Synthesis, characterization and biological evaluation of tricarbonyl M(I) (M = Re, ^{99m}Tc) complexes functionalized with melanin-binding pharmacophores

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Aiming to evaluate their potential as radioactive probes for *in vivo* targeting of melanotic melanoma and its metastases, we have synthesized ^{99m}Tc(I) tricarbonyl complexes (**Tc1–Tc8**) anchored by pyrazolyl-containing chelators with (N_3) or (N_2O) donor atom sets and functionalized with 2-aminoethyldiethylamine and 4-amino-*N*-(2-diethylaminoethyl)benzamide groups as melanin-binding pharmacophores. The chemical identification of the several ^{99m}Tc complexes has been accomplished by HPLC comparison with the Re congeners (**Re1–Re8**), which were synthesized at the macroscopic level and fully characterized by common analytical techniques. The biological evaluation of the ^{99m}Tc(I) complexes comprised the determination of their *in vitro* binding to synthetic melanin, measurement of cellular uptake in B16F1 murine melanoma cells, as well as biodistribution studies in B16F1 melanoma-bearing mice. All the tested complexes have shown a moderate to high *in vitro* affinity to melanin, with percentages of binding spanning between 60 and 94%. In agreement with the poor cellular uptake measured *in vitro*, the *in vivo* tumor uptake of the complexes was in general relatively low, ranging between 0.12 and 1.69% ID g^{-1} at 4 h p.i. However, some complexes have shown favorable tumor-to-organ ratios with values as high as 28 and 5.3 for tumor–muscle and tumor–blood ratios, respectively. This seems to indicate that some selectivity towards melanoma tissue was conserved, and encourages further optimization of the *in vitro*/*in vivo* biological properties of this type of complexes aimed at finding novel radioactive probes for non-invasive imaging of melanoma.

Introduction

Melanoma is a serious form of skin cancer that develops in the pigment-producing skin cells – melanocytes. Around 160 000 new cases of melanoma are diagnosed worldwide each year. Melanoma often starts as a single tumor or lesion that can spread to nearby lymph nodes and distant sites throughout the body – metastatic melanoma.[1,2] The early detection of melanoma and its metastases can considerably improve management and prognosis of the disease. The nuclear imaging techniques Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) are expected to provide an early and non-invasive detection of melanoma, given that radioactive probes suitable for a selective *in vivo* targeting of melanoma tissues are available. In the past few years, several approaches have been explored in the design of specific radioactive probes for *in vivo* targeting of melanoma tissue, based on monoclonal antibodies (MAbs),[3] peptide analogs of α-melanocyte stimulating hormone (α-MSH)[4] and small organic molecules such as precursors of melanin [5] or melanin-binders.[6–13] Melanin-binders constitute a heterogeneous drug family that comprises polycyclic aromatic compounds, polyamines and benzamide derivatives. So far, the best results have been found for radioiodinated benzamide derivatives that, in some cases, hold potential for SPECT imaging of malignant melanoma.[6–13] In particular, *N*-(2-diethylaminoethyl)-4-[^{123}I]iodobenzamide ([^{123}I]BZA) and its *ortho* analog ([^{123}I]BZA$_2$) allowed the detection of melanoma and its metastases with high sensitivity and selectivity in phase II clinical trials involving several melanoma patients.[9,11]

Due to several reasons, the cyclotron-produced ^{123}I is less convenient for SPECT imaging compared with the inexpensive and readily available ^{99m}Tc, which is the most used radionuclide for diagnostic applications in nuclear medicine. Taking these advantages into consideration, various research groups have pursued the finding of ^{99m}Tc-labelled benzamide derivatives as alternatives to radioiodinated benzamides. Until now, several ^{99m}Tc(V) complexes functionalized with benzamide derivatives, or their fragments, have been prepared and evaluated as potential SPECT probes for *in vivo* targeting of melanoma. This included complexes with the [^{99m}TcN]$^{2+}$ and [^{99m}TcO]$^{3+}$ cores anchored by N_2S_2 tetradentate bifunctional chelators, as well as [3 + 1] mixed-ligand Tc(V) oxocomplexes.[14–21] However, the tested complexes have shown in most cases only low to moderate tumor uptake, and did not emerge as an alternative to radioiodinated benzamides.

In the past few years, the so-called tricarbonyl approach has gained considerable attention, following the introduction by

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Alberto and co-workers of a convenient and fully aqueous-based kit preparation of the organometallic precursor *fac*-$[^{99m}Tc(OH_2)_3(CO)_3]^+$.[22] The *fac*-$[^{99m}Tc(CO)_3]^+$ core can be stabilized by a large variety of ligands and/or donor sets, without compromising the kinetic inertness of the resulting complexes. Such versatility allows fine-tuning of the physico-chemical properties of the complexes, and their adjustment to those of the carrier biomolecules to be labelled. These favorable features justify the exploration of this approach for labelling of a large variety of biomolecules. To the best of our knowledge, the *fac*-$[^{99m}Tc(CO)_3]^+$ core has only recently been explored in the ^{99m}Tc-labelling of melanin-binders, using cyclopentadienyl ligands as bifunctional chelators.[23] The reported results were encouraging in terms of tumor uptake in B16F1 melanoma-bearing mice, but the pharmacokinetics and biological profile of the evaluated organometallic complexes were rather unfavourable.

Using the tricarbonyl approach, we have embarked on the synthesis and biological evaluation of ^{99m}Tc(I) tricarbonyl complexes anchored by pyrazole-diamine (*N*,*N*,*N*-donors) or pyrazole-aminocarboxylic (*N*,*N*,*O*-donors) bifunctional chelators bearing 2-aminoethyldiethylamine and 4-amino-*N*-(2-diethylaminoethyl)benzamide (procainamide) groups as melanin-binding pharmacophores, aiming to evaluate their potential interest for the design of novel radioactive probes for the detection of malignant melanoma. Herein, we report on the synthesis and characterization of new pyrazole-diamine (**L^1**, **L^2**, **L^5**, **L^6**) or pyrazole-aminocarboxylic ligands (**L^3**, **L^4**, **L^7**, **L^8**) that carry the aforementioned melanin-binding pharmacophores at different positions of the chelator framework, *i.e.* at the 4-position of the pyrazol ring or at the central amine. These new ligands were used to prepare ^{99m}Tc(I)/Re(I) tricarbonyl complexes (**Tc1–Tc8**/**Re1–Re8**), which is expected to be useful for the design of melanoma-seeking radioactive probes. The Re complexes were fully characterized by common analytical techniques, and were used as surrogates for the ^{99m}Tc congeners. Reported herein is an evaluation of the potential of the ^{99m}Tc complexes for SPECT imaging of melanoma, achieved by evaluation of their *in vitro* binding to synthetic melanin, measurement of cellular uptake in B16F1 murine melanoma cells and biodistribution studies in B16F1 melanoma-bearing mice.

Results and discussion

Chemical synthesis: ligands and rhenium complexes

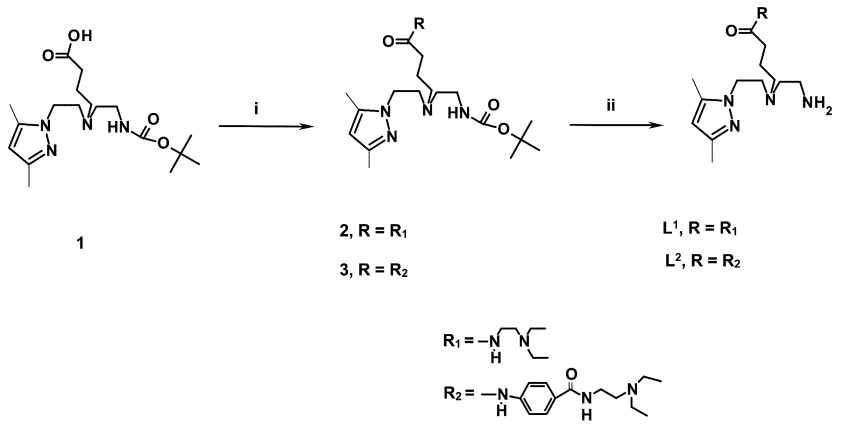
The mechanisms involved in the uptake of benzamide derivatives by melanoma tumor cells are not fully understood, but it has been shown that the compounds co-localize with melanin in the melanocytes of pigmented cells. Their uptake has been correlated to melanin binding, suggesting a non-receptor binding uptake mechanism. Moreover, for radioiodinated *N*-(2-dialkylaminoethyl)benzamides passive diffusion appears to be the dominant uptake mechanism on melanoma cells, it being reported in some cases that the tumor uptake is positively correlated with the lipophilicity of the compounds.[24–28] Therefore, ^{99m}Tc(I) tricarbonyl complexes bearing melanin-binding pharmacophores, to be explored as SPECT probes for melanoma detection, should also freely diffuse through the cell membrane to interact with melanin in the cytosol. The capability of the complexes to cross the cell membrane will be dependent on their physico-chemical properties, such as size, topology, charge and lipophilicity. As mentioned in the introductory section, we have focused on pyrazole-diamine or pyrazole-aminocarboxylic bifunctional chelators to stabilize the Re(I)/^{99m}Tc(I) tricarbonyl complexes functionalized with melanin-binding pharmacophores. By exploring these *N*,*N*,*N*- and *N*,*N*,*O*-tridentate chelators, we have taken into consideration their excellent coordination capability towards the *fac*-$[M(CO)_3]^+$ (M = Re, ^{99m}Tc) moiety, which enables the formation of complexes with excellent *in vitro* and *in vivo* stability.[29–31] Moreover, these compounds coordinate to the metal as neutral and monoanionic chelators, respectively, affording complexes with a different overall charge that might influence their ability to cross the cell membrane. Profiting from the versatility of pyrazole-diamine and pyrazole-aminocarboxylic ligands, in terms of possibilities of functionalization, we have prepared an enlarged family of bifunctional chelators containing 2-aminoethyldiethylamine and 4-amino-*N*-(2-diethylaminoethyl)benzamide (procainamide) as melanin-binding groups, which were introduced at the 4-position of the pyrazole ring or at the central amine.

The functionalization of the pyrazolyl-diamine ligands through the central amine started with a previously reported BOC-protected derivative (compound **1**) of 4-{(2-aminoethyl)-[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]amino}butanoic acid.[29] As shown in Scheme 1, the derivative contains a butyric pendant arm that has been used for the attachment of the melanin-binding pharmacophores by amide coupling reactions, using HBTU (*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate) as an activating reagent in the presence of triethylamine. The resulting compounds, **2** and **3**, were then treated with trifluoroacetic acid (TFA) to remove the BOC protecting group. The deprotection reaction afforded, almost quantitatively, the final ligands **L^1** and **L^2**, which were recovered as yellow oils.

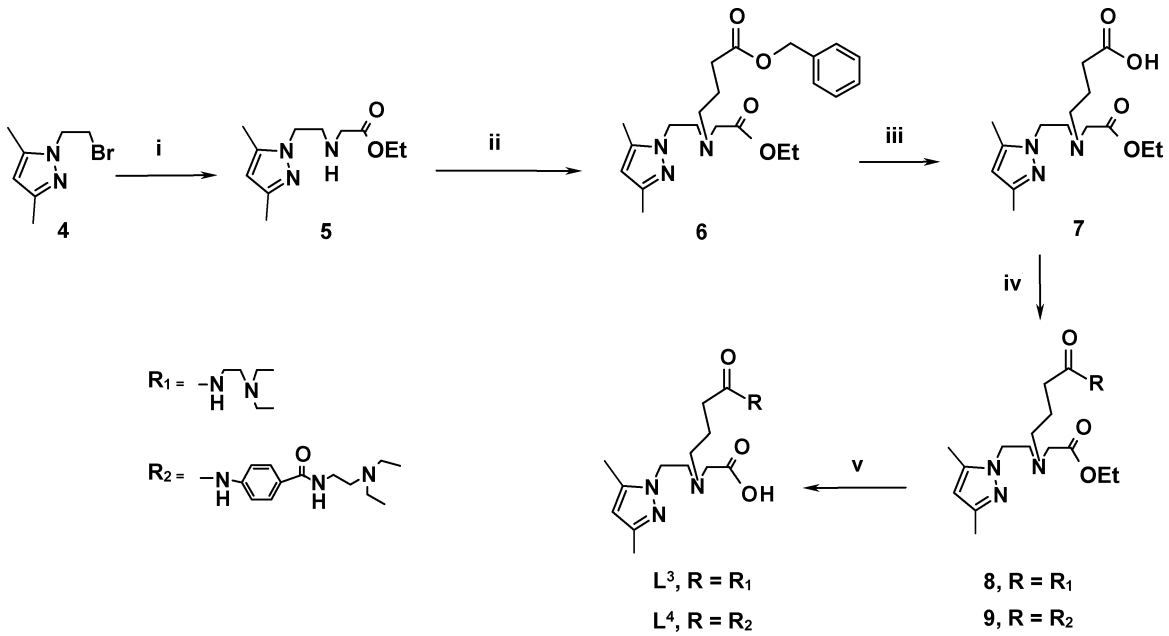
A similar strategy has been explored for coupling of the 2-aminoethyldiethylamine and 4-amino-*N*-(2-diethylaminoethyl)-benzamide pharmacophores to a butyric arm attached at the central amine of the pyrazole-aminocarboxylic ligands. As can be seen in Scheme 2, this involved the synthesis of a mixed ethyl/benzyl diester, **6**, which was converted to **7** by selective deprotection of the benzyl ester by hydrogenation over Pd/C. Compound **7** was then linked to the melanin-binding groups, as described above for **L^1** and **L^2**. The ethyl ester function of the resulting amide derivatives (**8** and **9**) was hydrolyzed with NaOH in refluxing THF–H_2O, yielding **L^3** and **L^4**. **L^3** and **L^4** were recovered as yellow oils, in moderate to high yield (60–98%), after neutralization of the respective reaction mixtures and extraction with dichloromethane.

The incorporation of the melanin-binding groups at the pyrazolyl rings of the pyrazole-diamine and pyrazoleamine-carboxylic chelators involved the synthesis of derivatives having a methylacetate substituent at the 4-position of the rings (Schemes 3 and 4).

For the pyrazole-diamine ligands, the precursor ethyl 2-(1-(2-bromoethyl)-3,5-dimethylpyrazolyl)acetate (**11**) was



Scheme 1 Synthesis of $\mathbf{L^1}$ and $\mathbf{L^2}$. Reagents and conditions: (i) *N*,*N*-Diethylethylenediamine or procainamide, HBTU, NEt_3, CH_3CN, 48 h, reflux; (ii) TFA, CH_2Cl_2, 2.5 h, r.t.

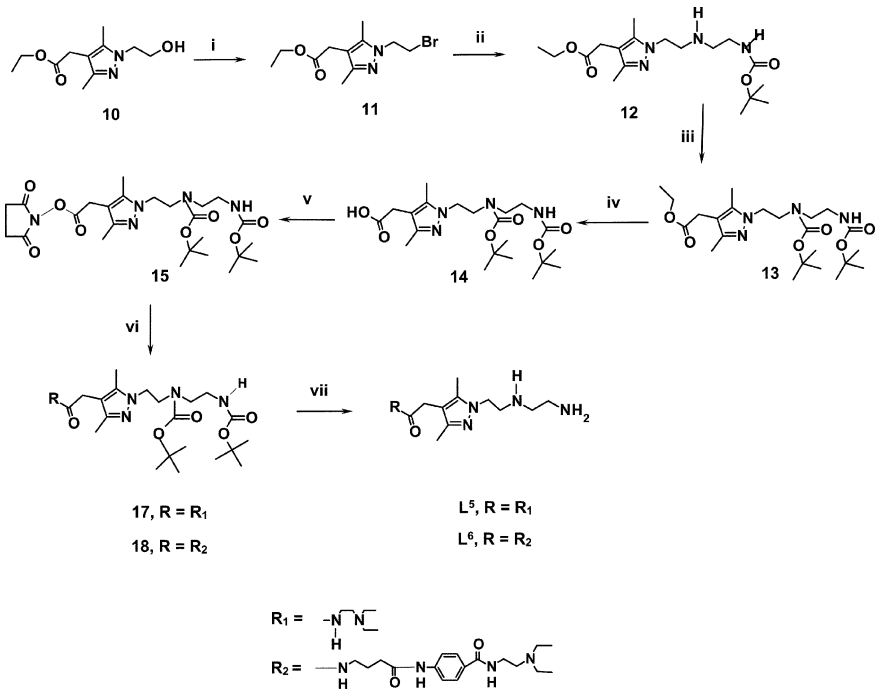


Scheme 2 Synthesis of $\mathbf{L^3}$ and $\mathbf{L^4}$. Reagents and conditions: (i) H_2NCH_2COOEt, CH_3CN, K_2CO_3, KI, reflux, 3 days; (ii) $Br(CH_2)_3COOCH_2C_6H_5$, CH_3CN, K_2CO_3, KI, reflux, 3 days; (iii) H_2, Pd/C, dry EtOH, 20 h; (iv) *N*,*N*-diethylethylenediamine or procainamide, HBTU, NEt_3, CH_3CN, 72 h, reflux; (v) NaOH, THF–H_2O, reflux, overnight.

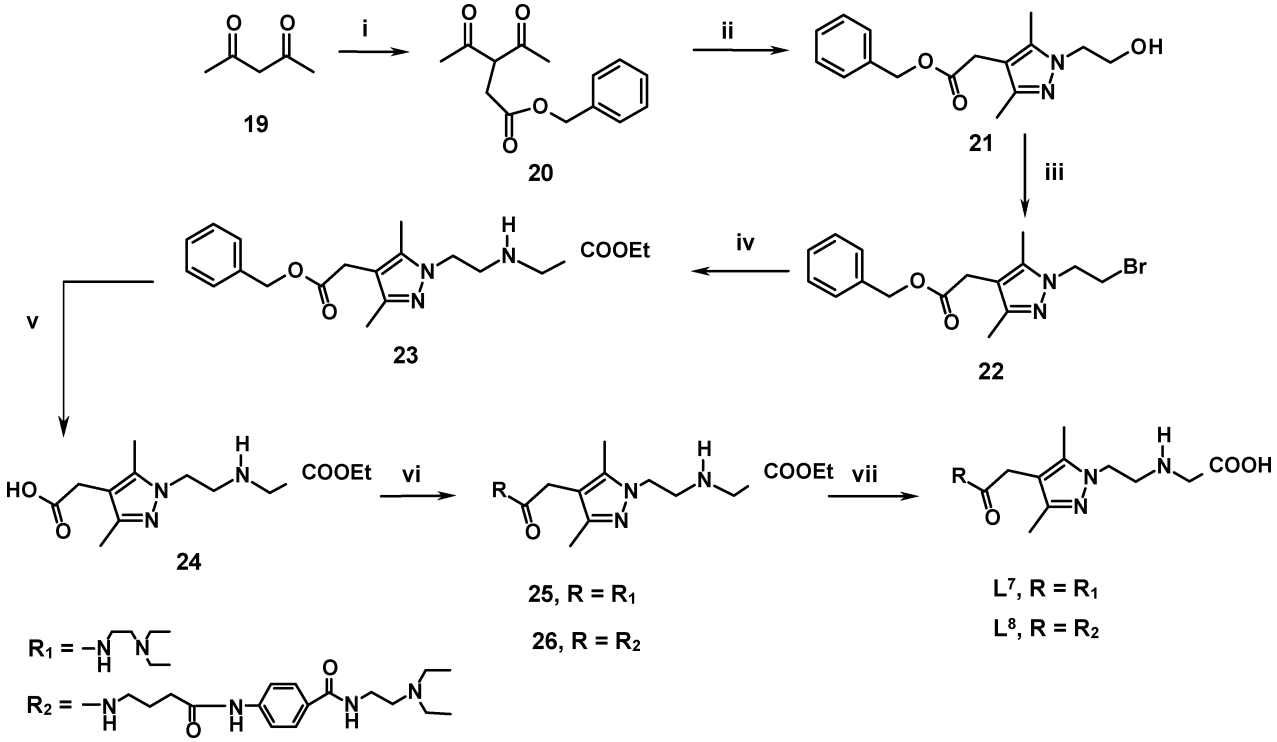
readily obtained by bromination of the corresponding alcohol (Scheme 3). Treatment of **11** with $BOCHNCH_2CH_2NH_2$ afforded a pyrazolyl-diamine derivative (**12**) bearing a (*tert*-butoxy)carbonyl protecting group at the terminal amine. Compound **12** reacted with di-*tert*-butyl-dicarbonate anhydride giving a bis-protected derivative (**13**) that has been converted to an NHS-activated ester (**15**), after deprotection of the ethyl ester group of **14**. Unlike 4-amino-*N*-(2-diethylaminoethyl)-benzamide, 2-aminoethyldiethylamine reacted smoothly with **15** leading to the formation of the amide derivative **17**. This difference in reactivity reflects the aromatic nature of the reacting amine group in the former case. To introduce the 4-amino-*N*-(2-diethylaminoethyl)benzamide group at the 4-position of the pyrazolyl ring, we have functionalized this pharmacophore with a 2-aminoethylcarboxy linker. The resulting derivative (**16**) was reacted with **15** to afford the desired compound (**18**). Removal of the BOC protecting groups from **17** and **18** led to the final pyrazolyl-diamine ligands, $\mathbf{L^5}$ and $\mathbf{L^6}$, containing melanin-binding pharmacophores at the 4-position of the pyrazolyl ring.

As shown in Scheme 4, the introduction of the melanin-binding groups at the 4-position of the pyrazole-aminocarboxylic ligands followed a similar strategy, starting from compound **24** which has a terminal ethyl ester function and a methylacetic group at the 4-position of the azolyl ring. Compound **24** was coupled to the two pharmacophores, upon activation with HBTU under basic conditions. Treatment of the resulting compounds, **25** and **26**, with NaOH gave $\mathbf{L^7}$ and $\mathbf{L^8}$, respectively. All the ligands bearing the pharmacophores at the 4-position of the pyrazolyl ring, $\mathbf{L^5}$–$\mathbf{L^8}$, are yellow oils that have been obtained in moderate to high yield (42–98%).

All the new ligands, $\mathbf{L^1}$–$\mathbf{L^8}$, were characterized by common spectroscopic techniques (IR, ^1H and ^{13}C NMR) and by



Scheme 3 Synthesis of L^5 and L^6. Reagents and conditions: (i) PBr_3, toluene, reflux, overnight; (ii) $NH_2(CH_2)_2NHBOC$, CH_3CN, NEt_3, NaI, reflux, 24 h; (iii) $(BOC)_2O$, THF, r.t., overnight; (iv) NaOH, THF–H_2O, reflux, overnight; (v) NHS, EDC, CH_2Cl_2, r.t., 24 h; (vi) 2-aminoethyldiethylamine or **16**, dry DMF, DIPEA, r.t., 48 h; (vii) TFA, CH_2Cl_2, r.t., 2.5 h.



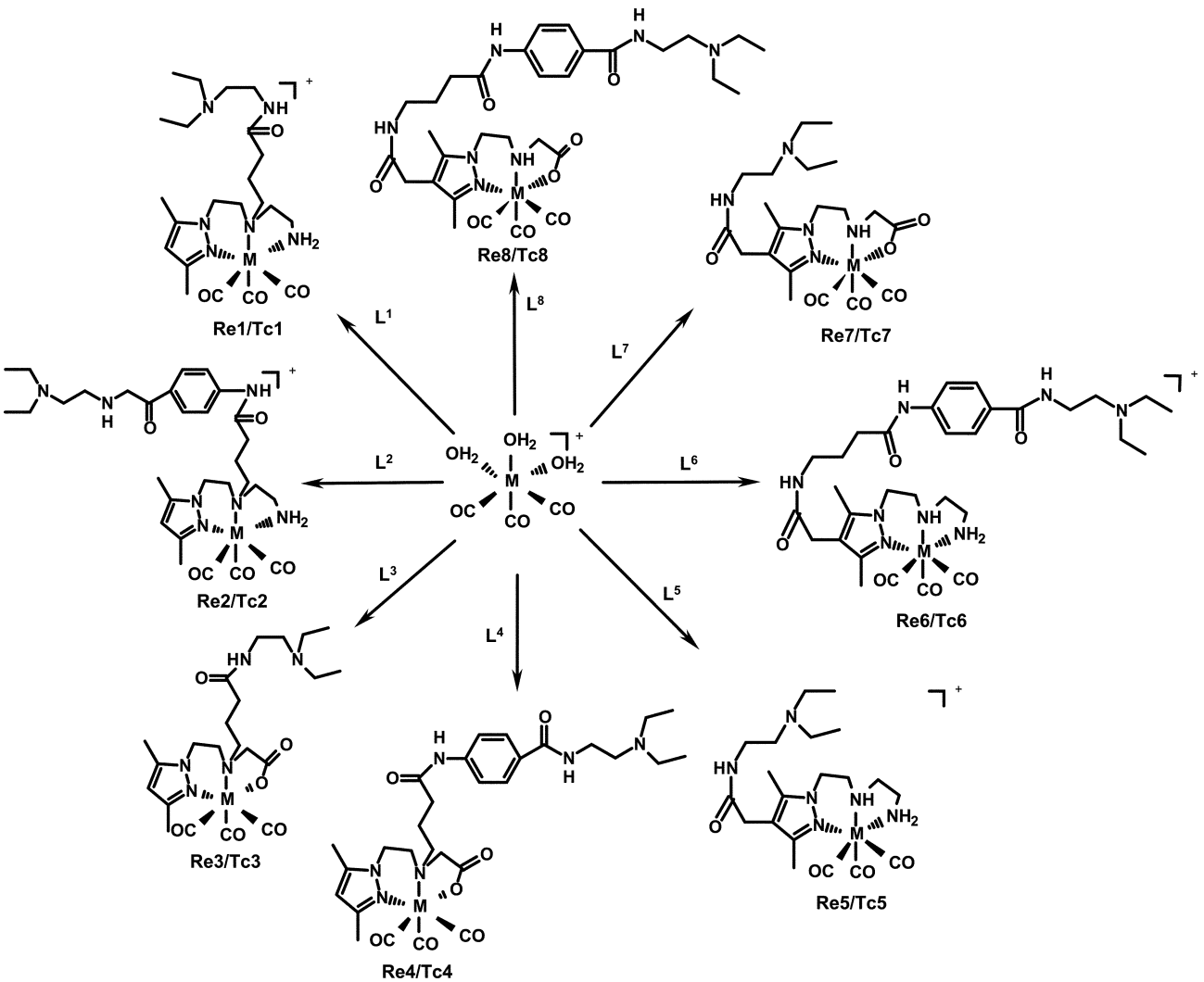
Scheme 4 Synthesis of L^7 and L^8. Reagents and conditions: (i) benzyl 2-bromobutyrate, NaH, THF, 0 °C, 3 h; (ii) hydrazine monohydrate, ethanol, r.t., overnight; (iii) CBr_4, PPh_3, THF, r.t., overnight; (iv) $H_2N(CH_2)_2COOEt$, CH_3CN, NaI, NEt_3, reflux, 24 h; (v) H_2, Pd/C, dry CH_2Cl_2, r.t., 20 h; (vi) 2-aminoethyldiethylamine or **16**, HBTU, NEt_3, dry CH_3CN, reflux, 24 h; (vii) NaOH, THF–H_2O, reflux, overnight.

HR-ESI-MS; such characterization confirmed the proposed formulations, namely the presence of the 2-aminoethyldiethylamine and 4-amino-*N*-(2-diethylaminoethyl)benzamide pharmacophores, and showed that all compounds were sufficiently pure to be used in the synthesis of the respective Re(I) and ^{99m}Tc(I) tricarbonyl complexes.

The synthesis of the rhenium complexes was performed by ligand exchange reactions of *fac*-[$Re(H_2O)_3(CO)_3$]Br with $\mathbf{L^1}$–$\mathbf{L^8}$ in refluxing methanol. These reactions led to cationic complexes (**Re1**, **Re2**, **Re5** and **Re6**) and neutral complexes (**Re3**, **Re4**, **Re7** and **Re8**) anchored by bifunctional pyrazolyl-diamine and pyrazolyl-aminocarboxylic ligands, respectively (Scheme 5). Complexes **Re1–Re8** were analyzed by spectroscopic techniques (IR and $^1H/^{13}C$ NMR) and mass spectrometry, which allowed for the unambiguous identification of their chemical structures. The IR spectra of **Re1–Re8** showed intense absorption bands between 1878 and 2032 cm^{-1}, easily assigned to the $\nu(C\equiv O)$ stretching modes of the *fac*-$[Re(CO)_3]^+$ unit. These frequencies compare well with those that we have previously reported for other Re(I) tricarbonyl complexes anchored by pyrazolyl-diamine or pyrazolyl-aminocarboxylic ligands.[29,30] The ^1H NMR data obtained for **Re1–Re8** corroborated a facial coordination of the ligands through the pyrazolyl ring, the central nitrogen atom and the terminal amine (**Re1**, **Re2**, **Re5** and **Re6**) or carboxylate (**Re3**, **Re4**, **Re7** and **Re8**) groups, since all the spectra have shown a set of multiplets for the methylenic protons of the framework of the pyrazolyl-diamine or pyrazolyl-aminocarboxylic ligands, which was consistent with the diastereotopic character of such protons. These multiplets exhibited chemical shifts significantly different from those of the corresponding protons in the respective free ligands. In contrast, the signals due to the protons of the 2-aminoethyl-diethylamine and 4-amino-*N*-(2-diethylaminoethyl)benzamide pharmacophores, as well as those due to the methylenic linkers attaching the pharmacophores to the ligand backbone, have shown splitting patterns and chemical shifts very similar for the free ligands and respective Re complexes. These findings confirmed that the pharmacophores were not interacting with the metal. In the positive mode, the HR-ESI-MS spectra of **Re1–Re8** have shown prominent peaks corresponding to the expected molecular-ions, with the correct isotope distribution pattern and without significant fragmentation.

Synthesis, characterization and *in vitro* evaluation of the ^{99m}Tc complexes

The synthesis of the ^{99m}Tc complexes (**Tc1–Tc8**) has been performed in aqueous solution by reaction of *fac*-$[^{99m}Tc(H_2O)_3(CO)_3]^+$ with the appropriate ligand ($\mathbf{L^1}$–$\mathbf{L^8}$)



Scheme 5 Synthesis of the Re and ^{99m}Tc complexes.

at 100 °C for 30 min (Scheme 5). The reactions were almost quantitative (radiochemical yield >95%) for concentrations of the ligands as low as 10^{-4} M. In the case of the pyrazolyl-aminocarboxylic ligands functionalized through the central amine, the pH of the reaction mixture strongly influenced the radiochemical purity of the respective complexes (**Tc3** and **Tc4**). **Tc3** and **Tc4** could be obtained with a radiochemical purity higher than 95% only when their synthesis was performed at relatively acidic pH, *i.e.* at pH = 5.5. At neutral pH, the same reactions were accompanied by the formation of another radioactive species, which was identified as [^{99m}Tc(CO)$_3$(3,5-Me$_2$pzCH$_2$CH$_2$NHCH$_2$COO)]. The formation of the latter complex resulted from the hydrolytic cleavage of the butyric pendant arm used to couple the melanin-binding groups to the chelator. This behavior has been previously found in labeling with *fac*-[^{99m}Tc(CO)$_3$]$^+$ of pyrazolyl-aminocarboxylic ligands functionalized with other biologically active fragments.[31] Hydrolytic cleavage of a C–N bond in a tridentate diethylenetriamine chelator coordinated to *fac*-[^{99m}Tc(CO)$_3$]$^+$ has been also reported by Alberto *et al.*[32] Unlike **Tc3** and **Tc4**, all the other ^{99m}Tc complexes could be obtained with high radiochemical purity (>95%) at pH 7. The chemical identity of **Tc1–Tc8** was ascertained by comparison of their HPLC profiles with those of the corresponding rhenium complexes (**Re1–Re8**). The respective retention times are presented in Table 1.

The *in vitro* evaluation of complexes **Tc1–Tc8** involved the study of their lipophilicity and their binding to synthetic melanin. As discussed above, the lipophilicity could be a decisive factor in the ability of the complexes to cross the membrane of melanoma cells, while binding to melanin was expected to promote their intracellular retention.

The lipophilicity of complexes **Tc1–Tc8** was assessed by measurement of the respective log $P_{o/w}$ values (*n*-octanol/0.1 M PBS, pH 7.4) using the multiple back-extraction method. The measured log $P_{o/w}$ values are shown in Table 1. Unlike **Tc4** (log $P_{o/w}$ = 1.02 ± 0.02), all the other complexes exhibited a hydrophilic character with log $P_{o/w}$ values ranging from −1.64 to −0.16. For the same pharmacophore, the neutral complexes are less hydrophilic than the cationic congeners, as would be expected. The complexes functionalized with the pharmacophores at the 4-position of the pyrazolyl ring are also more hydrophilic than those having the same pharmacophore at the pendant arm of the central amine. This trend reflects the presence, or not, of a central NH group, which contributes to the increases hydrophilicity of the complexes.

Table 1 HPLC retention times, log P values and *in vitro* binding to synthetic melanin for complexes **Tc1–Tc8**

Compound	Retention time/min[ab]	log P_o/P_w	% Bound to synthetic melanin
Tc1	17.8 (17.1)	−0.38 ± 0.04	79 ± 5
Tc2	18.5 (18.5)	−0.29 ± 0.08	94 ± 2
Tc3	19.4 (19.0)	−0.16 ± 0.03	77 ± 4
Tc4	20.3 (20.1)	1.02 ± 0.02	71 ± 3
Tc5	17.1 (16.5)	−1.64 ± 0.01	76 ± 2
Tc6	18.5 (18.1)	−1.24 ± 0.05	67 ± 5
Tc7	18.5 (18.2)	−0.93 ± 0.01	60 ± 5
Tc8	19.5 (19.2)	−0.31 ± 0.01	87 ± 7

[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 **Re1–Re8**. [c] Concentration of melanin 0.05 mg mL^{-1}, 1 h incubation.

The affinity for melanin of **Tc1–Tc8** was assessed *in vitro* by co-incubation of the complexes with synthetic melanin in distilled water. The experiments were optimized in terms of incubation time and melanin concentration. Relatively fast binding kinetics were observed, since almost identical percentages of binding were found after 1 h and 24 h of incubation. As shown in Table 2, the percentages of bound complexes spanned between 60 and 94%, for a melanin concentration of 0.5 mg per 10 mL after 1 h of incubation. These findings show that the complexes have a moderate to high affinity for melanin, which indicates that the metalation of the 2-amino-ethyldiethylamine and 4-amino-*N*-(2-diethylaminoethyl)-benzamide pharmacophores with the *fac*-[^{99m}Tc(CO)$_3$]$^+$ unit did not compromise their ability to interact with this pigment. Apparently, the charge of the complexes has some influence on their interaction with melanin, the percentage of binding of the cationic complexes being generally higher than the values found for the neutral counterparts. In contrast, we have verified that the lipophilicity of the complexes did not have a well-defined effect on their affinity to melanin. As proposed by other authors, the binding of benzamide derivatives to melanin is a reversible process that involves two classes of binding sites, one ionic and another one hydrophobic.[33] The behavior that has been observed for **Tc1–Tc8** indicates that for these complexes electrostatic interactions with melanin seem to be more determinant than the hydrophobic ones, which reflects the polyanionic nature of melanin.

Biological evaluation of the ^{99m}Tc complexes: *in vitro* cellular uptake and biodistribution studies

To evaluate the ability of the complexes to accumulate in melanoma tumor cells, *in vitro* cell uptake studies were performed for **Tc1–Tc8** in murine B16F1 melanoma cells, which were also used for the *in vivo* studies with tumor-bearing mice. As can be seen in Fig. 1, the measured cell uptake was almost independent of the incubation time (15–240 min), and the maximum cell uptake values ranged between 0.07 and 1.11%, when expressed as a percentage of total activity. These low uptake values, corresponding to internalized activity, have shown that all the complexes have a poor ability to accumulate into the cells.

We have pursued with the *in vivo* evaluation of the complexes in B16F1 melanoma-bearing mice, aiming to assess their biodistribution profile and pharmacokinetics, in terms of tumor uptake, excretory pathways, metabolic stability and target/non-target ratio. The biodistribution data obtained for **Tc1–Tc8** at 1 h and 4 h p.i., expressed in percent injected dose per gram tissue (% ID g^{-1}), are presented in Table 2.

With the exception of **Tc3**, all the complexes have shown a relatively fast blood clearance. Unlike other non-target tissues like the muscle, some retention of radioactivity was observed in the excretory organs, *i.e.* kidney and liver, which reflects the urinary and hepatobiliary excretion of **Tc1–Tc8**. A more pronounced kidney and liver uptake has been found for the

Table 2 Relevant biodistribution data (% ID g^{-1}) of **Tc1–Tc8** at 1 and 4 h post-injection (p.i.) in C57/B16 mice with palpable hind limb B16 melanoma nodules

Mean tissue concentrations (% ID g^{-1} organ)								
^{99m}Tc complex	Time (h, p.i.)	Muscle	Blood	Eyes	Tumor	Kidneys	Liver	Intestine
Tc1	1	0.42 ± 0.01	0.70 ± 0.06	1.09 ± 1.00	1.20 ± 0.08	18.52 ± 3.07	18.83 ± 6.48	15.35 ± 4.85
	4	0.28 ± 0.04	0.29 ± 0.03	0.45 ± 0.19	0.87 ± 0.18	12.61 ± 1.04	10.24 ± 0.88	15.19 ± 2.82
Tc2	1	0.12 ± 0.04	0.53 ± 0.22	—	1.24 ± 0.27	6.93 ± 1.06	46.15 ± 5.98	16.14 ± 3.15
	4	0.07 ± 0.02	0.26 ± 0.19	—	0.60 ± 0.29	4.28 ± 0.33	34.09 ± 3.29	26.24 ± 5.81
Tc3	1	0.17 ± 0.04	1.02 ± 0.08	0.33 ± 0.09	0.44 ± 0.16	2.4 ± 0.4	2.44 ± 0.05	12.94 ± 3.93
	4	0.24 ± 0.15	0.86 ± 0.68	0.39 ± 0.07	0.45 ± 0.24	1.89 ± 0.62	2.07 ± 0.82	15.71 ± 2.39
Tc4	1	0.06 ± 0.00	0.33 ± 0.06	0.17 ± 0.04	0.44 ± 0.22	0.93 ± 0.09	5.67 ± 1.01	38.86 ± 3.14
	4	0.08 ± 0.01	0.35 ± 0.05	0.25 ± 0.04	0.54 ± 0.04	1.55 ± 0.35	1.37 ± 0.46	20.81 ± 14.29
Tc5	1	0.15 ± 0.06	0.26 ± 0.03	0.62 ± 0.45	1.00 ± 0.22	40.21 ± 4.95	26.24 ± 2.62	6.18 ± 0.97
	4	0.10 ± 0.02	0.20 ± 0.20	0.23 ± 0.03	0.68 ± 0.23	34.42 ± 24.26	24.81 ± 0.68	9.28 ± 0.79
Tc6	1	0.16 ± 0.02	1.54 ± 0.28	0.70 ± 0.21	1.21 ± 0.02	57.14 ± 4.79	26.94 ± 0.84	8.45 ± 1.41
	4	0.06 ± 0.00	0.32 ± 0.08	0.31 ± 0.09	1.69 ± 0.39	16.65 ± 1.58	18.28 ± 1.24	16.87 ± 0.85
Tc7	1	0.13 ± 0.09	0.25 ± 0.05	0.14 ± 0.02	0.24 ± 0.04	1.01 ± 0.18	3.36 ± 1.20	12.14 ± 1.37
	4	0.04 ± 0.02	0.18 ± 0.07	0.14 ± 0.05	0.12 ± 0.03	0.67 ± 0.08	1.54 ± 0.17	16.03 ± 1.96
Tc8	1	0.05 ± 0.02	0.35 ± 0.02	0.39 ± 0.10	0.26 ± 0.04	1.15 ± 0.06	4.70 ± 2.64	29.97 ± 4.88
	4	0.03 ± 0.01	0.20 ± 0.02	0.28 ± 0.05	0.17 ± 0.03	0.62 ± 0.08	2.93 ± 2.79	34.87 ± 7.26

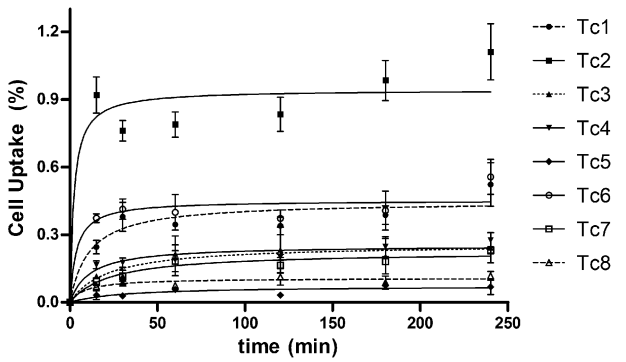


Fig. 1 *In vitro* cell uptake of complexes **Tc1–Tc8** in murine B16F1 melanoma cells.

cationic complexes (**Tc1**, **Tc2**, **Tc5** and **Tc6**) compared with the neutral congeners (**Tc3**, **Tc4**, **Tc7** and **Tc8**).

All the complexes have shown a negligible or modest tumor uptake, both at 1 h p.i. (0.24–1.21% ID g^{-1}) or at 4 h p.i. (0.17–1.69% ID g^{-1}). Nevertheless, the neutral complexes showed a reduced tumor uptake compared to the cationic ones, with uptake values at 4 h p.i. in the ranges 0.17–0.54% ID g^{-1} and 0.60–1.69% ID g^{-1}, respectively. Apparently, the increasing lipophilicity of the complexes did not improve their tumor uptake, as the neutral and lipophilic **Tc4** (log P = 1.02) has shown one of the lowest values of tumor uptake (0.54% ID g^{-1} at 4 h p.i.). In spite of their relatively poor tumor uptake, some of the complexes (*e.g.* **Tc2** and **Tc6**) have shown encouraging tumor-to-organ ratios, with tumor–muscle and tumor–blood ratios as high as 28 and 5.3, respectively, in the case of **Tc6**. Compared to radioiodinated benzamides, ^{99m}Tc(v) complexes functionalized with the same type of pharmacophore have commonly shown modest tumor uptake values,[14–22] as reported herein for the organometallic complexes **Tc1–Tc8**. So far, the best results have been reported by Eisenhut *et al.* for a $[^{99m}TcO]^{3+}$ complex anchored by a N_2S_2 chelator and bearing a 2-aminoethyldiethylamine group for melanin binding, which exhibited a considerable tumor uptake of 7.62% ID g^{-1} at 1 h p.i. in a murine melanoma model.[20] The melanoma uptake of ^{99m}Tc complexes bearing melanin-avid pharmacophores might depend on various factors, such as the nature of the Tc core and chelator, size, topology and lipophilicity of the complex, structure of the pharmacophore and position used for its attachment to the chelator framework. In particular, the lipophilicity should strongly influence their ability to freely diffuse across the cell membrane. In the published studies, no simple correlation has been found between melanoma uptake and lipophilicity, but all the evaluated ^{99m}Tc complexes having a hydrophilic character presented a low tumoral accumulation.[15–17] With the exception of **Tc4**, all the other complexes reported in this work are hydrophilic, which explains their relatively poor melanoma uptake.

The *in vivo* stability of **Tc1–Tc8** has been studied by HPLC analysis of urine and blood samples taken from mice injected with these complexes. Unlike **Tc6** and **Tc8**, all the other complexes have shown a remarkable resistance to metabolic transformation. In the latter case, the intact compounds were the unique radioactive species detected in the blood and urine. By contrast, significant amounts of metabolites were detected in the blood and/or urine of mice injected with **Tc6** and **Tc8**, as can be seen in the HPLC radiochromatograms presented in Fig. 2. For **Tc6**, the HPLC analysis of serum at 1 h p.i. has shown the presence of the intact complex (t_R = 18.90 min) together with a more lipophilic radioactive species (t_R = 22.38 min) in higher percentage (Fig. 2A). In the blood, only the original **Tc8** was found (see Fig. 2B). However, HPLC analysis of the urine of mice injected with **Tc6** and **Tc8** has shown that both complexes are transformed into more hydrophilic metabolites appearing at t_R = 17.87 min and t_R = 18.87 min, respectively. The common feature of **Tc6** and **Tc8** is the presence of a 4-amino-*N*-(2-diethylaminoethyl)benzamide pharmacophore that has been attached at the pyrazolyl ring through formation of an amide bond. As discussed above, the attachment of this pharmacophore required, in the case of

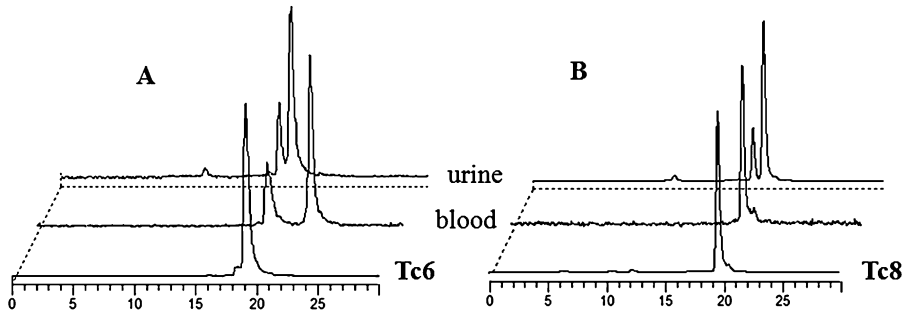


Fig. 2 HPLC analysis (radiometric detection) of blood and urine from mice injected with **Tc6 (A)** and **Tc8 (B)**, at 60 min p.i.

these complexes, the use of a propylenic spacer and the formation of an aliphatic amide. Eventually, the more hydrophilic metabolites detected in the urine may result from an enzymatic cleavage of such amide bonds. However, no further efforts were made to identify chemically the hydrophilic metabolites detected in the urine for **Tc6** and **Tc8**, or the more lipophilic species found in the blood for **Tc6**.

Conclusion

We have successfully synthesised an enlarged family of Re(I)/^{99m}Tc (I) tricarbonyl complexes anchored by bifunctional pyrazolyl-containing chelators of the N_3- or N_2O-types, bearing 2-aminoethyldiethylamine or 4-amino-*N*-(2-diethylaminoethyl)-benzamide groups as melanin-binders. Profiting from the versatility of these systems the melanin-avid fragments were introduced either at the 4-position of the azolyl ring or at the central amine of the chelator backbone. The *in vitro* evaluation of the ^{99m}Tc complexes in B16F1 murine melanoma cell lines has shown a poor cellular uptake. In line with this result, the *in vivo* tumor uptake of these complexes in B16F1 melanoma-bearing mice was rather disappointing. Nevertheless, the complexes anchored by the N_3-donors have shown, in general, an increased tumor uptake compared with the congeners anchored by the N_2O-donors. This trend reflects the highest binding affinity of the former for melanin, which is due most probably to the polyanionic nature of melanin and to the cationic character of the complexes with the N_3-donors. It is worthwhile to mention that some of these cationic compounds have shown favorable tumor–non-target ratios, which seems to indicate that they have some selectivity towards melanoma tissue. Such selectivity encourages the further optimization of the *in vitro*/*in vivo* biological properties of these types of complexes, particularly concerning their ability to cross the cell membrane and target intracellular melanin. To achieve this goal we are currently evaluating the possibility of synthesizing related, but more compact, complexes.

Experimental section

Chemistry

Unless otherwise stated, the synthesis of the ligands and complexes was carried out under a nitrogen atmosphere, using standard Schlenk techniques and dry solvents; the work-up procedures were performed under air. The compounds 4-(2-aminoethyl)[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]amino-butanoic acid (**1**),[29] ethyl 2-(1-(2-hydroxyethyl)-3,5-dimethyl-pyrazol)acetate[34] were prepared according to published methods. The starting material *fac*-[Re(H$_2$O)$_3$(CO)$_3$]Br was synthesized by the literature method.[35] 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. High-resolution mass spectra (HR-MS) were performed at the Faculty of Science of the University of Lisbon in a ApexQe FTICR Mass Spectrometer from Bruker Daltonics (Billerica, MA, USA) equipped with an electrospray ion source (ESI) and a 7 T actively shielded superconducting magnet.

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 a LC 290 tunable UV-Vis detector and to a Berthold LB-507A radiometric detector, using an analytic 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 – acetonitrile; method: 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.

Synthesis of pyrazolyl-diamine ligands (L^1 and L^2) functionalized with melanin-avid pharmacophores at the central amine group

***tert*-Butyl 2-((4-(2-(diethylamino)ethylamino)-4-oxobutyl)-(2-(3,5-dimethylpyrazol-1-yl)ethyl)amino)ethylcarbamate (2).** To a solution of 4-(2-aminoethyl)[2-(3,5-dimethyl-1*H*-pyrazol-1-yl)ethyl]amino}butanoic acid (**1**) (200 mg, 0.543 mmol) in dry CH_3CN was added *N*,*N*-diethylethylenediamine (76 μL, 0.543 mmol), HBTU (206 mg, 0.543 mmol) and NEt_3 (228 μL; 1.62 mmol). The reaction mixture was refluxed for 72 h; after cooling to room temperature, the solvent was removed under vacuum. The residue was purified by silica-gel column

chromatography using a gradient from 100% MeOH to MeOH/NH_4OH (98 : 2). Compound **2** (98%, 312 mg) was recovered from the collected fractions as a yellow oil, after removal of the solvent under reduced pressure. R_f (SiO_2 and MeOH) = 0.28; ^{1}H NMR ($CDCl_3$): δ 0.97 (6H, t, ($NCH_2C\underline{H}_3$), 1.39 (9H, s, CH_3 (BOC)), 1.66 (2H, t, CH_2), 2.07 (2H, t, CH_2), 2.17 (3H, s, CH_3-pz), 2.19 (3H, s, CH_3-pz), 2.47 (8H, m, CH_2), 2.67 (2H, t, CH_2), 2.95 (4H, q, CH_2), 3.25 (2H, t, CH_2), 3.95 (2H, t, CH_2), 5.17 (1H, b, NH-BOC), 5.75 (1H, s, H(4)-pz), 6.96 (1H, b, NH).

***tert*-Butyl 2-((4-(4-(2-(diethylamino)ethylcarbamoyl))phenyl-amino)-4-oxobutyl)(2-(3,5-dimethylpyrazol-1-yl)ethyl)amino)-ethylcarbamate (3).** Compound **3** was synthesized and recovered as described above for **2**, starting from compound **1** (120 mg, 0.543 mmol) and procainamide (89 mg, 0.326 mmol) and with reflux of the reaction mixture for 24 h. Compound **3** (41 mg, 13%) was obtained as a yellow oil after purification by silica-gel column chromatography with a gradient from CH_2Cl_2–MeOH (10 : 90) to 100% MeOH. R_f (SiO_2 and CH_2Cl_2–MeOH–NH_4OH (95 : 4 : 1)) = 0,4;^{1}H NMR (CD_3OD): δ 1.34 (15H, m, $NCH_2C\underline{H}_3$ + BOC); 2.08 (2H, t, CH_2); 2.13 (3H, s, CH_3-pz); 2.28 (3H, s, CH_3-pz); 2.62 (2H, t, CH_2); 3.33 (12H, m, 6 CH_2); 3.76 (4H, t, 2CH_2); 4.41 (2H, t, CH_2); 5.89 (1H, s, H(4)-pz); 7.67 (2H, d, Ph); 7.83 (2H, d, Ph).

4-((2-Aminoethyl)(2-(3,5-dimethylpyrazol-1-yl)ethyl)amino)-*N*-(2-diethylamino)ethyl)butanamide·CF_3COOH (L^1). To a solution of **2** (156 mg, 0.267 mmol) in CH_2Cl_2 (5 mL) was added trifluoroacetic acid (600 μL, 6.68 mmol), and the mixture was stirred for 4 h at room temperature. The solvent was removed under vacuum and the crude product was purified by RP-HPLC on a preparative Waters μ Bondapak C18 (150 × 19 mm) with a flow rate of 5.0 mL min^{-1} and using the method described above. Compound L^1 (98 mg, 77%) was recovered from the collected fractions as a transparent oil, after removal of the solvent under vacuum. IR υ_{max}(KBr)/cm^{-1} 1702 (C═O); ^{1}H NMR (CD_3OD): δ 1.36 (6H, t, 2 CH_3 ($NCH_2C\underline{H}_3$), 1.99 (2H, m, CH_2), 2.25 (3H, s, CH_3-pz), 2.35 (3H, s, CH_3-pz), 2.41 (2H, t, CH_2), 3.21 (2H, t, CH_2), 3.32 (10H, m, CH_2), 3.46 (2H, m, CH_2), 3.57 (2H, t, CH_2), 4.43 (2H, t, CH_2), 6.00 (1H, s, H(4)-pz); ^{13}C NMR (CD_3OD): δ 8.97 (CH_3), 10.72 (CH_3-pz), 13.08 (CH_3-pz), 21.24 (CH_2), 33.20 (CH_2), 35.57 (CH_2), 36.32 (CH_2), 44.37 (CH_2), 49.58 (CH_2), 51.36 (CH_2), 52.16 (CH_2), 53.95 (CH_2), 54.84 (CH_2), 107.01 (C_4-pz), 119.72 (C_{TFA}), 142.45 (C($H_{3/5}$-pz)), 149.45 ($C_{3/5}$-pz), 162.61 (C_{TFA}), 176.22 (C═O). HR ESI-MS (*m*/*z*): calcd for $[M+H]^+$, 367.31799; found, 367.32606.

4-(4-((2-Aminoethyl)(2-(3,5-dimethylpyrazol-1-yl)ethyl)amino)-butanamido)-*N*-(2-(diethylamino)ethyl)benzamide (L^2). L^2 was obtained as described above for L^1, starting from 41 mg (0.070 mmol) of compound **3**. L^2 (31 mg, 73%), was recovered as a yellow oil after removal of the solvent. IR υ_{max}(KBr)/cm^{-1} 1635, 1688 (C═O); ^{1}H NMR (CD_3OD): δ 1.34 (6H, t, 2 CH_3), 2.02 (2H, t, CH_2), 2.15 (3H, s, CH_3-pz), 2.29 (3H, s, CH_3-pz), 2.56 (2H, t, CH_2), 3.33 (12H, m, 6 CH_2), 3.74 (4H, t, 2CH_2), 4.39 (2H, t, CH_2), 5.91 (1H, s, H(4)-pz), 7.67 (2H, d, aromatic), 7.83 (2H, d, aromatic);^{13}C NMR (CD_3OD): 9.1 (CH_3), 10.9 (CH_3), 13.1 (CH_3), 23.9 (CH_2), 34.8 (CH_2), 36.3 (CH_2), 38.8 (CH_2), 47.4 (CH_2), 52.1 (CH_2), 52.8 (CH_2), 54.1 (CH_2), 54.2 (CH_2), 60.3 (CH_2), 106.3 (C(H_4-pz), 112.4 ($C_{aromatic}$), 115.97 (C_{TFA}), 116.3 ($C_{aromatic}$), 120.2 ($C_{aromatic}$), 129.4 ($C_{3/5}$-pz)), 141.1 ($C_{aromatic}$), 143.7 ($C_{3/5}$-pz), 165.33 (C_{TFA}), 170.6 (C═O), 174.6 (C═O). HR ESI-MS (*m*/*z*): calcd for $[M+H]^+$, 486.36922; found, 486.35510.

Synthesis of pyrazolyl-aminocarboxylic ligands (L^3 and L^4) functionalized with melanin-avid pharmacophores at the central amine group

Ethyl 2-(2-(3,5-dimethylpyrazol-1-yl)ethylamino)acetate (5). To a solution of 1-(2-bromoethyl)-3.5-dimethylpyrazole (**4**) (2.80 g, 13.7 mmol) in dry CH_3CN (35 mL) was added glycine ethyl ester (2.88 g, 20.6 mmol), K_2CO_3 (2.85 g, 20.6 mmol) and KI (171 mg, 1.03 mmol). The reaction mixture was refluxed for 3 days. After cooling to room temperature, the solvent was removed under vacuum. The residue obtained was applied on the top of a silica-gel column, which was eluted with a gradient of CH_2Cl_2–MeOH (95 : 5 to 85 : 15). Removal of the solvent from the collected fractions gave **5** (1.480 g, 48%) as a brown oil. R_f (SiO_2 and CH_2Cl_2–MeOH (90 : 10)) = 0.44; ^{1}H NMR ($CDCl_3$): δ 1.20 (3H, t, CH_3(Et)), 2.15 (3H, s, CH_3-pz), 2.18 (3H, s, CH_3-pz), 2.96 (2H, t, CH_2), 3.31 (2H, s, CH_2), 4.00 (2H, t, CH_2), 4.10 (2H, q, CH_2), 5.72 (1H, s, H(4-pz)).

Benzyl 4-((2-(3,5-dimethylpyrazol-1-yl)ethyl)(2-ethoxy-2-oxoethyl)amino)butanoate (6). To a solution of ethyl 2-(2-(3,5-dimethylpyrazol)ethylamino)acetate (**5**) (700 mg, 3.11 mmol) in dry CH_3CN (20 mL) was added NEt_3 (871 μL, 6.22 mmol) and KI (47 mg, 0.31 mmol). Then, a solution of benzyl 4-bromobutanoate (800 mg, 3.11 mmol) in dry CH_3CN (5 mL) was added dropwise. The reaction mixture was refluxed for 3 days. The residue obtained was applied on the top of a silica-gel column which was eluted with a gradient of CH_2Cl_2–MeOH (95 : 5 to 80 : 20). The removal of the solvent from the collected fractions gave **6** (422 mg, 34%) as a brown oil. R_f (SiO_2 and CH_2Cl_2–MeOH (90 : 10)) = 0.96; ^{1}H NMR ($CDCl_3$): δ 1.24 (3H, t, CH_3), 1.70 (2H, m, CH_2), 2.17 (3H, s, CH_3-pz), 2.18 (3H, s, CH_3-pz), 2.28 (2H, t, CH_2), 2.62 (2H, t, CH_2), 2.99 (2H, t, CH_2), 3.32 (2H, s, CH_2), 3.97 (2H, t, CH_2), 4.13 (2H, q, CH_2), 5.10 (2H, s, CH_2), 5.72 (1H, s, H_4-pz), 7.34 (5H, s, C_6H_5).

4-((2-(3,5-Dimethylpyrazol-1-yl)ethyl)(2-ethoxy-2-oxoethyl)-amino)butanoic acid (7). To a solution of benzyl 4-((2-(3,5-dimethylpyrazol)ethyl)(2-ethoxy-2 oxoethyl)amino)butanoate (**6**) (422 mg, 1.05 mmol) in dry ethanol (40 mL) was added palladium on charcoal, 10% (500 mg). The reaction mixture was saturated with H_2 and stirred at room temperature for 20 h. The catalyst was removed by vacuum filtration through a pad of celite; the supernatant was evaporated under vacuum to afford **7** (257 mg, 79%) as a transparent oil. ^{1}H NMR ($CDCl_3$): δ 1.22 (3H, t, CH_3), 1.72 (2H, m, CH_2), 2.17 (3H, s, CH_3-pz), 2.20 (3H, s, CH_3-pz), 2.35 (2H, t, CH_2), 2.68 (2H, t, CH_2), 2.99 (2H, t, CH_2), 3.24 (2H, s, CH_2), 3.73 (2H, t, CH_2), 4.10 (2H, q, CH_2), 5.75 (1H, s, H_4-pz).

Ethyl 2-((4-(2-(Diethylamino)ethylamino)-4-oxobutyl)(2-(3,5-dimethypyrazol-1-yl)ethyl)amino)acetate (8). Compound **8** was obtained as above described for **2**, starting from 128 mg

(0.41 mmol) of **7** and reflux of the reaction mixture for 48 h. The purification of **8** (70 mg, 42%) was performed by silica-gel column chromatography using a gradient from 100% MeOH to MeOH–NH_4OH (98 : 2); R_f (SiO_2 and MeOH) = 0.29; ^1H NMR ($CDCl_3$): δ 1.06 (6H, t, CH_3), 1.18 (3H, t, CH_3), 1.65 (2H, m, CH_2), 2.11 (3H, s, CH_3-pz), 2.13 (2H, m, CH_2), 2.17 (3H, s, CH_3-pz), 2.53 (2H, t, CH_2), 2.69 (6H, m, CH_2), 2.93 (2H, t, CH_2), 3.23 (2H, s, CH_2), 3.35 (2H, m, CH_2), 3.96 (2H, t, CH_2), 4.06 (2H, q, CH_2), 5.68 (1H, s, H_4-pz), 7.26 (1H, b, NH).

Ethyl 2-((4-(4-(2-(diethylamino)ethylcarbamoyl)phenylamino)-4-oxobutyl)(2-(3,5-dimethypyrazol-1-yl)ethyl)amino)acetate (9). Compound **9** was obtained as above described for **3**, starting from 450 mg (14.5 mmol) of **7** and with reflux of the reaction mixture for 72 h. The purification of **9** (107 mg, 14%) was performed by silica-gel column chromatography with $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2) as eluent; R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (70 : 28 : 2)) = 0.80; ^1H NMR ($CDCl_3$): δ 1.00 (6H, t, CH_3), 1.20 (3H, t, CH_3), 1.77 (2H, m, CH_2), 2.15 (3H, s, CH_3-pz), 2.19 (3H, s, CH_3-pz), 2.31 (2H, m, CH_2), 2.57 (8H, m, 4 CH_2), 2.93 (2H, t, CH_2), 3.21 (2H, s, CH_2), 3.43 (2H, m, CH_2), 3.97 (2H, t, CH_2), 4.09 (2H, q, CH_2), 5.78 (1H, s, H_{4-pz}), 6.93 (1H, bs, NH), 7.69 (4H, s, CH), 9.84 (1H, b, NH).

[(3-{[2-(Diethylamino)ethyl]carbamoyl}propyl)[2-(3,5-dimethyl-pyrazol-1-yl)ethyl]amino]acetic acid (L^3). To a solution of **8** (70 mg, 0.17 mmol) in THF were added 7 mL of an aqueous 0.02 M NaOH solution (molar ratio **8**/NaOH = 1 : 10), and the mixture was refluxed overnight. After cooling to room temperature, the solution was neutralized to pH 7 and the solvent removed under vacuum. The residue was extracted with CH_2Cl_2 and the resulting solution was dried under vacuum giving a yellow oil that was formulated as L^3 (64 mg, 98%); IR υ_{max}(KBr)/cm^{-1} 1581, 1659 (C═O); ^1H NMR (CD_3OD): δ 1.17 (6H, t, 2CH_3), 1.66 (2H, m, CH_2), 2.06 (3H, s, CH_3-pz), 2.13 (2H, m, CH_2), 2.19 (3H, s, CH_3-pz), 2.58 (2H, t, CH_2), 2.93 (2H, t, CH_2), 2.98 (6H, m, 3 CH_2), 3.14 (2H, s, CH_2), 3.48 (2H, t, CH_2), 4.04 (2H, t, CH_2), 5.73 (1H, s, H_4-pz); ^{13}C NMR (CD_3OD): δ 8.22 (2 CH_3), 9.78 (CH_3-pz), 12.07 (CH_3-pz), 22.62 (CH_2), 32.58 (CH_2), 34.90 (CH_2), 45.60 (CH_2), 47.27 (CH_2), 51.30 (CH_2), 53.62 (CH_2), 53.96 (CH_2), 61.65 (CH_2), 104.85 (C_4-pz), 140.51 ($C_{3/5}$-pz), 147.66 ($C_{3/5}$-pz); 175.69 (C═O); 176.52 (C═O). HR ESI-MS (m/z): calcd for [M + H]+, 382.24994; found, 382.28188.

Ethyl 2-((4-(4-(2-(diethylamino)ethylcarbamoyl)phenylamino)-4-oxobutyl)(2-(3,5-dimethypyrazol-1-yl)ethyl)amino)acetic acid (L^4). L^4 (60 mg, 60%) was prepared as above described for L^3, starting from 107 mg (0.27 mmol) of **9**. Compound **9** was isolated as a yellow oil after purification by silica-gel column chromatography with MeOH–NH_4OH (99 : 1) as eluent. R_f (SiO_2 and MeOH–NH_4OH (99 : 1)) = 0.56; IR υ_{max}(KBr)/cm^{-1} 1544, 1632, 1685 (C═O); ^1H NMR (CD_3OD): δ 1.33 (6H, t, CH_3); 1.97 (2H, m, CH_2), 2.14 (3H, s, CH_3-pz), 2.25 (3H, s, CH_3-pz), 2.53 (2H, t, CH_2), 3.24 (10H, m, CH_2), 3.55 (2H, s, CH_2), 3.74 (2H, t, CH_2), 4.28 (2H, t, CH_2), 5.83 (1H, s, H_4-pz), 6.93 (1H, b, NH), 7.69 (2H, d, CH-aromatic), 7.83 (2H, d, CH-aromatic). ^{13}C NMR ($CDCl_3$): δ 9.06 (2 CH_3), 10.64 (CH_3-pz), 13.19 (CH_3-pz), 21.25 (CH_2), 34.35 (CH_2), 36.15 (CH_2), 43.88 (CH_2), 48.94 (CH_2), 52.65 (CH_2), 54.81 (CH_2), 55.96 (CH_2), 57.54 (CH_2), 106.49 (C_4-pz), 120.17 ($C_{aromatic}$), 129.29 ($C_{aromatic}$), 129.33 ($C_{aromatic}$), 141.73 ($C_{3/5}$-pz)), 143.42 ($C_{aromatic}$), 149.69 ($C_{3/5}$-pz)), 170.24 (C═O), 170.93 (C═O), 173.14 (C═O). HR ESI-MS (m/z): calcd for $[M + H]^+$, 501.31565; found, 501.23801.

Synthesis of pyrazolyl-diamine ligands (L^5 and L6) functionalized with melanin-avid pharmacophores at the 4-position of the azolyl ring

Ethyl 2-(1-(2-bromoethyl)-3,5-dimethylpyrazol-1-yl)acetate (11). To a solution of ethyl 2-(1-(2-hydroxyethyl)-3,5-dimethyl-pyrazol-1-yl)acetate, **10** (7.500 g, 35 mmol) in toluene (150 mL) was added PBr_3 (6.58 mL, 70 mmol), and the reaction mixture was refluxed overnight. After cooling to room temperature, the solvent was removed under vacuum, the residue dissolved in $CHCl_3$ and the resulting solution washed with distilled water. The organic phase was dried over $MgSO_4$, filtered and evaporated under vacuum, giving a yellow oil that was formulated as compound **11**. Yield: 55% (5.570 g, 19.2 mmol). ^1H NMR ($CDCl_3$): δ 1.21 (3H, t, CH_3); 2.17 (3H, s, CH_3-pz); 2.21 (3H, s, CH_3-pz); 3.30 (2H, s, CH_2); 3.67 (2H, q, CH_2); 4.10 (2H, m, CH_2); 4.33 (2H, m, CH_2).

Ethyl 2-(1-(2-(2-(*tert*-butoxycarbonylamino)ethylamino)ethyl)-3,5-dimethylpyrazol-1-yl)acetate (12). To a solution of ethyl 2-(1-(2-bromoethyl)-3,5-dimethylpyrazol-1-yl)acetate (**11**) (2.770 g, 9.6 mmol) in dry CH_3CN (35 mL) was added *tert*-butyl 2-aminoethylcarbamate (2.31 g; 14.4 mmol), K_2CO_3 (1.990 g; 14.4 mmol) and KI (80 mg; 0.48 mmol). The reaction mixture was refluxed overnight, and after cooling to room temperature the solvent was removed under vacuum. The product was purified by silica-gel column chromatography using CH_2Cl_2–MeOH (90/10) as the eluent. Removal of the solvent from the collected fractions gave **12** (650 mg, 12%) as a yellow oil. R_f (SiO_2 and CH_2Cl_2–MeOH (90 : 10)) = 0.38; ^1H NMR ($CDCl_3$): δ 1.20 (3H, t, CH_3), 1.39 (9H, s, CH_3 (BOC)), 2.14 (3H, s, CH_3), 2.16 (3H, s, CH_3), 2.36 (2H, t, CH_2), 2.70 (2H, t, CH_2), 2.98 (2H, t, CH_2), 3.16 (2H, s, CH_2), 4.00 (2H, t, CH_2), 4.05 (2H, q, CH_2), 5.17 (1H, b, NH).

Ethyl 2-(1-(2-(*tert*-butoxycarbonyl)(2-(*tert*-butoxycarbonyl-amino)ethyl)amino)ethyl)-3,5-dimethylpyrazol-1-yl)acetate (13). To a solution of **12** (650 mg; 1.75 mmol) in dry THF (25 mL) was added dropwise, at 0 °C, a solution of di-*tert*-butyl dicarbonate (382 mg, 1.75 mmol) in dry THF (25 mL). The reaction mixture was stirred overnight, at room temperature. The solvent was removed under vacuum, and the crude product extracted with a mixture of aqueous Na_2CO_3/$CHCl_3$. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent was removed under vacuum, giving **13** (820 mg, 99%) as a yellow oil. ^1H NMR ($CDCl_3$): δ 1.21 (3H, t, CH_3), 1.39 (18H, m, CH_3 (BOC)), 2.16 (3H, m, CH_3-pz), 3.00 (4H, m, CH_2), 3.29 (2H, s, CH_2), 3.50 (2H, m, CH_2), 4.07 (4H, m, 2 CH_2), 4.80 (1H, b, NH).

2-(1-(2-*tert*-Butoxucarbonyl(2-(*tert*-butoxycarbonylamino)-ethyl)amino)ethyl)-3,5-dimethylpyrazol-1-yl)acetic acid (14). To a solution of **13** (830 mg, 1.75 mmol) in dry THF (10 mL) was

added a solution of NaOH (700 mg, 17.5 mmol) in H_2O (10 mL). The reaction mixture was refluxed overnight, after cooling to room temperature, the mixture was neutralized to pH 7 with aqueous 1 M HCl. The solvent was evaporated under vacuum and the residue extracted with CH_2Cl_2. After filtration, dichloromethane was removed under vacuum, giving **14** (749 mg, 97%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.37 (18H, m, CH_3 (BOC)), 2.11 (3H, m, CH_3-pz), 2.97 (4H, m, CH_2), 3.29 (2H, m, CH_2), 3.47 (2H, m, CH_2), 4.09 (2H, m, CH_2).

***tert*-Butyl *N*-(2-{[(*tert*-butoxy)carbonyl](2-{4-[2-(2,5-dioxo-pyrrolidin-1-yl)-2-oxoethyl]-3,5-dimethylpyrazol-1-yl}ethyl)amino}-ethyl)carbamate (15).** To a solution of **14** (400 mg, 0.90 mmol) in dry CH_2Cl_2 (15 mL) was added, at 0 °C, *N*-hydroxy-succinimide (NHS) (115 mg, 0.99 mmol) and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (190 mg, 0.99 mmol). The resulting mixture was stirred at room temperature for 72 h and, after this time, was washed with distilled water. The organic phase was separated and dried over $MgSO_4$; after filtration, the solvent was removed under vacuum, giving **15** (478 mg, 93%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 1.37 (18H, m, CH_3 (BOC)), 2.13 (3H, m, CH_3-pz), 2.77 (4H, s, CH_2), 2.85 (4H, m, CH_2), 3.48 (2H, m, CH_2), 3.60 (2H, m, CH_2), 4.09 (2H, m, CH_2).

4-(4-Aminobutanoyloxyamino)-*N*-(2-(diethylamino)ethyl)-benzamide (16). To a solution of 4-(*tert*-butoxycarbonylamino)-butanoic acid (2.00g, 7.06 mmol) in dry CH_3CN (30 mL) was added procainamaide (1.44 g, 5.30 mmol), HBTU (2.678 g, 7.06 mmol) and NEt_3 (2.96 mL, 21.2 mmol). The reaction mixture was refluxed for 48 h and, after cooling to room temperature, the solvent was removed under vacuum. The resulting amide derivative *tert*-butyl 4-(4-(2-(diethylamino)-ethylcarbamoyl)phenylaminooxy)-4-oxobutylcarbamate was purified by silica-gel column chromatography using methanol as eluent. The product obtained from the collected fractions was dissolved in CH_2Cl_2 (5 mL) and stirred for 4 h in the presence of TFA (5 mL). Removal of the solvent under vacuum gave **16** (773 mg, 90%) as a yellow oil. R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2)) = 0.42; ^{1}H NMR (CD_3OD): δ 1.26 (3H, t, $2CH_3$), 1.95 (2H, m, CH_2), 2.50 (2H, t, CH_2), 2.96 (2H, t, CH_2), 3.24 (4H, q, 2 CH_2), 3.32 (2H, m, CH_2), 3.68 (2H, m, CH_2), 7.62 (2H, m, $H_{aromatic}$), 7.75 (2H, m, $H_{aromatic}$).

***tert*-Butyl *N*-(2-{[(*tert*-butoxy)carbonyl]({2-[4-({[2-(diethyl-amino)ethyl]carbamoyl}methyl)-3,5-dimethylpyrazol-1-yl]ethyl})-amino}ethyl)carbamate (17).** To a solution of **15** (400 mg, 0.67 mmol) in dry DMF was added *N*,*N*-diethylethylene-diamine (103 μL; 0.73 mmol) and *N*,*N*-diisopropylethylamine (DIPEA) (183 μL; 1.05 mmol). The reaction mixture was stirred at room temperature for 72 h. After removal of the solvent, **17** (340 mg, 91%) was purified by silica-gel column chromatography with $CHCl_3$–MeOH–NH_4OH (88/10 : 2) as the eluent. R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (88/10 : 2)) = 0.59; ^{1}H NMR ($CDCl_3$): δ 0.86 (6H, m, CH_3), 1.34 (18H, m, (CH_3)), 2.10 (6H, m, $2CH_3$-pz), 2.51 (8H, m, 4 CH_2), 3.24 (6H, m, 3 CH_2), 3.49 (2H, m, CH_2), 4.09 (2H, m, CH_2).

***tert*-Butyl *N*-(2-{[(*tert*-butoxy)carbonyl](2-{4-[({3-[(4-{[2-(diethyl-amino)ethyl]carbamoyl}phenyl)carbamoyl]propyl}carbamoyl)-methyl]-3,5-dimethylpyrazol-1-yl}ethyl)amino}ethyl)carbamate (18).** Compound **18** was synthesized as described above for **17**, starting from **15** (333 mg, 0.58 mmol) and **16** (186 mg, 0.58 mmol). **18** (70 mg, 16%) was purified by silica-gel column chromatography using $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2) as the eluent. R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2)) = 0.64; ^{1}H NMR ($CDCl_3$): δ 0.97 (6H, t, $2CH_3$), 1.25 (9H, s, CH_3 (BOC)), 1.39 (9H, s, CH_3 (BOC)), 1.78 (2H, m, CH_2), 2.05 (3H, s, CH_3-pz), 2.11 (3H, s, CH_3-pz), 2.29 (2H, m, CH_2), 2.51 (4H, q, CH_2), 2.58 (2H, t, CH_2), 3.39 (12H, m, 6-CH_2), 4.09 (2H, m, CH_2), 6.61 (1H, b, NH), 6.83 (1H, b, NH), 6.97 (1H, b, NH), 7.69 (4H, s, $H_{aromatic}$), 9.87 (1H, b, NH-BOC).

2-(1-(2-(2-Aminoethylamino)ethyl)-3,5-dimethylpyrazol-1-yl)-*N*-(2-(diethylamino)ethyl)acetamide·CF_3COOH (L^5). L^5 (107 mg, 73%) was synthesized and recovered as described above for L^2, starting from **177** mg (0.33 mmol) of **17**. IR υ_{max}(KBr)/cm^{-1} 1710 (C=O); ^{1}H NMR (CD_3OD): δ 1.30 (6H, t, CH_3), 2.15 (3H, s, CH_3-pz), 2.23 (3H, s, CH_3-pz), 3.23 (6H, m, CH_2), 3.43 (6H, m, CH_2), 3.55 (4H, m, CH_2), 4.38 (2H, t, CH_2); ^{13}C NMR (CD_3OD): δ 9.12 (CH_3); 9.39 (CH_3-pz); 11.83 (CH_3-pz); 31.09 (CH_2); 35.87 (CH_2); 36.79 (CH_2); 44.95 (CH_2); 45.73 (CH_2); 48.72 (CH_2); 49.85 (CH_2); 52.34 (CH_2); 115.59 (C_4-pz); 140.48 ($C_{3/5}$-pz); 149.29 ($C_{3/5}$-pz), 162.92 (C_{TFA}), 175.23 (C=O). HR ESI-MS (*m*/*z*): calcd for $[M + H]^+$, 339.28669; found, 339.29361.

4-(4-(2-(1-(2-Aminoethylamino)ethyl)3,5-dimethylpyrazol-1-yl)-acetamido)butanamido)-*N*-(2-(diethylamino)ethyl)benzamide·CF_3COOH (L^6). L^6 (230 mg, 80%) was synthesized and recovered as described above for L^2, starting from 234 mg (0.44 mmol) of **18**. IR υ_{max}(KBr)/cm^{-1} 1646, 1699 (C=O); ^{1}H NMR ($CDCl_3$): δ 1.27 (6H, t, CH_3); 1.81 (2H, m, CH_2), 2.09 (3H, s, $2CH_3$-pz), 2.16 (3H, s, $2CH_3$-pz), 2.36 (2H, m, CH_2), 3.25 (14H, m, 7 CH_2), 3.49 (2H, m, CH_2), 3.68 (2H, m, CH_2), 4.34 (2H, m, CH_2), 7.63 (2H, d, $H_{aromatic}$), 7.75 (2H, d, $H_{aromatic}$); ^{13}C NMR ($CDCl_3$): δ 7.92 (CH_3); 8.21 (CH_3-pz), 10.49 (CH_3-pz), 25.15 (CH_2), 29.95 (CH_2), 34.06 (CH_2), 35.20 (CH_2), 35.62 (CH_2), 38.93 (CH_2), 43.83 (CH_2), 44.58 (CH_2), 46.97 (CH_2), 51.80 (CH_2), 53.92 (CH_2), 109.16 (C_{4-pz}), 114.37 (C_{TFA}), 119.14 ($C_{aromatic}$), 120.48 ($C_{aromatic}$), 128.14 ($C_{aromatic}$), 141.87 ($C_{3/5}$-pz), 142.51 ($C_{aromatic}$), 148.02 ($C_{3/5}$-pz)), 160.35 (C_{TFA}), 169.53 (C=O), 171.25 (C=O), 172.89 (C=O). HR ESI-MS (*m*/*z*): calcd for $[M + H]^+$, 543.37656; found, 543.39389.

Synthesis of pyrazolyl-amino carboxylic ligands (L^7 and L^8) functionalized with melanin-avid pharmacophores at the 4-position of the azolyl ring

Benzyl 3-acetyl-4-oxopentanoate (20). To a suspension of NaH (3.16 g, 79 mmol) in dry THF (85 mL) was added dropwise, at 0 °C, freshly distilled pentane-2,4-dione (7.50 mL, 72 mmol). The resulting suspension was stirred at 0 °C for 1 h; then, benzyl bromoacetate (1.36 mL, 72 mmol) was added dropwise at the same temperature. The reaction mixture was stirred for 3 h at 0 °C, and overnight at room temperature. After this time, the mixture was treated with

aqueous 2 M HCl (383 mL) and then extracted with diethyl ether. The organic phase was separated, dried over $MgSO_4$, filtered and the solvent removed under vacuum, giving **20** (16.0 g, 90%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 2.11 (3H, s, CH_3–C═O *enol*), 2.14 (3H, s, CH_3–C═O, *enol*), 2.23 (6 H, s, 2 CH_3–C═O *keto*), 2.91 (2H, d, CH_2COOEt, *enol*), 3.28 (2H, d, CH_2COOEt, *keto*), 5.09 (4H, m, $COOCH_2$, *enol* + *keto*), 7.32 (10H, m, $H_{aromatic}$, *keto* + *enol*).

Benzyl 2-(1-(2-hydroxyethyl)-3,5-dimethylpyrazol-1-yl)acetate (21). To a solution of **20** (9.00 g, 36 mmol) in ethanol (70 mL) was added dropwise, at 0 °C, a solution of 2-hydroxyethylhydrazine (2.46 mL, 36 mmol) in ethanol (30 mL). The reaction mixture was stirred overnight at room temperature and, after this time, the solvent was removed under vacuum affording **21** (5.0 g, 98%) as an orange solid. ^{1}H NMR ($CDCl_3$): δ 2.17 (6H, s, 2 CH_3-pz), 3.37 (2H, s, CH_2), 3.92 (2H, t, CH_2), 4.01 (2H, m, CH_2), 5.09 (2H, s, CH_2), 7.31 (5H, m, $H_{aromatic}$).

Benzyl 2-(1-(2-bromoethyl)-3,5-dimethylpyrazol-1-yl)acetate (22). To a solution of **21** (9.527 g, 36 mmol) in dry THF (70 mL) was added, at 0 °C, carbon tetrabromide (21.89 g, 66 mmol) and a solution of triphenylphosphine (19.04 g, 73 mmol) in dry THF (70 mL). The temperature was slowly raised and the reaction mixture was stirred overnight at room temperature. The solvent was evaporated to dryness and the residue was purified by silica-gel column chromatography with CH_2Cl_2–MeOH (95 : 5) as eluent. Compound **22** (3.235 g, 30%) was obtained as a transparent oil, after removal of the solvent from the collected fractions. ^{1}H NMR ($CDCl_3$): δ 2.15 (3H, s, CH_3-pz), 2.18 (3H, s, CH_3-pz), 3.37 (2H, s, CH_2), 3.63 (2H, t, CH_2), 4.29 (2H, m, CH_2), 5.08 (2H, s, CH_2), 7.31 (5H, m, $H_{aromatic}$).

Benzyl 2-(1-(2-(2-ethoxy-2-oxoethylamino)ethyl)-3,5-dimethylpyrazol-1-yl)acetate (23). To a solution of **22** (1.632 g, 5.25 mmol) in dry CH_3CN (35 mL) was added glycine ethyl ester (1.100 g, 7.88 mmol), NEt_3 (1.47 mL, 10.50 mmol) and KI. The resulting mixture was stirred overnight. After removal of the solvent, compound **23** (900 mg, 46%) was recovered as an yellow oil upon purification by silica-gel column chromatography with CH_2Cl_2–MeOH (95 : 5) as eluent. R_f (SiO_2 and CH_2Cl_2–MeOH (95 : 5)) = 0.38; ^{1}H NMR ($CDCl_3$): δ 1.26 (3H, t, CH_3), 2.18 (3H, s, CH_3), 2.19 (3H, s, CH_3), 3.02 (2H, t, CH_2), 3.38 (2H, t, CH_2), 3.39 (2H, t, CH_2), 4.07 (2H, t, CH_2), 4.16 (2H, q, CH_2), 5.12 (2H, s, CH_2), 7.34 (5H, m, $H_{aromatic}$).

2-(1-(2-(2-Ethoxy-2-oxoethylamino)ethyl)-3,5-dimethylpyrazol-1-yl)acetic acid (24). Compound **24** was synthesized and recovered as above described for **7**, starting from 900 mg (2.42 mmol) of **23** (630 mg, 92%). ^{1}H NMR ($CDCl_3$): δ 1.24 (3H, t, CH_3), 2.03 (3H, s, CH_3), 2.13 (3H, s, CH_3), 3.24 (2H, t, CH_2), 3.28 (2H, t, CH_2), 3.79 (2H, s, CH_2), 4.14 (2H, q, CH_2), 4.22 (2H, m, CH_2).

Ethyl 2-(2-(4-(2-(2-(diethylamino)ethylamino)-2-oxoethyl)-3,5-dimethylpyrazol-1-yl)ethylamino)acetate (25). Compound **25** was prepared as above described for **2**, starting from **24** (238 mg; 0.84 mmol) and *N*,*N*-diethylethylenediamine (130 μL, 0.92 mmol) and by refluxing the reaction mixture for 24 h. The purification of **25** (108 mg, 34%) was performed using silica-gel column chromatography using a gradient elution from $CHCl_3$–MeOH (80 : 18) to $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2). R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (85 : 13 : 2)) = 0.53; ^{1}H NMR ($CDCl_3$): δ 0.79 (6H, t, CH_3), 1.16 (3H, t, CH_3), 2.06 (3H, s, CH_3), 2.09 (3H, s, CH_3), 2.35 (6H, m, CH_2), 2.92 (2H, t, CH_2), 3.12 (2H, t, CH_2), 3.19 (2H, s, CH_2), 3.29 (2H, s, CH_2), 4.01 (2H, t, CH_2); 4.08 (2H, q, CH_2); 6.16 (1H, s, NH).

Ethyl 2-(2-(4-(2-(4-(4-(2-(diethylamino)ethylcarbamoyl)phenylamino)-4-oxobutylamino)-2-(oxoethyl)-3,5-dimethylpyrazol-1-yl)-ethylamino)acetate (26). Compound **26** was prepared as above described for **2**, starting from **24** (450 mg, 1.60 mmol) and **16** (509 mg, 1.60 mmol) and by refluxing the reaction mixture for 48 h. The purification of **25** (100 mg, 10%) was performed using silica-gel column chromatography using a gradient elution from $CHCl_3$–MeOH–NH_4OH (90 : 8 : 2) to MeOH–NH_4OH (98 : 2)). R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (85 : 13 : 2)) = 0.63; ^{1}H NMR ($CDCl_3$): δ 1.16 (9H, t, CH_3), 1.78 (2H, m, CH_2), 2.11 (3H, s, CH_3-pz), 2.14 (3H, s, CH_3-pz), 2.32 (2H, m, CH_2), 2.94 (8H, m, CH_2), 3.31 (4H, m, CH_2), 3.61 (4H, m, CH_2), 4.09 (4H, m, CH_2), 7.71 (4H, m, $H_{aromatic}$).

2-(2-(4-(2-(2-(diethylamino)ethylamino)-2-oxoethyl)-3,5-dimethylpyrazol-1-yl)ethylamino)acetic acid (L^7). L^7 (100 mg, 98%) was prepared and recovered in the form of a yellow oil as above described for L^3, starting from 108 mg (0.28 mmol) of **25**. IR υ_{max}(KBr)/cm^{-1} 1590, 1664 (C═O); ^{1}H NMR (CD_3OD): δ 1.31 (6H, t, CH_3), 2.13 (3H, s, CH_3), 2.24 (3H, s, CH_3), 3.22 (6H, m, CH_2), 3.38 (2H, s, CH_2), 3.47 (2H, t, CH_2), 3.55 (2H, s, CH_2), 3.59 (2H, s, CH_2), 4.37 (2H, t, CH_2); ^{13}C NMR (CD_3OD): δ 8.11 (CH_3), 8.51 (CH_3-pz), 10.80 (CH_3-pz), 30.32 (CH_2), 34.79 (CH_2), 44.37 (CH_2), 47.04 (CH_2), 48.01 (CH_2), 49.38 (CH_2), 51.40 (CH_2), 110.27 (C_4-pz), 139.20 ($C_{3/5}$-pz), 148.10 ($C_{3/5}$-pz), 169.85 (C═O), 173.66 (C═O). HR ESI-MS (*m*/*z*): calcd for $[M + H]^+$, 354.24884; found, 354.24885.

Ethyl 2-(2-(4-(2-(4-(4-(2-(diethylamino)ethylamino)ethyl)-phenylamino)-4-oxobutylamino)(2-oxoethyl)3,5-dimethypyrazol-1-yl)ethyl)amino)acetic acid (L^8). L^8 was synthesized as described above for L^3, starting from 100 mg (0.17 mmol) of **26**. L^8 (40 mg, 42%) was recovered as a yellow oil after purification by silica-gel column chromatography with gradient elution from $CHCl_3$–MeOH–NH_4OH (75 : 13 : 2) to MeOH–NH_4OH (98 : 2). R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (75 : 13 : 2)) = 0.42; IR υ_{max}(KBr)/cm^{-1} 1586, 1652, 1687 (C═O); ESI/MS (+) (*m*/*z*): 558.2 $[M + H]^+$; ^{1}H NMR (CD_3OD): δ 1.35 (6H, t, CH_3), 1.88 (2H, m, CH_2), 2.13 (3H, s, CH_3-pz), 2.22 (3H, s, CH_3-pz), 2.44 (2H, m, CH_2), 2.42 (2H, m, CH_2), 3.32 (12H, m, 6 CH_2), 3.64 (2H, m, CH_2), 3.78 (2H, m, CH_2), 4.33 (2H, m, CH_2), 7.68 (2H, d, $H_{aromatic}$), 7.84 (2H, d, $H_{aromatic}$); ^{13}C NMR ($CDCl_3$): δ 8.02 (CH_3), 8.33 (CH_3-pz), 10.76 (CH_3-pz), 25.04 (CH_2), 30.27 (CH_2), 34.16 (CH_2), 35.23 (CH_2), 38.99 (CH_2), 44.01 (CH_2), 47.08 (CH_2), 47.90 (CH_2), 48.41 (CH_2), 51.73 (CH_2), 110.74 (C_4-pz), 119.21 ($C_{aromatic}$), 128.31 ($C_{aromatic}$), 139.00 ($C_{aromatic}$), 139.47 ($C_{3/5}$-pz), 142.45 ($C_{aromatic}$), 147.98 ($C_{3/5}$-pz), 169.37 (C═O), 169.67 (C═O), 172.80 (C═O), 173.00 (C═O). HR ESI-MS (*m*/*z*): calcd for $[M + H]^+$, 354.24884; found, 354.24885.

General procedure for the synthesis of the Re complexes (Re1–Re8)

$[Re(H_2O)_3(CO)_3]Br$ was reacted with equimolar amounts of L^1–L^8 in refluxing methanol for 18 h. After this time, the solvent was removed under vacuum and the desired complexes were purified by washing with organic solvents or water, by column chromatography, recrystallization 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 the method described above.

***fac*-[Re(CO)$_3$(k^3-L^1)](CF$_3$COO) (Re1). Re1** (36 mg, 45%) was purified by recrystallization from MeOH–diethyl ether at 0 °C. IR υ_{max}(KBr)/cm^{-1} 1735 (C═O), 1931, 2028 (C≡O); ^{1}H NMR (CD_3OD): δ 1.34 (6H, t, CH_3), 2.05 (1H, m, CH_2), 2.20 (1H, m, CH_2), 2.37 (3H, s, CH_3-pz), 2.41 (2H, t, CH_2); 2.44 (3H, s, CH_3-pz), 2.58 (1H, m, CH_2), 2.72 (1H, m, CH), 2.90 (2H, m, CH_2), 3.17 (1H, m, CH_2), 3.31 (8H, m, CH_2), 3.41 (1H, m, CH_2), 3.60 (3H, m, CH_2), 3.72 (1H, m, CH_2); 4.10 (1H, b, NH_2); 4.21 (1H, m, CH_2), 4.56 (1H, m, CH_2), 5.54 (1H, b, NH), 6.19 (1H, s, H_4-pz); ^{13}C NMR ($CDCl_3$): 9.23 ($2CH_3$), 11.62 (CH_3-pz), 16.10 (CH_3-pz), 21.01 (CH_2), 33.33 (CH_2), 35.77 (CH_2), 43.72 (CH_2), 48.97 (CH_2), 49.83 (CH_2), 52.57 (CH_2), 53.88 (CH_2), 62.55 (CH_2), 67.30 (CH_2), 109.20 (C_4-pz), 119.87 (C_{TFA}), 145.36 ($C_{3/5}$-pz), 155.10 ($C_{3/5}$-pz), 165.35 (C_{TFA}), 175.90 (C═O), 193.72 (C≡O), 194.90 (C≡O), 195.27 (C≡O). HR ESI-MS (m/z): calcd for M^+, 637.25072; found, 637.27338.

***fac*-[Re(CO)$_3$(k^3-L^2)](CF$_3$COO) (Re2). Re2** (249 mg, 67%) was purified by washing of the crude with CH_2Cl_2 and with a mixture of *n*-hexane–diethyl ether. IR υ_{max}(KBr)/cm^{-1} 1667, 1701 (C═O), 1942, 2017 (C≡O); ^{1}H NMR (CD_3OD): δ 1.34 (6H, t, CH_3), 2.12 (2H, m, CH_2), 2.25 (2H, m, CH_2), 2.37 (3H, s, CH_3), 2.53 (3H, s, CH_3), 2.67 (3H, m, CH_2), 2.76 (1H, m, CH_2), 2.89 (2H, m, CH_2), 3.19 (1H, m, CH), 3.39 (4H, q, CH_2), 3.54 (2H, m, CH_2), 3.67–3.75 (3H, m, CH_2), 3.92 (1H, m, NH), 4.22 (1H, m, CH), 4.54 (1H, m, CH), 5.53 (1H, m, NH), 6.20 (1H, s, H_4-pz), 7.71–7.87 (4H, m, Ar); ^{13}C NMR (CD_3OD): 9.1 (CH_3), 11.5 (CH_3), 13.1 (CH_3), 16.0 (CH_2), 20.8 (CH_2), 34.2 (CH_2), 36.4 (CH_2), 43.7 (CH_2), 53.0 (CH_2), 53.8 (CH_2), 62.4 (CH_2), 67.2 (CH_2), 109.2 (C_4-pz), 116.3 ($C_{aromatic}$), 120.3 ($C_{aromatic}$), 120.67 (C_{TFA}), 129.4 ($C_{aromatic}$), 143.6 ($C_{3/5}$-pz), 145.3 ($C_{aromatic}$), 155.1 ($C_{3/5}$-pz), 165.78 (C_{TFA}), 170.6 (C═O), 174.6 (C═O), 190.72 (C≡O), 193.67 (C≡O), 195.32 (C≡O). ESI/MS (+) (m/z): 378.5 $[M]^{2+}$.

***fac*-[Re(CO)$_3$(k^3-L^3)] (Re3). Re3** (60 mg, 71%) was purified by washing of the crude with distilled water. IR υ_{max}(KBr)/cm^{-1} 1629, 1845 (C═O), 1971, 2020 (C≡O); ESI/MS (+) (m/z): 651.9 $[M + H]^+$; ^{1}H NMR (CD_3OD): δ 1.37 (6H, t, $2CH_3$); 2.12 (2H, m, CH_2); 2.36 (3H, s, CH_3-pz); 2.43 (2H, m, CH_2); 2.48 (3H, s, CH_3-pz); 2.56 (1H, m, CH_2); 3.43 (4H, m, CH_2); 3.53 (7H, m, CH_2); 3.71 (1H, s, CH_2); 3.73 (1H, s, CH_2); 4.30 (1H, m, CH_2); 4.48 (1H, m, CH_2); 6.16 (1H, s, H_4-pz); ^{13}C NMR (CD_3OD): δ 9.27 (CH_3), 11.46 (CH_3-pz), 15.86 (CH_3-pz), 20.51 (CH_2), 33.19 (CH_2), 35.80 (CH_2), 46.80 (CH_2), 48.15 (CH_2), 52.66 (CH_2), 56.64 (CH_2), 63.80 (CH_2), 66.31 (CH_2), 108.79 (C(H_4-pz), 145.13 ($C_{3/5}$-pz), 155.17 ($C_{3/5}$-pz)), 175.89 (C═O), 181.65 (C═O), 195.24 (C≡O), 196.40 (C≡O), 196.97 (C≡O). HR ESI-MS (m/z): calcd for $[M + H]^+$, 652.21637; found, 652.21636.

***fac*-[Re(CO)$_3$(k^3-L^4)] (Re4). Re4** (60 mg, 71%) was purified by silica-gel column chromatography using $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2) as eluent. R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2)) = 0.73; IR υ_{max}(KBr)/cm^{-1} 1653, 1687, 1865 (C═O), 1909, 2024 (C≡O); ^{1}H NMR (CD_3OD): δ 1.30 (6H, t, CH_3); 2.23 (2H, m, CH_2), 2.33 (3H, s, CH_3-pz), 2.46 (3H, s, CH_3-pz), 2.53 (3H, m, CH_2), 3.24 (2H, m, CH_2), 3.34 (2H, m, CH_2), 3.37–3.75 (9H, m, CH_2), 4.27 (1H, m, CH_2); 4.49 (1H, m, CH_2), 6.13 (1H, s, H_4-pz); 7.72 (2H, d, $C_{aromatic}$), 7.85 (2H, d, $C_{aromatic}$); ^{13}C NMR (CD_3OD): δ 9.23 (CH_3), 11.38 (CH_3-pz), 15.84 (CH_3-pz), 20.44 (CH_2), 34.27 (CH_2), 36.46 (CH_2), 46.76 (CH_2), 49.11 (CH_2), 53.06 (CH_2), 56.56 (CH_2), 63.80 (CH_2), 66.29 (CH_2), 108.79 (C_4-pz), 120.36 ($C_{aromatic}$), 129.33 ($C_{aromatic}$), 129.48 ($C_{aromatic}$), 143.6 ($C_{aromatic}$), 143.60 ($C_{3/5}$-pz), 145.12 ($C_{3/5}$-pz), 170.59 (C═O), 173.05 (C═O), 181.69 (C═O), 195.23 (C≡O), 196.27 (C≡O), 196.94 (C≡O). HR ESI-MS (m/z): calcd for $[M + H]^+$, 771.25397; found, 771.25395.

***fac*-[Re(CO)$_3$(k^3-L^5)](CF$_3$COO) (Re5). Re5** (12 mg, 20%) was purified by RP-HPLC, as described above. IR υ_{max}(KBr)/cm^{-1}: 1696 (C═O), 1923, 2026 (C≡O); ^{1}H NMR (CD_3OD): δ 1.28 (6H, t, 2 CH_3); 2.31 (3H, s, CH_3); 2.34 (1H, m, CH); 2.39 (3H, s, CH_3); 2.55 (4H, m, 2 CH_2); 2.87 (2H, s, CH_2); 3.24 (2H, m, CH_2); 3.46 (2H, s, CH_2); 3.58 (4H, q, $2CH_2$); 3.89 (1H, m, CH); 4.11 (1H, m, CH); 4.51 (1H, m, CH); ^{13}C NMR ($CDCl_3$): δ 7.92 (CH_3), 9.13 (CH_3-pz), 13.27 (CH_3-pz), 29.82 (CH_2), 34.69 (CH_2); 41.97 (CH_2), 46.98 (CH_2), 47.55 (CH_2), 47.83 (CH_2), 48.80 (CH_2), 51.00 (CH_2), 54.69 (CH_2), 112.39 (C_4-pz), 142.68 ($C_{3/5}$-pz), 152.44 ($C_{3/5}$-pz), 173.0 (C═O), 195.77 (C≡O), 197.00 (C≡O), 197.80 (C≡O). HR ESI-MS (m/z): calcd for M^+, 609.21941; found, 609.24015.

***fac*-[Re(CO)$_3$(k^3-L^6)](CF$_3$COO) (Re6). Re6** (150 mg, 98%) was purified by washing of the crude with CH_2Cl_2 and with a mixture of *n*-hexane–diethyl ether. IR υ_{max}(KBr)/cm^{-1} 1677, 1694 (C═O) 1923, 2032 (C≡O); ^{1}H NMR (CD_3OD): δ 1.21 (6H, t, CH_3), 1.81 (2H, m, CH_2), 2.16 (3H, s, CH_3-pz), 2.25 (3H, s, CH_3-pz), 2.32 (2H, m, CH_2), 2.42 (CH_2), 2.74 (2H, m, CH_2), 3.26 (10H, m, 5 CH_2), 3.64 (2H, m, CH_2), 3.98 (2H, m, CH_2), 3.97 (1H, m, CH_2), 4.41 (1H, m, CH_2), 7.59 (2H, d, $H_{aromatic}$), 7.71 (2H, d, $H_{aromatic}$); ^{13}C NMR ($CDCl_3$): δ 8.03 (CH_3), 9.22 (CH_3-pz), 13.33 (CH_3-pz), 25.16 (CH_2), 30.13 (CH_2), 34.15 (CH_2), 35.19 (CH_2), 38.95 (CH_2), 42.09 (CH_2), 47.05 (CH_2), 47.90 (CH_2), 48.80 (CH_2), 51.85 (CH_2), 54.70 (CH_2), 112.85 (C_4-pz), 119.11 ($C_{aromatic}$), 128.11 ($C_{aromatic}$), 128.29 ($C_{aromatic}$), 142.52 ($C_{3/5}$-pz), 142.57 ($C_{aromatic}$), 152.32 ($C_{3/5}$-pz), 169.46 (C═O), 171.85 (C═O), 172.88 (C═O), 192.97 (C≡O), 193.44 (C≡O), 194.17 (C≡O). HR ESI-MS (m/z): calcd for M^+, 813.30936; found, 813.34603.

***fac*-[Re(CO)$_3$(k^3-L^7)] (Re7). Re7** (18 mg, 24%) was purified by silica-gel column chromatography using $CHCl_3$–MeOH–NH_4OH (75 : 23 : 2) as eluent. R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2)) = 0.65; IR υ_{max}(KBr)/cm^{-1} 1665, 1870 (C═O), 1919, 2023 (C≡O); ^{1}H NMR (CD_3OD): δ 1.28 (6H, t, 2 CH_3), 2.32 (3H, s, CH_3), 2.39 (1H, m, CH), 2.45

(3H, s, CH_3), 3.20 (6H, m, CH_2), 3.35 (2H, s, CH_2), 3.45 (2H, s, CH_2), 3.57 (2H, t, CH_2), 3.66 (1H, m, CH_2); 4.17 (1H, m, CH_2); 4.47 (1H, m, CH_2); ^{13}C NMR ($CDCl_3$): δ 10.18 (CH_3), 11.11 (CH_3-pz), 13.19 (CH_3-pz), 31.24 (CH_2), 36.30 (CH_2), 47.47 (CH_2), 49.40 (CH_2), 50.45 (CH_2), 52.61 (CH_2), 56.24 (CH_2), 113.24 (C_4-pz), 143.62 ($C_{3/5}$-pz), 153.70 ($C_{3/5}$-pz), 174.18 (C═O), 182.82 (C═O), 195.33 (C≡O), 195.88 (C≡O), 196.35 (C≡O). HR ESI-MS (*m*/*z*): calcd for $[M + H]^+$, 624.18311; found, 624.18313.

***fac*-[Re(CO)$_3$(k^3-L^8)] (Re8). Re8** (26 mg, 56%) was purified by silica-gel column chromatography using $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2) as eluent. R_f (SiO_2 and $CHCl_3$–MeOH–NH_4OH (80 : 18 : 2)) = 0.73; IR υ_{max}(KBr)/cm^{-1} 1648, 1692, 1877 (C═O), 1945, 2019 (C≡O); ^{1}H NMR (CD_3OD): δ 1.17 (6H, t, CH_3), 1.87 (2H, m, CH_2), 2.26 (3H, s, CH_3-pz), 2.35 (2H, m, CH_2), 2.41 (3H, s, CH_3-pz), 2.89 (6H, m, CH_2), 3.33 (6H, m, CH_2); 3.61 (4H, m, CH_2), 4.11 (1H, m, CH_2), 4.48 (1H, m, CH_2), 7.67 (2H, d, $H_{aromatic}$), 7.80 (2H, d, $H_{aromatic}$); ^{13}C NMR ($CDCl_3$): δ 8.97 (CH_3), 9.42 (CH_3-pz), 12.76 (CH_3-pz), 25.13 (CH_2), 30.17 (CH_2), 34.03 (CH_2), 36.32 (CH_2), 38.93 (CH_2), 47.37 (CH_2), 49.09 (CH_2), 50.93 (CH_2), 51.57 (CH_2), 55.08 (CH_2), 112.48 (C_4-pz), 119.11 ($C_{aromatic}$), 128.09 ($C_{aromatic}$), 128.81 ($C_{aromatic}$), 142.30 ($C_{3/5}$-pz), 142.40 ($C_{aromatic}$), 152.46 ($C_{3/5}$-pz), 168.83 (C═O), 171.83 (C═O), 172.80 (C═O), 181.65 (C═O), 194.20 (C≡O), 195.18 (C≡O). HR ESI-MS (*m*/*z*): calcd for $[M + H]^+$, 827.27686; found, 827.27782.

Synthesis and *in vitro* evaluation of the ^{99m}Tc(I) complexes

Synthesis of Tc1–Tc8. *General method.* In a nitrogen-purged glass vial, 40 μL of a 4.4 × 10^{-5} M aqueous solution of **L^1–L^8** was added to 400 μL of a solution of the organometallic precursor *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ (1–2 mCi) in saline at pH 7.4 (**Tc1**, **Tc2**, **Tc5–Tc8**) or at pH 5.5 (**Tc3**, **Tc4**). The reaction mixture was then heated to 100 °C for 30 min, cooled to room temperature and the final solution analyzed by RP-HPLC.

Partition coefficient measurements. The log $P_{o/w}$ values of complexes **Tc1–Tc8** (Table 1) were determined by the "shake flask" method under physiological conditions (*n*-octanol–0.1 M PBS, pH 7.4).[36]

***In vitro* binding to melanin.** The binding affinity to melanin of **Tc1–Tc8** was assessed using synthetic tyrosine–melanin (Sigma). The general procedure used was as follows: A 100 μL aliquot of the radioactive preparations of **Tc1–Tc8** 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 tubes were centrifuged at 30 000 *g* for 10 min, and aliquots of the supernatant were counted in the gamma counter. Control tubes were also used containing the radioactive preparation without melanin. The difference between the activity of aliquots from the supernatants of the test tubes (with melanin) and the control tubes (without melanin) allowed the calculation of the percentage of unbound complexes.

Cell uptake studies

Cell culture. B16F1 murine melanoma cells (ECACC, UK) were grown in DMEM containing GlutaMax I supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin–streptomycin antibitiotic solution (all from Gibco, Alfagene, Lisbon). 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 (Gibco, Alfagene, Lisbon) and seeded farther apart.

Cellular uptake studies. Cellular uptake assays of the ^{99m}Tc complexes (**Tc1–Tc8**) were performed in B16F1 murine melanoma cells seeded at a density of 0.2 million per 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 × 10^5 cpm per 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.

***In vivo* evaluation of the ^{99m}Tc complexes.** 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 studies. Biodistribution of the radioconjugates was evaluated in melanoma-bearing C57BL/6 female mice (8–10 weeks old). Mice were previously implanted subcutaneously with 1 × 10^6 B16F1 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 retro-orbital sinus with complexes **Tc1–Tc8** (3–10 MBq) diluted in 100 μL of PBS pH 7.2. Mice were killed by cervical dislocation at 1, 4, and 24 h after injection. The dose administered and the radioactivity in the killed animals was 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.

Metabolism studies. The *in vivo* stability of **Tc1–Tc8** was evaluated by urine and murine serum HPLC analysis, using an

analytic Macherey-Nagel C18 reversed-phase column (Nucleosil 100–10, 250 × 3 mm) with a flow rate of 1 mL min^{-1} and the method described above. The urine was collected at the time of sacrifice 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 μm) prior to RP-HPLC analysis.

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