and hypoxic conditions.[21] **Tc-Ntm-2** showed preferential uptake in hypoxia, as shown in Table 2.

2.5.2. Biodistribution studies in animals bearing induced tumours

Biodistribution studies were carried out in C57 mice bearing induced Lewis carcinoma. This animal model was selected because histopathologic studies demonstrated high degree of hypoxia within the tumours.[21] Complete results of biodistribution for **Tc-Ntm-1** and **Tc-Ntm-2** complexes are available as Supplementary Material. Biological behaviour of **Tc-Ntm-2** was characterized

Table 2
Uptake of ^{99m}**Tc-Ntm-2** both in oxia and in hypoxia



Uptake hypoxia/oxia at 60 min (n = 3)
2.2 ± 0.3

Value is shown as mean ± S.D.
(*) Not different (p <0.05).

by low blood and liver activity (Table 1), rapid depuration and quantitative excretion through the urinary tract (58% of injected activity in urine at 2 h after administration) and intestines (30% at the same time). Activities in other organs and tissues were negligible. Uptake in tumour was low but high and statistical significant (p <0.05) tumour/muscle ratios were achieved at all time points (Table 3). Another favourable characteristic was fast clearance from blood that lead to tumour/blood ratios that increased significantly with time (Table 3). On the other hand, biodistribu-

Table 3
Tumour uptakes for developed complexes on C57 mice bearing Lewis carcinoma

	% Injected dose/g		
	30 min	60 min	120 min
Tc-Ntm-1			
Blood	4.83 ± 0.43	5.50 ± 1.10	3.11 ± 0.74
Muscle	0.96 ± 0.15	0.82 ± 0.02	0.69 ± 0.10
Tumour	1.10 ± 0.20	0.85 ± 0.22	1.33 ± 0.32
Ratios			
Tumour/Blood	0.23 ± 0.05	0.16 ± 0.07	0.44 ± 0.15
Tumour/Muscle	1.17 ± 0.27	1.04 ± 0.30	1.93 ± 0.34
Tc-Ntm-2			
Blood	1.20 ± 0.60	0.29 ± 0.04	0.15 ± 0.04
Muscle	0.19 ± 0.05	0.09 ± 0.03	0.08 ± 0.02
Tumour	0.63 ± 0.06(*)	0.22 ± 0.07(*)	0.28 ± 0.07(*)
Ratios			
Tumour/Blood	0.50 ± 0.10	0.76 ± 0.10	1.86 ± 0.09
Tumour/Muscle	3.30 ± 0.10	2.40 ± 0.11	3.11 ± 0.09

(*) Significant differences between tumour and muscle uptake. p <0.05 (Student's test).

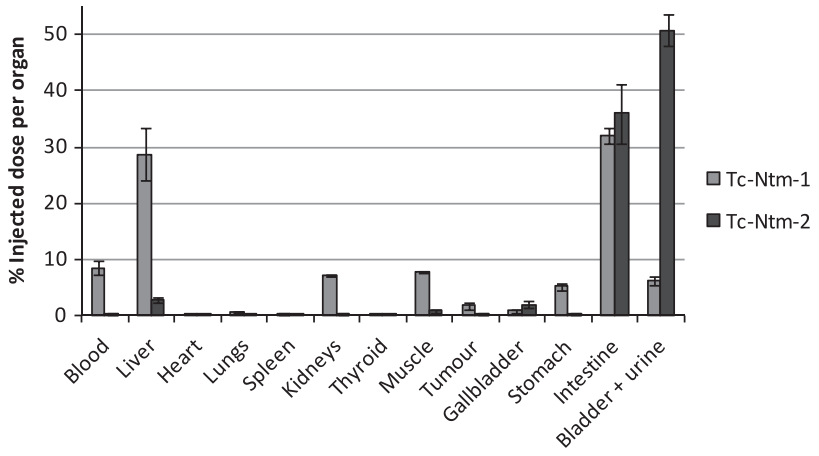
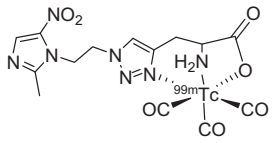


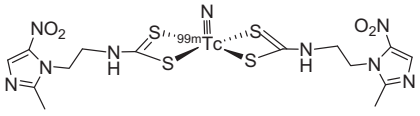
Figure 6. Biodistribution pattern of **Tc-Ntm-1** and **Tc-Ntm-2** complexes on C57 mice bearing Lewis carcinoma at 60 min post-injection.



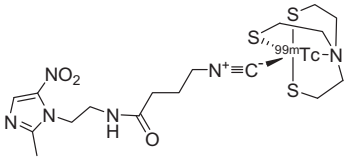
Tc-Ntm-2

Complex	Tc oxidation state	t/b[a] ratio	t/m[b] ratio
Tc-Ntm-2	I	1.86±0.09	3.11±0.09
Tc-dtc-1[c]	V	0.65±0.21	1.80±0.17
Tc-iso-1[d]	III	1.0±0.4	2.4±0.5

[a] t/b: tumour/blood ratio at 2 h after injection; [b] t/m: tumour/muscle ratio at 2 h after injection; [c] From reference [20]; [d] From reference [21]



Tc-dtc-1



Tc-iso-1

Figure 7. Comparison of tumour uptakes, expressed as tumour/blood and tumour/muscle ratios, of different bioreducible-pharmacophore containing Tc-complexes developed by our group.

tion of **Tc-Ntm-1** complex was less favourable, as expected from its high protein binding. Activity in blood and liver was high and uptake in kidneys (Table 1) and muscle was also higher compared with **Tc-Ntm-2** complex. Excretion occurs through hepatobiliary and renal systems. Although tumour uptake was considerably higher (Table 3) than the observed for **Tc-Ntm-2**, tumour/muscle ratios are lower and statistical analysis revealed that differences in uptake between tumour and muscle were not significant (p <0.05). Besides, tumour/blood ratios were unfavourable for all time points because of the high level of activity retained in blood. Figure 6 shows a comparison between biodistribution patterns of both complexes at 60 min post-injection and clearly demonstrates that **Tc-Ntm-1** and **Tc-Ntm-2** have completely different biological behaviour. While the complex formed by tridentate coordination of **Ntm-2** to technetium has a low uptake in all organs and tissues with rapid depuration, the complex formed by bidentate coordination of **Ntm-1** shows low depuration from blood, liver, kidneys and muscle, leading to an unfavourable biodistribution pattern for a potential radiopharmaceutical. This behaviour correlates with the high protein binding of the **Tc-Ntm-1** (Table 1) and could be associated with the bidentate coordination. Studies by Schibli et. al. on the influence of ligand denticity in ^{99m}Tc(I) tricarbonyl complexes indicate that clearance of bidentate coordinated complexes from blood is slower and activity is generally retained in all organs, especially in liver and kidneys.[31,35] A lower thermodynamic stability of this type of complexes is suggested as the reason for the high retention in vivo, where adequate functional groups of proteins can substitute the labile-bound water molecule in the coordination sphere.[36,37] Comparison between **Tc-Ntm-1** and **Tc-Ntm-2** support this idea. In fact, the addition of a third electron donor group to obtain a tridentate ligand had a great impact not only in vitro but also in vivo behaviour of the final complex.

3. Conclusion

This work presents the development of two novel 5-nitroimidazole derivatives and the corresponding ^{99m}Tc(I)-tricarbonyl complexes as potential radiopharmaceuticals for hypoxia imaging. Derivatization of the pharmacophore unit was made in order to introduce a bidentate or a tridentate chelator unit, obtaining final ligands **Ntm-1** and **Ntm-2**, respectively. Final ^{99m}Tc(I)-tricarbonyl complexes, **Tc-Ntm-1** and **Tc-Ntm-2**, were characterized in vitro and in vivo and results showed clearly that the complex with the tridentate ligand, **Tc-Ntm-2**, had a good and fast clearance from all organs and tissues, which correlates with a low protein binding. On the other hand, the bidentate-coordinated complex, **Tc-Ntm-1**, showed a high protein binding and revealed high retention of activity in blood, liver, kidneys, and muscle. Although several promising

^{99m}Tc(I)-tricarbonyl complexes have been reported using bidentate ligands,[5–7] literature also describes that in some cases a bidentate ligand could lead to low thermodynamic stability of the coordination compounds due to the substitution of the labile-bound water molecule in the coordination sphere. In our case, conclusion is that denticity of the ligand has a deep impact on physicochemical and biological behaviour of the final complex and tridentate chelating systems are preferable for pharmacophore derivatization.

Previously, we have studied the Tc(V)-nitride,[20] and Tc(III)-[4+1][21] cores as centres for coordination to the 5-nitroimidazolyl-pharmacophore where the dithiocarbamate and isonitrile containing complexes **Tc-dtc-1** and **Tc-iso-1** (Fig. 7), respectively, showed the best differential tumoural uptake in each group of compounds. Clearly, the Tc(I)-tricarbonyl complex, **Tc-Ntm-2**, displayed approximately 3-times and 1.5-times higher tumour/blood and tumour/muscle ratios, respectively, than the Tc(V)-nitride complex, **Tc-dtc-1**, at 2 h post-injection (Fig. 7). On the other hand, at the same experimental time the complex with Tc in the intermediate oxidation state, that is III, **Tc-iso-1**, displayed lower tumour/blood and tumour/muscle ratios than **Tc-Ntm-2** (Fig. 7). In conclusion, the new 5-nitroimidazolyl bioreducible pharmacophore containing Tc-complex **Tc-Ntm-2**, described herein, appears as the best radiopharmaceutical for hypoxic tumour diagnosis developed by our group.

4. Experimental

The precursors *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ and *fac*-[NEt$_4$]$_2$[Re(CO)$_3$Br$_3$] were prepared according to literature.[1,28–30] Distilled and dried solvents were employed for synthesis. All laboratory chemicals were reagent grade and were used without further purification. Solvents for chromatographic analysis were HPLC grade. Thin-layer chromatography was carried out on alumina or silica gel plates (Merck 60 F_{254}). Column chromatography was carried out on silica gel (Merck, 60–230 mesh) or neutral alumina (Merck 70–230 mesh).

[^{99m}Tc]NaTcO$_4$ was obtained from a commercial generator (Tecno Nuclear S.A., Argentine). The NMR spectra were recorded on a Bruker DRX-400 spectrometer in the indicated deuterated solvent. Chemical shifts are reported as δ values (parts per million) with respect to TMS. Coupling constants are reported in Hertz (Hz). The multiplicity is defined by s (singlet), t (triplet), or m (multiplet). The mass spectra (MS) were conducted in 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. Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and were uncorrected. Microanalyses were performed on a Fisons EA 1108 CHNS-O instrument and were within ±0.4% of the calculated compositions. HPLC analysis was developed on a LC-10 AS Shimadzu Liquid Chromatography System using a reverse phase column Phenomenex Luna 5m, C18 column (4.6 × 150 mm). Elution was performed with a binary gradient system at 1.0 mL/min flow rate using triehtylamine-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) 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 or in a scintillation counter, 3″ × 3″ NaI (Tl) crystal detector associated to an ORTEC monochanel analyzer.

4.1. Synthesis of [2-(2-methyl-5-nitro-1*H*-imidazol-1-yl)] ethylazide, Azd. Mtz

(0.5 g, 3.57 mmoles) and triphenylphosphine (1.4 g, 5.34 mmoles) in dried THF (25 mL) were stirred under ice bath. Azidotrimethylsilane (1.40 mL, 5.38 mmoles) was added to the solution and then diisopropyl azodicarboxylate (DIAD, 1.1 g, 5.4 mmoles) in dried THF (10 mL) was added maintaining the reaction mixture at 0 °C. After 12 h at room temperature the solvent was evaporated *in vacuo* and the residue was purified by column chromatography (SiO_2, CH_2Cl_2:MeOH, 95:5). Beige solid. ^{1}H NMR ($CDCl_3$) δ (ppm): 2.56 (s, 3H), 3.79 (t, 2H, J = 5.4), 4.46 (t, 2H, J = 5.4), 8.00 (s, 1H). ^{13}C NMR ($CDCl_3$) δ (ppm): 14.9, 46.0, 51.4, 129.0, 133.9, 151.7. IR (KBr) ν (cm^{-1}): 1386, 1528, 2064–2128. MS (EI, 70 eV) *m/z* (%): 196 ($M^{+\cdot}$), 151 ([M–NO_2–OH]$^+$), 122 ([M–NO_2–OH–NO]$^+$).

4.2. Synthesis of *N*-methyl-1-[1-(2-(2-methyl-5-nitro-1*H*-imidazole-1-yl)ethyl)-1*H*-1,2,3-triazole-4-yl]methylamine, Ntm-1

A solution of sodium ascorbate (0.30 g, 1.49 mmoles) and copper (II) sulphate (0.47 g, 2.97 mmoles) in distilled H_2O (15 mL) was added to a solution of **Azd** (1.50 g, 7.42 mmoles) and *N*-methylpropargylamine (0.63 mL, 7.42 mmoles) in *t*-butanol (15 mL) under stirring at room temperature. The mixture was stirred for 24 h and more sodium ascorbate (0.15 g, 0.75 mmoles) was added. After extra 24 h at room temperature the *t*-butanol was evaporated in vacuo, the residue was treated with aqueous solution of NaOH (33%, 60 mL) and the aqueous phase was extracted with EtOAc (4 × 60 mL). The organic phase was dried with Na_2SO_4 anhydrous and evaporated in vacuo. The brown-beige solid corresponds to the desired product (47%); mp: 86.5–88.9 °C. ^{1}H NMR (CD_3OD:D_2O) δ (ppm): 2.00 (s, 3H), 2.37 (s, 3H), 3.80 (s, 2H), 4.87 (t, J = 3.0, 2H), 4.91 (m, 2H), 7.79 (s, 1H), 7.98 (s, 1H). ^{13}C NMR (CD_3OD:D_2O) δ (ppm): 11.8, 33.9, 44.9, 46.1, 49.1, 123.9, 131.6, 138.7, 145.7, 151.0. IR (KBr) ν (cm^{-1}): 1366, 1527, 3402. MS (EI, 70 eV) *m/z* (%): 264 ([M–H]$^+$, 20), 248 ([M–OH]$^+$, 22), 218 ([M–HNO_2]$^+$, 9), 44 ([CH_3NHCH_2]$^+$, 100).

4.3. Synthesis of 2-amine-3-{1-[2-(2-methyl-5-nitro-1*H*-imidazole-1-yl)ethyl]-1*H*-triazole-4-yl}propanoic acid, Ntm-2

A solution of **Azd** (0.26 g, 1.32 mmoles), D,L-propargylglycine (0.15 mL, 1.32 mmoles), sodium ascorbate (0.53 g, 2.6 mmoles) and copper (II) sulphate (0.85 g, 5.3 mmoles) in *t*-butanol:distilled H_2O (60:15 mL) was stirring at room temperature for 24 h. Then cationic exchange resin (Chelex 100-Biorad, 0.5 g) was added and the mixture was stirred until disappearance of the blue colour (2 h). The resin was filtered and the volume of the solution was reduced in vacuo. Then ethyl ether:acetone (1:1, 10 mL) was added and the mixture was cooled at 4 °C for 12 h. The generated solid, corresponding to the desired product, was filtered and washed with cold ethanol. White solid (57%); mp: 134.0–137.0 °C. ^{1}H NMR (D_2O) δ (ppm): 1.83 (s, 3H), 3.16 (s, 2H), 3.89 (s, H), 4.77 (br s, 4H), 7.65 (s, H), 8.01 (s, 1H). ^{13}C NMR (D_2O) δ (ppm): 12.5, 26.1, 33.9, 46.3, 49.45, 62.2, 125.2, 129.0, 146.6, 151.7, 177.1. IR (KBr) ν (cm^{-1}): 1366, 1527, 1603, 3149. MS (EI, 70 eV) *m/z* (%): 309 ($M^{+\cdot}$, 3), 278 ([M–HNO]$^+$, 15), 265 (18).

4.4. ^{99m}Tc-labelling

4.4.1. Synthesis of ^{99m}Tc-Ntm-1 complex

Substitution of *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ was achieved by incubation of **Ntm-1** ligand (3 mg) dissolved in MeOH (100 μL) with the neutralized precursor solution (1.2 mL) at 70 °C for 30 min. Radiochemical purity was assessed by HPLC using the HPLC system

described previously. t_R = 15 and 16 min. Yield >90% (mixture of two species).

4.4.2. Synthesis of ^{99m}Tc-Ntm-2 complex

Substitution of *fac*-[^{99m}Tc(CO)$_3$(OH$_2$)$_3$]$^+$ was achieved by incubation of **Ntm-2** ligand (4 mg) dissolved in MeOH (100 μL) with the neutralized precursor solution (1.5 mL) at 70 °C for 30 min. Radiochemical purity was assessed by HPLC using the HPLC system described previously. t_R = 18 min. Yield >90%.

4.4.3. One pot synthesis of ^{99m}Tc-Ntm-2 complex

A solution of propargylglycine in H_2O (50μL, 1.131 mg/mL) was mixed with a solution of **Azd** in MeOH (65 μL, 1.286 mg/mL). A cooper acetate solution in H_2O (7.5 μL, 1.996 mg/mL) and a solution of sodium ascorbate in H_2O (15 μL, 1.980 mg/mL) were added and the mixture was heated for 1 h at 75 °C. After that the labelling process was done by addition of the neutralized precursor complex (100–200 μL) and heating for 1 h at 100 °C. Radiochemical purity is assessed by HPLC using the HPLC system described previously. t_R = 18 min. Yield >90%.

4.5. Preparation of rhenium analogues

4.5.1. Synthesis of Re(I) tricarbonyl complex with Ntm-1 ligand, Re-Ntm-1

A solution of **Ntm-1** ligand (64 nmoles) in MeOH:H_2O (1:1, 10 mL) was added to a solution of *fac*-[NEt$_4$]$_2$[Re(CO)$_3$Br$_3$] precursor (64 nmoles) in methanol:H_2O (1:1, 10 mL). The mixture was stirred at 70 °C for 3 h and the reaction progress was monitored by the HPLC system described previously, coupled to a UV detector. The solid product obtained was filtrated, washed, dried and analyzed by HPLC and elemental microanalysis. t_R (HPLC) = 16 min. Calculated analysis for $C_{13}H_{18}BrN_7O_5Re$: C: 25.3; H: 2.9; N, 15.9. Found: C: 26.3; H: 3.1; N: 15.5.

4.5.2. Synthesis of Re(I) tricarbonyl complex with Ntm-2 ligand, Re-Ntm-2

A solution of **Ntm-2** ligand (64 nmoles) in methanol:H_2O (1:1, 10 mL) was added to a solution of *fac*-[NEt$_4$]$_2$[Re(CO)$_3$Br$_3$] precursor (64 nmoles) in methanol:H_2O (1:1, 10 mL). The mixture was stirred at 70 °C for 3 h and the reaction progress was monitored by the HPLC system described previously, coupled to a UV detector. The desired product was purified by HPLC under conditions mentioned previously and analyzed by mass spectrometry. t_R (HPLC) = 18 min. MS (ESI) *m/z*: 971 ([2M–2 CO–2 CO_2–2 H–NO_2]$^+$).

4.6. Physicochemical studies

4.6.1. Stability

^{99m}Tc-complexes (**Tc-Ntm-1** or **Tc-Ntm-2**), in the labelling milieu, were incubated at room temperature and the radiochemical purity checked by HPLC analysis as described previously during 30 min for up to 4 h after labelling.

^{99m}Tc-complexes (**Tc-Ntm-1** or **Tc-Ntm-2**, 200 μL), in human plasma (1000 μL), were incubated at 37 °C for up to 120 min. After different incubation times (30, 60 and 120 min), samples (200 μL) were precipitated with ethanol (200 μL), centrifuged (12000 rpm, 5 min) and analyzed by HPLC as described previously.

4.6.2. 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 (**Tc-Ntm-1** or **Tc-Ntm-2**) 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*.

4.6.3. Protein binding

^{99m}Tc-complexes (**Tc-Ntm-1** or **Tc-Ntm-2**, 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 (GE Healthare), which have 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(Tl)-scintillation counter. Protein bound tracer was calculated as the percentage of activity eluted from the column.

4.7. 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 (Sigma) and 100 μg/mL streptomicine (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) 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 was measured in a solid scintillation counter and results were expressed as the ratio between the percentages of activity taken up by cells incubated in nitrogen (hypoxia) and incubated in normal conditions (oxia).

4.8. Biodistribution studies in animals bearing induced tumours

All animal studies were approved by the Ethics Committee of the Faculty of Chemistry, Universidad de la República (Uruguay). A culture of 3LL Lewis murine lung carcinoma cells was expanded and treated with trypsine previous to inoculation. A cell suspension in PBS containing 3×10^6 cells was prepared and injected subcutaneously in the right limb of C57B16 mice (8–10 weeks old). 20–30 days later the animals developed palpable tumour nodules ($1.5 \times 0.5 \times 0.5$ cm, approximately) and were used for biodistribution.

Three animals per group and per intervals were injected via a lateral vain in the tail with ^{99m}Tc-complexes (**Tc-Ntm-1** or **Tc-Ntm-2**, 0.1 mL, 0.037–0.37 MBq). At different intervals, 30, 60, and 120 min, 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 %injected dose/g of tissue ($x \pm \sigma_{n-1}$, $n = 3$).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.05.010.

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