^{99m}Tc Targeting of Sst_2-expressing Tumors by Tetraamine-octreotide: First Results in CA20948 Cells and Rat Models

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Abstract. *Background: Somatostatin subtype 2 receptor (sst_2) – targeted tumor imaging with [^{111}In-DTPA^0]octreotide is an established method for scintigraphic detection of neuroendocrine tumors. [^{99m}Tc]Demotide ([^{99m}Tc-N_4-bzdg^0,Tyr^3]octreotide), presented herein, is a potential sst_2-targeting radiotracer based on ^{99m}Tc. Materials and Methods: Demotide was synthesized in solution and labelled with ^{99m}Tc in alkaline medium. The in vitro properties of Demotide and [^{99m}Tc]Demotide were tested in sst_2-expressing CA20948 cells and rat tissues, while biodistribution was studied in Lewis rats bearing CA20948 tumors. Results: During labelling, [^{99m}Tc]Demotide was obtained in >98% yields and typical 1 mCi/nmol specific activities. Demotide showed in vitro sub-nM affinity for the sst_2. While rapidly internalizing into CA20948 cells, [^{99m}Tc]Demotide localized effectively in CA20948 implants (2.2±1.1%ID/g at 1 h post injection) and in target organs in rats via a sst-mediated process and cleared rapidly via the kidneys. Conclusion: The above favorable characteristics validate [^{99m}Tc]Demotide as a promising ^{99m}Tc-radiotracer for the targeted-imaging of sst_2-positive tumors.*

Several human tumors overexpress receptors for the peptide hormone somatostatin (sst), which comprise five subtypes: sst_{1-5} (1-3). The native hormone, however, is susceptible to rapid enzymatic degradation ($t_{½}$=2-3 min in blood) and is, therefore, unsuitable for *in vivo* applications. For this purpose, long-lived synthetic octapeptide analogs have been developed, such as octreotide, (D)Phe-c(Cys-Phe-(D)Trp-Lys-Thr-Cys)-Thr(ol), octreotate, (D)Phe-c(Cys-Phe-(D)Trp-Lys-Thr-Cys)-Thr and their Tyr^3-derivatives (4, 5).

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Peptide receptor scintigraphy with [^{111}In-DTPA^0]octreotide (OctreoScan®) is widely used nowadays in routine nuclear medicine practice for the visualization and staging of sst-positive neuroendocrine tumors (4). Despite the success of this compound as an imaging agent, a somatostatin analog incorporating ^{99m}Tc would be more desirable. For *in vivo* scintigraphic studies by planar imaging or single photon emission computed tomography (SPECT), ^{99m}Tc is the radionuclide of choice by virtue of its superior nuclear properties, cost-effectiveness and wide availability (6). In this regard, sst-avid peptide analogs radiolabelled with ^{99m}Tc are highly attractive alternatives to OctreoScan® for routine medical use (5, 6). Several attempts for the preparation of ^{99m}Tc-labelled somatostatin analogs over the last decade have met with varying success; labelling *via* HYNIC (hydrazinonicotinamide) or open chain tetraamines remain the two best approaches in terms of tumor-targeting and body clearance (6-12).

In a previous study, Maina *et al.* reported on ^{99m}Tc[N_4-(D)Phe^1]octreotide ([^{99m}Tc]SDZ 220-778), an octreotide analog carrying an open chain tetraamine group at the N-terminal amino acid *via* an isothiocyanate linker (10). This radiopeptide showed very promising results in terms of receptor affinity and tumor localization in a tumor-bearing rat model. Here, we present a novel synthetic somatostatin analog, Demotide ([N_4^0,Tyr^3]octreotide), wherein the 1,4,8,11-tetraazaundecane chelator is covalently attached at the N-terminal of [Tyr^3]octreotide *via* a *p*-benzylamino-diglycolic acid group. Furthermore, Phe^3 has been replaced by Tyr^3 to increase both the hydrophilicity and the internalization capability of the final radiopeptide (5, 8, 11, 13). The effects of these modifications on the biological performance of [^{99m}Tc]Demotide are studied using the same cell and animal models applied for [^{99m}Tc]SDZ 220-778.

Materials and Methods

Synthesis of Demotide. N,N′,N″,N‴-tetra-(*tert*-butoxycarbonyl)-6-{p-[(carboxymethoxy)acetyl]aminobenzyl}-1,4,8,11-tetraazaundecane (16.7 mg, 21.3 μmol) was coupled to the terminal (D)Phe^1 of [Tyr^3,Lys^5(Boc)]octreotide (10 mg, 7.0 μmol) in the presence of O-

(7-azabenzotriazolyl-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (9.0 mg, 23.6 μmol) and N-ethyldiisopropylamine (46.5 μmol, 8.0 μL) in DMF. The raw product was purified over silica gel using $CHCl_3$/MeOH 10/1 as eluent: Rf Boc-Demotide (SiO_2; $CHCl_3$/MeOH 10/1)= 0.4 (tetraamine precursor at 0.0); yield: 10.8 mg (81%); ES-MS calculated for $C_{92}H_{135}N_{15}O_{24}S_2$: 1899.3, found 1899.4 ($M^+$, 34).

For deprotection, the peptide conjugate was treated with trifluoroacetic acid (TFA) in the presence of thioanisol. The product was isolated on a Waters Prep Nova-Pak HP C-18 column applying the following elution system: 0 to 25 min 40 to 80% 0.1% TFA in MeOH and 60 to 20% 0.1% aqueous TFA at a flow rate of 10 mL/min. t_R: 12 min; TLC R_f Demotide [SiO_2, 1-butanol/acetic acid/H_2O/pyridine = 15/3/10/6 (v/v/v/v)] = 0.4; ES-MS calculated for $C_{67}H_{95}N_{15}O_{14}S_2$: 1398.7, found: 1398.8 (M^+, 100); yield = 9.3 mg (67% relative to [Tyr^3, Lys^5(Boc)]octreotide).

^{99m}Tc labelling. Labelling with ^{99m}Tc was based on a previously reported method (11). Briefly, to an Eppendorf vial containing 0.5 M phosphate buffer, pH 11.5 (50 μL), the following solutions were added: 0.1 M sodium citrate (5 μL), pertechnetate generator eluate (410 μL, ~15 mCi), peptide stock solution in 10 mM acetic acid (15 nmol, 15 μL) and a fresh $SnCl_2$ solution in ethanol (20 μL, 40 μg). The labelling mixture was left to react for 30 min at ambient temperature and then adjusted to pH 7.0 by the addition of 1 M HCl (10 mL).

Radiochemical analysis. Labelling yields were monitored by HPLC analysis on the Waters Symmetry Shield RP18 column, applying a linear gradient system at a 1 mL/min flow rate from 0% B to 60% B in 30 min, where solvent A was 0.1% trifluoroacetic acid in water and solvent B was pure acetonitrile. Under these conditions the columns recoveries exceeded 98%, with [^{99m}Tc]citrate and $^{99m}TcO_4^-$ eluting at 1.4-1.8 min and 3.8 min, respectively, followed by [^{99m}Tc]Demotide at 15.3 min. For detection of reduced hydrolyzed technetium ($^{99m}TcO_2$), paper chromatography was conducted, as previously reported (11).

Cell culture. Rat acinar pancreatic tumor CA20948 cells expressing the sst_2 (14) were grown as monolayers in Dulbecco's MEM GLUTAMAX-I (Gibco BRL, Life Technologies, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin, in humidified air containing 5% CO_2 at 37˚C. The cells were passaged once a week applying a trypsin/EDTA (0.05%/0.02% w/v) solution. All supplements were supplied by Biochrom KG Seromed® (Berlin, Germany).

In vitro receptor binding assays

Rat brain cortex membranes: Rat brain cortex membrane homogenates were prepared using a modified published protocol and competition binding studies were performed as detailed previously (11).

CA20948 cells: Competition binding assays were also performed in whole CA20948 cells seeded in 48-well plates (Greiner Labortechnik, Germany) at a density of 1-2 x 10^5 cells per well the day before the assay. On the day of the experiment, the cells were washed twice with 300 μL of ice-cooled binding buffer consisting of 50 mM HEPES, 125 mM NaCl, 7.5 mM KCl, 5.5 mM $MgCl_2$, 1 mM EGTA, 2 mg/L chymostatin, 100 mg/L soybean trypsin inhibitor, 50 mg/L bacitracin, 0.5% (w/v) BSA, pH 7.4. For the assay, 20,000 cpm of [^{125}I-Tyr^3]octreotide (100 μL) (11) was added to each well together with 100 μL of increasing amounts of Demotide and the cells were incubated for 1 h at 37˚C. Incubation was terminated by aspiration of the medium and the wells were rapidly rinsed twice with binding buffer at ambient temperature. The cells were solubilized with 1 N NaOH at 37˚C and cell-associated radioactivity was measured on the gamma counter. Non-specific binding was determined in the presence of 1 μM [Tyr^3]octreotide.

Radioligand internalization. The internalization rate of [^{99m}Tc] Demotide was studied in CA20948 cells, as described earlier (11). In short, cells were seeded in 6-well plates (Greiner Labortechnik) at a density of 8-9x10^5 cells per well, wherein they remained for 48 h. On the day of the experiment, the cells were washed twice with ice-cold internalization medium (Dulbecco's MEM GLUTAMAX-I supplemented by 1% (v/v) FBS) and then incubated with 300,000 cpm ^{99m}Tc-labelled peptide (corresponding to ~200 fmol total peptide) at 37˚C for 5, 15, 30, 60 and 120 min. The percentage of internalized radioligand was determined by washing the cells with acidic buffer (50 mM glycine buffer, pH 2.8, 0.1 M NaCl). Non-specific internalization was determined by parallel incubations in the presence of 1 μM [Tyr^3]octreotide.

Animal biodistribution studies

All animal studies were conducted in compliance with European and national guidelines.

Urine analysis by HPLC: Two male Wistar rats (180±20 g) were injected in the femoral vein with 500μ Ci [^{99m}Tc]Demotide in 200 μL phosphate-buffered saline (PBS). The animals were sacrificed 30 min later by Et_2O inhalation. Urine was immediately collected from their bladder with a syringe and was filtered through a Millex GV filter (0.22 μ). Aliquots thereof were analyzed by paper chromatography and HPLC.

Development of CA20948 tumors in Lewis rats: The CA20948 tumor suspension was diluted in equal amounts of Ham's F-12 K nutrient mixture supplemented by 10% (v/v) FBS and then gently centrifuged at 100-200 rpm. The supernatant was discarded and the washing process was repeated 2-3 times. The final sediment was diluted (1:1) in Ham's F-12 K nutrient mixture (10% (v/v) FBS) and 250 μL of this suspension was inoculated subcutaneously into each flank of 12 female Lewis rats of 8 weeks of age on the day of arrival (Charles River Laboratories, France). Twenty days post inoculation, palpable tumor masses had grown in all animals.

Tissue distribution in CA20948 tumor-bearing rats: Eight CA20948 tumor-bearing rats were injected into their femoral vein with a 100 μL bolus containing 25 μCi of [^{99m}Tc]Demotide (corresponding to *ca.* 10 pmol total peptide) in PBS (pH 7.4) while under a slight ether anesthesia. For *in vivo* receptor blockade, 100 μg [Tyr^3]octreotide were co-injected together with the radioligand. At predetermined time-intervals (1 and 4 h post injection (pi), the animals were euthanized by excising the heart while under ether anaesthesia and biodistribution was conducted as previously described (11). The data were calculated as percent of injected dose per gram of tissue (%ID/g) and the Student's *t*-test was used for statistical analysis with a p value <0.05 considered statistically significant.

Results

^{99m}Tc labelling. Labelling of Demotide with ^{99m}Tc was completed in >98% yields at ambient temperature. [^{99m}Tc]Demotide (Figure 1) formed in high purity, as

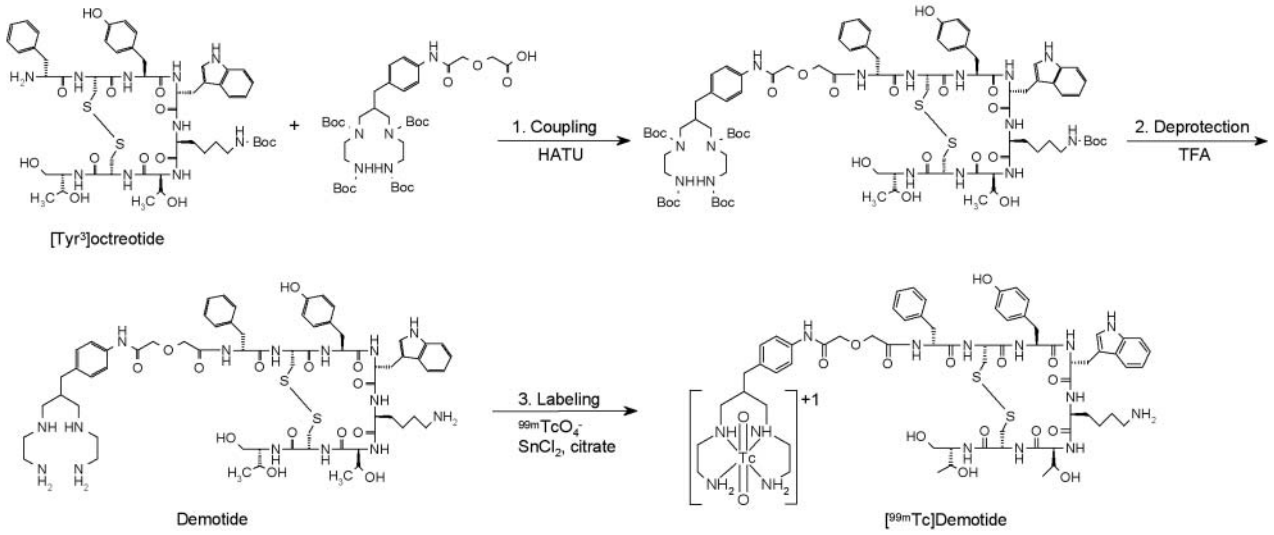

Figure 1. *Two-step synthesis of Demotide in solution and formation of [^{99m}Tc]Demotide after labelling with ^{99m}Tc.*

verified by HPLC and paper chromatography methods. Typical specific activities of 1 Ci/μmol peptide were easily accessible and are well within the range required for receptor-targeting applications employing ^{99m}Tc (11).

In vitro characterization of [^{99m}Tc]Demotide. The IC_{50} values calculated for Demotide from competition binding experiments in rat brain cortex membranes were 0.20±0.02 nM ([Tyr^3]octreotide: 0.46±0.07 nM), whereas the IC_{50} values for Demotide extracted from binding experiments in whole cells were 0.67±0.08 nM ([Tyr^3]octreotide: 0.60±0.09 nM), and demonstrate that introduction of the tetraamine chelator is well tolerated by the sst$_2$.

[^{99m}Tc]Demotide rapidly internalized into CA20948 cells, reaching a 70% plateau within 30-min incubation at 37˚C. Addition of excess [Tyr^3]octreotide (1 μM) to the medium reduced internalization (<10%), suggesting a sst$_2$-mediated process.

Animal studies. Tissue distribution data for [^{99m}Tc]Demotide in Lewis rats bearing sst$_2$-positive CA20948 tumors are given as %ID/g in Table I. A high radioactivity accumulation was evident in all somatostatin receptor-positive organs tested, including the pancreas, adrenals, stomach and intestines. This uptake represented mostly specific interaction with somatostatin receptors since, on co-injection of 100 μg [Tyr^3]octreotide, the uptake in these organs decreased very significantly ($p<0.005$) as compared to control values. Similarly, a high uptake was observed in the CA20948 tumor at 1 h pi. The tumor values did not change significantly between 1 and 4 h pi ($p>0.05$), in accordance with previous findings with [^{99m}Tc]SDZ 220-778 using the same animal model (10). By co-injection of excess of [Tyr^3]octreotide (Table I) uptake in the CA20948 tumor was reduced very significantly ($p<0.005$), suggesting again a receptor-mediated

Table I. *Biodistribution data (%ID/g, mean±SD) of [^{99m}Tc]Demotide in CA20948 tumor-bearing Lewis rats at 1 and 4 h pi (n=4)*[a].

Tissue/Time	1 h	4 h
Blood	0.32±0.03	0.03±0.00
Liver	1.03±0.06	0.67±0.09
Heart	0.25±0.02	0.04±0.00
Kidneys	3.99±0.49	2.55±0.36
Stomach	1.88±0.32	1.59±0.33*
Blocked stomach	nd[b]	0.41±0.06
Intestines	0.74±0.24	1.10±0.24*
Blocked intestines	nd	0.38±0.19
Spleen	0.41±0.06	0.22±0.04
Muscle	0.06±0.01	0.01±0.00
Lungs	0.90±0.10	0.25±0.01
Pancreas	24.01±2.28	13.34±1.20**
Blocked pancreas	nd	2.78±0.46
Adrenals	86.35±1.91	76.64±5.62**
Blocked adrenals	nd	5.56±1.22
Tumor	2.20±1.11	1.40±0.96**
Blocked tumor	nd	0.58±0.18
Tumor/blood	6.90	46.70
Tumor/liver	2.15	2.10
Tumor/kidneys	0.55	0.55
Tumor/muscle	37	140
Tumor/blocked tumor	nd	2.42

[a]Non-specific uptake was determined by co-injection of 100 μg [Tyr^3]octreotide (blocked animals); [b]not done.
*Significant difference between blocked and unblocked animals (Student's *t*-test $p<0.05$); **very significant difference ($p<0.005$).

process. [^{99m}Tc]Demotide was cleared very rapidly from the blood and was excreted from the body of mice predominantly *via* the kidneys into the urine, showing a small percentage of hepatobiliary excretion. Urine collected 30 min after injection of [^{99m}Tc]Demotide in healthy rats was found to contain the major excreted portion. Radiochemical analysis of such urine samples showed that [^{99m}Tc]Demotide was cleared into the urine as intact peptide. This finding is in agreement with previous reports on the *in vivo* stability of both similar [Tyr^3]octreotate derivatives (11) and of the [$^{99m}Tc^{V}(O)_2(N_4)]^+$-chelate (15). Due to the rapid clearance of [^{99m}Tc]Demotide from non-target tissues, high tumor to background ratios were easily achieved (Table I), illustrating its suitability for targeted tumor imaging.

Discussion

An open chain tetraamine attached at the N-terminus of [Tyr^3]octreotide *via* a long benzylaminodiglycolic acid spacer effectively binds ^{99m}Tc in >98% yields affording a highly pure radiopeptide, [^{99m}Tc]Demotide, in ~1 Ci/μmol specific activity. The new compound is an improvement on the previously reported [^{99m}Tc]SDZ 220-778, wherein coupling of the chelator is achieved *via* a thiourea bond. The latter is susceptible to Edman degradation in the acidic medium used during purification and storage of the peptide conjugate. In addition, introduction of the hydrophilic diglycolic acid chain and replacement of Phe^3 by Tyr^3 are expected to favor the *in vivo* profile of the new agent. In vitro Demotide showed sub-nM affinity binding to the sst_2. [^{99m}Tc]Demotide internalized rapidly and specifically in sst_2-expressing cells at 37˚C and displayed high and specific uptake in a sst_2-expressing tumor model in Lewis rats. Its clearance from the body of animals *via* the kidneys into the urine was more rapid than [^{99m}Tc]SDZ 220-778 and led to higher tumor to non-target ratios. These promising qualities are currently being tested in additional experimental models in order to eventually establish the suitability of [^{99m}Tc]Demotide for targeted tumor imaging in man.

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References

1 Hoyer D, Bell GI, Berelowitz M *et al*: Classification and nomenclature of somatostatin receptors. Trends Pharmacol Sci *16*: 86-88, 1995.

2 Reubi JC, Laissue J, Krenning E and Lamberts SW: Somatostatin receptors in human cancer: incidence, characteristics, functional correlates and clinical implications. J Steroid Biochem Mol Biol *43*: 27-35, 1992.

3 Reubi JC, Waser B, Schaer JC and Laissue JA: Somatostatin receptor sst1-sst5 expression in normal and neoplastic human tissues using receptor autoradiography with subtype-selective ligands. Eur J Nucl Med *28*: 836-846, 2001.

4 Bauer W, Briner U, Doepfner W *et al*: SMS 201-995: A potent and selective octapeptide analog of somatostatin with prolonged action. Life Sci *31*: 1133-1140, 1982.

5 Breeman WAP, de Jong M, Kwekkeboom DJ *et al*: Somatostatin receptor-mediated imaging and therapy: basic science, current knowledge, limitations and future perspectives. Eur J Nucl Med *28*: 1421-1429, 2001.

6 Liu S and Edwards D: ^{99m}Tc-labeled small peptides as diagnostic radiopharmaceuticals. Chem Rev *99*: 2235-2268, 1999.

7 Vallabhajosula S, Moyer BR, Lister-James J *et al*: Preclinical evaluation of technetium-99m-labeled somatostatin receptor binding peptides. J Nucl Med *37*: 1016-1022, 1996.

8 Decristoforo C, Melendez-Alafort L, Sosabowski JK and Mather SJ: ^{99m}Tc-HYNIC-[Tyr^3]-octreotide for imaging somatostatin-receptor-positive tumours: preclinical evaluation and comparison with ^{111}In-octreotide. J Nucl Med *41*: 1114-1119, 2000.

9 Decristoforo C, Mather SJ, Cholewinski W *et al*: ^{99m}Tc-EDDA/HYNIC-TOC: a new ^{99m}Tc-labeled radiopharmaceutical for imaging somatostatin receptor-positive tumours: first clinical results and intra-patient comparison with ^{111}In-labeled octreotide derivatives. Eur J Nucl Med *27*: 1318-1325, 2000.

10 Maina T, Stolz B, Albert R *et al*: Synthesis, radiochemical and biological evaluation of ^{99m}Tc[N_4-(D)Phe^1]octreotide, a new octreotide derivative with high affinity for somatostatin receptors. *In*: Technetium and Rhenium in Chemistry and Nuclear Medicine 4 (Nicolinin M, Badoli G, Mazzi U, eds.). Padova, SGEditoriali, pp. 395-400, 1995.

11 Maina T, Nock B, Nikolopoulou A *et al*: [^{99m}Tc]Demotate, a new ^{99m}Tc-based [Tyr^3]octreotate analogue for the detection of somatostatin receptor-positive tumours: synthesis and preclinical results. Eur J Nucl Med *29*: 742-753, 2002.

12 Decristoforo C, Maina T, Nock B *et al*: ^{99m}Tc-Demotate 1: first data in tumour patients – results of a pilot/phase I study. Eur J Nucl Med *30*: 1211-1219, 2003.

13 de Jong M, Breeman WAP, Bakker WH *et al*: Comparison of ^{111}In-labeled somatostatin analogs for tumour scintigraphy and radionuclide therapy. Cancer Res *58*: 437-441, 1998.

14 Bernard BF, Krenning E, Breeman WAP *et al*: Use of the rat pancreatic CA20948 cell line for the comparison of the radiolabelled peptides for receptor-targeted scintigraphy and radionuclide therapy. Nucl Med Commun *21*: 1079-1085, 2000.

15 Bläuenstein P, Pfeiffer G, Schubiger PA *et al*: Chemical and biological properties of a cationic Tc-tetraamine complex. Int J Appl Radiat Isot *36*: 315-317, 1985.

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