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N-Imidazolebenzyl-histidine Substitution in Somatostatin and in Its Octapeptide Analogue Modulates Receptor Selectivity and Function

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Supporting Information

ABSTRACT: Despite 3 decades of focused chemical, biological, structural, and clinical developments, unusual properties of somatostatin (SRIF, **1**) analogues are still being uncovered. Here we report the unexpected functional properties of **1** and the octapeptide cyclo(3−14)H-Cys-Phe-Phe-Trp[8]-Lys-Thr-Phe-Cys-OH (somatostatin numbering; OLT-8, **9**) substituted by imBzl-L- or -D-His at position 8. These analogues were tested for their binding affinity to the five human somatostatin receptors (sst_{1-5}), as well as for their functional properties (or functionalities) in an sst_3 internalization assay and in an sst_3 luciferase reporter gene assay. While substitution of Trp[8] in somatostatin by imBzl-L- or -D-His[8] results in sst_3 selectivity, substitution of Trp[8] in the octapeptide **9** by imBzl-L- or -D-His[8] results in loss of binding affinity for $sst_{1,2,4,5}$ and a radical functional switch from agonist to antagonist.

Peptide	Receptor Selectivity	Functionality	
	Binding Assay	sst_3 Luciferase Reporter Gene Assay	sst_3 Internalization Assay
cyclo(3-14)H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp[8]-Lys-Thr-Phe-Thr-Ser-Cys-OH (Somatostatin, SRIF)	Non-selective	Agonist	Agonist
cyclo(3-14)H-Ala-Gly-Cys-Lys-Asn-Phe-Phe-[imBzl-L or D-His[8]]-Lys-Thr-Phe-Thr-Ser-Cys-OH	sst_3 selective	Partial agonist	Agonist
cyclo(3-14)H-Cys-Phe-Phe-Trp[8]-Lys-Thr-Phe-Cys-OH (SRIF numbering)	Non-selective	Partial agonist	Agonist
cyclo(3-14)H-Cys-Phe-Phe-[imBzl-L or D-His[8]]-Lys-Thr-Phe-Cys-OH (SRIF numbering)	sst_3 selective	Partial agonist	Antagonist

INTRODUCTION

Somatostatin (SRIF) was isolated from sheep hypothalami, sequenced, and synthesized 38 years ago,[1] and its physiological role is still the subject of extensive studies because of its strong affinity for the five receptor subtypes and the broad use of its analogues in the clinic.[2−5] Non-peptide SRIF receptor-selective agonists have been described, but none of them have reached drug status yet.[6] Peptide-based receptor-selective ligands have been characterized including sst_1-selective agonists,[7−9] sst_2-selective agonists and antagonists,[10−13] sst_3-selective antagonists,[14] sst_4-selective agonists,[15] and sst_5-selective ligands.[16,17] From structural studies, we were able to propose bioactive conformations for sst_1−sst_4,[15,18−20] while the group of M. Ginanneschi developed a new pharmacophore model for sst_5.[16]

It has been shown that amino acid (AA) deletions not affecting the potency of SRIF analogues are located at the N- and C-termini with the sequence -Phe[7]-Trp[8]-Lys[9]-Thr[10]- being the critical amino acids for receptor binding and biological activity. Whereas substitution of D-amino acids for L-amino acids may increase the conformational stability of peptides, it also improves metabolic stability toward enzymatic degradation.

Soon after SRIF characterization, SAR studies were initiated and [DTrp[8]] substitution that increased potency in vitro and in vivo was discovered.[21] Shortly after, we and others[22−27] described shortened octapeptide and hexapeptide analogues with the scaffolds shown in Figure 1B−D.

Among the five existing SRIF receptor subtypes, we focused on sst_3 for several reasons: (a) this receptor is characterized by very strong internalization capabilities;[28] (b) there are well characterized radioligand agonists (nonselective) that can label the sst_3 receptor in vitro and in vivo;[29−31] (c) selective antagonists with high binding affinity that do not trigger receptor internalization have been described[14] that may be derivatized and used as antagonist radioligands; (d) in vivo animal models with sst_2- or sst_3-expressing tumors have recently been developed in our laboratories.[12] Because the [DTrp[8]] substitution seemed so favorable in vitro and in vivo, other aromatic residues were introduced in position 8.[14,15,32−34] We report here the binding affinities of a number of SRIF analogues with substitutions at position 8 within the scaffolds shown in Figure 1A and Figure 1B, respectively. We also report that these substitutions influence receptor selectivity and function (agonist versus antagonist).

RESULTS AND DISCUSSION

Understanding the mechanism of action and physiological functions of SRIF and its interactions with SRIF receptors is critical to the process of SRIF-based drug discovery and development. Studies carried out in our laboratory and those of others have led to the identification of sst-selective SRIF agonists and antagonists that can be used for structural, biochemical, and biological studies leading to clinical drug candidates. For example, the variable expression of the SRIF receptors among different tumors and the findings of the different SRIF receptors within subtypes of the same tumor are also justifying the search for receptor-selective analogues.[35] Because the physicochemical and

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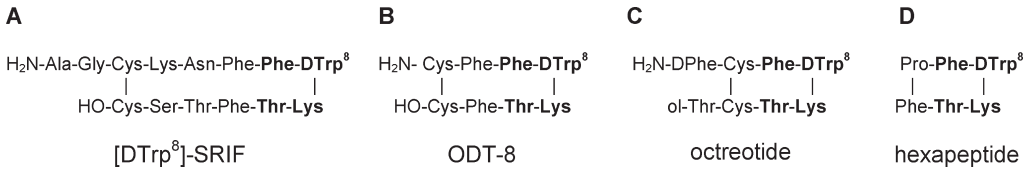

Figure 1. Most common scaffolds for SRIF analogues.

biological properties of these analogues are different depending on whether the desired analogues are for diagnostic or therapeutic purposes, we examined such properties as binding affinities and in vitro functionalities for the five known human somatostatin receptor subtypes (sst_{1-5}). In the process of characterizing the analogues shown in Table 1, some unusual binding and functional properties of position 8-substituted somatostatin (**1**) and cyclo(3−14)H-Cys-Phe-Phe-Trp-Lys-Thr-Phe-Cys-OH (somatostatin numbering, **9**)[36] analogues were uncovered. All of the analogues listed in Table 1 were synthesized automatically on a chloromethylated resin using the Boc strategy and diisopropylcarbodiimide/1-hydroxybenzotriazole (DIC/HOBt) for amide bond formation. The peptide resins were treated with hydrogen fluoride in the presence of scavengers to liberate the fully deblocked crude peptides. Cyclization of the cysteines was mediated by iodine in an acidic milieu (AcOH). Purification was carried out using multiple HPLC steps,[37] and the purity of the peptides was determined by HPLC,[37] capillary zone electrophoresis,[38] and mass spectrometry (Table 1 shows the results). Analogues were tested for binding affinity, selectivity, and functionality at the sst_3 receptor in an sst_3 internalization and an sst_3 luciferase reporter gene assay as described earlier (Table 1).[39]

The substitution of Trp^8 by $DTrp^8$ (**2**) in **1** sequence does not alter the binding affinity for all five sst_s. The introduction of Tyr^8 (**3**) or $DTyr^8$ (**4**) instead of Trp^8 in **1** generally results in a decrease of the binding affinity for all five receptor subtypes except for $DTyr^8$ (**4**) at sst_3. The introduction of $2Nal^8$ (**5**) or $D2Nal^8$ (**6**) instead of Trp^8 in **1** does not essentially influence the binding affinity at all five receptor subtypes. The substitution of $DTrp^8$, Tyr^8 (L- or D-), or $2Nal^8$ (L- or D-) for Trp^8 does not influence the subtype selectivity of the compounds. Moreover, all of these changes also have no influence on their functional behavior at sst_3. They are all agonists in the sst_3 internalization assay as well as in the sst_3 luciferase reporter gene assay (Figures 2 and 3A).

We also investigated the effect of some of the above substitutions in scaffold B (Figure 1) (**9**−**13**). The introduction of $DTrp^8$ (**10**) or $D2Nal^8$ (**11**) instead of Trp^8 in **9** results in a decrease of the binding affinity for all subtypes except for sst_3 and sst_5 (Table 1). The substitution of $DTrp^8$ or $D2Nal^8$ for Trp^8 (somatostatin numbering) does not influence the functional behavior of these peptides at sst_3. They are all agonists in the sst_3 internalization assay (Table 1, Figure 2); however, they shift from a partial agonist to an agonist in the sst_3 luciferase reporter gene assay (Figure 3B).

A different picture can be seen when imBzl-His (L- or D-) (Chart 1) is introduced at position 8 of **1** (**7** and **8**, respectively) as well as of **9** (**12** and **13**, respectively). Peptides **1** and **9** bind to all of the five receptors of somatostatin (pan-compounds), but their $imBzl\text{-}His^8$ substituted analogues (**7**, **8**, **12**, **13**) turn into sst_3-selective compounds (Table 1). Moreover, the binding affinities for $sst_{1,2,4,5}$ are completely lost, while the affinity for sst_3 is slightly decreased. In those cases, the imBzl-D-His-containing compound exhibits a slightly better affinity than that of the imBzl-His-containing analogue.

In the functional assays, the picture is also different. With regard to sst_3 internalization, the introduction of imBzl-His (D- or L-) at position 8 in **1** does not affect its property; both compounds remain agonists, although the imBzl-His-containing **7** is clearly less potent in stimulating sst_3 internalization than the imBzl-D-His-containing **8** (Figure 2). This might be explained with the weaker binding affinity of **7** compared to that of **8**. However, when the compounds are tested in the sst_3 luciferase reporter gene assay, these substitutions switch their functional behavior from an agonist to a partial agonist with an EC_{50} that is better for **8** than for **7** (Figure 5A).

In contrast to **1**, the introduction of imBzl-His (L- or D-) in **9** at position 8 corresponding to **7** and **8** in **1** results in **12** and **13** with a more dramatic functional change, namely, a switch from an agonist to an antagonist when tested for sst_3 internalization (Figure 4). In the sst_3 luciferase reporter gene assay, they behave like partial agonists, like **9**, but the EC_{50} values of analogues **12** and **13** are much higher (164 ± 29 and 299 ± 16 nM, respectively) than that of **9** (5.2 nM) (Figures 3B and 5B).

It is premature in the absence of clear structural results obtained by NMR in the presence of the cognate receptor to speculate about the role of the imBzl-His side chain in position 8 that is responsible for loss of function and retention of significant binding affinity.

Interestingly, it appears that the introduction of imBzl-His in **9** has more dramatic effects than when it is introduced in **1**. We can speculate in this case that the bioactive conformation of the octapeptide (**13**) is likely more constrained than that of the corresponding tetradecapeptide (**7**) and therefore less accommodating. Cyclo(3−14)Cbm-$DCys^3$-Phe^6-Tyr^7-DAgl(NMe,2-naphthoyl)^8-Lys^9-Thr^{10}-Phe^{11}-Cys^{14}-OH (sst_3-ODN-8; somatostatin numbering) that we published earlier[14] is also a position 8-substituted sst_3-selective analogue of **9**; it shows a more hydrophobic character than **12** or **13**, and the unusual amino acid DAgl(NMe,2naphthoyl) is not commercially available while the protected imBzl-His is. Structurally, one could speculate that both sst_3-ODN-8 and **9** assume similar conformations upon binding to sst_3.

We have shown that in somatostatin and its shortened analogues (Figure 1B−D), subtle substitution of a single amino acid can lead to changes in receptor binding affinity and selectivity and can switch agonists into antagonists. These findings indicate that we cannot assume, without experimental data, that a particular analogue will be an agonist or antagonist based solely on the chirality and structural similarities of each amino acid in its basic scaffold.[10,41] At the same time, such observations open new opportunities in drug design. Here we report that introduction of $imBzl\text{-}His^8$ (D- or L-) results in sst_3-selective compounds that

Table 1.[a]

compd	structure	physicochemical properties				binding affinity (IC_{50}, nM)					functional assay	
		$[M + H]^+_{calc}$	$[M + H]^+_{obs}$	HPLC	CE	sst_1	sst_2	sst_3	sst_4	sst_5	sst_3 IF internalization assay	sst_3 luciferase reporter gene assay (EC_{50}, nM)
	cyclo(3−14)H-Ala[1]-Gly[2]-Cys[3]-Lys[4]-Asn[5]-Phe[6]-Phe[7]-**Trp**[8]-Lys[9]-Thr[10]-Phe[11]-Thr[12]-											
1	Ser[13]-Cys[14]-OH (**SRIF**)	1637.73	1637.70	99	99	1.6 ± 0.36	0.67 ± 0.14	3.1 ± 1.4	1.7 ± 0.61	4.2 ± 0.67	agonist	Ago: 0.78 ± 0.1 (8)
2	[DTrp[8]]-SRIF	1637.73	1637.53	97	98	2.3 ± 0.78	1.1 ± 0.53	2.4 ± 0.64	2.3 ± 0.98	3.1 ± 0.46	agonist	Ago: 0.63 ± 0.23 (3)
3	[Tyr[8]]-SRIF	1614.71	1614.09	89	92	138 ± 28	35 ± 8.1	13 ± 3.0	25 ± 2.6	556 ± 145	agonist	Ago: 15.7 ± 0.7 (3)
4	[DTyr[8]]-SRIF	1614.71	1614.76	96	98	140 ± 20	4.1 ± 0.85	2.0 ± 0.51	16 ± 3.3	41 ± 9.6	agonist	Ago: 0.87 ± 0.07 (3)
5	[2Nal[8]]-SRIF	1648.73	1648.72	99	98	2.7 ± 0.43	15 ± 2.0	1.5 ± 0.49	12 ± 1.7	69 ± 11	agonist	Ago: 0.35 ± 0.09 (4)
6	[D2Nal[8]]-SRIF	1648.73	1648.95	99	99	4.1 ± 1.2	1.6 ± 0.22	1.0 ± 0.26	12 ± 2.0	2.4 ± 0.32	agonist	Ago: 0.23 ± 0.03 (3)
7	[imBzl-His[8]]-SRIF	1678.75	1678.69	92	90	>1000	>1000	131 ± 26	>1000	>1000	agonist	part. Ago: 315 ± 9 (3)
8	[imBzl-DHis[8]]-SRIF	1678.75	1678.62	84	90	>1000	106 ± 29	15 ± 4.1	>1000	>1000	agonist	part. Ago: 41 ± 7 (4)
	cyclo(3−14)H-Cys[3]-Phe[6]-Phe[7]-**Trp**[8]-Lys[9]-Thr[10]-Phe[11]-Cys[14]-OH (**9**)											
9	cyclo(3−14)H-Cys[3]-Phe[6]-Phe[7]-**DTrp**[8]-Lys[9]-Thr[10]-Phe[11]-Cys[14]-OH (**ODT-8**)	1079.45	1079.38	97	98	5.3 ± 0.7	130 ± 65	13 ± 0.7	0.7 ± 0.3	16 ± 1.8	agonist	part. Ago: 4.7 ± 1.1 (4)
10		1079.45	1079.04	95	98	27 ± 3.4	41 ± 8.7	13 ± 3.2	1.8 ± 0.7	2.6 ± 0.35	agonist	Ago: 3.33 ± 0.69 (3)
11	cyclo(3−14)H-Cys[3]-Phe[6]-Phe[7]-**D2Nal**[8]-Lys[9]-Thr[10]-Phe[11]-Cys[14]-OH (**ODN-8**)	1090.47	1090.54	99	98	607 ± 168	173 ± 41	6.7 ± 1.9	41 ± 19	6.0 ± 1.4	agonist	Ago: 0.53 ± 0.15 (3)
	cyclo(3−14)H-Cys[3]-Phe[6]-Phe[7]-**imBzl-His**[8]-Lys[9]-Thr[10]-Phe[11]-Cys[14]-OH											
12	[**imBzl-His**[8]]-(**9**)	1120.48	1120.45	99	99	>1000	>1000	164 ± 46	>1000 (2)	>1000	antagonist	part. Ago: 164 ± 29 (3)
	cyclo(3−14)H-Cys[3]-Phe[6]-Phe[7]-**imBzl-DHis**[8]-Lys[9]-Thr[10]-Phe[11]-Cys[14]-OH											
13	[**imBzl-DHis**[8]]-(**9**)	1120.48	1120.50	99	99	>1000	>1000	75 ± 2.9	590 ± 146	>1000	antagonist	part. Ago: 299 ± 16 (3)

[a] Mean ± SEM ($n \geq 3$).

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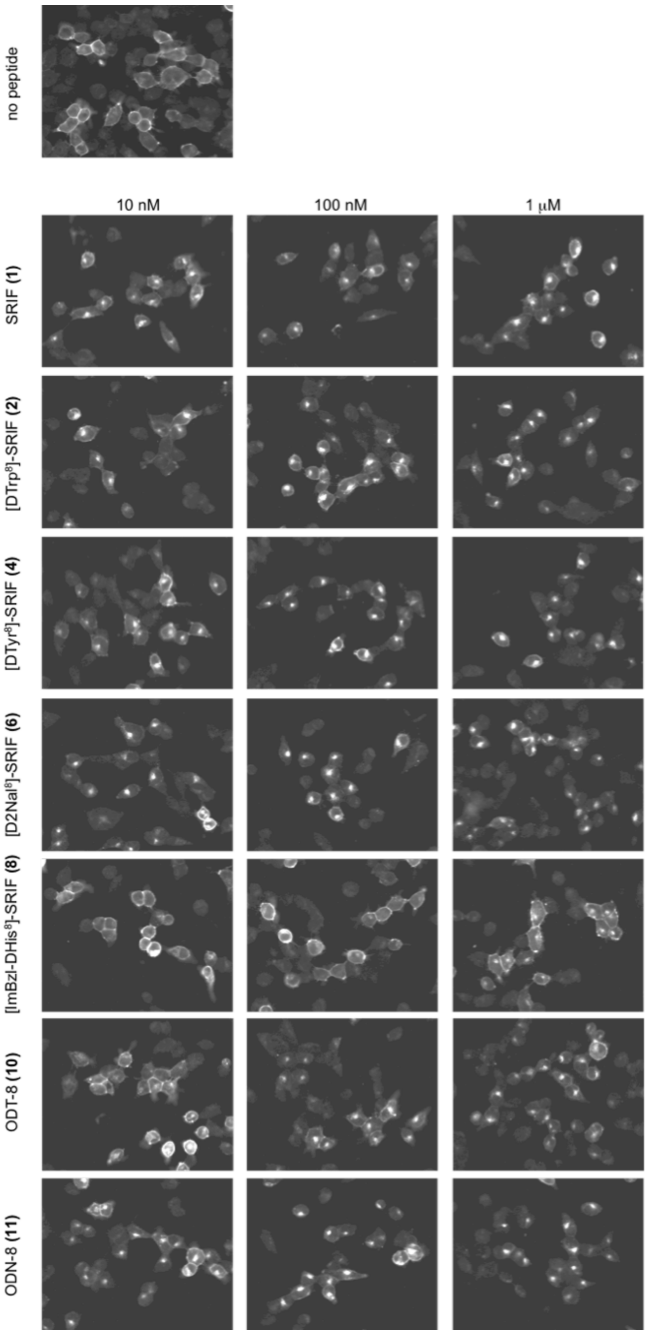


Figure 2. The sst_3 internalization assay shows that **2**, **4**, **6**, **8**, **10**, **11** are all agonists. HEK-sst_3 cells were treated for 30 min either with vehicle (no peptide) or with 10 nM, 100 nM, or 1 μM SRIF (**1**) or with **2**, **4**, **6**, **8**, **10**, **11**. Following incubation with the peptides, the cells were processed for immunocytochemistry as described in Supporting Information. All tested analogues are able to stimulate sst_3 internalization similarly to SRIF (**1**).

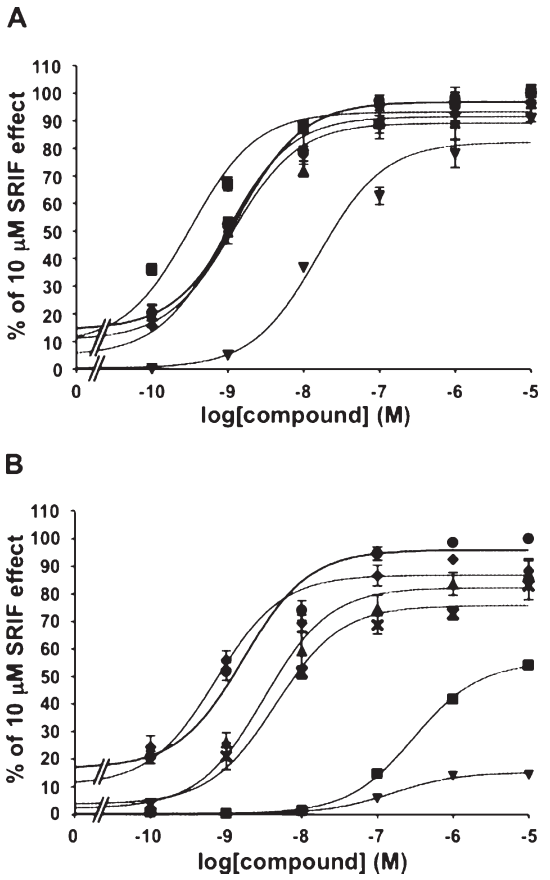
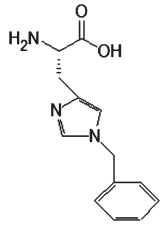


Figure 3. Analogues **2**, **3**, **4**, **6**, **9**, **10**, **11**, **12**, and **13** are tested in the luciferase reporter gene assay for agonism on the sst_3 receptor. The assay was performed as described in Supporting Information. CCL39-sst_3-Luci cells were treated with increasing concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) of SRIF (**1**) (●), **2** (▲), **3** (▼), **4** (◆), or **6** (■) (A) or SRIF (**1**) (●), **9** (×), **10** (▲), **11** (◆), **12** (▼), or **13** (■) (B). The stimulation of the luciferase reporter gene activity by the compounds is expressed as % stimulation of the 10 μM SRIF (**1**) effect. Shown are the dose−response curves of the analogues. While **2**, **3**, **4**, **6**, **9**, **10**, and **11** are full agonists, **12** and **13** behave like partial agonists.

Chart 1. Structure of imBzl-His[40]



might be important leads for further development. The recently published paper by Ramon et al. describes the single substitution of 3-(3′-quinolyl)alanine[8] for Trp[8] in somatostatin resulting in an analogue that is partially selective for sst_3 and sst_1.[42]

The nature of agonism versus antagonism of small peptide analogues is still complex. For example, a simple replacement of DXaa[1]-L-Cys[2] to LXaa[1]-D-Cys[2] in $sst_{2/3/5}$-selective analogues (Figure 1 C) is able to change the ligand from an agonist to an antagonist at sst_2.[10,41] A comparison of solution conformations between such SRIF agonists and antagonists does not reveal significant structural differences that might account for their different functional properties.

We recently published that addition of a DOTA chelator to an sst_3-selective competitive SRIF antagonist switches the analogue completely to an agonist in the sst_3 receptor internalization assay. This impressive switch in biological function after the addition of

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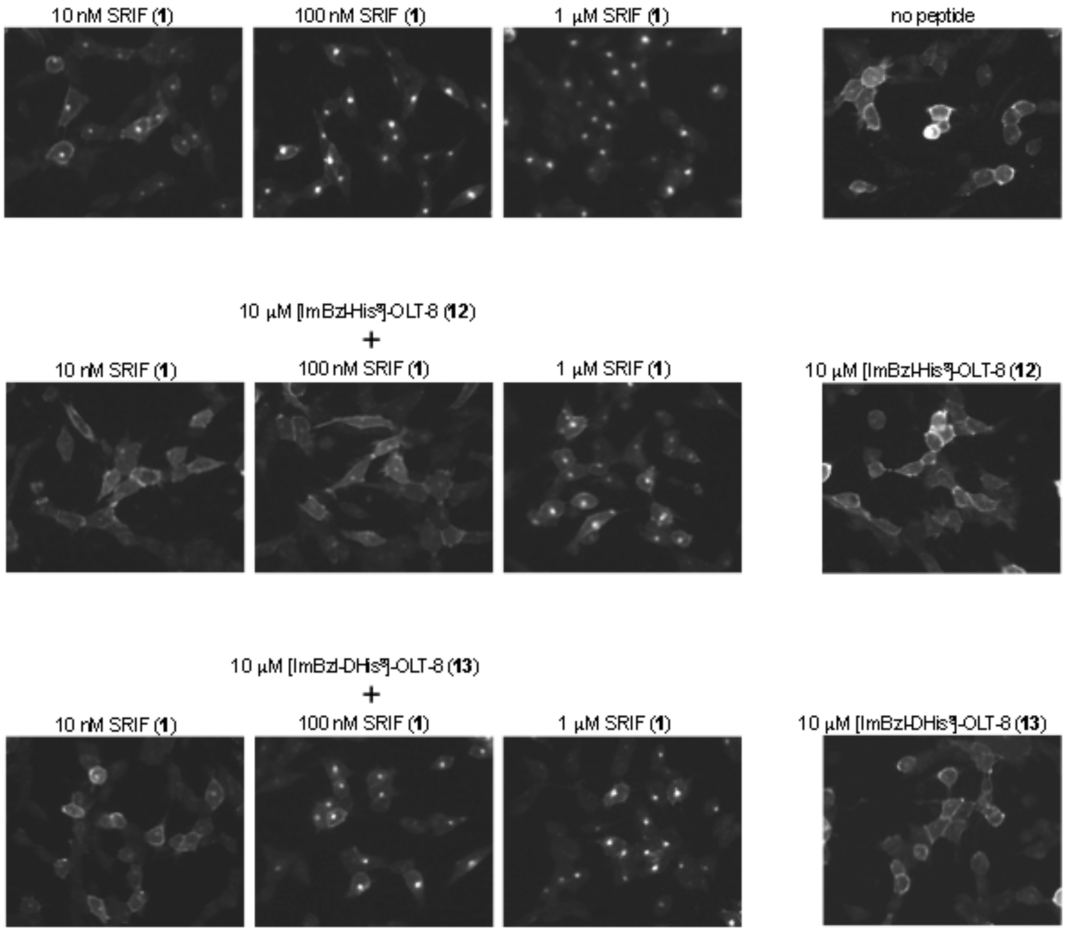

Figure 4. The sst_3 internalization assay to determine whether **12** and **13** are agonists or antagonists. HEK-sst_3 cells were treated for 30 min with vehicle (no peptide) or with 10 nM, 100 nM, or 1 μM SRIF (**1**) alone or with 10 nM, 100 nM, or 1 μM SRIF (**1**) in the presence of 10 μM **12** or **13** or with 10 μM **12** or **13** alone. Following incubation with the peptides, the cells were processed for immunocytochemistry as described in Supporting Information. While **12** and **13** are not able to stimulate sst_3 internalization at 10 μM, they are able to antagonize the SRIF stimulated sst_3 internalization effect.

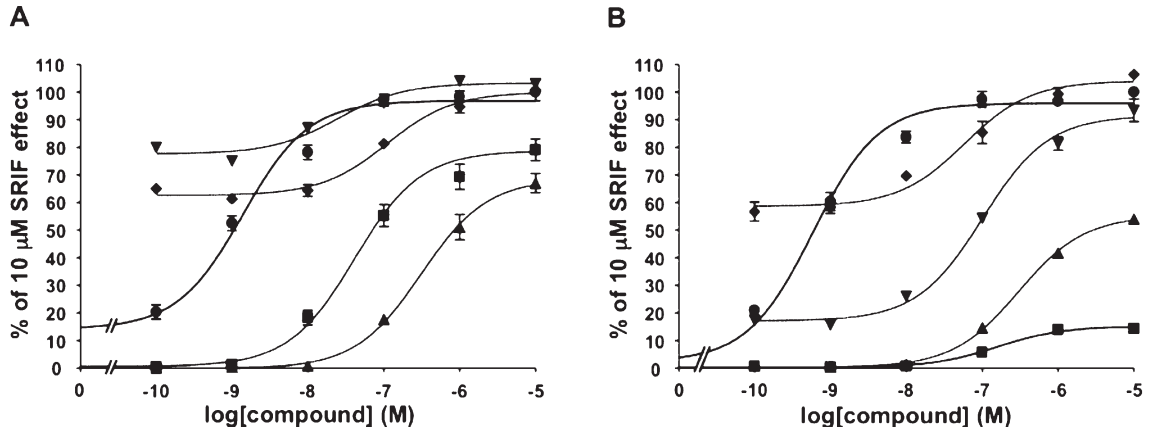

Figure 5. The sst_3 luciferase reporter gene assay to determine whether **7**, **8**, **12**, and **13** are agonists or antagonists. The assay was performed as described in Supporting Information. (A) CCL39-sst_3-Luci cells were treated either with increasing concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) of SRIF (**1**) (●), **7** (▲), **8** (■) or with increasing concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM and 10 μM) of SRIF (**1**) in the presence of 10 μM **7** (◆) or **8** (▼). (B) CCL39-sst_3-Luci cells were treated either with increasing concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM, and 10 μM) of SRIF (**1**) (●), **12** (■), **13** (▲) or with increasing concentrations (0.1 nM, 1 nM, 10 nM, 100 nM, 1 μM and 10 μM) of SRIF (**1**) in the presence of 10 μM **12** (▼) or **13** (◆). The stimulation of the luciferase reporter gene activity by the compounds is expressed as % stimulation of the 10 μM SRIF effect. Shown are the dose−response curves of the analogues. All four analogues **7**, **8**, **12**, and **13** behave like partial agonists, since they exhibit an agonistic effect on its own, but they are also able to partially antagonize the effect of SRIF (**1**).

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a chelator is unexpected and is, at the moment, hard to explain and understand from a structural point of view. In general, the conversion of a peptide agonist to a peptide antagonist has indeed been an empirical tour de force involving such modifications as deletions or the introduction of unnatural amino acids with different chirality.[43]

In conclusion, the data presented here add an additional degree of complexity when it comes to any attempt at rationalizing the governing parameters that will direct a particular biological active peptide analogue to be selective or not, agonist or not, antagonist or not, long acting or not, and probably, safe or not.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures and additional references. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

AA, amino acid; Agl, aminoglycine; Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; Bzl, benzyl; Bzl(3Br), 3-bromobenzyl; Z(2Br), 2-bromobenzyloxycarbonyl; Z(2Cl), 2-chlorobenzyloxycarbonyl; Cbm, carbamoyl; CZE, capillary zone electrophoresis; DIC, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; HOBt, l-hydroxybenzotriazole; ImBzl, N^{im}-benzyl; Mob, 4-methoxybenzyl; Nal, 3-(2-naphthyl)alanine; NMP, *N*-methylpyrrolidinone; SRIF, somatostatin; sst_s, SRIF receptors; TEA, triethylamine; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid

■ ADDITIONAL NOTE

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.

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