New Pansomatostatin Ligands and Their Chelated Versions: Affinity Profile, Agonist Activity, Internalization, and Tumor Targeting

Mihaela Ginj,1 Hanwen Zhang,1 Klaus-Peter Eisenwiener,1 Damian Wild,2 Stefan Schulz,4 Hans Rink,5 Renzo Cescato,3 Jean Claude Reubi,3 and Helmut R. Maecke1

Abstract

Purpose: Somatostatin receptor (sst) targeting is an established method to image and treat sst-positive tumors. Particularly, neuroendocrine tumors express the receptor subtype 2 in high density, but sst1, sst3, sst4, and sst5 are also expressed to some extent in different human tumors. Currently used targeting peptides mainly have sst2 affinity. We aimed at developing (radio)peptides that bind with high affinity to all receptor subtypes.

Experimental Design: Carbocyclic octapeptides were coupled with macrocyclic chelators for radiometal labeling. Affinity, internalization, and agonist potencies were determined on sst1- to sst5-expressing cell lines. Biodistribution was determined on nude mice bearing HEK-sst2 or AR4-2J and HEK-sst3 tumors.

Results: High affinity to all receptor subtypes was found. YIII-KE88 showed agonistic properties at all five sst receptor subtypes as it inhibits forskolin-stimulated cyclic AMP production. Surprisingly, very low or even absent sst2 receptor internalization was found compared with currently clinically established octapeptides, whereas the sst3 internalization was very efficient. Biodistribution studies of [111In]KE88 and [67Ga]KE88/[68Ga]KE88 reflected the in vitro data. In nude mice with s.c. implanted sst2 (HEK-sst2, AR4-2J)-expressing and sst3 (HEK-sst3)-expressing tumors, high and persistent uptake was found in sst3-expressing tumors, whereas the uptake in the sst2-expressing tumors was lower and showed fast washout. The kidney uptake was high but blockable by coinjection of lysine.

Conclusion: This peptide family shows pansomatostatin potency. As radiopeptides, they are the first to show a full pansomatostatin profile. Despite some drawback, they should be useful for imaging sst2-expressing tumors with short-lived radiometals, such as 68Ga, at early time points and for sst3-expressing tumors at later time points with longer-lived radiometals, such as 64Cu or 86Y.

Somatostatin [somatotropin release-inhibiting factor (SRIF)] is a tetradecapeptide with potent inhibitory actions on several tissues, such as the pituitary, the endocrine pancreas, and the gastrointestinal tract. SRIF also acts as neurotransmitter and neuromodulator (1, 2). The inhibition of the many secretory processes of hormones, such as growth factors, insulin, and glucagon, is an important property of SRIF and is mediated by at least five SRIF receptor subtypes (sst1–sst5), which belong to the G protein–coupled receptor family. Because of the short plasma half-life of <3 min, SRIF-14 (and SRIF-28) does not have therapeutic potential in the treatment of conditions related to pathologic secretory processes. Therefore, metabolically stabilized somatostatin-based peptides were developed (3–7). Two of them, octreotide (Sandostatin) and lanreotide (Somatuline), are being used in the clinic. They display high affinity only to sst2 and moderate affinity to sst3 and sst5. It is therefore conceivable to search for somatostatin analogues with a higher affinity binding profile to all five receptor subtypes (pansomatostatin character) with the aim to improve the established therapeutic potential or discover new indications. Recently, several such molecules have been discovered and developed, such as the cyclo-hexapeptide SOM 230 (8), which shows high affinity to sst1, sst2, sst3, and sst5 and has potent, long-lasting inhibitory effects on growth hormone and insulin-like growth factor-I release. As part of a small structure-activity relationship (SAR) study, we have synthesized a cyclooctapeptide with pansomatostatin characteristics (KE108; Table 1), which shows equivalent (sst1) or even higher affinity to sst2, sst3, sst4, and
Table 1. "Binding affinity" of different pansomatostatin ligands to all sst subtypes compared with SRIF-28 after normalization (mean ± SE; number of experiments in brackets)

<table>
<thead>
<tr>
<th>Code</th>
<th>Chemical structure</th>
<th>sst1</th>
<th>sst2</th>
<th>sst3</th>
<th>sst4</th>
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<tbody>
<tr>
<td>SRIF-28</td>
<td>Somatostatin-28</td>
<td>2.7 ± 0.3 (12)</td>
<td>2.3 ± 0.2 (12)</td>
<td>3.4 ± 0.3 (12)</td>
<td>2.7 ± 0.3 (12)</td>
<td>2.5 ± 0.3 (12)</td>
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<tr>
<td>KE108</td>
<td>Y-cyclo(dDab-RFFdWKTF)*</td>
<td>2.6 ± 0.4 (7)</td>
<td>0.9 ± 0.1 (7)</td>
<td>1.5 ± 0.2 (7)</td>
<td>1.6 ± 0.1 (7)</td>
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Relative values with SRIF-28 normalized to 1 as reference

<table>
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<tr>
<th>Code</th>
<th>Chemical structure</th>
<th>sst1</th>
<th>sst2</th>
<th>sst3</th>
<th>sst4</th>
<th>sst5</th>
</tr>
</thead>
<tbody>
<tr>
<td>KE108</td>
<td>Y-cyclo(dDab-RFFdWKTF)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>HR2215</td>
<td>cyclo(GABA-NFFdWKTF)</td>
<td>27 ± 3.0 (3)</td>
<td>4.1 ± 2 (4)</td>
<td>0.7 ± 0.1 (4)</td>
<td>2.3 ± 0.3 (4)</td>
<td>1.4 ± 0.3 (4)</td>
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<tr>
<td>HR2252</td>
<td>cyclo(GABA-RFFdWKTF)</td>
<td>3.2 ± 0.3 (4)</td>
<td>1.1 ± 0.1 (4)</td>
<td>0.7 ± 0.2 (4)</td>
<td>1.5 ± 0.2 (4)</td>
<td>0.5 ± 0.1 (4)</td>
</tr>
<tr>
<td>KE109</td>
<td>cyclo(GABA-RFFdWKTF)</td>
<td>7.7 ± 0.6 (2)</td>
<td>1.6 ± 0.1 (2)</td>
<td>0.6 ± 0.1 (2)</td>
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<tr>
<td>KE121</td>
<td>cyclo(dDab-RFFdWKTF)</td>
<td>1.6 ± 0.7 (4)</td>
<td>0.5 ± 0.2 (3)</td>
<td>0.3 ± 0.1 (3)</td>
<td>0.4 ± 0.2 (3)</td>
<td>0.2 ± 0.1 (3)</td>
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<tr>
<td>KE106B</td>
<td>cyclo(dDab-RFFdWKTF)</td>
<td>20 ± 2.9 (3)</td>
<td>2.0 ± 0.6 (3)</td>
<td>0.6 ± 0.1 (3)</td>
<td>0.5 ± 0.2 (3)</td>
<td>0.5 ± 0.1 (3)</td>
</tr>
<tr>
<td>KE88/Y</td>
<td>YIII-DOTA-cyclo(dDab-RFFdWKTF)</td>
<td>2.0 ± 0.8 (6)</td>
<td>4.3 ± 0.8 (6)</td>
<td>0.7 ± 0.2 (6)</td>
<td>0.5 ± 0.2 (6)</td>
<td>0.7 ± 0.2 (6)</td>
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<tr>
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<td>3.5 ± 1.6 (3)</td>
<td>1.8 ± 0.6 (3)</td>
<td>0.8 ± 0.3 (3)</td>
<td>1.8 ± 0.5 (3)</td>
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<tr>
<td>KE87/Y</td>
<td>YIII-DOTA-GA-cyclo(dDab-RFFdWKTF)</td>
<td>6.7 ± 2.1 (3)</td>
<td>2.7 ± 0.4 (3)</td>
<td>0.6 ± 0.1 (3)</td>
<td>1.6 ± 0.6 (3)</td>
<td>1.3 ± 0.4 (3)</td>
</tr>
<tr>
<td>KE131/Y</td>
<td>YIII-DOTA-cyclo(dDab-KFdWKTF)</td>
<td>3.9 ± 1.2 (3)</td>
<td>7.3 ± 1.3 (3)</td>
<td>0.9 ± 0.1 (3)</td>
<td>0.6 ± 0.1 (3)</td>
<td>1.1 ± 0.3 (3)</td>
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<tr>
<td>KE124/Y</td>
<td>YIII-DOTA-Ala-cyclo(dDab-RFFdWKTF)</td>
<td>6.7 ± 3.2 (3)</td>
<td>2.0 ± 1.0 (3)</td>
<td>0.6 ± 0.1 (3)</td>
<td>0.9 ± 0.2 (3)</td>
<td>0.5 ± 0.1 (3)</td>
</tr>
<tr>
<td>KE105</td>
<td>cyclo(dDab-RFFdWlamTFF)</td>
<td>12 ± 4.2 (3)</td>
<td>390 ± 106 (3)</td>
<td>80 ± 13 (3)</td>
<td>&gt;1000 (3)</td>
<td>111 ± 34 (3)</td>
</tr>
</tbody>
</table>

Note: The absolute values are given for SRIF-28 and KE108 in nmol/L. YIII stands for yttrium in the oxidation state 3+, to distinguish it from Y.

Abbreviations: Iamp, 4-((N-isopropyl)-aminoethylphenylalanine; dDab, D-diaminobutyric acid; GABA, γ-aminobutyric acid.

*Single-letter amino acid code.

Materials and Methods

Reagents. $^{67}$GaCl$_3$ and $^{111}$InCl$_3$ were purchased from Mallinkrodt. The prochelator DOTA(Bu)$_3$ was synthesized according to Heppeler et al. (14) and DOTAGA(Bu)$_4$ [1-(carboxy-3-carboxybutoxymethyl)-4,7,10-tetraazacyclododecane] was synthesized according to Eisenwiener et al. (15).

Chemical syntheses. Two groups of peptides were synthesized: (a) cyclic octapeptides incorporating an aminobutyric acid bridging unit and (b) octapeptides incorporating diaminobutyric acid (Dab) whose α-amino group allows the coupling of a chelator for radiometal labeling.

Synthesis of the pansomatostatin derivatives. The solid-phase peptide synthesis was carried out on a semiautomatic peptide synthesizer using the fluorenlymethoxybenzyl strategy (16). Details on the synthesis will be published elsewhere.

The synthesis of chelated versions of pansomatostatin ligands with our lead KE88 as an example is depicted in Scheme 1.

Formation of metal complexes and radiolabeling. The DOTA-SRIF analogues were synthesized by exchange with Y(NO$_3$)$_3$·5 H$_2$O or with Ga(NO$_3$)$_3$·9 H$_2$O, as described by Ginj et al. (16). The radiolabeling of these peptide conjugates was done according to Wild et al. (17) and the radipeptides were obtained in >97% radiochemical purity at specific activities of >37 GBq/μmol peptide.

Binding affinity determination to hss1-hss5. The hss1-hss5 binding affinity of the various compounds was measured as described previously using in vitro receptor autoradiography with 20-μm-thick sections from membrane pellets of the respective transfected cells (18).

Adenylyl cyclase activity. The effect of KE88/Y on forskolin-stimulated CAMP formation was done on hss1-hss5–transfected cells, as described previously (9, 19, 20).

In vitro receptor internalization studies. Immunoaffinity fluorescence microscopy–based internalization assay for sst2 and sst3 was done as previously described by Cescano et al. (21) using HEK-293 cells stably expressing the human sst2 and sst3. Cells were treated with KE88, YIII-DOTA-TOC, or YIII-DOTA-NOC (17) at a concentration of 10 nmol/L to 10 μmol/L for 30 min at 37°C in growth medium and then rinsed twice with 100 mmol/L phosphate buffer containing 0.15 mol/L sucrose (PS). After the cells were fixed and made permeable for 7 min with cold.
methanol (−20°C), they were rinsed twice with PBS, and nonspecific binding sites were blocked by incubating the cells in PBS containing 0.1% bovine serum albumin for 60 min at room temperature. The cells were subsequently incubated for 60 min at room temperature either with the sst2A-specific antibody (R2-88) or with the sst3-specific primary antibody (5, 50 pmol [111In]KE88 diluted 1:1,000 in PBS). The cells were washed three times with PBS containing 0.1% bovine serum albumin and then incubated for 60 min at room temperature in the dark with the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H+L; Molecular Probes, Inc.; Invitrogen) diluted 1:600 in PBS. Subsequently, the cells were washed thrice for 5 min each with PBS containing 0.1% bovine serum albumin, embedded with 1:1 PBS/glycerol, and covered with a glass coverslip. The cells were imaged using a Leica DM RB immunofluorescence microscope and an Olympus DP10 camera.

**Radioligand internalization.** The AR4-2J cell line and the HEK-293 cells stably expressing sst2, sst3, or sst5 receptors were maintained by serial passage in monolayers in DMEM supplemented with 10% fetal bovine serum, l-glutamine, penicillin-streptomycin, and 500 μg/mL G418 (for HEK cells) or amino acids and vitamins (for AR4-2J cells) in a humidified 5% CO2 atmosphere at 37°C (22). For all cell experiments, cells were seeded at a density of 0.9 to 1.1 million per well in six-well plates and incubated overnight in growth medium to obtain good cell adherence. The loss of cells during the internalization experiments was <10%.

Furthermore, the internalization rate was linearly corrected to 1 million cells per well in all cell experiments. The procedure for internalization was done according to Ginz et al. (16). To determine nonspecific membrane binding and internalization, cells were incubated with radioligand in the presence of 1 μM unlabeled ligand.

**Biodistribution and imaging studies in tumor-bearing nude mice.** Animals were kept, treated, and cared for in compliance with the guidelines of the Swiss regulations (approval 789). Five-week-old athymic female Swiss nude mice were implanted s.c. with 10 to 12 million AR4-2J or HEK-sst2 cells on one flank and HEK-sst3 cells on the other, freshly suspended in sterile PBS. Fourteen days after inoculation, the mice showed solid palpable tumor masses (tumor weight, 70-150 mg) and were used for biodistribution studies and imaging. Three to six mice were injected under ether anesthesia with 0.15 to 0.2 MBq of 50 pmol [111In]DTPA-TATE or 50 pmol [67Ga]DTPA-TATE or 50 pmol DOTA-TATE in 0.05 mL NaCl solution (0.9%, 0.1% bovine serum albumin) as a coinjection with the radioligand. The radioactivity uptake in the tumor and normal tissues of interest was expressed as percentage of the injected radioactivity per gram tissue (% IA/g).

To study the effect of lysine on the kidney uptake of [111In]KE88, four mice were coinjected with a 0.1 mL solution containing 10 mg lysine in PBS with the radioligand; they were sacrificed at 1 h after injection. Two mice were used for imaging studies. They were injected with 0.5 MBq of 50 pmol [111In]KE88. Fifteen min, 30 min, 1 h, 2 h, and 4 h after injection, the mice were anesthetized and images were acquired in the prone position using a γ-camera equipped with a medium energy parallel hole collimator (Bacsimac, Siemens).

**Statistical methods.** To compare differences between the radioligands, the Student’s t test was used. P < 0.05 was considered to be significant.

### Results

**Syntheses and radiolabeling.** The synthetic steps toward the new octapeptides and the structure of our lead KE88 are depicted in Scheme 1. The compounds were characterized by electrospray ionization-mass spectrometry and analytic high-performance liquid chromatography. The yields of the chelated peptides were ~40%. Complexation yields with the metal salts Y(NO3)3·5 H2O and Ga(NO3)3·9 H2O were >95%. Labeling yields using [111In]Cl3 and [67Ga]Cl3, respectively, were ≥97% at specific activities of 37 GBq/μmol peptide.

**sst affinity profiles.** Table 1 shows the normalized “IC50” values of the peptides studied in this work for the five sst subtypes with SRIF-28 as the control peptide set at 1. The table also contains KE105, a pansomatostatin peptide, which was published recently (9).

All peptides, except KE105, showed a broad SRIF receptor affinity profile with pansomatostatin character. The lead HR2215 exhibited 27 times lower affinity to sst1, 4.1 times lower affinity to sst2, and 2.3 times lower affinity to sst4 than SRIF-28. On sst3 and sst5, the two peptides were almost equipotent. The replacement of Asn in position 5 (numbering as for SRIF-14) by the positively charged Arg (HR2252) resulted in an improvement of affinity on all receptor subtypes, whereas Lys in position 5 instead of Arg resulted in a distinct decrease of sst2 affinity (data not shown). Phe7 replaced by Tyr (KE109 versus HR2252) led to a reduction of sst1 affinity by a factor of 2.4 and of sst2 affinity by ~50%, whereas this modification did not change the sst3 and sst5 affinity but improved the sst4 affinity 4-fold.

Introducing D-Dab (KE121) as a cyclizing unit instead of γ-aminobutyric acid improved the overall potency, giving values that were close to the sst1 affinity of SRIF-28 and showing a strikingly improved affinity to all other sst subtypes. Replacement of Phe7 in this compound for Tyr7 (KE106B versus KE121) resulted in a loss of affinity of all receptor subtypes except sst4. KE105 was studied for its potential sst1 selectivity. Indeed, the peptide showed good sst1 affinity and very low affinity to the other receptors. The affinities dropped by a factor of >10 on DOTA conjugation (data not shown).

We therefore selected KE121 as new lead for modification and chelator coupling. The DOTA coupling and YIII complexation has a minor influence on the sst1 affinity, but the sst2 affinity dropped 8-fold and sst3 and sst5 affinities dropped 2- and 3-fold, respectively. No change was observed on the sst4 affinity.

When replacing YIII with GaIII (KE88/Ga versus KE88/Y), some loss was observed on sst1 affinity but a 2.5-fold improvement on sst2, and no change on sst3 and sst5 but a 2.5-fold decrease on sst4 was measured.

Replacing Arg5 for Lys5 (KE131/Y versus KE88/Y) gave lower affinity values on sst1 and sst2 and no or little change on sst3, sst4, and sst5. A negative charge on the NH2 terminus due to the metal complex (KE87/Y versus KE88/Y, DOTAGA has four carboxylic acid groups) led to an affinity decrease except on sst2 and sst3.

β-Ala acts as a spacer like the propionic acid arm of DOTAGA but maintains the neutrality of the NH2-terminal metal complex (KE124/Y versus KE88/Y). This modification resulted in a 3-fold loss on sst1, an improvement on sst2, and little change on sst3, sst4, and sst5.

**Agonistic properties.** To test its agonistic properties, KE88/Y was studied for its effect on forskolin-stimulated cAMP accumulation in expressing cells using SRIF-28 as agonist control. Table 2 shows the similar EC50 values of KE88/Y in comparison with SRIF-28 for sst1, sst2, and sst5 and a very similar potency for sst3 and sst4.
**Internalization studies.** Figure 1A and B illustrates the peptide-induced receptor internalization, analyzed by immunofluorescence microscopy. The clinically studied $^{90}$YIII-DOTA-TOC was used as a control along with KE88. YIII-DOTA-TOC (IC$_{50}$ = 11.2 nmol/L) has good affinity to sst2 (18) and triggers sst2 internalization at 10 nmol/L, whereas KE88 induces sst2 internalization only at a concentration $>1,000$ nmol/L despite a 2.5-fold higher binding affinity. YIII-DOTA-NOC has high affinity to sst2, sst3, and sst5 and was used as control peptide for sst3 (17). It triggers sst3 receptor internalization at $\sim$10 nmol/L, at the same concentration as KE88 (Fig. 1B) despite a 15-fold lower binding affinity.

Radioligand internalization was studied at 0.167 nmol/L concentration in AR4-2J, HEK-sst2, HEK-sst3, and HEK-sst5 cell lines with $[^{111}$In$]$KE88, $[^{67}$Ga$]$KE88, $[^{111}$In$]$KE87, and $[^{111}$In$]$KE131. Table 3 shows the 4-h internalization values of the radiopeptides in AR4-2J, HEK-sst2, HEK-sst3, and HEK-sst5 cells. The uptake in the sst2-expressing cell lines is negligible under concentration conditions applied in molecular imaging in vivo. We did not study the binding on sst2-expressing cell lines as IC$_{50}$ values were determined on membranes, but as a “byproduct” of the internalization studies, we found practically 100% of the radioligand in the bound fraction. Conversely, a high internalization rate into HEK-sst3 is evident. $[^{67}$Ga$]$KE88 internalizes more efficiently than $[^{111}$In$]$KE88 ($P < 0.05$ at 2 and 4 h); there is no difference between $[^{111}$In$]$KE88 and $[^{111}$In$]$KE87, whereas $[^{111}$In$]$KE131 shows a somewhat lower rate of internalization. No internalization was observed into HEK-sst5.

**Biodistribution studies.** Table 4 summarizes the pharmacokinetics of $[^{111}$In$]$KE88 and $[^{67}$Ga$]$KE88 in a mouse xenograft tumor model carrying s.c. tumors in each flank, one exclusively expressing sst2 (HEK-sst2 or AR4-2J) and the other expressing sst3 (HEK-sst3). The most important finding of the biodistribution of $[^{111}$In$]$KE88 and $[^{67}$Ga$]$KE88 is the marked difference in the uptake kinetics of the two tumors. The HEK-sst3 tumor shows a high uptake of $[^{111}$In$]$KE88 peaking between 1 and 4 h after injection, the highest value amounting to $\sim$23% IA/g tumor at 1 h, whereas in HEK-sst2 the uptake was highest at 15 min (18.5 $\pm$ 0.65% IA/g); the washout from the HEK-sst2 tumor is fast, and at 24 h, only 1.14 $\pm$ 0.05% IA/g is left. Similarly, the uptake in the AR4-2J tumor is 8.5 $\pm$ 2.5% IA/g at 15 min followed by a fast washout amounting to only 1.2 $\pm$ 0.7% IA/g at 4 h after injection. In HEK-sst3, the uptake is long lasting being 23.2 $\pm$ 4.2% IA/g at 4 h and 14.9 $\pm$ 2.5% IA/g tumor at 24 h. The tumor uptake is specific and receptor mediated as

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**Table 2.** Effects of SRIF-28 and KE88/Y on forskolin-stimulated cAMP accumulation in CCL39 cells expressing sst1 to sst5

<table>
<thead>
<tr>
<th>Compound</th>
<th>cAMP</th>
<th>EC$_{50}$ (nmol/L)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>sst1</td>
<td>sst2</td>
</tr>
<tr>
<td>SRIF-28</td>
<td>3.49 $\pm$ 1.08 (4)</td>
<td>6.65 $\pm$ 2.85 (2)</td>
</tr>
<tr>
<td>KE88/Y</td>
<td>7.35 $\pm$ 4.52 (3)</td>
<td>9.73 $\pm$ 4.83 (3)</td>
</tr>
</tbody>
</table>
was shown by coinjection of 50 μg KE88 and 50 μg In\(^{111}\)-DTPA-TATE (sst2-selective ligand) or by coinjection of DOTA-TATE. The sst3 tumor uptake was reduced at 1 h after injection by 84% and the sst2 tumor uptake by 89.5%. \[^{67}\text{Ga}\]KE88 was also specifically taken up by the sst3 tumor to a significantly higher percentage at 4 h (30.1 ± 4.8% IA/g versus 23.2 ± 4.2% IA/g for \[^{111}\text{In}\]KE88; \(P < 0.05\)) but a somewhat faster washout with 15.3 ± 0.8% IA/g remaining at 24 h. In addition to the tumor, the pituitary shows specific uptake with a blocking efficiency of 60% at 1 h. The highest uptake of \[^{111}\text{In}\]KE88 was found in the kidney leading to low tumor to kidney ratios at all time points. For \[^{67}\text{Ga}\]KE88, this ratio is higher by a factor of 3 due to the higher tumor uptake of \[^{67}\text{Ga}\]KE88 but also to the significantly lower kidney uptake. The kidney uptake of \[^{111}\text{In}\]KE88 was efficiently lowered by >60% on coinjection of 10 mg lysine PBS solution. None of the other organs was influenced significantly by the lysine injection. The blood clearance of both radiopeptides was very fast with only 0.1% IA/g remaining in blood at 4 h. This results in excellent tumor (sst3 mainly) to blood ratios already at very early time points, such as 16.9 at 30 min, 32.7 at 1 h, 232 at 4 h, and 745 at 24 h for \[^{111}\text{In}\]KE88. Even for the HEK-sst2–expressing tumor, the tumor to blood ratio was ~20 at 1 h, 37 at 4 h, and 57 at 24 h. A similar high ratio was found for \[^{67}\text{Ga}\]KE88.

Interestingly, the washout from all normal organs except from the kidneys is very fast as well. These pharmacokinetic data are reflected in the planar images obtained with a \(\gamma\)-camera (Fig. 2).

**Discussion**

In the past, radiopeptides have been established as an important class of targeting vectors for the imaging and targeted...
radionuclide therapy of human tumors. There was consensus that agonists need to be selected as radioligands because they usually internalize into tumor cells. This represents an important mechanism for the active transport and accumulation of radiolabeled peptide analogues into the tumor cell. This is of importance for imaging and localization but more so for therapeutic application when a long residence time is mandatory for the success of this therapy modality.

The aim of this study was (a) to develop new pasmoceptors tatin ligands, (b) to understand some SARs, and (c) possibly to select a pharmacologically active peptide as a substitute for octreotide or lanreotide in the treatment of hypersecretory tumors. Of particular importance was the selection of the most promising peptides for radiometal labeling after peptide modification with a chelator.

In this study, according to the best of our knowledge, the first radiopeptides having a pansomatostatin binding profile are described. In addition, we show for the first time that SRIF receptor radiometalloligands can have high binding affinities and similar effects on receptor-mediated signaling but different effects on the triggering of receptor internalization.

Several important findings are emerging from these studies. (a) The new family of (metal) cyclooctapeptides shows high

**Table 3.** Internalization of $^{67}$Ga- or $^{111}$In-labeled peptides into AR4-2J, HEK-sst2, HEK-sst3, and HEK-sst5 cells after 4 h of incubation at 37°C

<table>
<thead>
<tr>
<th>Radiopeptide</th>
<th>% internalized into AR4-2J</th>
<th>% internalized into HEK-sst2</th>
<th>% internalized into HEK-sst3</th>
<th>% internalized into HEK-sst5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^{111} \text{In}] \text{KE88}$</td>
<td>&lt;0.4</td>
<td>&lt;0.5</td>
<td>32.2 ± 2.6</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>$[^{67} \text{Ga}] \text{KE88}$</td>
<td>—</td>
<td>&lt;0.5</td>
<td>40.6 ± 1.3</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>$[^{111} \text{In}] \text{KE87}$</td>
<td>&lt;0.4</td>
<td>2.3 ± 0.2</td>
<td>31.7 ± 2.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>$[^{111} \text{In}] \text{KE131}$</td>
<td>—</td>
<td>0.4 ± 0.1</td>
<td>23.3 ± 3.0</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

**Table 4.** Biodistribution in HEK-sst3 and AR4-2J tumor-bearing nude mice at 15 min, 30 min, 1 h, 4 h, and 24 h after injection of $[^{111} \text{In}] \text{KE88}$ and at 4 and 24 h after injection of $[^{67} \text{Ga}] \text{KE88}$

<table>
<thead>
<tr>
<th>Organ</th>
<th>$[^{111} \text{In}] \text{KE88}$</th>
<th>$[^{67} \text{Ga}] \text{KE88}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Blood</td>
<td>5.2 ± 0.7</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>Stomach</td>
<td>3.8 ± 0.7</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Kidney</td>
<td>70.9 ± 15.9</td>
<td>55.0 ± 2.0</td>
</tr>
<tr>
<td>Bowel</td>
<td>3.2 ± 0.5</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.8 ± 0.2</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.1 ± 0.2</td>
<td>0.8 ± 0.07</td>
</tr>
<tr>
<td>Liver</td>
<td>4.6 ± 0.9</td>
<td>2.4 ± 0.17</td>
</tr>
<tr>
<td>AR4-2J</td>
<td>8.5 ± 2.5</td>
<td>2.0 ± 0.03</td>
</tr>
<tr>
<td>HEK-sst2</td>
<td>18.5 ± 0.65</td>
<td>—</td>
</tr>
<tr>
<td>HEK-sst3</td>
<td>15.2 ± 1.6</td>
<td>22.0 ± 4.15</td>
</tr>
<tr>
<td>Lung</td>
<td>6.7 ± 1.2</td>
<td>2.9 ± 1.4</td>
</tr>
<tr>
<td>Heart</td>
<td>6.5 ± 4.5</td>
<td>0.6 ± 0.08</td>
</tr>
<tr>
<td>Adrenal</td>
<td>3.1 ± 0.4</td>
<td>1.3 ± 0.17</td>
</tr>
<tr>
<td>Bone</td>
<td>2.0 ± 0.4</td>
<td>0.7 ± 0.09</td>
</tr>
<tr>
<td>Pituitary</td>
<td>11.0 ± 2.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

**HEK-sst3 tumor to tissue ratios**

| Tumor to blood   | 2.92 | 16.92 | 32.7 | 4.75 | 232 | 745 | 301 | 765 |
| Tumor to kidney  | 0.21 | 0.40 | 0.36 | 0.10 | 0.40 | 0.44 | 1.20 | 1.25 |

**AR4-2J tumor to tissue ratios**

| Tumor to blood   | 1.63 | 1.54 | 2.15 | 1.75 | 12 | 25 | 8 | 25 |
| Tumor to kidney  | 0.12 | 0.04 | 0.02 | 0.04 | 0.02 | 0.01 | 0.03 | 0.04 |

**HEK-sst2 tumor to tissue ratios**

| Tumor to blood   | 3.56 | 19.4 | 37 | 57 |
| Tumor to kidney  | 0.26 | 0.21 | 0.06 | 0.03 |

**NOTE:** The results are expressed as % IA/g (mean ± SE; n ≥ 3).

*Blocked with 50 µg KE88 + 50 µg In$^{111}$-DTPA-TATE.

*Blocked with 50 µg DOTA-TATE.
potency, comparable with the endogenous SRIF-28, with regard to the binding affinity to sst1 to sst5. The peptides also seem to be full agonists on sst1 to sst5. (b) \[^{111}\text{In}]KE88 (and KE108) shows high potency with regard to internalization into sst3-expressing cells at concentrations relevant to \textit{in vivo} tumor targeting. The internalization is receptor mediated. (c) Conversely, \[^{111}\text{In}]KE88 and KE108 (21) show inefficient or no internalization into sst2 and sst5. They trigger receptor internalization at \sim 100\text{-fold} higher concentration than do currently established second-generation chelated and iodinated cyclohexaspeptides (16, 23, 24). None of the (radio)peptides triggers sst5 internalization, a property that was also found with other potent agonists, whereas the endogenous ligands SRIF-14 and SRIF-28 induced sst5 receptor internalization (21). (d) The inefficient sst2 internalization of these radiopeptides is contrary to what we (16, 17, 25) and others have found (26–30) using octreotide-derived radiopeptides promoting receptor and radioligand internalization at \textit{physiologic} receptor-subsaturating concentrations. It therefore seems that some SRIF analogues may be able to dissociate between their sst2 signaling properties and their internalizing ability. (e) As a consequence of the internalization properties, biodistribution and imaging with \[^{111}\text{In}]KE88, using a double tumor model, show the importance of efficient agonist internalization as an active uptake mechanism for prolonged tumor uptake.

With regard to the pansomatostatin ligand development, we encountered the fortunate situation that our lead peptide HR2215 (13) already had moderate to high affinity to all SRIF receptor subtypes. HR2215 was developed more than 20 years ago as a potential drug for the treatment of diabetes type 2. The replacement of Asn$^5$ in HR2215 (SRIF-14 numbering) by Arg (HR2252) resulted in a distinct improvement on all receptor subtypes. We conclude that the positive charge on aa$^5$ is important and responsible for the increased affinities. When replacing Arg$^5$ for Lys$^5$, there is a significant loss in sst2 and sst5 affinity but little difference in affinity to sst1, sst3, and sst4, again underlining the importance of a positive charge in this position. The substitution of Phe$^7$ by Tyr$^7$ (KE109) resulted in a drop of a factor of 7.7 in sst1 affinity (versus SRIF-28) but less than a factor of 2 in sst2 affinity, whereas the affinity for sst3, sst4, and sst5 is equal or even better for KE109. This substitution will allow iodination within the structure of the octapeptide and increases the hydrophilicity of the peptide.

The internalization data correspond to the \textit{in vivo} pharcokinetik data in the animal models. Whereas \[^{111}\text{In}]KE88 has a high and persistent uptake in the sst3 tumors, the uptake in the sst2 tumors was lower and a fast washout was observed, resulting in a very low tumor targeting as early as 4 h after injection. These biodistribution data consequently translate into the imaging results where a distinct and long-lasting localization of the sst3 tumor can be seen but the sst2 tumors can only be faintly visualized up to 60 min. Remarkably, at 4 h, only the sst3 tumor and the kidneys are clearly visible, a consequence of the fast washout of the tracer from all other organs.

The higher uptake of \[^{67}\text{Ga}]KE88 in the sst3 tumor at 4 h is most likely due to the faster rate of internalization of \[^{67}\text{Ga}]KE88 as measured \textit{in vitro}, a feature we have also found on sst2-expressing cells using DOTA-octreotide–based radiopeptides (31).

The kidney uptake of \[^{111}\text{In}]KE88 is very high at all time points, probably due to the 2-fold positive charge of the peptide. This hypothesis is corroborated by the finding that the uptake can be quite efficiently blocked by coinjection of lysine, a basic amino acid, which reduces kidney uptake by >60%. Also an interesting finding is the good blocking of the kidney by excess of cold peptide, possibly again a consequence of the 2-fold positive peptide charge as there is no indication of sst expression in the mouse kidneys. These nonoptimized kidney blocking effects indicate that more effective kidney blocking is feasible in case of potential human applications.

The kidney uptake of \[^{67}\text{Ga}]KE88 at 4 and 24 h is strikingly lower than the one of \[^{111}\text{In}]KE88, a phenomenon that we have also seen in a variety of cyclic octapeptides coupled with DOTA (14, 31). We have explained the improved kidney uptake of radiogallium-labeled somatostatin-based octapeptides with the different coordination chemistry of the monoamide-conjugated Ga$^{\text{III}}$-DOTA complex. The hexacoordination of Ga$^{\text{III}}$ affords a free carboxymethyl arm, which may alter the kidney handling of the peptide (14).
The results show that it is worthwhile to search for new radiolabeled pasmatoctatin ligands for the targeting of tumors. Obviously, for this family of peptides, there is dissociation between strong binding, induction of signaling, and the ability to induce radiolang receptor endocytosis on sst2.

We do not know at this time how to explain the findings that the same high affinity radioligand internalizes into one sst subtype very efficiently but not into the other, at least not under conditions relevant to nuclear oncology applications. It is not clear which steric and/or electronic features are responsible for these somewhat surprising pharmacologic properties and the differences between the molecules studied in this work and the octreotide-based radiopeptides studied by us and others. One structural difference between the two different groups, the natural peptides SRIF-14 and SRIF-28, the octapeptides currently used in the clinic, and these peptides is the mode of cyclization. The new peptides are carbocyclic and not cyclized via the Cys-Cys disulfide bridge. Future studies may show if this structural feature and concomitant decrease in conformational flexibility is responsible for these pharmacologic properties.

**Conclusion**

These studies clearly show the importance of agonist-induced ligand internalization for targeted visualization of G protein–coupled receptor-positive tumors using radiouclide-coupled somatostatin analogues. The double tumor models show the fast washout of the pasmatoctatin radioligand from tumor xenografts expressing the sst2 receptor, whereas high and persistent uptake was found in the sst3-expressing tumor, which obviously internalizes the radioligand also in vivo. The KE88-based radioligands may be interesting and suitable for imaging sst2-expressing tumors at early time points [e.g., with the metallic positron emitter $^{64}$Ga ($t_{1/2} = 68$ min; ref. 32)] and sst3-expressing tumors at later imaging time points using a longer-lived radionuclide [e.g., $^{64}$Cu ($t_{1/2} = 12.7$ h; ref. 23) and $^{68}$Y ($t_{1/2} = 14.74$ h; ref. 33)].

Obviously, agonistic properties are not sufficient for an adequate tumor targeting and a single thermodynamic constant is not sufficient to describe the efficacy of a ligand binding to a G protein–coupled receptor (34). Efficacy in terms of extended residence in a tumor targeted with an agonistic ligand for imaging and targeted therapy seems to depend on efficient internalization. These results do not contradict our recently reported findings on the use of radiolabeled somatostatin antagonists as new tools in nuclear oncology (20). As shown, antagonists recognize potentially many more receptor binding sites than agonists and re Binding after dissociation of the radioantagonist may be the efficient mechanism explaining a slow tumor washout. At the moment, peptidic antagonists are not available for every peptide receptor of interest and therefore need to be developed and studied with regard to tumor residence time. The present studies irrefutably underline the importance of internalization when using agonists for tumor targeting. In addition to the imaging and targeted radionuclide therapy aspects, some of the peptides compiled in Table 1 may be very good candidates for replacing octreotide or lanreotide in the treatment of symptoms of sst-overexpressing tumors.

**Acknowledgments**

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