Original Article

Synthesis, Labeling and Quality Control of a New Neuropeptide Y Analogue for Diagnosis of Breast Tumors

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(Received 14 June 2008, Revised 1 July 2008, Accepted 5 July 2008)

ABSTRACT

Introduction: Over expression of selected peptide receptors in human tumors has been shown to represent clinically relevant targets for cancer diagnosis and therapy. The aim of this work was to investigate Neuropeptide Y (NPY) as a new radiopharmaceutical for diagnosis of breast cancer.

Methods: A neuropeptide Y analogues with Y_1 receptor preference and agonistic properties was synthesized by solid phase method. After conjugation with diethylenetriaminepentaacetic acid (DTPA) labeling with ^{111}In was performed. For labeled peptide, yield of labeling, stability in human serum, receptor binding in cell surface with internalization in SK-N-MC cells, and biodistribution in normal rat were determined.

Results: Peptide was synthesized and labeled with more than 95% purity. Radiolabeled peptide was stable in human serum and specifically binds and internalized in the cells with Y_1 receptor (4h = 22%). A rapid clearance from blood pool and urinary with hepatobiliary excretion were observed.

Conclusion: Our results showed that this peptide can be considered as a candidate for diagnosis of breast tumors.

Key words: Neuropeptide Y, DTPA, In-111, Tumor

Iran J Nucl Med 2008; 16(1): 20-24

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INTRODUCTION

Peptides can be considered as ideal agents for diagnostic and therapeutic applications, adopting Paul Ehrlichs concept of the "magic bullet". Tumor tissues expressing receptors for a peptide can be addressed specifically by the peptide or differently labeled analogues (1). To date, radiolabeled receptor-binding peptides have emerged as a new class of radiopharmaceuticals (2,3). However, there are several prerequisites for peptides used for scintigraphy. The corresponding receptors have to be expressed on the target in suitable amounts, and it is crucial that the radiolabeled analogue retains a high affinity. Specificity is to be considered as well. Whereas the natural ligand usually binds with similar affinities to several receptor subtypes, chemically modified synthetic analogues often display pronounced subtype selectivity for only one or few receptor subtypes (2). Peptides that are taken up by receptor-mediated internalization may be preferred, as these results in accumulation of the radioligand in the target, providing an increased signal for scintigraphy (4). A main concern of radiolabeled peptides is their stability under physiological conditions, i.e., peptide fragmentation by peptidases and the stability of the radiometal-chelator complex (5). As the natural peptides usually exhibit a short half-life in plasma, metabolically more stable analogues had to be developed.

Neuropeptide Y (NPY) is a 36-amino acid peptide of the pancreatic polypeptide family. It is expressed in the peripheral and central nervous system and is one of the most abundant neuropeptides in the brain (6). Several physiological activities such as induction of food intake, inhibition of anxiety, increase in memory retention, presynaptic retention of neurotransmitter release, and vasoconstriction have been attributed to NPY (7). Its receptors are produced in a number of neuroblastoma and the thereof derived cell lines, making them optimal targets for tumor scintigraphy. Five distinct NPY receptors have been cloned, which have been named Y_1, Y_2, Y_4, Y_5, and Y_6 receptor subtype (8). In vitro receptor autoradiography has been shown a NPY receptor incidence of 85% in primary human breast carcinomas and of 100% in lymph node metastases of receptor positive primaries, predominantly as Y_1 subtype, whereas non neoplastic human breast expressed Y_2 preferentially (9). In this study we prepared a new NPY analogues conjugated with DTPA with a preference for the Y_1-receptor. Synthesis, labeling, stability, internalization and biodistribution studies are presented.

METHODS

All chemicals were obtained from commercial sources and used without further purification. Aminoacides and resin were obtained from NovaBiochem. 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. CC250/4 Nucleosil 120-3C18 column from Macherey-Nagel were used for HPLC. The gradient systems consisted of 0.1% trifluoroacetic acid (TFA)/water (solvent A), acetonitrile (solvent B), flow: 0.75 mL/min, λ = 280 nm. Quantitative gamma counting was performed on ORTEC Model 4001 M γ-system well counter. SK-N-MC cell line was obtained from Pasteur Institute of Iran and DMEM medium from Gibco®.

Synthesis

The peptide was synthesized by standard Fmoc solid-phase synthesis on Rink amide MBHA resin with substitution of 0.8 mmol/g. Coupling of each amino acid was performed in the presence of 3 molar excess of Fmoc-amino acid, 3 molar excess of *N*-hydroxybenzotriazole (HOBT), 3 molar excess of diisopropylcarbodiimide (DIC), and 5 molar excess of *N*-etthyldiisopropylamine (DIPEA) in *N*-methylpyrrolidone (NMP) for 2 h. Completeness of coupling reactions was monitored by the Kaiser test and the Fmoc groups were removed by adding 20% piperidine/*N*,*N*-dimethylformamide (DMF). The fully protected peptide was cleaved from the resin with 20% acetic acid. 3 mol DTPA was coupled with 3 molar DIC to the N-terminus of peptide. After deprotection and precipitation, product was purified and characterized by RP-HPLC and mass spectroscopy.

Labeling and quality control

Radiolabeling was performed by dissolving 20 µg DTPA-NPY in ammonium acetate buffer (300 µl, 0.5 mol/l, pH 5.5) followed by addition of $^{111}InCl_3$ (2 mCi), and incubation for 15 min at 95°C. A 1.5 molar excess of $^{111}InCl_3$ was added and incubated again for 15 min. Subsequently, ^{111}In-DTPA-NPY was purified utilizing a SepPak C18 cartridge preconditioned with 10 ml methanol and 10 ml water; the cartridge was eluted with 3ml water, followed by 2 ml ethanol to yield pure ^{111}In-DTPA-NPY. The labeled peptide was analyzed by analytical HPLC and ITLC on silica gel 60 (Merck) using normal saline as a mobile phase.

Stability in human serum

The stability of ^{111}In-DTPA-NPY was challenged by adding 1 ml of radiolabelled peptide with activity between 10 and 100 µCi to a vial contain 1ml fresh human serum. The reaction mixtures were incubated at 37°C for 24 hand analyzed by placing 100 µl of reaction mixture on a PD10 column. After washing the column with PBS contain 0.1% BSA activity bound to serum protein and peptide was measured with well-type gamma counter.

Binding affinity and internalization

I. The SK-N-MC cells cultured in DMEM supplemented with 10% FBS, 2 mM glutamine and penicillin-streptomycin. Cells were maintained in a humidified 5% CO_2/air atmosphere at 37 ºC. For all cell experiments, the cells were seeded at a density of 1 million cells per well in 6-well plates and incubated overnight with internalization medium (DMEM with 1% FBS). Medium was removed from the 6-well plates and cells were washed once with 2 mL of internalization medium. Furthermore, 1.5 mL internalization medium was added to each well and the plates were incubated at 37 °C for about 1 h. Afterward, about 150 KBq (2.5 pmol 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 membrane binding and internalization, cells were incubated with the radioligand in the presence of 150 µL, 1 µmol/L DTPA-NPY. The cellular uptake was stopped at appropriate time's periods (30 min, 1 h, 2 h, 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 the internalized fraction were measured radiometrically in a γ-counter.

Biodistribution

Six-week-old male Lewis rats were injected anesthesia with 20 MBq of 0.35 nmol (0.5 µg total peptide mass in 150 µL), ^{111}In-DTPA-NPY in saline into the tail vein. At 1, 4 and 24 h time periods after injection, rats were sacrificed under ether anesthesia. Organs and tissues (blood, heart, lung, spleen, kidneys, stomach, pancreas, ileum, colon, liver, muscle, bone, and brain) were excised from the sacrificed animals. The tissues were weighed, and the radioactivity was determined by γ-counting. Results were expressed as percentage of injected dose per gram of tissue (% ID/g).

RESULTS AND DISCUSSION

After standard solid-phase synthesis of peptide with sequence of YPSKPDFPGEDAPAEDLARYYSALRHYINLITRP RY the coupling of DTPA to the N-terminus of peptide were done in solution in an overall yield of 30%. The composition and structural identity of DTPA-NPY was verified by analytic HPLC and MS (Fig. 1, 2).

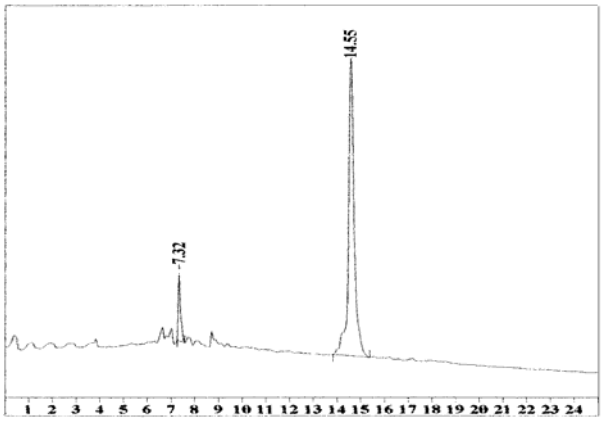

Fig. 1: RP-HPLC analysis of DTPA-NPY conjugate.

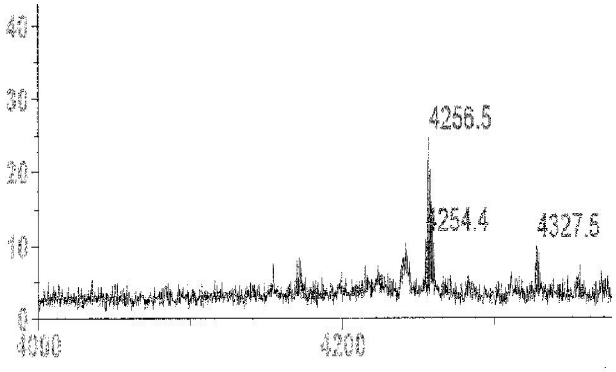

Fig. 2: Mass spectrometric analysis of synthesized peptide.

The purity was > 90% as confirmed by HPLC method and labeling yield of ^{111}In-DTPA-NPY, was >95% by HPLC and also ITLC (Fig. 3).

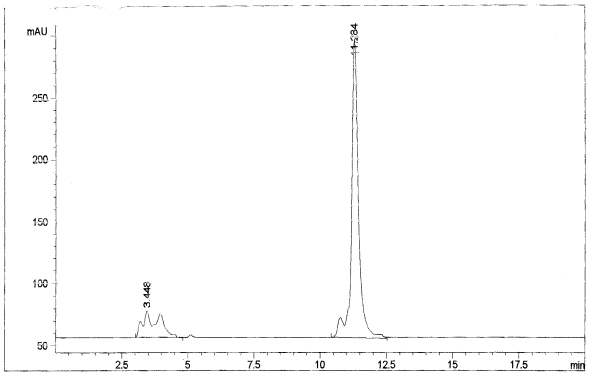

Fig. 3: RP-HPLC analysis of ^{111}In-DTPA-NPY.

The HPLC elution times were 3.44 min for $^{111}InCl_3$ and 12.84 min for ^{111}In-peptide. Results show with post labeling approach combined with the advantages of solid phase peptide synthesis we can obtain a highly pure labeled peptide conjugate.

The amount of ^{99m}Tc that was displaced from the labeled peptide to human serum after 24 hour was 25 ± 5% (Fig. 4). This shows the stability of our conjugate which can not be replaced with plasma proteins and also prevents to enzymatic destruction.

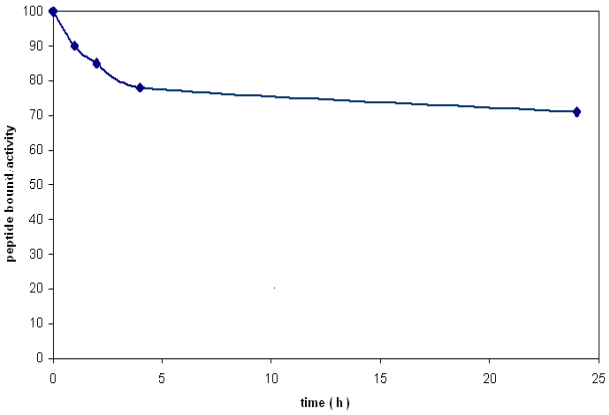

Fig. 4: Stability of ^{111}In-DTPA-NPY after different time of incubation with human serum.

Figure 5 shows the results of the time dependent and specific internalization of radioligand into SK-N-MC cells. At 30 min radioligand showed 5.36 ± 0.56% specific cell uptake that increased to 22.03 ± 1.68% up to 4 h. This receptor mediated internalization is due to specific binding to the Y_1 receptor and may lead to high uptake and retention in the target tissue.

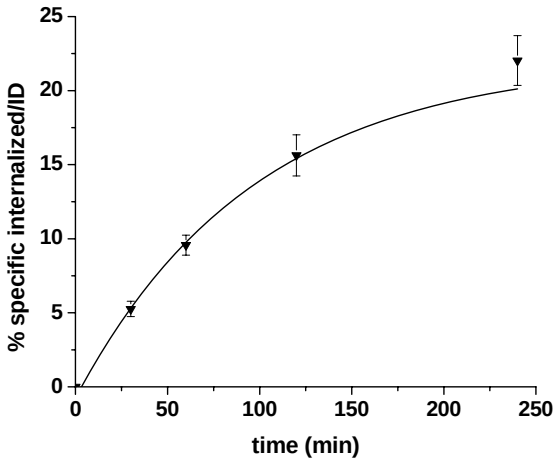

Fig. 5: Internalization rate of ^{111}In-DTPA-NPY into SK-N-MC cells.

The tissue distribution of ^{111}In-DTPA-NPY in rat after intravenous injection is shown in Figure 6. The peptide exhibited a rapid clearance from the blood pool. The levels after 24 h in both kidneys and liver might indicate urinary and hepatobiliary excretion. As seen by the low activity in the brain, the peptide is not crossing the intact blood-brain barrier. High uptake of radioactivity in organs, including stomach and intestine also were observed that can be caused by specific binding of the labeled peptide to its receptor.

CONCLUSION

We presented the preparation of a chemically and metabolically stable candidate, which maintains high binding affinity to its receptor. Therefore, 111In-DTPA-NPY has promising characteristics for future application in breast tumor diagnosis.

AKNOWLEDGEMENT

The author wishes to thank H. Talebi and M. Zoghi of the radioisotope department (AEOI) for assistant in quality control tests.

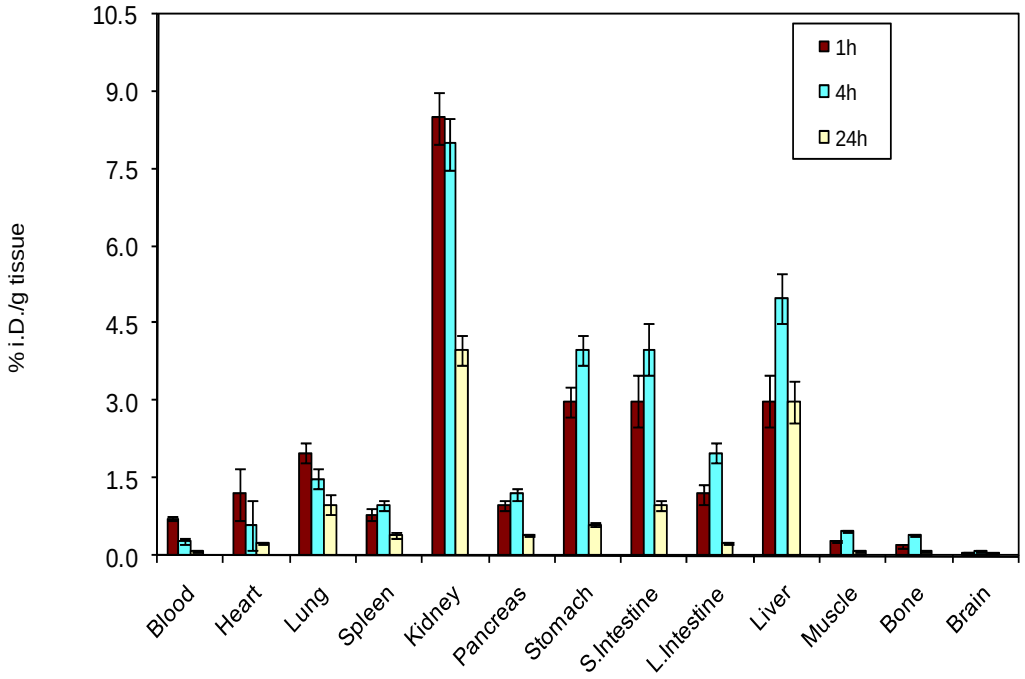

Fig. 6: Biodistribution of ^{111}In-DTPA-NPY in rats at time of 1, 4 and 24 h after injection.

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