Synthesis of a Substance P Antagonist with a Somatostatin Scaffold: Factors Affecting Agonism/Antagonism at GPCRs and the Role of Pseudosymmetry

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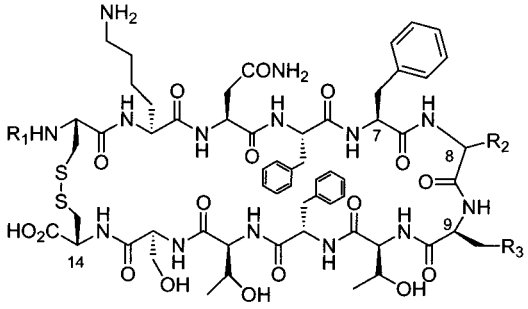
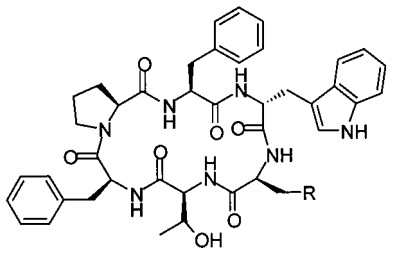
Introduction. Somatostatin-14 (SRIF, **1**; Figure 1) is a cyclic tetradecapeptide that was first isolated,



1 R_1= Ala-Gly, R_2 = L-Trp (sidechain), R_3 = -$(CH_2)_3NH_2$
2 R_1= Ala-Gly, R_2 = D-Trp (sidechain), R_3 = -$(CH_2)_3NH_2$
6 R_1= Ala-Gly, R_2 = D-Trp (sidechain), R_3 = *p*-F-Ph
7 R_1= H, R_2 = D-Trp (sidechain), R_3 = *p*-F-Ph
8 R_1= Ala-Gly, R_2= L-Trp (sidechain), R_3 = *p*-F-Ph

Figure 1. Analogues derived from SRIF.

characterized,[1] and synthesized at the Salk Institute.[2] Its bioactive conformation contains a β-turn composed of the tetrapeptide sequence -Phe^7-Trp^8-Lys^9-Thr^{10}-.[3,4] Replacement of Trp^8 by D-Trp^8, generating **2**, caused a 10-fold increase in potency,[5] attributed by Veber et al. to the stabilization of the bioactive conformation of the β-turn.[6] The importance of the β-turn was subsequently demonstrated through the elaboration of cyclic hexapeptides, such as L-363,301 (**3**), a potent, biostable SRIF agonist (Figure 2).[7] We synthesized **4**, the first SRIF peptidomimetic, where the β-D-glucose scaffold replaces the peptide backbone of **3**. Mimics of three of the four side chains of the β-turn of **3** (Phe^7, Trp^8, Lys^9) were attached to the sugar via ether linkages at positions 2, 1, and 6, respectively.[8] This glycoside displayed weak agonist affinity (15 μM) for somatostatin receptors on AtT-20 cells. Subsequently we generated more potent analogues (IC_{50} ca. 100 nM) at the human somatostatin receptor subtype 4 (hSSTR4).[9,10]



L-363,301 (**3**) R = -$(CH_2)_3NH_2$ (SRIF agonist)
(**5**) R = *p*-F-Ph (NK-1 antagonist)

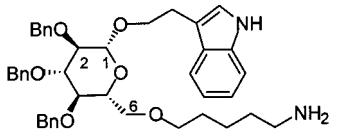


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Figure 2. A β-D-glucose peptidomimetic and cyclic hexapeptides.

Glycoside **4** was also a potent antagonist of substance P (SP) at the hNK1 receptor (IC_{50} 120 nM),[11] an unexpected observation at that time.[12−14] These results indicated that the β-glycoside interacts within the transmembrane binding domain for small molecules, which was found to be a common feature of G-protein coupled receptors (GPCRs).[15] We believe that glycosides, like cyclic hexapeptides, are *privileged structures* and thus able to bind several receptors.[16]

The unexpected binding of the peptidomimetic **4** at both the SSTR and NK1 receptors suggested that the topology of the binding sites of the two receptors must have significant similarities. Promiscuity in the binding of **4** to different receptors may, in part, be due to its pseudosymmetry, which confers on the sugar the freedom to adopt different binding orientations to fit diverse receptors (e.g., **4** is also a β_2-adrenergic blocking agent).[11] Earlier, we had reported that pseudosymmetry allows two closely related glycosides to bind given SSTRs differently.[8,9] Importantly, molecular modeling suggests that the binding to the NK1 receptor involves the C1 and C2 side chains of **4**, whereas the binding of the SRIF receptor involves C1 and C6.[17] Because the cyclic hexapeptides lack such pseudosymmetry, the potent peptidal SSTR agonist L-363,301 required an amino acid substitution (Lys^9 to *p*-F-Phe^9) to generate a potent hNK1 receptor antagonist **5** (IC_{50} 28 ± 14 nM).[18] The latter retained little or no affinity for the somatostatin receptors on AtT-20 cells.[18] From a broad screening perspective, these results show that a scaffold exhibiting pseudosymmetry can confer the potential advantage of polyvalency in the screening of a new compound in diverse assays.

Toward a better understanding of the factors determining agonism/antagonism at GPCRs, it was important to learn whether **6** and **7** (Figure 1), which incorporate the scaffold of the potent SRIF agonist **2**, would be agonists or antagonists at the NK receptors. Our successful conversion of **3** into the NK1 receptor ligand **5** did not, however, provide any assurance that **6** and/or **7** would also bind NK receptors since **5** is a

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cyclic hexapeptide, a structure considered privileged. Also the potential for steric hindrance was a concern with the larger peptides **6** and **7**. We synthesized **6** and **7**,[19] in the hope that the β-turn of these cyclic tetradeca- and dodecapeptides would bind in the conserved ligand-binding site of the transmembrane region, with the end opposite to the β-turn projecting toward the loops.

Results and Discussion. Pleasingly we found that **6** and **7** do indeed bind the hNK1 receptor,[20] albeit with lesser affinity than **5** (Table 1),[21] probably reflecting an unfavorable interaction due to the larger size of the ligand. On the other hand, since much of the sequence variation between receptor subtypes occurs in the loop regions,[22] the larger cyclic peptides (i.e., **6** and **7**) held the promise of modulating the receptor subtype binding affinity pattern. This proved to be the case since compounds **6** and **7**, unlike **5**, are highly potent ligands at the hNK2 receptors (Table 1).[23] It is of interest that D-Trp[8] SRIF (**2**), unlike **1**, displays modest affinity at the hNK1 and hNK2 receptors, consistent with our earlier suggestion that SRIF and NK receptors incorporate similarities and that SRIF analogues can reach the loop regions of the SP receptors. Compounds **2** and **8** have strikingly similar binding patterns at the hNK1 and hNK2 receptors (see below, and Table 1).

Table 1. IC_{50} of SRIF and Its Analogues at NK Receptors[a]

peptide	hNK1	hNK2
1	did not bind	did not bind
2	7% inhib @ 1 μM	850 ± 170 nM
6[b]	220 ± 40 nM	27 ± 4 nM
7[b]	250 ± 30 nM	46 ± 3 nM
8[c]	44% inhib @ 1 μM	730 nM

[a] [^{125}I]SP binding to human NK1 or NK2 receptors stably expressed in CHO cells; determinations are means ± SD (n = 3). [b] Compounds **6** and **7** were antagonists as measured by the ability to shift the EC_{50} for SP-stimulated IP1 accumulation 4−6-fold to the right at 2 μM. [c] Agonism/antagonism not determined.

Having established that **6** and **7** bind the NK receptors permitted us to investigate the most important question of this research: whether **6** and **7** are agonists or antagonists of SP. Both compounds were found to be *antagonists* of SP at the hNK1 receptor, with no reduction in the maximal response to SP.[20] Neither **6** nor **7** demonstrated any agonist activity in the cells as measured by IP1 accumulation when tested at 2 μM in the absence of SP.

We also synthesized **8**,[19] the *p*-F-Phe[9] analogue of SRIF **1**, which displayed weak affinity for both hNK1 and hNK2 receptors (Table 1). The weak affinity at the hNK receptors did not allow us to obtain reliable results in functional assays to demonstrate that **8**, like **6** and **7**, is an antagonist. Compounds **6**−**8**, unlike **5**, revealed some, albeit low, binding affinities at hSSTR1−4,[24,25] presumably because all but one of the amino acids (i.e., Lys[9]) of **2** or **1** are present in **6**−**8**, respectively.

These results confirm that the SSTRs and NK receptors must have very similar binding regions in order to accommodate similar large scaffolds (e.g, **1**, **2**, **6**−**8**). Their opposite response to these peptides may be explained by differences in the mode of action of the two receptors. Indeed, a large number of analogues of **2** have been designed and synthesized based on the bioactive conformation of SRIF and the importance of the above-mentioned β-turn for binding to hSSTRs. These compounds, which include **3**, MK-678, octreotide, retro-enantio analogues,[26] and potent subtype-specific ligands, are agonists.[27,28] Application of the two receptor state[29,30] and the multiple receptor[31,32] state concepts teaches that the resting state of the SSTRs must therefore be close to the activated state. Attention has recently been drawn to receptors that have high affinity for a G-protein in the absence of agonists (precoupled receptors), which also show a proclivity for agonism. These include the adenosine A_1 receptor, the C_{5a} receptor, and others.[32] Such a preactivation is supported by the fact that the G-protein can in some cases be coeluted with the receptor[33,34] and is often typified by an agonist response of the receptor toward ligands of various scaffolds. We propose that the SSTRs are also precoupled to their G-proteins and that it is this lower energy state of the *receptor·G-protein complex* that induces the agonism exhibited by the above ligands. Although the SSTRs thus exist predominantly in an activated state, they are nevertheless also in an equilibrium with nonactivated states as called for by the two or multiple state receptor models. This is consistent with the scarce reports of SRIF antagonists.[35−37] For example, AC-178,335 was reported to have in vitro and in vivo properties consistent with SRIF antagonism,[38] which may reflect its greater affinity for the resting form than the precoupled form of the SSTRs.

In contrast, both agonists and antagonists have been reported for the NK receptor, and their interconversion can be effected much more readily. Indeed, SP, like SRIF, is an agonist at its receptors, but the former is readily converted into such antagonists as spantide, spantide II, and others.[39] Further, Ward et al. demonstrated that in the case of peptides incorporating a spirolactam moiety, agonism can be converted into antagonism at the NK1 receptor by reversing the chirality of the core spirolactam used as the element of constraint.[40,41] The propensity of many ligands to be NK receptor antagonists is not understood but must somehow reflect a greater affinity of the NK receptor ligands to bind the resting state of the receptors.

Because of the apparent ease of conversion of agonists to antagonists of the NK receptors through conformational changes, we also studied the NMR spectra of **6**−**8** to ascertain whether the conformation of the peptide in the β-turn region had been affected by the transformation of **2** into **6**. We found that Trp[8] shields the aromatic protons of *p*-F-Phe[9] of **6** in a manner analogous to that seen for Lys[9] in **2**.[42] Further, as in the case of **1** and **2**, this shielding is more pronounced for the D-Trp[8] diastereomers (**6** and **7**) than the L-Trp[8] diastereomer **8**. This indicates that it is unlikely that the conversion of **2** to **6** produced a significant change in the conformation of these respective β-turns which, per se, might have changed agonism, at the SSTRs, to antagonism at the hNK1 receptor.

Having shown that **6**−**8** could indeed bind the NK receptors as antagonists (for **6** and **7**), we also assessed whether these large peptides could reach the transmembrane binding site of the nonpeptide antagonist interacting with the residues on the NK1 receptor (e.g., His197, His265, Gln165). Such nonpeptidal NK1 receptor antagonists as CP-96,345 and **4** have reduced affinity for certain mutants of the NK1 receptor, for

Table 2. IC_{50} of SRIF Analogues at Mutant NK1 Receptors[a]

peptide	WT (NK1)	Q165A (NK1)	H197A (NK1)	H265A (NK1)
6	240 ± 40 nM	30% inhib @ 1 μM	950 ± 240 nM	6100 ± 500 nM
7	260 ± 20 nM	37% inhib @ 1 μM	900 ± 280 nM	9600 ± 3400 nM
8	1400 ± 200 nM	3600 ± 1600 nM	2700 ± 1500 nM	8200 ± 3000 nM
SP	1.4 ± 0.4 nM	1.7 ± 1.0 nM	0.9 ± 0.1 nM	1.1 ± 0.2 nM

[a] [^{125}I]SP binding to wild type (WT) or mutant NK1 receptors transiently expressed in COS cells; determinations are means ± SD (n = 3).

which the affinity of the agonist SP is unchanged. These observations have given rise to the suggestion that peptides such as SP and nonpeptidic antagonists such as CP-96,345 have separate binding sites[43] that appear to be overlapping.[44,45] We found that, taken together, the peptides **6**−**8**, unlike SP but like CP-96,345, displayed a diminished affinity for the mutant hNK1 receptors compared to the wild-type receptor (Table 2).[46] Thus, even though **6**−**8** are relatively large peptides, they access the binding site within the transmembrane domain of the hNK1 receptor.

As mentioned above, our results also show that, unlike cyclic hexapeptide **5** which is selective for the hNK1 receptor, the SRIF-derived analogues (**6** and **7**) are more potent at the hNK2 receptor. Little is known about the NK2 receptor and about factors determining NK subtype selectivity. Nevertheless, mutational analysis has shown that the first and second extracellular loops and the second transmembrane helix of the NK2 receptor are involved in the binding of SP-related agonists.[23] Further, comparison of NK1 and NK2 receptor sequences reveals many conserved residues in the three regions required for peptide agonist binding. The nonpeptide antagonist binding site of the NK1 and NK2 receptors are in the same region of the receptors but do not appear to be identical to each other.[47,48]

Conclusion. The results previously reported from these laboratories, combined with those described herein, while building on the multiple receptor state concepts, reveal also some of the subtle aspects determining agonism versus antagonism. Thus one single chemical entity, peptidomimetic **4**, is an SRIF agonist but binds also the NK1 receptor (but as an *antagonist*). That conversion of the SRIF agonist cyclic hexapeptide **3** into an NK1 receptor antagonist **5** required the replacement of Lys^9 by *p*-F-Phe^9 is now explained by the fact that cyclic hexapeptides lack the pseudosymmetry of **4**. This concept also has relevance in lead discovery. D-Trp^8 SRIF (**2**) itself has now been similarly converted into a SP antagonist, which however reveals a preference for the NK2 receptor. Our speculation,[11] that the SRIF and NK receptors have much in common, was validated by the discovery that unlike SRIF (**1**), D-Trp^8 SRIF (**2**) has modest affinity at the hNK2 receptor, comparable to that of **8**.

Previous studies showed that GPCRs have an evolutionarily conserved ligand binding site. We propose that this site provides regions that accommodate the projections of side chains from the exposed surface of β- and γ-turns and their components or mimics. The exposed elements are recognized because they are compact and provide stable interaction surfaces. Since they are also observed within well-packed proteins and in smaller peptides, they are likely to be stabilized through interactions with the receptor (induced fit).[49] In addition, other structural features, such as helices, may be recognized by a different class of receptors (e.g., Mdm2 recognizes the p53 helix).[50] We had reported earlier that **4** is also a β_2-adrenergic blocking agent.[11] Significantly, this provided the first direct evidence that there is a linkage between peptides and biogenic amines in signal transduction by GPCRs. It is this ability for GPCRs to recognize privileged ligand scaffolds that suggests that there are common binding motifs also inherent to the receptors, making them complementary to the *privileged platforms*.

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Supporting Information Available: General experimental procedure for the preparation of **6**−**8**, along with ^{1}H NMR, HRMS, and amino acid analysis data. This material is available free of charge via the Internet at http://pubs.acs.org.

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