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Preclinical study of a new ^{177}Lu-labeled somatostatin receptor antagonist in HT-29 human colorectal cancer cells

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ABSTRACT

Objective(s): Somatostatin receptor-positive neuroendocrine tumors have been targeted using various peptide analogs radiolabeled with therapeutic radionuclides for years. The better biomedical properties of radioantagonists as higher tumor uptake make these radioligands more attractive than agonists for somatostatin receptor-targeted radionuclide therapy. In this study, we tried to evaluate the efficiency of Luthetium-177 (^{177}Lu) radiolabeled DOTA-Peptide 2 (^{177}Lu-DOTA-Peptide 2) as a new radioantagonist in HT-29 human colorectal cancer in vitro and in vivo.

Methods: DOTA conjugated antagonistic peptide with the sequence of p-Cl-Phe-Cyclo(D-Cys-L-BzThi-D-Aph-Lys-Thr-Cys)-D-Tyr-NH2 (DOTA-Peptide 2) was labeled with ^{177}Lu. In vitro assays (saturation binding assay and internalization test) and animal biodistribution were performed in human colon adenocarcinoma cells (HT-29) and HT-29 tumor-bearing nude mice.

Results: ^{177}Lu-DOTA-Peptide 2 showed high stability in acetate buffer and human plasma (>97%). Antagonistic property of ^{177}Lu-DOTA-Peptide 2 was confirmed by low internalization in HT-29 cells (<5%). The desired dissociation constant (K_d =11.14 nM) and effective tumor uptake (10.89 percentage of injected dose per gram of tumor) showed high binding affinity of ^{177}Lu-DOTA-Peptide 2 to somatostatin receptors.

Conclusion: ^{177}Lu-DOTA-Peptide 2 demonstrated selective and high binding affinity to somatostatin receptors overexpressed on the surface of HT-29 cancer cells, which could make this radiopeptide suitable for somatostatin receptor-targeted radionuclide therapy.

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Introduction

Therapeutic radiopharmaceuticals based on beta-emitting radionuclides have been utilized to treat various diseases for decades (1). Proteins and their fragments (monoclonal antibodies, nanobodies, peptides) and small molecules (steroids, phosphonate ligands and, etc.) conjugated to therapeutic radionuclides such as iodine-131 (^{131}I), lutetium-177 (^{177}Lu) and yttrium-90 (^{90}Y) have been developed for the treatment of benign and malignant disorders(2).

Nowadays, targeted radionuclide therapy as a specific treatment of cancers is performed to expose the tumor cells by high doses of ionizing radiation, reduce the radiation toxic effects on healthy cells, and simultaneous destruction of primary and metastatic tumors (3). Overexpression of peptide receptors on the surface of cancer cells has established the peptide-receptor radionuclide therapy (PRRT) as an efficient method to treat a variety of cancers (4–6). ^{177}Lu-labeled peptide analogues like bombesin (7), prostate-specific membrane antigen (PSMA) (8), substance P (9), Cholecystokinin (CCK) (10), α-melanocyte-stimulating hormone (α-MSH) (11) have been assessed for treatment of many types of cancers based on the desirable ^{177}Lu therapeutic properties.

Somatostatin (SST) receptors overexpressed in neuroendocrine tumors (NET) are the most widely used targets for PRRT and varied sequences of somatostatin labeled with therapeutic radionuclides have been designed for the treatment of NETs. Of all SST analogs, DOTA-Tyr3-octreotide (DOTA-TOC) and DOTA-Tyr3-octreotate (DOTA-TATE) radiolabeled with therapeutic radionuclides are the most commonly used SST receptor agonists applied in PRRT so that the ^{177}Lu-DOTA-TATE was approved by U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA)(4,12).

Despite the extensive studies on SST receptor agonists, it has been indicated that SST receptor antagonists with lower receptor internalization show higher tumor uptake compared to corresponding agonists. Different new ^{177}Lu-labeled somatostatin antagonists like ^{177}Lu-DOTA-BASS and ^{177}Lu-DOTA-JR11 have been developed in recent years due to their desirable properties including the better pharmacokinetic the as well as higher tumor uptake and effective dose(13).

In the present study, we assessed our new published ^{177}Lu-labeled antagonistic peptide (^{177}Lu-DOTA-Peptide 2, Figure 1) (14) for stability, receptor binding affinity to SST receptors on human colorectal adenocarcinoma cell line HT-29, and biodistribution in HT-29 tumor-bearing mice models.

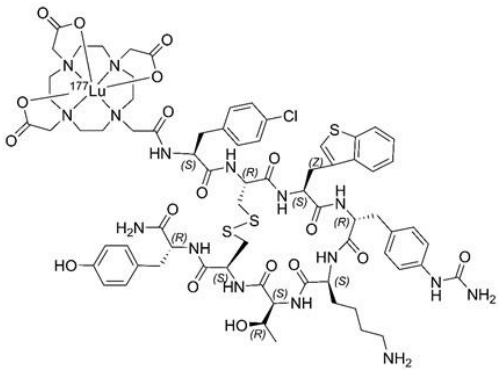

Figure 1. Chemical structure of ^{177}Lu-DOTA-Peptide 2

Methods

Materials

The antagonistic peptide (DOTA-Peptide 2) with the sequence of ([(1, 4, 7, 10-Tricarboxymethyl-1, 4, 7, 10-tetrazacyclododec-1-yl) acetyl]-(L) p-Chlorophenylalanyl-(D) Cysteinyl-(L)-3-Benzo-Thienylalanyl (L-BzThi)-(D)-4-Aminocarbamoyl-phenylalanyl(D-Aph)-(L)-Lysyl-(L)-Threoninyl-(L)-Cysteinyl-(D)-Tyrosine-NH2-cyclic disulfide) was synthesized using the previously reported method(14).

^{177}Lu Trichloride ($^{177}LuCl_3$) and DOTA-TATE were prepared by Pars Isotope Co. (Tehran, Iran). Human colon adenocarcinoma cells (HT-29) was purchased from the Pasteur Institute of Iran (Tehran, Iran). All chemicals and solvents used in our work were of analytical reagent (AR) grade .

Instant thin-layer chromatography (ITLC) was performed using TLC–silica gel sheets (Gelman Sciences, Washington, DC, USA) which were plotted using a MiniGITA TLC Scanner (Elysia-raytest GmbH, Germany). Radiochemical purity (RCP) of radiopeptide was evaluated by high-performance liquid chromatography (C18-RPHPLC, Sykam S7131, Eresing, Germany) equipped with a Gabi radioactivity detector (Raytest-Gabi, Straubenhardt, Germany).

Radiosynthesis and stability study of ^{177}Lu-DOTA-Peptide 2

Radiosynthesis and stability study of ^{177}Lu-DOTA-Peptide 2 Radiolabeling of DOTA-Peptide 2, stability in ammonium acetate buffer and human plasma and quality control procedures were performed based on the previous study (14). Briefly, $^{177}LuCl_3$ solution (25 µL, 120 MBq) was added to DOTA-Peptide 2 in ammonium acetate buffer (0.25 M, pH=5.0) and heated at 95°C for 30 min. RCP was determined by Radio-HPLC (0.1% TFA (solvent A) acetonitrile (solvent B), gradient program: 0–8 min, 20%–65% solvent B, flow rate: 1.0 mL/min) and Radio-TLC (citrate buffer 0.1 M (pH=5.0) as mobile phase).

In-vitro stability of ^{177}Lu-DOTA-Peptide 2 (20 µL, 5 MBq) in ammonium acetate buffer solution (500 µL, 0.25 M, pH=5) was evaluated for 3 days post-labeling at room temperature (25°C) by radio-TLC. The plasma stability of ^{177}Lu-DOTA-Peptide 2 (50 µL, 12 MBq) was assessed at 37 ˚C for 1, 4 and 24 h as well. After each interval, plasma proteins were precipitated by cold ethanol, the mixture was centrifuged RCP of supernatant was reported by ITLC (14).

Cell culture and animal models

The HT-29 cells were cultured in RPMI-1640 (Gibco, UK) complemented with 10% fetal bovine serum, 2% L-Glutamine solution 2 mM, penicillin (50 U/mL) and streptomycin (50 µg/mL) in a humidified atmosphere at 37°C with 5% CO_2(15). All animal experiments were performed in accordance with the national research council's guide and the investigation was approved by the ethical committee at Tehran University of Medical Sciences (Code no: IR.TUMS.VCR.REC.1397.894). To develop tumor-bearing mice models, male athymic nude mice (8 week, 18-23 gr, Pasteur Institute of Iran) were subcutaneously injected with HT-29 cells (7×10^6 cells in 0.1 mL PBS) into the right shoulders .

Cell binding affinity test

The HT-29 cells were seeded in 6-well plates (approximately 10^5 cells/ 2 mL RPMI per well) and incubated at 37˚C for 24 h. Afterwards, the cells were treated with different concentrations (1-100 nM) of ^{177}Lu-DOTA-Peptide 2 and incubated for 60 min at 37°C. To estimate the non-specific binding of radiopeptide to SST receptors, a 1,000-fold molar excess of octreotide was added to some wells 30 min before the addition of ^{177}Lu-DOTA-Peptide 2. The cells were harvested after washing twice with PBS and the bound activity was measured using a gamma counter (EG&G/ORTEC, Model 4001M). Dissociation constant (K_d) and the total concentration of SST receptors expressed on HT-29 cells (B_{max}) values were calculated by plotting the specific binding versus the concentration of ^{177}Lu-DOTA-Peptide 2 (nM) using software (Prism; GraphPad) (16, 17). All experiments were conducted in triplicate.

Internalization Study

The internalization study of ^{177}Lu-DOTA-Peptide 2 was performed in HT-29 cells. Approximately 10^5 cells per well were seeded in 6-well plates (triplicate for each time) and incubated for 24 h at 37˚C with 5% CO2. The cells were treated with 2.5 pmol of radioligand and washed with PBS at 0.5, 1, 2, 4 and 6 h after treatment. The cells were incubated with 1 mL glycine buffer 0.1 M (pH=2.8) twice for 5 min and then rinsed with 1 mL PBS. Finally, 1 mL NaOH 1 N was added to lyse the cells (three times) and the internalized radioactivity was measured in a gamma counter. Specific internalization was evaluated in the presence of an excess amount of octreotide (5 nmol). The percentage of the applied radioactivity calculated as internalization results (16, 18).

Biodistribution studies

Approximately 3 weeks after cell implantation, biodistribution studies were performed in HT-29-bearing nude mice (19). Mice models were intravenously injected with ^{177}Lu-DOTA-Peptide 2 (10 MBq) in 100 µL sterile saline. At 1, 4, and 24 h after injection, mice were sacrificed under anaesthesia, removed organs were weighted and heir radioactivity was counted using a gamma counter. Blocking studies were carried out by the intravenous injection of octreotide (50 µg) in 100 µL normal saline. To compare, biodistribution study of ^{177}Lu-DOTA-TATE (10 MBq in 100 µL) was done in the tumor mice as well. The results were reported as the percentage of injected dose per gram (%ID/g) of organ or tissue mass (20).

Results

Radiosynthesis and stability study of ^{177}Lu-DOTA-Peptide 2

RCP of ^{177}Lu-DOTA-Peptide 2 was obtained >98% by radio-HPLC (Rt: radiopeptide 17.38 min, free ^{177}Lu 5.23 min) and radio-TLC (Rf: radiolabeled peptide 0.3-0.4, free ^{177}Lu 0.9- 1.0) procedures.

The stability assessment of ^{177}Lu-DOTA-Peptide 2 in ammonium acetate buffer solution showed more than 95% of RCP during 3 days. The RCP of ^{177}Lu-DOTA-Peptide 2 in human plasma was calculated > 97% for 24 h at 37˚C (Table 1).

Table 1. Stability study of ^{177}Lu-DOTA-Peptide 2 in acetate buffer and human plasma. Values represent mean±SD, (n=3)

Time (h) / Medium	1	4	24	48	72
Acetate buffer	99.3±0.32	99.2±0.21	98.9±0.43	98.3±0.56	96.7±0.87
Human plasma	99.2±0.17	98.3±0.35	97.6±0.44	-	-

Determination of K_d and B_{max}

Human colon adenocarcinoma cells (HT-29) were utilized for saturation binding assay. As shown in figure 2, K_d and Bmax values for ^{177}Lu-DOTA-Peptide 2 were determined by specific binding curve and obtained 11.14±2.10 nM and 0.25±0.01 pmol/10^6 cells, respectively.

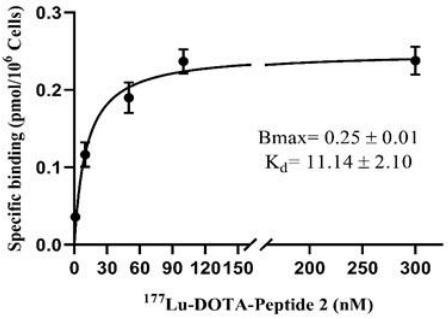

Figure 2. Saturation binding curve for ^{177}Lu-DOTA-Peptide 2 in HT-29 cells

Internalization study

Cell internalization was studied using HT-29 cells at 0.5, 1, 2, 4 and 6 h after treatment with ^{177}Lu-DOTA-Peptide 2. As shown in Figure 3, negligible internalization (~ 5%) was observed for ^{177}Lu-DOTA-Peptide 2 in HT-29 cells.

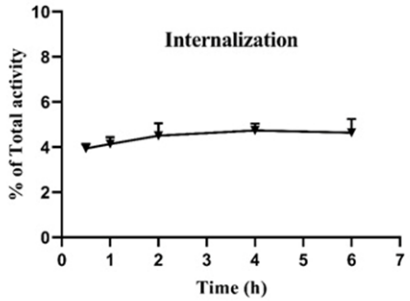

Figure 3. Internalization of ^{177}Lu-DOTA-Peptide 2 in HT-29 cells

Biodistribution in tumor-bearing mice

The distribution of radioactivity and tumor-to-organ ratios for ^{177}Lu-DOTA-Peptide 2 in the tumor-bearing mice at 1, 4 and 24 h after injection were shown in Table 2 and Figure 4, respectively.

The radiopeptide cleared fast from circulation and a significant uptake was observed in pancreas of tumor-bearing mice. Because of the hydrophilic nature of radiopeptide, renal clearance was the main elimination pathway.

Table 2. Biodistribution of ^{177}Lu-DOTA-Peptide 2 and ^{177}Lu-DOTA-TATE in HT-29 tumor-bearing mice. Values represent mean±SD, (n=3)

%ID/g organ	^{177}Lu-DOTA-Peptide 2			*Blocking Tumor	^{177}Lu-DOTA-TATE
Organ	1 h	4 h	24 h	4 h	4 h
Blood	0.31±0.04	0.11±0.02	0.02±0.01	0.10±0.07	0.71±0.06
Liver	6.66±1.90	2.54±0.70	0.73±0.40	3.03±0.50	2.74±0.60
Kidneys	14.57±4.20	10.85±3.30	7.79±2.80	13.34±6.20	9.48±4.10
Stomach	19.58±4.10	15.60±3.70	8.4±2.10	2.2±1.20	4.32±1.71
Heart	1.33±0.45	1.17±0.15	0.32±0.07	0.8±0.02	1.04±0.08
Spleen	3.33±1.30	2.8±1.10	0.89±0.40	0.78±0.40	1.72±1.60
Pancreas	50.22±10.60	41.56±7.70	19.63±8.70	5.22±3.80	17.05±9.50
Lung	10.12±4.37	5.91±2.45	4.28±2.07	1.08±0.60	3.08±1.10
Intestine	4.19±4.40	3.55±2.35	1.31±0.80	2.10±2.47	3.52±1.97
Muscle	0.3±0.10	0.12±0.10	0.11±0.07	0.18±0.10	0.15±0.08
Tumor	7.87±1.30	10.89±3.45	8.83±2.17	1.22±0.70	6.28±1.65
Whole body	2.8±0.67	1.31±0.12	1.19±0.40	2.64±0.82	2.08±0.71

*Octreotide (50 µg) in 0.1 mL saline as a co-injection

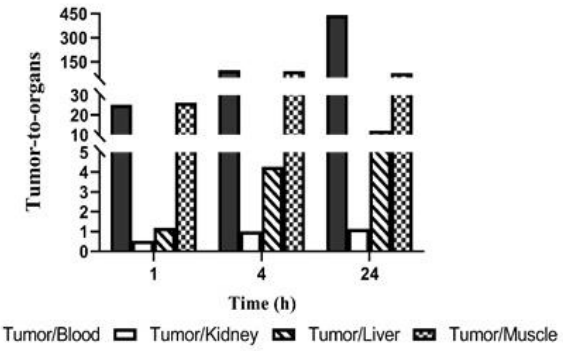

Figure 4. Tumor-to-organ ratios at 1, 4 and 24 h after injection of ^{177}Lu-DOTA-Peptide 2

Discussion

It has been reported plenty of preclinical and clinical studies on SST receptor agonistic and antagonistic peptides for targeted diagnosis and therapy of NETs (21, 22). Studies have revealed that binding of SST antagonists to their receptors leads to lower desensitization and internalization than agonists which provide a large number of binding sites and decreased dissociation rate than agonists (23).

In this research, we radiolabeled DOTA-Peptide 2 as an SST antagonistic peptide with ^{177}Lu to evaluate its preclinical behavior in mice models (14). DOTA is known as the most used chelator for ^{177}Lu radiolabeling which can form stable coordination complexes (log KML=23.5) (24). Therefore, RCP of ^{177}Lu-DOTA-Peptide 2 showed high radiolabeling yield and favorable stability in buffer solution and human plasma.

The expression analysis of SST receptors in HT-29 human colon adenocarcinoma cells using various methods like reverse transcription polymerase chain reaction (RT-PCR) (25), western-blot (15), fluorescence imaging (19) and immunocytochemistry (26) showed the expression of all SST receptor subtypes (SSTR1-5) in HT-29 cells. Since the acceptable in-vitro results were obtained in our previous study for C6 glioma cells (K_d=12.06 nM) (14), the binding affinity of our radiopeptide was evaluated by saturation binding assay and internalization study using HT-29 cells. Considerable binding affinity to SST receptors was observed for ^{177}Lu-DOTA-Peptide 2 (K_d=11.14 nM) which was comparable with the obtained K_d of ^{177}Lu-DOTA-BASS (8.16 nM) (27) as a favorable SSTR2 antagonist with the similar radiometal and chelating agent.

As expected, low internalization of radiopeptide (5%) in HT-29 cells after 6 h confirmed the antagonistic behavior of this radioligand. Intracellular localization of therapeutic radiopharmaceuticals could be advantageous in targeted radionuclide therapy. A receptor antagonist remains bound to the cell membrane on average at a larger distance from the nucleus and its DNA content which it might reduce the number of β-particles effectively reaching the DNA to induce damage. But due to high tissue rang of beta particles (~ 700 μm for ^{177}Lu) and crossfire effect by β-emitting radionuclides, therapeutic effect could be obtained by radioantagonists and without cellular internalization (28, 29).

Renal clearance was the main elimination pathway for ^{177}Lu-DOTA-Peptide 2 due to the hydrophilic nature of the radiopeptide, hepatobiliary excretion was observed in biodistribution assay using HT-29 xenograft mice models though (30). The main differences between the biodistribution of an antagonist (^{177}Lu-DOTA-Peptide 2) and agonist (^{177}Lu-DOTA-TATE) were observed in organs with the expression of somatostatin receptors like stomach and pancreas. Due to the higher tendency of antagonists to bind the SST receptors than agonists, it is expected that higher radioactivity is accumulated in these organs. Therefore, higher uptake of ^{177}Lu-DOTA-Peptide 2 in mentioned organs than ^{177}Lu-DOTA-TATE could confirm the antagonistic property of our radioligand. This phenomenon was also reported for antagonistic radiopeptides as ^{177}Lu-DOTA-JR11 (31) and ^{177}Lu-DOTA-sst2-ANT (27). On the other hand, higher accumulation of radiopeptide in SST expressing organs can cause more damages to them. But, it has been reported that tumor effective dose by radioantagonists will be 2-times more than radioagonists only using 50% of applied activity for peptide receptor radionuclide therapy. Therefore, reduced radiation to non-target organs as well as higher tumor efficient dose could be achieved by lower standard dose for agonists. This property is one of the advantages of radioantagonists (32). The most accumulation of ^{177}Lu-DOTA-Peptide 2 in tumor was obtained at 4 h post-injection (10.89% ID/g), whereas this value was significantly decreased to 1.22% after injection of Octreotide as an SSTRs blocking agent ($p<0.05$). Octreotide is a somatostatin analogue which is considered as the gold standard for systemic therapy of advanced neuroendocrine tumors because of the capability of this ligand to bind to SST receptors with a high affinity. Therefore, octreotide was used in blocking tests to ensure the occupation of all SST receptors on the surface of cells. The reduction in tumor uptake indicated the specific binding of ^{177}Lu-DOTA-Peptide 2 to SSTRs. Decreased tumor uptake for ^{177}Lu-DOTA-TATE (6.28%) compared to ^{177}Lu-DOTA-Peptide 2 (10.89%) may be due to agonistic property of this radiopeptide.

Conclusion

Treatment of neuroendocrine tumors using SSTR-targeting radiopeptides have been utilized for years. Radiolabeled SSTR antagonists have been developed for SSTR overexpressed tumors due to desirable characteristics of antagonists. In this study, ^{177}Lu-DOTA-Peptide 2 as an SST radioantagonist manifested high in vitro stability and good affinity to SSTRs. Acceptable tumor uptake and the high tumor-to-blood ratio of ^{177}Lu-DOTA-Peptide 2 could introduce this radiopeptide as a therapeutic agent for colorectal adenocarcinoma in human.

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