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Tetraamine-modified octreotide and octreotate: labeling with ^{99m}Tc and preclinical comparison in AR4-2J cells and AR4-2J tumor-bearing mice

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Abstract: Two somatostatin analogues, [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4, were compared with [^{99m}Tc]Demotate 1, a previously reported somatostatin receptor subtype 2 (sst_2) targeting tracer. Conjugates were prepared by coupling an open-chain tetraamine chelator to D-Phe^1 of [Tyr^3]-octreotide or [Tyr^3]-octreotate, respectively, via a *p*-benzylaminodiglycolic acid spacer adopting solid-phase peptide synthesis techniques. Peptide conjugates were collected in a highly pure form after chromatographic purification. Eventually, [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 were obtained in ~1 Ci/μmol specific activity and >96% purity after labeling under alkaline conditions. Demotide and Demotate 4 exhibited similar high binding affinities for the sst_2 expressed in AR4-2J cells with IC_{50} values 0.16 and 0.10 nM, respectively. The (radio)metallated analogues [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 showed equally high affinities to the sst_2 during saturation binding assays in AR4-2J cell membranes (K_ds 0.08 and 0.07 nM, respectively). During incubation at 37 °C with AR4-2J cells, the radiopeptides internalized effectively via a receptor-mediated process, with [^{99m}Tc]Demotate 4 exhibiting a faster internalization rate than [^{99m}Tc]Demotide. After injection in athymic mice bearing sst_2-expressing AR4-2J tumors, the radiotracers showed high and specific uptake in the tumor (>25%ID/g at 1 h) and in the sst_2–positive organs. However, both [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 showed unfavorably higher background activity, especially in the abdomen, in comparison to [^{99m}Tc]Demotate 1 and are, therefore, less suited than [^{99m}Tc]Demotate 1 for sst_2-targeted tumor imaging in man. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: receptor-targeted imaging; ^{99m}Tc labeling; tetraamine chelator; [Tyr^3]-octreotate; [Tyr^3]-octreotide

INTRODUCTION

Somatostatin receptor scintigraphy with [^{111}In-DTPA^0]-octreotide (DTPA, diethylenetriaminepentaacetic acid) (OctreoScan®) has been established as the method of choice in the diagnosis and staging of neuroendocrine tumors [1–3]. At the same time, several other cyclic octapeptide somatostatin analogues labeled with a wide variety of metallic radionuclides useful for SPET (single photon emission tomography), PET (positron emission tomography) or therapeutic applications have been developed. Stable binding of bi- and trivalent radiometals, such as $^{111}In^{+3}$, $^{90}Y^{+3}$, $^{68}Ga^{+3}$, $^{64}Cu^{+2}$ and radiolanthanides, is achieved after covalent coupling of suitable chelators, such as mono- or bifunctional DTPA, or DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) at their *N*-terminus [1–6].

Owing to the fact that ^{99m}Tc remains the gold standard of diagnostic nuclear medicine by virtue of its optimal nuclear characteristics ($t_{1/2}$ = 6 h, gamma photons 140 keV), low cost and easy availability via commercial ^{99}Mo/^{99m}Tc generators, the search for ^{99m}Tc-based sst_2-targeting radiotracers has been equally intensive [7]. Efforts have been additionally motivated by the prospect of implementing a logistically convenient one-day clinical protocol using a ^{99m}Tc-based radiotracer capable of localizing at the tumor sites fast enough to ideally match the radionuclide's half-life. Most of the ^{99m}Tc-labeled somatostatin analogues reported so far are based on octreotide or other cyclic somatostatin peptide analogues and are modified at the *N*-terminus with a large variety of chelators to ensure stable binding of ^{99m}Tc. Thus, tetradentate N_3S [8,9] or mixed-ligand HYNIC/EDDA-Tricine (HYNIC, hydrazinonicotinamide; EDDA, ethylenediamine-*N*,*N*′-diacetic acid; tricine, *N*-[tris(hydroxymethyl)]glycine) systems [10,11] stabilizing the metal at oxidation state +5 have led to promising radiotracers that are either already commercially available or are currently undergoing clinical trials.

We have been recently engaged in the development of cyclic somatostatin analogues functionalized at the *N*-terminus with an open-chain tetraamine chelator for stable ^{99m}Tc binding. This donor atom set was selected because of previous reports on its ability to form *in vivo*

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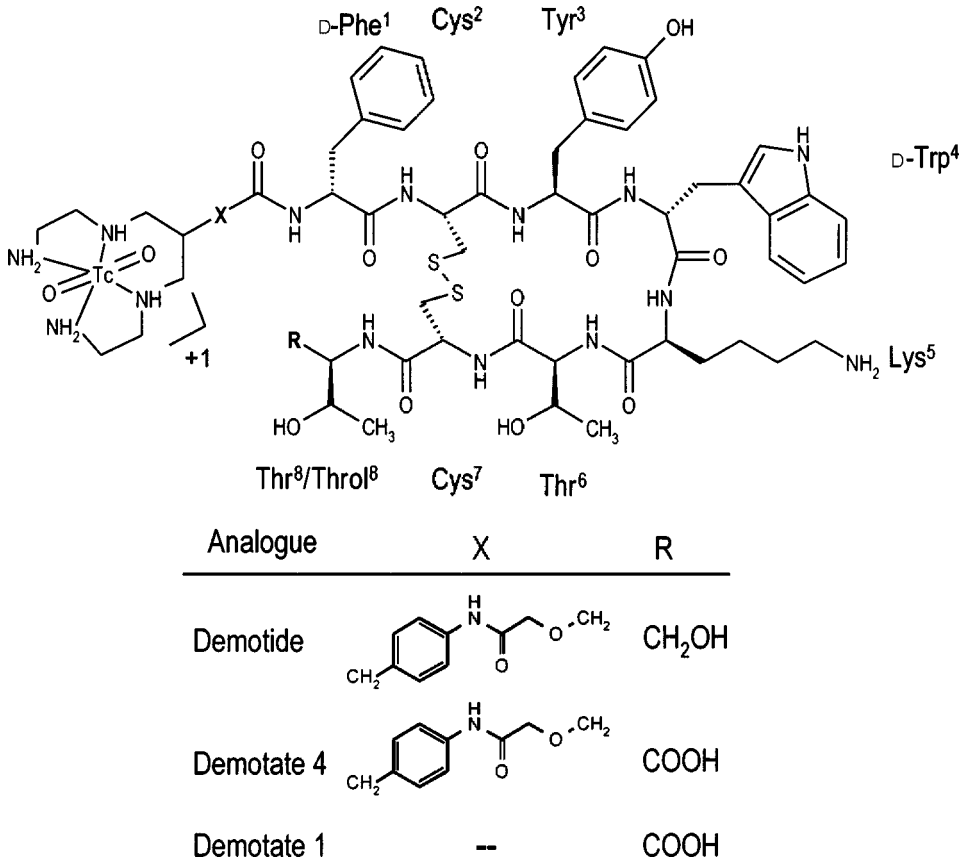

Figure 1 Chemical structure of [^{99m}Tc] labeled peptide analogues.

stable isostructural polar monocationic complexes with technetium and rhenium [12–15], while the corresponding ^{99m}Tc-chelate is easily produced in specific activities suitable for receptor targeting applications [12]. Furthermore, the reported similarities of technetium and rhenium chemistries versus open-chain tetraamines offer the exciting prospect of eventually using diagnostic ^{99m}Tc-therapeutic ^{188}Re somatostatin-based radiopeptide pairs in the scintigraphic detection and treatment of sst$_2$-positive tumors [12,15].

The favorable qualities of the ^{99m}Tc-chelate could be first confirmed in [^{99m}Tc]Demotate 1, a [Tyr^3]-octreotate analogue carrying a tetraamine chelator directly attached at the N-terminal D-Phe^1 residue via a peptide bond (Figure 1). [^{99m}Tc]Demotate 1 showed high affinity for the sst$_2$ *in vitro* and a high tumor uptake in pathological animal models [16] and in patients [17,18]. The positive charge of the metal chelate at the immediate vicinity of the peptide is suspected to have favored receptor binding and tumor uptake of [^{99m}Tc]Demotate 1. In the present study, we were interested to see if introduction of a long spacer between the metal chelate and [Tyr^3]-octreotate affected the biological performance of the resulting radiotracer. Therefore, we developed [^{99m}Tc]Demotate 4, wherein the tetraamine chelator is coupled to the *N*-terminus of the peptide via the long *p*-benzylaminodiglycolic acid spacer (Figure 1). Data on the biological properties of [^{99m}Tc]Demotate 4 extracted from experiments in rat acinar pancreatic AR4-2J cells and AR4-2J tumor-bearing mice are compared with those of [^{99m}Tc]Demotate 1 [16]. Furthermore, the effect of changing from octreotide [Thr(ol)^8] to octreotate (Thr^8) on the biological performance of the related radiotracers could be studied by directly comparing [^{99m}Tc]Demotate 4 with [^{99m}Tc]Demotide. The latter is a [Tyr^3]-octreotide derivative carrying a tetraamine chelator coupled via the *p*-benzylaminodiglycolic acid spacer at its *N*-terminus (Figure 1) having recently shown promising sst$_2$-targeting properties in a rat model [19].

MATERIALS AND METHODS

Safety Note!

Technetium-99m is a weak γ-emitter ($E_\gamma = 140$ keV) with a half-life of 6.03 h. Technetium-99g has a half-life of 2.1×10^5 y and decays by emitting weak β-radiation ($E_{max} = 0.28$ MeV). All manipulations of solutions and solids containing these radionuclides were performed behind sufficient lead protection while normal safety procedures were followed at all times to prevent contamination.

Materials

All chemicals were reagent grade and were used without further purification unless otherwise stated. [Tyr^3]-octreotide was purchased from Bachem (Bubendorf, Switzerland) and [Tyr^3,Lys^5]-(Boc)]octreotide (Boc, *tert*-butoxycarbonyl) was kindly provided by the International Atomic Energy Agency (IAEA, Vienna). [Tyr^3]-octreotate was synthesized on solid phase, as described below. The [(n-C_4H_9)$_4$N][Re^{V}OCl$_4$] precursor was synthesized as previously described [20] using KRe^{VII}O$_4$ (Aldrich, Athens) as rhenium source. Iodine-125 was purchased from MDS Nordion, SA (Fleurus, Belgium). Technetium-99m in the form of [^{99m}Tc]NaTcO$_4$ was eluted in physiological saline from a commercial ^{99}Mo/^{99m}Tc generator (Cis International, Gif-Sur-Yvette, France). NH$_4^{99g}$TcO$_4$ was purchased from Oak Ridge National Laboratories (Roane County, TN) as an impure black solid, which was treated prior to use, as previously described [16]. Paper chromatography was conducted on Whatman 3-mm paper strips. Solvents for high-performance liquid chromatography (HPLC) were HPLC grade; they were filtered through 0.22-μm membrane filters (Millipore, Milford, MA) and degassed by helium flux prior to use. The Bradford reagent (Cat. N^o B-6916) supplied by Sigma Diagnostics (St. Louis, MO) was utilized for protein measurements with bovine serum albumin (BSA) as a standard. Rat acinar pancreatic carcinoma AR4-2J cells used in this study were kindly offered by Dr. R. Kleene (Philipps University, Marburg, Germany). All culture media were supplied by Gibco BRL, Life Technologies (Grand Island, NY) and all supplements by Biochrom KG Seromed® (Berlin, Germany). Animal experiments were carried out in compliance to European and national regulations. For metabolism experiments, in-house male Swiss albino mice (30 ± 5 g) were used. For experimental tumor models female Swiss-nu/nu mice of 6 weeks of age on arrival (Charles River Laboratories, L'Arbresle, France) were employed and the animals were kept under aseptic conditions until biodistribution was performed.

Instrumentation

Preparative HPLC was conducted on a Waters system (Waters Prep LC 4000) coupled to a photodiode array UV detector (Waters 996). The Millennium software by Waters was applied for controlling the system and processing the data. Separations were carried out on a Prep Nova-Pak HP C-18 (25 mm × 100 mm, 6 μm) column (Waters, Vienna). Analyses were performed on a Waters 600E chromatography system coupled to the above PDA-UV detector and/or to a GABI gamma detector from Raytest (RSM Analytische Instrumente GmbH, Straubenhardt, Germany). As stationary phase the Waters Symmetry Shield RP18 column (5 μm, 3.9 mm × 150 mm) was used. Electrospray ionization mass spectroscopy (ESI-MS) was conducted on a AQA Navigator instrument (Finnigan, San Jose, CA) for peptide conjugates and on a LCQ (ThermoFinnigan, San Jose, CA) ion trap instrument for [Re^V]metallated compounds. For radioactivity measurements an automatic well-type gamma counter was used (NaI(Tl) crystal, Canberra Packard Auto-Gamma 5000 series instrument). A Brandel M-48 cell harvester (Adi Hassel Ingenieur Büro, Munich, Germany) was employed in the binding experiments. For imaging, a small field of view experimental gamma camera was employed. The system was based on a position-sensitive photomultiplier tube (Hamamatsu R2486), a pixelized CsI(Tl) scintillation crystal and CAMAC electronics [21].

Synthesis of Peptide Conjugates

Demotide was prepared in solution as previously reported [19]. For the synthesis of Demotate 4, the linear [Tyr^3]-octreotate amino acid sequence D-Phe-L-Cys-L-Tyr-D-Trp-L-Lys-L-Thr-L-Cys-L-Thr-OH was assembled on the acid-sensitive 2-chlorotrityl chloride resin employing typical Fmoc chemistry. Conjugation of *N,N′,N″,N‴*-tetra-(*tert*-butoxycarbonyl)-6-{*p*-[(carboxymethoxy)acetyl]aminobenzyl}-1,4,8,11-tetraazaundecane to the N-terminal D-Phe^1 of the immobilized peptide chain was completed as previously described [16]. Cleavage of the conjugate from the resin and deprotection was achieved by a 5-h treatment in a solution of trifluoroacetic acid (TFA)/dichloromethane (DCM)/1,2-ethanedithiol/water/anisole (8/1/0.7/0.2/0.1, 20 ml/g peptide resin) followed by a 72-h cyclization process in the presence of a 20% dimethylsulphoxide (DMSO) aqueous solution (3 mg/ml peptide) at ambient temperature. Purification of the resulting crude product was performed by gel chromatography and semipreparative HPLC, as previously reported [16].

Yield: 27%; HPLC (Symmetry Shield RP18 eluted at 1 ml/min with 0% B to 60% B in 30 min, A = 0.1% aqueous TFA and B = pure acetonitrile) t_R = 15.1 min, purity ≥97%; ES-MS calculated for $C_{67}H_{93}N_{15}O_{15}S_2$: 1412.7, found 1412.6 ($M^+$, 43), 706.9 ($MH^{2+}$, 100).

Radiolabeling

Radioiodination of [Tyr^3]-octreotide was performed according to published methods [22]. Labeling with ^{99m}Tc was performed according to a previously reported protocol [16]. Briefly, to an Eppendorf vial containing 0.5 m phosphate buffer pH 11.5 (50 μl) the following solutions were consecutively added: 0.1 M sodium citrate (5 μl), pertechnetate generator eluate (410 μl, ~15 mCi), peptide stock solution (15 nmol, 15 μl) and finally a freshly prepared $SnCl_2$ solution in ethanol (20 μl, 40 μg). The labeling reaction mixture was left to react for 30 min at ambient temperature and then adjusted to pH 7.0 by addition of 1 M HCl (10 μl). Preparation of [^{99g}Tc/^{99m}Tc]peptides was based on a similar procedure using a higher amount of $SnCl_2$ [16].

The isostructural Re^V-metallated peptide conjugates were prepared after reaction of Demotide and Demotate 4 with excess of [(n-C_4H_9)$_4$N][Re^{V}OCl$_4$] precursor [20] in methanol (MeOH) in the presence of Hünig's base (*N*-ethyldiisopropylamine). The metallated conjugates were purified by HPLC.

[Re^V]Demotide. Analytical HPLC, purity/t_R: ≥98%/14.7 min; MW: 1614.60 for Re-185 and 1616.60 for Re-187; ESI-MS, (m/z): 1598.70 and 1600.60, respectively ([M-H_2O]$^+$).

[Re^V]Demotate 4. Analytical HPLC purity/t_R: ≥98%/15.2 min; MW: 1628.60 for Re-185 and 1630.60 for Re-187; ESI-MS, (m/z): 1612.60 and 1614.60, respectively ([M-H_2O]$^+$).

Radiochemical Analysis

Labeling yields were monitored by HPLC analysis on a Symmetry Shield RP18 column eluted at 1 ml/min with

a linear gradient system starting from 0% B to 60% B in 30 min (A = 0.1% aqueous TFA and B = pure acetonitrile). Under these conditions [^{99m}Tc]citrate and $^{99m}TcO_4^-$ eluted at 1.4–1.8 min and 3.8 min, respectively, followed by [^{99m}Tc]Demotate 4 at 15.7 min and [^{99m}Tc]Demotide at 15.3 min. Column recoveries were >90%. The presence of reduced hydrolyzed technetium ($^{99m}TcO_2$) was monitored by paper chromatography. An aliquot (<1 μl) of the labeled product was applied on the paper strip, which was developed up to 10 cm from the origin with 1 M ammonium acetate/pure MeOH 1/1 (v/v). The paper was left to dry in the open and then cut into two pieces: 1st = start (origin + 1 cm) and 2nd = front (the rest of the strip): $^{99m}TcO_4^-$, [^{99m}Tc]citrate and [^{99m}Tc]peptide conjugate. The radioactivity content of each piece was measured in the gamma counter [16,19]. Samples from the open labeling reaction vial were analyzed up to 6 h post labeling. Analytical HPLC profiles of resulting radiopeptides were compared after co-injection with the respective Re^V-metallated authentic samples employing parallel UV/Vis (for Re species) and radiometric (for ^{99m}Tc species) detection modes.

Cell Culture

Rat acinar pancreatic carcinoma AR4-2J cells predominantly expressing the sst$_2$ [23] were maintained in Ham's F-12 K nutrient mixture supplemented by 20% (v/v) fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, and grown to confluence in humidified air containing 5% CO_2 at 37 °C. Subculturing was performed once a week employing a trypsin/EDTA (0.05%/0.02% w/v) solution.

Receptor Binding Assays

Binding studies were conducted in AR4-2J cell membranes, prepared as previously described [16]. Competition binding experiments were performed according to a published protocol [16] using [^{125}I-Tyr^3]-octreotide (2200 Ci/mmol) as the radioligand and [Tyr^3]-octreotide/[Tyr^3]-octreotate as control peptides. Briefly, AR4-2J cell membrane homogenate, corresponding to 15 μg protein, was incubated with 30 000 cpm (~0.1 nM) of radioligand in the presence of increasing concentrations of tested peptide in a total volume of 300 μl of HEPES buffer (50 mM HEPES pH 7.6, 0.3% BSA, 5 mM $MgCl_2$, 10 μM bacitracin) for 40 min at 37 °C. For saturation binding experiments, AR4-2J cell membrane suspension corresponding to 15 μg protein was mixed with HEPES buffer containing increasing concentrations of the respective [^{99g}Tc/^{99m}Tc]peptide following a published protocol [16]. Analysis of binding data was performed using the PRISM™ 2 program (GraphPad Software, San Diego, CA).

Internalization Assays

Internalization experiments were performed using AR4-2J cells seeded in 35-mm-diameter dishes (Greiner Labortechnik, Frickenhausen, Germany) at a density of 8–9 × 10^5 cells per well, wherein they remained for 48 h. On the day of the experiment, cells were washed twice with ice-cold internalization medium [F-12K nutrient mixture supplemented by 1% (v/v) FBS]. Internalization medium (1.2 ml) was added to each well together with 300,000 cpm ^{99m}Tc labeled peptide (in 150 μl PBS/0.5% BSA buffer, corresponding to ~200 fmol total peptide) and incubated at 37 °C in triplicates for the indicated periods of time. The percentage of internalized radioligand was determined by acid washing as previously described [16]. Non-specific internalization was determined by parallel incubations in the presence of 1 μM [Tyr^3]-octreotide (for [^{99m}Tc]Demotide) or [Tyr^3]-octreotate (for [^{99m}Tc]Demotate 4).

Analysis of Urine Samples

A 100-μL bolus containing >100 μCi ^{99m}Tc labeled peptide was injected in the tail vein of healthy Swiss Albino mice. The animals were kept in metabolic cages for 30 min with free access to water but not to food and were then sacrificed by ether asphyxiation. Urine was immediately collected from their bladder with a syringe and was combined with the urine found in the metabolic cages, centrifuged at 35 000 rpm for 10 min and filtered through a 0.22 μ Millex-GV filter (Millipore). Aliquots were analyzed by HPLC applying the same conditions as those described for the radiochemical analysis of [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4. Paper chromatography was conducted in parallel on paper strips developed with acetone (R_f $^{99m}TcO_4^-$ = 1).

Incubation in Murine Plasma

Radiopeptide samples were incubated at 37 °C with freshly isolated murine plasma. Aliquots withdrawn at 5-, 15-, 30- and 60-min time points were diluted in ethanol (EtOH) in a 2/1 v/v EtOH/aliquot ratio and then centrifuged at 35 000 rpm for 10 min and passed through a 0.22 μ Millex-GV filter (Millipore). Supernatant samples were analyzed by HPLC and paper chromatography as described above.

Biodistribution Studies

Biodistribution was studied in female Swiss-nu/nu mice bearing AR4-2J tumor xenografts [16]. Each animal received a 100-μl bolus containing 4–5 μCi radiolabeled peptide in PBS buffer pH 7.4 (corresponding to ca 4–5 pmol of total peptide) via the tail vein. Animals were sacrificed in groups of four at 1 and 4 h post injection (pi) by cardiac puncture while under a slight ether anaesthesia. Two additional groups of animals received intravenously (iv) 50 μg [Tyr^3]-octreotide (for [^{99m}Tc]Demotide) or [Tyr^3]-octreotate (for [^{99m}Tc]Demotate 4) together with the radioligand (blocked animals). These were also sacrificed at 1 and 4 h pi. Samples of blood, urine and organs of interest were immediately collected, weighed and transferred in a gamma counter for determination of their radioactivity content using appropriate standard solutions. Tissue distribution data were calculated as percent injected dose per gram (%ID/g) applying a suitable algorithm. Statistical evaluation was performed using one-way ANOVA analysis, followed by comparison among means using the Student's *t*-test ($p < 0.05$ was considered statistically significant).

Imaging

Experimental AR4-2J tumors were grown in the femur of two athymic mice two weeks after inoculation. The animals were injected intravenously with a bolus containing

[^{99m}Tc]Demotide (200 μCi, 200 μl) alone or together with 50 μg [Tyr^3]-octreotide (blocked animal) and were sacrificed by ether asphyxiation at 1 h pi. The animals were positioned under the head of an experimental gamma camera, suitable for both planar and tomographic imaging under a small field of view [21]. Acquisition of gamma camera images was completed and correction for ^{99m}Tc decay was performed in projection data. Dissection of the animals was subsequently performed to correlate scintigraphic findings with anatomical and tissue distribution data.

RESULTS AND DISCUSSION

Several studies have shown that replacement of the *C*-terminal Throl^8 by Thr^8 in the octreotide sequence improves the pharmacological properties of the respective radiolabeled analogues. Thus, octreotate derivatives show higher affinity for the sst_2, increased internalization, and faster body clearance in animal models [1,2,5]. We have previously reported on [^{99m}Tc]Demotate 1, wherein direct conjugation of a tetraamine chelator on D-Phe^1 of [Tyr^3]-octreotate allows for stable binding of ^{99m}Tc [16]. In this study, we compared [^{99m}Tc]Demotate 4 and [^{99m}Tc]Demotide [19], both carrying a *p*-benzylaminodiglycolic acid spacer between the ^{99m}Tc-chelate and the peptide, but differing in their *C*-terminus [Thr^8 and Thr(ol)^8, respectively]. By using the same cells and animal models previously employed for the evaluation of [^{99m}Tc]Demotate 1, it was possible to directly study the effects of these modifications on the biological response of resulting peptides.

Radiolabeling – Radiochemical Analysis

As expected, labeling with ^{99m}Tc easily produced [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 with >96% yields after incorporation of the radionuclide by the tetraamine chelator and formation of a single radioactive species, as demonstrated by HPLC analysis (Figure 2). Chromatographic comparison with Re^V-authentic samples was consistent with the formation of the respective [^{99m}Tc(O)$_2$(N$_4$)]$^+$-chelates in agreement with data reporting on the formation of isostructural technetium and rhenium complexes with open-chain tetraamines [12–14]. Specific activities of 1 Ci/μmol were readily accessible, which are considered satisfactory for effective receptor targeting with ^{99m}Tc [16]. Radiopeptides remained >96% intact in the open labeling reaction vial up to 6 hours post labeling, as verified by combined paper chromatography and HPLC methods. The above qualities reflect the easiness of producing and handling the new tetraamine-modified radiotracers in a clinical environment [16].

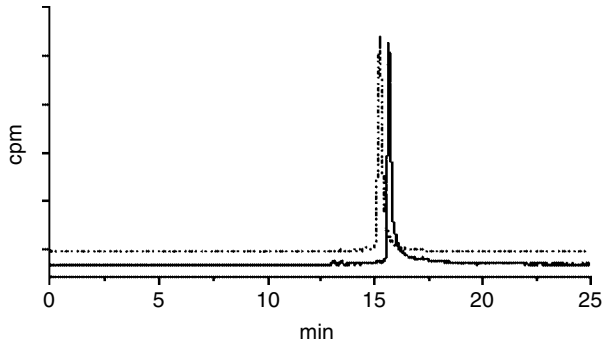

Figure 2 HPLC gamma traces of products formed during labeling (····· [^{99m}Tc]Demotide and – [^{99m}Tc]Demotate 4).

Receptor Affinity Studies

As summarized in Figure 3, both Demotide and Demotate 4 were capable of inhibiting the specific binding of [^{125}I-Tyr^3]-octreotide to the sst_2 in AR4-2J cell membranes [23] in a concentration-dependent manner. The IC_{50} values determined for Demotide and Demotate 4 were lower than the values calculated for the respective control compounds [Tyr^3]-octreotide and [Tyr^3]-octreotate (Figure 3). This finding shows that coupling of the tetraamine chelator is well tolerated by the sst_2 and is in agreement with data from previous studies with similar tetraamine-functionalized cyclic octapeptide analogues [16].

Saturation binding experiments in AR4-2J cell membranes revealed the high affinity of (radio)metallated peptide conjugates [^{99m}Tc/^{99g}Tc]Demotide and [^{99m}Tc/^{99g}Tc]Demotate 4 to the sst_2. Thus, specific binding of both analogues was rapid and saturable, resulting in equilibrium dissociation constants (K_d) of 0.08 ± 0.02 nM for [^{99m}Tc/^{99g}Tc]Demotide and 0.07 ± 0.009 nM for [^{99m}Tc/^{99g}Tc]Demotate 4 with the respective B_{max} values amounting to 297 ± 15.4 fmol/mg and

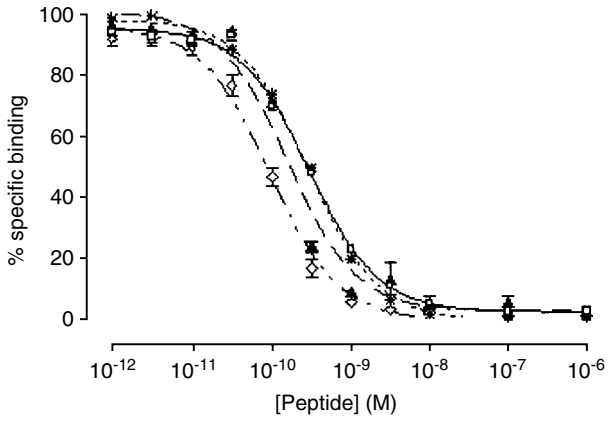

Figure 3 Competitive inhibition of [^{125}I-Tyr^3]-octreotide binding to AR4-2J cell membranes by increasing concentrations of [Tyr^3]-octreotide (□), [Tyr^3]-octreotate (∗), Demotide (▲) and Demotate 4 (◇). Values are means ± S.D. of three independent experiments performed in triplicates. IC_{50} values were: [Tyr^3]-octreotide, 0.30 ± 0.02 nM; [Tyr^3]-octreotate, 0.31 ± 0.02 nM; Demotide, 0.16 ± 0.05 nM and Demotate 4, 0.10 ± 0.04 nM.

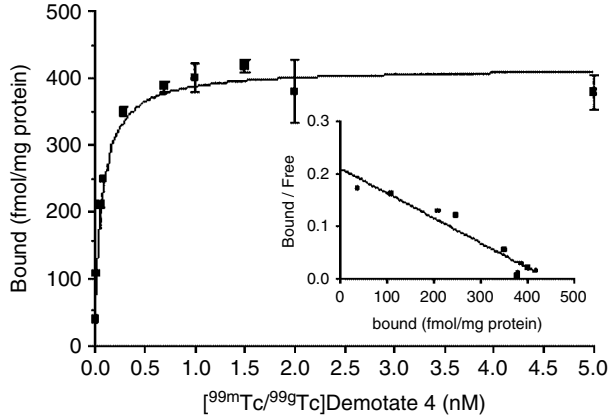

Figure 4 Concentration dependence of [^{99m}Tc/^{99g}Tc]Demotate 4 binding to sst$_2$ receptors (*inset*, Scatchard transformation) in AR4-2J cell membranes, with $K_d = 0.07 \pm 0.009$ nM and $B_{max} = 414 \pm 9.8$ fmol/mg protein. Data represent the mean ± S.D. of three different determinations performed in triplicates.

414 ± 9.8 fmol/mg of protein. All data were best fit to a single-site analysis and show that the presence of the monocationic metal chelate at the *N*-terminus of parent peptides does not interfere with their interaction with the sst$_2$, as reported also for similar somatostatin analogues [16]. A representative saturation curve for [^{99m}Tc/^{99g}Tc]Demotate 4 is shown in Figure 4.

Internalization

To be useful for scintigraphic diagnosis and radionuclide therapy, radiopeptides must internalize into tumor cells, wherein the radionuclide is expected to be retained for sufficient time. [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 internalized in a time-dependent and receptor-specific manner during incubation at 37 °C with AR4-2J cells. Their time course of intracellular migration expressed as % of specific cell-associated activity is presented by the curve of Figure 5. It is interesting to note that, while [^{99m}Tc]Demotate 4 reached the ~80% internalization plateau within 15 min, [^{99m}Tc]Demotide needed >1 h. Similar trends have also been reported for other octreotide/octreotate analogue pairs [1,2,5]. As shown by the reduction of intracellular activity to <10% caused during incubation in the presence of 1 μM of respective unmodified peptides, the process is receptor-mediated.

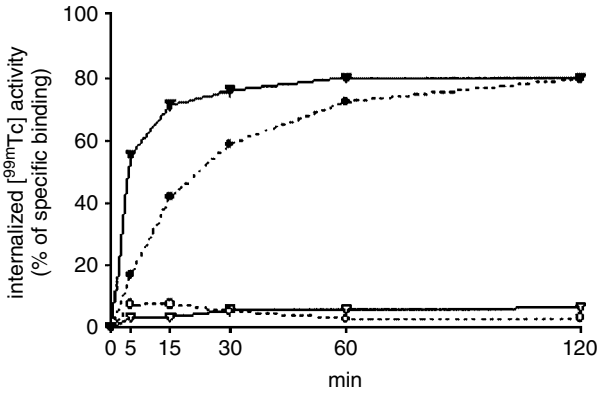

Figure 5 Internalization of [^{99m}Tc] labeled peptide analogues in AR4-2J cells. Cells were incubated with [^{99m}Tc]Demotide (●) or [^{99m}Tc]Demotate 4 (▼) at 37 °C for various time intervals. Results are expressed as the percentage of specifically bound radioactivity, located in the cells. Nonspecific internalization is also presented herein (open circles and triangles).

Metabolism

For effective localization at the target site, radiopeptides must remain intact in the biological milieu for time enough after administration to ensure sufficient delivery to the target. In this respect, plasma stability is of particular relevance. Both [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 remained intact during 2 hours incubation in fresh murine plasma at 37 °C. Furthermore, chromatographic analysis of urine collected 30 minutes after injection of these radiopeptides in healthy mice revealed that radioactivity released in the urine is in the form of intact [^{99m}Tc]Demotide or [^{99m}Tc]Demotate 4. This finding is in agreement with data reported for similar radiolabeled somatostatin analogues [16] and previous studies reporting on the high *in vivo* stability of the ^{99m}Tc-chelate [12].

Biodistribution Studies

Data of [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 biodistribution in AR4-2J tumor-bearing mice are summarized in Table 1, as %ID/g at 1 and 4 h pi. Both analogues localized rapidly and efficiently in the physiological somatostatin receptor expressing tissues, like pancreas, adrenals and gastrointestinal tract (stomach and intestines). Although both compounds displayed similar values in most of these tissues ($p > 0.1$), in the pancreas [^{99m}Tc]Demotate 4 showed significantly higher uptake at 1 h ($p < 0.0001$) and 4 h p.i. ($p < 0.01$) as compared to [^{99m}Tc]Demotide (Table 1). The higher pancreatic uptake of [^{99m}Tc]Demotate 4 versus [^{99m}Tc]Demotide may be related to the preferential affinity of [Tyr^3]-octreotate for the sst$_2$ and variations reported in the distribution of somatostatin receptor subtype expression in normal tissues [1,2,5,24]. The significant reduction ($p < 0.01$) in the accumulation of radioactivity in the sst-expressing organs, caused by co-injection of excess (50 μg) parent peptide together with [^{99m}Tc]Demotide or [^{99m}Tc]Demotate 4, suggests a receptor-mediated uptake. Similarly, in the sst$_2$-positive tumor high uptake was attained by both radiopeptides. This uptake was shown to be receptor-specific by the significant reduction in tumor values observed in the blocked animals.

Table 1 Biodistribution results of [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 in AR4-2J tumor-bearing athymic mice

Organs	[^{99m}Tc]Demotide		[^{99m}Tc]Demotate 4	
	1 h pi	4 h pi	1 h pi	4 h pi
Blood	1.13 ± 0.31	0.27 ± 0.05	0.72 ± 0.16	0.24 ± 0.03
Liver	5.88 ± 0.74	4.76 ± 0.70	3.71 ± 0.60	4.72 ± 0.25
Heart	1.08 ± 0.30	0.48 ± 0.10	0.96 ± 0.29	0.39 ± 0.07
Kidneys	19.77 ± 1.64	17.70 ± 4.28	16.57 ± 3.32	10.77 ± 0.69
Stomach	13.92 ± 4.11	17.78 ± 5.47	26.26 ± 1.17	9.88 ± 1.74
Blocked stomach	*3.14 ± 1.59***	*4.16 ± 1.14***	*2.25 ± 1.80****	*0.71 ± 0.11****
Intestine	4.70 ± 1.13	5.50 ± 2.74	6.09 ± 1.52	8.72 ± 0.26
Blocked intestine	*2.36 ± 0.53****	*3.37 ± 1.42*	*2.28 ± 0.42****	*4.10 ± 0.65****
Spleen	5.27 ± 1.79	4.09 ± 2.03	2.34 ± 0.73	1.52 ± 0.36
Muscle	0.27 ± 0.06	0.08 ± 0.03	0.17 ± 0.04	0.06 ± 0.02
Lung	5.13 ± 1.56	3.15 ± 0.93	6.73 ± 2.25	2.04 ± 0.51
Pancreas	7.61 ± 2.29	6.23 ± 2.44	51.01 ± 3.18	14.55 ± 4.79
Blocked pancreas	*1.73 ± 0.58****	*0.32 ± 0.07****	*2.01 ± 1.29****	*0.45 ± 0.07***
Adrenals	18.66 ± 6.69	16.66 ± 2.53	24.69 ± 4.62	14.40 ± 3.28
Blocked adrenals	*5.13 ± 1.91****	*1.86 ± 0.79****	*3.95 ± 1.16****	*1.87 ± 0.68***
AR4-2J tumor	27.87 ± 2.07	23.61 ± 3.99	29.46 ± 2.63	25.43 ± 2.08
Blocked tumor	*2.30 ± 0.67****	*1.13 ± 0.30****	*2.96 ± 0.91****	*1.73 ± 0.08****

Data are expressed as %ID/g tissue and presented as mean ± SD ($n = 4$). Blocking was achieved by co-injection of 50 μg [Tyr^3]-octreotide or [Tyr^3]-octreotate, respectively. Statistical analysis was performed using the Student's *t*-test; a *p*-value of <0.01 (**) was considered very significant, while a *p*-value of <0.001 (***) was considered extremely significant.

The radiotracers were cleared very rapidly from blood, muscle and all nontarget tissues. They were excreted predominantly via the kidneys and the urinary system with the major part of radioactivity found in the urine within 1 hour pi. [^{99m}Tc]Demotate 4 cleared more rapidly from the body and showed lower hepatobiliary excretion in comparison to [^{99m}Tc]Demotide. This can be the effect of the extra carboxylic function at the *C*-terminus of [^{99m}Tc]Demotate 4, which is deprotonated in the biological fluid (pH 7.4), thereby rendering the whole molecule more hydrophilic. Despite the superior pharmacokinetic profile [^{99m}Tc]Demotate 4 versus [^{99m}Tc]Demotide, both radiotracers, containing the *p*-benzylaminodiglycolic acid spacer, showed a less favorable overall profile than the previously reported [^{99m}Tc]Demotate 1, because of an overall higher background activity, especially in the abdomen. In [^{99m}Tc]Demotate 1, the ^{99m}Tc-metal chelate was directly attached to the D-Phe^1 residue of [Tyr^3]-octreotate in an approach that favors body clearance without affecting interaction with the sst_2 *in vitro* or *in vivo* [16].

Imaging

Planar static whole body images obtained at 1 h after injection of [^{99m}Tc]Demotide in AR4-2J tumor-bearing mice are shown in Figure 6. Clear delineation of the sst_2-positive tumor was possible combined with rapid clearance of the tracer by mainly renal excretion. The tumor could not be visualized in the mouse receiving a high dose of [Tyr^3]-octreotide together with [^{99m}Tc]Demotide as a result of *in vivo* receptor blockade. Some liver uptake could be also seen consistent with biodistribution data.

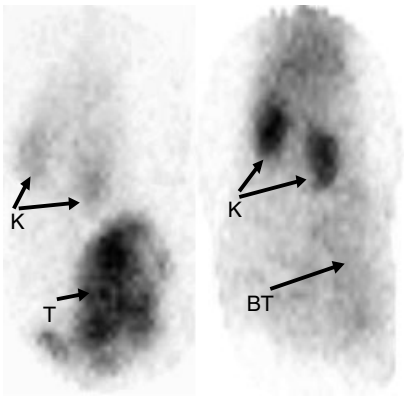

Figure 6 Planar whole body images of AR4-2J tumor-bearing athymic mice 60 min following the injection of 0.2 mCi of [^{99m}Tc]Demotide. Left: control; right: blockade by co-injection of 50 μg of [Tyr^3]-octreotide. Symbols indicate position of (K) kidneys, (T) tumor and (BT) blocked tumor.

CONCLUSIONS

In conclusion, this study has demonstrated that certain modifications in the chemical structure of the [Tyr^3]-octreotide backbone, such as insertion

of an extra negative charge at the *C*-terminus [replacement of Thr(ol)^8 by Thr^8] or introduction of a long spacer between the chelator and the peptide chain can exert significant effects in the imaging characteristics of the respective radiolabeled [Tyr^3]-octreotide/[Tyr^3]-octreotate derivatives. [^{99m}Tc]Demotide and [^{99m}Tc]Demotate 4 have both shown suitable properties for noninvasive *in vivo* targeting of sst$_2$-positive tumors. However, the previously reported and clinically tested [^{99m}Tc]Demotate 1, wherein the monocationic polar ^{99m}Tc-chelate is directly attached to the D-Phe^1 of [Tyr^3]-octreotate, remains the radiotracer of choice for clinical application by virtue of its faster body clearance leading to images of higher contrast.

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