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Evaluation of a new Bombesin Analogue Labeled with ^{99m}Tc as Potential Targeted Tumor Scintigraphic Agent

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Abstract

Background: Bombesin shows high affinity for Gastrin-releasing peptide (GRP) receptors which over expressed on the cell surfaces of several human tumors particularly in prostate and breast cancers. The aim of this study was labeling of designed analogue with ^{99m}Tc via HYNIC and Tricine /EDDA and evaluation as potential targeted tumor scintigraphic agent.

Materials and Methods: HYNIC-Bombesin was prepared by solid phase synthesis using Fmoc strategy and radiolabeled with ^{99m}Tc at 100 °C for 10 min by exchange method and radiochemical analysis involved ITLC and HPLC methods. The stability of radiopeptide was checked in the presence of human serum at 37 °C up to 24 h. Internalization was studied with the human GRP receptor cell line PC-3. Biodistribution study was performed in mice.

Results: Radiochemical purities of >98% was obtained. Radiopeptide showed high stability in serum. Radioligand internalization into PC-3 cells was high and specific. Biodistribution study demonstrated that ^{99m}Tc-HYNIC peptide cleared fast from blood and most non-targeted tissues and was excreted mainly by renal pathway and was uptake significantly in GRPr positive tissues such as pancreas.

Conclusion: Easy radiolabeling of peptide conjugate together with favorable in vitro and in vivo characteristics might be a useful peptide radiopharmaceutical in diagnosis of GRPr positive tumors.

Keywords: Bombesin; GRP; Tumor; Labeling

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Introduction

Recently, peptide-based radiopharmaceuticals are becoming of increasing for receptor targeted scintigraphy and radiotherapy in nuclear oncology (1, 2). Bombesin/Gastrin-releasing peptide (BB/GRP) receptors recently have shown great promise for targeted radionuclide therapy and diagnosis of GRPr positive tumors. Over-expression of BB/GRP receptors in human cancers including prostate, breast and small cell lung cancers make these receptors promising molecular targets for radiolabeled BB/GRP analogues (1-7).

Technetium-99m is the gold standard for diagnosis due to its ideal nuclear properties and labeling chemistry. The rapid clearance from blood and the fast tumor accumulation of small peptides which make these peptides favorable for use shorter-lived radioisotopes such as ^{99m}Tc. Several approaches to label peptides with bifunctional chelators (BFCs) for ^{99m}Tc labeling have been published, and among them HYNIC is of interest as a ^{99m}Tc-binding unit because of its monodenticity which is necessary the coordination sphere of the technetium (v) core to be completed by different coligands that may be useful for the fine tuning of the biodistribution. The HYNIC-biomolecules including antibodies and peptide was described before to label successfully with ^{99m}Tc (5, 8). Among the studied the BB analogues, analogues of full-length BB (1-14) and truncated analogues based

on the c-terminal amino acid sequence BB (7-14) can be recognized. The difficulties with ^{99m}Tc-labeled BB analogues are often high abdominal accumulation, the poor in vivo stability and loss of binding affinity upon coupling with a chelator/or introduction of a radiolabel. These problems can be minimized by introduction/substitution of specific amino acids, chelating group and more important spacer chain (9-13). ^{99m}Tc-EDDA/HYNIC-[Lys^3]-bombesin to target gastrin-releasing peptide receptor-positive tumors was studied in an animal model (14) and in human (15). The D-Phe^{13} versus leu^{13} modification and replacement of D-Tyr^6 instead of Asn^{6}can lead to improve binding affinity, pharmacokinetic characteristics and decrease enzymatic metabolism (11, 13, 16).

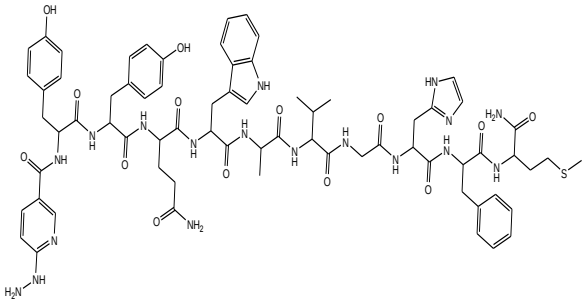

Figure 1. Structural formula of [HYNIC- D-Tyr^5- D-Tyr^6-D-Phe^{13}] BB (5-14)

The aim of the present study was to prepare, radio-chemical evaluation and in vivo/in vitro study of a new ^{99m}Tc-labeled HYNIC- peptide conjugate, [^{99m}Tc/EDDA/tricin/HYNIC-D-Tyr^5-D-Tyr^6-D-Phe^{13}] BB (5-14) for targeting GRP receptor positive tumors.

Materials and Methods

Reagents and instrumentation

Rink amide 4-Methylbenzhydrylamine (MBHA) resin and all Fmoc-protected amino acids were obtained from Nova Biochem. Other reagents were purchased from Fluka, solulink and used without further purification. The reactive side chains of the amino acids were masked with one of the following groups: Trp, *t*-butoxycarbonyl; His, trityl; Tyr, t-butyl. The cell culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), amino acids, vitamins and penicillin/streptomycin from Gibco. Sodium pertechnetate (Na $^{99m}TcO_4$) obtained from commercial ^{99}Mo/ ^{99m}Tc generator (Radioisotope Division, AEOI). Analytical reverse phase high performance liquid chromatography (RP-HPLC) was performed on a JASCO 880-PU intelligent pump HPLC system equipped with a multiwavelength detector and a flow-through Raytest-Gabi γ-detector. CC 250/4.6 Nucleosil 120-5 C18 column from Teknokroma was used for analytical HPLC, and a VP 250/10 Nucleosil 100-5 C18 column was used for semipreparative HPLC. The gradient systems consisted of 0.1% trifluoroacetic acid/water (Solvent A) and acetonitrile (Solvent B). For analytic HPLC, Gradient I was used: 0 min 95% A (5% B), 5 min 95% A (5% B), 30 min 0% A (100% B), 33 min 0% A (100% B), 35 min 95% A (5% B), flow =1 ml/min, λ=280 nm. For semipreparative HPLC Gradient II was used: 0 min 80% A (20% B), 2 min 80% A (20% B), 17 min 50% A (50% B), 19 min 0%A (100% B), 21 min 0% A (100% B), 25 min 80% A (20% B) flow=2 ml/min, λ=280 nm. Mass spectrum was recorded on a HP 1100 series LC/MSD. Quantitative gamma counting was performed on an ORTEC Model 4001 M γ-system well counter.

Synthesis

The peptide-chelator conjugate was synthesized by standard Fmoc solid phase synthesis on Rink Amide MBHA resin with substitution, 0.69 mmol/g. Coupling of each amino acid was performed in the presence of 3 mol excess of Fmoc-amino acid, 3 mol excess of N-hydroxybenzotriazole (HOBt), 3 mol excess of Diisopropylcarbodiimide (DIC) and 5 mol excess of diisopropyletylamine (DIPEA) in Dimethyl-formamide (DMF). Coupling success was checked by the established 2, 4, 6-trinitro- benzenesulfonicacid (TNBSA) test. Cleavage of the Fmoc group was achieved by repetitive treatment with 20% piperidine in DMF. Coupling of HYNIC to peptide was performed in the presence of 1.2 mol excess of HYNIC-BOC 2.5 mol excess of (2-(7-Aza-1H-benzotriazol-1-yl)-1, 1, 3, 3-tetramethyluronium hexa fluorophophate) (HATU), 5 mol excess of DIPEA in DMF.

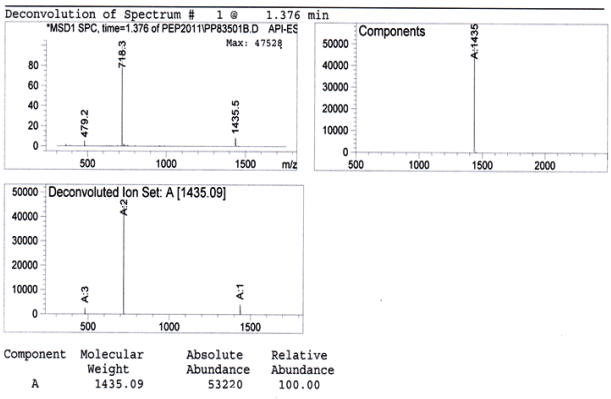

Figure 2. Mass spectrum of [HYNIC- D-Tyr^5- D-Tyr^6-D-Phe^{13}] BB (5-14)

The peptide HYNIC conjugate was removed from the

resin and amino acid side chains were deprotected by treatment with a cocktail of trifluoro acetic acid (TFA), triisopro-pylsilane and water (95:2.5:2.5). After removing the organic solvents in vacuo, the crude product was precipitated with cold diethyl ether. The crude peptide HYNIC conjugate was dissolved in water and purified by semi-preparative (Gradient II) RP-HPLC, next the purified product was characterized by LC/MSD and analytic HPLC.

Radiolabeling with ^{99m}Tc

Radiolabeling of peptide HYNIC conjugate was performed by adding 20 µg (13.9 nmol) of the stock solution new HYNIC–BB derivative (1mmol/l in water) and 15mg (84µmol) of tricine and5 mg (28µmol) of EDDA in 0.5 mL of water. 40 µg $SnCl_2$ (20 µl of 2 mg/ml $SnCl_2$, $2H_2O$ in nitrogen-purged 0.1 M HCl) were added to this solution. Finally, 370-1300MBq of $^{99m}TcO_4^-$ in 0.5 mL saline was added to the solution and incubated for 10 min at 100 ºC. After cooling down to room temperature, the reaction mixture was analyzed.

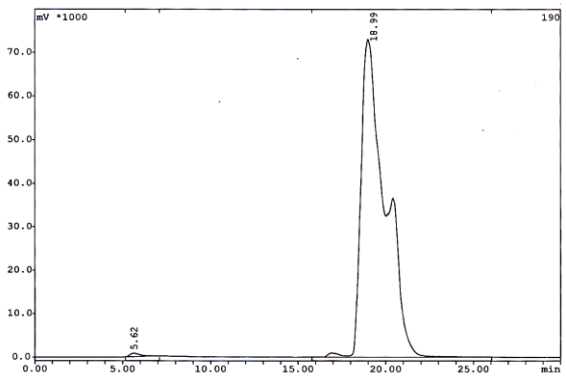

Figure 3. RP-HPLC radiochromatogram of [^{99m}Tc/tricine/EDDA-HYNIC^0, D-Tyr^5- D-Tyr^6-D-Phe^{13}] Bombesin (5-14)

Radiochemical analysis

After cooling up to room temperature, the radiolabeling yield of the labeled peptide was determined by analytical RP-HPLC (Gradient I) and ITLC on silica gel 60 (Merck) using different mobile phases: 2-butanone for free $^{99m}Tco_4^-$ (Rf =1), 0.1 M sodium citrate (pH 5) to determine the non-peptide bound ^{99m}Tc coligand with $^{99m}Tco_4^-$ (Rf =1) and methanol/1M ammonium acetate 1/1 for ^{99m}Tc colloid (Rf =0). The radioactivity was quantified by cutting the strip (1.5×10 cm^2) into 1 cm pieces and counting in a well type gamma counter.

Human serum stability

To 1mL of freshly prepared human serum, we added 100µl (18.5-35MBq) radiolabeled BB derivative and mixture was incubated at 37 ˚C. 100µl aliquots was removed and treated with 200 µl of ethanol (11) at different times up to 24 h. Sample was centrifuged at 3000 rpm for 15 min to precipitate serum proteins. The radioactivity of precipitate serum proteins and supernatant was quantified in a well type gamma counter.

Cell culture

The human androgen-independent prostate carcinoma cell line PC-3 was obtained from National Cell Bank of Iran (NCBI) affiliated to Pasteur Institute of Iran. The cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1% L-glutamine (2mM), 1% penicillin (100IU/ml) /streptomycin (100µg/ml) and 1% amphotericin B (0.25 µg/ml). The cells were incubated at 37 ˚C in a humidified atmosphere containing 5% CO_2 and were subcultured weekly detaching with trypsin/EDTA solution (0.25%).

Internalization Assay

Medium was removed from the 6-well plates contain PC-3 cells with density of 1 million cells per well and cells were washed once with 2 ml of internalization medium (DMEM with 1% FBS). Furthermore, 1.5 ml internalization medium was added to each well, and the plates were incubated at 37 °C for about 1 h. Afterwards, about 150 kBq (2.5 p mol total peptide mass per well) was added to the medium, and the cells were incubated at 37 °C for various time periods. To determine nonspecific internalization, we incubated cells with the radioligand in the presence of 150 µl, 1 µmol/L Bombesin. The cellular uptake was stopped at appropriate time periods (30 min, 1 h, 2 h and 4 h) by removing medium from the cells and washing twice with 1 ml of ice-cold phosphate-buffered saline (PBS). An acid wash for 10 min with a glycine buffer (pH 2.8) on ice was also performed twice. This step was to distinguish between membrane-bound (acid releasable) and internalized (acid resistant) radioligand. Finally, the cells were treated with 1 N NaOH. The culture medium, the receptor-bound and internalized fractions were measured radiometrically in a gamma counter.

Table 1. Data for [HYNIC- D-Tyr^5- D-Tyr^6-D-Phe^{13}] BB (5-14) from LC/MSD analysis

Compound	Calculated mass (g)	Observed mass (g)
[HYNIC]-peptide	1434.61	1435.09 $[M+H]^+$;100%

Biodistribution

Animal experiments were performed in compliance with the regulations of our institution and with generally accepted guidelines governing such work. Mice were injected with 20 MBq (0.35 nmol) of ^{99m}Tc-Bombesin was injected via tail vein. In order to

determine the non-specific uptake of the radio-peptides and in receptor-positive organs, a group of 3 animals were injected with 100 µg cold peptide in 50 µL saline as a co-injection with the radio- peptides (blocked animals). After 1, 4 and 24 h, the mice in groups of 3 animals were killed, organs of interest were collected, weighed and radioactivity was measured in a gamma-counter. The percentage of the injected dose per gram (%ID/g) was calculated for each tissue.

Statistical analyses

The calculations of means and standard deviations for internalization and biodistribution were performed on Microsoft Excel. Student's *t* test was used to determine statistical significance. Differences at the 95% confidence level ($P<0.05$) were considered significant.

Results

The composition and structural identity of [HYNIC-D-Tyr^5-D-Tyr^6-D-Phe^{13}] BB (5-14) (Figure 1) was verified by LC-MSD (Figure 2) (Table 1).^{99m}Tc-radiolabeling yield of HYNIC-peptide using tricine /EDDA exchange labeling was higher than 98% by HPLC and also ITLC at a specific activity of ~80 GBq/µmol. The HPLC Chromatographic profile of [^{99m}Tc/ tricine /EDDA/HYNIC^0, D-Tyr^5-D-Tyr^6-D-Phe^{13}] Bombesin (5-14) is presented in Figure 3. After 24 h in human serum, the radiochemical purity remained >80%.

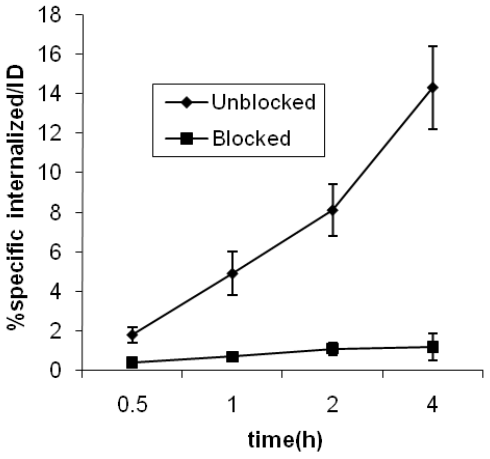

Figure 4. Internalization rate of [^{99m}Tc/tricine/EDDA-HYNIC^0, D-Tyr^5-D-Tyr^6-D-Phe^{13}] Bombesin (5-14) in unblocked and blocked PC-3 cells (mean ± SD, n =3)

The result of the in-vitro assay of the radioligand into PC-3 cell showed rapid receptor-specific internalization (4.9 ± 1.1% at 1 h and 14.3 % ± 2.1 at 4 h). As it shows the significant differences of uptake between blocked and unblocked cells in various time periods are very noticeable ($P<0.05$) (Figure 4).

The results of biodistribution in mice are summarized in Table 2 and Figure 5 .The results of biodistribution demonstrated a rapid clearance from the blood and most tissues with predominantly renal excretion. The highest non-specific uptake was found in kidneys. A significant uptake of radioactivity in receptor-positive organ, including pancreas was observed. The specificity was confirmed by blocking the receptor through prior injection of cold peptide diminished the activity in pancreas. Reduction uptake percentages were 80.9% in the pancreas (0.89% ID/g vs. 0.17% ID/g, $P<0.05$).

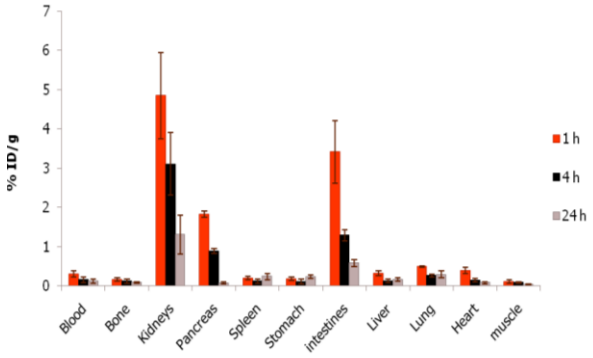

Figure 5. Biodistribution findings in mice (% Injected Dose per Gram Organ ± SD, n =3) the uptake reduction in non-targeted tissues was not significant by the blocking dose.

Discussion

Previous works have indicated that HYNIC acts as a Monodentate ligand to form a mixed ligand ^{99m}Tc complex in the presence of suitable coligands (5, 8). More stable from ^{99m}Tc- HYNIC peptide may result in improved tumor targeting and body retention. HYNIC makes ^{99m}Tc-radiolabeling in high specific activity possible followed by using various coligands, which permit control of the hydrophilicity and pharmacokinetics of radiopeptide (5, 17).

High specific activity achieves with low concentration of the HYNIC peptide conjugate with utilizing exchange labeling technology via tricine and EDDA. In comparison to report regarding ^{99m}Tc-EDDA/ HYNIC complex which have relatively high uptake in pancreas (14) the uptake of our new radiopeptide into GRP receptor-positive pancreas after 4 h was significantly lower. This lower uptake may be due to influence from peptide sequence. Compare with our pervious compound (18) this study showed higher rate of internalization after 4 h in PC-3 cells (14.3 ± 2.1% versus 10.9 ± 1.3%). It could be due to modification of D-Phe^{13} versus Leu^{13} and replacement of (D-Tyr)$_2$instead of Gly^5 and Asn^6. As

the most of BB analogues display high abdominal accumulation which may represent a problem in their clinical for diagnostic imaging and targeted therapy (12) our compound similar pervious study (14) with modified lipophilicity has a good improvement in renal excretion, significant and specific pancreas. Another important advantage of this work is feasibility to prepare a freeze dried kit formulation for routine clinical use in nuclear medicine.

Table 2. Biodistribution of [99mTc/tricine/EDDA-HYNIC0, D-Tyr5-D-Tyr6-D-Phe13] Bombesin (5-14) in mice at 4 h after injection (% Injected Dose per Gram Organ ± SD, n =3)

Organ	Unblocked	Blocked
Blood	0.16±0.07	0.17±0.06
Bone	0.12±0.06	0.14±0.04
Kidneys	3.11±0.8	3.23 ±0.6
Pancreas	0.89±0.07	0. 17±0.05
Spleen	0.12± 0.06	0.14± 0.07
Stomach	0. 11±0.06	0.09±0.02
Intestines	1.29±0.14	1.18±0.16
Liver	0.13±0.05	0.15±0.06
Lung	0.26±0.04	0.31±0.08
Heart	0.15±0.04	0.17±0.02
Muscle	0.08±0.02	0.07±0.03

^{99m}Tc-EDDA/HYNIC-[Lys^3]-bombesin to target gastrin -releasing peptide receptor-positive tumors was in an animal model (14) and in human (15).

The prepared radiopeptide similar previous studies (14, 15) showed a suitable accumulation of radioactivity in pancreas as a positive GRP receptors targeted tissue followed by excretion via the kidneys. These promising Characteristics and feasibility to prepare a freeze dried kit formulation make our new designed labeled peptide conjugate as a suitable candidate for targeted GRP receptor positive tumors scintigraphic agent.

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