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Original article

^{99m}Tc(I)/Re(I) tricarbonyl complexes for *in vivo* targeting of melanotic melanoma: Synthesis and biological evaluation

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ABSTRACT

The ^{99m}Tc (I) tricarbonyl complexes *fac*-[^{99m}Tc(κ^3-**L**)(CO)$_3$] (**Tc1**–**Tc6**) containing *N*-ethylpyrrolidine and *N,N*-diethylethylamine groups for melanin binding, were evaluated *in vitro* and *in vivo* as radioactive probes for the targeting of melanotic melanoma. Aiming at the modification of their size, topology and lipophilicity, **Tc1**–**Tc6** were obtained based on an S,N,O-donor bifunctional chelator (BFC) derived from cysteamine and on pyridyl- and pyrazolyl-containing N,N,O-donor BFCs. **Tc1**–**Tc6** were chemically identified by HPLC comparison with the Re congeners (**Re1**–**Re6**) that were synthesized at the macroscopic level and fully characterized by common analytical techniques. With the exception of **Tc5** and **Tc6**, these ^{99m}Tc complexes are moderately lipophilic, and bind to melanin with moderate to high affinity (23–87%). The cell uptake of **Tc1**–**Tc6**, expressed as a percentage of total activity per million cells, spanned between 0.86 and 21.02% for the melanotic B16-F1 cell line and between 0.49% and 13.58% for the amelanotic A375 cell line. In the B16-F1 cell line, **Tc1**, **Tc3** and **Tc4** showed moderate cellular uptake values (>10% at 4 h of incubation). In the amelanotic A375 cell line, only **Tc4** has shown a moderate cell uptake (>10% at 4 h of incubation), with all the other compounds displaying a relatively poor uptake, i.e. inferior to 5%. Competition studies with haloperidol have shown that the involvement of sigma receptors in cellular uptake and retention is likely to occur for **Tc4**. Complex **Tc1**, stabilized with the S,N,O-donor BFC and containing a *N,N*-diethylethylamine group, presented the most promising biological profile for *in vivo* targeting of melanoma, showing a moderate tumor uptake of 2.17% ID/g at 1 h p.i in a B16-F1 melanoma-bearing mouse and rather favorable target/non-target ratios with values as high as 16.9 and 5.2 for tumor/muscle and tumor/blood ratios, respectively.

1. Introduction

Malignant melanoma is one of the most lethal cancers due to its high cellular proliferation rate and the early occurrence of metastases. Around 160,000 new cases of melanoma are diagnosed in the world each year, and although procedures for effective treatment of melanoma are still not available, increased surveillance with early diagnosis and accurate staging of the disease is an important approach to increase survival [1,2]. The nuclear imaging techniques Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) may allow such early diagnosis, provided that radioactive probes are available for *in vivo* targeting of melanoma and its metastases.

At present, 2-[^{18}F]fluoro-2-deoxy-D-glucose (^{18}F-FDG) is the unique radioactive probe in clinical use for the detection of melanoma. However, ^{18}F-FDG is a nonspecific PET tumor imaging agent with serious limitations for the diagnostic of melanoma due to its poor sensitivity to detect micrometastic sites [3,4]. For this reason, alternative target-specific probes for the diagnostic of melanoma, by means of PET or SPECT, have been investigated in the past few years. In this area of research, particular effort has been devoted to the design of compounds having the ability to interact with intracellular melanin, which is a very attractive target for melanoma diagnosis due to the extensive pigmentation of most melanoma cells [5]. A considerable part of this research work involved ^{123}I-labeled benzamide derivatives, which led in some instances to encouraging results justifying their clinical evaluation as SPECT probes for *in vivo* detection of melanoma [6–13]. Radioiodinated benzamides seem to be localized in the melanocytes of pigmented cells and their uptake has been correlated to melanin binding,

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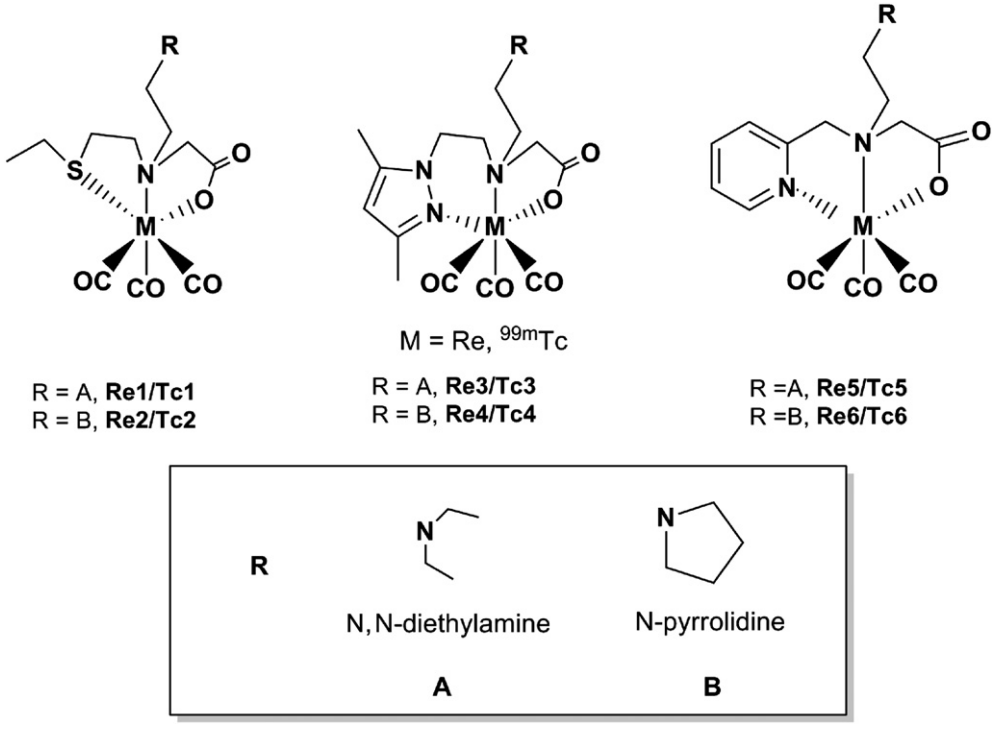
suggesting a non-receptor uptake mechanism that, however, is not fully understood [10,14–18].

^{123}I-labeled molecules present several disadvantages for clinical diagnosis by SPECT compared with ^{99m}Tc-based radiopharmaceuticals, since ^{99m}Tc has more favorable nuclear properties for routine clinical imaging and is available at lower costs from a ^{99}Mo-^{99m}Tc generator [19]. Therefore, several research groups embarked in the search of ^{99m}Tc complexes for the *in vivo* detection of melanoma in alternative to radioiodinated benzamides. This involved mainly the synthesis of ^{99m}Tc complexes functionalized with benzamide derivatives or their fragments, with the aim of obtaining compounds still behaving as melanin binders and with the ability to reach this pigment at intracellular level. For this purpose, a variety of complexes have been studied, including Tc(V) complexes with the $[^{99m}TcN]^{2+}$ [20–22] and $[^{99m}TcO]^{3+}$ [19,23–25] cores stabilized by N_2S_2 tetradentate BFC's, [3 + 1] mixed ligand Tc(V) oxocomplexes [26,27] and, more recently, organometallic complexes with the *fac*-$[^{99m}Tc(CO)_3]^{+}$ [12,28] core. None of these complexes has emerged as a good alternative to radioiodinated benzamides, despite some encouraging results found for Tc(V) oxocomplexes bearing the *N,N*-diethylamine group as a melanin-binding pharmacophore.

Looking for compounds with interest for the design of SPECT probes for the *in vivo* detection of melanotic melanoma, our research group has described recently Re(I) and Tc(I) complexes stabilized by pyrazole-diamine and pyrazole-aminocarboxylic chelators, acting respectively as (N,N,N) and (N,N,O) donors, and containing the 4-amino-*N*-(2-diethylaminoethyl)benzamide group or its fragments [28]. Most of these complexes have shown a hydrophilic character and high *in vitro* affinity for melanin, but presented a low *in vitro* cell uptake in murine melanoma cells, as well as a negligible tumor uptake in melanoma-bearing mice. Searching for complexes with a better ability to target intracellular melanin in melanotic melanoma cells, we have focused on related Re(I) and ^{99m}Tc(I) tricarbonyl complexes (**Re1** and **Tc1**, respectively) (Scheme 1) of low molecular weight and with augmented liphophilicity, anchored by a new linear (N,S,O)-donor ligand framework derived from cysteamine [29]. The preliminary *in vitro* evaluation of **Tc1** has shown that this complex displays a moderate cell uptake in B16-F1 murine melanoma cells. These encouraging findings prompted us to pursue with the biological evaluation of **Tc1**, and to extend our studies to congener neutral complexes of pyridyl- and pyrazolyl-containing (N,N,O)-donor bifunctional chelators [30,31] functionalized with N,N-diethylamine and *N*-pyrrolidine groups for melanin binding (Scheme 1). By exploring different classes of chelators, we expected to have a better ability to tune the physico-chemical properties of the final complexes, such as size, topology and lipophilicity, which can be determinant of their ability to freely diffuse across the cell membrane and to interact with the putative cytosolic target. In these complexes, the metallic chelate containing the *fac*-$[^{99m}Tc(CO)_3]^{+}$ unit would replace the aromatic part of benzamides while the pendant and protonable *N,N*-dialkylalkyl group should enhance their affinity towards the melanin pigment.

Herein, we report on the synthesis and characterization of new Re (**Re2**–**Re6**) and ^{99m}Tc (**Tc2**–**Tc6**) tricarbonyl complexes, anchored by (S,N,O)- or (N,N,O)-donor BFCs and bearing *N,N*-diethylamine and *N*-pyrrolidine groups that have been linked at the central amine of the chelators using an ethylenic linker (Scheme 1). We also compare the biological behavior of **Tc2**–**Tc6** with the one exhibited by the previously reported **Tc1**, aiming to have an insight into their relevance for the design of ^{99m}Tc-labeled probes for *in vivo* targeting of melanotic melanoma. To obtain such insight, the biological evaluation of these ^{99m}Tc complexes comprised the measurement of their binding affinity to synthetic melanin, cellular uptake studies in the melanotic murine B16-F1 cell line and the amelanotic A375 human cell line, as well as biodistribution studies in melanoma-bearing C57BL/6 female mice. In addition, competition studies with haloperidol, a nonselective σ1–σ2 inhibitor, were also performed to ascertain the possible influence of sigma receptors in the cell uptake of the complexes.



Scheme 1. Molecular structures of the Re and ^{99m}Tc complexes containing *N,N*-diethylethylamine and *N*-ethylpyrrolidine groups for melanin binding.

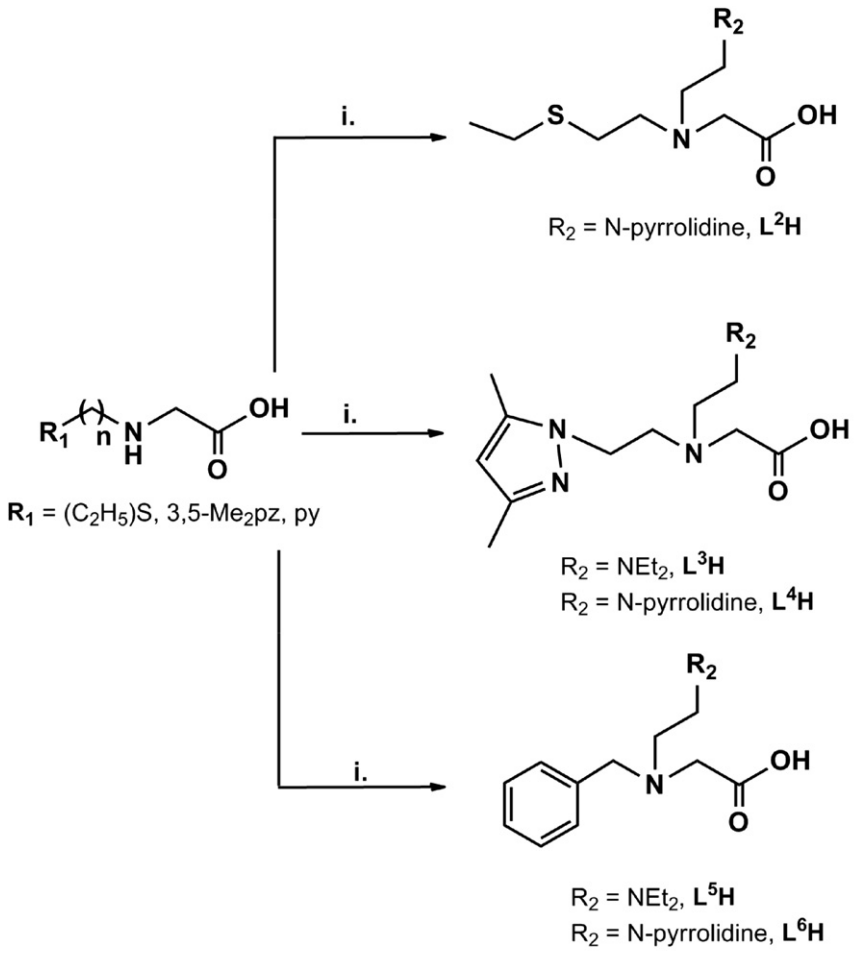
2. Results and discussion

2.1. Synthesis and characterization of the bifunctional chelators and respective Re(I) and ^{99m}Tc(I) tricarbonyl complexes

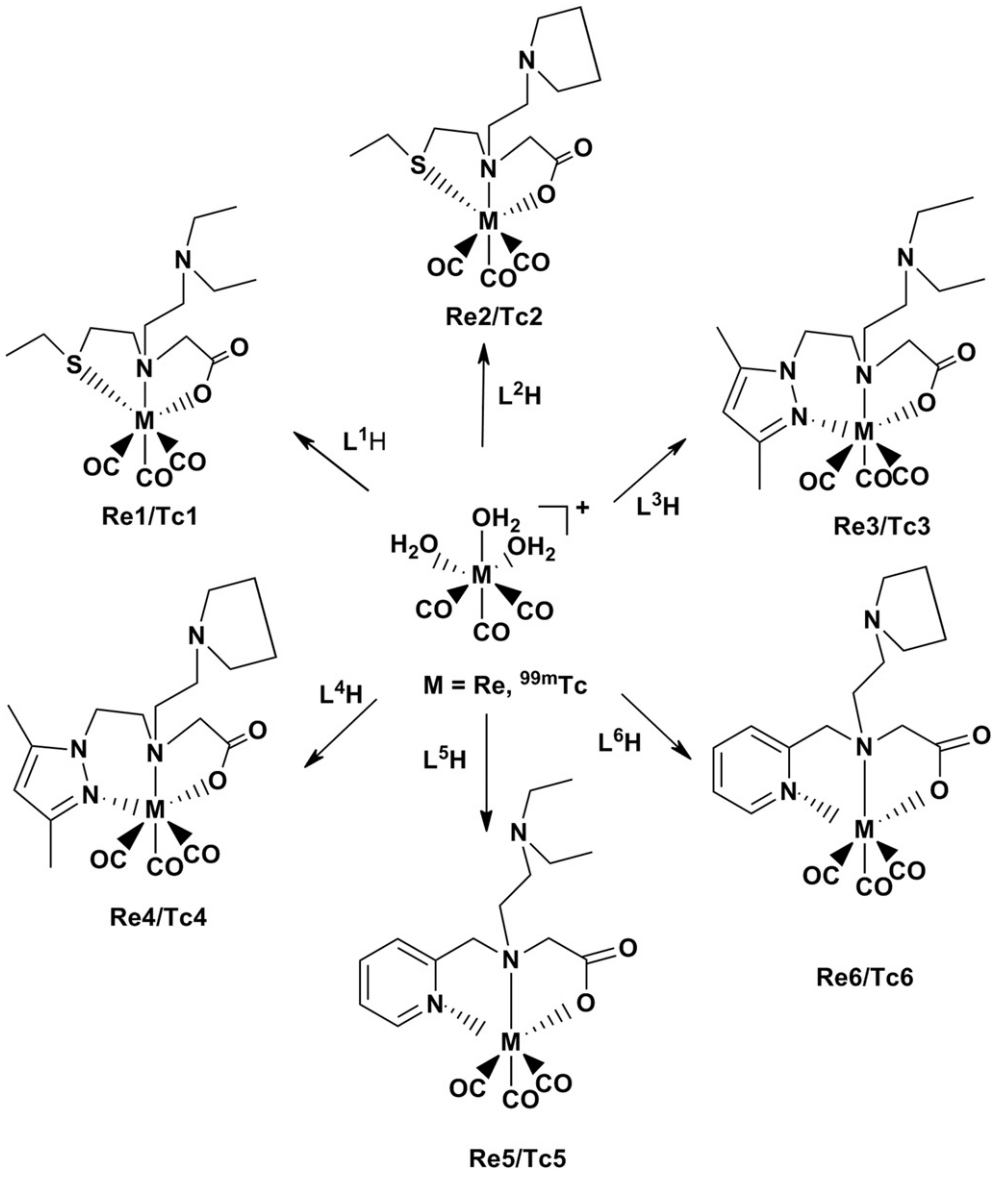
The functionalization of the three classes of (S,N,O)- or (N,N,O)-donor BFCs with the two different *N,N*-dialkylalkyl groups, *N,N*-diethylethylamine and *N*-ethylpyrrolidine, has been done in a convergent way using a common synthetic approach. As shown in Scheme 2, this involved the N-alkylation of the central amine of each chelator with an appropriate chloro derivative of the *N*-dialkylalkyl groups. These *N*-alkylation reactions were performed under reflux in a THF/H_2O mixture at pH 12, by reacting 2-[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethylamino]acetic acid, 2-[(pyridin-2-yl)methylamino]acetic acid or 2-[2-(ethylthio)ethylamino]acetic acid with 1-(2-chloroethyl)pyrrolidine or 2-chloro-*N,N*-diethylethanamine (Scheme 2). The resulting new ligands, **L^2H**–**L^6H**, were obtained in low to moderate yield (15–36%) after adequate work-up.

The new chelators, **L^2H**–**L^6H**, were reacted with *fac*-[$Re(H_2O)_3(CO)_3$]Br in refluxing methanol, affording the Re(I) tricarbonyl complexes **Re2**–**Re7** (Scheme 3). **Re2**–**Re7** were purified by column chromatography or by RP-HPLC, being recovered in low to moderate yield (25%–71%). These Re complexes were applied as reference compounds to assign the chemical identity of the ^{99m}Tc congeners by means of HPLC comparison, a common and well accepted practice in radiopharmaceutical chemistry due to the physico-chemical similarities of these group 7 metals.

All the new compounds, **L^2H**–**L^6H** and **Re2**–**Re6**, were characterized by the common spectroscopic techniques (IR, ^{1}H and ^{13}C NMR), by ESI-MS and elemental analysis. The collected analytical data confirmed the proposed formulations. In particular, the IR spectra of **Re2**–**Re6** showed the presence of two strong ν(C≡O) bands characteristic of the *fac*-$[Re(CO)_3]^+$ moiety, with values ranging from 1866 to 2037 cm^{-1} and comparable to those previously reported for other Re(I) tricarbonyl complexes anchored by the same BFCs [30,32]. The ^{1}H NMR spectra of **Re3**–**Re6** showed the presence of a set of well-defined multiplets in the aliphatic region, between 2.59 and 4.80 ppm, presenting a splitting pattern and relative intensities consistent with the diastereotopic character of the methylenic protons from the coordinating backbone of the respective (N,N,O)-donor chelators. In these spectra, the aromatic resonances from the pyridyl and pyrazolyl rings range between 7.53-8.83 ppm and 5.83–5.84 ppm, respectively, being downfield shifted compared to the same resonances in the corresponding free ligands. Altogether, these data corroborate the tridentate and facial



Scheme 2. Synthesis of the ligands.



Scheme 3. Synthesis of the Re and ^{99m}Tc complexes.

coordination of $\mathbf{L^2}$–$\mathbf{L^6}$. For complex **Re2**, the ^{1}H and ^{13}C NMR spectra recorded at room temperature indicated the presence of two interconverting species, due to sulfur inversion as we have reported for the similar compound, **Re1**, containing the same S,N,O-donor atom set but bearing a *N,N*-diethylamine substituent. The ^{1}H NMR spectra of **Re2** were run at variable temperature, from −40 °C to 60 °C , but the effect of the temperature on the resonances of each invertomer could not be followed properly due to the complexity of the spectra. The ^{13}C NMR spectrum obtained at 20 °C presented two relatively narrow resonances for each methylenic carbon in agreement with the presence of two interconverting invertomers. By raising the temperature to 60 °C, each pair of these resonances merged to single signals, which are relatively narrow for the $\underline{C}H_2$ atoms of the linker and *N*-pyrrolidine ring but are rather broad for the methylenic carbons linked to the coordinating S, N, and O atoms. These data show that a fast-exchange limit spectrum could not be obtained at this temperature, indicating also that the sulfur inversion affects more strongly the chemical environment of the atoms that are closer to the metallic center.

The ^{99m}Tc complexes, **Tc2**–**Tc6**, were synthesized in aqueous solution at pH 5.5 by reaction of *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$ with the appropriate ligand ($\mathbf{L^2}$–$\mathbf{L^6}$) at 100 °C for 30 min, as previously reported for **Tc1** [29]. Under these conditions, all complexes were obtained with a high radiochemical yield (≥95%) being used in the *in vitro* and *in vivo* biological studies without further purification. The chemical identification of **Tc2**–**Tc6** has been done by comparison of their HPLC profiles with those of the corresponding rhenium complexes (**Re2**–**Re6**). The *in vitro* evaluation of **Tc2**–**Tc6** involved the measurement of their lipophilicity and the evaluation of their binding to synthetic melanin (Table 1).

The lipophilicity, expressed as the distribution coefficient (log $D_{o/w}$) in octanol/0.1 M phosphate buffer (pH 7.4), was determined using the multiple back-extraction method [33]. With the exception of **Tc5** and **Tc6**, displaying log $D_{o/w}$ values of 0,00 ± 0,01 and −0,02 ± 0,03 respectively, all the other complexes are lipophilic with log $D_{o/w}$ values ranging between 0.87 and 1.12.

The measurement of the *in vitro* affinity of **Tc2**–**Tc6** to melanin involved the incubation of the complexes at room temperature for

Table 1
HPLC retention times, log D values and *in vitro* binding to synthetic melanin for complexes **Tc1**–**Tc6**.

Compound	Retention time (min)[a,b]	log $D_{o/w}$ (pH 7.4)	% Bound to synthetic melanin
Tc1[29]	17.80 (17.80)	0.53 ± 0.01	23 ± 2
Tc2	16.83 (16.85)[a]	1.12 ± 0.06	36 ± 2
Tc3	18.85 (18.82)[a]	0.87 ± 0.03	74 ± 2
Tc4	17.76 (17.56)[a]	1.10 ± 0.03	87 ± 3
Tc5	16.40 (16.35)[a]	0.00 ± 0.01	48 ± 4
Tc6	15.50 (15.40)[a]	−0.02 ± 0.03	62 ± 3[c]

[a] Using a gradient of aqueous 0.1% CF_3COOH and methanol as the solvent.
[b] The values in parentheses are for the Re complexes **Re2**–**Re7**.
[c] Concentration of melanin 0,5 mg/10 mL, 1 h incubation.

1 h with a suspension of synthetic melanin in distilled water, as described elsewhere [28]. The percentage of binding, spanning between 36 and 87%, depends very much on the donor-atom set ((S,N,O) *vs* (N_{pz},N,O) *vs* (N_{py},N,O)) of the BFC used to stabilize the organometallic core, being less influenced by the nature of the pendant tertiary amine. The complexes with the (S,N,O)-donor ligands, **Tc2** and the previously reported **Tc1** [29], presented the lowest affinity to melanin with binding percentages of 36 ± 2% and 23 ± 2%, respectively. **Tc3** and **Tc4** anchored by pyrazolyl-containing (N,N,O)-donor chelators have shown the highest binding affinity with values of 74 ± 2% and 87 ± 3%, respectively. Melanin is a polymer rich in negatively charged groups and melanin-binders interact with this pigment mainly through ionic interactions. However, such ionic interactions can be strengthened by other attractions such as van der Waals and/or charge-transfer processes involving the aromatic structures of the melanin-binders and the indole monomers of melanin [16]. Probably, the occurrence of charge-transfer processes between the pyrazolyl rings of **Tc3** and **Tc4** and the indole groups of melanin can account for the higher melanin affinity observed for these complexes.

2.2. Cell uptake studies

Cellular uptake studies were performed for **Tc1**–**Tc6** in the melanotic murine B16-F1 cell line *versus* the amelanotic A375 human cell line, in order to have an insight into the role of melanin in the cellular accumulation of the complexes. The B16-F1 and A375 melanoma cells were incubated with the radioactive complexes at different time points, up to 240 min. As can be seen in Fig. 1, the cell uptake at 37 °C was time-dependent and showed plateau values spanning between 0.86 and 21.02% for the B16-F1 cell line and between 0.49% and 13.58% for the A375 cell line, when expressed as a percentage of total activity per million cells.

In the B16-F1 cell line, **Tc1**, **Tc3** and **Tc4** showed moderate cellular uptake values (>10% at 4 h of incubation), being **Tc4** the complex with the best ability to accumulate in this cell line (uptake of 21.02 ± 0.71% at 4 h). In the amelanotic A375 cell line, **Tc4** has shown a moderate cell uptake (>10% at 4 h of incubation), but all the other compounds have shown a relatively poor uptake, i.e. inferior to 5%. **Tc6** showed the lowest cell uptake in both cell lines with almost negligible uptake values (<1% at 4 h of incubation), which most probably reflects its hydrophilic character. All tested complexes have shown a higher uptake in the melanotic cell line compared to the amelanotic cell line. This finding might indicate that the cellular retention of the compounds involves their interaction with intracellular melanin. However, it is possible that other intracellular targets could play a role in the cell accumulation of the compounds, since **Tc1** presented the lowest *in vitro* affinity for melanin (23 ± 2%) but still displayed a reasonable cell uptake.

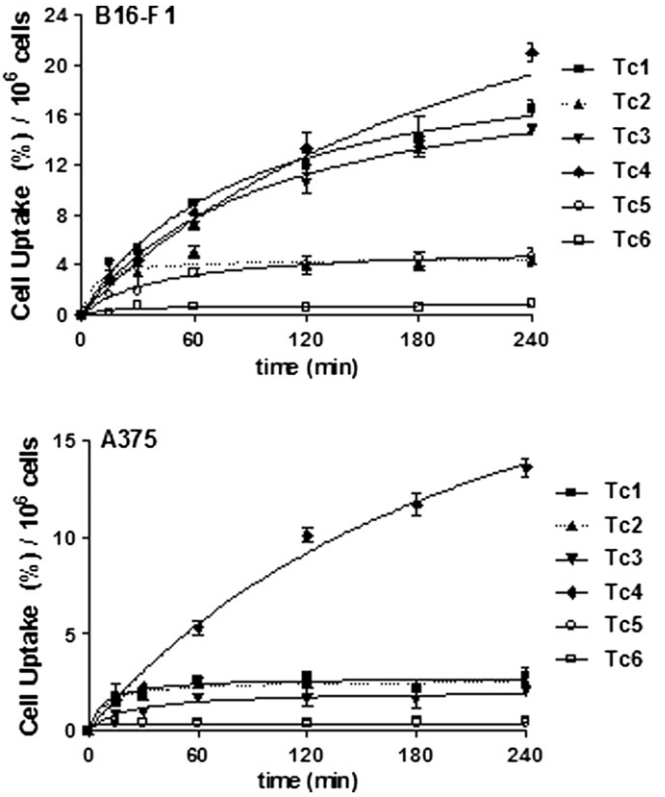


Fig. 1. Cell uptake of **Tc1**–**Tc6** in murine B16-F1 melanoma cells and in human amelanotic A375 melanoma cells, expressed as percentage of applied radioactivity per million cells.

It has been shown that benzamide derivatives, particularly those containing *N*-pyrrolidinyl substituents as is the case of **Tc4**, can recognize σ-receptors [15,34–36], which are a class of proteins existing in at least two subtypes (σ_1 and σ_2) [37]. The function of these receptors is poorly understood but it is known that they are over-expressed in different neoplastic tissues, namely in melanoma cell lines [15,36,37]. Hence, we decided to evaluate if σ-receptors could be involved in the cell uptake of the complexes reported herein. Such evaluation has been performed only for the complexes which presented a moderate cell uptake, i.e. **Tc1**, **Tc3** and **Tc4** in the B16-F1 cell line and **Tc4** in the A375 cell line, and involved competition studies using haloperidol. Haloperidol is a non selective inhibitor of both σ_1 and σ_2 receptor subtypes [38]. It is well known that the A375 amelanotic melanoma cell line expresses high levels of σ-receptors, as described in the literature [39]. However, to the best of our knowledge, it has not been reported previously the presence or not of σ-receptors in the B16-F1 cell line. Therefore, we have evaluated the levels of σ_1-receptor expression in the B16-F1 cell line by Western blot analysis using a commercially available antibody, as described in the experimental section. As a control, the Western blot analysis was also performed for A375 and other tumor cell lines (PC-3 [40] and MCF-7 [39,41]) that are known to express σ-receptors. The results obtained are presented in Fig. 2 and confirmed the presence of a 30 KDa band corresponding to the σ1 receptor, in all cell lines, including the B16-F1.

The difference in size between the band in the A375 cell line sample and the band in the B16-F1 samples is due to the occurrence of splicing variants of the receptor (which present slighty different molecular weights) [37,40,41].

As can be seen in Fig. 3, the incubation with haloperidol led to a decrease of the cellular uptake of **Tc1**, **Tc3** and **Tc4** in the B16-F1

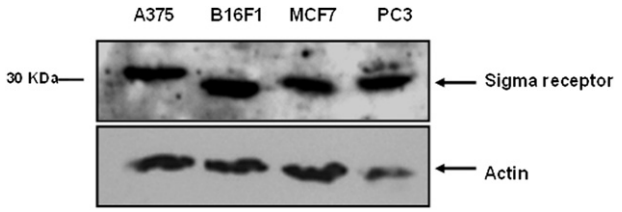


Fig. 2. Evaluation of sigma 1 receptor expression in different cancer cell lines by western blot analysis.

cell line for concentrations of haloperidol $>10^{-9}$ M, being such decrease more prominent for **Tc4**. **Tc4** has shown an even more pronounced decrease of cellular uptake in the A375 cell line, which started for concentrations of haloperidol as low as 10^{-14} M (Fig. 3). Haloperidol is a neuroleptic with potential toxic effects that can affect the cell viability, as reported earlier for the M4 Beu cell line [7]. Therefore, the changes on the cellular uptake of the ^{99m}Tc complexes could arise from alterations in cell viability. To discard this possibility, we have checked the eventual cytotoxic effects of haloperidol against the B16-F1 and A375 cell lines, in the range of haloperidol concentrations (10^{-14}–10^{-4} M) used in the competition studies. The cell viability was evaluated after incubation with different concentrations of haloperidol, at 37 °C for 4 h, using a colorimetric method based on the tetrazolium salt MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]). The MTT assays showed that the cytotoxic effects of haloperidol occur uniquely for concentrations higher than 10^{-4} or 10^{-5} M for the B16-F1 and A375 cell lines, respectively. Hence, the decrease of the cellular uptake of the ^{99m}Tc complexes in both cell lines is not due to any alteration of the cell viability but probably results from the blockade of σ receptors by haloperidol. These findings indicate that the cellular uptake of these complexes may involve the interaction with sigma receptors, particularly in the case of **Tc4**.

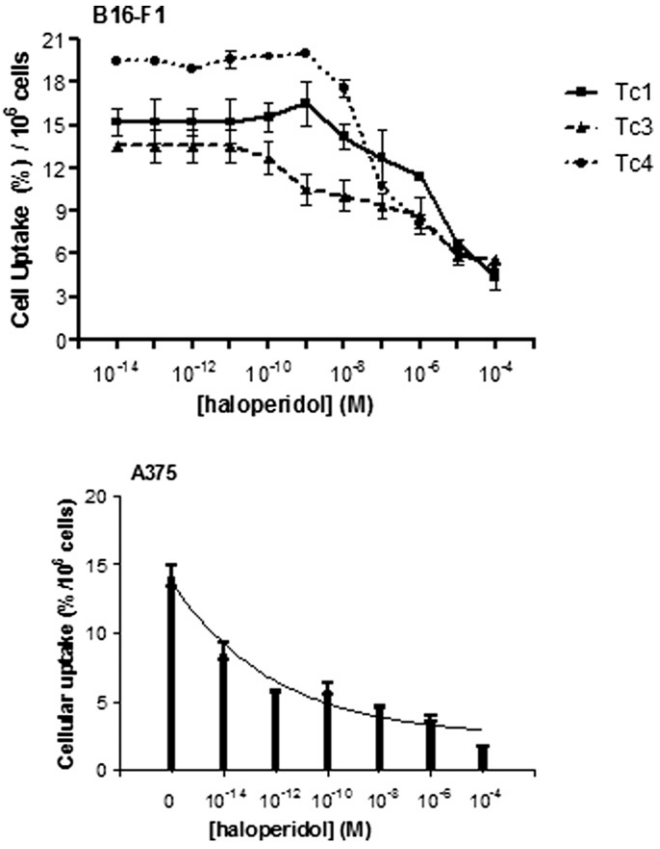


Fig. 3. Effect of haloperidol concentration in the cell uptake of complexes **Tc1**, **Tc3** and **Tc4** in murine B16-F1 melanoma cells and in the cell uptake of complex **Tc4** in human amelanotic A375 melanoma cells.

2.3. Biodistribution and imaging studies

The *in vivo* stability and the biodistribution of complexes **Tc1**–**Tc6** were studied in B16-F1 melanoma-bearing mice, in order to have a further insight into their relevance to design radioactive probes for the targeting of melanotic melanoma.

The *in vivo* stability of **Tc1**–**Tc6** has been assessed by HPLC analysis of the urine and blood of mice injected with these complexes. These studies have shown that the intact complexes correspond to the major part of the activity detected in the plasma and in the urine, indicating that **Tc1**–**Tc6** are quite resistant to metabolic transformation (Fig. S1).

The biodistribution data obtained for **Tc1**–**Tc6** at 1 h and 4 h p.i, expressed in percent injected dose per gram tissue (%ID/g), are presented in Table 2. All the complexes presented a relatively fast blood clearance and a moderate rate of excretion, with low retention of radioactivity in non-target tissues, with the exception of the organs involved in the excretion of the compounds, i.e. kidney, liver and intestine. The biodistribution data indicate that **Tc1**–**Tc6** undergo both urinary and hepatobiliary excretion, being however the hepatobiliar excretory pathway dominant as indicated by the highest accumulation of activity in the intestine that spans between 19.44 and 28.5 %ID/g at 4 h p.i.

Complexes **Tc1**–**Tc6** showed low to moderate tumor uptake values in the range 0.17 ± 0.06 to 2.17 ± 0.42 %ID/g at 1 h p.i. **Tc5** and **Tc6**, stabilized by pyridyl-containing BFCs, have shown the poorest ability to target *in vivo* melanoma tissues in agreement with their almost negligible *in vitro* cell uptake. **Tc1** and **Tc4,** which have shown the highest *in vitro* cell uptake, exhibited also the highest tumor uptake with values of 2.17 ± 0.42 and 1.74 ± 0.24 %ID/g at 1 h p.i., respectively. **Tc1** has shown higher tumor retention than **Tc4**, being observed a tumor uptake of 1.69 ± 0.35 and 1.22 ± 0.29 %ID/g at 4 h p.i. for **Tc1** and **Tc4**, respectively. At 1 h p.i., the tumor uptake of **Tc1** is roughly two-fold higher than the tumor uptake of the best performing ^{99m}Tc(I) tricarbonyl complexes, containing the 4-amino-*N*-(2-diethylaminoethyl)benzamide pharmacophore, previously reported by our research group [28].

The highest tumor uptake found for **Tc1**, together with its favorable target/muscle ratio, prompted the use of this complex for imaging studies in a B16-F1 melanoma-bearing mice. As can be seen in Fig. 4, **Tc1** clearly detected a melanoma tumor with low retention of radioactivity by the surrounding non-target tissues. However, a considerable accumulation of radioactivity was also visualized in the gastrointestinal tract as expected from the biodistribution results.

3. Conclusion

Within our interest on ^{99m}Tc(I) organometallic complexes as potential SPECT probes for *in vivo* imaging of melanotic melanoma, we have evaluated a series of complexes (**Tc1**–**Tc6**) with (S,N,O)- and (N,N,O)-donor BFCs, bearing thioether, pyridyl or pyrazolyl coordinating functions and functionalized with *N*-diethyl and *N*-pyrrolidine groups for melanin binding. All the new ^{99m}Tc complexes were obtained in high yield, and their chemical identity was assessed by HPLC comparison with the Re congeners (**Re1**–**Re6**). The ability of **Tc1**–**Tc6** to target melanoma cells, *in vitro* or *in vivo*, is quite dependent on the nature of the BFCs. **Tc5** and **Tc6**, stabilized with the pyridyl-containing (N,N,O)-chelators, presented

Table 2
Relevant biodistribution data (% ID g^{-1}) of **Tc1**–**Tc6** at 1 and 4 h post-injection (p.i.) in C57/B16 mice ($n = 3–5$) with palpable hind limb B16 melanoma nodules.

Mean tissue concentrations (%ID/g organ)									
^{99m}Tc complex	Time (h, pi)	Muscle	Blood	Eyes	Tumor	Kidneys	Liver	Intestine	Excretion (% ID)
Tc1	1	0.42 ± 0.07	0.87 ± 0.13	1.54 ± 0.15	2.17 ± 0.42	2.39 ± 0.13	4.96 ± 0.40	20.59 ± 1.36	31.6 ± 11.1
	4	0.10 ± 0.01	0.40 ± 0.05	1.41 ± 0.27	1.69 ± 0.35	1.32 ± 0.34	3.66 ± 0.69	19.44 ± 1.38	56.9 ± 7.2
Tc2	1	0.37 ± 0.05	0.68 ± 0.21	1.38 ± 0.30	1.44 ± 0.32	1.64 ± 0.17	3.53 ± 0.80	22.92 ± 2.49	33.70 ± 2.48
	4	0.05 ± 0.01	0.25 ± 0.05	0.82 ± 0.56	0.89 ± 0.38	0.67 ± 0.23	1.36 ± 0.24	17.55 ± 4.07	51.10 ± 4.62
Tc3	1	0.88 ± 0.08	0.99 ± 0.17	1.71 ± 0.40	0.99 ± 0.19	3.92 ± 0.95	5.46 ± 1.54	25.91 ± 1.51	16.5 ± 12.6
	4	0.16 ± 0.08	0.41 ± 0.12	1.11 ± 0.19	0.79 ± 0.76	1.10 ± 0.26	2.86 ± 1.03	23.41 ± 2.78	37.3 ± 11.5
Tc4	1	0.88 ± 0.24	0.82 ± 0.21	1.34 ± 0.24	1.74 ± 0.24	7.21 ± 2.09	3.72 ± 0.53	26.80 ± 2.06	28.25 ± 2.32
	4	0.15 ± 0.02	0.26 ± 0.08	1.24 ± 0.10	1.22 ± 0.29	1.35 ± 0.28	2.19 ± 1.09	28.56 ± 2.15	41.18 ± 5.78
Tc5	1	0.63 ± 0.22	0.48 ± 0.13	0.56 ± 0.05	0.97 ± 0.15	4.77 ± 0.68	4.24 ± 0.64	18.74 ± 1.9	51.5 ± 3.0
	4	0.22 ± 0.06	0.25 ± 0.06	0.63 ± 0.19	0.81 ± 0.20	2.53 ± 0.35	2.31 ± 0.45	21.39 ± 2.04	58.2 ± 7.7
Tc6	1	0.51 ± 0.13	0.79 ± 0.15	0.89 ± 0.17	0.42 ± 0.06	3.10 ± 0.52	6.29 ± 0.69	17.39 ± 0.53	47.0 ± 2.9
	4	0.28 ± 0.06	0.33 ± 0.08	1.49 ± 1.05	0.27 ± 0.05	1.81 ± 0.23	5.08 ± 1.30	21.84 ± 6.71	50.3 ± 14.0

the lowest *in vitro* cell uptake and a very poor *in vivo* tumor uptake, most probably due to the low log $D_{o/w}$ values of these complexes. **Tc1** and **Tc4**, containing respectively a cysteine-based (S,N,O)-chelator and a pyrazolyl-containing (N,N,O)-chelator, have shown the more favorable biological profile for *in vivo* targeting of melanotic melanoma, particularly **Tc1** which has shown the highest tumor uptake and encouraging target/non-target ratios. The reasons for the highest tumor uptake observed for **Tc1** are not clear, since **Tc1** presented the lowest binding affinity towards melanin among all the tested compounds. An alternative to justify the best biological performance of **Tc1** could be the involvement of σ receptors. However, such possibility seems more probable for **Tc4**, as shown by competitive studies with haloperidol. **Tc1** allowed the visualization of a melanotic melanoma in a tumor-bearing mice but presented a moderate tumor uptake and a relatively high accumulation in the gastrointestinal tract. Therefore, similarly to other ^{99m}Tc complexes reported in the literature, **Tc1** is not an alternative to radioiodinated benzamides. Nevertheless, our results showed that ^{99m}Tc complexes stabilized with a new (S,N,O)-donor BFC derived from cysteamine have an enhanced ability to accumulate in tumor cells, most probably due to their small size, lipophilicity and favorable topology. These findings indicate that this new BFC deserves to be further explored to obtain other ^{99m}Tc complexes containing different small molecules directed against intracellular targets with relevance for the detection of neoplastic tissues.

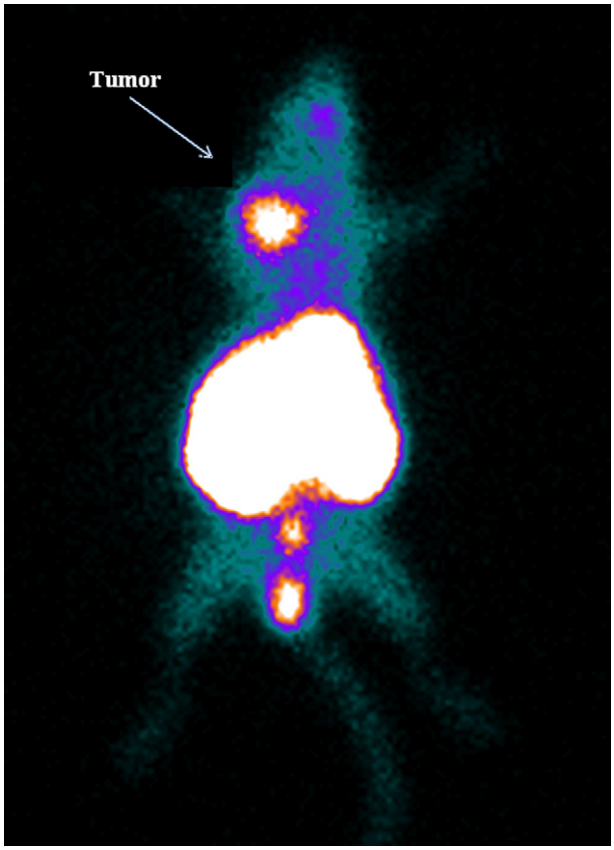


Fig. 4. Scintigraphic image of **Tc1** in C57/B16 mice with palpable hind limb B16 melanoma nodules.

4. Experimental procedures

Unless otherwise stated, the synthesis and work-up of the ligands and complexes were performed under air. The compounds 2-[2-(ethylthio)ethylamino]acetic acid [29], 2-[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethylamino]acetic acid [30], 2-[(pyridin-2-yl)methylamino]acetic acid [31] and 2-[(2-(diethylamino)ethyl)(2-(ethylthio)ethyl)amino]acetic acid (**L^1H**) [29] were prepared according to published methods. The starting material *fac*-[$Re(H_2O)_3(CO)_3$]Br [42] was synthesized by the literature method. Na[$^{99m}TcO_4$] was eluted from a commercial ^{99}Mo/^{99m}Tc generator, using 0.9% saline. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 300 MHz spectrometer; ^{1}H and ^{13}C chemical shifts are given in ppm and were referenced with the residual solvent resonances relative to $SiMe_4$. IR spectra were recorded as KBr pellets on a Bruker, Tensor 27 spectrometer. Electrospray ionisation mass spectrometry (ESI-MS) was performed at ITN on a QITMS instrument in positive ion mode. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates. Column chromatography was performed with silica gel 60 (Merck). HPLC analysis of the Re and ^{99m}Tc complexes was performed on a Perkin–Elmer LC pump 200 coupled to an LC 290 tunable UV–Vis detector and to a Berthold LB-507A radiometric detector, using an analytical Macherey–Nagel C18 reversed-phase column (Nucleosil 100–10, 250 × 3 mm) with a flow rate of 1 mL min^{-1}. HPLC purifications were performed using a preparative Waters μ Bondapak C18 column (150 × 19 mm) at a flow rate of 5.0 mL min^{-1}; UV detection, 254 nm; eluents, A – aqueous 0.1% CF_3COOH solution, B – MeOH. The HPLC analysis and purifications were done with gradient elution, using the following methods:

I) 0–5 min, 75% A; 5–30 min, 75%-50% A; 30–30.1 min, 50%-0% A; 30.1–38 min 0% A.
II) 0–3 min, 100% A; 3–3.1 min, 100%-75% A; 3.1–9 min, 75% A; 9–9.1 min 75%-66% A; 9.1–20 min, 66%-0% A; 20–25 min, 0% A; 25–25.1 min, 0%-100% A; 25.1–30 min, 100% A.

III) 0–3 min, 100% A; 3–3.1 min, 100%-75% A; 3.1–9 min, 75% A; 9–9.1 min 75%-66% A; 9,1–20 min, 66%-0% A; 20–25 min, 0% A; 25–25.1 min, 0%-100% A; 25.1–30 min, 100% A.

4.1. Synthesis of 2-[(2-(ethylthio)ethyl)(2-(pyrrolidin-1-yl)ethyl) amino]acetic acid, L^2H

To a solution of 2-[2-(ethylthio)ethylamino]acetic acid (579 mg, 3.50 mmol) in H_2O (15 mL) was added a solution of 1-(2-chloroethyl)pyrrolidine (595 mg, 3.50 mmol) in THF (15 mL). The pH was adjusted to 10–12 by addition of 5 M NaOH, and the reaction mixture was refluxed overnight. The solvent was removed under vacuum and the residue purified by silica gel column chromatography with $CHCl_3$/MeOH/NH_4OH (78/20/2) as eluent. Compound **L^2H** (16%, 145 mg) was recovered from the collected fractions as a yellow oil, after removal of the solvent under reduced pressure.

R_f (SiO_2, $CHCl_3$/MeOH/NH_4OH (80/18/2)) = 0.80; IR υ_{max}/cm^{-1}: 1587 (C≡O); ^1H NMR (CD_3OD): δ_H 1.23 (3H, t, J = 7.2 Hz, CH_3), 2.09 (4H, m, 2 CH_2), 2.60 (2H, q, J = 7.2 Hz, CH_2), 2.63 (2H, t, J = 6.0 Hz, CH_2), 2.85 (4H, m, 2 CH_2), 3.18 (2H, s, CH_2), 3.19 (2H, m, CH_2), 3.33 (4H, m, 2 CH_2), ^{13}C NMR (CD_3OD): δ_c 13.35 (CH_3), 21.92 (CH_2), 24.96 (CH_2), 28.55 (CH_2), 51.02 (CH_2), 52.38 (CH_2), 52.50 (CH_2), 56.00 (CH_2), 57.05 (CH_2), 178.02 (C=O); ESI/MS (+) (m/z): 261.2 $[M + H]^+$, calcd for $C_{12}H_{26}N_2O_2S$ = 260.2; Anal. Calcd. for $C_{12}H_{23}N_2O_2S.NH_4$: C 51.95, H 9.81, N 15.15, S 11.56, found: C 51.94, H 9.16, N 14.86, S 11.85.

4.2. Synthesis of 2-[(2-(diethylamino)ethyl)(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)amino]acetic acid, L^3H

L^3H (36%, 85 mg) was prepared and purified as above described for **L^2H**, starting from 150 mg (0.76 mmol) of 2-[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethylamino]acetic acid and 131 mg (0.76 mmol) of 2-chloro-*N,N*-diethylethanamine.

R_f (SiO_2, $CHCl_3$/MeOH/NH_4OH (78/20/2)) = 0.61; IR υ_{max}/cm^{-1}: 1579 (CO); ^1H NMR (CD_3OD): δ_H 1.19 (6H, t, J = 7.2 Hz, 2-CH_3), 2.17 (3H, s, CH_3-pz), 2.22 (3H, s, CH_3-pz), 2.72 (2H, m, CH_2), 2.87 (6H, m, 3 CH_2), 3.02 (2H, t, J = 5.7 Hz, CH_2), 3.19 (2H, s, CH_2), 4.02 (2H, t, J = 5.7 Hz, CH_2), 5.83 (1H, s, H(4-pz)); ^{13}C NMR (CD_3OD): δ_c 8.08 (2 CH_3), 10.95 (CH_3-pz), 13.29 (CH_3-pz), 46.94 (CH_2), 48.73 (CH_2), 49.87 (CH_2), 53.41 (CH_2), 54.27 (CH_2), 58.40 (CH_2), 106.09 (C(H_4-pz)), 142.37 (C($H_{3/5}$-pz)), 149.08 (C($H_{3/5}$-pz)), 179.65 (C=O); ESI/MS (+) (m/z): 297.0 $[M + H]^+$ calcd for $C_{15}H_{28}N_4O_2$ = 296.2; Anal. Calcd. for $C_{15}H_{27}N_4O_2.NH_4$: C 57.48, H 9.97, N 22.34, found: C 57.59, H 9.47, N 22.42.

4.3. Synthesis of 2-[(2-(3,5-dimethyl-1H-pyrazol-1-yl)ethyl)(2-(pyrrolidin-1-yl)ethyl)amino]acetic acid, L^4H

L^4H (17%, 120 mg) was prepared and purified as above described for **L^2H**, starting from 480 mg (2.40 mmol) of 2-[2-(3,5-dimethyl-1H-pyrazol-1-yl)ethylamino]acetic acid and 414 mg (2.40 mmol) of 1-(2-chloroethyl)pyrrolidine.

R_f (SiO_2, $CHCl_3$/MeOH/NH_4OH (70/28/2)) = 0.58; IR υ_{max}/cm^{-1}: 1584 (CO); ^1H NMR (CD_3OD): δ_H 2.00 (4H, m, 2 CH_2), 2.17 (3H, s, CH_3-pz), 2.24 (3H, s, CH_3-pz), 2.82 (2H, t, J = 5.6 Hz, CH_2), 3.07 (4H, m, 2 CH_2), 3.12 (2H, m, CH_2), 3.28 (2H, t, J = 7.2 Hz, CH_2), 3.35 (2H, s, CH_2), 4.01 (2H, t, J = 5.7 Hz, CH_2), 5.84 (1H, s, H(4-pz)); ^{13}C NMR (CD_3OD): δ_c 9.28 (CH_3-pz), 12.13 (CH_3-pz), 22.80 (CH_2), 46.34 (CH_2), 52.70 (CH_2), 53.48 (CH_2), 53.57 (CH_2), 56.00 (CH_2), 57.39 (CH_2), 105.00 (C(H_4-pz)), 141.20 (C($H_{3/5}$-pz)), 147.85 (C($H_{3/5}$-pz)), 178.79 (C=O); ESI/MS (+) (m/z): 295.2 $[M + H]^+$, calcd for $C_{15}H_{26}N_4O_2$ = 294.2; Anal. Calcd. for $C_{15}H_{27}N_4O_2.NH_4$: C 57.85, H 9.39, N 22.49, found: C 57.79, H 9.07, N 22.42.

4.4. Synthesis of 2-[(2-(diethylamino)ethyl)(pyridin-2-ylmethyl) amino]acetic acid, L^5

L^5H (24%, 200 mg) was prepared and purified as above described for **L^2H**, starting from 500 mg (3.01 mmol) of 2-[(pyridin-2-yl)methylamino]acetic acid and 518 mg (3.01 mmol) of 2-chloro-*N,N*-diethylethanamine.

R_f (SiO_2, $CHCl_3$/MeOH/NH_4OH (70/28/2)) = 0.56; IR υ_{max}/cm^{-1}: 1594 (C≡O); ^1H NMR (CD_3OD): δ_H 1.33 (6H, t, J = 7.2 Hz, 2-CH_3), 2.98 (2H, t, J = 5.4 Hz, CH_2), 3.15 (4H, q, J = 5.5 Hz, 2 CH_2), 3.20 (2H, s, CH_2), 3.22 (2H, m, CH_2), 3.97 (2H, s, CH_2), 7.34 (1H, t, J = 6.0 Hz, H_{py}), 7.54 (1H, d, J = 7.8 Hz, H_{py}), 7.84 (1H, t, J = 7.8 Hz, H_{py}), 8.53 (1H, d, J = 4.8 Hz, H_{py}); ^{13}C NMR (CD_3OD): δ_c 7.93 (CH_3), 46.92 (CH_2), 50.94 (CH_2), 52.11 (CH_2), 58.11 (CH_2), 60.38 (CH_2), 122.98 (C_{py}), 123.59 (C_{py}), 137.83 (C_{py}), 148.75 (C_{py}), 158.50 (C_{py}), 178.45 (C=O); ESI/MS (+) (m/z): 266.1 $[M + H]^+$, calcd for $C_{14}H_{23}N_3O_2$ = 265.2; Anal. Calcd. for $C_{14}H_{22}N_3O_2.NH_4$: C 59.49, H 9.29, N 19.85, found: C 59.27, H 9.04, N 19.84.

4.5. Synthesis of 2-[(pyridin-2-ylmethyl)(2-(pyrrolidin-1-yl)ethyl) amino]acetic acid, L^6H

L^6H (15%, 142 mg) was prepared and purified as above described for **L^2H**, starting from 600 mg (3.60 mmol) of 2-[(pyridin-2-yl) methylamino]acetic acid and 612 mg (3.60 mmol) of 1-(2-chloroethyl)pyrrolidine.

R_f (SiO_2, $CHCl_3$/MeOH/NH_4OH (70/28/2)) = 0.43; IR υ_{max}/cm^{-1}: 1595 (CO); ^1H NMR (CD_3OD): δ_H 2.10 (4H, m, 2-CH_2), 2.99 (2H, t, J = 5.4 Hz, CH_2), 3.20 (2H, s, CH_2), 3.26 (2H, t, J = 5.4 Hz, CH_2), 3.33 (4H, m, 2 CH_2), 3.95 (2H, s, CH_2), 7.33 (1H, t, J = 6.1 Hz, H_{py}), 7.56 (1H, d, J = 7.8 Hz, H_{py}), 7.83 (1H, t, J = 7.8 Hz, H_{py}), 8.50 (1H, d, J = 4.8 Hz, H_{py}); ^{13}C NMR (CD_3OD): δ_c 22.83 (CH_2), 51.65 (CH_2), 53.15 (CH_2), 53.32 (CH_2), 58.12 (CH_2), 61.08 (CH_2), 122.93 (C_{py}), 123.84 (C_{py}), 137.76 (C_{py}), 148.72 (C_{py}), 158.80 (C_{py}), 178.36 (C=O); ESI/MS (+) (m/z): 264.1 $[M + H]^+$, calcd for $C_{14}H_{21}N_3O_2$ = 263,1; Anal. Calcd. for $C_{14}H_{20}N_3O_2.NH_4$: C 59.98, H 8.63, N 19.98, found: C 59.91, H 8.97, N 19.71.

4.6. General procedure for the synthesis of the Re complexes (**Re2**–**Re6**)

$[Re(H_2O)_3(CO)_3]Br$ was reacted with equimolar amounts of **L^2H**–**L^6H** in refluxing methanol for 18 h. After this time, the solvent was removed under vacuum and the desired complexes were purified by column chromatography or by RP-HPLC. In the case of RP-HPLC, the purification was achieved using a preparative Waters μ Bondapak C18 (150 × 19 mm) with a flow rate of 5.0 mL min^{-1} and using gradient elution with the methods indicated above for each compound.

4.6.1. ***fac*-[Re(CO)$_3$(k^3-L^2)](Re2). Re2** (59%, 76 mg) was purified by RP-HPLC using method I

IR υ_{max}/cm^{-1}: 1677(CO), 1906, 2031 (C≡O); ^1H NMR (CD_3OD): T = 20 °C δ_H 1.34–1.45 (3H, m, CH_3); 2.11 (4H, m, 2 CH_2); 2.58-4.04 (16H, m); ^{13}C NMR (CD_3OD): T = 20 °C δ_c 12.10, 12.24 (CH_3), 22.84, 28.71, 31.87, 32.27, 33.71, 49.63, 54.37, 54.64, 60.21, 60.82, 61.86, 63.49 (CH_2), 114.31 (C_{TFA}), 160.39 (C_{TFA}), 180.72, 180.96 (C=O), 191.45, 191.80, 193.61, 195.22 (C≡O); T = 60 °C δ_c 11.28 (CH_3), 21.99 (CH_2), 28.98 (br, CH_2), 31.68, 31.99, 33.19 (br, CH_2), 48.98 (CH_2), 53.84 (CH_2), 59.49 (CH_2), 61.35, 62.45 (br, CH_2), 63.40 (br, CH_2), 114.27 (C_{TFA}), 160.34 (C_{TFA}), 180.67 (C=O), 190.76, 193.57 (C≡O); ESI/MS (+) (m/z): 531.2 $[M + H]^+$, calcd for $ReC_{15}H_{23}N_2O_5S$ = 530.1; Anal. Calcd. for $ReC_{15}H_{23}N_2O_5S$. 2 CF_3COOH: C 30.12, H 3.33, N 3.70, S 4.23, found: C 30.32, H 4.34, N 4.05, S 4.43.

4.6.2. ***fac-[Re(CO)$_3$(k^3-L^3)](Re3). Re3*** *(61%, 42 mg) was purified by RP-HPLC using method II*

IR υ_{max}/cm^{-1}: 1675(CO); 1934, 2024 (C≡O); ^{1}H NMR (CD_3OD): δ_H 1.37 (6H, t, J = 7.2 Hz, 2-CH_3), 2.33 (3H, s, CH_3-pz), 2.48 (3H, s, CH_3-pz), 2.64 (2H, m, CH_2), 3.45 (1H, m, CH_2), 3.48 (4H, m, 2 CH_2), 3.58 (3H, m, 2CH_2), 3.84 (2H, m, 2CH_2), 4.36 (2H, m, CH_2), 6.16 (1H, s, $H_{4\text{-}pz}$); ^{13}C NMR (CD_3OD): δ_c 9.18 (CH_3), 11.35 (CH_3-pz), 15.95 (CH_3-pz), 46.52 (CH_2), 46.97 (CH_2), 51.17 (CH_2), 57.49 (CH_2), 60.27 (CH_2), 63.47 (CH_2), 108.99 (C(H_4-pz)), 115.47 (C_{TFA}), 145.36 (C($H_{3/5}$-pz)), 155.50 (C($H_{3/5}$-pz)), 160.14 (C_{TFA}), 180.77 (C═O), 195.58 (C≡O), 196.61 (C≡O), 197.35 (C≡O); ESI/MS (+) (m/z): 566.9 $[M + H]^+$ calcd for $ReC_{18}H_{27}N_4O_5$ = 566.2; Anal. Calcd. for $ReC_{18}H_{27}N_4O_5.CF_3COOH$: C 35.24, H 4.15, N 8.24, found: C 35.16, H 4.45 N 8.37.

4.6.3. ***fac-[Re(CO)$_3$(k^3-L^4)](Re4). Re4*** *(79%, 83 mg) was purified by RP-HPLC using method I*

IR υ_{max}/cm^{-1}: 1677 (CO), 1901, 2026 (C≡O), ^{1}H NMR (CD_3OD): δ_H 2.03 (4H, m, 2CH_2), 2.33 (3H, s, CH_3-pz), 2.48 (3H, s, CH_3-pz), 2.59 (2H, m, CH_2), 3.35 (1H, m, CH_2), 3.47 (4H, m, 2 CH_2), 3.53 (1H, m, CH_2), 3.74 (2H, m, CH_2), 3.82 (1H, m, CH_2), 3.90 (1H, m, CH_2), 4.35 (2H, m, CH_2), 6.16 (1H, s, $H_{4\text{-}pz}$); ^{13}C NMR (CD_3OD): δ_c 9.34 (CH_3-pz), 13.97 (CH_3-pz), 22.01 (CH_2), 44.53 (CH_2), 48.15 (CH_2), 53.76 (CH_2), 55.20 (CH_2), 58.83 (CH_2), 61.48 (CH_2), 106.93 (C(H_4-pz)), 116.27 (C_{TFA}), 143.37 (C($H_{3/5}$-pz)), 153.44 (C($H_{3/5}$-pz)), 160.14 (C_{TFA}), 178.86 (C═O), 192.85 (C≡O), 193.87 (C≡O), 194.48 (C≡O); ESI/MS (+) (m/z): 565.2 $[M + H]^+$, calcd for $ReC_{18}H_{25}N_4O_5$ = 564,1; Anal. Calcd. for $ReC_{18}H_{25}N_4O_5.2CF_3COOH$: C 33.38, H 3.44, N 7.08, found: C 33.26, H 3.45, N 7.37.

4.6.4. ***fac-[Re(CO)$_3$(k^3-L^5)](Re5). Re5*** *(55%, 50 mg) was purified by silica-gel column chromatography using $CHCl_3$/MeOH/NH_4OH (70/28/2) as eluent*

Rf (SiO_2 and $CHCl_3$/MeOH/NH_4OH (70/28/2)) = 0.72.IR υ_{max}/cm^{-1}: 1652 (CO), 1876, 2019 (C≡O); ^{1}H NMR (CD_3OD): δ_H 1.10 (6H, t, J = 7.2 Hz, 2-CH_3), 2.68 (4H, m, 2 CH_2), 2.94 (2H, m, CH_2), 3.49 (1H, d, J = 17.1 Hz, CH_2), 3.70 (2H, m, CH_2), 4.03 (1H, d, J = 17.1 Hz, CH_2), 4.60 (1H, d, J = 15.6 Hz, CH_2), 4.76 (1H, d, J = 15.6 Hz, CH_2), 7.53 (1H, t, J = 6.0 Hz, H_{py}), 7.69 (1H, d, J = 7.8 Hz, H_{py}), 8.08 (1H, t, J = 7.8 Hz, H_{py}), 8.80 (1H, d, J = 4.8 Hz, H_{py}); ^{13}C NMR (CD_3OD): δ_c 11.69 (CH_3), 48.12 (CH_2), 50.17 (CH_2), 62.09 (CH_2), 68.05 (CH_2), 69.64 (CH_2), 125.00 (C_{py}), 127.00 (C_{py}), 141.69 (C_{py}), 153.55 (C_{py}), 160.72 (C_{py}), 183.17 (C═O), 195.63 (C≡O), 196.39 (C≡O), 196.61 (C≡O); ESI/MS (+) (m/z): 536.0 $[M + H]^+$, calcd for $ReC_{17}H_{22}N_3O_5$ = 535.0; Anal. Calcd. for $ReC_{17}H_{22}N_3O_5$: C 38.20, H 4.15, N 7.86, found: C 38.52, H 4.34, N 7.62.

4.6.5. ***fac-[Re(CO)$_3$(k^3-L^6)](Re6). Re6*** *(39%, 55 mg) was purified by RP-HPLC using method* ***I***

IR υ_{max}/cm^{-1}: 1679(CO), 1906, 2029 (C≡O); ^{1}H NMR (CD_3OD): δ_H 2.13 (4H, m, 2-CH_2), 3.57 (1H, d, J = 17.1 Hz, CH_2), 3.90 (6H, m, 3CH_2), 4.03 (3H, m, 2 CH_2), 4.65 (1H, d, J = 15.3 Hz, CH_2), 4.80 (1H, d, J = 15.3 Hz, CH_2), 7.58 (1H, t, J = 6.3 Hz, H_{py}), 7.72 (1H, d, J = 7.8 Hz, H_{py}), 8.15 (1H, t, J = 7.8 Hz, H_{py}), 8.83 (1H, d, J = 5.1 Hz, H_{py}); ^{13}C NMR (CD_3OD): δ_c 22.01 (CH_2), 48.83 (CH_2), 53.82 (CH_2), 59.53 (CH_2), 62.55 (CH_2), 67.12 (CH_2), 115.34 (C_{TFA}), 123.19 (C_{py}), 125.30 (C_{py}), 139.94 (C_{py}), 151.60 (C_{py}), 157.92 (C_{py}), 160.02 (C_{TFA}), 180.38 (C═O), 194.85 (C≡O), 195.49 (C≡O), 195.71 (C≡O); ESI/MS (+) (m/z): 534.2 $[M + H]^+$, calcd for $ReC_{17}H_{20}N_3O_5$ = 533.1; Anal. Calcd. for $ReC_{17}H_{20}N_3O_5.3CF_3COOH$: C 31.55, H 2.76, N 4.80, found: C 31.53, H 4.89, N 2.91.

*4.7. Synthesis of the ^{99m}Tc(I) complexes (**Tc1**–**Tc6**)*

The ^{99m}Tc complexes were obtained as previously described for **Tc1** [29], using the following general method: In a nitrogen-purged glass vial, 40 μL of a 4.4 x 10^{-5} M aqueous solution of $\mathbf{L^1H}$–$\mathbf{L^6H}$ were added to 400 μL of a solution of the organometallic precursor *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$ (1–2 mCi) in saline at pH 5.5. The reaction mixture was then heated to 100 °C for 30 min, cooled to room temperature and the pH adjusted to pH = 7.4. The final solutions were analyzed by RP-HPLC using a Macherey–Nagel C18 reversed-phase column (Nucleosil 10 μm, 250 × 4 mm) and a gradient of aqueous 0.1% CF_3COOH (A) and methanol (B) with a flow rate of 1.0 mL min^{-1} and using method **III**.

4.8. Determination of distribution coefficients

The log $D_{o/w}$ values of complexes **Tc1**–**Tc6** were determined by the "shake flask" method under physiological conditions (n-octanol–0.1 M PBS, pH 7.4) [33].

4.9. In vitro binding to melanin

The binding affinity to melanin of **Tc1**–**Tc6** was assessed using synthetic tyrosine–melanin (*Sigma–Aldrich*, St Louis, Missouri, USA). The general procedure used was as follows: A 100 μL aliquot of the radioactive preparations of **Tc1**–**Tc6** was added to a melanin suspension (0.5 mg/10 ml) in distilled water. The reaction mixture was incubated at room temperature for 1 h with stirring. After incubation, the samples were centrifuged at 30,000 g for 10 min, and aliquots of the supernatant were counted in the gamma counter. Control samples were also used containing the radioactive preparation without melanin. The difference between the activity of aliquots from the supernatants of the test samples (with melanin) and the control samples (without melanin) allowed the calculation of the percentage of unbound complexes.

4.10. Cell culture

B16-F1 murine melanotic melanoma (ECACC, UK) and A375 human amelanotic melanoma cells (ATCC, Spain) were grown in DMEM containing GlutaMax I supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin antibitiotic solution (all from Invitrogen). Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C (Heraeus, Germany), with the medium changed every other day. The cells were adherent in monolayers and, when confluent, were harvested from the cell culture flasks with trypsin–EDTA and seeded farther apart.

4.11. Cellular uptake studies and cytotoxicity assays

Cellular uptake assays of the ^{99m}Tc complexes (**Tc1**–**Tc6**) were performed in B16-F1 and A375 cells seeded at a density of 0.2 million/0.5 mL culture medium per well in 24-well tissue culture plates and allowed to attach overnight. After that period, the medium was removed and replaced by fresh medium containing approximately 2 × 10^5cpm/0.5 mL of each ^{99m}Tc complex. The cells were incubated again under humidified 5% CO_2 atmosphere, at 37 °C for a period of 15 min to 4 h. After 0.25, 0.5, 1, 2, 3 and 4 h incubation period the cells were washed twice with cold PBS, lysed with 0.1 M NaOH and the cellular extracts were counted for radioactivity. Each experiment was performed in quadruplicate. Cellular uptake data were expressed as an average value plus the standard deviation.

The cell uptake of complexes **Tc1, Tc3** and **Tc4** in B16-F1 murine melanoma and A375 human amelanotic melanoma cells was also evaluated in competition experiments with haloperidol. In these competition experiments intact cells were incubated with the test complex in the presence of different concentrations of haloperidol

(10^{-14} to 10^{-4} M) for 4 h. After this time, the cell uptake was measured as described above.

The cytotoxicity of haloperidol against the two different cell lines was evaluated using a colorimetric method based on the tetrazolium salt MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is reduced by viable cells to yield purple formazan crystals. Cells were seeded in 96-well plates at a density of 50 x 10^5 cells per well, allowed to attach overnight and then incubated for 4 h in the presence of various concentrations of haloperidol, dissolved in ethanol (the maximum percentage of ethanol did not exceed 2%). At the end of the incubation period the medium was removed and the cells were incubated with MTT (0.5 mg/mL in culture medium; 200 μl) for 3 h at 37 °C and 5% CO_2. The purple formazan crystals formed inside the cells were then dissolved in 200 μl of DMSO by thorough shaking, and the absorbance was read at 570 nm, using a plate spectrophotometer (Power Wave Xs; Bio-Tek). Each test was performed with at least six replicates and repeated at least 2 times. The cell viability was calculated dividing the absorbance of each well by that of the control wells (cells treated with medium and without addition of haloperidol).

4.12. Evaluation of Sigma-1 receptor expression

Western blot experiments were performed to evaluate the levels of expression of Sigma-1 receptor in the following cell lines: A375, PC-3, MCF-7 and B16-F1. Cells were lysed in Cell Lytic-MT Extraction reagent (Sigma) supplemented with Complete protease inhibitor cocktail tablets (Roche). After 15 min on ice, lysates were centrifuged at 14,000 g for 10 min at 4 °C to pellet the cellular debris and the supernatants removed for further use. The total protein content was determined by using the DC Protein Assay Kit (Biorad) and aliquots of protein (30 μg) from each sample were analyzed using standard western blot procedures. Briefly, protein extracts were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred electrophoretically onto nitrocellulose membranes. The blots were blocked with (PBS + 0.1% Tween20) containing 5% nonfat dry milk for 1 h. Then, the blotting membranes were incubated with primary antibodies against Sigma-1 (1:200, goat polyclonal, Abcam) and actin (1:8000, mouse monoclonal, Sigma) overnight. Membranes were washed with PBS-T and incubated for 1 h with secondary antibody (donkey anti-goat IgG-HRP, Santa Cruz Biotechnologyand goat anti-mouse IgG-HRP, Biorad) diluted 1:3000. Finally, membranes were developed using the SuperSignal WetsPico Substrate kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

4.13. Biodistribution and imaging studies

All animal experiments were performed in compliance with Portuguese regulations for animal treatment. The animals were housed in a temperature- and humidity-controlled room with a 12 h light/12 h dark schedule.

Biodistribution of complexes **Tc1**–**Tc6** was evaluated in melanoma-bearing C57BL/6 female mice (8–10 weeks old). Mice were previously implanted subcutaneously with 1 × 10^6 B16-F1 cells to generate a primary skin melanoma. Ten to 12 days after the inoculation, tumors reached a weight of 0.2–1 g. Animals were intravenously injected into the retroorbital sinus with the test complex (3–10 MBq) diluted in 100 μL of PBS pH 7.2. Mice were sacrificed by cervical dislocation at 1 and 4 h after injection. The dose administered and the radioactivity in the killed animals were measured using a dose calibrator (Curiemeter IGC-3, Aloka, Tokyo, Japan or Carpintec CRC-15W, Ramsey, USA). The difference between the radioactivity in the injected and sacrificed animals was assumed to be due to excretion. Tumors and normal tissues of interest were dissected, rinsed to remove excess blood, weighed, and their radioactivity was measured using a γ-counter (LB2111, Berthold, Germany). The uptake in the tumor and healthy tissues was calculated and expressed as a percentage of the injected radioactivity dose per gram of tissue. For blood, bone, muscle, and skin, total activity was estimated assuming that these organs constitute 6, 10, 40, and 15% of the total body weight, respectively. Urine was also collected and pooled together at the time the animals were killed.

For imaging, the animals were injected with complex **Tc1** (29 MBq) and sacrificed at 1 h p.i.. A set of static images (256 × 256 matrix, Zoom 2, 2 min) were acquired, by placing the animals over a γ camera (GE 400AC; Maxicamera, Milwaukie, USA) coupled with a high-resolution parallel collimator and controlled with GENIE Acquisition computer.

4.14. In vivo stability studies

The *in vivo* stability of **Tc1-Tc6** was evaluated by urine and murine serum HPLC analysis, using the elution conditions above described for the analysis of these ^{99m}Tc complexes. The urine was collected at sacrifice time and filtered through a Millex GV filter (0.22 μm) before RP-HPLC analysis. Blood collected from mice was immediately centrifuged for 15 min at 3000 rpm at 4 °C, and the serum was separated. Aliquots of 100 μL of serum were treated with 200 μL of ethanol to precipitate the proteins. Samples were centrifuged at 4000 rpm for 15 min, at 4 °C. Supernatant was collected and passed through a Millex GV filter (0.22 lm) prior to RP-HPLC analysis.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2012.02.014.

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