Available online at www.sciencedirect.com
ScienceDirect
Nuclear Medicine and Biology 35 (2008) 91–99
NUCLEAR MEDICINE — AND — BIOLOGY
www.elsevier.com/locate/nucmedbio

A $^{99m}Tc(CO)_3$-labeled pyrazolyl–α-melanocyte-stimulating hormone analog conjugate for melanoma targeting

Paula D. Raposinho[a], João D.G. Correia[a], Susana Alves[a], Maria F. Botelho[b], Ana C. Santos[b], Isabel Santos[a,*]

[a]*Departamento de Química, ITN, Estrada Nacional 10, 2686-953 Sacavém, Portugal*
[b]*Instituto de Biofísica/Biomatemática, IBILI-Faculdade de Medicina de Coimbra, 300-548 Coimbra, Portugal*

Received 6 June 2007; received in revised form 26 July 2007; accepted 11 August 2007

Abstract

Melanoma primary tumors can be, in most cases, removed surgically, whereas there is no satisfactory treatment for metastatic melanoma, being almost always lethal at this stage. Therefore, early detection of primary melanoma tumors is essential. The finding that melanocortin-1 receptor (MC1R) is overexpressed in isolated melanoma cells and melanoma tissues led to the radiolabeling of several α-melanocyte-stimulating hormone (α-MSH) analogs for early detection and treatment of melanoma. We have coupled the α-MSH analog Ac-Nle-Asp-His-D-Phe-Arg-Trp-Gly-Lys-NH_2, through the ε-amino group of Lys^{11}, to a pyrazolyl-containing chelator (pz). The resulting pz–α-MSH analog reacted with the *fac*-$[^{99m}Tc(CO)_3]^+$ moiety, giving [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-$^{99m}Tc(CO)_3$)]α-MSH_{4-11} in high yield, high specific activity and high radiochemical purity. This radioconjugate, which presents remarkable stability in vitro, exhibited time- and temperature-dependent internalization (4 h at 37°C; 56.7% maximum internalization) and high cellular retention (only 38% was released from the cell after 5 h) in murine melanoma B16F1 cells. A significant tumor uptake [4.2±0.9%ID/g, at 4 h postinjection (p.i.)] was also obtained in melanoma-bearing C57BL6 mice. The in vivo affinity and specificity of the radioconjugate to MC1R were demonstrated by receptor-blocking studies with the potent NDP-MSH agonist (63.5% reduction in tumor uptake at 4 h p.i.).

Keywords: ^{99m}Tc; α-MSH; Melanoma; MC1R

1. Introduction

Over the last decades, the incidence of melanoma has increased more rapidly than that of any other malignancy, becoming a major health issue in many countries. Early detection of primary melanoma tumors is essential, since current treatments do not substantially enhance patient survival once metastasis has occurred. Therefore, the development of new melanoma-specific radiopharmaceuticals for imaging (γ or β^+ emitters) or internal radiotherapy (β^- or α particles) is a subject of great interest and intense research. The use of radiolabeled antibodies or antibody fragments for melanoma targeting presents several disadvantages, which are related not only to a lack of true melanoma-associated antigens but also to pharmacokinetic constraints, namely, slow clearance and reduced tumor uptake [1]. Since α-melanocyte-stimulating hormone (α-MSH) receptors, namely, the melanocortin-1 receptor (MC1R), are expressed in mouse melanoma cells and human melanocytes [2–4], the development of new radiolabeled α-MSH analogs for the diagnosis and treatment of melanoma has been strongly explored in recent years [5–16]. This approach seems quite promising, since receptor agonists of that type are rapidly internalized upon interaction with the receptor. The tridecapeptide α-MSH (Fig. 1), which is produced in the brain and pituitary gland from proteolytic cleavage of proopiomelanocortin, is the most potent and naturally occurring melanotropic peptide and the most active peptide of MC1R [17]. The *MC1R* gene is a major determinant of skin and hair pigmentation, sun sensitivity and susceptibility to skin cancer. Structure–bioactivity studies revealed that the amino acid sequence His-Phe-Arg-Trp in α-MSH is sufficient

* Corresponding author. Tel.: +351 21 994 62 01; fax: +351 21 955 01 17.
E-mail address: isantos@itn.pt (I. Santos).

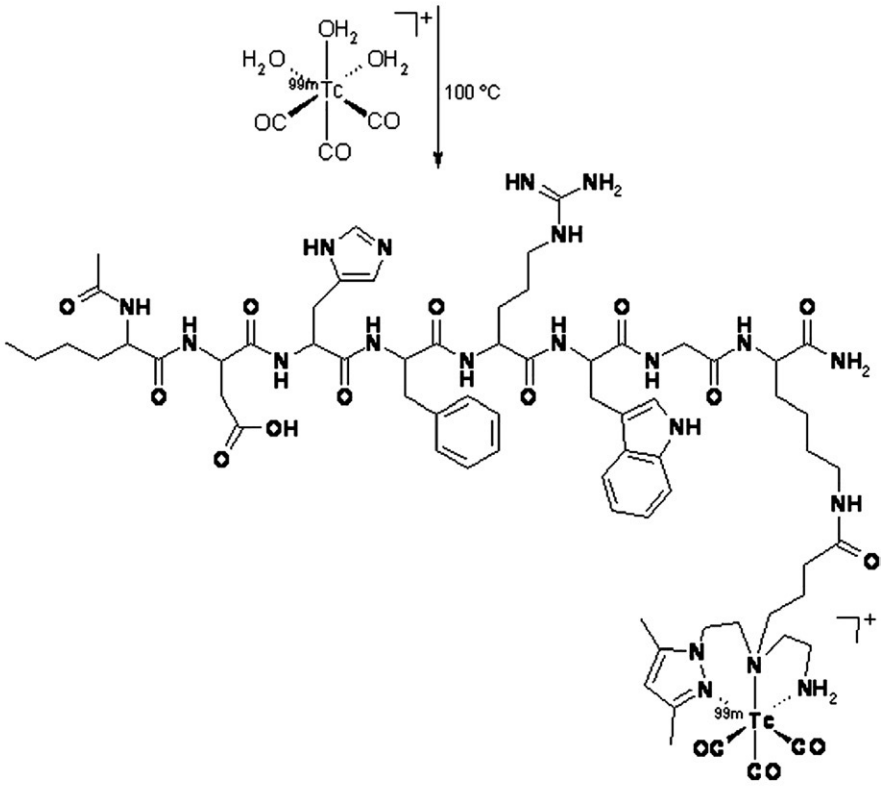
0969-8051/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nucmedbio.2007.08.001

Ac-Ser-Tyr-Ser- Met^4 -Glu^5-His – Phe^7 -Arg-Trp -Gly-Lys-Pro-Val-NH_2 **(α-MSH)**

Ac-Ser-Tyr-Ser-**Nle**^{4}-Glu^5-His-***D*Phe**^{7}-Arg-Trp-Gly-Lys-Pro-Val-NH_2 **(NDP-MSH)**

Ac-**Nle**^{4}-**Asp**^{5}-His-***D*Phe**^{7}-Arg-Trp-Gly-Lys-NH_2

Ac-**Nle**^{4}-**Asp**^{5}-His-***D*Phe**^{7}-Arg-Trp-Gly-Lys(pz)-NH_2



[Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]-αMSH$_{4\text{-}11}$

Fig. 1. Structure of α-MSH, NDP-MSH, [Ac-Nle^4,Asp^5,D-Phe^7]α-MSH$_{4–11}$, [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz)]α-MSH$_{4–11}$ and the radiocomplex [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4–11}$.

for receptor recognition and that the replacement of Met^4 and Phe^7 with Nle and D-Phe, respectively, led to the more potent NDP-MSH analog (Fig. 1), which is enzymatically stable and has a longer half-life than the α-MSH native peptide [18]. The [Ac-Nle^4,Asp^5,D-Phe^7]α-MSH$_{4–11}$ peptide (Fig. 1), another α-MSH analog with a high affinity for MC1R, has been radiolabeled with ^{111}In, ^{67}Ga/^{68}Ga and ^{64}Cu using 1,4,7,10-tetraazacyclododecane-*N*,*N*′,*N*″,*N*‴-tetracetic acid (DOTA) as bifunctional chelator and has been evaluated for melanoma targeting [6,7]. Despite the good tumor uptake shown by those radiocomplexes in melanoma-bearing mice, their potential use as melanoma-targeting agents is limited due to not only high kidney retention but also the high cost of radionuclides. Another α-MSH analog, which has nanomolar affinity for MC1R, is the rhenium-mediated cyclized Re-[Cys^{3,4,10},D-Phe^7]α-MSH$_{3–13}$ (ReCCMSH) peptide, which has been conjugated to the chelator DOTA through the terminal amine, labeled with ^{111}In and evaluated in a murine tumor model system [8]. In order to optimize the in vivo biological properties of this analog, Lys^{11} has been further replaced with an Arg residue, yielding the peptide ReCCMSH(Arg^{11}), which presents both a lower nonspecific retention of radioactivity in the kidneys and a higher tumor uptake than ReCCMSH [9]. The peptide ReCCMSH(Arg^{11}) has also been labeled with β-emitter radioisotopes (^{188}Re, ^{90}Y and ^{177}Lu), and its usefulness has been evaluated for internal radiotherapy [10–12]. More recently, the same peptide has been labeled with ^{64}Cu through a cross-bridged cyclam bifunctional chelator (CBTE2A), yielding a highly stable complex that is potentially useful for positron emission tomography imaging of melanoma [13].

Owing to its almost ideal physicochemical characteristics ($t_{1/2}$=6 h, E_γ=140 keV, 85%), widespread availability and low cost [19], technetium-99m is the most widely used

radionuclide in diagnostic nuclear medicine [single photon emission computed tomography (SPECT)]. Despite this fact, the labeling of α-MSH analogs with ^{99m}Tc for melanoma targeting is still limited to a few examples [14–16].

The organometallic *fac*-[^{99m}Tc(CO)$_3$]$^+$ core, which was recently introduced for the development of new target-specific radiopharmaceuticals, is a quite versatile synthon for labeling different types of bioactive molecules, including peptides, with a wide variety of bifunctional chelating agents [20–28]. Our interest in the radiolabeling of tumor-seeking peptides with the *fac*-[^{99m}Tc(CO)$_3$]$^+$ core, using a tridentate bifunctional ligand containing a pyrazolyl-diamine backbone (pz) [25–28], prompted us to apply the same approach to the labeling of the potent and stable peptide [Ac-Nle^4,Asp^5,D-Phe^7]α-MSH$_{4–11}$. In this article, we report on the preparation and characterization of the new conjugate [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz)]α-MSH$_{4–11}$, as well as on its radiolabeling with the *fac*-[^{99m}Tc(CO)$_3$]$^+$ core. We also describe for this radiolabeled α-MSH analog in vitro/in vivo stability studies, internalization/externalization in B16F1 cells and biodistribution in murine melanoma-bearing mice.

2. Methods

2.1. Materials

NDP-MSH and MSH analogs were purchased from Neosystem (Strasbourg, France). Bovine serum albumin (BSA) was purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM) containing GlutaMax I, fetal bovine serum, penicillin/streptomycin antibiotic solution, trypsin EDTA and phosphate-buffered saline (PBS; pH 7.2) were obtained from Gibco-Invitrogen (Alfagene, Lisbon).

2.2. Pyrazolyl–peptide conjugate synthesis and characterization

The synthesis of [Ac-Nle^4,Asp^5,D-Phe^7]α-MSH$_{4–11}$ peptide was performed by Neosystem by using solid-phase peptide techniques and by employing standard fmoc chemistry. The tridentate pyrazolyl ligand was synthesized as previously described [25,26] and was coupled to the peptide by acetylation of the ε-amino group of Lys^{11} ([Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz)]α-MSH$_{4–11}$). After final reversed-phase high-performance liquid chromatography (RP-HPLC) purification (97%), the conjugates were characterized by electrospray ionization–mass spectrometry (ESI-MS) (calculated *m*/*z* for [M+2H]$^+$: 1349.7; found: 1349.7).

2.3. Radiolabeling

Na[^{99m}TcO$_4$] was eluted from a ^{99}Mo/^{99m}Tc generator, using 0.9% saline. Using an Isolink kit (Mallinckrodt-Tyco, Inc.), the preparation of *fac*-[^{99m}Tc(CO)$_3$ (H$_2$O)$_3$]$^+$ was performed according to a previously described procedure [25,26]. In a nitrogen-purged glass vial, 100 μl of a 10^{-4} M solution of the conjugate [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz)]α-MSH$_{4–11}$ was added to 900 μl of a solution of *fac*-[^{99m}Tc(CO)$_3$ (H$_2$O)$_3$]$^+$ (37–74 MBq) in PBS. The reaction mixture was incubated at 100°C for 30–60 min and then analyzed by RP-HPLC, using an analytical C-18 reversed-phase column. The radiolabeled compound was purified by semipreparative RP-HPLC. The activity corresponding to the ^{99m}Tc(CO)$_3$ conjugate was collected in a 50-ml Falcon flask containing 200 μl of PBS with 0.2% BSA, or MEM with 0.2% BSA, for biodistribution and internalization studies, respectively. The solutions were concentrated to a final volume of 200 μl under nitrogen stream, and the product was controlled by analytical RP-HPLC to confirm its purity and stability after purification and evaporation.

2.4. HPLC analysis

RP-HPLC was performed on a Perkin-Elmer system (LC-Pump, Series 200) coupled to a UV–Vis detector [(LC 290 (Perkin-Elmer) or SDP-10AV (Shimadzu)] and a γ detector (LB 507 or LB 509; Berthold, Germany) for the ^{99m}Tc compounds. Analytical separations were achieved on a Hypersil ODS column (250/4 mm, 10 μm). Semipreparative separations of the radioactive complexes were achieved on a Hypersil ODS column (250/8 mm, 10 μm). The columns were eluted with a binary gradient system with a flow rate of 1.0 ml/min (analytical) or 2.0 ml/min (semipreparative). Mobile Phase A, TFA 0.5%; Mobile Phase B, CH_3CN 0.5% TFA. Method: 0–3 min, 0% B; 3–3.1 min, 0–25% B; 3.1–9 min, 25% B; 9–9.1 min, 25–34% B; 9.1–14.1 min, 34–100% B; 14.1–19 min, 100% B; 19–21 min, 100–0% B; 21–30 min, 0% B.

2.5. Partition coefficient

Partition coefficient was evaluated by the "shake-flask" method [29]. The radioconjugate was added to a mixture of octanol (1 ml) and 0.1 M PBS (pH 7.4; 1 ml), which have been previously saturated with each other by stirring. This mixture was vortexed and centrifuged (3000 rpm, 10 min, room temperature) to allow phase separation. Aliquots of both octanol and PBS were counted in a γ counter. The partition coefficient ($P_{o/w}$) was calculated by dividing the counts in the octanol phase by those in the buffer, and the results were expressed as log $P_{o/w}$.

2.6. In vitro stability

The in vitro stability of the radioconjugate was determined in fresh human plasma. The ^{99m}Tc-labeled complex (100 μl, ≈10 MBq) was added to fresh human plasma (1 ml), and the mixture was incubated at 37°C. At appropriate time points (5 min, 45 min and 4 h), 100-μl aliquots (in duplicate) were sampled and treated with 200 μl of ethanol to precipitate the proteins. Samples were centrifuged at 3000 rpm for 15 min at 4°C, and the supernatant was analyzed by HPLC. The stability of the radiolabeled conjugate in the solutions containing 0.2% BSA was checked

by HPLC (column: Supelguard LC 3 DP, 2 cm×4.6 mm ID; eluents: A, 10% isopropanol and 90% TFA 0.1%; B, 90% isopropanol and 10% TFA 0.1%; flux: 1 ml/min) and instant thin-layer chromatography (ITLC; 5% 6 N HCl/MeOH).

2.7. Cell culture

B16F1 murine melanoma cells (ECACC, England, 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-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 (Gibco-Invitrogen) and seeded farther apart.

2.8. Internalization and cellular retention studies

Internalization assays of the radioconjugate were performed in B16F1 murine melanoma cells seeded at a density of 0.2 million/well in 24-well tissue culture plates and allowed to attach overnight. The cells were incubated at room temperature or 37°C for a period of 5 min to 4 h, with about 200,000 cpm of the conjugate in 0.5 ml of assay medium (modified Eagle's medium with 25 mM HEPES and 0.2% BSA). Incubation was terminated by washing the cells with ice-cold assay medium. Cell-surface-bound radioligand was removed by two steps of acid wash (50 mM glycine·HCl/100 mM NaCl, pH 2.8) at room temperature for 5 min. pH was neutralized with cold PBS with 0.2% BSA and, subsequently, the cells were lysed by a 10-min incubation with 1 N NaOH at 37°C to determine the internalized radioligand. The cell-retaining properties of the internalized radioconjugate were determined by incubating B16F1 cells with the radiolabeled compound for 3 h at 37°C, washing them with cold assay medium, removing the membrane-bound radioactivity with acid buffer wash and monitoring radioactivity release into the culture media (0.5 ml) at 37°C. At different times points over a 5-h incubation period, the radioactivities in the medium and in the cells were separately collected and counted.

2.9. Biodistribution

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 the [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ complex was estimated in healthy and melanoma-bearing C57BL/6 female mice (8–10 weeks old). Mice had been previously implanted subcutaneously with 1×10^6 B16F1 cells to generate primary skin melanoma. Ten to 12 days after the inoculation, tumors reached a weight of 0.2–1 g.

Animals were intravenously injected with the radiolabeled complex (4–13 MBq) diluted in 100 μl of PBS (pH 7.2) into their retro-orbital sinus. To confirm specific uptake, 10 μg of NDP-MSH was coinjected with the radioactive complex. Mice were sacrificed by cervical dislocation at 1, 4 and 24 h postinjection (p.i.). The administered dose and the radioactivity in the sacrificed animals were measured with a dose calibrator [Curiemeter IGC-3 (Aloka, Tokyo, Japan) or CRC-15W (Capintec, Ramsey, USA)]. The difference in radioactivity between the injected animal and the sacrificed animal was assumed to be due to excretion. Tumors and normal tissues of interest were dissected, rinsed to remove excess blood and weighed, and their radioactivity was measured using a γ counter (LB 2111; Berthold). The uptake in the tumor and healthy tissues of interest was calculated and expressed as a percentage of the injected radioactivity dose per gram of tissue (%ID/g). For blood, bone, muscle and skin, total activity was estimated by 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 of sacrifice. For imaging, the animals were anesthetized using a mixture of ketamine and xylazine (80 and 20 mg/ml, respectively), injected with the radioconjugate (29 MBq) and placed over a γ camera (GE 400AC; Maxicamera, Milwaukie, USA) coupled with a high-resolution parallel collimator and controlled with GENIE Acquisition computer. A set of dynamic images (60 images of 128×128 matrix, Zoom 2, with individual duration of 30 s), followed by static images (256×256 matrix, Zoom 2, 2 min), each lasting for 30 min until 2 h, was acquired.

2.10. In vivo stability by HPLC analysis

The in vivo stability of the complex was assessed by urine and murine serum RP-HPLC analysis, under conditions identical to those used for analyzing the original radiolabeled complex. The samples were taken 1 h p.i. The urine collected at the time of sacrifice was filtered through a Millex GV filter (0.22 μm) before RP-HPLC analysis. Blood collected from mice was immediately centrifuged at 3000 rpm for 15 min at 4°C, and the serum was separated. The serum was treated with ethanol in a 2:1 (vol/vol) ratio to precipitate the proteins. After centrifugation at 3000 rpm for 15 min at 4°C, the supernatant was collected and analyzed by RP-HPLC.

3. Results and discussion

The development of radiolabeled α-MSH-receptor-targeting peptides for melanoma diagnosis and therapy is attractive, since receptor agonists are rapidly internalized upon binding. In order to overcome the short half-life exhibited by α-MSH in humans, Met^4 and Phe^7 residues were replaced by Nle and D-Phe, respectively, leading to the NDP-MSH analog (Fig. 1) that is enzymatically stable, is more potent and has prolonged biological activity [30,31].

The [Ac-Nle^4,Asp^5,D-Phe^7]α-MSH$_{4–11}$ peptide, shorter than the NDP-MSH peptide and with the Glu^5 replaced with Asp^5, still retains a high affinity for MC1R (IC_{50}=0.27 vs. 0.21 nM for NDP-MSH; B16F1 cells and [^{125}I]NDP-MSH as radioligands) [6]. Herein, we report on the labeling of the potent and stable [Ac-Nle^4,Asp^5,D-Phe^7]α-MSH$_{4–11}$ peptide conjugated to a tridentate pyrazolyl-containing ligand (pz) chelator. Since the affinity of this peptide to the MC1R was not negatively influenced by attachment of the DOTA chelator to the ε-amino group of Lys^{11} (IC_{50}=1.37 nM) [6], we chose this amino group to couple the pz chelator (Fig. 1).

The conjugate [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz)]α-MSH$_{4–11}$ was prepared by standard solid-phase synthetic methods, purified by preparative RP-HPLC (purity ≥97%) and characterized by ESI-MS (*m*/*z*=1349.7 [M+2H]$^+$). The complex [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4–11}$ was obtained in an almost quantitative yield (>98%) upon the addition of the precursor [^{99m}Tc(CO)$_3$(H$_2$O)$_3$]$^+$ to a vial containing the conjugate [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz)]α-MSH$_{4–11}$ (final concentration, 5×10^{-5} M), followed by heating (Fig. 1).

To increase the specific activity of the ^{99m}Tc-labeled conjugate to in turn maximize cellular internalization and tumor uptake, the radioactive complex (rt=16 min) was separated from the nonmetallated conjugate (rt=14.1 min) by semipreparative RP-HPLC (Fig. 2). The fraction corresponding to the radioconjugate was collected in a Falcon vial containing PBS (for biodistribution studies) or MEM (cell internalization studies) solutions with 0.2% BSA to avoid the adsorption of the radiolabeled peptide to the vial after the evaporation of acetonitrile. The stability of the radioconjugate in PBS and MEM solutions containing 0.2% BSA was evaluated by HPLC (Supelguard LC 3 DP, 2 cm×4.6 mm ID column) and ITLC. The HPLC chromatogram of the radioconjugate, after being incubated for 24 h at room temperature with a 0.2% BSA solution in PBS, revealed only the presence of the radioconjugate (γ detection; rt=5.6 min) and BSA (UV detection; rt=10 min). No radiochemical impurities were detected, confirming that there is no interaction with BSA and/or transmetallation.

The same conclusion was drawn by analyzing the ITLC radiochromatogram (eluent: 5% 6 N HCl solution in methanol) of the same sample. No hydrolyzed ^{99m}Tc species (R_f=0), labeled BSA (R_f=0), [^{99m}TcO$_4$]$^-$ (R_f=0.85) or *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$] precursor (R_f=0.80) was detected, with the radioconjugate being the only radiochemical species present (R_f=0.55).

The results obtained with both HPLC and ITLC indicate a high stability of the radioconjugate in the presence of BSA and confirm the high ability of the pyrazolyl-diamine chelator to stabilize the *fac*-[^{99m}Tc(CO)$_3$]$^+$ moiety, avoiding transmetallation and/or oxidation of ^{99m}Tc [25–28].

To assess the resistance of the radioconjugate to proteolytic degradation caused by endogenous peptidases and to predict its in vivo stability, we have performed stability assays in fresh human plasma at 37°C. Analysis by RP-HPLC at different time points postincubation (0 min, 5 min, 45 min and 4 h) indicated high plasma stability with

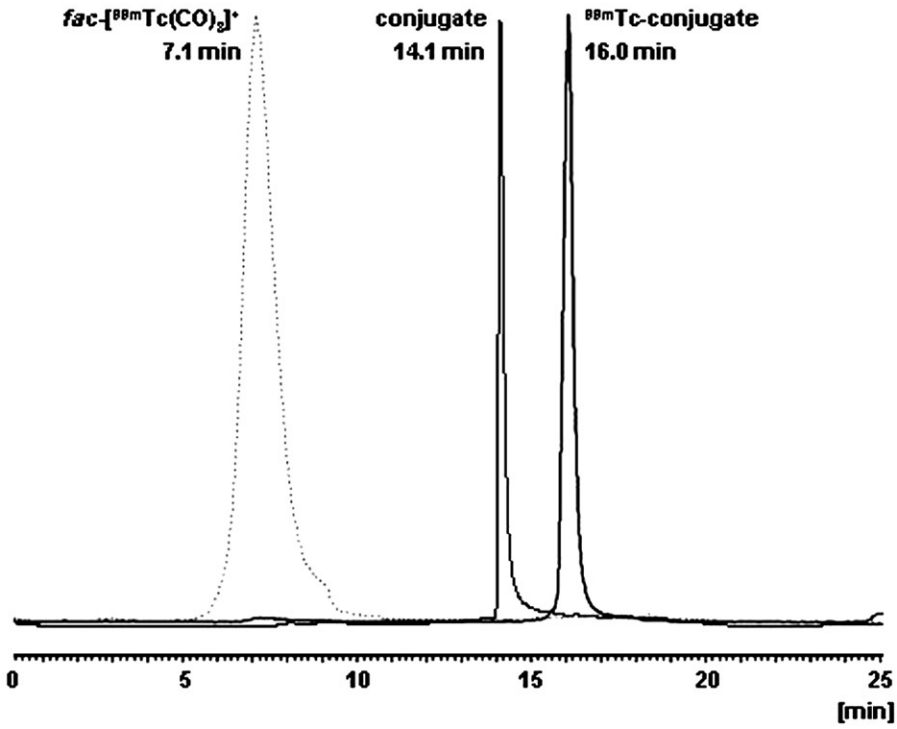


Fig. 2. Superimposition of RP-HPLC chromatograms of the precursor *fac*-[^{99m}Tc(CO)$_3$(H$_2$O)$_3$], [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4–11}$ (γ detection) and the peptide conjugate [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz)]α-MSH$_{4–11}$ (UV detection, 280 nm).

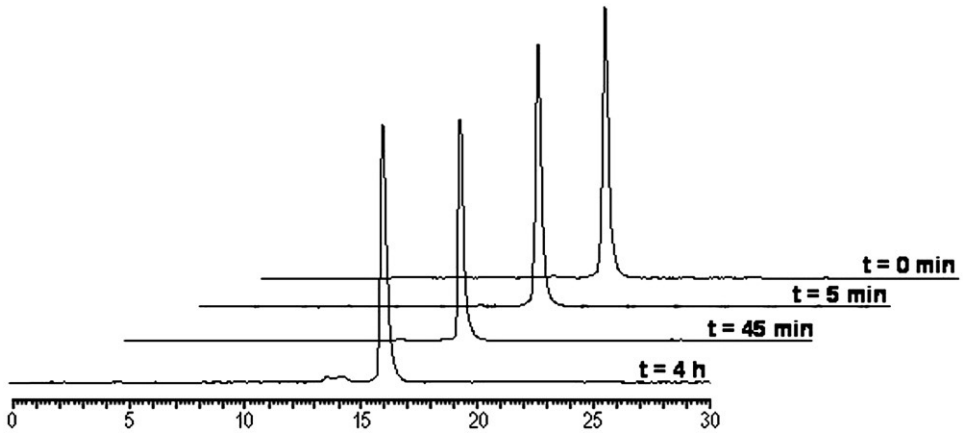


Fig. 3. RP-HPLC profile of [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ (rt=16.0 min) after incubation in fresh human plasma at different time points.

negligible degradation of the radioconjugate (Fig. 3). In fact, the radioconjugate presents a purity of ≥98% even after a 4-h postincubation in human plasma. Again, it has been shown that the pyrazolyl-diamine-chelating unit is able to stabilize the metal center against transmetallation reactions to serum-based proteins.

Determination of the partition coefficient in physiological conditions allowed us to conclude that the radioconjugate was moderately hydrophilic (log $P_{o/w}$=−0.420±0.003).

Of great importance to predicting the tumor-targeting properties of the [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ complex is the degree of internalization in B16F1 murine melanoma cells. Internalization studies, performed both at room temperature and at 37°C, revealed that cellular uptake was temperature dependent and time dependent (Fig. 4). The highest level of internalization was reached at 4 h postincubation (37°C), when 56.7% of the total cell-associated activity for the administered radioconjugate was taken up and internalized by the cells. This value corresponds to 5.4% of the total activity applied.

The cellular retention of the radioconjugate was evaluated in the same type of cells at 37°C, after 3 h of internalization. As shown also in Fig. 4C, the retention of the compound was significantly high, retaining ca. 62% of the cell-associated activity internalized in the cells even after 4 h.

The in vitro internalization and cellular retention studies in B16F1 cells for the ^{99m}Tc complex reflect agonistic binding behavior, which is consistent with data previously reported for other radiolabeled α-MSH analogs [7,15].

Biodistribution studies at 1, 4 and 24 h after intravenous injection of the [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ complex in B16F1 melanoma-bearing mice are summarized in Table 1.

The labeled peptide is moderately cleared from the bloodstream (1.5±0.6%ID/organ, 4 h p.i.) and major organs, except those related with the excretion pathways.

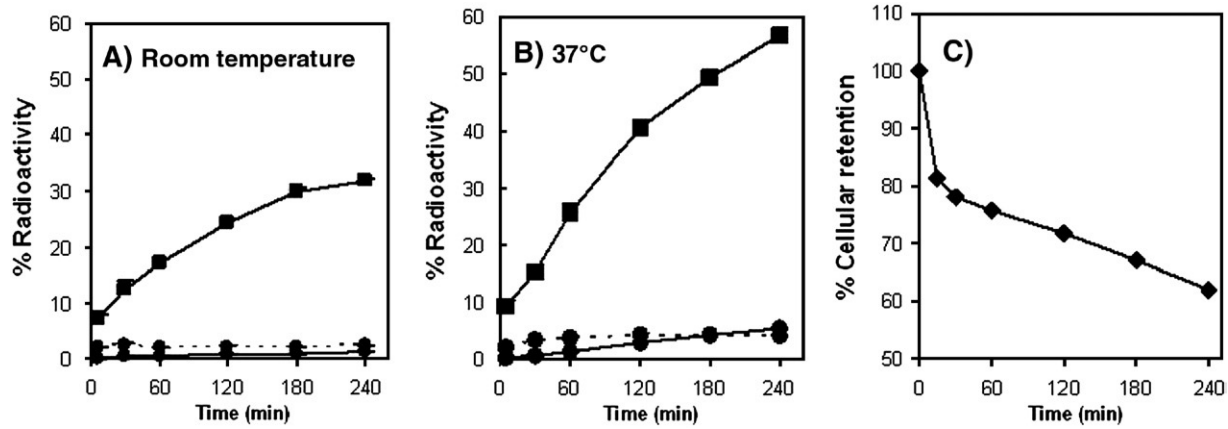


Fig. 4. Internalization (A and B) and cellular retention at 37°C (C) for [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]αMSH$_{4-11}$ in B16F1 cells at different time points. (■) Internalized activity expressed as a fraction of bound activity (activity on the membrane surface and inside the cell). (● Dark line) Internalized activity expressed as a percentage of applied activity. (● Dotted line) Surface-bound activity as a percentage of applied activity.

Table 1
Biodistribution studies of the complex [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ in B16F1 murine melanoma-bearing C57BL/6 mice at 1, 4 and 24 h p.i. (n=3–5)

Tissue/organ	%ID/g±S.D.			
	1 h	4 h	4 h with NDP [a]	24 h
Tumor	5.88±2.11	4.24±0.94	1.55±0.28	2.37±0.56
Blood	3.32±0.51	1.62±0.64	0.82±0.14	0.23±0.01
Liver	10.8±1.6	6.7±2.1	3.6±0.6	1.89±0.24
Intestine	9.1±0.8	14.4±1.6	15.3±1.9	0.20±0.04
Spleen	1.26±0.24	0.88±0.30	0.60±0.06	0.24±0.05
Heart	0.85±0.11	0.58±0.19	0.26±0.02	0.11±0.01
Lung	2.88±0.55	1.21±0.45	2.16±1.07	0.16±0.03
Kidney	9.7±0.9	4.5±2.4	4.9±1.8	0.73±0.05
Muscle	0.31±0.03	0.18±0.14	0.11±0.02	0.04±0.00
Bone	0.66±0.10	0.40±0.20	0.25±0.03	0.11±0.03
Stomach	2.92±1.7	1.06±0.96	0.45±0.25	0.06±0.03
Pancreas	0.57±0.10	0.46±0.32	0.34±0.11	0.07±0.01
Skin	1.00 ± 0.13	0.48±0.26	0.44±0.08	0.20±0.04
Tumor/normal tissue uptake ratio				
Tumor/blood	1.8	2.6	1.9	10.3
Tumor/muscle	19.0	23.5	14.1	59.2
Total excretion (%)	57.3±2.7	69.1±8.3	65.9±2.5	94.1±1.2

[a] Coinjection of the radioconjugate with NDP.

A rapid overall excretion at 1 h p.i. (57%) suggests the urinary route as the main excretory route, in accordance with the hydrophilic character of the radioconjugate. Nevertheless, a significant percentage of radioactivity in the hepatobiliary tract remained (10.7±0.4%ID/organ and 14.0±0.4%ID/organ at 1 h p.i.; and 5.7±2.5%ID/organ and 15.0±2.0%ID/organ at 4 h p.i., for the liver and intestine, respectively), indicating the importance of this path in the total elimination of the radioactive complex. The low stomach uptake indicates minimal, if any, in vivo oxidation of the complex to [^{99m}TcO$_4$]$^-$.

A moderate tumor uptake (5.88±2.11%ID/g and 4.24±0.94%ID/g at 1 and 4 h p.i., respectively) with a high retention (2.37±0.56%ID/g at 24 h p.i.) of [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ was found, in agreement with the relatively slow washout observed in B16F1 cell studies. The in vivo specificity of the complex [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ for the MC1R was evaluated by coadministration of the radioconjugate and cold NDP peptide (linear α-MSH peptide analog with picomolar affinity for MC1R expressed in murine melanoma cells). This receptor-blocking study revealed that the tumor uptake was reduced by 63.5% (1.55±0.28%ID/g vs. 4.24±0.94%ID/g) in the presence of NDP, without significant changes in the biodistribution profile of the radioconjugate in normal tissues. These results led us to conclude that the radiopeptide keeps its biological activity upon conjugation to pz and ^{99m}Tc labeling, and confirm an MC1R-mediated uptake mechanism. The tumor/blood and tumor/muscle uptake ratios of [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ were 2.6 and 23.5, respectively (Table 1). The increase of these ratios over time also indicates receptor-mediated transport and subsequent intracellular trapping of this radiocomplex in the MC1R-expressing tumor.

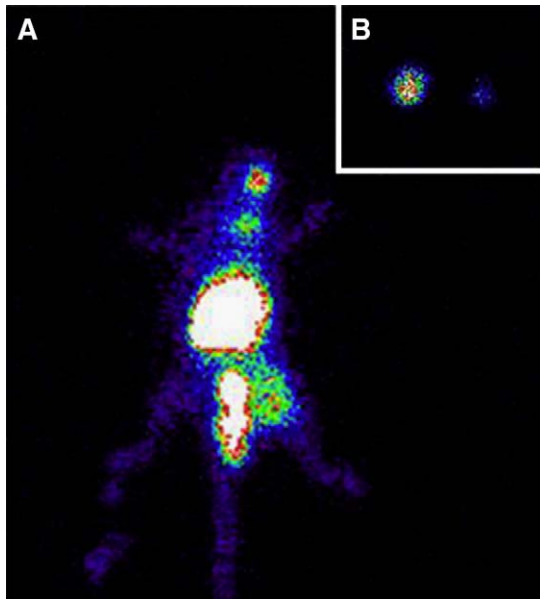


Fig. 5. (A) SPECT images of B16F1 melanoma-bearing mice injected with [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4-11}$ at 2 h p.i., before sacrifice. (B) Image of the excised tumor and of an excised muscle sample at 2 h p.i.

The radioconjugate did not show nonspecific radioactivity accumulation in the kidney, as the uptake decreased

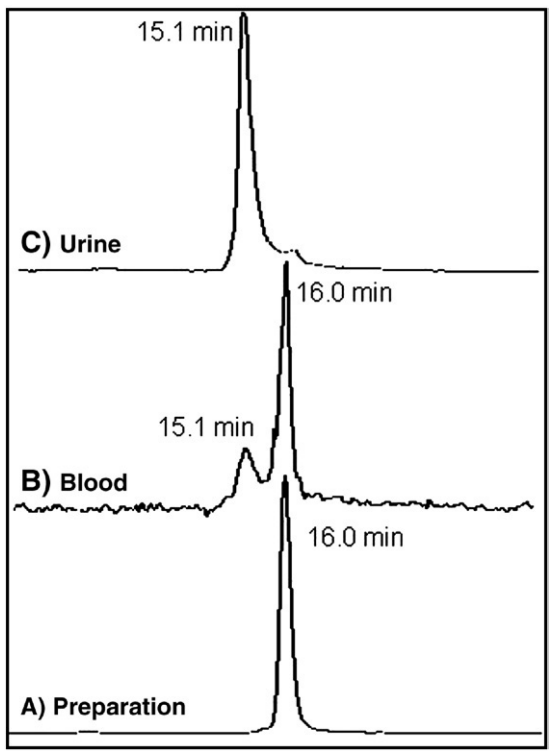


Fig. 6. RP-HPLC chromatograms of the injected preparation (A), blood serum (B) and urine (C) samples collected at 1 h p.i.

rapidly along excretion and only 0.73±0.05%ID/g was retained in that organ 24 h p.i., presenting at that time point a good tumor/kidney ratio (3.24). This ratio is a good starting point for the development of a radioactive probe for melanoma targeting, since low kidney retention is an essential feature of a peptide-based radiopharmaceutical [32]. Note that when the same α-MSH analog was conjugated to DOTA and was labeled with ^{111}In and ^{67}Ga, a less favorable behavior concerning kidney uptake was observed. In fact, the best tumor/kidney ratio achieved was obtained 4 h p.i., and the values were 1.49 and 2.37 for ^{111}In- and ^{67}Ga-labeled peptide, respectively [6]. Furthermore, this ratio was even worse (<1) when the same DOTA–peptide conjugate was labeled with ^{64}Cu [7]. High renal uptake was also observed for labeled α-MSH analogs based on the cyclic ReCCMSH [8–16].

SPECT imaging has confirmed the biodistribution data described above. Although the radioactivity accumulation in the excretory pathways was relatively high, the B16F1 tumor could still be visualized with good tumor-to-contralateral background at 2 h p.i., as displayed in Fig. 5. In fact, the tumor began to be visualized at 30 min, and its uptake increased overtime.

In vivo stability studies using healthy C57BL6 mice have shown that, in blood samples (1 h p.i.), most of the radioactivity found corresponds to the complex [Ac-Nle^4, Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4–11}$ (rt=16.0 min). Only a minor amount of the metabolite appears in the blood at rt=15.1 min, which is the main component found in the urine (Fig. 6).

These results indicate that metal complexation via the tridentate pyrazolyl-diamine backbone overcomes potential binding/transmetallation to coordinating residues in circulating proteins such as histidine, cysteine or methionine. They also indicate that significant excretion through the renal–urinary pathway is most probably due to the metabolization of the radioconjugate, with formation of a more hydrophilic species (rt=15.1 min).

4. Concluding remarks

Combining the advantages of the *fac*-[^{99m}Tc(CO)$_3$]$^+$ moiety and a suitable and well-defined bifunctional chelating agent containing a pyrazolyl-diamine backbone (pz), we have prepared the new and stable melanoma-targeting complex [Ac-Nle^4,Asp^5,D-Phe^7,Lys^{11}(pz-^{99m}Tc(CO)$_3$)]α-MSH$_{4–11}$. This radioconjugate was obtained in high yield and with high radiochemical purity, presenting significant cellular internalization and retention. A moderate MC1R-mediated tumor uptake was found in biodistribution and SPECT imaging studies. Despite being eliminated mainly through the urinary route, the radioconjugate still presents considerable hepatobiliary excretion. Improvement of pharmacokinetics may be achieved by using a more hydrophilic pyrazolyl-containing chelator and/or by using different spacers, namely, carbohydrates.

Acknowledgments

Mallinckrodt-Tyco, Inc., is acknowledged for financial support.

References

[1] Carlson JA, Slominski A, Linette GP, Mihm MC, Ross JS. Biomarkers in melanoma: staging, prognosis and detection of early metastases. Expert Rev Mol Diagn 2003;3:303–30.
[2] Ghanem GE, Comunale G, Libert A, Vercammen-Grandjean A, Lejeune FJ. Evidence for alpha-melanocyte-stimulating hormone (alpha-MSH) receptors on human malignant melanoma cells. Int J Cancer 1988;41:248–55.
[3] Siegrist W, Solca F, Stutz S, Giuffre L, Carrel S, Girard J, et al. Characterization of receptors for alpha-melanocyte-stimulating hormone on human melanoma cells. Cancer Res 1989;49:6352–8.
[4] Siegrist W, Stutz S, Eberle AN. Homologous and heterologous regulation of α-melanocyte-stimulating hormone receptor in human and mouse melanoma cell lines. Cancer Res 1994;54:2604–10.
[5] Eberle AN, Froidevaux S. Radiolabeled alpha-melanocyte-stimulating hormone analogs for receptor-mediated targeting of melanoma: from tritium to indium. J Mol Recognit 2003;16:248–54.
[6] Froidevaux S, Calame-Christe M, Shuhmacker J, Tanner H, Saffrich R, Henze M, et al. A gallium-labeled DOTA-alpha-melanocyte-stimulating hormone analog for PET imaging of melanoma metastases. J Nucl Med 2004;45:116–23.
[7] Cheng Z, Xiong Z, Subbarayan M, Chen X, Gambhir SS. ^{64}Cu-labeled alpha-melanocyte-stimulating hormone analog for microPET imaging of melanocortin 1 receptor expression. Bioconjug Chem 2007;18: 765–72.
[8] Chen J-Q, Cheng Z, Owen NK, Hoffman TJ, Miao Y, Jurisson SS, et al. Evaluation of an ^{111}In-DOTA-rhenium cyclized α-MSH analog: a novel cyclic-peptide analog with improved tumor-targeting properties. J Nucl Med 2001;42:1847–55.
[9] Cheng Z, Chen J, Miao Y, Owen NK, Quinn TP, Jurisson SS. Modification of the structure of a metallopeptide: synthesis and biological evaluation of ^{111}In-labeled DOTA-conjugated rhenium-cyclized α-MSH analogues. J Med Chem 2002;45:3048–56.
[10] Miao Y, Owen NK, Whitener D, Gallazzi F, Hoffman TJ, Quinn TP. In vivo evaluation of ^{188}Re-labeled alpha-melanocyte stimulating hormone peptide analogs for melanoma therapy. Int J Cancer 2002; 101:480–7.
[11] Miao Y, Whitener D, Feng W, Owen NK, Chen J, Quinn TP. Evaluation of the human melanoma targeting properties of radiolabeled α-melanocyte stimulating hormone peptide analogues. Bioconjug Chem 2003;14:1177–84.
[12] Miao Y, Hoffman TJ, Quinn TP. Tumor-targeting properties of ^{90}Y- and ^{177}Lu-labeled α-melanocyte stimulating hormone peptide analogues in a murine melanoma model. Nucl Med Biol 2005;32:485–93.
[13] Wei L, Butcher C, Miao Y, Gallazzi F, Quinn TP, Welch MJ, et al. Synthesis and biologic evaluation of ^{64}Cu-labeled rhenium-cyclized α-MSH peptide analog using a cross-bridged cyclam chelator. J Nucl Med 2007;48:64–72.
[14] Giblin MF, Wang N, Hoffman TJ, Jurisson SS, Quinn TP. Design and characterization of alpha-melanotropin peptide analogs cyclized through rhenium and technetium metal coordination. PNAS 1998;95: 12814–8.
[15] Chen J-Q, Cheng Z, Hoffman TJ, Jurisson SS, Quinn TP. Melanoma-targeting properties of (99m)technetium-labeled cyclic alpha-melanocyte-stimulating hormone peptide analogues. Cancer Res 2000;60: 5649–58.
[16] Miao Y, Benwell K, Quinn TP. ^{99m}Tc-and ^{111}In-labeled α-melanocyte-stimulating hormone peptides as imaging probes for primary and pulmonary metastatic melanoma detection. J Nucl Med 2007;48: 73–80.

[17] Garcia-Borrón JC, Sánchez-Laorden BL, Jiménez-Cervantes C. Melanocortin-1 receptor structure and functional regulation. Pigment Cell Res 2005;18:393–410.

[18] Holder JR, Haskell-Luevano C. Melanocortin ligands: 30 years of structure–activity relationship (SAR) studies. Med Res Rev 2004;24: 325–56.

[19] Liu S, Edwards DS. ^{99m}Tc-labeled small peptides as diagnostic radiopharmaceuticals. Chem Rev 1999;99:2235–68.

[20] Alberto R, Pak JK, Staveren DV, Mundwiler S, Benny P. Mono-, bi-, or tridentate ligands? The labelling of peptides with ^{99m}Tc-carbonyls. Biopolymers 2004;76:324–33.

[21] Alberto R. New organometallic complexes for radiopharmaceutical imaging. Top Curr Chem 2005;252:1–44.

[22] Schibli R, Schubiger AP. Current use and future potential of \organometallic radiopharmaceuticals. Eur J Nucl Med 2002;29: 1529–42.

[23] Santos I, Paulo A, Correia JDG. Rhenium and technetium complexes anchored by phosphines and scorpionates for radiopharmaceutical applications. Top Curr Chem 2005;252:45–84.

[24] Banerjee SR, Maresca KP, Francesconi L, Valliant J, Babich JW, Zubieta J. New directions in the coordination chemistry of ^{99m}Tc: a reflection on technetium core structures and a strategy for new chelate design. Nucl Med Biol 2005;32:1–20.

[25] Alves S, Paulo A, Correia JDG, Domingos Â, Santos I. Coordination capabilities of pyrazolyl containing ligands towards the *fac*-[Re $(CO)_3]^+$ moiety. J Chem Soc, Dalton Trans 2002;24:4714–9.

[26] Alves S, Paulo A, Correia JDG, Gano L, Smith CJ, Hoffman TJ, et al. Pyrazolyl derivatives as bifunctional chelators for labeling tumor-seeking peptides with the *fac*-$[M(CO)_3]^+$ moiety (M=^{99m}Tc, Re): synthesis, characterization, and biological behavior. Bioconjug Chem 2005;16:438–49.

[27] Alves S, Correia JDG, Santos I, Veerendra B, Sieckman GL, Hoffman TJ, et al. Pyrazolyl conjugates of bombesin: a new tridentate ligand framework for stabilization of the *fac*-$[M(CO)_3]^+$ moiety. Nucl Med Biol 2006;33:625–34.

[28] Alves S, Correia JDG, Gano L, Rold TL, Prasanphanich A, Haubner R, et al. In vitro and in vivo evaluation of a novel ^{99m}Tc$(CO)_3$–pyrazolyl conjugate of cyclo-(Arg-Gly-Asp-D-Tyr-Lys). Bioconjug Chem 2007; 18:530–7.

[29] Troutner DE, Volkert WA, Hoffman TJ, Holmes RA. A neutral lipophilic complex of ^{99m}Tc with a multidentate amine oxime. Int J Appl Radiat Isot 1984;35:10467–70.

[30] Sawyer TK, Sanfilippo PJ, Hruby VJ, Engel MH, Heward CB, Burnett JB, et al. 4-Norleucine, 7-D-phenylalanine-alpha-melanocyte-stimulating hormone: a highly potent alpha-melanotropin with ultralong biological activity. PNAS 1980;77:5754–8.

[31] Haskell-Luevano C, Miwa H, Dickinson C, Hadley ME, Hruby VJ, Yamada T, et al. Characterizations of the unusual dissociation properties of melanotropin peptides from the melanocortin receptor, hMC1R. J Med Chem 1996;39:432–5.

[32] Trejtnar F, Laznicek M. Analysis of renal handling of radiopharmaceuticals. Q J Nucl Med 2002;46:181–94.