Preclinical Evaluation of New and Highly Potent Analogues of Octreotide for Predictive Imaging and Targeted Radiotherapy

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ABSTRACT

Purpose: Molecular imaging and targeted radiotherapy are emerging fields in nuclear oncology. Five human somatostatin receptors (hsstr1-hsstr5) are known to be overexpressed to some degree on various tumors, ssr2 being the most important one. Clinically used somatostatin based radiopeptides target exclusively ssr2. The aim of this study was to develop novel analogues with a broader ssr profile for diagnostic (positron emission tomography and single-photon emission computed tomography) and radiotherapeutic applications.

Experimental Design: The following promising structures emerged from a parallel synthetic approach: [1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)9,1-Na+3,Tyr3]-octreotide (1, DOTA-NOC-ATE) and [DOTA8,BzTh3,Tyr8]-octreotide (2, DOTA-BOCATE). The conjugates were labeled with cold and radioactive 111In. Pharmacologic properties were compared with 111In-DOTA,Tyr3]-octreotide ([111In-DOTA]-TOC).

Results: The receptor affinity profile showed high affinity of both peptides to hsstr2, hsstr3, and hsstr5 and some intermediate affinity to hsstr4, whereas 111In-DOTA]-TOC shows affinity only to ssr2. The internalization is fast in ssr2 expressing AR4-2J and in transfected ssr3 expressing human embryonic kidney 293 cells. Both radiopeptides internalize much more efficiently than 111In-DOTA]-TOC. Animal biodistribution studies showed very high and specific uptake of 111In-1 and 111In-2 in s.c. implanted AR4-2J tumors (Lewis rats) and in somatostatin receptor expressing normal tissues. The uptake was at least 2-fold higher in these tissues and in the tumor compared with 111In-DOTA]-TOC. In addition, the kidney uptake was significantly lower for both radiopeptides.

Conclusions: These data suggest that the novel radiopeptides are superior to 111In/90Y-DOTA]-TOC and show great promise for the clinical application in the imaging of somatostatin receptor–positive tumors and their targeted radiotherapy.

INTRODUCTION

Targeted radiotherapy using different vector molecules like monoclonal antibodies, peptides, and others has made remarkable progress in recent years. The breakthrough has centered around the Food and Drug Administration–approved therapeutic radiolabeled monoclonal antibody, Zevalin, an anti-CD20 antibody labeled with 90Y (1). Nevertheless, peptides have several advantages over the antibodies (faster clearance, rapid tissue penetration, no antigenicity, readily synthesized and GMP produced, etc.) and the most commonly used receptor-targeting agents are a variety of somatostatin analogs. The molecular basis for the use of radiolabeled somatostatin analogues in peptide receptor–mediated radionuclide therapy is provided by the overexpression of the five somatostatin receptors (ssr1-ssr5) on a variety of human tumors, especially neuroendocrine tumors and their metastases (2, 3). It is now more than a decade since the first radiolabeled analogue of somatostatin, 111In-diyethylentriaminepentaacetic acid (DTPA)-octreotide (OctreoScan), was approved for scintigraphy of patients with neuroendocrine tumors (4) and it is still one of the best imaging agents (5). Because a β particle emitter, such as 90Y, in most cases seems more suitable for tumor therapy (peptide receptor–mediated radionuclide therapy) than the Auger electron emitter 111In, derivatives like 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA)-[Tyr3]-octreotide (DOTA-TOC) have been developed, enabling stable labeling with 90Y, 111In, or 177Lu (6, 7). Numerous preclinical and clinical studies with 111In- and 90Y-labeled DOTA-TOC have shown the effective targeting and therapy using these conjugates (7–13). In addition, replacement of the alcohol group at the COOH terminus of the octapeptide by a carboxylic acid group led to increased ssr2 affinity (14, 15), if peptides are labeled with YIII- and CuII-based radiometals and 177Lu-[DOTA8,Tyr3,Thr8]-octreotide ([177Lu-DOTA]-TATE) showed higher tumor uptake than 111In-DTPA]-octreotide in six patients with somatostatin receptor–positive tumors (16). These new radiopeptides show distinctly higher ssr2 affinity compared with OctreoScan and InIII/YIII-DOTA]-TOC. Nevertheless, they bind with high affinity only to ssr2.

Expression of different somatostatin receptor subtypes in human tumors has been extensively investigated using different methods: mRNA detection [Northern blots (17), in situ hybridization (18), RNase protection assays, and reverse transcription-PCR (19)], immunohistochemical studies (20) and receptor autoradiography with subtype-selective ligands (3).
Although each of these methods has its own disadvantage and the results from all these studies cannot always be compared with each other, they all lead to some common important conclusions. That is, there is a considerable heterogeneity in the expression of individual SSTR within and between different tumors, SSTR2 being predominantly expressed in a variety of tumors. However, in a significant number of tumors this was being absent or expressed in lower density. For example, correlating immunohistochemical and mRNA detection data with OctreoScan scintigraphy, Papotti et al. (21) found SSTR2, SSTR3, and SSTR5 in human lung tumors. Growth hormone producing adenomas frequently have SSTR2 and SSTR5; a predominant SSTR3 expression has been reported in inactive pituitary adenomas (3, 22) and in thymomas (23); recently, a high incidence of SSTR1, SSTR2, and SSTR3 has been revealed in human cervical and endometrial cancers (24). The expression of SSTR1, 2, 3 and 5 is frequently found in gastro-entero-pancreatic tumors (25), medullary thyroid cancers (17) and in epithelial ovarian cancers (26). Therefore, it is obvious that new radiolabeled somatostatin analogues with improved binding affinity profiles are needed.

To extend not only the present range of targeted cancers but also to increase the tumor uptake, given the presence of different receptor subtypes on the same tumor cell or in the same tumor entity, we started a program to design and synthesize radiopeptides with affinity to all SSTR. Our strategy was based on two methods: (a) progressive extension of the peptide cycle from octreotide to somatostatin-14 (27) and (b) modification of octreotide. The second approach was realized through parallel solid phase synthesis, mainly by exchanging the amino acids in the positions 3 and 8 of octreotide. We have already reported a first compound resulting from this small library, $^{111}$In/$^{90}$Y-DOTA-NOC, with improved biological properties, currently in clinical trials (28).

In the present study, we investigated the biological activity profile of two new $^{111}$In/$^{90}$Y-labeled DOTA-peptide conjugates, [DOTA$^{9,13}$-Nal$^{3}$,Thr$^{8}$]-octreotide (1, DOTA-NOC-ATE) and [DOTA$^{9,13}$,BzThi$^{3}$,Thr$^{8}$]-octreotide (2, DOTA-BOC-ATE) by means of receptor binding affinity, rate of internalization, cellular retention, and biodistribution in a tumor-bearing rat model. These two compounds are also part of the library acquired through octreotide modification, and their evaluation has been done in comparison with $^{111}$In/$^{90}$Y-DOTA-TOC which in our laboratory is the gold standard of somatostatin receptor imaging and, labeled to $^{90}$Y, of targeted radiotherapy.

**MATERIALS AND METHODS**

All chemicals including 9-fluorenylmethoxycarbonyl-protected amino acids were obtained from commercial sources and used without further purification. Tritylchloride resin was obtained from PepChem (Tübingen, Germany) and $^{111}$InCl$_3$ from Mallinckrodt Medical (Petten, the Netherlands). The prochelator DOTA(tBu)$_3$ was synthesized according to Heppeler et al. (6). The reactive side chains of the amino acids were masked with one of the following groups: Cys, acetamidomethyl; Lys, t-butoxycarbonyl; Thr, t-buty1; Trp, t-butoxycarbonyl. Analytic reverse phase-high performance liquid chromatography (HPLC) was carried out on a Hewlett-Packard 1050 HPLC system equipped with a multiwavelength detector and a flow-through Berthold LB506CI γ-detector. Preparative HPLC was done on a Bischof HPLC system (Metrohm AG, Herisau, Switzerland) with HPLC pumps 2250 and a Lambda 1010 UV detector. CC250/4 Nucleosil 120-3C18 columns from Macherey-Nagel (Düren, Germany) were used for analytic HPLC and a VP250/21 Nucleosil 200-5C15 column for preparative HPLC. The gradient systems consisted of mixtures of acetonitrile and water with 0.1% trifluoroacetic acid. Quantitative γ-counting was done on a COBRA 5003 γ-system well counter from Packard Instrument Co. (Hombrechtikon, Switzerland). Electrospray ionization-mass spectrometry was carried out with a Finnigan SSQ 7000 spectrometer (Bremen, Germany).

**Synthesis.** The peptide-chelator conjugates were synthesized by standard 9-fluorenylmethoxycarbonyl-solid phase synthesis (29) on tritylchloride resin (substitution, 0.8 mmol/g) on a Rink Engineering peptide-synthesizer Switch 24 (RinkCombiChem Technologies, Bubendorf, Switzerland) according to the general procedure described previously (28) affording compounds 1 and 2 (Fig. 1), which could be labeled with natural or radioactive indium or yttrium. The compounds were characterized by electrospray ionization-mass spectrometry and reverse phase-HPLC.

![Fig. 1 Structural formulæ of DOTA-[1-Nal$^{3}$, Thr$^{8}$]-octreotide (DOTA-NOC-ATE, 1) and DOTA-[BzThi$^{3}$, Thr$^{8}$]-octreotide (DOTA-BOC-ATE, 2).](image-url)
Formation of Metal Complexes. The DOTA-somatostatin analogues were complexed with InCl₃ (anhydrous) and Y(NO₃)₃·5H₂O as described by Wild et al. (28). The radiopeptides were also synthesized according to Wild et al. (28) and obtained in >99% radiochemical purity at specific activities of >37 GBq/μmol peptide. For internalization experiments, the DOTA peptides were labeled to a specific activity of about 37 GBq/μmol peptide and then excess InCl₃ was added to afford structurally characterized homogenous ligands.

Determination of Somatostatin Receptor Affinity Profiles. CHO-K1 and CCL39 cells stably expressing human sstr1-5 were grown as described previously (14). All culture reagents were supplied by Life Technologies (Grand Island, NY). Cell membrane pellets were prepared and receptor autoradiography was done on pellet sections (mounted on microscope slides), as described in detail previously (14). For each of the tested compounds, complete displacement experiments were done with the universal somatostatin radioligand [¹²⁵I]-[D-Leu⁸,D-Trp⁷,Tyr³]-somatostatin-28 using increasing concentrations of the MetalloDOTA-peptide ranging from 0.1 to 1,000 nmol/L. Somatostatin-28 was run in parallel as control using the same increasing concentrations. IC₅₀ values were calculated after quantification of the data using a computer-assisted image processing system. Tissue standards (autoradiographic [¹²⁵I] microscales, Amersham, Buckinghamshire, United Kingdom) containing known amounts of isotopes, cross-calibrated to tissue-equivalent ligand concentrations, were used for quantification (14). The concentrations of the peptide solutions were measured by UV spectroscopy (ε(NO₃-ATE, 280 nm = 9,855 and εBOC-ATE, 280 nm = 7,570).

Cell Culture, Radioligand Internalization, and Cellular Retention Studies. The AR4-2J cell line was maintained by serial passage in monolayers in DMEM, supplemented with 10% fetal bovine serum, amino acids, vitamins, and penicillin-streptomycin, in a humidified 5% CO₂ atmosphere at 37°C. Human embryonic kidney 293 cells stably expressing rat sstr3 receptors were a gift from Dr. S. Schulz (Magdeburg, Germany; ref. 30) and were grown in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin, and G418 (500 μg/mL) in a humidified 5% CO₂ atmosphere at 37°C. Cell numbers were counted under the microscope with a “Neubauer’s counting chamber”. For all cell experiments, the cells were seeded at a density of 0.8 to 1.1 million cells per well in 6-well plates and incubated over night with internalization buffer to obtain a good cell adherence. The loss of cells during the internalization experiments was <10%. When different radiolabeled peptides were compared in cell experiments, the same cell suspension was used. Furthermore, the internalization rate was linearly corrected to 1 million cells per well in all AR4-2J cell experiments. Medium was removed from the 6-well plates and cells were washed once with 2 mL of internalization buffer [DMEM, 1% fetal bovine serum, amino acids, and vitamins (pH 7.4)]. Furthermore, 1.5 mL internalization buffer were added to each well and incubated at 37°C for about 1 hour. Thereafter ~500,000 cpm or 0.02 MBq per well [¹¹¹In] labeled peptides (2.5 pmol per well) to a final concentration of 1.67 nmol/L were added to the medium and the cells were incubated at 37°C for the indicated time periods in triplicates. To determine nonspecific membrane binding and internalization, cells were incubated with radioligand in the presence of 1 μmol/L [¹¹¹In]-I. Cellular uptake was stopped by removing medium from the cells and by washing twice with 1 mL of ice-cold PBS. Acid wash for 10 minutes with a glycine-buffer (pH 2.8) on ice was also done twice. This procedure was done to distinguish between membrane-bound (acid releasable) and internalized (acid resistant) radioligand. Finally, the cells were treated with 1 N NaOH. The culture medium, the receptor bound and the internalized fraction were measured radiometrically in a γ-counter (Packard, Cobra II). Internalization into AR4-2J cells was also studied using three different concentrations of [¹¹¹In]-1 and [¹¹¹In]-2 (6.67 nmol/L or 10 pmol per well, 1.67 nmol/L or 2.5 pmol per well, and 0.167 nmol/L or 0.25 pmol per well).

For cellular retention studies AR4-2J cells (1 million) were incubated with 2.5 pmol per well (1.67 nmol/L) [¹¹¹In/InIII]-labeled DOTA-NOC-ATE, DOTA-BOC-ATE, or DOTA-TOC for 120 and 240 minutes, respectively, then the medium was removed and the wells were washed twice with 1 mL ice-cold PBS. In each experiment, an acid wash for 10 minutes on ice with a glycine-buffer of pH 2.8 was done to remove the receptor-bound ligand. Cells were then incubated again at 37°C with fresh internalization buffer [DMEM containing 1% fetal bovine serum (pH 7.4)]. After different time points, the external medium was removed for quantification of radioactivity in a γ-counter and replaced with fresh 37°C medium. The cells were solubilized in 1 N NaOH, removed and the internalized radioactivity was quantified in a γ-counter. The recycled fraction was expressed as percentage of the total internalized amount per 1 million cells and the integrity of the externalized peptides was determined using HPLC after removing the solvent by a centrifugal evaporator.

Biodistribution and Imaging Studies in Rats. Animals were kept, treated, and cared for in compliance with the guidelines of the Swiss regulations (approval 789). Five-week-old male Lewis rats were implanted s.c. with 10 to 12 millions AR4-2J cells freshly suspended in sterile PBS. Fourteen days after inoculation, the rats showed solid palpable tumor masses (tumor weight, 0.4-0.7 g) and were used for the experiments. Rats were injected under ether anesthesia with 2 to 3 MBq of 0.34 nmol (0.5 μg total peptide mass) [¹¹¹In]-1, [¹¹¹In]-2 and [¹¹¹In]-DOTA-TOC, respectively, in 0.05 mL NaCl solution 0.9% into the femoral vein. At 4, 24, and 48 hours after injection rats were sacrificed under ether anesthesia. Organs and blood were collected and the radioactivity in these samples was determined using a γ-counter.

To determine the nonspecific uptake of the radiopeptides, rats were injected with 25 μg [¹¹¹In]-2 in 0.05 mL NaCl solution 0.9% as a coinjection with the radioligand. To study a potential sstr2, sstr3, and sstr5 related uptake of [¹¹¹In]-I in the somatostatin receptor–positive tissues, blocking studies were designed with different somatostatin analogs: DTPA-TATE [sstr2-selective ligand, IC₅₀(sstr2) = 3.9 ± 1 nmol/L], [¹¹¹In] DTPA-TATE [sstr2-selective ligand, IC₅₀(sstr2) = 1.3 ± 0.2 nmol/L], and [¹¹¹In]-2 (sstr3, and sstr5 affinity). In a first series, 25 μg of these peptides were coinfected with 2 to 3 MBq...
[111In]-1 (0.34 nmol in 0.05 mL NaCl solution 0.9%) into the femoral vein of AR4-2J tumor-bearing male Lewis rats. In a second series, increased amounts of DTPA-TATE (50 μg) and 25 μg of [111In-DTPA-TATE] were coinjected. Rats were sacrificed at 4 hours and the organs of interest collected and counted for radioactivity. The radioactivity uptake in the tumor and normal tissues of interest was expressed as a percentage of the injected radioactivity dose per gram tissue (% ID/g).

Two rats were used for imaging studies. One rat was injected with 3 MBq of 0.34 nmol [111In]-1 and the other one was coinjected with the same amount and type of radioligand and 25 μg [InIII]-2 into the femoral vein. Four hours after injection, rats were anaesthetised and images were acquired in the prone position using a γ-camera equipped with a medium energy parallel hole collimator (Basicam, Siemens, Erlangen, Germany).

**Statistical Methods.** To compare differences between the radiopeptides the Student’s t test was used.

**RESULTS**

**Synthesis and Labeling.** The DOTA-coupled octapeptides 1 and 2 (Fig. 1) were obtained by solid phase synthesis on a tritylchloride resin. The overall yield of the DOTA peptides was about 30%. Uncomplexed and metal-complexed DOTA peptides were characterized by analytic HPLC and electrospray ionization-mass spectrometry. Table 1 lists the calculated and measured molecular weights and also the HPLC retention time of the conjugates and their labeled versions.

**Receptor Binding and Affinity Profiles.** Table 2 shows the IC50 values of the radiopeptides studied in this work as their InIII complexed versions in comparison with [InIII-DOTA]-TOC for the five somatostatin receptor subtypes. Additionally, the binding profile of the compounds used for the blocking studies in rats are also listed along with the unmodified octapeptides in Table 2. The values were obtained by performing complete displacement experiments with the universal somatostatin radio-ligand [125I][Leu8, D-Trp22, Tyr25]-somatostatin-28 on membranes from cells expressing the receptor subtypes and were compared with somatostatin-28. All compounds bind specifically to sstr2, with IC50 values ranging from 0.8 to 4.6 nmol/L. High binding affinities for sstr3 were found for [InIII]-2 (IC50 = 5.5 ± 0.8 nmol/L) and [InIII]-1 (IC50 = 13 ± 4.0 nmol/L), whereas [InIII-DOTA]-TOC showed very low sstr3 affinity (IC50 = 120 ± 26 nmol/L). [InIII]-1 and [InIII]-2 displayed moderate affinity for sstr4 (IC50 = 160 ± 3.8 and 135 ± 32 nmol/L, respectively).

**Table 1.** Analytic data of compounds 1, 2, [InIII]-1, and [InIII]-2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Calculated MW</th>
<th>Measured MW</th>
<th>RP-HPLC* retention time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOTA-NOC-ATE, 1</td>
<td>1,469.68</td>
<td>1,508.2 (M + K+)</td>
<td>22.73</td>
</tr>
<tr>
<td>DOTA-BOC-ATE, 2</td>
<td>1,475.71</td>
<td>1,515.4 (M + K+)</td>
<td>22.45</td>
</tr>
<tr>
<td>[InIII]-DOTA-NOC-</td>
<td>1,580.6</td>
<td>1,582.5 (M + H+)</td>
<td>23.15</td>
</tr>
<tr>
<td>[InIII]-DOTA-BOC-</td>
<td>1,587.7</td>
<td>1,589.2 (M + H+)</td>
<td>22.9</td>
</tr>
<tr>
<td>[InIII]-DOTA-TOC</td>
<td>1,533.64</td>
<td>1,534.8 (M + H+)</td>
<td>18.23</td>
</tr>
</tbody>
</table>

*Elution system: flow, 0.75 mL/min; solvent A, 0.1% trifluoroacetic acid in H2O; solvent B, acetonitrile; linear gradient: 0 to 30 minutes, 90% A to 40% A.

Specific sstr5 affinity was found for [InIII]-1 and [InIII]-2 (IC50 = 4.3 ± 0.5 and 3.9 ± 0.2 nmol/L, respectively).

**In vitro Internalization Studies in AR4-2J and rsstr3-HEK Human Embryonic Kidney Cells.** Figure 2A shows the results of the specific internalization of [111In]-1 and [111In]-2 in comparison with [111In-DOTA]-TOC into AR4-2J rat pancreatic tumor cells. The highest rate of internalization corresponds to [111In]-1 with 25.1 ± 1.3% specific cellular uptake at 4 hours, followed by [111In]-2 with 17.8 ± 0.8% and [111In-DOTA]-TOC with 12.5 ± 0.7%. The percentage of internalized peptide at 30 minutes as a function of concentration showed a linear dependence (data not shown). Figure 2B shows the internalization of [111In]-1 and [111In]-2 in sstr3 transfected human embryonic kidney cell lines compared with [111In-DOTA]-TOC. During 4 hours, [111In]-2 internalized 20.1% of the added radiopeptide and [111In]-1 13.5%, whereas [111In-DOTA]-TOC showed <0.8% specific internalization.
Comparison of cellular retention over time between $[^{111}\text{In}]$- and $[^{111}\text{In-DOTA}]$-TOC in AR4-2J cells at 37°C, after 2 hours of internalization (A) and 4 hours of internalization (B). % Radioactivity retained in the cell from the total internalized conjugate.

**Cellular Retention.** Cellular retention of $[^{111}\text{In}]$-[SS-28], $[^{111}\text{In-DOTA}]$-[NOC-ATE], $[^{111}\text{In-DOTA}]$-[BOC-ATE], and $[^{111}\text{In-DOTA}]-$TOC was analyzed and compared in AR4-2J cells. In these experiments, the radiopeptides were allowed to internalize for 120 and 240 minutes, respectively; cells were then washed twice with PBS before removing the receptor-bound ligand with glycine buffer (pH 2.8). Warm medium (37°C) was then added and removed after 15, 30, 60, 120, and 240 minutes and measured for radioactivity. Figure 3 illustrates the cellular radioactivity retention of these three compounds in AR4-2J cells over time after two different internalization times. There is no significant difference between the cellular retention after 2 and 4 hours of internalization for the studied conjugates, respectively, except for $[^{111}\text{In}]$-SS which maintained after 4 hours 30 ± 4.0% ($P < 0.05$) of the 2 hours of internalized fraction and 41.6 ± 6.0% of the 4 hours of internalization fraction, respectively. As shown in Fig. 3, after 4 hours, the percentage of cellular retention for all three conjugates reaches a plateau. HPLC study of the externalized $[^{111}\text{In}]$-SS gave no indication of metabolites (data not shown).

**Biodistribution and Imaging Studies in Rats. Pharmacokinetics.** The 4, 24, and 48 hours uptake values of $[^{111}\text{In}]$-2 and $[^{111}\text{In-DOTA}]-$TOC gave no indication of metabolites (data not shown).

**Comparison.** Figure 4 shows a comparison of the biodistribution properties between $[^{111}\text{In}]$-1, $[^{111}\text{In}]$-2 and $[^{111}\text{In-DOTA}]-$TOC in AR4-2J cells at 37°C, after 2 hours of internalization (A) and 4 hours of internalization (B). % Radioactivity retained in the cell from the total internalized conjugate.

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**Table 2** Affinity profiles (IC$_{50}$) of a series of somatostatin analogues for human somatostatin receptors sstr1 to sstr5

<table>
<thead>
<tr>
<th>Compound</th>
<th>hsstr1</th>
<th>hsstr2</th>
<th>hsstr3</th>
<th>hsstr4</th>
<th>hsstr5</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS-28</td>
<td>3.6 ± 0.7</td>
<td>2.3 ± 0.6</td>
<td>3.3 ± 0.5</td>
<td>2.7 ± 0.5</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>$[^{111}\text{In-DOTA}]$-NOC-ATE</td>
<td>&gt;10,000</td>
<td>3 ± 0.35</td>
<td>13 ± 4</td>
<td>160 ± 3.8</td>
<td>43.5 ± 0.5</td>
</tr>
<tr>
<td>$[^{111}\text{In-DOTA}]$-BOC-ATE</td>
<td>&gt;1,000</td>
<td>1.4 ± 0.37</td>
<td>5.5 ± 0.8</td>
<td>135 ± 32</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>NOC-ATE</td>
<td>&gt;1,000</td>
<td>3.6 ± 1.6</td>
<td>302 ± 137</td>
<td>260 ± 95</td>
<td>16.7 ± 9.9</td>
</tr>
<tr>
<td>BOC-ATE</td>
<td>&gt;1,000</td>
<td>3.8 ± 0.4</td>
<td>33 ± 5.5</td>
<td>80 ± 20</td>
<td>3.6 ± 1.5</td>
</tr>
<tr>
<td>Octreotide*</td>
<td>&gt;10,000</td>
<td>2.0 ± 0.7</td>
<td>187 ± 55</td>
<td>&gt;10,000</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>$[^{111}\text{In-DOTA}]$-TOC*</td>
<td>&gt;10,000</td>
<td>4.6 ± 0.2</td>
<td>120 ± 26</td>
<td>230 ± 82</td>
<td>130 ± 17</td>
</tr>
<tr>
<td>DTPA-TATE*</td>
<td>&gt;10,000</td>
<td>3.9 ± 1</td>
<td>&gt;10,000</td>
<td>&gt;1,000</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>$[^{111}\text{In-DTPA}]$-TATE*</td>
<td>&gt;10,000</td>
<td>1.3 ± 0.2</td>
<td>&gt;10,000</td>
<td>433 ± 16</td>
<td>&lt;1,000</td>
</tr>
</tbody>
</table>

**NOTE.** IC$_{50}$ values are in nmol/L (mean ± SE) and are the mean of at least three experiments. Somatostatin-28 is used as reference.

*Data from Reubi et al. (14).
**Selective Blocking.** To estimate the uptake in sstr-positive organs which may be due to receptor subtype expression other than sstr2, in vivo blocking studies were done in AR4-2J tumor-bearing rats using different blocking agents like [DTPA-Tyr<sup>11</sup>]-octreotide (DTPA-TATE) and [In<sup>111</sup>]DTPA-[TATE], two sstr2-specific ligands and [In<sup>111</sup>]-NOC-ATE, [In<sup>111</sup>]-BOC-ATE, and [In<sup>111</sup>]-TOC (see Table 2 for IC<sub>50</sub> values). Two series of experiments were done, using different amounts of these blocking compounds (Table 4A and B). As already mentioned in Table 3, 25 µg [In<sup>111</sup>]-2 per rat are enough for 95% blocking of the uptake in tumor and sstr-positive organs (<i>P</i> < 0.001). Table 4A displays the selective blocking of tumor, adrenals, pancreas, and stomach when using only 25 µg DTPA-TATE. As the AR4-2J rat pancreatic tumor expresses only sstr2, the blocking with DTPA-TATE should be as effective as that with [In<sup>111</sup>]-2. In our assay, however, only 70% of the tumor uptake were blocked by the sstr2-selective ligand. In the second selective blocking experiment (Table 4B), increased amounts of DTPA-TATE (50 µg per rat) were employed as well as the sstr2-selective ligand [In<sup>111</sup>]-DTPA-[TATE] (25 µg per rat) with its improved affinity to this receptor (see Table 2). The two sstr2 ligands were found to be equispotent in decreasing the tumor uptake (85%) but slightly different in their effect on the pancreas and adrenal uptake. The kidney uptake is not influenced by any of these added ligands.

**Rat Images.** Figure 5 shows the γ-scintigraphy of two Lewis rats s.c. bearing AR4-2J tumors on the thorax 4 hours after injection of [In<sup>114</sup>]-1 with (Fig. 5A) and without (Fig. 5B) coinjection of excess of [In<sup>111</sup>]-2. Coinjection led to a visible increase in tumor uptake.
DISCUSSION

This study describes the design, synthesis, and preclinical evaluation of two new somatostatin-based DOTA-coupled peptides for the labeling with a variety of hard acid radiometals like $^{111}$In, $^{67}$Ga, $^{90}$Y, and the lanthanides.

The pharmacologic profiles as well as the biological properties of the $^{111}$In-labeled peptides are compared with $^{[111}In, 90Y$-DOTA-Tyr$^3$-octreotide (6, 9). The two peptide structures emerged from a parallel synthesis approach with modifications at position 3 of the octapeptide octreotide and the COOH-terminal replacement of Thr(ol) for Thr (14, 15).

The rationale to develop these new peptides came from the desire to develop radiopharmaceuticals for a broader spectrum of targeted tumors and a potentially higher uptake in tumors expressing different receptor subtypes concomitantly.

A first radiopharmaceutical emerging from this library of compounds was $^{[111}In, 90Y$-DOTA-[1-Nal$^3$]-octreotide which showed improved affinities towards sstr2, sstr3, and sstr5 compared with $^{[111}In$-DOTA-[Tyr$^3$]-octreotide (28). The new compounds have several advantages over existing radiopeptides.

First, the affinity for sstr2 is as high as the best radiopeptides studied thus far (14). In addition, high affinity to sstr3 and sstr5 was found as well and some emerging sstr4 affinity, thus representing the broadest sstr profile of any somatostatin-based radioligands whereas maintaining a very high sstr2 affinity. The structural features determining the broader affinity profile are not fully understood yet. We observed earlier that the modification of Phe$^2$ by Tyr$^3$ in DOTA-coupled octa- and decapeptides led to a 3-fold improved sstr2 affinity but the sstr3 affinity dropped by a factor of $>10$ (14, 28). The higher lipophilicity of benzoxybenzyl-Ala (BzThi) and 1-Nal probably improves sstr3 and sstr5 affinity. The sstr2 affinity increase is most likely a combination of the increased lipophilicity and the internalization of the Thr(ol) versus Thr replacement and the increased lipophilicity at aa$^3$ position.

A comparison of the affinity profiles of the metallo-chelated peptides with the unmodified peptides BOC-ATE and NOC-ATE showed that BOC-ATE could represent an improved alternative for octreotide (Sandostatin) in the treatment of acromegaly and/or the carcinoid syndrome as it has a higher potency on the receptor subtypes 2-5. Also, the coupling of a DOTA-based metal complex to the N$^1$-terminus may improve the affinity to some receptor subtypes. $^{[InIII}$-DOTA-NOC-ATE and $^{[In}$-DOTA-BOC-ATE are at least equipotent to the nonchelated peptide on sstr2 but distinctly more potent on sstr3. On sstr5 $^{[InII}$-DOTA-BOC-ATE is equipotent to BOC-ATE but $^{[InII}$-DOTA-NOC-ATE is more potent than NOC-ATE.

Somatostatin receptors belong to the family of G-protein coupled receptors (31). There are several important consequences of the coupling of agonists to this type of receptors like desensitization, Ca$^{2+}$ mobilization, cyclic AMP production, and internalization. The latter is of special relevance to the targeting aspects of using G-protein coupled receptor targeting radioligands as it allows long retention times on the tumor which is of special importance in therapeutic applications but may also contribute to the diagnostic sensitivity due to an increased tumor-to-background ratio with time. Both radiopeptides internalize distinctly faster than $^{[InII}$-DOTA-TOC in AR4-2J tumor cells. The eflux rate of both radiopeptides from AR4-2J cells is similar, the total amount of cell released radiopharmaceutical is lower if more time is allowed for internalization, indicating that pathways inside the cell slow down the eflux of radiopeptides (32). $^{[111}$In]-1 and $^{[111}$In]-2 internalize very efficiently into sstr3 cells. $^{[111}$In]-2 being clearly superior to $^{[111}$In]-1 which may be explained with the higher affinity of $^{[111}$In]-2 on sstr3 (see Table 2). $^{[111}$In-DOTA]-TOC having a

**Table 4** Radioactivity uptake in AR4-2J tumor-bearing rats, 4 hours after coinjection of $^{[111}$In-DOTA]-NOC-ATE and different blocking agents ($n = 4$ rats per blocking experiment)

<table>
<thead>
<tr>
<th>Blocking compound</th>
<th>Amount injected per rat (µg)</th>
<th>Radioactivity uptake in organs and tumor (%ID/g tissue ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AR4-2J tumor</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>4.01 ± 0.49</td>
</tr>
<tr>
<td>[InIII-DOTA]-BOC-ATE</td>
<td>25</td>
<td>0.30 ± 0.03*</td>
</tr>
<tr>
<td>(A) DTPA-TATE</td>
<td>25</td>
<td>1.26 ± 0.16†</td>
</tr>
<tr>
<td>(B) DTPA-TATE</td>
<td>50</td>
<td>0.6 ± 0.08*</td>
</tr>
<tr>
<td>[InIII-DTPA]-TATE</td>
<td>25</td>
<td>0.56 ± 0.07*</td>
</tr>
</tbody>
</table>

*P < 0.001.

†0.05 > P > 0.001 compared with nonblocked data series.

---

Fig. 4 Biodistribution comparison between $^{[111}$In-DOTA]-NOC-ATE (■), $^{[111}$In-DOTA]-BOC-ATE (▲) and $^{[111}$In-DOTA]-TOC ( ■) in AR4-2J tumor-bearing rats at 4 hours after injection.
low affinity to sstr3 shows negligible internalization. These
data clearly show that $[^{111}\text{In}]$-1 and $[^{111}\text{In}]$-2 have a much
higher potential to target tumors with sstr3 expression, either
alone or concomitantly with other subtypes. The high sstr5
affinity indicates that this also holds for sstr5 expressing
tumors.

The efflux rate of the new radiopeptides is faster than the
one of $[^{111}\text{In-DOTA}]$-TOC, an effect that we cannot explain at
the moment. As the externalized radiopeptides are intact, a faster
metabolic degradation cannot be the justification for this.
Nevertheless, the amount retained in the cell remains higher
for $[^{111}\text{In}]$-1 and $[^{111}\text{In}]$-2 than for $[^{111}\text{In-DOTA}]$-TOC, due to
the higher internalization rate of the first two. In internalization,
we found a close approximation to a steady state after 4 hours of
internalization of both new radiopeptides and also the efflux
kinetics showed a distinct leveling off after 4 hours. We have
explained similar results recently assuming rapid recycling of the
radiopeptides to the extracellular medium and reactivation of the
receptors by the intact externalized peptides followed by
reendocytosis (28). This explanation is also in accordance with
the work of Koenig et al. (33).

The in vivo pharmacokinetics in the AR4-2J rat model of
$[^{111}\text{In}]$-1 and $[^{111}\text{In}]$-2 shows a rapid and specific targeting of
the somatostatin receptor 2 expressing tumor and somatostatin
receptor–positive tissue like the pancreas (Fig. 5B for $[^{111}\text{In}]$-1).

The specificity of this uptake is shown by the blocking
experiment which shows lack of uptake in the tumor and the
pancreas, even if only 25 µg $[^{111}\text{In}]$-2 are coinjected with 0.5 µg
$[^{111}\text{In}]$-1 (Fig. 5A).

A quantitative analysis of the biodistribution at 4, 24, and
48 hours showed a high uptake in the tumor which was similar
for $[^{111}\text{In}]$-1 and $[^{111}\text{In}]$-2 but 2-fold higher than for $[^{111}\text{In-
DOTA}]$-TOC at 4 hours. The uptake in other somatotropin
release-inhibiting factor receptor–positive tissues like the
pancreas, adrenals, stomach, and bowel was also very high,
specific, and receptor mediated as shown by the blocking
experiment. Already a coinjection of 25 µg $[^{113}\text{In}]$-2 blocks the
tumor uptake by >90%, the adrenals by 97%, the pancreas by
95%, the stomach by 97%, and the bowel by 88%, illustrating
the potential advantage of the broader affinity profile of this
ligand. The almost 7- and 4-fold higher uptake in the adrenals
and 5- and 4-fold higher uptake in the pancreas may be
explained by the expression of sstr3 and sstr5 in these organs
(34). The more efficient blocking capability of $[^{113}\text{In}]$-2 compared
with the sstr2-selective ligands DTPA-octreotate and $[^{113}\text{In-
DTPA}]$-octreotate also indicates that this radioligand is superior
to other somatostatin-based radiopeptides. The residence time
of $[^{111}\text{In}]$-1 and $[^{111}\text{In}]$-2 in the tumor is somewhat shorter
than that of other somatostatin-based radiopeptides and
parallels the efflux rate from AR4-2J cells compared with

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Fig. 5  Four hours post injection scintigraphy
of two Lewis rats bearing AR4-2J s.c. tumors:
rat with a coinjection of $[^{111}\text{In-DOTA}]$-NOC-
ATE and excess $[^{113}\text{In-DOTA}]$-BOC-ATE (A)
and rat injected only with $[^{111}\text{In-DOTA}]$-NOC-
ATE (B).
radiopeptides like \([114Cu-TETA-Tyr^3, Thr^8]\)-octreotide (15).

In addition, \([111In]\)-radioligand for SOM230, \([111In]\)-evaluation in phase 1 clinical trials (36). Because there is no advanced is SOM230, a cyclohexapeptide that has high binding octreotide or lanreotide are currently being developed. Most therapeutic potential and a more universal binding profile than makes these two radiopeptides very promising candidates for somatostatin receptor targeted radiotherapy.

New cold somatostatin-based peptides with superior therapeutic potential and a more universal binding profile than octreotide or lanreotide are currently being developed. Most advanced is SOM230, a cyclohexapeptide that has high binding affinity to sstr1, sstr2, sstr3, sstr5 and is currently under evaluation in phase 1 clinical trials (36). Because there is no radioligand for SOM230, \([111In]-1\) and \([111In]-2\) may be candidates to identify patients adequate for SOM230 treatment. In addition, \([111In]-1\) and \([111In]-2\) may be much better alternatives to predict the usefulness of cold octreotide (Sandostatin) or lanreotide (Somatuline) therapy than OctreoScan, which has a much less adequate binding profile for this purpose.

**CONCLUSION**

We have designed and characterized chemically and pharmacologically two new DOTA-based peptides for diagnostic and therapeutic applications, \([111In-DOTA-Na^3, Thr^3]\)-octreotide and \([111In-DOTA-BzTh^3, Thr^3]\)-octreotide. The peptides were compared with our clinical gold standard \([111In, 90Y-DOTA-Tyr^3]\)-octreotide. They show superior pharmacologic properties when compared with the latter. Both peptides are currently prepared for clinical studies.

The combined preclinical data indicate that \([111In, 90Y-DOTA]-NOC-ATE and \([111In, 90Y-DOTA]-BOC-ATE\) are very promising new somatostatin-based radioligands for the diagnosis and targeted radiotherapy of a broader range of tumors expressing somatostatin receptors. They represent the first somatostatin-based radiopeptides, which show high affinity to sstr2, sstr3, and sstr5 and intermediate affinity to sstr4. For the first time, efficient internalization into a sstr3 expressing cell line using radiometallabeled somatostatin analogues was shown.

We also propose \([111In-DOTA]-NOC-ATE\) and \([111In-DOTA]-BOC-ATE\) as imaging agents to predict the successful use of cold octreotide or lanreotide therapy, as OctreoScan has very low affinity to sstr3 and sstr5.

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