

# Oxotechnetium and Oxorhenium 3+1 Mixed Ligand Complexes as Potential Melanoma Targeting Agents

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## Abstract

*Tc-99m "3+1" mixed ligand complexes with potential affinity for melanoma have been designed by an integrated approach using N-alkyl substituted benzamides as leader structure. This paper presents the preparation of a series of complexes with general formula  $Tc-99m O[(CH_3CH_2)_2N(CH_2)_2N(CH_2CH_2)_2S_2][RS]$  and their "in vivo" evaluation as potential melanoma targeting agents. Tc-99m complexes Tc1, Tc2, Tc3 and Tc4 were prepared by combining the tridentate ligand N,N-bis(2-mercaptoethyl)-N',N'-diethylethylenediamine with 4 different monodentate thiols. Labelling was performed by substitution using Tc-99m-glucoheptonate as precursor. All complexes were obtained with high yield (85%) and high radiochemical purity (90%). Identity of Tc compounds was corroborated by HPLC coinjection with the analogous rhenium complexes. Biodistribution studies were performed on the murine C57B16 mouse melanoma model obtained by subcutaneous inoculation of melanoma cells B16F1. After intravenous injection, all complexes showed high initial blood, lung and liver uptake but clearance after 12-24 hours was almost complete. Initial tumour uptake was relatively high (0.83.4 % dose /g at 2 hvs. post-injection) and retention until 24 hours significant (0.450.88 % dose/g). Tumour/blood and tumour/muscle ratios were favourable from 6 to 24 hours after injection due to fast blood and soft tissue clearance. Complex Tc2 showed the best tumour/blood and tumour/muscle ratios at 12 and 24 hours post-injection (1.9-2.4 and 7.5-12.0, respectively). Early and late static gamma-camera images acquired for this compound allowed delineation of the tumour with tumour/soft tissue ratios 7.4 at 12 hours. post/inj.) Complex Tc2 was also administered subcutaneously in the peritumoral region of melanoma bearing mice, in order to avoid high liver and hepatobiliary doses. In this condition, a very high percentage of the injected dose remained in the tumour, even after 24 hours (21.5%/g) with considerably higher tumour/blood and tumour/soft tissue ratios. These*

*results are promising and further development and future studies should include preparation of analogous Re-186 or Re-188 complexes and evaluation of their therapeutic potential, especially in early stages of melanoma growth.*

*Key words: Tc-99m radiopharmaceuticals, melanoma targeting- technetium/ rhenium compounds, therapeutic radiopharmaceuticals.*

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## Introduction

Malignant melanoma has become a great public health problem due to a notably high increase in its incidence during the past few years (1,2). New molecular probes for early detection of metastatic lesions, together with more effective therapeutic options are required to effectively address this problem (3,4).

Nuclear Medicine offers interesting alternatives in both fields. Research activities in recent years have followed two main approaches while looking for novel melanoma-targeting agents: the study of alpha melanocyte stimulating hormone (-MSH) peptide analogues (5,6,7) and the evaluation of N- (dialkylaminoalkyl) benzamides derivatives (8,9,10).

Tc-99m is the preferred radionuclide for these investigations, due to its ideal nuclear properties for diagnosis in Nuclear Medicine ( $t_{1/2} = 6$  h,  $E = 140$  KeV). Furthermore, the analogy in chemistry between technetium and rhenium, together with the availability of radionuclides of rhenium with appropriate nuclear properties for therapy (Re-186,  $t_{1/2} = 90.6$  h,  $E_{max} = 1.1$  MeV,  $E = 137$  KeV; Re-188,  $t_{1/2} = 17$  h,  $E_{max} = 2.1$  MeV,  $E = 155$  KeV), have mainly contributed to this preference, since the studies of Tc-99m complexes may be further expanded to the preparation of analogous rhenium compounds for therapy.

Based on the structure of the [I-123]-N-(2-diethylaminoethyl)-4-yodobenzamide (IBZA), a radiolabelled benzamide that displays high "in vivo" melanoma uptake in animal models and which has entered phase II clinical trials (11,12), various research groups have pursued the development of Tc-99m derivatives that demonstrate melanoma uptake.

Iodine atom has initially been replaced by different technetium chelating structures using a pendent approach. Nitrido technetium bis(dithiocarbamate) compounds (13)

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N-functionalized nitrido (14) and oxo-technetium bis(aminothioli) complexes (15) have been reported. Biological results showed in all cases only suboptimal tumour uptake.

More recently integration of the oxotechnetium core within the N-(2-diethylaminoethyl) part of the ethylbenzamide pharmacophore has been proposed. Replacement of the aromatic moiety by the metal chelate unit [Tc(O)(SNS)(S)] (16) or by the tetradentate chelate amine-amide dithiol (AADT) (17) leads to a series of compounds with promising properties, thereby suggesting that small technetium 99m complexes could be useful as potential melanoma imaging agents (18).

Our group has been working on the development of potential radiopharmaceuticals based on oxotechnetium and oxorhenium "3+1" mixed ligand complexes (19,20,21). Due to structural similarities, [SNS+S] mixed ligand complexes with potential affinity for melanoma can be designed by an integrated approach using N-alkyl benzamides as leader structure (Figure 1).

We describe herein the preparation of a series of complexes

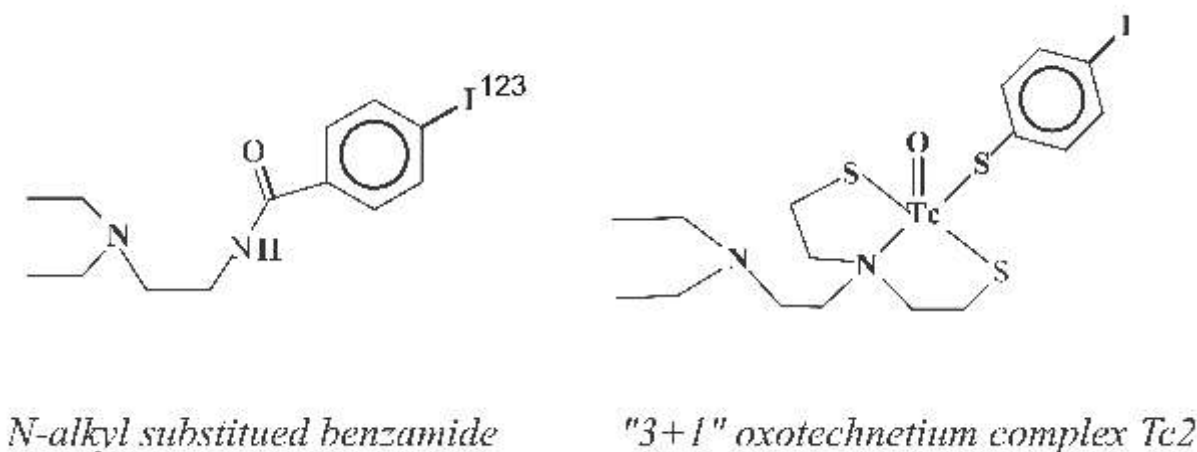
$C_6H_4S\}}(Re-3)$  and

$\{ReO[(CH_3CH_2)_2N(CH_2)_2N(CH_2CH)_2S_2][C_4H_9S]\}(Re-4)$  were prepared and characterised following the procedures described elsewhere (23,24). Solvents used for chromatographic analysis were of HPLC grade.

[Tc-99m]NaTcO<sub>4</sub> was obtained from an Elumatic III generator (Cis-Bio-International).

HPLC analysis was performed using a reverse phase Spherex 5 C18 column (4.6 x 150 mm); mobile phase: A= phosphate buffer pH 7.4 with 2 % triethylamine, B = methanol, system: t=0 0% B; t= 0-10 min linear gradient to 100% B; t= 10-25 min 100% B; flow rate 1.0 mL/min) and a LC-10 AS Shimadzu Liquid Chromatograph. Detection was either accomplished with a photodiode array detector (SPD-M10A, Shimadzu) or a 3"x3" NaI(Tl) crystal scintillation detector (Parken).

Activity measurements were performed either in a Capintec CRC- 5R dose calibrator or in a scintillation counter, using a 3"x3" NaI (Tl) crystal detector associated to an ORTEC monochanel analyzer.



**Figure 1.** Design of Tc-99m complexes that mimic N-alkyl substituted benzamides

with general formula Tc-99m  $O[(CH_3CH_2)_2N(CH_2)_2N(CH_2CH)_2S_2][RS]$  and their "in vivo" evaluation as potential melanoma targeting agents.

## Materials and methods

### Reagents

All laboratory chemicals were reagent grade and used without further purification. N,N-bis(2-mercaptoethyl)-N',N'-diethylethylenediamine was synthesised following a previously described method (22). Thiophenol, p-iodothiophenol, p-methoxythiophenol and butylmercaptane were purchased from from Fluka (Fluka Chemie AG).

Non-radioactive rhenium complexes  $\{ReO[(CH_3CH_2)_2N(CH_2)_2N(CH_2CH)_2S_2][C_6H_4S]\}(Re-1)$ ,  $\{ReO[(CH_3CH_2)_2N(CH_2)_2N(CH_2CH)_2S_2][p-I-C_6H_4S]\}(Re-2)$ ,  $\{ReO[(CH_3CH_2)_2N(CH_2)_2N(CH_2CH)_2S_2][p-OCH_3-$

### Preparation of Tc-99m complexes

Radiolabelling with Tc-99m was accomplished by using [Tc-99m]Tc-glucoheptonate as precursor. A vial containing a lyophilised mixture of 200 mg calcium glucoheptonate and 0.2 mg SnCl<sub>2</sub>.2H<sub>2</sub>O was reconstituted with 5 ml water and 0.5 ml of this solution was mixed with 0.5-1 mL [Tc-99m]NaTcO<sub>4</sub> with an activity of 185 - 1850 MBq (5 - 50 mCi). Radiochemical purity was checked by ascending chromatography using Whatman 1 paper and either acetone or NaCl 0.9% as mobile phases. A mixture of  $2 \times 10^{-5}$  moles of N,N-bis(2-mercaptoethyl)-N',N'-diethylethylenediamine and either thiophenol (Tc1), p-yodo-thiophenol (Tc2), p-methoxythiophenol (Tc3) were left to react with the previously prepared Tc-99m glucoheptonate (radiochemical purity >95%) for 10 minutes at room temperature. The lipophilic species were extracted with CH<sub>2</sub>Cl<sub>2</sub> and analysed by HPLC. For complex

| ORGAN     | <i>Tc1</i>  |             |             |             |             | <i>Tc2</i>  |             |             |             |             |
|-----------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
|           | 0.5 hs.     | 2 hs.       | 6hs.        | 12hs.       | 24hs.       | 0.5 hs.     | 2 hs.       | 6hs.        | 12hs.       | 24hs.       |
| Blood     | 4.3 ± 0.8   | 2.7 ± 0.8   | 1.0 ± 0.1   | 0.64 ± 0.23 | 0.30 ± 0.01 | 4.7 ± 0.2   | 2.9 ± 0.5   | 1.2 ± 0.2   | 1.1 ± 0.5   | 0.60 ± 0.16 |
| Liver     | 34.4 ± 1.8  | 25.5 ± 1.3  | 3.4 ± 0.5   | 1.6 ± 0.3   | 1.0 ± 0.1   | 27.3 ± 2.0  | 13.7 ± 1.6  | 5.7 ± 1.3   | 1.7 ± 0.4   | 1.2 ± 0.5   |
| Lungs     | 1.7 ± 0.5   | 0.43 ± 0.28 | 0.27 ± 0.09 | 0.22 ± 0.13 | 0.13 ± 0.01 | 2.1 ± 0.6   | 1.0 ± 0.2   | 0.99 ± 0.08 | 1.1 ± 0.4   | 0.22 ± 0.07 |
| Spleen    | 0.24 ± 0.05 | 0.08 ± 0.02 | 0.03 ± 0.01 | 0.04 ± 0.02 | 0.02 ± 0.01 | 0.38 ± 0.17 | 0.10 ± 0.01 | 0.06 ± 0.02 | 0.06 ± 0.02 | 0.05 ± 0.02 |
| Thyroid   | 0.12 ± 0.01 | 0.03 ± 0.01 | 0.03 ± 0.01 | 0.02 ± 0.01 | 0.02 ± 0.01 | 0.21 ± 0.09 | 0.10 ± 0.01 | 0.04 ± 0.01 | 0.01 ± 0.01 | 0.01 ± 0.01 |
| Stomach   | 2.1 ± 0.9   | 2.6 ± 0.2   | 0.16 ± 0.04 | 0.42 ± 0.01 | 0.81 ± 0.37 | 1.1 ± 0.5   | 0.80 ± 0.04 | 0.26 ± 0.04 | 0.25 ± 0.08 | 0.16 ± 0.04 |
| Intestine | 19.5 ± 1.8  | 39.1 ± 4.3  | 33.4 ± 4.3  | 14.9 ± 5.7  | 12.3 ± 0.6  | 15.0 ± 1.9  | 43.3 ± 3.5  | 61.2 ± 2.8  | 9.2 ± 0.6   | 4.0 ± 0.7   |
| Bladder   | 8.0 ± 0.7   | 15.5 ± 1.6  | 20.5 ± 0.3  | 0.47 ± 0.10 | 0.08 ± 0.01 | 15.3 ± 1.0  | 29.7 ± 2.3  | 22.6 ± 1.7  | 0.06 ± 0.04 | 0.46 ± 0.16 |
| Urine     | -           | -           | 5.4 ± 0.1   | 25.8 ± 2.4  | 20.5 ± 1.7  | -           | -           | 5.0 ± 0.5   | 30.0 ± 0.5  | 34.6 ± 2.6  |
| Faeces    | -           | -           | 35.2 ± 0.5  | 45.4 ± 4.2  | 62.6 ± 5.1  | -           | -           | 1.8 ± 0.4   | 51.5 ± 0.8  | 53.9 ± 5.3  |

| ORGAN     | <i>Tc3</i> |            |           |           |             | <i>Tc4</i> |            |            |           |           |
|-----------|------------|------------|-----------|-----------|-------------|------------|------------|------------|-----------|-----------|
|           | 0.5 hs.    | 2 hs.      | 6hs.      | 12hs.     | 24hs.       | 0.5 hs.    | 2 hs.      | 6hs.       | 12hs.     | 24hs.     |
| Blood     | 5.3-1.1    | 2.80±0.2   | 1.4-0.1   | 1.3-0.3   | 0.7±0.1     | 5.7±0.6    | 5.7-0.7    | 3.8-1.0    | 1.7-0.4   | 1.4-0.3   |
| Liver     | 35.3±2.6   | 11.4-1.5   | 3.2-0.3   | 2.9-0.6   | 2.6±0.2     | 29.9-2.5   | 17.5±1.4   | 9.0-2.4    | 5.8-0.5   | 3.7-0.5   |
| Lungs     | 1.6-0.1    | 0.6-0.1    | 0.25-0.04 | 0.34-0.05 | 0.19±0.03   | 3.2±0.9    | 1.0-0.1    | 0.90-0.07  | 0.6-0.1   | 0.5-0.2   |
| Spleen    | 0.32-0.06  | 0.12-0.03  | 0.09-0.01 | 0.04-0.01 | 0.05±0.01   | 0.50±0.10  | 0.26-0.04  | 0.18-0.02  | 0.20-0.07 | 0.22-0.03 |
| Thyroid   | 0.23=0.09  | 0.07=0.05  | 0.04=0.01 | 0.02=0.01 | 0.01±0.01   | 0.17±0.02  | 0.18=0.04  | 0.6±0.02   | 0.05=0.01 | 0.02=0.01 |
| Stomach   | 3.8 0.5    | 1.8 0.2    | 0.23 0.02 | 3.4 0.8   | 1.4 ± 0.2   | 3.5 ± 0.3  | 4.7 0.2    | 1.9 0.4    | 1.6 0.5   | 0.69 0.08 |
| Intestine | 17.2±2.7   | 50.5±2.7   | 36.7±3.1  | 12.7±0.9  | 29.1=2.0    | 30.2=0.9   | 46.7±3.6   | 56.8=1.5   | 15.5±2.7  | 5.5=1.7   |
| Bladder   | 3.7 0.9    | 20.0 ± 2.4 | 4.5 0.7   | 0.01 0.01 | 0.14 ± 0.02 | 2.5 ± 0.3  | 12.3 ± 1.9 | 30.3 ± 1.4 | 0.01 0.01 | 0.17 0.06 |
| Urine     | -          | -          | 24.5±3.5  | 12.1±2.2  | 36.9=2.9    | -          | -          | 0.92=0.07  | 4.7=0.4   | 32.6=1.4  |
| Faeces    | -          | -          | 22.7+4.6  | 58.2+3.3  | 54.1-1.9    | -          | -          | 1.9-0.2    | 57.6+4.3  | 50.8-2.1  |

**Table 1.-** Biodistribution results after intravenous injection of complex Tc1-Tc4 (% Dose/ organ ± <sub>n</sub>, n=3)

*Tc4* butylmercaptane was used as coligand in a molar ratio coligand/ligand of 5 (25).

The characterization of the Tc-99m complexes (*Tc1*, *Tc2*, *Tc3*, *Tc4*) was accomplished by chromatographic correlation (HPLC) with the corresponding rhenium complexes (*Re1*, *Re2*, *Re3*, *Re4*).

### In vivo evaluation

All Animal studies were approved by the Ethics Committee of the Faculty of Chemistry from Uruguay. In vivo evaluation of Tc-99m complexes was performed by biodistribution using animals bearing induced melanoma.

Murine melanoma cell line B16F1 (American Type Cultured Collection) was defrosted in water bath at 40°C and diluted with DMEM medium. After centrifugation at 1200 rpm, cells were suspended in DMEM medium containing 10% fetal bovine serum and cultured at 37°C in 5% CO<sub>2</sub> incubator for 2-5 days. Confluent cell culture was treated with trypsin at 37°C, washed and re-suspended in PBS previous to inoculation (26). Viability of the cells was assessed by trypan blue and cell count determined in a Neubauer chamber.

A cell suspension in PBS containing 2.5x10<sup>6</sup> cells/ml was prepared and 200 µl were injected subcutaneously in the right limb of C57B16 mice (8-10 weeks old). Ten to 15 days later the animals developed palpable tumour nodules and were used for biodistribution, either after systemic administration or after peritumoral injection of the Tc-99m

complexes.

Three animals per group were injected via a lateral tail vein with the HPLC purified complex reconstituted with 25:75 methanol: saline (0.1 ml, 35350 KBq). At different intervals after injection (0.5 to 24 hours) the animals were sacrificed by neck dislocation. Whole organs of interest, whole tumour and samples of blood and muscle were collected, weighed and assayed for radioactivity. Animals were kept in metabolic cages in order to collect total urine volume and faeces during biodistribution period (6-24 hours). Corrections by different sample geometry were applied when necessary. Results were expressed as percentage of dose/organ or percentage of dose/g. The tumour/blood and tumour/muscle ratios were calculated from the corresponding percent dose/gram values.

Three animals per group were injected subcutaneously in the peritumoral region with the HPLC purified complex *Tc2*, reconstituted with 25:75 methanol: saline (0.1 ml, 250 - 350 KBq). At different intervals after injection (0.5 to 24 hours) the animals were sacrificed by neck dislocation. Whole organs of interest, whole tumour and samples of blood and muscle were collected, weighed and assayed for radioactivity. Animals were kept in metabolic cages in order to collect total urine volume and faeces during biodistribution period (6-24 hours). Corrections by different sample geometry were applied when necessary. Results were expressed as % Dose/organ organ or % Dose/g. The tumour/blood and tumour/muscle ratios were

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calculated from the corresponding percent dose/ gram values.

**Gamma-camera imaging**

Imaging was performed using a rectangular field (21.2 x 15.7 inches) gamma/camera (Sophy Camera DSX) equipped with a low energy, high resolution, parallel hole collimator. Static images from C57B16 mice bearing induced melanoma were acquired at 2 and 12 hours after intravenous injection of HPLC purified complex *Tc2* reconstituted with 25:75 methanol: saline (0.1 ml, 10-15 MBq). A 256x256 matrix with a 2.66 zoom factor was employed and images were digitally stored for further analysis. Identical regions of interest were drawn on the limb containing the tumour and the contralateral one and activity ratios were calculated using the activity/pixel.

**Results****Preparation of Tc-99m complexes**

Tc-99m complexes were prepared by a simple procedure at room temperature as shown in Figure 2. The labelling yield, measured as percentage of activity extracted by dichloromethane, was more than 85% and the radiochemical purity, evaluated by HPLC analysis was more than 90%.

In order to corroborate the identity of compounds Tc 1-Tc 4, HPLC coinjection with the corresponding rhenium compounds (Re 1- Re 4) was performed. Both radioactivity (for tracer) and UV-vis (for carrier) detectors exhibited identical chromatographic profiles, suggesting that the same chemical structure was formed under both chelating conditions.

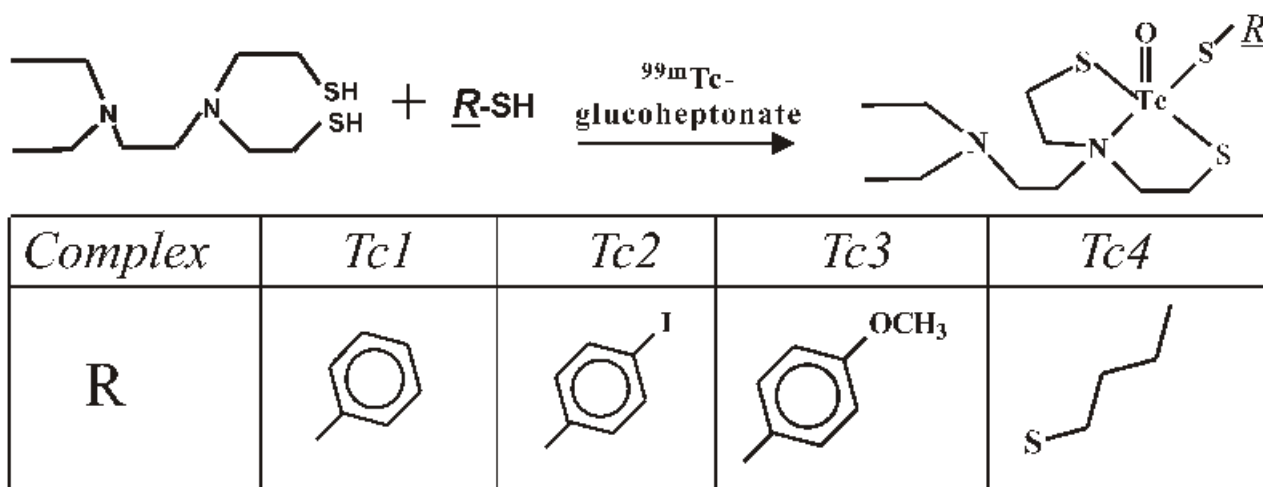


Figure 2. Preparation of Complexes Tc1 Tc4

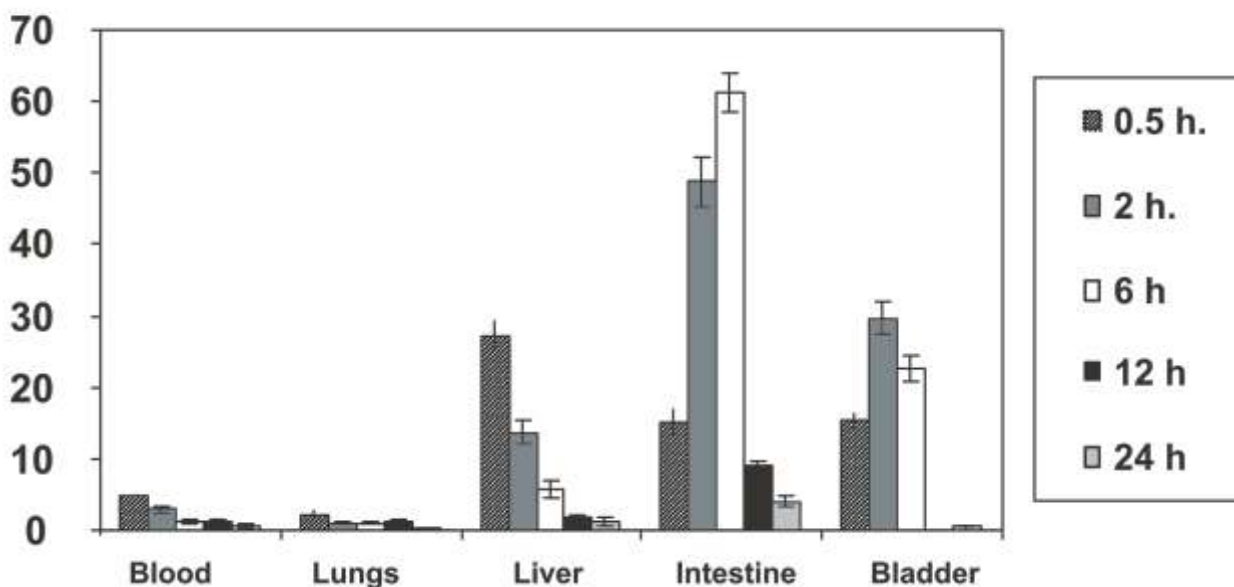
**% Dose/ organ**

Figure 3. Bio-distribution profile of Complex Tc2 in C57B16 mice between 0.5 and 24 hours post-injection

| ORGAN      | <i>Tc1</i> |           |           |           |           | <i>Tc2</i> |           |           |           |           |
|------------|------------|-----------|-----------|-----------|-----------|------------|-----------|-----------|-----------|-----------|
|            | 0.5 hs.    | 2 hs.     | 6hs.      | 12hs.     | 24hs.     | 0.5 hs.    | 2 hs.     | 6hs.      | 12hs.     | 24hs.     |
| Tumour (T) | 1.6±0.5    | 0.72±0.31 | 0.81±0.12 | 0.93±0.11 | 0.45±0.08 | 1.4±0.5    | 1.6±0.5   | 0.82±0.30 | 1.3±0.7   | 0.88±0.12 |
| Muscle (M) | 0.70±0.20  | 0.24±0.10 | 0.14±0.01 | 0.16±0.03 | 0.07±0.02 | 1.3±0.8    | 0.41±0.10 | 0.13±0.01 | 0.18±0.08 | 0.07±0.02 |
| Blood (B)  | 2.1±0.5    | 1.2±0.6   | 0.66±0.07 | 0.37±0.12 | 0.19±0.02 | 4.0±1.7    | 2.0±0.5   | 0.81±0.16 | 0.67±0.12 | 0.40±0.08 |
| T/B        | 0.88±0.26  | 0.63±0.14 | 1.3±0.3   | 2.6±0.5   | 2.5±0.8   | 0.36±0.08  | 0.79±0.10 | 1.0±0.3   | 1.9±0.2   | 2.4±0.7   |
| T/M        | 2.3±0.3    | 3.0±0.7   | 5.7±0.4   | 5.9±0.7   | 6.5±1.9   | 1.3±0.6    | 3.9±0.4   | 6.1±1.4   | 7.5±0.3   | 12.0±1.4  |

| ORGAN      | <i>Tc3</i> |           |           |           |           | <i>Tc4</i> |           |           |           |           |
|------------|------------|-----------|-----------|-----------|-----------|------------|-----------|-----------|-----------|-----------|
|            | 0.5 hs.    | 2 hs.     | 6hs.      | 12hs.     | 24hs.     | 0.5 hs.    | 2 hs.     | 6hs.      | 12hs.     | 24hs.     |
| Tumour (T) | 1.8±0.2    | 3.4±0.2   | 1.4±0.3   | 2.0±0.3   | 0.70±0.14 | 2.0±0.3    | 3.17±0.11 | 1.3±0.2   | 1.6±0.4   | 0.87±0.17 |
| Muscle (M) | 1.1±0.1    | 0.54±0.10 | 0.24±0.03 | 0.33±0.03 | 0.11±0.04 | 1.4±0.4    | 1.0±0.3   | 0.43±0.11 | 0.36±0.11 | 0.16±0.01 |
| Blood (B)  | 3.1±0.7    | 2.2±0.2   | 0.99±0.12 | 0.85±0.31 | 0.50±0.08 | 2.8±0.5    | 4.3±0.5   | 2.3±0.2   | 1.2±0.3   | 0.75±0.02 |
| T/B        | 0.32±0.16  | 1.5±0.2   | 1.4±0.2   | 2.37±0.38 | 1.5±0.3   | 0.72±0.12  | 0.75±0.08 | 0.58±0.04 | 1.3±0.4   | 1.1±0.3   |
| T/M        | 1.71±0.09  | 6.4±1.2   | 5.7±0.8   | 5.9±0.5   | 6.6±1.4   | 1.5±0.3    | 3.3±0.9   | 3.1±0.4   | 4.6±0.9   | 5.4±1.0   |

**TABLE 2.** Tumour uptake and tumour/tissue ratios after intravenous injection of complex Tc1-Tc4 (% Dose/g  $\pm$   $n=3$ )

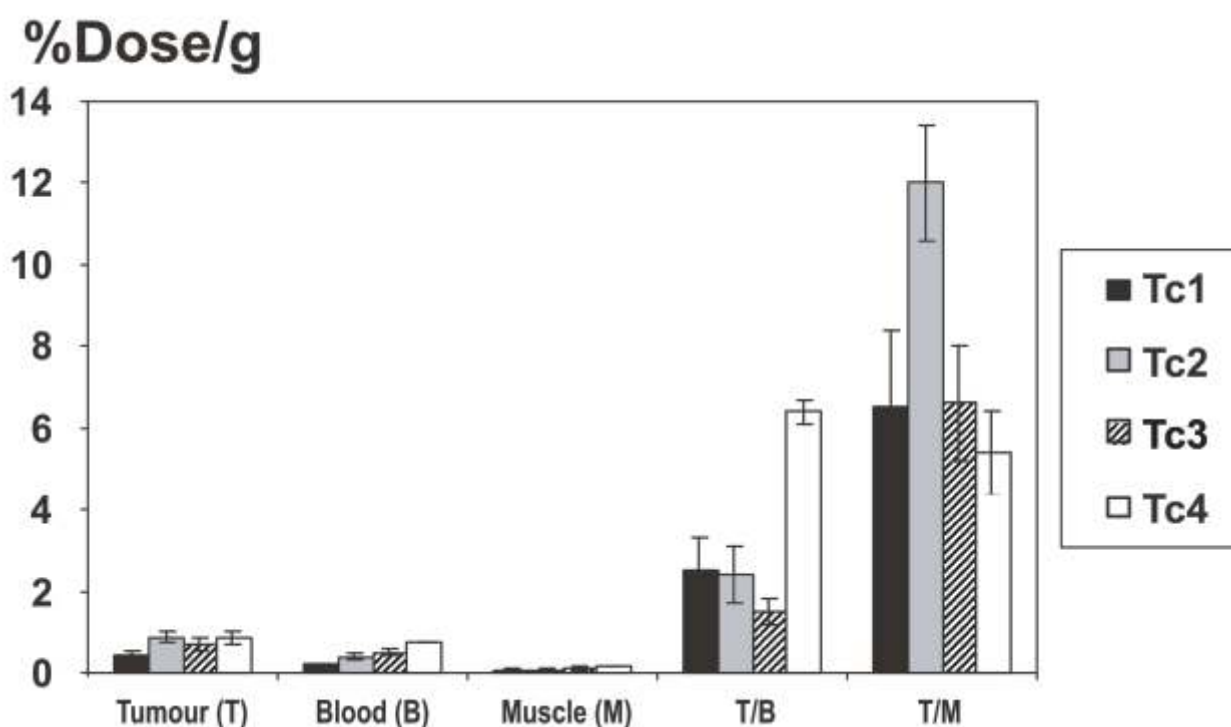
### In vivo evaluation

Biodistribution studies of the Tc-99m complexes were performed on the murine C57B16 mouse melanoma model obtained by subcutaneous inoculation of murine melanoma cells B16F1. Ten to fifteen days after cell inoculation the animals developed palpable tumors (1x1x0.5 cm) and were used for biodistribution studies.

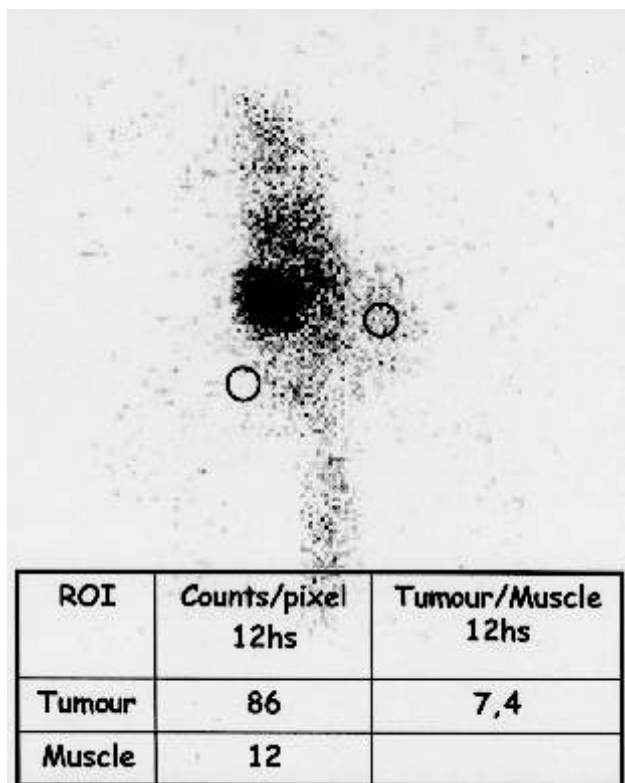
Complexes *Tc1-Tc4*, purified by HPLC and diluted in saline: methanol 75:25 were injected intravenously. Table 1 shows percentage dose per organ in the most significant organs and tissues as a function of time. All complexes have high initial blood, lung and liver uptake (4.3-5.7%, 1.6-3.2%, 27.3-35.3, respectively) as expected from lipophilic compounds but clearance after 12-24 hours is almost

complete. Excretion occurs mainly through the hepatobiliary tract, determining high intestinal activity (aprox. 30-60% at 6 hs. post-inj). Urinary elimination is lower (15-30% at 6 hs. post-inj). Total elimination within the studied period is very high (61-88.5% of the injected dose within 24 hours). Stomach and thyroid values are within acceptable levels (aprox. 1-3% and 0.1-0.2%, respectively at 30 min. and 0.1-1% and 0.01-0.02% respectively at 24 hs.) demonstrating minimal "in vivo" reoxidation. Figure 3 shows general biodistribution profile for complex *Tc2*.

Table 2 summarises tumour uptake (expressed as percentage of dose/g) as well as tumour/blood and tumour/muscle ratios. All complexes show relatively high



**Figure 4.** Tumour uptake and comparison to blood and muscle of complexes Tc1-Tc4 at 24 hours post-injection.



**Figure 5.** Gamma-camera image of complex Tc2 in C57B16 mice at 12 hours post-injection

initial tumour uptake (0.8–3.4 % dose/g at 2 hs. p/inj.) and significant retention until 24 hours (0.450.88 % dose/g at 2

hs. p/inj.). Blood and soft tissue clearance is fast, and this is reflected in tumour/blood and tumour/muscle ratios, which are very favourable from 6 to 24 hours after injection. Figure 4 shows comparative tumour uptake data for complexes Tc1-Tc 4 at 24 hours post-injection. Although complexes Tc2 and Tc3 exhibit the highest tumour uptake at all biodistribution times, the best tumour/blood and tumour/muscle ratios at 12 and 24 hours post-injection correspond to complex Tc2 (1.9-2.4 and 7.5-12.0, respectively) due to its faster blood clearance.

Table 3 shows biodistribution results for complex Tc2, after subcutaneous administration in the peritumoral region. General biodistribution and excretion data are expressed as percentage Dose per organ, while uptake in tumour, blood and soft tissue together with tumour/blood and tumour/muscle ratios are calculated from percentage of dose/g. Comparison with data obtained after intravenous injection of complex Tc2 (Tables 1 and 2) demonstrate that although a significant amount of radioactivity also reaches circulation after peritumoral administration, a very high percentage of the injected dose remains in tumour even 24 hours after administration (21.5%/g). Tumour/ blood ratios are 10–15 times higher and Tumour/soft tissue 25-30 times higher.

#### Gamma-camera imaging

Static gamma-camera images were acquired for complex Tc2 at 2 and 12 hours after intravenous administration. Early and late gamma-camera studies allowed clear delineation of the tumour and tumour/soft tissue ratio calculated from images was similar to that obtained from

| ORGAN        | % Dose/ organ $\pm$ $n-1$ $n=3$ ) |                  |
|--------------|-----------------------------------|------------------|
|              | 12 hours                          | 24 hours         |
| Blood        | 2.5 $\pm$ 1.4                     | 1.9 $\pm$ 0.9    |
| Liver        | 1.6 $\pm$ 0.6                     | 1.3 $\pm$ 0.2    |
| Lungs        | 0.87 $\pm$ 0.03                   | 0.07 $\pm$ 0.001 |
| Spleen       | 0.016 $\pm$ 0.006                 | 0.03 $\pm$ 0.01  |
| Kidneys      | 0.7 $\pm$ 0.2                     | 1.0 $\pm$ 0.2    |
| Stomach      | 0.54 $\pm$ 0.07                   | 1.2 $\pm$ 0.4    |
| Intestine    | 9.5 $\pm$ 0.1                     | 7.9 $\pm$ 0.3    |
| Urine        | 25.4 $\pm$ 6.9                    | 31.7 $\pm$ 2.0   |
| Faeces       | 21.1 $\pm$ 5.9                    | 21.9 $\pm$ 1.4   |
|              | % Dose/g $\pm$ $n-1$ $n=3$ )      |                  |
| Blood        | 0.6 $\pm$ 0.2                     | 1.0 $\pm$ 0.3    |
| Tumor        | 19.5 $\pm$ 0.5                    | 21.5 $\pm$ 1.3   |
| Muscle       | 0.12 $\pm$ 0.06                   | 0.06 $\pm$ 0.005 |
| Tumor/Blood  | 32.5                              | 17.9             |
| Tumor/muscle | 195                               | 358              |

**TABLE 3.-** Biodistribution results after subcutaneous peritumoral administration of complex Tc2

biodistribution studies. Figure 5 shows a representative image acquired at 12 hours post-injection.

## Discussion

“3+1” mixed ligand complexes are formed by simultaneous coordination of a tridentate ligand and a monodentate coligand on an oxotechnetium or oxorhenium precursor. These complexes are quite flexible, giving the possibility of tailoring the physicochemical and biological properties through changes introduced in the structure either of the ligand or the coligand.

Using this strategy a number of potential radiopharmaceuticals for brain perfusion, neuroreceptor imaging, etc., have been designed (27,28,29). The present study proposes the application of the “3+1” mixed ligand approach to the development of melanoma-targeting agents. The combination of the tridentate aminothiols N,N-bis(2-mercaptoethyl)-N',N'-diethylethylene-diamine with 4 different monodentate thiols result in the formation of complexes that mimic N-alkylsubstituted benzamides, compounds with high affinity for melanoma (Figure 1). Coligands 1-3 are aromatic thiols having either hydrogen, iodine or methoxy group in para position of the aromatic ring and were selected in order to study the effect of substituents in melanoma uptake. Coligand 4 is an aliphatic thiol and will allow to determine if the aromatic ring is important for “in vivo” behaviour.

Tc-99m labelling was performed by substitution using glucoheptonate as precursor, by a well known procedure (25). The expected compounds were obtained with high yield and radiochemical purity.

The structure of this type of compounds has been extensively studied using nonradioactive rhenium analogs as model for technetium. Rhenium, as technetium's third row congener, exhibits many of the chemical properties of technetium and consequently Tc and Re complexes with identical ligands have essentially identical coordination parameters. The tridentate ligand coordinates on the  $(MO)^{+3}$  core through the 2 thiol groups and the tertiary amine leaving an open coordination site cis to the oxo group, that is occupied by the monodentate thiol. The final complex is neutral due to ionization of both ligand and coligand.

Identity of Tc-99m compounds is usually corroborated by simultaneous injection with analogous rhenium complexes on HPLC. Radioactivity (for Tc-99m) and UV-vis (for Re) detection is used and identical chromatographic profiles demonstrate that both complexes have the same chemical structure.

Biological evaluation was performed on C57B16 mouse bearing induced melanoma. This animal model has been widely used in the evaluation of potential melanoma targeting radiopharmaceuticals (16,17,18). Biodistribution results after intravenous administration of all Tc-99m complexes showed melanoma uptake and retention and favourable tumour/blood and tumour/muscle ratios from 6-24 hours post-injection, specially for complexes bearing an Iodine in the para position of the aromatic ring. Closer structural similarity to IBZA, a compound that displays

high melanoma affinity can justify the “in vivo” behaviour. In order to corroborate biodistribution results, static gamma-camera images were acquired for complex Tc2 at 2 and 12 hours post-injection. Gamma-camera images allowed visualisation of the tumour and calculated tumour/soft tissue ratios were consistent with those obtained from biodistribution studies. However, high gastrointestinal activity due to preferential hepatobiliary excretion is an important drawback for imaging purposes since liver and spleen are likely sites for the occurrence of metastases. Potential therapeutic application of systemically administered analogous Re-186 or Re-188 compounds are also precluded due to dosimetric considerations.

Another interesting alternative proposed lately for therapy of melanoma and other skin tumours is topical application of therapeutic radiopharmaceuticals. Skin patches containing Re-188 labelled tin particles or Y-90 ferric hydroxide macroaggregates retained by filtration or Ho-166 nitrate fixed in a polyurethane matrix have been prepared and evaluated in animal models with considerable success (30,31,32). Although our complexes are not adequate for topical application since they can cross the skin due to their lipophilicity, previously described results encouraged us to explore the possibility of the peritumoral subcutaneous administration to enhance tumour uptake and minimise background activity in other organs. This third option is not optimal for diagnosis but can eventually be used for therapy.

Complex Tc2, the one that gave the best tumour/blood and tumour/muscle ratios, was selected for this study. Long biodistribution times of 12 and 24 hours were chosen, in order to corroborate both tumour uptake and leakage of radioactivity to blood and other organs of interest, specially liver and gastrointestinal tract. Although a significant amount of radioactivity reaches circulation, a very high percentage of the injected dose remains in tumour even 24 hours after administration leading to very high tumour/blood and tumour/muscle ratios.

## Conclusion

A family of Tc-99m 3+1 mixed ligand complexes with potential for melanoma imaging was designed, prepared and evaluated *in vivo* in mice, bearing induced melanoma. The compounds were obtained with high yield and radiochemical purity. Biodistribution and Nuclear Medicine imaging results showed melanoma uptake and retention and favourable tumour/blood and tumour/muscle ratios from 6-24 hours post-injection, especially for complexes bearing an Iodine in the para position of the aromatic ring. In spite of promising melanoma uptake, lipophilicity determined high activity in other important organs, specially liver and gastrointestinal tract, a fact that will probably prevent diagnostic applications of Tc-99m complexes or therapeutic use of analogous rhenium compounds due to dosimetric considerations. In order to overcome this difficulty we proposed the subcutaneous peritumoral administration as an alternative, specially for

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therapeutic purposes.

High tumour uptake was achieved at 12 and 24 hrs after and ratios to other organs or tissues were very favourable. These results are promising and further developmental studies should include preparation of analogous Re-186 or Re-188 complexes and evaluation of their therapeutic potential, especially in early stages of melanoma growth.

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