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Research Article

Synthesis, radio-LC–MS analysis and biodistribution in mice of ^{99m}Tc–NIM–BAT

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Summary

S,*S*′-*bis*-trityl-*N*-BOC-1,2-ethylenedicysteamine (*S*,*S*′-*bis*-trityl-*N*-BOC–BAT) was conjugated to 2-nitroimidazole (NIM) through a propylene spacer in order to provide a precursor for a potential technetium-99 m labelled hypoxia tracer. For labelling with technetium-99 m, a two-step one-pot procedure was developed consisting of deprotection of the ligand by heating in mild acidic conditions and subsequent exchange labelling in the presence of $SnCl_2$, tartrate and $^{99m}TcO_4^-$.

The labelling reaction mixture was analyzed using electrospray radio-LC–MS and the observed mass spectrum corresponding to the main radiometric peak was in accordance with the predicted structure of oxo–Tc(V)–NIM–BAT.

^{99m}Tc–NIM–BAT was purified using RP–HPLC and its biodistribution was evaluated in normal mice at 10 min and 4 h p.i. ^{99m}Tc–NIM–BAT was cleared from plasma mainly by hepatobiliary excretion.

Key Words: hypoxia; technetium-99m; *bis*-amino *bis*-thiol; bifunctional chelating agent

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Introduction

Accurate measurement of tumor hypoxia can be clinically useful to predict sensitivity to radiation therapy and to evaluate interventions that aim to increase tumor oxygenation or to identify tumors that are amenable to hypoxia-directed gene therapy or hypoxia-selective therapy, e.g.with tirapazamine.[1]

Several hypoxia markers contain a nitroimidazole moiety as the reactive chemical species. Nitroimidazoles are reduced intracellularly in all cells, but in the absence of an adequate supply of oxygen, they undergo further reduction to more reactive products which bind to cell components. In this way, they are trapped in hypoxic tissue.[1,2]

Examples of PET hypoxia imaging agents are the fluorine-18 labelled 2-nitroimidazole derivatives ^{18}FNIM[3], ^{18}FETNIM[4] and ^{18}F-EF1[5] besides ^{62}Cu-ATSM[6], which lacks the 2-nitroimidazole moiety. For SPECT imaging of hypoxia, the 2-nitroimidazole containing iodine-123 labelled compound IAZA has been used clinically.[7] In addition, several technetium-99 m complexes have been proposed and evaluated[8–14] among which ^{99m}Tc-HL91 (oxo-^{99m}Tc(V)-4,9-diaza-3,3,10,10-tetramethyldodecan-2,11-dione dioxime, ^{99m}Tc-BnAO, Prognox) which does not contain the bioreductive 2-nitroimidazole moiety.[15] In a clinical study, ^{99m}Tc-HL91 was found to be useful for tumor visualization[16,17] but despite these encouraging results commercialization of this agent was not pursued.

In a search for a new technetium-99 m labelled hypoxia imaging agent we have conjugated the thiol protected bifunctional chelating agent 1,2-ethylenedicysteamine (BAT) to 2-nitroimidazole using a propylene spacer. The corresponding ^{99m}Tc-complex was characterized with LC–MS and its biodistribution was studied in mice.

Results and discussion

The *S*,*S*′-*bis*-trityl-*N*-BOC protected conjugate of 1,2-ethylenedicysteamine and 2-nitroimidazole was synthesized by reaction of protected BAT with 1-bromo-3-chloropropane and subsequent alkylation of 2-nitroimidazole using the *N*-derivatized BAT intermediate (Figure 1).

Deprotection and labelling of the conjugate with ^{99m}Tc can be performed in the conventional way. In this case, thiols and the BOC-protected amine are first deprotected, e.g. using a mixture of

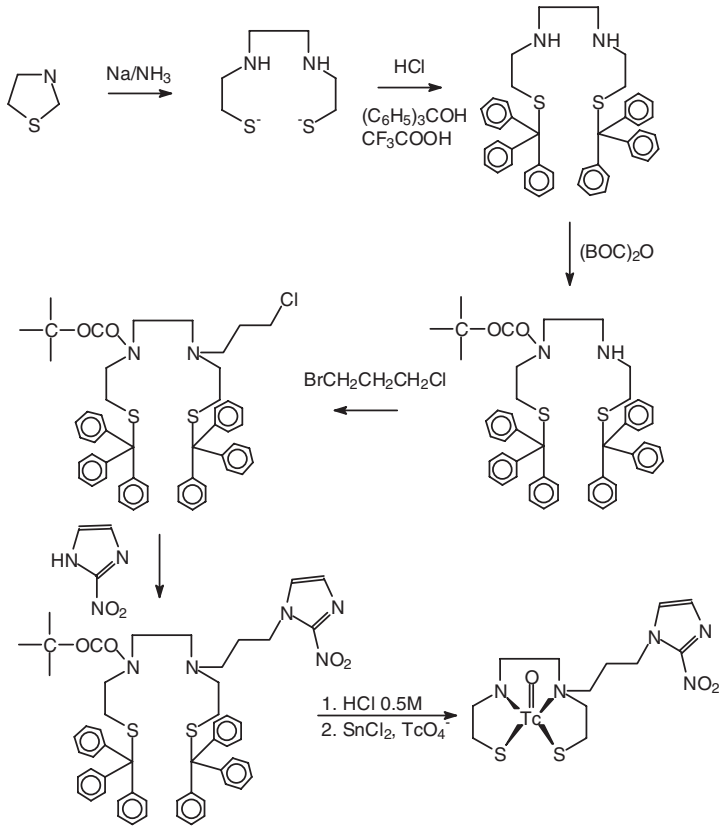

Figure 1. Synthesis of *S*,*S*′-*bis*-trityl-*N*-BOC–NIM–BAT and Tc–NIM–BAT (proposed structure)

trifluoroacetic acid, anisole and methansesulfonic acid, and the deprotected conjugate is then isolated, purified and stored in this form for labelling at a later moment. However, we have now developed a simpler one-pot two-step procedure to deprotect and label the *S*,*S*′-*bis*-trityl-*N*-BOC protected conjugate. In this procedure, the ligand was deprotected just before use in a labelling vial by heating in acidic conditions. The deprotected conjugate was subsequently labelled with technetium-99 m in a second heating step after addition of solutions of phosphate buffer pH 7, EDTA and tartrate, $SnCl_2$ and $^{99m}TcO_4^-$. Using this two-step one-pot labelling procedure, the ^{99m}Tc–NIM–BAT complex was obtained with a radiochemical purity of 80%, which is comparable to the radiochemical purity obtained using the two-pot labelling procedure. The trityl group is thus an interesting protection group for thiols of technetium chelating agents as it remains stable

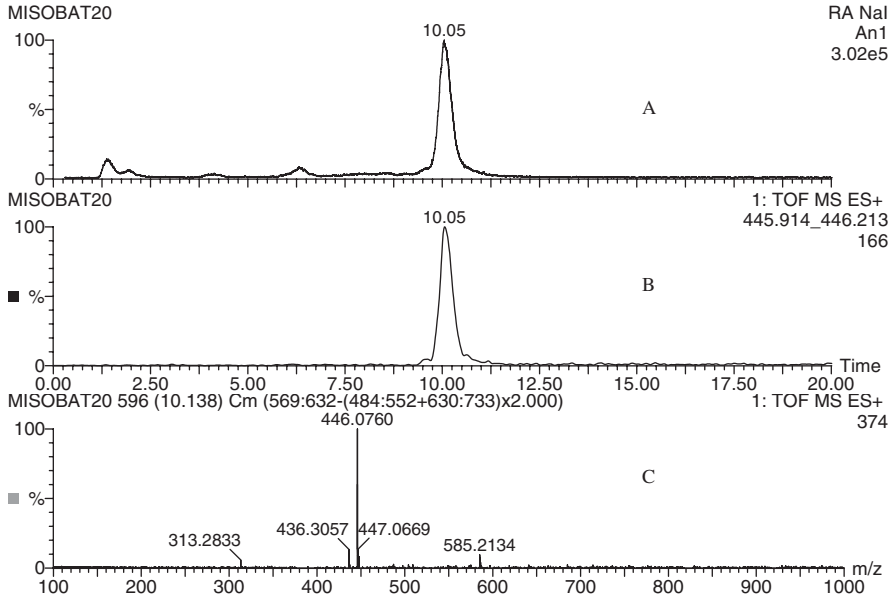

Figure 2. Radio-LC–MS analysis of Tc–NIM–BAT: (A) Radiometric trace. (B) Single ion chromatogram (444.914–446.213 Da). (C) Background subtracted summed spectrum over Rt = 9.1 to 10.95 min

during conjugation reaction and can nevertheless be removed in a convenient way by heating in mild acidic conditions. This allows flexible coupling of the BAT bifunctional chelating agent to a variety of biologically interesting molecules which in this way can be labelled efficiently with technetium-99 m.

Radio-LC–MS analysis of the reaction mixture after labelling of the BAT–NIM conjugate with ^{99m}Tc showed one major peak on the radiometric channel (Figure 2A). The background subtracted summed mass spectrum over this peak showed the presence of a molecular ion peak corresponding to the expected oxo-technetium(V)–NIM–BAT complex (Figure 2C). The accurate mass (theoretical 446.0137 Da, found 446.0760 Da) also corresponds to the expected structure (Figure 1) and was determined with a relative error of only 2.9 ppm.

The single ion mass chromatogram (445.914–446.213 Da) further shows only one peak which has an identical retention time as the peak of the radiometric trace (Figure 2B). These mass spectrometric data provide a strong identity confirmation of the supposed structure of Tc–NIM–BAT.

Table 1. Biodistribution of ^{99m}Tc–NIM–BAT in mice

	% of ID ($n = 4$)						% of ID/g ($n = 4$)					
	10 min p.i.			4 h p.i.			10 min p.i.			4 h p.i.		
	mean	±	SD	mean	±	SD	mean	±	SD	mean	±	SD
Urine	0.3	±	0.2	8.8	±	7.2	—	—	—	—	—	—
Kidneys	1.7	±	0.2	0.9	±	0.3	2.9	±	0.3	1.8	±	0.6
Liver	39.2	±	3.2	18.7	±	4.2	17.8	±	2.0	9.0	±	2.6
Spleen	0.1	±	0.03	0.02	±	0.01	0.9	±	0.1	0.1	±	0.0
Lungs	0.5	±	0.1	0.1	±	0.1	1.9	±	0.3	0.4	±	0.3
Heart	0.2	±	0.0	0.04	±	0.01	1.6	±	0.3	0.2	±	0.1
Intestines	25.9	±	6.2	59.0	±	16.0	7.00	±	1.6	17.7	±	2.7
Stomach	1.5	±	0.8	0.7	±	0.1	3.1	±	2.4	1.3	±	0.2
Cerebrum	0.02	±	0.01	0.01	±	0.00	0.07	±	0.02	0.03	±	0.02
Cerebellum	0.01	±	0.00	0.01	±	0.00	0.10	±	0.00	0.05	±	0.04
Blood	3.0	±	0.59	0.7	±	0.3	1.2	±	0.2	0.3	±	0.1
Carcass	22.2	±	2.9	3.5	±	1.7	0.8	±	0.1	0.1	±	0.1

The results of the biodistribution study in mice are summarized in Table 1. As can be expected for a lipophilic compound, ^{99m}Tc–NIM–BAT undergoes extensive hepatobiliary excretion.

At 4 h p.i. about 60% of the injected activity is excreted to the intestines and about 10% is excreted with the urine. A fraction of 22% of the injected dose of ^{99m}Tc–NIM–BAT is retained in the carcass at 10 min p.i. and is cleared as a function of time, resulting in a residual activity of only 3.5% of the injected dose at 4 h p.i. From the %ID/g values it can be concluded that this compound distributes evenly over the different organs and tissues with exception of the excretory organs liver (and intestines) and kidneys, the latter however to a minor degree.

Experimental

All reagents were obtained commercially from Acros (Geel, Belgium), Fluka (Bornem, Belgium) or Merck (Darmstadt, Germany).

^{1}H-NMR spectra were obtained using a Varian 200 MHz spectrometer (Varian, Palo Alto, CA, USA). Chemical shifts are reported in ppm relative to the internal standard tetramethylsilane (TMS, $\delta = 0$).

Mass spectra were acquired on a Micromass LCT mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray interface.

HPLC analysis and purification was done using an XTerra RP18 5 μ column (4.6 mm × 250 mm, Waters, Milford, USA) eluted with gradient mixtures of ammonium acetate 0.1 M and acetonitrile (linear gradient;

0 min 0% acetonitrile; 30 min 90% acetonitrile). The eluate was monitored for radioactivity using a 2-in NaI(Tl) scintillation detector.

Radio-LC–MS

Radio-LC–MS analysis was performed on a system consisting of a Waters separation module (Waters, Milford USA), an XTerra MS C18 column (50 mm × 2.1 mm, Waters) and a 2-in NaI(Tl) radiation detector and a Micromass LCT (time-of-flight) mass spectrometer equipped with electrospray interface in series. Masslynx software (Micromass) was used for acquisition and data processing.

The column was eluted at a flow rate of 300 µl/min with gradient mixtures of acetonitrile and $HCOONH_4$ 1% (linear gradient from 0% acetonitrile at 0 min to 80% at 20 min).

A solution of Kryptofix 2.2.2 (0.01% m/v) in CH_3CN/H_2O (50:50, v/v) was added to the column eluent at a flow rate of 1 µl/min and served as lock mass for accurate mass determination. For each analysis a volume of 50 µl of the radiolabelling reaction mixture was injected on the column.

1, 2-*Ethylenedicysteamine (*<u>**2**</u>*)*

Thiazolidine (<u>**1**</u>, 8.91 g, 100 mmol) was dissolved in liquid ammonia (60 ml) in a two-necked flask fitted to a Dewar cooler, and the mixture was stirred vigorously. Sodium (4.84 g, 210 mmol) was added until a blue color persisted for at least 15 min. Ammonium chloride was then added in portions until decoloration persisted and the resulting reaction mixture was kept at room temperature to allow slow evaporation of ammonia. Cold water (200 ml) was added followed by the addition of HCl 12 M to pH 1. After washing with three portions of 100 ml ethyl acetate, the water layer was evaporated at reduced pressure. The white residue was dried overnight at 60°C under vacuum and used without purification in the next reaction step.

MS ESI+: m/z $(M+H)^+$ found 181 (calculated, $C_6H_{17}N_2S_2$, 181).

S,S′-Bis-triphenylmethyl-1,2-ethylenedicysteamine (<u>**3**</u>*)*

The residue obtained in the previous reaction step was dissolved in 120 ml trifluoroacetic acid at room temperature. Triphenylmethanol

(26.03 g, 100 mmol) was added in portions to the reaction mixture which was subsequently stirred at room temperature for 15 min. Evaporation of the reaction mixture under reduced pressure yielded a red oil. The evaporation was repeated three times after addition of hexane (3×100 ml) to remove traces of trifluoroacetic acid. The residual oil was dispersed in a mixture of water (200 ml) and ethyl acetate (200 ml) and the mixture was neutralized with 10% $NaHCO_3$ solution. The organic phase was separated, dried over anhydrous magnesium sulfate, concentrated under reduced pressure and chromatographed on a Kieselgel 60 column eluted with 5% MeOH in EtOAc. The appropriate fractions were pooled and evaporated to give 16.6 g (24.9 mmol, 49.9%) of a cream-colored foam. TLC (5% MeOH in EtOAc): R_f 0.55.

^{1}H-NMR ($CDCl_3$) δ 1.79 (br s, 2 H, N*H*), 2.3–2.6 (m, 4 H, SC*H*$_2$CH$_2$N), 2.45 (s, 4 H NC*H*$_2$C*H*$_2$N), 2.84 (m, 4 H, SCH$_2$C*H*$_2$N).

MS ESI+: *m*/*z* $(M+H)^+$ found 664.38 (calculated, $C_{44}H_{45}N_2S_2$, 664.29).

*S,S′-Bis-triphenylmethyl-N-(tert-butoxycarbonyl)-1,2-ethylenedicysteamine (**4**)*

To a solution of **3** (7.51 g, 11.26 mmol) and diisopropylethylamine (DIEA, 2.2 ml, 12 mmol) in 250 ml CH_2Cl_2 was added dropwise a solution of $(BOC)_2O$ (2.53 g, 11.26 mmol) in CH_2Cl_2 (50 ml) at 0°C. The mixture was stirred at 0°C for 1 h, concentrated and purified by column chromatography (Kieselgel 60, hexane/EtOAc/NEt_3 80:15:5, v/v/v) to give 4.21 g of a white foam (48.8%).

^{1}H-NMR ($CDCl_3$) δ 1.38 (s, 9 H, C(C*H*$_3$)$_3$), 2.27–2.58 (m, 8 H, C*H*$_2$NC*H*$_2$ +2x SC*H*$_2$CH$_2$N), 2.97 (m, 4 H, C*H*$_2$N-BocC*H*$_2$), 7.1–7.4 (m, 30 H, Ar*H*).

MS ESI+: *m*/*z* $(M+H)^+$ found 764.96 (calculated, $C_{49}H_{53}N_2O_2S_2$, 765.34).

*S,S′-Bis-triphenylmethyl-N-(tert-butoxycarbonyl)-N′-(3-chloropropyl)-1,2-ethylenedicysteamine (**5**)*

The amine **4** (3.45 g, 4.5 mmol) and DIEA (4 ml, 22.5 mmol) were dissolved in dry acetonitrile (30 ml) and to this solution was added 1-bromo-3-chloropropane (3.6 g, 22.5 mmol). The resulting solution was refluxed overnight, concentrated in vacuo and chromatographed (Kieselgel 60, hexane/ethyl acetate, 4:1, v/v). This yielded 1.5 g (40%) of the desired product **5** which was isolated as a colorless oil.

^{1}H NMR ($CDCl_3$) δ 1.38 (s, 9 H, C(CH_3)$_3$), 1.62 (m, 2 H, $CH_2CH_2CH_2Cl$), 2.2–2.3 (m, 8 H, -CH_2NCH_2- + 2x SCH_2CH_2N), 2.93 (m, 4 H, CH_2N-BocCH_2), 3.46 (t, 2 H, CH_2Cl), 3.55 (t, 1 H, $NCH_2CH_2CH_2Cl$), 3.69 (t, 1 H, $NCH_2CH_2CH_2Cl$), 7.2-7.4 (m, 30 H, Ar*H*).

MS ESI+: *m/z* $(M+H)^+$ found 840.92 (calculated, $C_{52}H_{58}ClN_2O_2S_2$, 841.35).

*S,S′-Bis-triphenylmethyl-N-(tert-butoxycarbonyl)-N′-(3-(2′-nitro-1′H-imidazoyl)-propyl)-1,2-ethylenedicysteamine (**6**)*

A solution of **5** (149 mg, 0.18 mmol), 2-nitroimidazole (30.3 mg, 0.18 mmol), KI (30 mg, 0.18 mmol) and K_2CO_3 (252 mg, 1.8 mmol) in 5 ml DMF was heated at 110°C for 2 h. After removal of DMF, the residue was purified by chromatography (Kieselgel 60, hexane/EtOAc/NEt_3, 4:1:0.25, v/v/v) to yield 100 mg (55%) of a yellow powder.

^{1}H NMR ($CDCl_3$) δ 1.37 (s, 9 H, C(CH_3)$_3$), 1.76 (m, 2 H, $CH_2CH_2CH_2$), 2.1–2.4 (m, 10 H, CH_2NCH_2 + 2x SCH_2CH_2N + $NCH_2CH_2CH_2$Im), 3.0 (m, 4 H, CH_2N-BocCH_2), 4.34 (m, 2 H, CH_2Im), 7.0–7.4 (m, 32 H, 2 × Im*H*, 30 × Ar*H*).

MS ESI+: *m/z* $(M+H)^+$ found 918.37 (calculated, $C_{55}H_{60}N_5O_4S_2$, 918.40).

Labelling with ^{99m}Tc and purification

Method A. An aliquot of 1 mg of ligand **6** was dissolved in a mixture of 0.3 ml ethanol and 0.5 ml HCl 0.5 M and heated in a boiling water-bath during 10 min. After cooling, 1 ml of a solution containing 0.5 ml phosphate buffer 0.5 M pH 7, 0.25 ml Na_2EDTA 0.1 M and 0.25 ml KNa-tartrate solution (40 mg/ml) was added, immediately followed by the addition of 100 µg $SnCl_2 \cdot 2H_2O$ in 25 µl HCl 0.05 N and 1 ml ^{99m}Tc-pertechnetate solution (200 MBq, monday morning eluate). The labelling vial was heated again in a boiling water-bath for 10 min, cooled and the solution filtered through a 0.45-µm membrane filter. The reaction mixture was then analyzed with the HPLC system described higher (Rt ^{99m}Tc–NIM–BAT = 23 min).

Method B. An amount of 918 mg (1 mmol) of **6** was dissolved in a mixture of TFA (1 ml) and anisole (0.1 ml) at room temperature. The solution was cooled to 0°C and methanesulfonic acid (0.4 ml) was added dropwise. The resulting solution was stirred at room temperature for

1.5 h under N_2 and concentrated in vacuo. Water (5 ml) and diethyl ether (5 ml) were added to the residue, the water layer was separated, neutralized with 10% $NaHCO_3$ solution and extracted with CH_2Cl_2 (3×5 ml). The CH_2Cl_2 layers were combined, dried over anhydrous $MgSO_4$ and concentrated in vacuo. The yellow solid formed was dried overnight under vacuum at 60°C. Yield: 125 mg (30%).

MS ESI+: m/z $(M+H)^+$ found 334 (calculated, $C_{12}H_{24}N_5O_2S_2$, 334). For labelling with ^{99m}Tc, 100 µg of the deprotected ligand was added to a solution containing 100 µl ethanol, 100 µg $SnCl_2 \cdot 2H_2O$, 0.5 ml HCl 0.5 N and 0.5 ml generator eluate (containing 370 MBq $^{99m}TcO_4^-$). The labelling reaction mixture was heated for 10 min in a boiling water-bath. The reaction mixture was then analyzed with the HPLC system described higher (Rt Tc–NIM–BAT = 23 min).

Biodistribution in mice

Solutions of ^{99m}Tc–NIM–BAT obtained after HPLC purification were diluted with normal saline to a concentration of 148 kBq/ml. Biodistribution was studied in normal male NMRI mice (body mass 30–40 g). The animal studies were performed in accordance with the Belgian code of practice for the care and use of animals. The mice were sedated by i.m. injection of 0.1 ml of a solution containing 34 µg/ml fentanyl and 16 mg/ml ketamine. A volume of 0.1 ml of the diluted tracer solution was injected in the mice via a tail vein. The mice were sacrificed by decapitation at 10 min or 4 h post injection (p.i.) ($n = 4$ at each time point). Blood was collected in a tared tube and weighed. All organs and other body parts were dissected, weighed and their radioactivity was counted in a 3 in NaI(Tl) well crystal, coupled to a multichannel analyzer (Wallac, Turku, Finland). Corrections were made for background radiation and physical decay during counting. Results were expressed as percentage of injected dose (% of ID), equal to the sum of the net counts in all organs and where possible as percentage of injected dose per gram of tissue (% of ID/g). For calculation of total blood activity, blood mass was assumed to be 7% of the body mass.

Conclusion

We have conjugated *S*,*S*′-*bis*-trityl-*N*-BOC protected 1,2-ethylenedicysteamine with 2-nitroimidazole. An interesting one-pot two-step method

for the labelling with technetium-99 m was developed starting from the protected ligand, yielding a high yield of the oxo-technetium(V)–NIM–BAT complex of which the identity was confirmed with radio-LC–MS. In mice, this lipophilic compound showed extensive hepatobiliary excretion.

The evaluation of the potential of this new ^{99m}Tc-labelled conjugate as a hypoxia tracer will be published in a separate paper.

Acknowledgements

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