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Exploration of the DTrp-NMeLys Motif in the Search for Potent Somatostatin Antagonists

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Abstract—Previous studies from this laboratory demonstrated that *N*-methylation at Lys[5] residue in somatostatin octapeptide antagonist analogues increased the GH release inhibition potency by as much as 300%. We have now further investigated *N*-methylation of this Lys[5] residue in conjunction with a number of N- and C-terminal modifications previously found to give highly potent somatostatin receptor antagonists. Synthetic analogues were tested in a functional assay for their ability to inhibit somatostatin-inhibited GH release from rat pituitary cells in culture and to displace ^{125}I-labeled somatostatin from CHO cells transfected with the five known human somatostatin receptors. Several interesting observations resulted from the study. Replacement of liphophilic Nal[8] at the C-terminus with a hydrophilic His[8] resulted in the increased affinity and selectivity for type 2 receptor to give the most potent antagonist analogue yet discovered (K_i, 1.5 nM), although in the rat pituitary cells inhibitory activity on somatostatin inhibited GH release decreased somewhat. A His[3] substitution within the cyclic portion of the analogues retained pituitary cell potency and affinity for type 2 receptor as did substitution with Bip[8] and Fpa[1]. Replacement of Cpa[1] with Iph[1] did not effect the affinity for type 2 receptor significantly, but did decrease the effects on rat cell GH release. Iph[3] within-ring substitution increased the selectivity for sst_2 appreciably although the affinity for that receptor was considerably decreased. Substitution of Npa[3] resulted in good selectivity for sst_2 receptor. Replacement of Nal[8] with D-Trp[8] also increased the selectivity for type 2 receptor. Use of a 'bivalent ligand' approach in which two peptides were joined by 4,4′-biphenyldicarbonyl as a spacer destroyed the affinity for all the subtypes, however, the bivalent ligand formed with the Ahp spacer displayed significant affinity and high selectivity for the type 2 receptor.

Introduction

Naturally occurring somatostatin (SRIF), is a tetradecapeptide originally discovered by Brazeau et al.[1] and it has been shown to have potent inhibitory effects on many secretory processes in tissues such as pituitary, pancreas or gastrointestinal tract. SRIF also acts as a neuromodulator in the central nervous system.[2] These biological effects, which are thus far all inhibitory in nature, are elicited through a G-protein coupled receptor mechanism(s), and five different receptor subtypes have been characterized (sst_1–sst_5).[3] These all have similar affinities for the endogenous SRIF ligands but have differing distribution in various tissues.[4] The development of potent, smaller SRIF agonists led to the discovery of differing affinities of the various truncated ligands for the different subtypes.[5,6] It has been demonstrated that only the Trp[4]–Lys[5] residue pair is required for receptor recognition and bioactivity.[7–9] This crucial dipeptide unit forms part of a β-bend which is usually stabilized via substitution of D- for L-Trp, cyclization of the backbone, a disulfide bridge, or all constraints.[10] One unintended consequence of such structural simplification, carried out before the discovery of multiple receptor subtypes, was the loss of broad spectrum binding affinity. This is typified by the high type 2 but low type 1, 3, 4, and 5 affinities of peptides in the octreotide (Sandostatin®) series.[11] Thus, the many basic biological studies with this type of analogue failed to detect effects mediated by all but one of the somatostatin receptor types. Since then, much work in our laboratory has gone into the re-introduction of broader spectrum binding[12] into small, biologically stable peptides on the one hand and the development of peptides and peptidomimetics[13] with discrete specificity for a particular receptor, on the other.

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Thus far, of the five receptor subtypes for somatostatin, only sst_2 and sst_5 have been associated with specific physiological functions. Type 2 has a predominant role in mediating the release of GH as evidenced by positive correlation between the binding affinities of a large number of small agonist analogues and their ability to inhibit GH secretion from cultured rat anterior pituitary cells. Inhibition of insulin secretion is thought to be mediated through sst_5.[14] However, elucidating the physiological role of each of the receptor subtypes would be greatly enhanced by the development of receptor subtype-specific antagonists. Also, SRIF antagonists might provide a novel route to increasing endogenous levels of some hormones, notably growth hormone, insulin, and several gastrointestinal hormones.[15]

One interesting approach, just made easier with new solid-phase chemistry,[16] was the introduction of peptide backbone constraint imposed by *N*-alkylation. This could yield useful information about bioactive conformation since *N*-methyl amino acid substitutions have often been used to increase the potency and/or selectivity of a peptide ligand[17,18] due to the steric bulkiness of the methyl group and also removal, of intramolecular hydrogen bonding points. *N*-Methylation also stabilizes an analogue towards proteolytic degradation.

With respect to somatostatin analogues, previous studies[19] from this laboratory revealed that methylation at both D-Trp^4 and -Lys^5 produced a number of interesting effects on receptor affinity and selectivity and in this report we focus on *N*-Me–Lys^5 antagonistic structures.

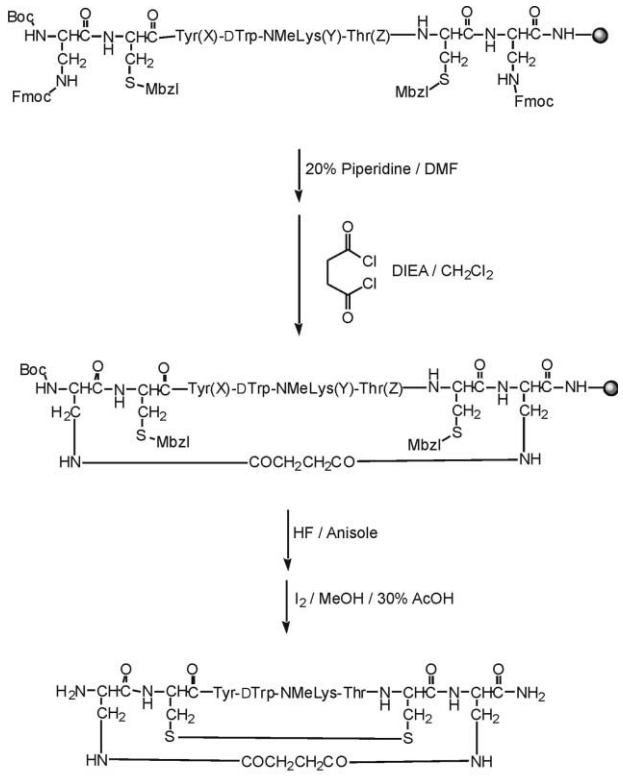
Results and Discussion

The search for synthetic peptide analogues of somatostatin (SRIF) which exhibit selective affinities for the five known receptor subtypes (sst_{1-5}) has generated a large number of potent agonists. Some of these agonists display good affinities and subtype selectivities for types 1, 2, 3, and 5, including analogues created by *N*-methyl amino acid substitutions in a standard octapeptide analogue format. A previous report from this laboratory demonstrated that the somatostatin analogue, Cpa-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-Nal-NH_2 had the highest anti-somatostatin potency (0.5 nM) at rat pituitary cells reported in the literature at that time and it was about 3 times more potent than its unmethylated congener. Therefore, the high potency of the above peptide was attributed to *N*-MeLys^5 residue, which seems to slightly perturb the conformation of the peptide at the active site. The presence of *N*-MeLys^5 residue in conjunction with some other previously successful sequence alterations was, therefore, further explored in a search for potent and/or additionally selective somatostatin antagonists.

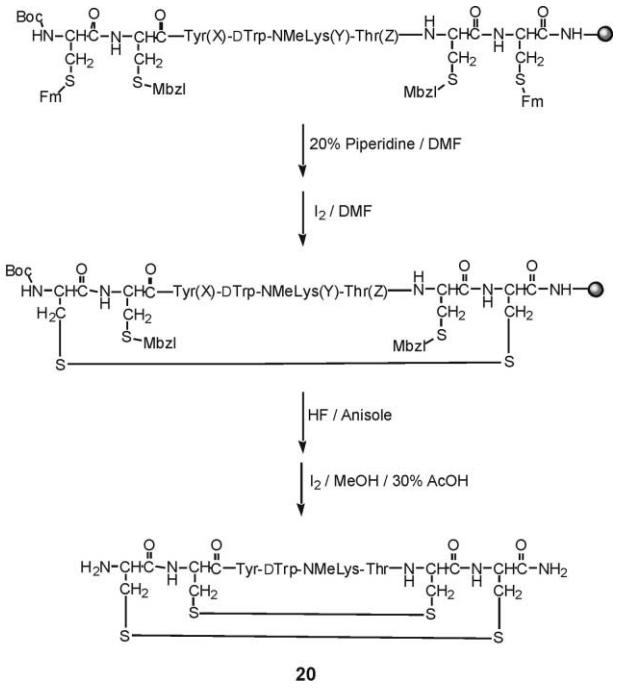
Synthetic analogues were tested for their ability to inhibit somatostatin-inhibited GH release from rat pituitary cells in culture and to displace ^{125}I-labeled somatostatin from CHO cells transfected with the five known human somatostatin receptors. Several interesting observations resulted from the study. Replacement of liphophilic Nal^8 at the C-terminus with a hydrophilic His^8 (**1**) resulted in increased affinity and selectivity for type 2 receptor, this was unexpected since prior successful substitutions at this position appeared to indicate that hydrophobic aromatic amino acids were preferred. This increased type 2 affinity did not translate into higher functional antagonism at rat pituitary cells and possibly reflects slight species differences in ligand-receptor binding affinities. Pal^3 substitution (**2**) in analogue **1** also resulted in high type 2 binding affinity. Replacement of Thr^6 with the hydrophobic Abu^6 (**3**) resulted in considerable loss of affinity for type 2 and concomitant decreased GH release potency. When the C-terminus of **1** was replaced by Tyr^8 (**4**), it resulted in high selectivity for sst_2, but with decreased affinity and potency. Substitution of Bip^8 and Fpa^1 (**5**) resulted in the high type 2 selectivity and very high potency at the rat pituitary cells which clearly seem to favor neutral, hydrophobic aromatic amino acids at the C-terminus. Thr^8 and Tyr^3 substitution in **3**, gave the peptide (**6**) with increased affinity for the type 2 receptor and also with better rat GH release potency. Substitution of Nal^1 (**7**) in **6** turned the peptide into a weak agonist although its affinity for the type 2 receptor only halved. Replacement of Cpa^1 with Iph^1 (**8**) did not affect the affinity for the type 2 receptor much, but it decreased rat GH release potency by 6-fold. His^3 substitution (**9**) in the previously reported most potent peptide sequence (Cpa-cyclo[DCys-Tyr-DTrp-*N*-MeLys-Thr-Cys]-Nal-NH_2) retained the potency and affinity for the type 2 receptor with little decrease in the selectivity. However, a large halogen substituted aromatic amino acid, Iph^3 (**10**) decreased the affinity and potency for GH release by about 20-fold. Npa^3 replacement (**11**) had increased selectivity for sst_2 with half the potency of the original peptide. Substitution of Cpa^3 (**12**) resulted in decreased affinity and potency just like in **10**. C-Terminus D-Trp^8 replacement (**14**) decreased the type 2 affinity and potency by 50%. Apa^3 substitution (**13**) retained the affinity for type 2, but lost 3-fold rat GH release potency. In an extended cyclic system, methylation at Lys^5 yielded the analogue **16** with high selectivity for type 3 receptor. However, *para* amino substitutions (**15**) in the phenylalanine residues of **16** resulted in the total loss of affinity for type 3 receptor.

Conformational restriction, imposed by a second cyclization (Scheme 1) of the side chains of Dap residues at both the N- and C-terminus using the succinic moiety as a linker (**17**) destroyed the affinity for all the subtypes. Appreciable loss of affinity was also observed for analogues that had been cyclized using succinyl and maleinyl moieties as in **18** and **19**. Bicyclic analogue **20** was synthesized by a new selective on-resin cyclization of C- and N-terminus Cys residues (Scheme 2). Biological evaluation of this analogue **20** revealed that it had poor affinity for the type 2 receptor.

Having studied the effect of substitution of various residues at both the sides of active center and also conformational restriction by bicyclic analogue formation,



Scheme 1. Schematic representation of cyclization of Dap side chain using succinyl moiety; X = 2,6-dichlorobenzyl; Y = 2-chlorobenzyloxycarbonyl; Z = benzyl.



Scheme 2. Schematic representation of selective oxidation of Cys residues; X = 2,6-dichlorobenzyl; Y = 2-chlorobenzyloxycarbonyl; Z = benzyl.

it was decided to begin to explore the effect of dimerization of the peptide ligands using linkers of varied flexibility and hydrophilicity. This strategy has been used often to increase the selectivity and potency for a chosen target[20,21] receptor. In the first attempt, 4,4-biphenyldicarboxylic acid was used to generate the bivalent peptide ligand **22** on the resin. Biological evaluation revealed that **22** lost affinity for all the subtypes. In another approach towards generating a bivalent ligand, the C-terminus of one pharmacophore was connected to the N-terminus of the other using 7-aminoheptanoic acid as a linking agent. The generated bivalent peptide ligand **23** bound to type 2 receptor with only moderate binding affinity but good type 2 selectivity thus suggesting that the correct choice of linking group and spacing might well eventually produce potent and selective constructs.

Conclusions

Of all the N- and C-terminal aromatic amino acids necessary for the conversion of somatostatin agonists to potent antagonists, His[8] at the C-terminus results in the highest affinity analogue yet found for the human sst_2 receptor. Although potent in the functional rat receptor assay, this analogue was not as effective as several previously synthesized analogues containing neutral, hydrophobic aromatic amino acids in this position, suggesting small species differences in receptor/ligand interactions. Other new analogues were found, however, to inhibit somatostatin effects on rat pituitary cell GH release with subnanomolar IC_{50} values and several of these selective and potent type 2 antagonists should provide useful tools for the investigation of somatostatin functions in animal models. Some of the most potent analogues are also potential drug candidates, for instance, for children with growth deficiency and elderly patients with GH deficiency caused by increased hypothalamic somatostatin release.

Experimental

Abbreviations

The nomenclature for the somatostatin receptor subtypes is in accordance with the recommendations of IUPHAR,[22] in which sst_4 refers to the receptor originally cloned by Bruno et al.,[23] and sst_5 refers to the receptor cloned by O'Carroll et al.[24] Abbreviations of the common amino acids are in accordance with the recommendations of IUPAC-IUB.[25] Additional abbreviations: Ahp, 7-aminoheptanoic acid; Cpa, 3-(4-chlorophenyl)alanine; Dap, 2,3-diaminopropionic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCM, dichloromethane; DIC, diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMF, dimethylformamide; Fm, flourenylmethyl; MTBD, 1,3,4,6,7,8-hexahydro-1-methyl-2H-pyrimido[1,2-a]pyrimidine; Nal, 3-(2-naphthyl)alanine; *o*-NBS, 2-nitrobenzenesulfonyl; Pal, 3-(3-pyridyl)alanine; TBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*,*N*-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Tfm, 4-trifluoromethylphenylalanine.

Table 1. *N*-Methyl analogue structures and analytical data

No.	*N*-Me Sequence	Mass spectrum (M $-H^+$)		HPLC
		Calcd[a]	Obsd[b]	(t_{R-1})[c]
1	Cpa-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-His-NH_2	1132.8	1133.4	12.5
2	Cpa-cyclo(DCys-Pal-DTrp-NMeLys-Thr-Cys)-His-NH_2	1118.6	1119.0	5.3
3	Cpa-cyclo(DCys-Pal-DTrp-NMeLys-Abu-Cys)-His-NH_2	1102.6	1102.7	6.6
4	Cpa-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-Tyr-NH_2	1158.8	1156.2	31.8
5	Fpa-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-Bip-NH_2	1203.3	1202.6	21.6
6	Cpa-cyclo(DCys-Tyr-DTrp-NMeLys-Abu-Cys)-Thr-NH_2	1080.7	1082.4	19.6
7	Nal-cyclo(DCys-Tyr-DTrp-NMeLys-Abu-Cys)-Thr-NH_2	1096.3	1097.5	20.8
8	Iph-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1284.3	1284.3	26.3
9	Cpa-cyclo(DCys-His-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1166.8	1167.1	19.5
10	Cpa-cyclo(DCys-Iph-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1302.7	1303.2	34.9
11	Cpa-cyclo(DCys-Npa-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1221.9	1222.2	30.0
12	Cpa-cyclo(DCys-Cpa-DTrp-NMeLys-Thr-Cys)-Nal-NH_2	1211.3	1211.2	32.3
13	Cpa-cyclo(DCys-Apa-DTrp-NMeLys-Thr-Cys)-DTrp-NH_2	1180.9	1181.6	16.1
14	Cpa-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-DTrp-NH_2	1181.8	1182.5	22.1
15	Cyclo(DCys-DApa-Tyr-DTrp-NMeLys-Thr-Apa-Cys)-NH_2	1138.4	1139.9	6.0
16	Cyclo(DCys-DPhe-Tyr-DTrp-NMeLys-Thr-Phe-Cys)-NH_2	1108.4	1109.9	17.8
17	Cyclo[Dap(suc)cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-Dap]-NH_2	1070.2	1069.8	11.6
18	Cpa-cyclo(DDap(suc)-Pal-DTrp-NMeLys-Thr-Dap)-Nal-NH_2	1228.8	1228.3	15.8
19	Cpa-cyclo(DDap(maleinyl)-Pal-DTrp-NMeLys-Thr-Dap)-Nal-NH_2	1226.6	1227.2	17.2
20	Cyclo[Cys-cyclo(DCys-Tyr-DTrp-NMeLys-Thr-Cys)-Cys]-NH_2	1018.3	1018.8	13.5
21	Cyclo[Cys-cyclo(DCys-Pal-DTrp-NMeLys-Thr-Cys)-NMeDCys]-NH_2	1018.1	1018.1	4.9
22	4.4′-Biphenyldicarbonyl-(NH-X)$_2$	2591.9	2591.1	42.8
23	Y-Ahp-Z	2495.9	2494.8	37.7

[a]Theoretical molecular weight (M$-H^+$, Da).
[b]Observed molecular weight (M$-H^+$, Da).
[c]Reversed-phase HPLC (C-18, 5 μm, 4.6×250 mm, λ = 215 nm) retention times (min). Each compound was found to have a purity of >98% by HPLC.
[c]HPLC elution system: (A); 0.1% TFA; (B); 0.1% TFA in 80% MeCN, 20% B to 80% B at 1% min^{-1} and 1.5 mL min^{-1}. X = Cpa-Cyclo[DCys-Tyr-DTrp-NMeLys-Thr-Cys]-Nal-NH_2. Y = Nal-cyclo[Cys-Thr-NMeLys-DTrp-Tyr-DCys]-Cpa. Z = Cpa-Cyclo[DCys-Tyr-DTrp-NMeLys-Thr-Cys]-Nal-NH_2.

Materials

4-Methylbenzhydrylamine hydrochloride resin (0.25 or 0.5 mequiv g^{-1}) was obtained from Advanced ChemTech Inc., Louisville, KY, USA. N^{α}-*tert*-Butyloxycarbonyl (Boc) protected amino acids were purchased from Bachem Inc., Torrance, CA, USA, Advanced ChemTech Inc., and Synthetech Inc., Albany, OR, USA. The reactive side chains of the amino acids were masked with one of the following groups: Cys, 4-methylbenzyloxycarbonyl; Lys, 2-chlorobenzyloxycarbonyl; Thr, *O*-benzyl; Tyr, *O*-2,6-dichlorobenzyl. All reagents and solvents were ACS grade or better and used without further purification.

Peptide synthesis

The somatostatin antagonists were assembled on 4-methylbenzhydrylamine functionalized, 1% cross-linked polystyrene resin (0.25 or 0.5 mequiv g^{-1}), in 0.25 mmol scale on a CS Bio (model 136) synthesizer, using the following protocol: deblocking, 35% TFA (2 min, 20 min); DCM wash cycle (three washes); neutralization, 10% DIEA (1 min, 5 min); DMF wash cycle; DCM wash cycle (two washes); double coupling; first with 1,3-diisopropyl carbodiimide esters (3 equiv), 30 min in DCM; DCM wash (three washes); second coupling with preformed TBTU esters (3 equiv), 90 min in DMF, with a catalytic amount of DIEA; DMF wash (one wash); DCM wash (three washes). Coupling reactions were monitored qualitatively.

Selective oxidation of cys residues (20 and 21)

The peptides were assembled on MBHA resin as described above. The side-chain protecting group, Fm was selectively removed by agitating the resin with 20% piperidine in DMF for 4–6 h. The resin was thoroughly washed with DMF and DCM (twice). It was again suspended in DMF. To this suspension was added dropwise a 10% iodine solution in MeOH until a persistence of yellow color was maintained. It was shaken for 15 min and then the resin was washed with DMF followed by DCM (two washes).

*N*α Protection

After deblocking the amino group at the desired methylation site, the resin was suspended in DCM (20 mL). To this suspension, collidine (3 equiv) and *o*-nitrobenzenesulfonyl chloride (3 equiv) were added and the mixture was shaken using Advanced ChemTech (model 200) synthesizer for 2 h. Then the resin was subjected to DCM wash (two washes) and DMF wash (three washes). Protection was monitored by qualitatively by the ninhydrin test.

*N*α Methylation

The *o*-nitrobenzenesulfonamide protected resin was suspended in DMF (20 mL), to which MTBD (3 equiv) and methyl 4-nitrobenzenesulfonate or dimethyl sulfate (for Cys[11]) were added. The mixture was shaken using

Advanced ChemTech (model 200) synthesizer for 0.5 h and the resin was subjected to DMF wash (four washes).

Deprotection

Once the desired residue was methylated, the resin was again suspended in DMF (20 mL). DBU (3 equiv) and 2-mercaptoethanol (3 equiv) were added to the suspension and the mixture was agitated for 0.5 h in Advanced ChemTech (model 200) synthesizer. Then the resin was thoroughly washed with DMF (five washes).

Peptide cleavage

The peptides were cleaved from the resin support with simultaneous side-chain deprotection by acidolysis using anhydrous hydrogen fluoride containing the scavenger anisole (~30% v/v) for 45 min at 0 °C. The peptides were cyclized in 90% acetic acid (~600 mL) with a slight excess of I_2 (15 min). Excess I_2 was then removed by the addition of ascorbic acid.

Purification

The crude peptides were purified by preparative RP-HPLC on C-18 bonded silica gel using axial compression columns (Dynamax-300 Å, 5 or 8 μm, 21.4×250 mm). A linear gradient elution system at a flow rate of 20 mL min^{-1} was employed: A; 0.1% TFA, B; 0.1% TFA in 80% MeCN, 20% B to 50% B at 1% min^{-1}. The separations were monitored by analytical RP-HPLC at 215 nm. The fractions containing the product were pooled, concentrated in vacuo and subjected to lyophilization. Each peptide was obtained as a fluffy white powder of constant weight by lyophilization from aqueous acetic acid. The purity of the final peptides was assessed at 215 nm by analytical RP-HPLC. Analytical RP-HPLCs were recorded using a Vydac C-18 support (4.6×250 mm, 5 μm, 300 Å pore size, Liquid Separations Group). The linear gradient system was used at a flow rate of 1.5 mL min−^{1}: HPLC, A, 0.1% TFA; B, 0.1% TFA in 80% MeCN; 20% B to 80% B at 1% min−^{1}. Column eluent was monitored at 215 nm. The retention time and purity of each peptide was assessed by the Rainin Dynamax HPLC Method Manager. Each peptide was found to have a purity of ≥98%. The HPLC retention time results are given in Table 1.

Amino acid analysis

The peptides were hydrolyzed in vacuo (110 °C; 20 h) in 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole (Pierce). Amino acid analyses were performed on the hydrolyzates following derivatization with *o*-phthaldialdehyde reagent (Sigma Chemical Co.) using an automatic HPLC system (Rainin Instrument Co.) fitted with a 100×4.6 mm, 3 μm C18 axial compression column with integral guard column (Microsorb AAAnalysis™, Type O; Rainin Instrument Co.) The derivatized primary amino acids were eluted using a binary gradient of buffer A; 0.10 M sodium acetate containing 4.5% v/v methanol and 0.5% v/v tetrahydrofuran at pH 7.2 and buffer B; methanol. The gradient sequence; 0% A at 0 min; 35% A at 16.5 min; 90% A at 30 min and 90% A at 33 min was used with a flow rate of 1.0 mL min^{-1} at ambient temperature. Eluent was monitored at 340 nm and integrated by the Dynamax HPLC Method Manager (Rainin). Standard retention times were as follows: Asp, 6.6 min; Arg, 19.9 min; Trp, 25.4 min and Lys, 29.5 min. Each peptide produced the expected analytical results for the primary amino acids. Cysteine was not quantified. (Results not shown.)

Mass spectrometry

The peptides were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry using a LaserMat 2000 mass spectrometer (Thermal Bioanalysis, San Jose, CA, USA) using α-cyano-4-hydroxycinnamic acid as the matrix with Substance P (1348.7 Da) as an internal standard. In each case, the spectra consisted of a major $M-H^+$ ion peak for the internal standard, the expected analyte $M-H^+$ peak, and a few peaks associated with the matrix (<500 Da). The results are given in Table 1.

Antagonism of in vitro SRIF inhibition of GH release

Anterior pituitaries from adult male rats were collected and dispersed by a previously described trypsin/DNase method.[26] The dispersed cells were diluted with sterile-filtered Dulbecco's modified Eagle medium (MEM, Gibco Laboratories, Grand Island, NY, USA), which was supplemented with 2.5% fetal calf serum (Gibco), 3% horse serum (Gibco), 10% fresh rat serum (stored on ice for no longer than 1 h) from the pituitary donors, 1% MEM nonessential amino acids (Gibco), gentamycin (10 ng mL^{-1}; Sigma) and nystatin (10,000 U mL^{-1}; Gibco). The cells were randomly plated at a density of approximately 200,000 cells/well (Costar cluster 24; Rochester Scientific Co., Rochester, NY, USA). The plated cells were maintained in the above Dulbecco's medium in a humidified atmosphere of 95% air/5% CO_2 at 37 °C for 4–5 days. In preparation for a hormone challenge, the cells were washed with medium 199 (Gibco, 3×1 mL). Each dose of analogue (6 doses/plate) was tested in the presence of SRIF (1 nM) in triplicate wells in a total volume of 1 mL medium 199 containing 1% BSA (fraction V; Sigma Chemical Co.). All wells contained GHRH(1–29)NH_2 (1 nM). A GHRH(1–29)NH_2 (1 nM) stimulated control group and an SRIF (1 nM) with GHRH(1–29)NH_2 (1 nM) inhibited control group were included on each cell culture plate. After incubation in an air/carbon dioxide atmosphere (95/5%, 3 h at 37 °C), the medium was removed and stored at −20 °C until assayed for hormone content. Growth hormone in the media was measured by a standard double antibody RIA using components generously supplied by the NHPP, NIDDK, NICHHD and USDA. Antagonist IC_{50}'s versus SRIF (1 nM) were calculated using Sigmaplot (Jandel Scientific, San Rafael, CA, USA). Values are expressed as the mean IC_{50} (nM)±SEM from (*n*) separate dose–response curves and are given in Table 2.

Table 2. Binding affinities (K_I) for the cloned human sst_{1-5} receptors and antagonist data

Peptide no.	K_I[a] ± SEM (nM)					Antagonist[b] IC_{50} ± SEM (nM)
	$hsst_1$	$hsst_2$	$hsst_3$	$hsst_4$	$hsst_5$	
SRIF-14	2.0±0.35	0.25±0.03	1.2±0.2	2.0±0.3	1.4±0.3	N/A[c]
SRIF-28	1.9±0.4	0.31±0.06	1.3±0.3	5.4±2.5	0.4±0.1	N/A[c]
1	1000	1.48±0.6	124.9	1000	226±15	10.0±2.6
2	1000	5.57±0.56	58	nd[d]	1000	13.0±0.6
3	1286	24.8±2.2	106	nd[d]	1000	83±9
4	1000	26.3±5.6	1000	1000	1000	70.8±34
5	1000	8.05±3.1	336	1000	229	1.1±0.0
6	1000	16.5±1.78	114±13.5	1000	226±108	23.4±0.7
7	1000	35±8.2	198±13.5	364	96.4±23.1	Weak agonist
8	1000	6.83±0.56	85.25±36.75	1000	48.3±16.3	3.5±1.6
9	1000	6.07±0.27	36.8±3.2	1000	51.1±8.2	0.9±0.3
10	1000	86.0.±33	439±32	1000	1000	11±3.3
11	1000	24.5±13.5	109±20	1000	441	1.1±0.3
12	1000	56.4±12.2	195±69	1000	533	12.0±5.3
13	1000	4.42±1.2	99.5±18.5	1000	37.9	1.6±0.4
14	1000	12.4±6.2	129±27	1000	267	0.9±0.1
15	1000	393±131	1000	1000	1000	nd[d]
16	1000	581	38.9	1000	1000	nd[d]
17	1000	473	1000	914	1000	nd[d]
18	1000	1000	352	1000	1000	nd[d]
19	1000	390±136	1000	1000	552	nd[d]
20	1000	301±170	1000	837±162	1000	nd[d]
21	1000	1000	1000	1000	1000	nd[d]
22	1000	830±24	1000	1000	1000	nd[d]
23	1000	34.7±3.6	137±8	1000	1000	24.9±4.1

[a]Expressed as the mean±SEM, single values indicate the results of one binding experiment.
[b]Rat in vitro antagonist IC_{50} (nM) versus SRIF (1.0 nM), expressed as the mean±SEM of (*n*) separate dose–response curves.
[c]Not applicable.
[d]Not determined.

Functional expression of the cloned human somatostatin receptors

The genomic clones containing the human somatostatin receptors ($hsst_{1-5}$)[27–30] were kindly provided by Dr. Graeme I. Bell (University of Chicago). The $hsst_1$, $hsst_2$, $hsst_3$, $hsst_4$ and $hsst_5$ cDNAs were isolated as a 1.5-kb *Pst*I-*Xmn*I fragment, 1.7-kb *Bam*HI-*Hin*dIII fragment, 2.0-kb *Nco*I-*Hin*dIII fragment, 1.4-kb *Nhe*I-*Nde*I fragment, and a 1.2-kb *Hin*dIII-*Xba*I fragment, respectively, each containing the entire coding region of the full-length receptors. These fragments were independently subcloned into the corresponding restriction endonuclease sites in the mammalian expression vector pCMV5, downstream from the human cytomegalovirus (CMV) promoter, to produce the expression plasmids pCMV5/$hsst_1$, pCMV5/$hsst_2$, pCMV5/$hsst_3$, pCMV5/$hsst_4$ and pCMV5/$hsst_5$. For transfection into CHO-K1 cells, a plasmid, pRSV-neo (American Type Culture Collection, Rockville, MD, USA), carrying the neomycin mammalian cell selectable marker was added.

Receptor expression and transfection

Transfections were performed by the calcium phosphate method. CHO-K1 cells were maintained in α-minimum essential medium (α-MEM; Gibco) supplemented with 10% fetal calf serum and transfected with each of the expression plasmids using calcium phosphate precipitation. Clones that had inherited the expression plasmid were selected in α-MEM supplemented with 500 μg mL^{-1} of geneticin (G418; Gibco). Independent CHO-K1 clones were picked by glass-ring cloning and expanded in culture in the selective media. Membranes were prepared from the isolated clones and hsst expression was initially assessed for binding with [^{125}I]Tyr^{11}-SRIF and [^{125}I]MK-678 (for sst_2).

Radioligand binding assays

Cell membranes of the five receptor types were obtained from homogenates (Polytron setting 6, 15 s) of the corresponding CHO-K1 cells, in ice-cold Tris–HCl (50 mM) and centrifuged (39,000*g*, 10 min×2), with an intermediate resuspension in fresh buffer. The final pellets were resuspended in Tris–HCl (10 mM) for assay. Aliquots of the membranes were incubated (30 min at 37 °C) with 0.05 nM [^{125}I]Tyr^{11}-SRIF (types 1,3,4,5) or [^{125}I]MK-678 (type 2) in 50 nM HEPES (pH 7.4) containing BSA (10 mg mL^{-1}); $MgCl_2$ (5 mM), Trasylol (200 kIU mL^{-1}), bacitracin (0.02 mg mL^{-1}), and phenylmethanesulfonyl fluoride (0.02 mg mL^{-1}). The final assay volume was 0.3 mL and incubations were terminated by rapid filtration through GF/C filters presoaked in 0.3% poly(ethylenimine) using a Brandel rapid filtration module. Each tube and filter was then washed with aliquots of cold buffer (3×5 mL).

Specific binding was defined as the total radioligand bound minus that bound in the presence of 1.0 μM SRIF. The following total radioligand binding and nonspecific binding (nsb) values were typically obtained with these assay systems: $hsst_1$, 7000 cpm total versus 3500 cpm nsb; $hsst_2$, 9000 cpm total versus 1000 cpm

nsb; $hsst_3$, 8000 cpm total versus 1000 cpm nsb; $hsst_4$, 6000 cpm total versus 3500 cpm nsb; and $hsst_5$, 7500 cpm total versus 3500 cpm nsb. The binding affinities expressed as K_i values ± SEM (nM) for each of the five receptor subtypes are given in Table 2.

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