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Synthesis and biological characterisation of novel dithiocarbamate containing 5-nitroimidazole ^{99m}Tc-complexes as potential agents for targeting hypoxia

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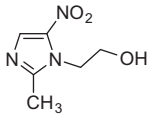
ABSTRACT

With the aim to develop new potential ^{99m}Tc-radiopharmaceuticals for imaging hypoxia based on the formation of Tc-nitrido complexes, two novel dithiocarbamate containing metronidazole derivatives (L1 and L2) have been prepared and characterised. The synthesis of L1 and L2 was achieved in excellent yield and high purity. Labelling with ^{99m}Tc was successfully performed using a low ligand concentration (approximately 2–3 mg) and the desired products were obtained with high radiochemical purity (>90%). Lipophilicity, plasma protein binding, and biodistribution in normal- and tumour-bearing-CD1 mice studies were performed to asses the potentiality for nuclear medicine oncology. According to the physicochemical and biological behaviour both in healthy animals and in animals bearing solid tumours complex **dtcTc1** could be considered as a starting point for the development of new radiopharmaceuticals for imaging hypoxia.

Hypoxic regions in tumours are formed when tumour growth exceeds the capacity of accompanying blood vasculature to deliver adequate quantities of oxygen to the growing mass of cells.[1] The resistance of hypoxic cells to conventional radiotherapy and chemotherapy[2,3] is a major obstacle for the complete remission of tumours. Due to the inherent drawbacks of invasive techniques to detect hypoxia[4] nuclear medicine imaging, based on the administration of small amounts of radioactive compounds, the so-called radiopharmaceuticals, followed by the detection of the radiation escaping from the body, is considered an interesting alternative. Nitroimidazoles are reduced selectively under low oxygen pressure and consequently accumulate into the hypoxic regions of tumours, providing the attractive possibility of employing them as ligands for the preparation of potential radiopharmaceuticals targeting hypoxic tissues.[5–7] The 5-nitroimidazole metronidazole (Mtz, Fig. 1), which radiosensitizes hypoxic tumour cells in vivo, has been selected due to its affinity for hypoxic tumours as starting material for the preparation of ^{99m}Tc radiopharmaceuticals.[8–14]

^{99m}Tc is the radionuclide of choice in diagnostic nuclear medicine due to its ideal nuclear properties for imaging ($t_{1/2}$ = 6 h, E_γ = 140 keV). In technetium radiopharmaceuticals the metal is bound to the bioactive group by means of a coordination compound using the so-called pendant approach,[15] which literally means to combine the pharmacophore with appropriate chelating groups to bind the metal, separating them through a spacer to prevent interference with the biological activity. Selection of the chelating groups is essential to achieve adequate stability and pharmacokinetics of the resulting compound. A variety of chelating groups and co-ligands, as well as Tc oxidation states, have been investigated for the radiolabelling of biologically active compounds. The Tc-nitrido core, initially developed by Baldas and Bonnyman,[16] is an auspicious tool for the design of novel radiopharmaceuticals due to their intrinsic structural robustness.[17] The co-ordination sphere of these complexes is either build up by two molecules of bidentate σ-donor ligands or a combination of 'pseudotridentate' σ-donors π-acceptors diphosphinoamines (PNP) and bidentate σ-donor ligands (X–Y). Various bidentate σ-donor ligands can be used but the bis(dithiocarbamate)^{99m}Tc-nitrido complexes, which are square pyramidal, neutral and highly lipophilic compounds are specially useful for the preparation of radiopharmaceuticals with adequate cell penetration. A remarkable example is ^{99m}TcN-NOEt, a symmetric ^{99m}Tc-nitrido complex with outstanding properties for myocardial imaging (see Fig. 2).[18]



Mtz
(metronizadole)

Figure 1. Chemical structure of the radiosensitizer metronidazole.

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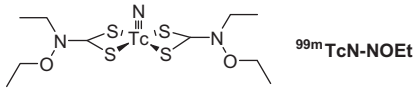
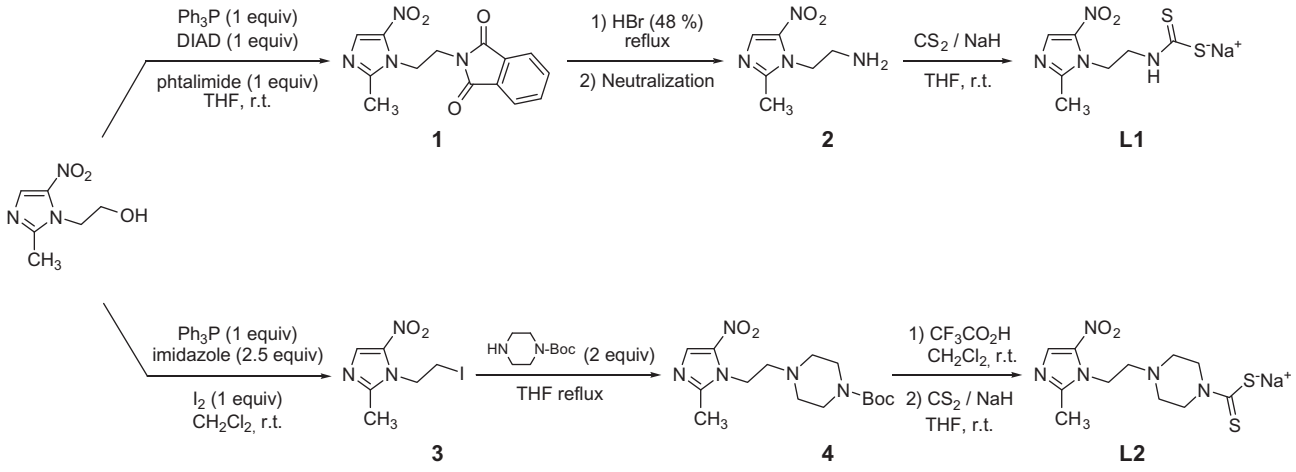


Figure 2. Example of bis(dithiocarbamate)^{99m}Tc≡N complex.

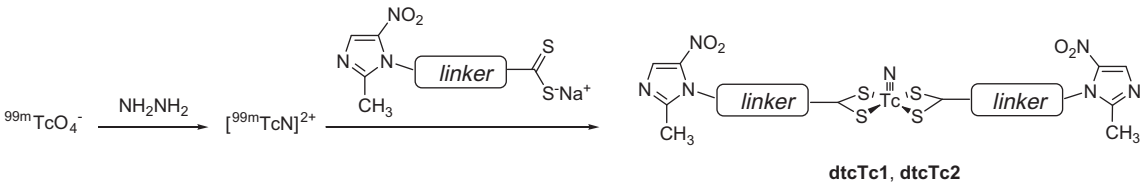
With the aim to develop new potential ^{99m}Tc-radiopharmaceuticals for imaging hypoxia, we have selected the 5-nitroimidazol-1-yl moiety as the bioactive group and the dithiocarbamate group as chelator for the technetium. The 5-nitroimidazole Mtz (Fig 1), with a pendant $-CH_2CH_2OH$ linker, acts as a suitable substrate for facile derivatisation to a dithiocarbamate containing ligand. Herein, we report the synthesis of two new dithiocarbamate containing 5-nitroimidazoles, **L1** and **L2**, and its ^{99m}Tc-complexes bearing the ^{99m}Tc≡N core, **dtcTc1** and **dtcTc2**. To the best of our knowledge, this report constitutes the first of its kind in using the dithiocarbamate as donor moiety in combination with a nitroimidazolic pharmacophore for the preparation of ^{99m}Tc complexes for targeting tumour hypoxia. A preliminary evaluation of the main physicochemical and biological properties is also presented in order to asses the potentiality of our approach.

The syntheses of the ligands **L1** and **L2** (Scheme 1) were achieved by two synthetic routes. In the first route Mtz was transformed into its amino-analogue **2** using a previously described Mitsunobu methodology[19] and then transformed into the corresponding dithiocarbamate **L1** by reaction with CS_2 in presence of NaH in aprotic milieu. This procedure affords the desired product in excellent yield and high purity by evaporation of the organic solvent. However, when the process was assayed in aqueous milieu (CS_2/aqueous NaOH (50%)) the isolation and purification of **L1** was unsuccessful. In the second route, ligand **L2** was prepared in a four-step procedure from Mtz with good global yield. In the first step Mtz was converted, via phosphonium ion (Ph_3P/imidazole/I_2),[20] into the corresponding iodide derivative, **3**, in excellent yield. Then **3** was transformed, via nucleophilic substitution, into the protected amine **4** which was deprotected in presence of CF_3CO_2H and transformed into the desired dithiocarbamate, **L2**, in the same condition that it was used for **L1**. The intermediates, **1–4**,[21] and products, **L1** and **L2**, were characterised by NMR (^{1}H and ^{13}C) and IR spectroscopies and MS spectrometry.[22] The presence of the $-N-CS_2$ moiety was clearly evidenced in the ^{13}C NMR and IR of **L1** and **L2** by a signal near to 200 ppm and the signals near to 1120 and 1000 cm^{-1}, corresponding to the thiocarbonyl carbon and the C=S and –N–C from the dithiocarbamate moiety, respectively. The ligands **L1** and **L2** were used as such without further purification. Preparation of the ^{99m}Tc nitrido complexes was accomplished by a two step procedure as follows: first, the $[^{99m}Tc{\equiv}N]^{2+}$ intermediate was obtained using an optimised protocol (Scheme 2). One millilitre of freshly eluted $^{99m}Tc^{VII}O_4^-$ (37–50 MBq) was added to a commercial kit (Cis-Bio int.) containing succinic acid dihydrazide as nitrido donor (5.0 mg), Na_2EDTA (5.0 mg), and $SnCl_2{\cdot}H_2O$ (0.1 mg) as reducing agent; the mixture was vortexed for one minute and kept at room temperature for 5 min. Formation of the $[^{99m}Tc{\equiv}N]^{2+}$ intermediate species was controlled by paper chromatography using acetone as solvent (R_f $[^{99m}Tc{\equiv}N]^{2+}$-intermediate = 0, R_f $^{99m}TcO_4^- = 1$); a minimum radiochemical purity of 90% was required to proceed to the second step.

Substitution was achieved by addition of ligands **L1** or **L2** to the precursor. The following reaction parameters: ligand concentration (studied: 0.0035–0.030 mmol/0.1 mL), temperature (studied: 50–90 °C), and time (studied: 20–40 min) were assayed to obtain maximum complexation yield. The desired complexes **dtcTc1** and **dtcTc2** were finally obtained by substitution of **L1** (0.011 mmol/0.1 mL) or **L2** (0.012 mmol/0.1 mL for **L2**) on the precursor followed by incubation at 75 °C during 30 min. The radiochemical purity for both complexes was determined by RP-HPLC (Fig. S1, Supplementary data, Table 1) and found to be above 90%.



Scheme 1. Synthesis of metronidazole dithiocarbamates.



Scheme 2. Synthesis of ^{99m}Tc-complexes.

Table 1
Physicochemical parameters for ^{99m}Tc-complexes

Complex	t_R[a] (min)	Stability in plasma[b] (%)	PBPP[c]	log P[d]
dtcTc1	7.5	>90.0	31 ± 3%	0.63 ± 0.04
dtcTc2	8.5	>87.0	75 ± 4%	0.7 ± 0.1

[a] HPLC conditions: reverse-phase Waters LC-18 column, 250 mm × 4.6 mm ID, particle size 5 μM, (Waters Corporation, Massachusetts, USA); mobile phase A: TFA/water, 0.1:99.9 (v/v), mobile phase B: TFA/acetonitrile, 0.1:99.9 (v/v), 0–3 min: 100% A (0% B), 3–5 min: 100% A→0% A (0% B→100% B), 5–20% A (100% B); flow rate 1.0 mL min^{-1}; γ-detection.
[b] Radioactivity after 4.0 h of incubation with human plasma at 37 °C.
[c] PBPP: percentage of binding to plasmatic proteins.
[d] log $P = S_{octanol}/S_{buffer\ (pH\ 7.4)}$.

Stability of **dtcTc1** and **dtcTc2** complexes in human plasma at 37 °C was studied using RP-HPLC (Table 1). Both complexes exhibit significant stability since the radiochemical purity remained near to 90% for at least 4 h. Protein binding (PBPP) of the studied complexes, **dtcTc1** and **dtcTc2**, was determined using blood plasma by size exclusion chromatography (Table 1). Low protein binding values are associated with fast blood clearance. High values, on the other hand, indicate normally poor in vivo stability, high blood activity and high hepatobiliary excretion. Lipophilicity is also a relevant indication of pharmacokinetic behaviour and determines the excretion pattern of the radiopharmaceutical. It was determined as the partition coefficient between 1-octanol and saline (Table 1). According to PBPP **dtcTc1** complex could probably have a more favourable biodistribution pattern than **dtcTc2**.

The biodistribution studies were carried out using healthy CD1 mice between 30 min and 6 hours post-injection for **dtcTc1** and 120 and 240 min for **dtcTc2** complex. Biological behaviour of **dtcTc1** was characterised by high initial blood and muscle activity, rapid depuration and quantitative excretion through the urinary tract (70% of injected activity in urine at 6 h after administration, Fig. S2a). Activity from other organs and tissues was also washed out with time. On the other hand, biodistribution of complex **dtcTc2** was less favourable as expected from high protein binding. Activity uptake in liver was very high, above 50% of injected dose and urinary excretion very low (9% at 4 h post-injection) (Fig. S2b).

Table 2
Tumour/blood and tumour/muscle ratios of ^{99m}Tc-complex **dtcTc1** at various time points

Time post-injection (min)	Tumour/blood	Tumour/muscle
30	0.28 ± 0.03	1.63 ± 0.26
60	0.30 ± 0.17	1.29 ± 0.10
120	0.65 ± 0.21	1.80 ± 0.17
240	1.15 ± 0.32	2.43 ± 0.75

Table 3
Tumour/blood and tumour/muscle ratios of ^{99m}Tc-complex **dtcTc2** at 240 min post-injection

Tumour/blood	Tumour/muscle
0.28 ± 0.07	1.50 ± 0.37

The results of the biodistribution of complexes **dtcTc1** and **dtcTc2**, in C57 mice bearing induced Lewis carcinoma, are represented in Fig. 3. Overall in vivo behaviour was very similar to the observed in healthy animals. The tumour retains about 1.0% of injected dose per gram for **dtcTc1** (Fig. S3) and this remains nearly constant over the period of study up to 240 min post-injection. The striking observation in the present study was the desirable feature of steady retention of the tumour activity throughout the period of study. Another favourable characteristic was fast clearance from blood and soft tissues that led to tumour/blood and tumour/muscle ratios that increased significantly with time (Table 2) reaching values of 1.15 and 2.43, respectively, at 240 min post-injection. Student's test was applied to asses the significance of the difference in uptake between tumour and muscle and results demonstrate that uptake in tumour of complex **dtcTc1** is significantly higher in comparison to muscle ($p = 0.05$) at 120 and 240 min post-injection.

On the other hand, the results of biodistribution for **dtcTc2**, in C57 mice bearing Lewis carcinoma, showed a very different biological behaviour. Uptake in tumour was significantly lower than the observed for **dtcTc1**, that is, 0.40 ± 0.10% of injected dose per gram of tumour at 4 h after administration. As shown in Fig. 3, the

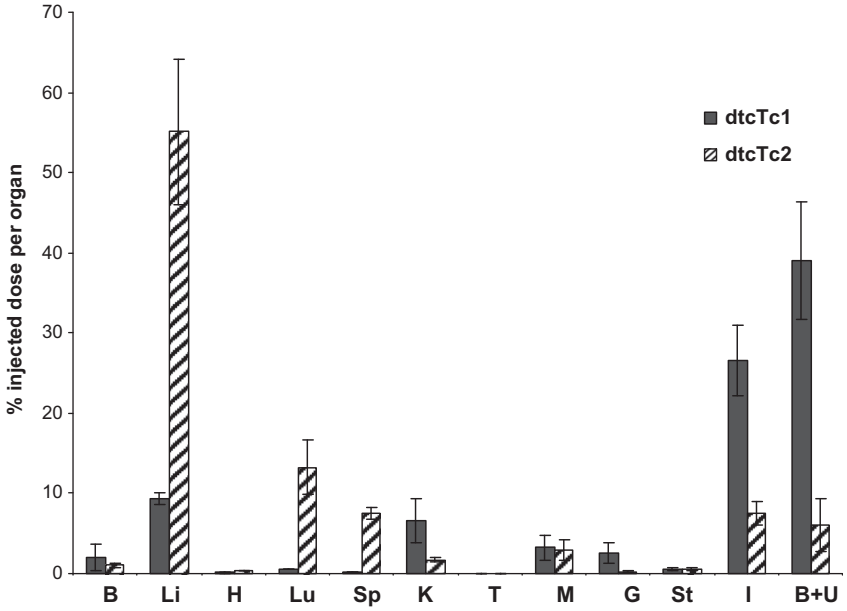


Figure 3. Biodistribution pattern of ^{99m}TcN–metronidazole complexes **dtcTc1** and **dtcTc2** on C57 mice bearing Lewis carcinoma, $n = 3$, at 240 min post-injection. B: blood; Li: liver; H: heart; Lu: lung; Sp: spleen; K: kidney; T: thyroid; M: muscle; G: gallbladder; St: stomach; I: intestine; B + U: bladder + urine.

activity at 240 min was mainly uptaken by liver and intestinal tract indicating that **dtcTc2** is excreted predominantly through the hepatobiliary system, without tumour retention. A minor portion of the injected dose is also excreted renally, as seen from the activity associated with the kidneys, urine, and bladder. Although tumour/muscle ratio resulted a little favourable at 240 min post-injection (Table 3) statistical analysis showed that this difference in uptake was not significant ($p = 0.05$).

Clearly, the inclusion in complex **dtcTc2** of a piperazine linker, between the 5-nitroimidazole pharmacophore and the dithiocarbamate chelating group, altered significantly the physicochemical properties and biological behaviour in comparison to **dtcTc1** (Table 1). The differential tumour and -organs uptake could be partially due to a higher **dtcTc2**-lipophilicity, which promotes hepatic uptake but the main factor seems to be protein binding of **dtcTc2** to plasmatic proteins that could endorse high hepatic uptake, preferential hepatobiliary excretion and lower tumour accumulation.

To conclude, the ligands **L1** and **L2** were designed combining the 5-nitroimidazole bioreductive moiety with the chelator dithiocarbamate. These were successfully synthesised and utilised in the preparation of two novel ^{99m}Tc complexes, **dtcTc1** and **dtcTc2**, with potentiality for hypoxia imaging. ^{99m}Tc complexes were obtained with high radiochemical purity (>90%), but only **dtcTc1** showed adequate chemical and biological stability together with low protein binding and intermediate lipophilicity. Overall biodistribution in normal animals was very favourable due to fast clearance from blood and soft tissues via kidney. Finally, according to the biological behaviour of complex **dtcTc1** in animals bearing solid tumours, this system could be considered as a starting point for the development of new radiopharmaceuticals for imaging hypoxia. We are currently investigating other biological aspects and other labelling methods in the search for compounds with improved properties.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.130.

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22. **L1**—Oil, that solidifies in vacuum. ^{1}H NMR (DMSO-d_6/D_2O, 9:1) δ (ppm): 2.57 (s, 3H, CH_3), 3.80 (t, 2H, J = 5.4 Hz, CH_2), 4.46 (t, 2H, J = 5.4 Hz, CH_2), 8.00 (s, 1H, CH); ^{13}C NMR (DMSO-d_6/D_2O, 9:1) δ (ppm): 14.9 (CH_3), 45.9 (CH_2), 51.4 (CH_2), 130.0 (CH), 133.9 (CNO_2), 151.7 (C), 190.4 (CS_2); IR (KBr) ν (cm^{-1}): 1001 (NH–CS_2), 1118 (–(C═S)–NH), 1373 (CH), 1374 (NO_2), 1576 (NO_2), 3520 (NH); MS (EI, 70 eV), m/z: 246 ($M^{+\cdot}$+H–Na); $C_7H_9N_4NaO_2S_2$. **L2**—Oil, that solidifies in vacuum. ^{1}H NMR (CD_3OD) δ (ppm): 2.55 (br t, 4H, J = 6.2 Hz, CH_2–N), 2.58 (s, 3H, CH_3), 2.73(br t, 4H, J = 6.2 Hz, N–CH_2), 3.33 (t, 2H, J = 6.0 Hz, CH_2), 4.54 (t, 2H, J = 6.0 Hz, CH_2), 7.93 (s, 1H, CH); ^{13}C (MeOD) δ (ppm): 15.3 (CH_3), 39.9 (CH_2), 51.4 (CH_2), 54.3 (CH_2–N), 58.5 (N–CH_2), 133.0 (C), 142.9 (CNO_2), 158.7 (C), 206.3 (CS_2); IR (KBr) ν (cm^{-1}): 1005 (NH–CS_2), 1124 (–(C═S)–NH), 1370 (CH), 1380 (NO_2), 1579 (NO_2), 3528 (NH); MS (EI, 70 eV), m/z: 315 ($M^{+\cdot}$+H–Na); $C_{11}H_{16}N_5NaO_2S_2$.