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Design and *in vitro* Characterization of Highly Sst₂-selective Somatostatin Antagonists Suitable for Radio-Targeting

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Abstract

Radiolabeled sst₂ and sst₃ antagonists are better candidates for tumor targeting than agonists with comparable binding characteristics.¹ Because the majority of neuroendocrine tumors express sst₂, we used the known antagonists Acetyl-pNO₂Phe²-c[D-Cys³-Tyr⁷-D-Trp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-D-Tyr¹⁵-NH₂ (**1**)^{2,3} and H-Cpa²-c[D-Cys³-Tyr⁷-D-Trp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-2-Nal¹⁵-NH₂ (**7**)⁴ as leads for analogues with increased sst₂ binding affinity and selectivity. Among the 32 analogues reported here, DOTA-pNO₂Phe-c[D-Cys-Tyr-D-Aph(Cbm)-Lys-Thr-Cys]-D-Tyr-NH₂ (**3**) and DOTA-Cpa-c[D-Cys-Aph(Hor)-D-Aph(Cbm)-Lys-Thr-Cys]-D-Tyr-NH₂ (**31**) had the highest sst₂ binding affinity and selectivity. All of the analogues tested kept their sst₂ antagonistic properties (i.e. did not affect calcium release *in vitro* and competitively antagonized the agonistic effect of [Tyr³]-octreotide). Moreover, in an immunofluorescence-based internalization assay, the new analogues prevented sst₂ internalization induced by the sst₂ agonist [Tyr³]-octreotide, without being active by themselves. In conclusion, several analogues (in particular **3**, **31** and **32**) have outstanding sst₂ binding and functional antagonistic properties and, because of their DOTA moiety, are excellent candidates for *in vivo* targeting of sst₂-expressing cancers.

Introduction

The limitations of therapeutic applications of somatostatin (SRIF) due to its rapid proteolytic degradation and multiple targets have led to the search for peptide analogues with higher metabolic stability and improved selectivity with respect to the five SRIF receptor subtypes. Long-acting preparations of octreotide⁵ and lanreotide⁶ are now available for use in the treatment of acromegaly, neuroendocrine tumors, and various gastrointestinal disorders. Moreover, targeting neuroendocrine tumors expressing SRIF receptor subtypes with radiolabeled SRIF agonists is an established diagnostic and therapeutic approach in oncology. Somatostatin receptor scintigraphy with ¹¹¹In-DTPA-octreotide (¹¹¹In-diethylenetriaminepentaacetyl-octreotide) is the current imaging technique for the localization of neuroendocrine tumors⁷ whereas ¹⁷⁷Lu or ⁹⁰Y-1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetyl-[Tyr³]-octreotate (⁹⁰Y-DOTA-TATE) are the radioligands used for tumor radiotherapy.^{8,9} Radio-guided surgery for the *in situ* detection of neuroendocrine tumors during surgery also utilizes ¹¹¹In-DTPA-octreotide as a tool for tumor localization. The development

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of more selective analogues with high affinity and enhanced uptake by the SRIF receptor containing tumor cells is expected to open new and more sensitive avenues for radiotherapy and radio-guided surgery.¹⁰⁻¹² Classically, this can be achieved using established approaches used for drug design whereby the physico-chemical properties of the analogues are systematically modulated leading to stepwise improvements. For example, hydrophobicity, ionic charges, stabilization of secondary structures and, in the case of somatostatin analogues for radiotherapy, exhaustive modifications of the chelator moiety and of the radioactive metal have been reported.¹³⁻¹⁹

While SRIF agonists readily internalize into tumor cells, permitting accumulation of radioactivity, radiolabeled antagonists do not, and therefore have not been considered for tumor targeting until recently.¹ We reported that the macrocyclic chelator DOTA-coupled sst₃ and sst₂-selective antagonists did not trigger sst₃ or sst₂ internalization, prevented agonist-stimulated internalization, yet were excellent *in vivo* tumor markers.^{1,20} Potent agonists with strong binding and internalization properties showed a much lower and shorter-lasting uptake in SRIF receptor expressing tumors than the tested antagonists. The amount of uptake of the antagonist radioligand was particularly high in the tested tumors: 60% IA/g uptake has indeed never been achieved before by any radiolabeled SRIF receptor agonist, not even by those developed most recently.^{11,12} Not only was the uptake at the peak time point very high, but also the long-lasting accumulation of the antagonist radioligand up to 72 h after injection was a remarkable result and represented a considerable advantage over radio-targeting with established agonists. We concluded that SRIF antagonist radiotracers are, therefore, preferable over agonists for the *in vivo* targeting of sst₃- or sst₂-expressing tumors. The use of potent radiolabeled antagonists for *in vivo* tumor targeting may considerably improve the sensitivity of diagnostic procedures, the staging of the disease, the detection of unexpected tumor sites and the efficacy of receptor-mediated radiotherapy and complementary procedures.^{10,21-23}

To generate pure sst₂ antagonists for therapeutic applications and because the great majority of neuroendocrine tumors express predominantly sst₂, we have focused the present study on the development of potent, highly sst₂-selective unlabeled and DOTA-labeled antagonists. This was achieved with the introduction of novel amino acid derivatives within the sequence of octreotide amide, and documented with binding assays to the five human sst_s and several functional assays such as internalization assays and calcium release.

Results and Discussion

All of the analogues shown in Table 1 were synthesized either manually or automatically on a MBHA resin using the Boc-strategy, diisopropylcarbodiimide (DIC)/HOBt (1-hydroxybenzotriazole) for amide bond formation and trifluoroacetic acid (TFA) for Boc removal. The peptide resins were treated with hydrogen fluoride (HF) in the presence of scavengers to liberate the fully deprotected crude linear peptides. Cyclization of the cysteines was mediated by iodine in an acidic milieu. Purification was carried out using multiple HPLC steps.²⁴ DOTA was coupled to the Lys(Fmoc)⁹ protected analogues in solution. The purity of the peptides was characterized by HPLC,²⁴ capillary zone electrophoresis²⁵ and mass spectrometry. The observed monoisotopic mass ($M + H$)⁺ values of each peptide correspond to the calculated mass (M) values. Results are shown in Table 1.

To investigate their sst_s binding properties, the peptides were tested for their ability to bind to cryostat sections from membrane pellets of cells expressing the five human sst_s (Table 2). For each of the tested compounds, complete displacement experiments with the universal SRIF radioligand [Leu⁸,DTrp²², ¹²⁵I-Tyr²⁵]SRIF-28. Results are shown in Table 2.

Inverting chirality at positions 2 and 3 in the octreotide scaffold (H- D-Phe^2 -c[Cys³-Phe⁷- D-Trp^8 -Lys⁹-Thr¹⁰-Cys¹⁴]-Thr¹⁵-ol, SRIF numbering) was reported to be the key structural modification converting an SRIF agonist into an antagonist.² Additional substitutions resulted in partially selective antagonists Acetyl-pNO₂Phe-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-D-Tyr-NH₂² or H-Cpa-c[D-Cys-Tyr-D-Trp-Lys-Thr-Cys]-2NaI-NH₂.⁴ These antagonists display preferentially high binding affinity for sst₂, and lower or no affinity to sst₃, sst₄ and sst₅. None of the analogues bind to sst₁. Using these lead compounds, we have designed SRIF antagonists that were more affine (> 3-fold) and more sst₂-selective than those reported so far. Guided by earlier observations whereby amide bond-rich moieties are favorably recognized by GPCR, most of the analogues reported have carbamoyl functionalities.²⁶ We like to believe that this empirically-based approach to drug design is to be distinguished from what is referred to as SAR studies. Indeed, a state-of-the-art and comprehensive SAR study will include conformational considerations”, whereas a “drug design” strategy may be based on intuitive, systematic and iterative substitutions, an understanding (although partial) of the fundamentals of peptide/protein interactions, deletions and changes in chirality among others, with the exclusion of structural requirements. On the other hand, it is only with the knowledge of the 3D NMR structures of the improved, structurally constrained and bioactive analogues resulting from such empirical strategies (applying a drug design approach) that a consensus pharmacophore can be determined and used for true SAR. This is exemplified in a manuscript to be submitted elsewhere (Grace et al., 2008).

This being said, analogues of antagonists like Acetyl-pNO₂Phe²-c[D-Cys³-Tyr⁷-D-Trp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-D-Tyr¹⁵-NH₂² (**1**) and H-Cpa²-c[D-Cys³-Tyr⁷-D-Trp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-2NaI¹⁵-NH₂⁴ (**7**) were synthesized to investigate the effect of different substitutions on binding affinity, receptor-subtype selectivity, overall hydrophilicity as well as agonism and antagonism.

The substitution of the N-terminal acetyl group by DOTA in **1** (IC₅₀ = 3.6 nM at sst₂) resulted in **2**, which bound to sst₂ with an IC₅₀ = 1.5 nM, suggesting that the DOTA moiety, which is crucial for radiolabeling with ¹¹¹In, ⁹⁰Y, or ¹⁷⁷Lu for *in vivo* targeting, is well tolerated by sst₂ (Table 2). This conclusion is confirmed further with several additional examples. The introduction of D-Aph(Cbm)⁸ in place of D-Trp⁸ in **2** yielded **3** (IC₅₀ = 0.75 nM). It is noteworthy that these two substitutions are cumulative, thus resulting in the most potent sst₂ antagonist in this series, with no measurable binding affinity to any of the other receptors. Further replacement of D-Tyr¹⁵ in **3** by 2NaI¹⁵ yielded **5** with a similar binding affinity for sst₂ (IC₅₀ = 1.3 nM). Analogue **4**, a peptide with the same sequence as **5** but without DOTA at its N-terminus, still had excellent binding affinity for sst₂ (IC₅₀ = 2.6 nM) and also bound measurably to sst₃ (IC₅₀ = 384 nM). Substitution of Tyr in position 7 by Aph(Hor) resulting in **6** had no effect on sst₂ binding affinity and selectivity when compared with the parent **4** (IC₅₀ = 2.6 and 2.7 nM at sst₂ and 384 nM and 451 nM at sst₃, respectively, and no binding affinity at the other three receptors) (Table 2).

We also used H-Cpa²-c[D-Cys³-Tyr⁷-D-Trp⁸-Lys⁹-Thr¹⁰-Cys¹⁴]-2NaI¹⁵-NH₂ (**7**) published by Hocart et al.⁴ as a second lead for sst₂-selective antagonists. This antagonist has IC₅₀ values in our binding assay equal 5.7, 112 and 218 nM at sst_{2/3/5}, respectively, as compared to the reported values of 26, 93 and 48 nM. In our assays, **7** is more potent than reported at sst₂ by a factor of five and less potent at sst₅ by the same factor. This points to the danger of comparing results from one laboratory to another when engaged in SAR studies.

Whereas N^α-methylation of Lys⁹ in **7**⁴ to yield **8**²⁷ increased binding affinity by a modest 5-fold in the assay used by Hocart et al.⁴ (K_i = 26 nM and 5.51 nM, respectively) with no improvement at sst₃ or sst₅, (K_i = ca. 50-100 nM), our *in vitro* binding assay could not confirm this improvement at sst₂ and, as a result, we did not pursue the use of this substitution in the design of additional sst₂-selective analogues. Instead, we synthesized **9** with an L-Agl

(NMe,benzoyl)⁷ in an attempt to constrain the orientation of the side chain at position 7. The use of such aminoglycine derivatives (betides)^{28,29} had been taken advantage of in the design of an sst₃-selective antagonist.^{30,31} While **9** lost some binding affinity for sst₂ (3-fold) as compared to that of **7**, it also lost comparable binding affinity for sst₃ and sst₅. This observation further suggests that position 7 is critical for all three sst_{2,3,5}. In fact, **10** with the D-Agl (NMe,benzoyl)⁷ lost binding affinity at sst₂ while retaining similar binding affinities as **7** at sst_{3/4/5}, thus accomplishing one of our goals of identifying the or those residues/conformations responsible for binding to any particular receptor (i.e., sst₂ in this case).

Whereas substituting Phe by Leu at position 7 in **7** yielded **11** that lost 10-fold binding affinity for sst₂ and selectivity, substitution by Aph(Cbm) yielded **12** which exhibited similar binding affinity and selectivity as **7** at the five sst_s. N-terminal carbamoylation of **12** to yield **13** improved binding affinity slightly at sst_{3/4} with some loss of binding affinity for sst₂ as compared to **12**. Addition of DOTA to **12** resulted in **14** whose sst₂ binding affinity is similar to that of **12** and increased selectivity for sst₂.

Interestingly, addition of a spacer in **14** between DOTA and the octapeptide such as βAla in **15** and Peg in **16** was unexpectedly detrimental^{32,33} in terms of sst₂ binding affinity, yet favorable for sst₃ and neutral at sst_{1/4/5}.

From our observation that 2Nal¹⁵ may contribute to the sst₃, sst₄, and sst₅ binding pocket, **17** (missing this residue) was synthesized and found to have similar binding affinities when compared to the parent **12**. Substitution of 2Nal¹⁵ in **12** by different other residues such as Cha in **18**, Aph(Hor) in **19**, D-Aph(Cbm) in **20** and Aph(Cbm) in **21** did not markedly influence affinity at sst₂ or selectivity. This is noteworthy in that there is only a three-fold difference in binding affinity at sst₂ for **20** (D-configuration and IC₅₀ = 5.4 nM) and **21** (L-configuration and IC₅₀ = 15 nM) where the C-terminal amino acid is of the D or L configuration, respectively. This supports the earlier observation that D-Tyr (as in **1** and **2**) or 2Nal (as in **4** and **5**) are both equally accepted. On the other hand, extension of the sequence of **20** by Glycine-OH as in **22**, leads to significant loss of affinity at all receptors.

In order to modulate the overall hydrophilicity of **7** (with Tyr at position 7), we introduced the following carbamates (Aph(Cbm)⁷) in **12**, (Aph(CONH-OCH₃)⁷) in **23** and (Aph(CONH-OH)⁷) in **24** at position 7. Whereas binding affinities for these analogues are not different from that of the parent **7**, the order of elution of these analogues on HPLC at neutral pH suggests that **24** (RT = 31.6 min) may be more hydrophilic than **7** (RT = 34.8 min), **12** (RT = 31.9 min) and **23** (RT = 34.2 min). Since hydrophilicity may be a critical criterion for a clinically relevant radioligand, subtle differences in structure may favor one of these analogues when selecting a clinical candidate. The fact that **12**, **23** and **24** are not superior to **7** in terms of sst₂ binding affinity and selectivity supports our previous finding that residue 7 is not an essential contributor to the sst₂ pharmacophore.³⁴

We then investigated the effect of substitutions at position 8. There is literature precedent suggesting that 5F-Trp is a favorable substitution for Trp^{8,35}. When introduced in **12**, to yield **25** and **26**, we observed a slight improvement in binding affinity for the three sst_{2/3/5} as expected for the 5F-D-Trp-containing **25** and less so for the corresponding L-isomer-containing **26**. No increase in selectivity however, was seen for either analogue.

It was therefore very rewarding to find out that substitution of D-Trp⁸ in **7** by D-Aph(Cbm)⁸ yielding **27** was clearly superior in terms of sst₂ selectivity with improved binding affinity. Further derivatization with the addition of DOTA at the N-terminus yielded **28** with an additional increase in binding affinity to sst₂ and greater than 500-fold selectivity at all other receptors.

Substitution of Tyr⁷ in **27** and **28** with Aph(Hor) yielded **29** and **30**. Whereas **29** retained high binding affinity at sst₂ it also exhibited moderate binding affinity for sst₃; the binding affinity at sst₃ was lost upon the introduction of DOTA (**30**). Substitution of Tyr⁷ in **2** with ITyr yielded **32**, the binding affinity of which was similar to that of **2** at sst₂.

We then substituted 2NaI¹⁵ in **30** by ¹²⁵I-Tyr¹⁵ to yield **31**. Of all analogues presented here, **31** (because of its hydrophilicity, RT = 13.2 min) may be the preferred candidate for bio-distribution and ultimately clinical investigation over **3** (RT = 13.6 min), **5** (RT = 26.1 min), **28** (RT = 26.7 min), **29** (RT = 27.7 min) or **32** (RT = 25.0 min) that are equally potent and selective in the binding assay. It is remarkable that the dipeptide sequence -Aph(Hor)-¹²⁵I-Aph(Cbm)- found in **29-31** is identical to that found in degarelix (Fe-200486),²⁶ a gonadotropin releasing hormone antagonist where it played a critical role in stabilizing a turn and in extending duration of action.

Put in perspective, the most affine DOTA-containing antagonists presented here (**3** and **31**) have binding affinities three to four-fold higher than that of SRIF-28 with no detectable binding affinity at any of the other four sst_s and are therefore potential candidates for clinical use.

All of the analogues tested here are antagonists in the calcium release assay in HEK293 cells stably expressing the human sst₂. Testing them alone, they do not affect calcium release up to 10 μM. However, the agonistic effect of the sst₂ agonist [Tyr³]-octreotide can be competitively antagonized with a 100-fold excess of each of the analogues applied individually. Figure 1 illustrates the antagonistic properties of some of the sst₂ antagonists using the calcium release assay.

The antagonistic property of the analogues **3**, **31** and **32** was also confirmed in an immunofluorescence-based internalization assay²⁰ with HEK293 cells stably expressing the human sst₂. Figure 2 illustrates that although the control agonist [Tyr³]-octreotide can induce sst₂ internalization, the tested sst₂-selective antagonists have no effect when given alone, even at a concentration of 10 μM. Moreover, they prevent sst₂ internalization induced by [Tyr³]-octreotide. Figure 3 shows the antagonistic properties of another analogue (**32**) in the ELISA internalization assay.

To conclude, a great majority of the analogues reported here have a high affinity binding in the nanomolar range for sst₂ and often a high selectivity for sst₂ as well. The best compounds were **3** and **31** (with IC₅₀ values below 1 nM) followed by **32**, **5**, **28**, **2** and **29**. All of these antagonists are of particular interest, since they all include a DOTA moiety, making them candidates for *in vivo* tumor targeting. The labeling properties of the analogues and the specific *in vivo* uptake in tumors will be discussed in a subsequent publication (Maecke *et al.*, in preparation).

Experimental Section

Starting Materials

MBHA resin with a capacity of 0.3-0.4 mequiv/g was used in the solid phase syntheses. All Boc-N^α-protected amino acids with side chain protection: Cys(Mob), Lys(ε-2Cl-Z), Lys(Fmoc), Thr(Bzl), Tyr(2Br-Z) and ITyr(3Br-Bzl) are commercially available (Bachem Inc., Torrance, CA; Chem Impex, Wood Dale, IL; Reanal, Budapest, Hungary) except Boc-Aph(Cbm)-OH, Boc-¹²⁵I-Aph(Cbm)-OH, Boc-Aph(Cbm-OCH₃)-OH, Boc-Aph(Cbm-OH)-OH, Boc-Aph(Hor)-OH,²⁶ Fmoc-D/L-Agl(NMe,Boc)-OH,²⁸ Fmoc-D-Agl(Boc)-OH,³⁶ Boc-5F-Trp-OH, Boc-5F-¹²⁵I-Trp-OH which were synthesized in our laboratory. 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraacetic acid mono(N-hydroxysuccinimide).ester.

3CH₃COOH.HPF₆ (DOTA-NHS) was purchased from Macrocyclics Inc. (Dallas, TX). All reagents and solvents were ACS grade and were used without further purification.

Peptide Synthesis

Peptides were synthesized by the solid-phase approach either manually or on a CS-Bio Peptide Synthesizer Model CS536.³⁷ A 3-equiv excess of Boc-amino acid (1.2 mmol) based on the original substitution of the resin was used for each coupling. Peptide couplings were mediated for 1 h by DIC/HOBt (1.2 mmol/1.8 mmol) in dimethylformamide (DMF) and monitored by the qualitative ninhydrin test.³⁸ Boc removal was achieved with trifluoroacetic acid (TFA) (60% in CH₂Cl₂, 1-2% ethanedithiol or *m*-cresol) for 20 min. An isopropyl alcohol (1% *m*-cresol) wash followed TFA treatment and then successive washes with triethylamine (TEA) solution (10% in CH₂Cl₂), methanol, triethylamine solution, methanol and CH₂Cl₂ completed the neutralization sequence. The ureido group (Cbm) at the N-terminus of **13** was introduced on the resin. The N-terminal Boc group of the fully assembled peptide was deprotected with TFA in the usual manner,²⁶ after neutralization, the carbamoylation proceeded with NaOCN (100 mg, 0.65 mmol) in *N*-methylpyrrolidinone (NMP) (4 mL) and glacial acetic acid 3 mL per gram of initial resin. The mixture was agitated at room temperature for 30 min and ninhydrin test indicated a complete reaction. The completed peptide was then unprotected and cleaved from the resin by HF containing the scavengers anisole (10% v/v) and methyl sulfide (5% v/v) for 60 min at 0 °C. The diethyl ether precipitated crude peptides were cyclized in 75% acetic acid (200 mL) by addition of iodine (10% solution in methanol) until the appearance of a stable orange color. Forty minutes later, ascorbic acid was added to quench the excess of iodine.

For the synthesis of **9**, we used unresolved Fmoc-*D/L*-Agl(NMe,Boc)-OH and the two diastereomers were separated readily during the standard HPLC purification steps.^{24,39} The optical configuration of the two diastereomers was tentatively inferred from a comparison of the HPLC elution behavior with analogue synthesized separately as diastereomers of known optical configuration. In short: after coupling Fmoc-*D*-Agl(Boc)-OH in position 7, the side chain protecting Boc group was removed with 60% TFA, washed, neutralized and to the 0.9 g peptide resin (0.36 mmol/g) swollen in dichloromethane, Dod-Cl (130 mg; 0.5 mmol) was added along with DIEPA (500 μL). The mixture was shaken for an hour to complete the alkylation. The resin was washed, and shaken after the addition of formaldehyde (2 mL, 37% solution) in NMP (18 mL) and acetic acid (100 μL). After 5 min, sodium cyanoborohydride (300 mg) was added and the mixture was shaken for 60 min. After the removal of the Dod group with TFA (60%) for 30 min, benzoyl chloride (500 μL) was used to acylate the free secondary amino group of the side chain.⁴⁰ Removal of the N^α-Fmoc protecting group with 20% piperidine in NMP in two successive 5 and 15 min treatments was followed by the standard elongation protocol until completion of the peptide. The peptide was cleaved, deprotected and cyclized as described above. On HPLC, this *D* configuration diastereomer coeluted with the earlier eluting diastereomer from the synthesis performed with the unresolved amino acid, therefore the slower eluting peptide (**9**) was tentatively identified as the L-Agl(NMe, benzoyl)⁷ containing analogue.

Generally, for the synthesis of the DOTA-peptide-conjugates, the side chain of Lys⁹ was protected with an Fmoc protecting group that stays on after HF cleavage. To a solution of the RP-HPLC purified [Lys(Fmoc)⁹]-sst₂-antagonist (~20 μM) in dry DMF (800 μL) was added a solution of DOTA-NHS-ester (38 mg, 48 μM) in DMF (160 μL) and *N,N'*-Diisopropylethylamine (DIPEA) (40 μL, 24 μM). The mixture was stirred at room temperature for 5 hours. The progress of the reaction was followed by analytical HPLC. After completion of the reaction, a preparative RP-HPLC purification was performed yielding the pure DOTA-[Lys(Fmoc)]⁹-sst₂-antagonist. Removal of the Fmoc protecting group from the Lys side chain

was achieved with 20% piperidine/DMF solution resulting in the DOTA-sst₂-antagonist, which was further purified by preparative RP-HPLC.

Purification of Peptides

The crude, lyophilized peptides were purified by preparative RP-HPLC²⁴ on a 5 cm × 30 cm cartridge, packed in the laboratory with reversed-phase 300Å Vydac C₁₈ silica (15-20 µm particle size). The peptides eluted with a flow rate of 100 mL/min using a linear gradient of 1% B per 3 min increase from the baseline % B (eluent A = 0.25 N TEAP pH 2.25, eluent B = 60% CH₃CN, 40% A). All peptides were subjected to a second purification step carried out with eluents A = 0.1% TFA in water and B = 60% CH₃CN/40% A on the same cartridge using a linear gradient of 1% B per min increase from the baseline % B. Analytical HPLC screening of the purification was performed on a Vydac C₁₈ column (0.46 × 25 cm, 5 µm particle size, 300 Å pore size) connected to a Rheodyne injector, two Waters pumps Model 501, System Controller Programmer, Kratos 750 UV detector, and Houston Instruments D-5000 strip chart recorder. The fractions containing the product were pooled and subjected to lyophilization.

Characterization of SRIF Analogues (Table 1)

The purity of the final peptides was determined by analytical RP-HPLC performed with a linear gradient using 0.1 M TEAP pH 2.5 as eluent A and 60% CH₃CN/40% A as eluent B on a Hewlett-Packard Series II 1090 Liquid Chromatograph connected to a Vydac C₁₈ column (0.21 × 15 cm, 5 µm particle size, 300 Å pore size), Controller Model 362 and a Think Jet printer. Capillary zone electrophoresis (CZE) analysis was performed as described earlier.²⁵ Each peptide was found to have a purity of >95% by HPLC and CZE. Mass spectra (MALDI-MS) were measured on an ABI-PerSeptive DE-STR instrument. The instrument employs a nitrogen laser (337 nm) at a repetition rate of 20 Hz. The applied accelerating voltage was 20 kV. Spectra were recorded in delayed extraction mode (300 ns delay). All spectra were recorded in the positive reflector mode. Spectra were sums of 100 laser shots. Matrix α -cyano-4-hydroxycinnamic acid was prepared as saturated solutions in 0.3% trifluoroacetic acid and 50% acetonitrile. The observed monoisotopic (M + H)⁺ values of each peptide corresponded with the calculated (M + H)⁺ values.

Reagents

All reagents were of the best grade available and were purchased from common suppliers. [Tyr³]-octreotide⁴¹ was from Novartis Inc. (Basel, Switzerland). All other peptides, including Coy-14²⁷ were synthesized at the Salk Institute. The sst_{2A}-specific antibody R2-88 antibody to the sst_{2A} receptor was generated as previously described and has been extensively characterized.⁴² The secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H+L) was from Molecular Probes, Inc. (Eugene, OR), the monoclonal anti-T7 antibody from Novagen (Madison, WI), the goat anti-mouse IgG horseradish peroxidase conjugate from Bio-Rad Laboratories, Inc. (Hercules, OR); the Fluo-4NW Calcium Assay kit was from Molecular Probes, Inc. (Eugene, OR), substrate mix for horseradish peroxidase (ABTS) was from Bio-Rad Laboratories, Inc. (Hercules, OR), lactalbumin hydrolysate was from HyClone (Logan, UT).

Cell lines

CHO-K1, CCL39 cells stably expressing the cloned five human sst_s and the HEK293 cell line expressing the T7-epitope tagged human sst_{2A} (HEK-sst₂) were grown as described previously.^{8,20} All culture reagents were from Gibco BRL, Life Technologies, (Grand Island, NY).

Receptor autoradiography

Cell membrane pellets were prepared as previously described⁸ and stored at -80 °C. Receptor autoradiography was performed on 20-µm thick cryostat (Microm HM 500, Walldorf, Germany) sections of the membrane pellets, mounted on microscope slides, and then stored at -20 °C. For each of the tested compounds, complete displacement experiments with the universal SRIF radioligand [Leu⁸, D-Trp²², ¹²⁵I-Tyr²⁵]-SRIF-28 (¹²⁵I-[LTT]-SRIF-28) (2,000 Ci/mmol; Anawa, Wangen, Switzerland) using 15,000 cpm/100 µl and increasing concentrations of the unlabelled peptide ranging from 0.1 - 1000 nM were performed. As control, unlabelled SRIF-28 was run in parallel using the same increasing concentrations. The sections were incubated with ¹²⁵I-[LTT]-SRIF-28 for 2 hours at room temperature in 170 mmol/L Tris-HCl buffer (pH 8.2), containing 1% BSA, 40 mg/L bacitracin, and 10 mmol/L MgCl₂ to inhibit endogenous proteases. The incubated sections were washed twice for 5 min in cold 170 mmol/L Tris-HCl (pH 8.2) containing 0.25% BSA. After a brief dip in distilled water to remove excess salts, the sections were dried quickly and exposed for 1 week to Kodak BioMax MR film. IC₅₀ values were calculated after quantification of the data using a computer-assisted image processing system as described previously.⁴³ Tissue standards (Autoradiographic [¹²⁵I] microscales, GE Healthcare; Little Chalfont, UK) that contain known amounts of isotope, cross-calibrated to tissue-equivalent ligand concentrations were used for quantification.⁴⁴

Immunofluorescence-based sst₂ Internalization Assay

Immunofluorescence microscopy-based internalization assay for sst₂ was performed with HEK-sst₂ using the sst₂-specific antibody R2-88 as described earlier.²⁰ HEK-sst₂ cells were treated either with vehicle alone, the sst₂ agonist [Tyr³]-octreotide at a concentration of 100 nM, [Tyr³]-octreotide at a concentration of 100 nM in the presence of an excess of the SRIF analogues to be tested (100 times the concentration of [Tyr³]-octreotide), or with the SRIF analogues to be tested alone at a concentration of 10 µM, and then processed for immunofluorescence microscopy as described previously.²⁰

Quantitative assay for sst₂ internalization (ELISA)

Receptor internalization was determined using an ELISA to quantitate T7-epitope-tagged human sst₂ on the cell surface. HEK-sst₂ cells were seeded on poly-D-lysine (20 µg/mL) coated 24-well plates (250'000 cells per well) in growth medium and cultured for 1 day at 37 °C and 5% CO₂. On the day of the assay, cells were incubated with the monoclonal anti-T7 antibody at a dilution of 1:3000 for 2 h at room temperature in DMEM containing 5 g/L lactalbumin hydrolysate + 20 mM HEPES, pH 7.4 (DMEM-LH) to label cell surface receptors. After washing with DMEM-LH to remove unbound antibody, cells were incubated for 30 min at 37 °C and 5% CO₂ either without or with the SRIF analogues to be tested, added at the concentrations indicated. Incubations were terminated by placing the plates in an ice bath. Cells were then washed twice with cold PBS and fixed for 10 min at room temperature with 3% paraformaldehyde in PBS (pH 7.4). Nonspecific binding sites were blocked by incubating the cells for 60 min at room temperature with PBS containing 1% bovine serum albumin (BSA; Fraction V; SERVA, Heidelberg, Germany). Cells were then incubated for 60 min at room temperature with goat anti-mouse IgG horseradish peroxidase conjugate (1:1000) in PBS containing 1% BSA. After 3 additional washes with PBS, antibody binding was measured by adding 0.3 mL substrate mix for horseradish peroxidase (ABTS). The OD₄₀₅ was measured after an approximately 30 min incubation at room temperature. The amount of sst₂ remaining at the cell surface after ligand treatment was calculated as the absorbance measured in treated cells expressed as a percentage of the absorbance in untreated cells. Nonspecific absorbance was determined in experiments in which HEK293-sst₂ cells were incubated without the anti-

T7 antibody. Each data point represents the mean $\pm$ SEM of three experiments performed in duplicate.

Calcium release assay

Intracellular calcium release was measured in HEK-sst₂ using the Fluo-4NW Calcium Assay kit as described previously.^{45,46} In brief, HEK-sst₂ cells were seeded (25,000 cells per well) in poly-D-lysine (20 μ g/mL) coated 96 well plates and cultured for 1 day at 37 °C and 5% CO₂ in culture medium. On the day of the experiment, the cells were washed with assay buffer (1 $\times$ HBSS, 20 mM HEPES) containing 2.5 mM probenecid, and then incubated with 100 μ L/well Fluo-4NW dye in assay buffer containing 2.5 mM probenecid for 30 min at 37 °C and 5% CO₂ and an additional 30 min at room temperature. To measure the intracellular calcium mobilization after stimulation with the SRIF analogues to be tested, the dye-loaded cells were transferred to a SpectraMax M2^e (Molecular Devices, Sunnyvale, CA). Intracellular calcium mobilization was recorded in a kinetic for 60 sec at room temperature monitoring fluorescence emission at 520 nm (with λ_{ex} = 485 nm) in the presence of the analogues at the concentrations indicated. Maximum fluorescence (F-max) was measured after the addition of 25 μ M ionomycin. Baseline (F-baseline) measurements were taken for dye-loaded, untreated cells. Data are shown as percentage of maximum calcium response (F-max - F-baseline = 100 % of maximum calcium response) as reported previously.^{45,46} All experiments were repeated at least three times in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

The abbreviations for the common amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (Eur. J. Biochem. 1984, 138:9-37). The symbols represent the L-isomer except when indicated otherwise. Additional abbreviations:

Agl	aminoglycine
Aph	4-aminophenylalanine
3-Br-Bzl	3-bromobenzyl
Boc	t-Butoxycarbonyl
BSA	bovine serum albumin
Bzl	benzyl
Cbm	carbamoyl
Cha	cyclohexylalanine

Cpa	4-Cl-phenylalanine
CZE	capillary zone electrophoresis
DIC	N,N'-diisopropylcarbodiimide
DIPEA	N,N'-Diisopropylethylamine
DMEM-LH	Dulbecco's modified Eagle's medium-lactalbumine hydrolysate/HEPES
DMF	dimethylformamide
Dod	4,4'-dimethoxydityl
DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
DTPA	diethylenetriaminepentaacetic acid
ELISA	enzyme-linked immunosorbent assay
Fmoc	9-Fluorenylmethoxycarbonyl
HBSS	Hank's balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethansulfonic acid
HOBt	1-hydroxybenzotriazole
MBHA	methyl-benzhydryl resin
Mob	4-methoxybenzyl
2Nal	3-(2-naphthyl)-alanine
NMP	N-methylpyrrolidinone
NMR	nuclear magnetic resonance
3D	three-dimensional
Peg	12-amino-4,7,10-trioxadodecanoic acid
PBS	Phosphate buffered saline
pNO₂-Phe	para-nitrophenylalanine
SAR	structure activity relationships
SRIF	somatostatin-14
sst_s	SRIF receptors
SRIF-28	somatostatin-28
TATE	[Tyr ³ ,Thr ⁸]-octreotide = [Tyr ³]-octreotate
TEA	triethylamine
TEAP	triethylammonium phosphate
TFA	trifluoroacetic acid
TOC	[Tyr ³]-octreotide

Z(2Br)	2-bromobenzyloxycarbonyl
Z(2Cl)	2-chlorobenzyloxycarbonyl

References

- (1). Ginj M, Zhang H, Waser B, Cescato R, Wild D, Erchegyi J, Rivier J, Mäcke HR, Reubi JC. Radiolabeled somatostatin receptor antagonists are preferable to agonists for *in vivo* peptide receptor targeting of tumors. *Proc. Natl. Acad. Sci. USA* 2006;103:16436–16441. [PubMed: 17056720]
- (2). Bass RT, Buckwalter BL, Patel BP, Pausch MH, Price LA, Strnad J, Hadcock JR. Identification and characterization of novel somatostatin antagonists. *Mol. Pharmacol* 1996;50:709–715. [PubMed: 8863814]
- (3). Bass RT, Buckwalter BL, Patel BP, Pausch MH, Price LA, Strnad J, Hadcock JR. Identification and characterization of novel somatostatin antagonists. *Mol. Pharmacol* 1997;51:170. Erratum
- (4). Hocart SJ, Jain R, Murphy WA, Taylor JE, Coy DH. Highly potent cyclic disulfide antagonists of somatostatin. *J. Med. Chem* 1999;42:1863–1871. [PubMed: 10354394]
- (5). Bauer W, Briner U, Doepfner W, Haller R, Huguenin R, Marbach P, Petcher TJ, Pless J. SMS 201-995: a very potent and selective octapeptide analog of somatostatin with prolonged action. *Life Sci* 1982;31:1133–1140. [PubMed: 6128648]
- (6). Murphy WA, Lance VA, Moreau S, Moreau J-P, Coy DH. Inhibition of rat prostate tumor growth by an octapeptide analog of somatostatin. *Life Sci* 1987;40:2515–2522. [PubMed: 2885701]
- (7). Kwekkeboom D, Krenning EP, de Jong M. Peptide receptor imaging and therapy. *J. Nucl. Med* 2000;41:1704–1713. [PubMed: 11038002]
- (8). Reubi JC, Schaer JC, Waser B, Wenger S, Heppeler A, Schmitt JS, Mäcke HR. Affinity profiles for human somatostatin receptor sst1-sst5 of somatostatin radiotracers selected for scintigraphic and radiotherapeutic use. *Eur. J. Nucl. Med* 2000;27:273–282. [PubMed: 10774879]
- (9). Kwekkeboom DJ, Bakker WH, Kooij PP, Konijnenberg MW, Srinivasan A, Erion JL, Schmidt MA, Bugaj JL, de Jong M, Krenning EP. [177Lu-DOTAOTyr3]octreotate: comparison with [111In-DTPA]octreotide in patients. *Eur. J. Nucl. Med* 2001;28:1319–1325. [PubMed: 11585290]
- (10). Gulec SA, Baum R. Radio-guided surgery in neuroendocrine tumors. *J. Surg. Oncol* 2007;96:309–315. [PubMed: 17726664]
- (11). Wild D, Schmitt JS, Ginj M, Macke HR, Bernard BF, Krenning E, De Jong M, Wenger S, Reubi JC. DOTA-NOC, a high-affinity ligand of somatostatin receptor subtypes 2, 3 and 5 for labelling with various radiometals. *Eur. J. Nucl. Med. Mol. Imaging* 2003;30:1338–1347. [PubMed: 12937948]
- (12). Ginj M, Chen J, Walter MA, Eltschinger V, Reubi JC, Maecke HR. Preclinical evaluation of new and highly potent analogues of octreotide for predictive imaging and targeted radiotherapy. *Clin. Cancer Res* 2005;11:1136–1145. [PubMed: 15709181]
- (13). Wehrmann C, Senftleben S, Zachert C, Muller D, Baum RP. Results of individual patient dosimetry in peptide receptor radionuclide therapy with 177Lu DOTA-TATE and 177Lu DOTA-NOC. *Cancer Biother. Radiopharm* 2007;22:406–416. [PubMed: 17651048]
- (14). Gabriel M, Decristoforo C, Kendler D, Dobrozemsky G, Heute D, Uprimny C, Kovacs P, Von Guggenberg E, Bale R, Virgolini IJ. Ga-DOTA-Tyr3-octreotide PET in neuroendocrine tumors: comparison with somatostatin receptor scintigraphy and CT. *J. Nucl. Med* 2007;48:508–518. [PubMed: 17401086]
- (15). Van Essen M, Krenning EP, De Jong M, Valkema R, Kwekkeboom DJ. Peptide Receptor Radionuclide Therapy with radiolabelled somatostatin analogues in patients with somatostatin receptor positive tumours. *Acta Oncol* 2007;46:723–734. [PubMed: 17653893]
- (16). Kaltsas GA, Papadogias D, Makras P, Grossman AB. Treatment of advanced neuroendocrine tumours with radiolabelled somatostatin analogues. *Endocr. Relat. Cancer* 2005;12:683–699. [PubMed: 16322317]
- (17). Kwekkeboom DJ, Mueller-Brand J, Paganelli G, Anthony LB, Pauwels S, Kvols LK, O'Dorisio T, Valkema R, Bodei L, Chinol M, Maecke HR, Krenning EP. Overview of results of Peptide

receptor radionuclide therapy with 3 radiolabeled somatostatin analogs. *J. Nucl. Med* 2005;46:62S–66S. [PubMed: 15653653]

- (18). Kwekkeboom DJ, Teunissen JJ, Kam BL, Valkema R, de Herder WW, Krenning EP. Treatment of patients who have endocrine gastroenteropancreatic tumors with radiolabeled somatostatin analogues. *Hematol. Oncol. Clin. North Am* 2007;21:561–573. x. [PubMed: 17548040]
- (19). Heppeler A, Froidevaux S, Eberle AN, Maecke HR. Receptor targeting for tumor localisation and therapy with radiopeptides. *Curr. Med. Chem* 2000;7:971–994. [PubMed: 10911025]
- (20). Cescato R, Schulz S, Waser B, Eltschinger V, Rivier JE, Wester HJ, Culler M, Ginj M, Liu Q, Schonbrunn A, Reubi JC. Internalization of sst₂, sst₃, and sst₅ Receptors: Effects of Somatostatin Agonists and Antagonists. *J. Nucl. Med* 2006;47:502–511. [PubMed: 16513620]
- (21). Li S, Beheshti M. The radionuclide molecular imaging and therapy of neuroendocrine tumors. *Curr. Cancer Drug Targets* 2005;5:139–148. [PubMed: 15810878]
- (22). Sundin A, Garske U, Orlefors H. Nuclear imaging of neuroendocrine tumours. *Best Pract. Res. Clin. Endocrinol. Metab* 2007;21:69–85. [PubMed: 17382266]
- (23). Forrer F, Valkema R, Kwekkeboom DJ, de Jong M, Krenning EP. Neuroendocrine tumors. Peptide receptor radionuclide therapy. *Best Pract. Res. Clin. Endocrinol. Metab* 2007;21:111–129. [PubMed: 17382268]
- (24). Miller C, Rivier J. Peptide chemistry: Development of high-performance liquid chromatography and capillary zone electrophoresis. *Biopolymers Pept. Sci* 1996;40:265–317.
- (25). Miller C, Rivier J. Analysis of synthetic peptides by capillary zone electrophoresis in organic/aqueous buffers. *J. Pept. Res* 1998;51:444–451. [PubMed: 9650719]
- (26). Jiang G, Stalewski J, Galyean R, Dykert J, Schteingart C, Broqua P, Aebi A, Aubert ML, Semple G, Robson P, Akinsanya K, Haigh R, Riviere P, Trojnar J, Junien JL, Rivier JE. GnRH antagonists: A new generation of long acting analogues incorporating urea functions at positions 5 and 6. *J. Med. Chem* 2001;44:453–467. [PubMed: 11462984]
- (27). Rajeswaran WG, Hocart SJ, Murphy WA, Taylor JE, Coy DH. Highly potent and subtype selective ligands derived by *N*-methyl scan of a somatostatin antagonist. *J. Med. Chem* 2001;44:1305–1311. [PubMed: 11312929]
- (28). Jiang G-C, Simon L, Rivier JE. Orthogonally protected *N*-methyl-substituted α -aminoglycines. *Prot. Pep. Lett* 1996;3:219–224.
- (29). Rivier JE, Jiang G-C, Koerber SC, Porter J, Craig AG, Hoeger C. Betidamino acids: Versatile and constrained scaffolds for drug discovery. *Proc. Natl. Acad. Sci. USA* 1996;93:2031–2036. [PubMed: 8700880]
- (30). Reubi JC, Schaer J-C, Wenger S, Hoeger C, Erchegyi J, Waser B, Rivier J. SST3-selective potent peptidic somatostatin receptor antagonists. *Proc. Natl. Acad. Sci. USA* 2000;97:13973–13978. [PubMed: 11095748]
- (31). Erchegyi, J.; Wenger, S.; Waser, B.; Eltschinger, V.; Cescato, R.; Reubi, JC.; Koerber, SC.; Grace, RCR.; Riek, R.; Rivier, JE. Use of betidamino acids in drug design; Understanding Biology Using Peptides, The 19th American Peptide Symposium; American Peptide Society: San Diego, CA. 2005; p. 517-518.
- (32). Chen X, Hou Y, Tohme M, Park R, Khankaldyyan V, Gonzales-Gomez I, Bading JR, Laug WE, Conti PS. Pegylated Arg-Gly-Asp peptide: ⁶⁴Cu labeling and PET imaging of brain tumor alphavbeta3-integrin expression. *J. Nucl. Med* 2004;45:1776–1783. [PubMed: 15471848]
- (33). Antunes P, Ginj M, Zhang H, Waser B, Baum RP, Reubi JC, Maecke H. Are radiogallium-labelled DOTA-conjugated somatostatin analogues superior to those labelled with other radiometals? *Eur. J. Nucl. Med. Mol. Imaging*. 2007Epub ahead of print
- (34). Grace CRR, Erchegyi J, Koerber SC, Reubi JC, Rivier J, Riek R. Novel sst₂-selective somatostatin agonists. Three-dimensional consensus structure by NMR. *J. Med. Chem* 2006;49:4487–4496. [PubMed: 16854054]
- (35). Meyers CA, Coy DH, Huang WY, Schally AV, Redding TW. Highly active position eight analogues of somatostatin and separation of peptide diastereomers by partition chromatography. *Biochemistry* 1978;17:2326–2330. [PubMed: 678512]
- (36). Synniewski M, Penke B, Simon L, Rivier J. (R)-tert-Butoxycarbonylamino-fluorenylmethoxycarbonyl-glycine from (S)-Benzyloxycarbonyl-serine or from papain resolution

of the corresponding amide or methyl ester. *J. Org. Chem* 2000;65:6595–6600. [PubMed: 11052107]

- (37). Stewart, JM.; Young, JD. *Solid Phase Peptide Synthesis*. Vol. 2nd ed.. Pierce Chemical Co.; Rockford, IL: 1984. *Solid Phase Peptide Synthesis*; p. 176
- (38). Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem* 1970;34:595–598. [PubMed: 5443684]
- (39). Hoeger CA, Galyean RF, Boublik J, McClintock RA, Rivier JE. Preparative reversed phase high performance liquid chromatography. II. Effects of buffer pH on the purification of synthetic peptides. *Biochromatography* 1987;2:134–142.
- (40). Kaljuste K, Undén A. New method for the synthesis of N-methyl amino acids containing peptides by reductive methylation of amino groups on the solid phase. *Int. J. Pept. Prot. Res* 1993;42:118–124.
- (41). Reubi JC. Evidence for two somatostatin-14 receptor types in rat brain cortex. *Neurosci. Lett* 1984;49:259–263. [PubMed: 6149498]
- (42). Gu YZ, Schonbrunn A. Coupling specificity between somatostatin receptor sst2A and G proteins: isolation of the receptor-G protein complex with a receptor antibody. *Mol. Endocrinol* 1997;11:527–537. [PubMed: 9139797]
- (43). Reubi JC, Kvols LK, Waser B, Nagorney D, Heitz PU, Charboneau JW, Reading CC, Moertel C. Detection of somatostatin receptors in surgical and percutaneous needle biopsy samples of carcinoids and islet cell carcinomas. *Cancer Res* 1990;50:5969–5977. [PubMed: 2168286]
- (44). Reubi JC. *In vitro* identification of vasoactive intestinal peptide receptors in human tumors: Implications for tumor imaging. *J. Nucl. Med* 1995;36:1846–1853. [PubMed: 7562054]
- (45). Magrys A, Anekonda T, Ren G, Adamus G. The role of anti-alpha-enolase autoantibodies in pathogenicity of autoimmune-mediated retinopathy. *J. Clin. Immunol* 2007;27:181–192. [PubMed: 17235687]
- (46). Michel N, Ganter K, Venzke S, Bitzegeio J, Fackler OT, Keppeler OT. The Nef protein of human immunodeficiency virus is a broad-spectrum modulator of chemokine receptor cell surface levels that acts independently of classical motifs for receptor endocytosis and G-protein signaling. *Mol. Biol. Cell* 2006;17:3578–3590. [PubMed: 16775006]

Figure 1.

The SRIF analogues **3**, **31** and Coy-14 behave like antagonists when tested in the calcium release assay. The calcium release assay was performed as described in Materials and Methods. HEK-sst₂ cells were treated either with 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, and 10 μmol/L [Tyr³]-octreotide (●) alone, or with 10 nmol/L, 100 nmol/L, 1 μmol/L, and 10 μmol/L [Tyr³]-octreotide in the presence of an increasing concentration (10 nmol/L, 100 nmol/L, 1 μmol/L, and 10 μmol/L) of **3** (■) or **31** (▲), or the specific sst₂-antagonist Coy-14 (◆). **3**, **31** and Coy-14 are antagonists since they shift the dose-response curve of [Tyr³]-octreotide to a higher molar range. Tested alone at 1 μmol/L and 10 μmol/L **3** (×), **31** (○) and Coy-14 (◇) have no effect on calcium release in HEK-sst₂ cells.

Figure 2.

Sst₂ internalization induced by [Tyr³]-octreotide is efficiently antagonized by the three SRIF analogues **32**, **3**, and **31**. The immunofluorescence-based internalization assay was performed as described in Materials and Methods. HEK-sst₂ cells were treated for 30 min either with vehicle (no peptide, a), or with 100 nmol/L [Tyr³]-octreotide (b), a concentration inducing a sub-maximal internalization effect. Panels (d, f, h) show HEK-sst₂ cells treated with 100 nmol/L [Tyr³]-octreotide in the presence of 10 μmol/L of **32**, **3**, and **31**. The effect of **32**, **3**, and **31** alone at a concentration of 10 μmol/L is shown in panels (c, e, g). As controls, panel (i) shows cells treated with 100 nmol/L [Tyr³]-octreotide in the presence of 10 μmol/L of the specific sst₂-antagonist Coy-14, and panel (j) shows cells treated with 100 nmol/L [Tyr³]-octreotide in

the presence of 10 $\mu\text{mol/L}$ of the sst₃-antagonist sst₃-ODN-8. A clear punctate perinuclear staining is detectable for [Tyr³]-octreotide. This punctate staining is efficiently abolished by an excess of the analogues **32**, **3**, **31**, as well as the established antagonist Coy-14. However, **32**, **3**, and **31** given alone have no effect on sst₂ internalization. Sst₃-ODN-8, as negative control, is not able to antagonize the [Tyr³]-octreotide effect.

Figure 3.

The SRIF analogues **32** and Coy-14 behave like antagonists when tested in the ELISA-based internalization assay. HEK-sst₂ cells were pre-incubated with the mouse monoclonal HA epitope antibody (1:1,000) at room temperature for 2 h and then processed for ELISA as described in Material and Methods. HEK-sst₂ cells were treated either with 0.1 nmol/L, 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μ mol/L, and 10 μ mol/L [Tyr³]-octreotide (●) alone, or with 1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μ mol/L, and 10 μ mol/L [Tyr³]-octreotide in the presence of an increasing concentration (1 nmol/L, 10 nmol/L, 100 nmol/L, 1 μ mol/L, and 10 μ mol/L) of **32** (■) or the specific sst₂-antagonist Coy-14 (▲). **32** and Coy-14 are antagonists since they shift the dose dependent internalization curve of [Tyr³]-octreotide to a higher molar range. Tested alone at 100 nmol/L, 1 μ mol/L and 10 μ mol/L **32** (□) and Coy-14 (△) do not stimulate sst₂ internalization in HEK-sst₂ cells.

Physico-chemical Properties of Sst₂ Antagonists

Table 1

Structure of SRIF analogues												
Residues are numbered according to SRIF numbering ¹³												
H-Ala ¹ -Gly ² -c(Cys ³ -Lys ⁴ -Asn ⁵ -Phe ⁶ -Phe ⁷ -Trp ⁸ -Lys ⁹ -Thr ¹⁰ -Cys ¹¹ -Thr ¹² -Ser ¹³ -Cys ¹⁴ -Ile ¹⁵ -OH (SRIF))												
Substitution in H-pPhe ² -c(Cys ³ -Phe ⁷ -D-Trp ⁸ -Lys ⁹ -Thr ¹⁰ -Cys ¹¹ -Thr ¹² -Ile ¹⁵ -OH (octreotide))												
N-terminus	2	3	7	8	9	10	14	15	C-terminus	Purity	MS ^c	
										HPLC ^d /CZE ^b	M _{calc}	M+H _{obs}
12 Ac-	pNO ₂ -Phe-	pCys-	Tyr-	pTrp-	Lys-	Thr-Cys-	pTyr-	NH ₂		99	1196.44	1197.59
2 DOTA-	pNO ₂ -Phe-	pCys-	Tyr-	pTrp-	Lys-	Thr-Cys-	pTyr-	NH ₂		95	1540.61	1541.46
3 DOTA-	pNO ₂ -Phe-	pCys-	Tyr-	pAph(Cbm)-	Lys-	Thr-Cys-	pTyr-	NH ₂		99	1559.63	1560.83
4 H ₂ N-	pNO ₂ -Phe-	pCys-	Tyr-	pAph(Cbm)-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1207.47	1208.54
5 DOTA-	pNO ₂ -Phe-	pCys-	Tyr-	pAph(Cbm)-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1593.65	1594.17
6 H ₂ N-	pNO ₂ -Phe-	pCys-	Aph(Hor)-	pAph(Cbm)-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1346.50	1347.59
74 H ₂ N-	Cpa-	pCys-	Tyr-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1177.43	1178.43
827 H ₂ N-	Cpa-	pCys-	Tyr-	DTrp-	NMeLys-	Thr-Cys-	2Nal-	NH ₂		97	1191.45	1192.52
9 H ₂ N-	Cpa-	pCys-	L-Arg(NMe, benzoyl)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		95	1204.45	1205.51
10 H ₂ N-	Cpa-	pCys-	D-Arg(NMe, benzoyl)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		98	1204.45	1205.48
11 H ₂ N-	Cpa-	pCys-	Leu-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1127.45	1128.46
12 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		97	1219.45	1220.12
13 Cbm-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		96	1262.46	1263.40
14 DOTA-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		98	1605.64	1606.50
15 DOTA-β-Ala-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1676.67	1677.67
16 DOTA-Peg-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1809.62	1810.24
17 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	-	NH ₂		99	1022.37	1023.49
18 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	Cha-	NH ₂		96	1175.48	1176.36
19 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	Aph(Hor)-	NH ₂		99	1324.47	1325.55
20 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	pAph(Cbm)-	NH ₂		99	1227.45	1228.45
21 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	Aph(Cbm)-	NH ₂		99	1227.45	1228.37
22 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	pTrp-	Lys-	Thr-Cys-	pAph(Cbm)-	Gly-OH		98	1285.46	1286.34
23 H ₂ N-	Cpa-	pCys-	Aph(CONH-OCH ₃)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1249.46	1250.56
24 H ₂ N-	Cpa-	pCys-	Aph(CONH-OH)-	pTrp-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1235.44	1236.47
25 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	5F- <i>o</i> -Trp-	Lys-	Thr-Cys-	2Nal-	NH ₂		96	1237.44	1238.44
26 H ₂ N-	Cpa-	pCys-	Aph(Cbm)-	5F- <i>o</i> -Trp-	Lys-	Thr-Cys-	2Nal-	NH ₂		96	1237.44	1238.24
27 H ₂ N-	Cpa-	pCys-	Tyr-	pAph(Cbm)-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1196.43	1197.36
28 DOTA-	Cpa-	pCys-	Tyr-	pAph(Cbm)-	Lys-	Thr-Cys-	2Nal-	NH ₂		99	1582.62	1583.72
29 H ₂ N-	Cpa-	pCys-	Aph(Hor)-	pAph(Cbm)-	Lys-	Thr-Cys-	2Nal-	NH ₂		91	1335.47	1336.44
30 DOTA-	Cpa-	pCys-	Aph(Hor)-	pAph(Cbm)-	Lys-	Thr-Cys-	2Nal-	NH ₂		95	1721.65	1722.56
31 DOTA-	Cpa-	pCys-	Aph(Hor)-	pAph(Cbm)-	Lys-	Thr-Cys-	pTyr-	NH ₂		96	1687.64	1688.83
32 DOTA-	pNO ₂ -Phe-	pCys-	ITyr-	pTrp-	Lys-	Thr-Cys-	pTyr-	NH ₂		99	1666.52	1667.74

^a Percent purity determined by HPLC using buffer system: A = TEAP (pH 2.5) and B = 60% CH₃CN/40% A with a gradient slope of 1% B/min, at flow rate of 0.2 mL/min on a Vydac C₁₈ column (0.21 × 15 cm, 5-μm particle size, 300 Å pore size). Detection at 214 nm.

^b Capillary zone electrophoresis (CZE) was done using a Beckman P/ACE System 2050 controlled by an IBM Personal System/2 Model 50Z and using a ChromJet integrator. Field strength of 15 kV at 30 °C, mobile phase: 100 mM sodium phosphate (85:15, H₂O:CH₃CN) pH 2.50, on a Supelco P175 capillary (363 μm OD × 75 μm ID × 50 cm length). Detection at 214 nm.

^c The calculated m/z of the monoisotope compared with the observed [M + H]⁺ monoisotopic mass.

Table 2

Sst₁₋₅ Binding Affinities (IC₅₀s, nM) and Function of Sst₂-Selective Analogues

	IC ₅₀ (nM) ^a					Functional Characterization	
	sst ₁	sst ₂	sst ₃	sst ₄	sst ₅	sst ₂ - internalization	Calcium assay
SRIF-28	2.7 ± 0.2	2.7 ± 0.2	3.3 ± 0.4	2.6 ± 0.4	2.4 ± 0.2	-	-
1	>1000	3.6 ± 0.4	>1000	349 ± 30	276 ± 119	Antagonist	-
2	>1000	1.5 ± 0.4	>1000	287 ± 27	>1000	Antagonist	Antagonist
3	>1000	0.75 ± 0.2	>1000	>1000	>1000	Antagonist	Antagonist
4	>1000	2.6 ± 0.7	384 ± 97	>1000	>1000	Antagonist	-
5	>1000	1.3 ± 0.2	>1000	>1000	>1000	Antagonist	Antagonist
6	>1000	2.7 ± 0.6	451 ± 80	>1000	>1000	-	-
7	>1000	5.7 ± 1.5	112 ± 32	296 ± 19	218 ± 63	Antagonist	Antagonist
8	>1000	10 ± 3.5	61 ± 14	715 ± 137	53 ± 19	Antagonist	Antagonist
9	>1000	17 ± 5	827 ± 244	>1000	442 ± 254	Antagonist	-
10	>1000	158 ± 37	102 ± 10	116 ± 47	728 ± 272	-	-
11	>1000	58 ± 21	340 ± 77	908 ± 138	657 ± 299	-	-
12	>1000	6.9 ± 0.7	155 ± 29	479 ± 8	149 ± 37	Antagonist	Antagonist
13	>1000	23 ± 4.3	54 ± 15	136 ± 7.5	111 ± 17	-	-
14	>1000	9.8 ± 1.2	972 ± 212	831 ± 82	>1000	Antagonist	Antagonist
15	>1000	46 ± 13	124 ± 53	>1000	>1000	Antagonist	Antagonist
16	>1000	40 ± 1.5	88 ± 13	728 ± 158	895 ± 294	Antagonist	Antagonist
17	>1000	5.9 ± 1.8	138 ± 52	>1000	461 ± 106	Antagonist	-
18	>1000	4.1 ± 0.9	255 ± 79	>1000	247 ± 66	Antagonist	Antagonist
19	>1000	27 ± 3.8	162 ± 19	>1000	320 ± 69	-	-
20	>1000	5.4 ± 1	328 ± 69	800 ± 295	191 ± 49	-	-
21	>1000	15 ± 3	336 ± 46	551 ± 151	560 ± 144	-	-
22	>1000	52 ± 4.7	661 ± 115	>1000	810 ± 200	-	-
23	>1000	9.3 ± 0.9	157 ± 49	883 ± 174	313 ± 35	-	-
24	>1000	9.3 ± 1.4	120 ± 45	813 ± 152	426 ± 189	-	-
25	>1000	4.9 ± 1.5	50 ± 5.8	287 ± 64	94 ± 34	-	-
26	>1000	23 ± 3.7	90 ± 11	905 ± 132	618 ± 248	Antagonist	Antagonist
27	>1000	3.7 ± 1.3	346 ± 81	>1000	>1000	-	-
28	>1000	1.4 ± 0.5	>1000	>1000	>1000	Antagonist	Antagonist
29	>1000	2.4 ± 0.6	83 ± 2.0	>1000	>1000	Antagonist	Antagonist
30	>1000	1.7 ± 0.2	>1000	>1000	>1000	Antagonist	Antagonist
31	>1000	0.7 ± 0.12	>1000	>1000	>1000	Antagonist	Antagonist
32	>1000	1.2 ± 0.4	>1000	455 ± 125	>1000	Antagonist	Antagonist

^aThe IC₅₀ values (nM) were derived from competitive radioligand displacement assays reflecting the affinities of the analogues for the cloned SRIF receptors using the non-selective [Leu⁸,DTrp²², 125I-Tyr²⁵]SRIF-28, as the radioligand. Mean value ± SEM when n ≥ 3.