Synthesis, development and preclinical comparison of two new peptide based freeze-dried kit formulation ^{99m}Tc-EDDA-Tricine-HYNIC-TOC and ^{99m}Tc-EDDA-Tricine-HYNIC-TATE for somatostatin receptor positive tumor scintigraphy

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Received 7 Feb. 2006; Revised 27 Jun. 2006; Accepted 19 July 2006

ABSTRACT

Somatostatin receptor (sstr) scintigraphy with [^{111}In diethylenetriaminepentaacetic acid]-octreotide ([^{111}In-DTPA]-OC) has became a routine diagnostic procedure in oncology. However, it suffers from some drawbacks concerning the limited availability, suboptimal imaging properties and elevated radiation burden of ^{111}In.

In this study synthesis, conjugation and preclinical evaluation of two new freeze-dried kit formulation based on somatostatin analogues, Tyr^3-Octreotide (TOC) and Tyr^3-octreotate (TATE), designed for the labeling with ^{99m}Tc are described.

After cleavage from the resin and preparation of the cyclized peptides, these compounds were conjugated with 6-hydrazinopyridine-3-carboxylic acid (HYNIC) in solution. Radiolabeling of HYNIC peptide conjugates was performed at high specific activity using one-step kits formulation based on tricine and ethylenediamine-*N*,*N*′-diacetic acid (EDDA) as co-ligands.

Both, 6-hydrazinopyridine-3-carboxylic acid^0-Tyr^3-Octreotide (HYNIC-TOC) and 6-hydrazinopyridine-3-carboxylic acid^0-Tyr^3-Octreotate (HYNIC-TATE), showed a specific and high rate of internalization after 4 h in AR4-2J rat pancreatic tumor cells, (11.2±0.8 and 18.1±1.2 respectively).

Biodistribution studies in AR4-2J tumor-bearing rats showed rapid clearance of both analogues from all sstr-negative tissues except the kidneys. The specific uptake in tumor and sstr-positive tissues especially pituitary, pancreas and adrenals were observed. After 4 h the adrenals to pancreas uptake ratio for HYNIC-TOC was higher than that of HYNIC-TATE. Although both compounds had high kidney and low liver excretion, for HYNIC-TATE, it was lower. The results suggest these two new peptide based freeze-dried kits might be of great promise for clinical application in imaging of somatostatin receptor-positive tumors.

Keywords: ^{99m}Tc, Somatostatin, Tyr^3-octreotide, Tyr^3-Octreotate, HYNIC, Kit formulation

INTRODUCTION

Peptides are important regulators of growth and cellular functions not only in normal tissues but also in tumors. Somatostatin receptors are known to be expressed in a large number of human tumors and represent the basis for *in-vivo* tumor targeting. Stable somatostatin derivatives such as octreotide or lantreotide are the most frequently used radiopharmaceuticals acting through specific binding to somatostatin receptors; however, they do not bind with high affinity to all five receptor subtypes. The development of [^{111}In-DTPA]-OC served as a sensitive and specific technique for somatostatin receptor scintigraphy, which can detect *in-vivo* somatostatin receptors in various tumors, mainly of endocrine origin. Presently, [^{111}In-DTPA]-OC (octreoscan: Mallinckrodt, Inc.) is commercially available for imaging, however; the use of ^{111}In as a radiolabel results in high cost, limited availability, suboptimal image quality and a high radiation burden to the patient (1). These disadvantages could be overcome by the use of ^{99m}Tc labeled somatostatin analogues, especially if they show faster tumor visualization and allow implementation of a one-day imaging protocol. Thus, ^{99m}Tc labeling of somatostatin analogues

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has been intensively studied and attempts have been made for direct labeling after reduction of the disulfide bridge and the use of bifunctional chelators (2). The use of HYNIC as a technetium ligand for labeling of somatostatin analogues has previously been reported (3). In this article, synthesis, formulation and comparison of two different somatostatin analogues, HYNIC-TOC and HYNIC-TATE after labeling with ^{99m}Tc as targeted for somatostatin receptor-positive tumors is described. Labeling was performed using EDDA and tricine as co-ligands. The rate of internalization in AR4-2J tumor cells and animal biodistribution in rat bearing AR4-2J tumor in comparison with each other were studied.

MATERIALS AND METHODS

Materials

All chemicals were obtained from commercial sources and used without further purification. Trityl chloride-resin, O-*t*-butylthreoninol-2-chlorotrityl resin and most 9-fluorenylmethoxy-carbonyl (fmoc) protected amino acids were purchased from Nova Biochem (Switzerland). The reactive side chains of the amino acids were masked with one of the following groups: Cystein, acetamidomethyl (Cys-Acm); Lysine, *t*-butoxycarbonyl (Lys-BOC); Threonine, *t*-butyl (Thr-tBu); Tryptophane, *t*-butoxycarbonyl (Trp-BOC). The prochelator tert-butoxy carbonyl hydrazinonicotinic acid (BOC-HYNIC) was synthesized by the reported method in literature (4). 1- Hydroxy-benzotriazol (HOBT), diisopropylcarbodiimide (DIC), N-methylpyrolidone (NMP), piperidine, N, N-dimethylformamide (DMF), ethylenediamine-*N*, *N'* -diacetic acid (EDDA) and tricine were purchased from Fluka. The cell culture medium was Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, amino acids, vitamins and penicillin/ streptomycin from Gibco. Sodium pertechnetate (Na $^{99m}TcO_4$) obtained from commercial ^{99m}Mo/ ^{99m}Tc generator (Radio-isotope Division, Atomic Energy Organization of Iran).

Analytical reverse phase-high performance liquid chromatography (HPLC) was carried out on a JASCO 880-PU intelligent pump HPLC system (Tokyo, Japan) equipped with a multiwavelength detector and a flow-through γ-detector (Raytest-Gabi). CC 250/4.6 Nucleosil 120-3 C_{18} columns from Macherey-Nagel (Germany) were used for analytical HPLC and a VP 250/10 Nucleosil 100-5 C_{18} column from Macherey-Nagel (Germany) for preparative HPLC. The gradient systems consisted of 0.1% trifluoroacetic acid (TFA)/water (solvent A), acetonitrile (solvent B). For analytical HPLC, gradient I was used: 0 min 95% A (5% B), 5 min 95% A (5% B), 25 min 0% A (100% B), 27 min 0% A (100% B), 30 min 95% A (5% B), flow: 1 mL/min, λ = 280 nm; and for preparative HPLC gradient II: 0 min 95% A (5% B), 30 min 50% A (50% B), 32 min 0% A (100% B), 34 min 0% A (100% B), 37 min 95% A (5% B), flow: 2 mL/min, λ=280 nm. Quantitative gamma counting was performed on a well type gamma counter (ORTEC Model 4001 M Minibin & power supply).

Synthesis

The peptides were synthesized by standard Fmoc solid phase synthesis on trityl chloride resin (substitution, 0.8 mmol /g) and o-*t*- Butyl-threoninol-2-chlorotrityl resin (substitution, 0.6 mmol /g) on a semiautomatic peptide synthesizer (5). Coupling of each amino acid was performed in the presence of 3 molar excess of Fmoc-amino acid, 4.5 molar excess of HOBT, 3.3 molar excess of DIC and 8 molar excess of DIPEA in NMP for 1.5 h. The reaction was monitored by the ninhydrin test and the Fmoc groups were removed by treatment with 20% piperidine in DMF for 30 min. After coupling the last amino acid peptide chain with all protecting groups were cleaved from the resin by 2 h. treatment in a solution of 20% acetic acid in DMF. Peptide chain was cycled by formation of cys-cys disulfide bond in solution through treatment with 10eq iodine for 30 min. BOC-HYNIC was coupled to the N-terminal of cyclic peptide. Removals of lateral protecting groups were achieved by treatment with a solution of trifluoroacetic acid (90%), water (5%), and thioanisole (5%) at ambient temperature for 10 min. The solutions was then concentrated to a small volume and peptide was precipitated by addition of ether. Purification was achieved by preparative HPLC.

Kit formulation

Purified HYNIC-peptide conjugates were dissolved in distilled water. A solution of tricine in distilled water was also prepared separately. EDDA was dissolved in water by gentle heating and adjusting pH to 7.0 and $SnCl_2$. 2 H_2O was dissolved in 0.1 N HCl. After mixing two solutions and adjusting pH of the final solution to 7, 1 mL of this solution, containing 10 mg EDDA, 20 mg Tricine, 20 µg $SnCl_2.2H_2O$ and 20 µg HYNIC-peptide, were dispensed in a sterile glass vial under air protection. The glass vial was immediately frozen, lyophilized and closed afterward under vacuum.

Labeling

Radiolabeling was performed by addition 0.5 ml of sterile 0.9% saline solution to the freeze-dried kit formulation followed by addition of 0.5 ml sodium pertechnetate ($^{99m}TcO_4^-$ 1GBq in sterile 0.9% saline solution) and heating the mixture for 10 min at 100ºC.

Quality control

Radiochemical purity (RCP) of two ^{99m}Tc labeled kit, after cooling down to room temperature and up to 24 h after radiolabeling was assessed by analytical HPLC (gradient I) and Instant thin-layer chromatography on silica gel 60 (TLC-SG). TLC-SG was performed using different mobile phases. 2-Butanone was used to determine the amount of free $^{99m}TcO_4^-$ (R_f = 1), 0.1 M sodium citrate of pH 5 was used to determine the nonpeptide-bound ^{99m}Tc coligand and $^{99m}TcO_4^-$ (R_f = 1), methanol/1 M ammonium acetate 1/1 was used for ^{99m}Tc- colloid (R_f = 0).

Internalization

The internalization experiments were performed according to the reported method (6). Briefly, the AR4-2J cells line with sst2 receptors were maintained by serial passage in monolayer in DMEM medium, in a humidified 5% CO_2/air atmosphere at 37°C. For all cell experiments, the cells were seeded at a density of 0.8 to 1.1 million cells per well in six-well plates and incubated over night with internalization medium. Medium was removed from the six-well plates and cells were washed once with 2mL of internalization buffer (DMEM, 1% fetal bovine serum, amino acids and vitamins, pH 7.4). Furthermore, 1.5 mL of internalization buffer was added to each well and the plates were incubated at 37°C for about 1 h. Afterward, about 150 KBq (2.5 pmol per well) of the peptides were added to the medium and the cells were incubated at 37°C for indicated time periods in triplicates. To determine non-specific membrane binding and internalization, cells were incubated with radioligand in the presence of 1 µmol/L octreotide. Cellular uptake was stopped by removing medium from the cells and by washing with 1 mL of ice-cold phosphate-buffered saline (PBS). Twice acid wash for 10 min with a 0.1 M glycine buffer of pH 2.8 on ice was also performed twice. Finally, the cells were treated with 1 N NaOH. The culture medium, the receptor bound and the internalized fraction were measured radiometrically in a gamma counter.

Biodistribution

Animal experiments were performed in compliance with the regulations of our institutions and with generally accepted guidelines governing such work. Male lewis rats (200-250 g) bearing AR4-2J tumor (0.5-3 g) were used in the experiments. Rats under ether anesthesia were treated with 20 MBq (0.35 nmol) ^{99m}Tc-peptide diluted in saline (total injected volume = 150 µL; total peptide mass = 0.5 µg). In order to determine the non-specific uptake of the radiopeptides, in tumor or receptor-positive organs, a group of 3 animals were injected with 100 µg Tyr^3-octreotide in 50 µL saline as a co-injection with the radipeptides. After 4 h, the rats in groups of 3 animals were scarified, 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

Synthesis and labeling

Peptides were synthesized in an overall yield of about 30% (Figure 1). The purity of conjugated peptides were more than 95% as confirmed by HPLC. The labeling for two freeze-dried kits formulation were more than 90% by HPLC.

Quality control

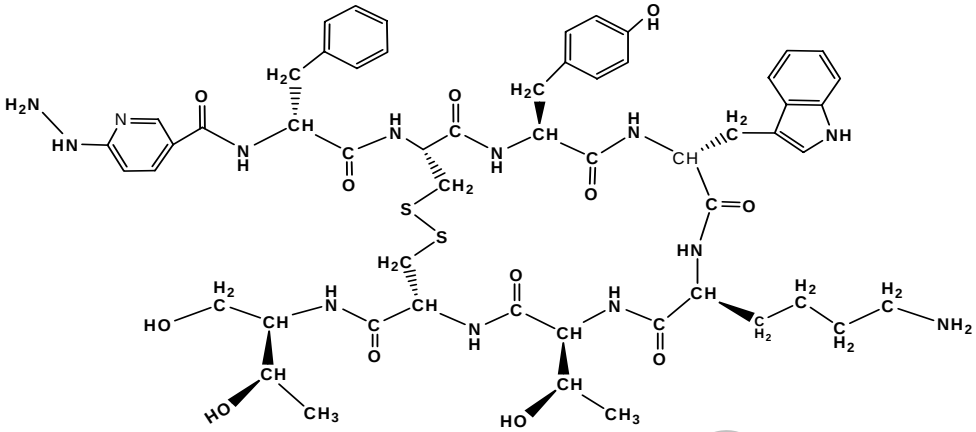
The retention time of ^{99m}Tc-EDDA-Tricine-HYNIC-TOC and ^{99m}Tc-EDDA-Tricine-HYNIC-TATE were 18.3 min and 18 min respectively. The peak corresponding to ^{99m}Tc-coligand complex and $^{99m}TcO_4^-$ eluted at 5 min (Figure 2). Radiochemical purity for both compounds were more than %95 by HPLC and also TLC.

Internalization

Figure 3 shows the results with respect to the time-dependent internalization of ^{99m}Tc-EDDA/tricine-HYNIC-TATE and ^{99m}Tc-EDDA/tricine-HYNIC-TOC into AR4-2J rat pancreatic tumors cells during a 240-min incubation period at 37°C. At 30 min, ^{99m}Tc-EDDA/tricine-HYNIC-TATE showed 5.2% ± 0.5% specific cell uptake of the total activity administered which increased to 18.1% ± 1.2% uptake at 4 h. ^{99m}Tc-EDDA/tricine-HYNIC-TOC showed 1.4% ± 0.3% internalization at 30 min, rising to 11.2% ± 0.8% at 4 h. Two compounds showed a significant difference in their rate of receptor-specific internalization after 4 h ($p < 10^{-4}$). The internalization was strongly reduced in the presence of 1 µmol/L of octreotide. Nonspecific internalization was < 5% after 4 h, and the surface-bound peptide (acid removable) was < 3% of the added activity after 4 h.

Biodistribution

Four hour uptake values of ^{99m}Tc-EDDA/tricine-HYNIC-TATE and ^{99m}Tc-EDDA/tricine-HYNIC-TOC in somatostatin receptor-positive organs



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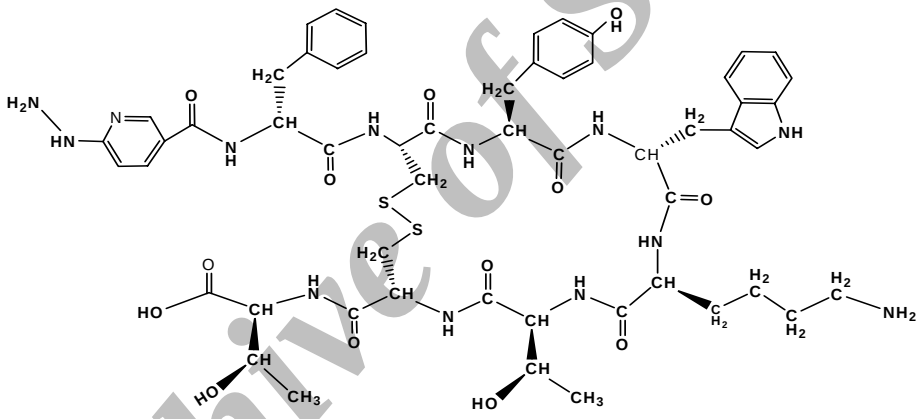

(2)

Figure 1. Chemical structure of the synthesized HYNIC-peptide conjugates. (1) HYNIC-D-Phe^{1}-Tyr^{3}-Octreotide (2) HYNIC-D-Phe^{1}-Tyr^{3}-Octreotate

including pancreas, adrenals, and pituitary, stomach and AR4-2J rat pancreatic tumors, as well as the kidneys, liver, spleen, bone, blood, intestine, lung and heart are shown in table 1 and Figure 4. Both radiopeptides displayed rapid blood clearance with less than 0.05% ID/g remaining in the blood after 4 h. There was also fast clearance from all somatostatin receptor-negative tissues except the kidneys. Both compounds showed a high uptake of radioactivity in receptor-positive organs. This uptake was shown to be receptor specific, given the significant reduction of the uptake in the group of animals receiving an excess of unlabeled Tyr^{3}-octreotide. The uptake in no target tissues was not influenced by the blocking dose. The uptake in tumor after 4 h for ^{99m}Tc-EDDA/tricine-HYNIC-TATE (3.72 ± 0.75) and ^{99m}Tc-EDDA/tricine-HYNIC-TOC (3.50 ± 0.60) were comparable.

DISCUSSION

Somatostatin receptors belong to the family of G-protein coupled receptors (7). There are several important consequences of coupling of agonist to this type of receptors such as desensitization, cyclic AMP production, and internalization. The later is of special relevance to the targeting aspects of using G-protein coupled receptor radioligands (8). It allows long retention times on the tumor that is of special importance in therapeutic applications. It may also contributes to the diagnostic sensitivity due to an increased

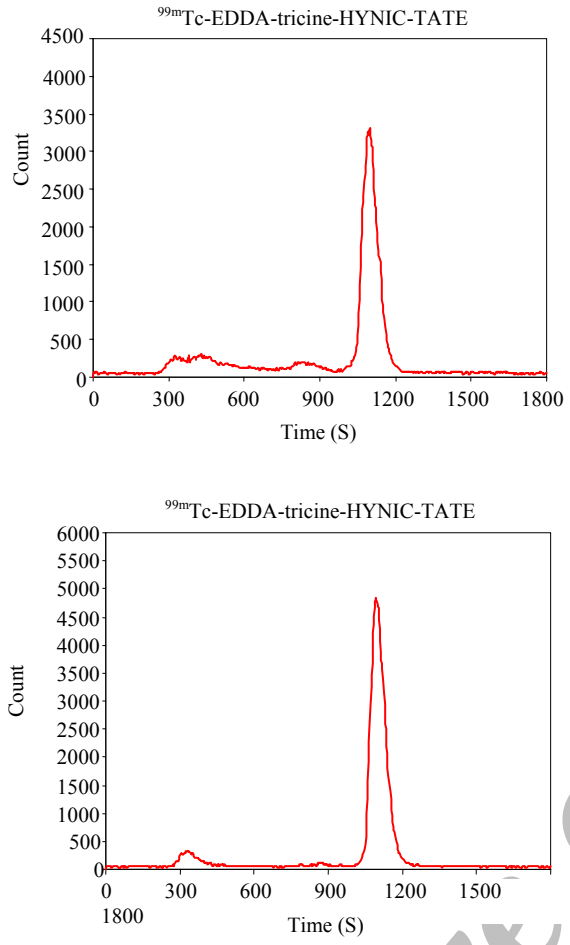

Figure 2. Radiochromatogram of two peptide conjugate after labeling with ^{99m}Tc, analyzed by reverse-phase HPLC and gamma detector.

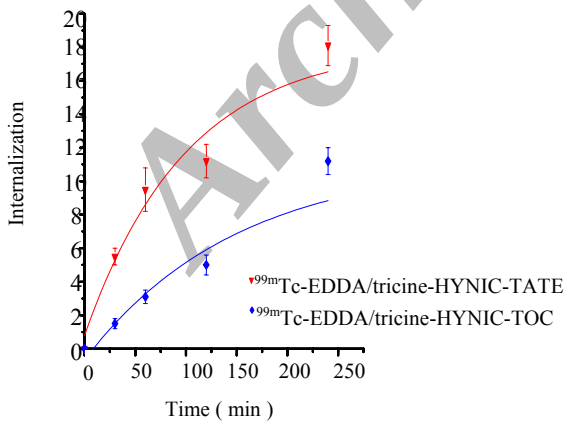

Figure 3. Comparison of the internalization rate of ^{99m}Tc-EDDA/tricine-HYNIC-TATE and ^{99m}Tc-EDDA/tricine-HYNIC-TOC in AR4-2J cells. Values and standard deviations are the result of three independent experiments with triplicates in each experiment and are expressed as specific internalization (percentage of dose added to 1 million cells at 2.5 pmol concentration, 37°C). Significant difference in their rate of internalization after 4 h was obtained ($p < 10^{-4}$).

tumor-to-background ratio with time (9). In order to develop radiopeptides with a broader spectrum of targeted tumors and a potentially higher uptake in tumors a parallel synthesis approach was taken (10). In octreotide, replacement of threoninol with threonine and pheylalanine in position 3 by tyrosine improved affinity for $sstr_2$ receptors (11). Replacement of pheylalanine in position 3 of octreotide by naphtylalanine in 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-coupled peptide led to an improved affinity for $sstr_2$, $sstr_3$ and $sstr_5$ (12). In our study synthesis, conjugation, kit formulation, labeling and preclinical evaluation of two somatostatin based peptides were described.

In another study comparison of these two analogs with different kit formulation and conjugate preparation methods has been described (13). In contrast to our work, in their biodistribution studies the uptake for pituitary, as a positive somatostatin receptor organ has not been reported. Their results show a very high uptake in pancreas that was correlated with the rate of internalization for HYNIC-TATE. They also have reported a high uptake in stomach and a lower uptake in adrenal in comparison with pancreas for HYNIC-TOC. Their studies show low differences between both compounds in liver and lung uptake.

In our study HYNIC was conjugated with Tyr^3-octreotide and Tyr^3-octreotate after cleavage of peptide from resin and its subsequent cyclization in solution. In preparation of conjugate, higher yields could be obtained by reducing the number of reaction steps after conjugation of HYNIC with peptide. In contrast to another report we prepared new one-step kits formulation with the minimum amount of $SnCl_2$ and final adjustment of PH at 7. The results showed that the labeling procedure was simpler and the yield was higher.

In internalization study, both peptides showed a rapid and receptor-specific internalization into AR4-2J rat pancreatic tumor cells. Our data showed that ^{99m}Tc-EDDA/tricine-HYNIC-TATE has a higher potential to bind and internalize in tumor cells with $sstr_2$ expression, no correlation could be found between the rate of internalization at 4 h and uptake in pancreas for this compound.

A quantitative analysis of the biodistribution at 4 hours showed high uptake in tumor and positive somatostatin receptor tissues like pancreas, adrenals and especially pituitary for both compounds. The results of the blocking experiment showed that these uptakes were specific, and receptor mediated. Except for kidneys, which are the main organ of excretion, fast clearances from all sstr-negative tissues were observed. There was not significant difference between the tumor uptakes of HYNIC-TOC and HYNIC-TATE

Table 1. Biodistribution in AR4-2J tumor bearing rats at 4 hours (n = 3 rats) after injection of ^{99m}Tc-EDDA/tricine-HYNIC-TATE and ^{99m}Tc-EDDA/tricine-HYNIC-TOC

Organ \ Time	^{99m}Tc-EDDA/tricine-HYNIC-TATE (%ID/g tissue±SD)		^{99m}Tc-EDDA/tricine-HYNIC-TOC (%ID/g tissue±SD)	
	4-h unblocked	4-h blocked*	4-h unblocked	4-h blocked*
Blood	0.02 ± 0.01	0.04 ± 0.00	0.04 ± 0.01	0.09 ± 0.00
Tumor	3.72 ± 0.75	0.23 ± 0.04†	3.50 ± 0.60	0.16 ± 0.03†
Kidney	1.807 ± 0.2	1.758± 0.3	1.990 ± 0.45	2.342 ± 0.7
Adrenals	0.998 ± 0.3‡	0.157 ± 0.04†	3.423 ± 0.6	0.358 ± 0.20†
Pancreas	1.58 ± 0.18	0.252 ± 0.05†	1.861 ± 0.2	0.216 ± 0.1†
Spleen	0.10 ± 0.01	0.08 ± 0.00	0.17 ± 0.01	0.17 ± 0.04
Stomach	0.21 ± 0.04	0.04 ± 0.00†	0.35 ± 0.05	0.13 ± 0.01†
Intestine	0.20 ± 0.06	0.16 ± 0.02	0.25 ± 0.07	0.12 ± 0.01
Liver	0.07 ± 0.00‡	0.05 ± 0.00	0.14 ± 0.03	0.15 ± 0.01
Lung	0.09 ± 0.01	0.06 ± 0.00	0.19 ± 0.08	0.16 ± 0.01
Heart	0.02 ± 0.00	0.03 ± 0.00	0.05 ± 0.02	0.07 ± 0.01
Bone	0.02 ± 0.01	0.01± 0.00	0.06 ± 0.02	0.04 ± 0.01
Pituitary	4.14 ± 1.01	0.43 ± 0.06†	4.32 ± 0.98	0.65 ± 0.1†

* Blocked with 100 µg Tyr^3-octreotide as a co-injection with the radiopeptide.
‡ P < 0.05 vs. ^{99m}Tc-EDDA/tricine-HYNIC-TOC
† *P* < 0.05 vs. 4 h- unblocked

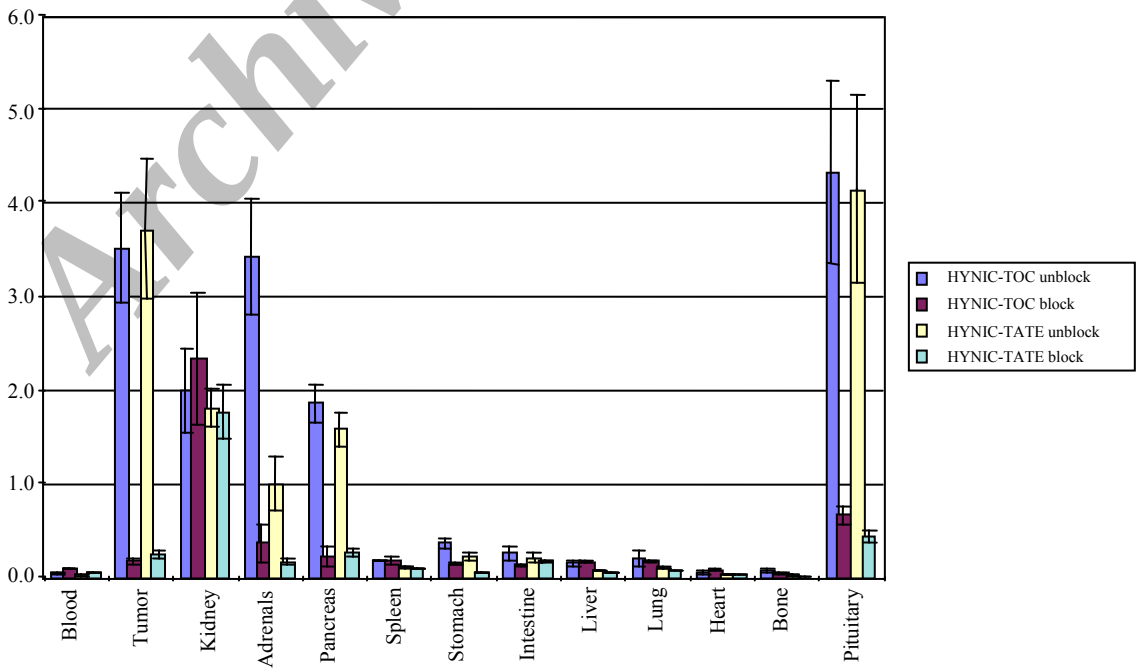

Figure 4. Biodistribution in AR4-2J tumor bearing rats at 4 hours after injection

(p = 0.395). A higher adrenal to pancreas uptake ratio of HYNIC-TOC to HYNIC-TATE could be explained by the lower expression of $sstr_2$ in comparison with $sstr_3$ and $sstr_5$ for these organs (14). Although both compounds showed low liver and high kidney uptakes, lower uptake of HYNIC-TATE in comparison to HYNIC-TOC could be explained by higher hydrophilicity of HYNIC-TATE. By conjugation with DOTA as a chelator for beta emitter radionuclides, due to higher tumor-to-kidney ratio, Tyr^3-octreotate could be a good candidate for somatostatin receptor targeted radiotherapy.

CONCLUSION

Synthesis and chemical characterization of two ^{99m}Tc-labeled somatostatin analogues and comparison of their pharmacological properties are described. Both analogues are good candidates for somatostatin receptor scintigraphy. It seems that in comparison with ^{99m}Tc-EDDA/tricine-HYNIC-TOC, and due to lower uptake in liver and kidney, ^{99m}Tc-EDDA/tricine-HYNIC-TATE has a lower background activity in abdominal and a better picture of tumors in this region could be taken. Clinical comparison of these two peptide-based radiopharmaceuticals is under investigation.

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