Development of a $^{99m}$Tc-labeled lactam bridge-cyclized alpha-MSH derivative peptide as a possible single photon imaging agent for melanoma tumors

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Abstract

Objective Melanocortin-1 (MC1) receptor is an attractive melanoma-specific target which has been used for melanoma imaging and therapy. In this work, a new lactam bridge ω-MSH analog was labeled with $^{99m}$Tc via HYNIC and EDDA/tricine as coligands including gamma aminobutyric acid (GABA) as a three carbon chain spacer between HYNIC and the N-terminus of the cyclic peptide. Also, stability in human serum, receptor bound internalization, in vivo tumor uptake, and tissue biodistribution were thoroughly investigated.

Methods HYNIC-GABA-Nle-CycMSHhept was synthesized using a standard Fmoc strategy. Labeling was performed at 95 °C and analysis involved instant thin layer chromatography and high performance liquid chromatography methods. The receptor bound internalization rate was studied in MC1 receptor expressing B16/F10 cells. Biodistribution of radiopeptide was studied in nude mice bearing B16/F10 tumor.

Results Labeling yield of >98 % ($n = 3$) was obtained corresponding to a specific activity of 81 MBq/nmol. Peptide conjugate showed efficient stability in the presence of human serum. The radioligand showed specific internalization into B16/F10 cells (12.45 ± 1.1 % at 4 h). In biodistribution studies, a receptor-specific uptake was observed in MC1 receptor-positive organs so that after 2 h the uptake in mouse tumor was 5.10 ± 0.08 % ID/g, while low accumulation in the kidney uptake was observed (4.58 ± 0.68 % ID/g at 2 h after injection).

Conclusions The obtained results show that the presented new designed labeled peptide conjugate may be a suitable candidate for diagnosis of malignant tumors.

Keywords α-MSH · $^{99m}$Tc · B16/F10 cells · Tumor imaging

Introduction

The molecular basis for the use of radiopeptides was found to be that peptide receptors are over-expressed by certain tumors [1–3]. Up to now, several types of peptides have been introduced for tumor targeting, and many types of cancer cells have demonstrated overexpression of various peptide receptors [4, 5]. Alpha-Melanocyte stimulating hormone (α-MSH) is a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH$_2$) produced in pituitary gland that binds to Melanocortin-1 (MC1) receptor on the surface of normal melanocytes, for the purpose of regulating melanin production involved with skin pigmentation. The involvement of MC1 receptor during the proliferation of melanoma cells, suggests that α-MSH and its analogs may be candidates for peptide-based diagnosis or therapy of this tumor type [6–8]. Over 80 % of human metastatic melanomas were reported to over-express MC1 receptors, and this disease causes greater than 75 % of skin cancer deaths [9–11]. The need for new radiopharmaceuticals able to diagnose tumor in the early stages is evident. Native α-MSH has an extremely short
biological half-life because it is easily hydrolyzed by proteases and also the methionine residue in α-MSH can be oxidized [12]. Newer generation agents have been designed to enhance their potency toward MC1 receptor, including peptides with linear structures [13–16] and cyclic structures [17–19]. However, development of new analogs with higher MC1 receptor affinity, stability, and prolonged activity of the α-MSH analog which resulted in greater tumor uptake and lower kidney uptake is promising.

Previous studies on the α-MSH peptide agonists for the MC1 receptor revealed that the lactam bridge-cyclized α-MSH peptide with a 12-amino acid in the peptide ring [Lys-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Arg-Pro-Val-Asp] exhibiting high MC1 receptor-mediated tumor uptake [18, 20]. Also utilization of the concept of pseudoisosteric cyclization by replacing Met4 and Gly10 by a Cys4, Cys10 disulfide bridge, to give a cyclic disulfide containing 7-aminoacid peptide ring analog. [Cys-Glu-His-Phe-Arg-Trp-Cys] (CycMSHhept), which was found to possess superpotent bioactivity in the frog skin bioassay [21, 22]. A correlation between ring size and biological potency in the frog skin bioassay system was found in 7-aminoacid peptide ring CycMSHhept disulfide containing melanotropin analogs [23, 24]. In evaluation of the effect of ring size on the potencies of the cyclic lactam analogs, it has been reported that potency and prolongation decreased with reduction of the ring size in 6-amino acid in the peptide ring [Asp-His-D-Phe-Arg-Trp-Lys] (CycMSHhex) from 23 to 20 membered ring compounds [25].

Different amino acids and hydrocarbon linkers displayed profound favorable effects on the receptor-binding affinities and pharmacokinetics of radiolabeled RGD [26], α-MSH peptides [18, 20, 27], and bombesins [28]. Miao et al. [18] reported that introduction of a negatively charged linker (Gly-Glu) between the DOTA and CycMSH peptide sequence decreased the renal uptake by 44 % without affecting the tumor uptake at 4 h post injection. Guo et al. [27] also reported that introduction of a neutral Gly–Gly linker between the DOTA and Nle-CycMSHhex maintained high melanoma uptake, while reducing kidney and liver uptake of 111In-DOTA-GGNle-CycMSHhex. α-MSH lactam bridge-cyclic analogs have been radio-labeled with different radionuclides including 111In [18], 67Ga [29], 64Cu [30], and 99mTc [31, 32]. Guo et al. [31] recently evaluated the radiochemical and biological behavior of 99mTc(EDDA)-HYNIC-GGNle-CycMSHhex and concluded that the HYNIC moiety in the radioactive molecule promoted its stability as well as enhancing renal excretion. Our efforts to pursue a 99mTc-labeled peptide for tumor targeting, based on the previously reported unique structure of DOTA-Nle-CycMSHhex, have led us to prepare a HYNIC-conjugated Nle-CycMSH peptide. Also to examine the exhaustive effects of the amino acid linker on the melanoma targeting and pharmacokinetic properties of peptide, gamma aminobutyric acid (GABA) as the three carbon chain spacer was inserted between the HYNIC and the Nle-CycMSH peptide to generate HYNIC-GABA-Nle-CycMSH. Furthermore, based on the explanation above by which reduction of the ring size in cyclic MSHhex potency of peptide reduced, the size of the ring was increased by introduction of a Gly10 from the main sequence of α-MSH peptide between Trp9 and Lys11 to yield HYNIC-GABA-Nle-CycMSHhept (Asp-His-D-Phe-Arg-Trp-Gly-Lys). This study presents the synthesis of HYNIC-GABA-Nle-CycMSHhept, the optimum radiolabeling conditions with 99mTc via tricine and EDDA as coligands, and further characterization of the 99mTc-HYNIC-GABA-Nle-CycMSHhept using an in vivo C57 mice bearing B16/F10 tumor model.

**Experimental**

**Materials**

Sieber amide resin and all of the Fmoc-protected amino acids were commercially available. The pro-chelator HYNIC-Boc was synthesized according to Abrams et al. [33]. Other reagents were purchased from Sigma-Aldrich (Munich, Germany) and used without further purification. The reactive side chains of the amino acids protecting agents were masked with one of the following groups: Trp, tert-butoxycarbonyl (Boc); Lys, N0-tert-butoxycarbonyl (Boc); Lys, N0-methyltrityl (Mt); Arg, 2,2,4,6,7 pentamethyldihydrobenzofurane-5-sulfonyl (pbf); His, N3-Triyl (Trt); and Asp, 2-phenylisopropylester (o-2-hipr). The cell culture medium was Roswell Park Memorial Institute (RPMI-1640) supplemented with 10 % fetal bovine serum (FBS), amino acids, vitamins, and penicillin/streptomycin (Gibco, Eggenthal, Germany). Sodium pertechnetate (Na99mTcO4) was obtained from a commercial 99Mo/99mTc-generator (Radioisotope Division, NSTRI, Tehran, Iran). Analytical reverse phase high performance liquid chromatography (RP-HPLC) was performed on a HPLC system (Sykam S7131, Eresing, Germany) equipped with a multichannel UV detector set at = 280 nm, a flow-through gamma-detector (Raytest-Gabi, Straubenhardt, Germany), and a C18 analytical column (CC 250/4.6 Reprosil-pur ODS-3.5 μm). The mobile phase consisted of 0.1 % trifluoroacetic acid/water (Solvent A) and acetonitrile (Solvent B), and the gradient system used a flow rate of 1 mL/min at an A:B ratio of: 95:5 at 0 min, 95:5 at 5 min, 0:100 at 25 min, 0:100 at 27 min, 95:5 at 30 min, and 95:5 at 35 min. A mass spectrometer (1100/Brucker Daltonic; Agilent, Bremen, Germany) with a VL instrument (LC/MS) was also used. Quantitative gamma counting was performed on an
The peptide was synthesized by standard Fmoc solid-phase synthesis on Sibor amide resin with substitution, 0.65 mmol/g [34]. Coupling of each amino acid was performed in the presence of 3 mol excess of Fmoc-amino acid, N-hydroxybenzotriazole (HOBT) and diisopropylcarbodiimide (DIC) in dimethylformamide (DMF) and 5 mol excess of diisopropylmethyamine (DIPEA) in DMF. The Kaiser test determined the end of a coupling reaction. Fmoc groups were removed by adding 20 % (v/v) piperidine in DMF (10 mL). The protecting groups Mtt and 2-phenylisopropyl were removed by 1 % TFA in advance of the peptide cyclization reaction, and protected peptide was cleaved from the resin by treatment with a mixture of 2.5 % TFA and 5 % of triisopropylsilane in dichloromethane (DCM) (10 mL). Each protected peptide was cyclized by coupling the carboxylic group from aspartic acid with the epsilon amino group on lysine. The cyclization reaction was achieved by an overnight reaction with peptide in DMF (2 mL) using benzotriazole-1-yl-oxytris-pyrrolidino-phosphonium-hexafluorophosphate (PyBOP) as a coupling agent in the presence of DIPEA. HYNIC-Boc (1.2 mol) is coupled with O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (1.2 mmol) and DIPEA (3.6 mmol) in DMF (5 mL) to the N-terminus of the peptide (1 mmol). The amino acid side chains of the peptide HYNIC conjugate were also deprotected by treatment with TFA, thioanisole, water, and triisopropylsilane (92.5:2.5:2.5:2.5) for 30 min at 25 °C. After removing the organic solvents in a vacuum, the crude product was precipitated by adding a solution of cold petroleum ether and diisopropyl ether (50:50).

99mTc-labeling of HYNIC-peptide

Labeling was performed in accordance with a previous report [35, 36]. Briefly, a stock solution was prepared by dissolving the HYNIC-peptide in distilled water. Radiolabeling of peptide was performed by adding to water (0.5 mL), the stock solution (20 μg of peptide; 20 mL), tricine (15 mg), and EDDA (5 mg). To this mixture was added nitrogen-purged stannous chloride dihydrate solution (0.1 M HCl; 2 mg/mL; 20 μL). Finally, 99mTc-pertechnetate (370-1110 MBq) in saline (0.5 mL) was added to the solution in an inert atmosphere and incubated for 10 min at 95 °C. After the contents were cooled to the ambient temperature, quality control tests were performed as described below.

Radiochemical analysis

99mTc-HYNIC-peptide was assayed by analytical RP-HPLC in the above-mentioned analytical condition. Instant thin layer chromatography (ITLC) strips (silica gel 60; Merck) were loaded with sample and developed using three different mobile phases: (1) methylethylketone for 99mTc-pertechnetate ($R_f = 1.0$); (2) aqueous sodium citrate (0.1 M; pH 5) to determine the non-peptide bound 99mTc-coligand and 99mTc-pertechnetate ($R_f = 1.0$); and (3) [1:1] methanol: aqueous ammonium acetate (1 M) for 99mTc-colloid ($R_f = 0.0$). The radioactivity was quantified by cutting a strip (10 cm) into 1 cm pieces and then counting each piece in a gamma counter.

Stability

In vitro stability was evaluated in NaCl 0.9 % (W/V) and in human serum. Aliquots were taken out at 1, 4, 6, 12, and 24 h post labeling at room temperature and analyzed by HPLC and ITLC. The level of protein-bound radioactivity versus non-bound radioactivity was evaluated using a precipitation method. To a freshly prepared human serum (1 mL), was added 99mTc-HYNIC-peptide (86 MBq; 0.1 mL), and the mixture was incubated at 37 °C for 24 h. Samples of 100 μl aliquots were removed and treated with ethanol (100 μL). Each sample was centrifuged for 5 min at 3000 rpm to separate the precipitated serum protein. Supernatant was removed with a syringe, and radioactivity in the supernatant (containing soluble 99mTc-ligands) was compared with that in sediment (containing insoluble 99mTc, HYNIC-peptide) to determine the percentage of radioligand and/or 99mTc-proteins. The supernatant was analyzed with HPLC to determine the stability of labeled compound at 24 h.

The evaluation of in vivo stability of radiolabeled peptide was conducted in normal balb/c mice by injecting 20 MBq (100 μL) 99mTc-HYNIC-peptide intravenously through the tail vein. After an hour, a fraction of urine was collected and centrifuged at 15,000 rpm for 10 min and analyzed by HPLC.

Partition coefficient (hydrophilicity)

To determine the partition coefficient value, 99mTc-HYNIC-peptide in phosphate-buffered saline (PBS) (0.5 mL) was mixed with n-octanol (0.5 mL) in a 2 ml microtube. The tube was vigorously vortexed over a period of 2 min and centrifuged at 4000 × g for 5 min. From each layer, three samples were taken (100 mL) and then counted in the gamma counter. The average radioactivity values from each of the aqueous and octanol layers were calculated and then were used to compute the octanol-to-water
partition coefficient ($P_{oct}$) by dividing the radiolabeled peptide value (octanol phase) by the aqueous phase value.

**Cell culture**

The B16/F10 murine melanoma cells were cultured in RPMI-1640 supplemented with 10 % (v/v) FBS, glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 µg/ml). Cells were maintained in a humidified 5 % CO$_2$/air atmosphere at 37 °C. For all cell experiments, the cells were seeded (1 million cells per well) in six well plates and then incubated overnight with internalization medium (RPMI-1640 with 1 % FBS).

**Binding and internalization study**

Medium was removed by pipette from each of the six well plates containing adhered B16/F10 cells, with a density of 1 million cells per well and then the cells were washed once with 2 ml of internalization medium (RPMI-1640 with 1 % FBS; 2 mL). Furthermore, a 1.5 mL sample of internalization medium was added to each well, the plates were incubated at 37 °C for 1 h, and then $^{99m}$Tc-HYNIC-peptide (150 KBq; 2.5 pmol total peptide per well) was added. The cells were incubated at 37 °C for various time periods (0.5–4 h). To determine non-specific membrane binding and internalization, we also incubated cells with the radioligand in the presence of α-MSH peptide (150 µL; 1 µmol/L). In separate experiments, the cellular binding and uptake reaction was stopped at 0.5, 1, 2, and 4 h by removing medium from the adhered cells, washing them twice with cold PBS (~4 °C; 1 mL), and then an acid wash with a duration of 10 min was performed twice with cold glycine buffer (pH 2.8; ~4 °C; 1 mL). The last step was designed to distinguish between membrane-bound (acid releasable) and internalized (acid resistant) radioligand. Finally, the cells were treated with NaOH (1 M; 3 mL). All the fractions (reaction medium; PBS washes; acid washes; cells) at the different time points were counted in a gamma counter.

**Biodistribution assay**

Animal experiments were performed in compliance with the regulations of NSTRI institution and with generally accepted guidelines governing such work. Groups of three C57 nu/nu mice (8–10-weeks old, Pasteur Institute, Amol, Iran) were used in each experiment. A suspension of B16/F10 cells (1x10$^7$) in PBS (0.1 mL) was subcutaneously injected in the right flank of each mouse. Seven to 10 days after inoculation, the tumors developed and then $^{99m}$Tc-HYNIC-peptide (20 MBq, 100 µL) was injected via a tail vein. Also a group of three mice were injected with (α-MSH) peptide (100 µg) in saline (0.9 %; 50 mL) as a co-injection as a single dose in a one syringe. The aims are to (1) determine the distribution of radiotracer in non-target tissues that contain MC1 receptors and (2) broadly understand the MC1 receptor density in target versus non-target tissues, by challenging receptor binding of $^{99m}$Tc-HYNIC-peptide with α-MSH peptide.

After 1, 2, 4, and 24 h, the groups of mice were sacrificed, after which the organs of interest were excised, weighed, and counted in the gamma counter. The percentage of the injected dose per gram (% ID/g) was calculated for each tissue.

**Scintigraphy studies**

To better understand the whole body localization, the behavior of $^{99m}$Tc-HYNIC-peptide was evaluated by the static images of the mice, each of which received (20 MBq, 100 µL) of labeled peptide via a tail vein. Also for blocking study mice were injected with (α-MSH) peptide (100 µg) in saline (0.9 %; 50 µL) using a co-injection as a single dose in a one syringe. Before the imaging mice were anesthetized with 0.05 mL ketamine 10 % (3.3 mg) and 0.05 mL xylazine 2 % (1.33 mg) intraperitoneally. After about 5 min, the animal was fixed on a board by covering it with pieces of cloth for immobilization during the scanning.

Scintigraphy imaging study was obtained using a single head gamma camera (small area mobile, 140 keV, Siemens, Germany) equipped with high sensitivity parallel whole collimator. Whole body image was obtained using a 256 x 256 matrix size with 5000 k counts at 1 h post injection. For image acquisition, a 10 % acceptance window around the 140 keV photo peak was used.

**Statistical methods**

The data are reported as mean ± standard deviation. A Student t test was used to determine the statistical significance, which was defined as $P < 0.05$.

**Results and discussion**

**Synthesis and labeling**

MC1 receptors are known to be over-expressed in human skin melanomas [6–8]. The aim of this study was to target the MC1 receptor in vitro using a tumor cell line and in vivo using a mouse tumor model, using an analog of α-MSH containing a HYNIC-coupled lactam bridge-cyclic structure. HYNIC chelator was covalently attached to GABA-bonded onto the N-terminus of Nle-CycMSH$_{hept}$,
the desired conjugating group for the chosen radionuclide $^{99m}$Tc.

$[\text{HYNIC-GABA-Nle}]$-$\text{CycMSH}_{\text{hept}}$ was synthesized by Fmoc strategy, and it was obtained in an overall yield of 35 % based on the removal of the first Fmoc group. The composition and structural identity of HYNIC-peptide were verified by analytical RP-HPLC in the above-mentioned analytical condition and LC–MS (Table 1). ESI-Mass analysis was consistent with the calculated molecular weight for the $[\text{HYNIC-GABA-Nle}]$-$\text{CycMSH}_{\text{hept}}$. Calculated mass for this new derivative is 1258.65 g/mol, and LC–MS analysis confirmed a $[\text{M} + \text{H}]^+$ molecular ion of 1259.399.

A variety of bifunctional chelating agents (BFCA) have been used to label proteins, peptides, and other biologically active molecules with $^{99m}$Tc [36, 37]. More recently, HYNIC has become a more popular BFCA because in the presence of various coligands having an effect on the hydrophilicity and pharmacokinetics of radiopeptides, high specific activity products can be prepared [38]. Of the various coligands, tricine achieves the best radiolabeling efficiency. It has been reported that the composition for the $^{99m}$Tc-HYNIC-tricine complex most likely is, $[^{99m}$Tc($\text{HYNIC}$)(tricine)$_2$], with the complex unstable in solution and found to exist in multiple forms that could be attributed to coordination isomerism in its structure [38]. Various isomers can result from different bonding modalities of the hydrazine functionality of the HYNIC and the two tricine coligands [38]. Both Tc-hydrazido and Tc-di-azenido bonds have been previously reported and characterized by X-ray crystallography [39, 40]. These different bonding modalities from the tricine and hydrazine ligands are expected to produce numerous structural and stereoisomers. [38]. The coligand EDDA is also of particular interest because of being a potentially tetradentate ligand. It is expected to form a more symmetrical and stable complex with technetium when compared to the tricine [38]. It has been shown that using both coligands together to produce $^{99m}$Tc-HYNIC-peptide via a transmetalation type of reaction produces very good results [41]. In this study, 20 μg of HYNIC-peptide was used with tricine and EDDA together as a coligand in amounts of 15 mg and 5 mg, respectively, in a final volume of the labeled solution. The structure of synthesized ligand and the proposed structure after preparation of $^{99m}$Tc/Tricine/EDDA/HYNIC-GABA-Nle-$\text{CycMSH}_{\text{hept}}$ have been shown (Fig. 1).

### Quality control

The radiochemical purity of $^{99m}$Tc-HYNIC-peptide was evaluated by RP-HPLC using the gradient systems consisting of 0.1 % trifluoroacetic acid (TFA) in water (Solvent A) and acetonitrile (Solvent B). The radiolabeling yield was $>98$ % ($n = 3$), acquired via HPLC and also ITLC with a small amount of $^{99m}$Tc-pertechnetate ($<0.5$ %), $^{99m}$Tc-radiocolloid ($<0.2$ %), and $^{99m}$Tc-coligands ($<1.0$ %), and the specific activity was determined to be 81 MBq/nmol. The HPLC elution times were 3.34 min for free pertechnetate and 16 min for labeled peptide (Fig. 2). In comparison to reported data in the literature regarding $^{99m}$Tc/tricine-HYNIC complex instability [38] due to the exchange labeling method, a single major peak was observed without any impurities due to isomeric forms of the $^{99m}$Tc-HYNIC-peptide.

### Stability and hydrophilicity

The $^{99m}$Tc-HYNIC-peptide was stable up to 24 h post labeling at the ambient temperature. High labeling yield and stability may be due to optimization of formulation in the amount of materials, and also in exchanging the labeling method.

In serum protein binding studies, 17.9 ± 1.56 % of the activity was associated with the precipitate obtained after ethanol addition, indicating the low binding of complex with serum proteins due to a specific adsorption or transchelation. The ethanol fraction was characterized by HPLC where a single peak was observed at the same time (90 %) as that of the complex (both peaks having the same retention time). Radiochemical purity yield started at 98 %, and then declined to 90 % at 24 h, indicating 8 % decomposition over this time period (Table 2). $^{99m}$Tc-HYNIC-peptide showed metabolic stability in human serum up to 24 h after labeling and incubation (Fig. 3). In contrast to the high stability for cyclic lactam analog achieved in our work, Raposinho et al. [42] reported a relatively low metabolic stability for the linear derivative.

### Table 1

Analytical data of $[\text{HYNIC-GABA-Nle}]$-$\text{CycMSH}_{\text{hept}}$

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass spectrum</th>
<th>RP-HPLC</th>
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<tbody>
<tr>
<td></td>
<td>Calculated mass (g/mol)</td>
<td>Observed mass (g/mol)</td>
</tr>
<tr>
<td>HYNIC-peptide</td>
<td>1258.65</td>
<td>1259.399 [M+H]$^+$</td>
</tr>
</tbody>
</table>

Analytical HPLC performed on a C18 column using a gradient of 0.1 % aqueous TFA (Solvent A) in acetonitrile (Solvent B) in 30 min at 1 mL/min. The following gradient was used. A:B ratio of: 95:5 at 0 min, 95:5 at 5 min, 0:100 at 25 min, 0:100 at 27 min, 95:5 at 30 min, and 95:5 at 35 min.
The high stability of this lactam bridge-cyclic $^{99m}$Tc-HYNIC-peptide could be attributed to its stable secondary structure such as a beta-turn with an intramolecular hydrogen bond. Due to this structural peculiarity, the cyclic peptide has less conformational freedom and higher stabilities than the linear peptide [21, 25, 43, 44].

The partition coefficient for $^{99m}$Tc-HYNIC-peptide was calculated and found to be $(\log P) = 1.31 \pm 0.12$ which is a good indicator of its hydrophilicity.

Internalization and binding

Figure 4 shows the results related to time dependency and specific internalization of $^{99m}$Tc-HYNIC-peptide into B16/F10 cells. High rate of internalization was observed in this $^{99m}$Tc-HYNIC-peptide ($12.45 \pm 1.1\ %$ up to 4 h) which was not unexpected since CycMSH sequence offers agonistic property to the compound [25, 36]. Previous studies in a series of $^{99m}$Tc-labeled CycMSH$_{hept}$ demonstrate internalization and receptor-mediated trapping of labeled compounds [18, 19, 31, 39]. As it shows the significant differences of uptake between blocked and unblocked cells in various time periods are very noticeable ($P < 0.05$).

Garcia et al. [32] recently evaluated a $^{99m}$Tc labeled HYNIC-MSH analogs, while HYNIC without spacer is connected directly to Nle in the peptide structure. They reported at 60 min, only $1.3\ %$ of $^{99m}$Tc-HYNIC-cycMSH/EDDA was internalized in the B16/F1 melanoma cells ($0.5 \times 10^6$). In comparison to this we obtained a $7.1\ %$ of internalization after 1 h in B16/F10 melanoma cells ($1 \times 10^6$). Since the cell line and the method are not similar, it is difficult to compare the results with each other, nevertheless this high rate of internalization may be due to the structural modification of the $^{99m}$Tc-HYNIC-peptide. This can be explained by the fact that by placing a bifunctional chelating agent (HYNIC) farther from the receptor-binding region of peptide with the use of a three carbon chain spacer (GABA), the negative effect of
chelator on the receptor binding is reduced. So, the more specific receptor binding of the compound, the higher the radioactivity internalization.

It has been shown that peptide with positive charge in their sequence tends to interact faster and more effectively with proteins which are not targeted to a specific receptor [42]. Also a somewhat different amphiphilic-like structure has been recognized in a series of enkephalin analogs previously [45]. Considering to reach a fast and receptor-specific internalization which is also an indication of the balance of lipophilicity for the complex may explain the insertion of a GABA linker as a hydrocarbon spacer in the structure of a MSH conjugate.

Biodistribution

Results from biodistribution studies using the $^{99m}$Tc-HYNIC-peptide are presented in Table 3 as the percentage of injected dose per gram of tissue (% ID/g). $^{99m}$Tc-HYNIC-peptide displayed rapid blood clearance with $0.62\pm0.19$ % ID/g at 1 h. Clearance from the blood circulation was fast with $0.18\%$ ID/g remaining in the blood at 4 h, and the whole body clearance proceeded via the urinary system. The HPLC analysis of urine at 1 h post injection was performed to identify the urine metabolites. The radiochromatogram of urine analysis is shown in Fig. 5. The profile shows that $^{99m}$Tc-HYNIC-peptide has remained intact in urine at 1 h post injection which confirms the in vivo stability of complex.

Clearance from MC1 receptor-negative tissues was also rapid. In normal tissues, the uptake of $^{99m}$Tc-HYNIC-peptide was lower than $1.00\%$ ID/g except for kidneys, at 1, 2, 4, and 24 h post injection. The uptake value of $^{99m}$Tc-HYNIC-peptide for skin was $0.84\pm0.14\%$ ID/g at 1 h, while this value decreased to $0.43\pm0.09\%$ ID/g at 2 h post injection which shows the rapid clearance of the $^{99m}$Tc-HYNIC-peptide.

At 1 h after injection, the kidney uptake was $5.72\pm0.40\%$ ID/g which decreased to $1.47\pm0.30\%$ ID/g at 24 h after injection. In an in vivo blocking study by co-injection of cold peptide at 1 h post injection, the renal uptake activity did not reduce, indicating that the renal uptake was not MC1 receptor mediated and the kidney is the major excretion pathway for the $^{99m}$Tc-HYNIC-peptide.

The tumor uptake was $4.90\pm0.47\%$ ID/g at 1 h post injection and reached its peak value of $5.10\pm0.08\%$ ID/g at 2 h post injection. High tumor/blood (7.90) and high

Table 2 In vitro stability of $^{99m}$Tc/Tricine/EDDA/[HYNIC-GABA-Nle]-CycMSHhept in human plasma (37 °C)

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Radiochemical purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98.3 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>97.4 ± 0.3</td>
</tr>
<tr>
<td>6</td>
<td>96.7 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>94.6 ± 0.4</td>
</tr>
<tr>
<td>24</td>
<td>90.5 ± 0.3</td>
</tr>
</tbody>
</table>

The data are expressed as mean ± standard deviation ($n=3$)
tumor/skin (5.83) uptake ratios were achieved as early as 1 h post injection. In the blocking study at 1 h post injection, tumor uptake was significantly reduced from 4.90 % ID/g in the control group to 1.63 % ID/g in the blocked group which shows that the tumor uptake was MC1 receptor mediated. On the other hand, the uptake reduction in normal tissues due to blocking was not significant. In blocked skin, activity reduction was not significant (0.84 ± 0.14 % ID/g versus 0.62 ± 0.12 % ID/g) which proves non-specific targeting in this organ.

Uptake values compared to those of a previously reported compound $^{99m}\text{Tc(EDDA)-HYNIC-GGNle-CycMSH}_{\text{hex}}$ [31], with the value lower than 1.02 % ID/g except for the kidneys at 2, 4, and 24 h post injection, for this $^{99m}\text{Tc-HYNIC-peptide},$ $^{99m}\text{Tc(EDDA)/HYNIC-GABANle-CycMSH}_{\text{hept}},$ the uptake of radioactivity in normal tissues was lower than 0.51 % ID/g except for the kidneys at 2, 4, and 24 h post injection. Interestingly, the introduction of a GABA linker reduced kidney uptake of HYNIC-GABANle-CycMSH$_{\text{hept}}$, compared to HYNIC-GGNle-CycMSH$_{\text{hex}}$ (4.58 ± 0.68 % ID/g versus 7.52 ± 0.69 % ID/g at 2 h post injection, respectively). It has been reported that different pathways may play roles in the mechanism of the renal uptake of radiolabeled peptides such as the electrostatic interaction between the positively charged peptides and negatively charged tubule cells, [46], endocytosis in tubular cells [47], and transmembrane glycoproteins such as megalin [48]. The introduction of a negatively charged glutamic acid as a linker has reduced the renal uptakes of radiolabeled cyclized $\alpha$-MSH peptides [49]. The reduction in kidney uptake might be attributed to shielding of the electrostatic interaction between positively charged Arg in the moiety of cyclized $\alpha$-MSH peptides and negatively charged surface of tubule cells or could be explained by one of the above mechanisms. Further investigation for better understanding of the mechanism is required.

The tumor uptake of $^{99m}\text{Tc/Tricine/EDDA/HYNIC-GABANle-CycMSH}_{\text{hept}}$ in B16/F10 melanoma-bearing mice was lower than that reported for $^{99m}\text{Tc(EDDA)-HYNIC-GGNle-CycMSH}_{\text{hex}}$ [31] which was studied in B16/F1 melanoma-bearing mice (5.10 ± 0.08 % ID/g versus 14.14 ± 4.90 % ID/g at 2 h post injection, respectively). As it shows the comparison of uptake values in
different cell lines, it is not conclusive and in order to reach a better conclusion, further studies in similar cell lines are necessary.

The tumor uptake of $^{99m}$Tc/Tricine/EDDA/HYNIC-GABA-Nle-CycMSH$_{hept}$ was more than two fold than that reported for $^{111}$In-DOTA-GlyGlu-CycMSH [20] which was studied in B16/F10 metastatic melanoma-bearing lung mice, (5.10 ± 0.08 % versus 2.00 ± 0.74 % ID/g at 2 h post injection, respectively).

The tumor accumulation of $^{99m}$Tc/Tricine/EDDA/HYNIC-GABA-Nle-CycMSH$_{hept}$ and its efficient pharmacokinetic behavior such as low tendency to accumulate in liver and kidney followed by fast clearance due to low lipophilicity and high stability are the major advantages of $^{99m}$Tc-HYNIC-peptide for melanoma detection.

**Imaging**

Since the highest tumor uptake and highest urinary clearance occurred at 1-2 h post injection for $^{99m}$Tc-HYNIC-peptide, in order to visualize tumor localization, the planer gamma imaging studies also were performed at 1 h post

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**Table 3** Biodistribution of $^{99m}$Tc/Tricine/EDDA/[HYNIC-GABA-Nle]-CycMSH$_{hept}$ in B16/F10 tumor-bearing nude mice (% injected dose/g organ ± SD, n = 3)

<table>
<thead>
<tr>
<th>Organ</th>
<th>1 h</th>
<th>1 h block</th>
<th>2 h</th>
<th>4 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.62 ± 0.19</td>
<td>0.88 ± 0.16</td>
<td>0.29 ± 0.08</td>
<td>0.18 ± 0.07</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>Heart</td>
<td>0.29 ± 0.05</td>
<td>0.48 ± 0.18</td>
<td>0.14 ± 0.02</td>
<td>0.10 ± 0.03</td>
<td>0.02 ± 0.04</td>
</tr>
<tr>
<td>Lung</td>
<td>0.64 ± 0.08</td>
<td>0.56 ± 0.26</td>
<td>0.34 ± 0.09</td>
<td>0.19 ± 0.11</td>
<td>0.02 ± 0.05</td>
</tr>
<tr>
<td>Liver</td>
<td>0.65 ± 0.11</td>
<td>0.56 ± 0.10</td>
<td>0.51 ± 0.07</td>
<td>0.41 ± 0.18</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.25 ± 0.03</td>
<td>0.61 ± 0.12</td>
<td>0.16 ± 0.02</td>
<td>0.12 ± 0.02</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.86 ± 0.14</td>
<td>0.75 ± 0.22</td>
<td>0.49 ± 0.14</td>
<td>0.33 ± 0.09</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>Intestines</td>
<td>0.61 ± 0.10</td>
<td>0.85 ± 0.28</td>
<td>0.48 ± 0.10</td>
<td>0.31 ± 0.15</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>Kidneys</td>
<td>5.72 ± 0.40</td>
<td>6.49 ± 0.56</td>
<td>4.58 ± 0.68</td>
<td>4.06 ± 0.33</td>
<td>1.47 ± 0.30</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.14 ± 0.03</td>
<td>0.11 ± 0.04</td>
<td>0.07 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Skin</td>
<td>0.84 ± 0.14</td>
<td>0.62 ± 0.12</td>
<td>0.43 ± 0.09</td>
<td>0.39 ± 0.09</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Tumor</td>
<td>4.90 ± 0.47</td>
<td>1.63 ± 0.46</td>
<td>5.10 ± 0.08</td>
<td>4.27 ± 0.09</td>
<td>1.03 ± 0.05</td>
</tr>
</tbody>
</table>

**Fig. 5** Radioactive HPLC profiles of urine analysis of mice 1 h post injection of $^{99m}$Tc/Tricine/EDDA/HYNIC-GABA-Nle-CycMSH$_{hept}$
In this study, the synthesis, radiolabeling, and biodistribution of \([\text{HYNIC-GABA-Nle}_2]-\text{CycMSH}_{\text{hept}}\) were shown. The radiolabeling step was completed within a very short time (10 min) in high specific activity, and no significant impurities were detected by HPLC. Furthermore, \(^{99m}\text{Tc}\text{-HYNIC-peptide}\) was prepared by labeling of peptide with a GABA linker, HYNIC, and tricine/EDDA as coligands. The \(^{99m}\text{Tc}\text{-HYNIC-peptide}\) demonstrated an excellent radiochemical stability even up to 24 h post labeling. Presented \(^{99m}\text{Tc}\text{-HYNIC-peptide}\) had a specific cell binding and internalization followed by a stable behavior in human serum at 37 °C for at least 24 h. The \(^{99m}\text{Tc}\text{-HYNIC-peptide}\) showed low accumulation in the kidney, high accumulation in tumor as a positive MC1 receptor-targeted tissue followed by excretion via the kidney. These promising characteristics make this \(^{99m}\text{Tc}\text{-HYNIC-peptide}\) a suitable candidate for the diagnosis of malignant tumors.

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References


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Fig. 6 Posterior whole body scan image of C57 nu/nu mice bearing B16/F10 tumor 1 h after injection of \(^{99m}\text{Tc}\text{-Tricine/EDDA/[HYNIC-GABA-Nle}_2]-\text{CycMSH}_{\text{hept}}\). a Unblock. b Block


