Further Studies at Neuropeptide S Position 5: Discovery of Novel Neuropeptide S Receptor Antagonists

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Neuropeptide S (NPS) regulates various biological functions by activating the NPS receptor (NPSR). Previous studies demonstrated that the substitution of Gly^5 with D-amino acids generates NPSR antagonists. Eleven [D-Xaa^5]NPS derivatives were synthesized and pharmacologically tested measuring $[Ca^{2+}]_i$ in HEK293$_{mNPSR}$ cells. The results confirmed that the [D-Xaa^5] substitution promotes antagonist activity with potency inversely related to the side chain size and allowed identification of the novel potent NPSR peptide antagonist [^{t}Bu-D-Gly^5]NPS.

Introduction

Neuropeptide S (NPS, human sequence SFRNGVGTG-MKKTSFQRAK) is the endogenous ligand of the 7TM receptor NPSR.[1] Via selective NPSR activation, NPS regulates several biological functions including wakefulness,[1,2] stress and anxiety,[1−5] locomotor activity,[1−3,6] food intake,[6−8] memory processes,[5,9] and drug abuse.[10−13] To investigate these NPS sensitive biological functions and to identify the therapeutic potential of drugs interacting with the NPSR, potent and selective ligands are required. To identify NPSR ligands, structure−activity relationship (SAR) studies were performed on the NPS sequence that allowed demonstration of the crucial importance of the sequence Phe^2-Arg^3-Asn^4 for receptor binding and activation[14−16] and of the sequence Gly^5-Val^6-Gly^7 for shaping NPS into the biologically active conformation.[14,16,17] A few SAR studies were then performed on Phe^2 (ref 18), Arg^3, and Asn^4 (ref 19). More recently, a SAR study focused on Gly^5 was carried out.[20] This investigation demonstrated that the introduction in NPS position 5 of a chiral center with relative configuration D produces important changes in peptide potency and, particularly, in its efficacy. In fact, the replacement of Gly^5 with D-Leu or D-Cys generated NPSR partial agonists while that with D-Met or D-Val produced pure and fairly potent NPSR antagonists. The NPSR antagonistic properties of [D-Val^5]NPS were confirmed in vivo in the mouse locomotor activity assay, where the peptide at 10 nmol blocked the stimulatory effect elicited by the supraspinal administration of 0.1 nmol of NPS.[20] These findings prompted us to further investigate position 5 with the aim of understanding the chemical requirements of the D-amino acid side chain that are instrumental for generating NPSR antagonism.

Results and Discussion

Eleven novel peptides (Table 1) were synthesized in good yield and with a purity grade not less than 95% following procedures previously described.[18] NPS, [D-Val^5]NPS (used as reference NPSR antagonist), and the novel peptides were pharmacologically evaluated in a calcium mobilization assay using HEK293 cells stably expressing the mouse NPSR (HEK293$_{mNPSR}$). The protocols and the experimental conditions used in the present study have been previously illustrated in detail.[20,21] However, to facilitate drug diffusion into the wells in antagonist type experiments, the present studies were performed at 37 °C and three cycles of mixing (25 μL from each well moved up and down 3 times) were performed immediately after antagonist injection to the wells. In addition, inhibition response curves to putative antagonists were determined against the stimulatory effect of 30 nM NPS.

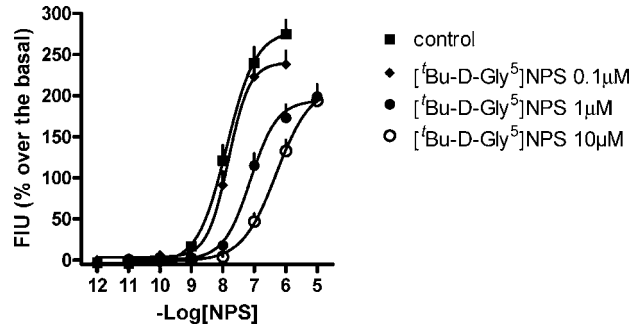

Figure 1. Concentration−response curve to NPS obtained in the absence (control) and presence of increasing concentrations of [^{t}Bu-D-Gly^5]NPS in calcium mobilization experiments performed in HEK293$_{mNPSR}$ cells. Data are the mean ± SEM of four separate experiments performed in duplicate.

NPS increased the intracellular calcium concentrations in a concentration-dependent manner with pEC_{50} and E_{max} values of 8.32 and 295% over basal, respectively (Table 1). Confirming previous findings,[20] [D-Val^5]NPS did not evoke any effect per se but inhibited in a concentration dependent manner the stimulatory effect of 30 nM NPS, thus behaving as an NPSR antagonist. A pK_B value of 6.54 was derived from these experiments. The replacement of the isopropyl group (as in Val) with a *sec*-butyl group (**1** and **2**) produced a similar moderate reduction of potency independently from the side chain chiral center. Similar results were obtained substituting a methyl of the isopropyl group with an oxidril function (**3** and **4**). In this

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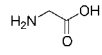
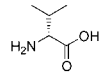
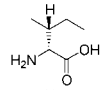
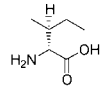
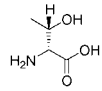
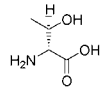
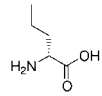
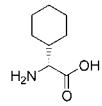
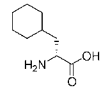
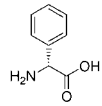
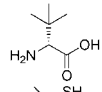
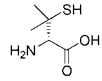
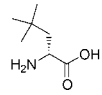
Table 1. Effects of NPS and [D-Xaa^5]NPS Analogues in HEK293 Cells Expressing the Mouse NPSR[a]

Compound	Xaa^5		Agonist		Antagonist
	Chemical formula	Name	pEC_{50} ($CL_{95\%}$)	E_{max} ± sem	pK_B ($CL_{95\%}$)
NPS		Gly	8.32 (8.00 - 8.64)	295 ± 22%	-
[D-Val^5]NPS		D-Val	inactive		6.54 (5.99 – 6.99)
1		D-Ile	inactive		5.72 (5.10 – 6.34)
2		D-allo-Ile	inactive		6.04 (5.52 – 6.56)
3		D-Thr	6.10 (5.79 – 6.41)	25 ± 5%*	6.09 (5.79 - 6.39)
4		D-allo-Thr	inactive		6.04 (5.51 – 6.57)
5		D-Nva	5.57 (5.02 – 6.12)	24 ± 7%*	5.68 (5.38 - 5.98)
6		cyclohexyl-D-Gly	inactive		< 5
7		D-Cha	inactive		< 5
8		D-Phg	6.18 (5.81 – 6.55)	15 ± 2%*	6.12 (5.81 - 6.43)
9		^{t}Bu-D-Gly	inactive		7.06 (6.48 – 7.64)
10		D-Pen	inactive		7.08 (6.46 - 7.70)
11		^{t}Bu-D-Ala	5.78 (5.06 - 6.50)	104 ± 2%*	6.32 (5.63 - 7.01)

[a] pEC_{50}: the negative logarithm to the base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. $CL_{95\%}$: 95% confidence limits. E_{max}: the maximal effect elicited by the agonist expressed as % over the baseline. SEM: standard error of the mean. Inactive: inactive up to 10 μM. The antagonist potencies (pK_B) of the peptides were assessed in inhibition−response curves against the stimulatory effect elicited by 30 nM NPS: (*) $p < 0.05$ vs NPS, according to one-way ANOVA followed by the Dunnett test. Data are mean values of at least four separate experiments performed in duplicate.

latter case, the side chain chiral center seems to exert an effect on peptide efficacy, since [D-allo-Thr^5]NPS behaves as a pure NPSR antagonist while [D-Thr^5]NPS as a low efficacy partial agonist. However, the difference in efficacy between **3** and **4** ([D-Thr^5]NPS $\alpha = 0.08$; [D-allo-Thr^5]NPS $\alpha = 0$) is too little to be meaningful. A linear three-carbon side chain (**5**) produced an analogue that behaved as a NPSR low efficacy partial agonist 10-fold less potent than [D-Val^5]NPS. These findings indicated that the isopropyl moiety is highly important for NPSR antagonist binding and that the replacement of one of its methyl groups with ethyl or oxidril functions produced a reduction of potency. Moreover, the three carbon atoms of the D-Val side chain must have a ramified (isopropyl) rather than linear (*n*-propyl, as in **5**) shape; in fact, the latter generates a clear reduction of peptide potency. The introduction in position 5 of a cyclohexyl or methylcyclohexyl moiety (**6** and **7**) generated inactive derivatives, while the introduction of a phenyl ring (**8**) produced only a 3-fold reduction in potency compared to the isopropyl moiety of [D-Val^5]NPS. These data suggest that the increase in the side chain size (as in **6** and **7**) decreases peptide potency. This is further suggested by the results obtained with **8**. In fact, the aromaticity of the phenyl ring of **8** reduced the side chain size and changed its shape compared with the cyclohexyl moiety of **6**, and this may explain the moderate potency of [D-Phg^5]NPS compared to the inactivity of [cyclohexyl-D-Gly^5]NPS. Next, the effect of the insertion in the D-Val^5 isopropyl moiety of a CH_3 (**9**) or SH (**10**) group was evaluated. In both cases the chemical change did not modify the pharmacological activity of the peptides; i.e., they behaved as pure antagonists, with a 3-fold increase of potency. Finally, the

Table 2. Selectivity Profile of [^{t}Bu-D-Gly^5]NPS at Eight Different Human G-Protein-Coupled Receptors[a]

			control		10 μM [^{t}Bu-D-Gly^5]NPS	
cell line	receptor	agonist	pEC_{50}	$E_{max} \pm$ SEM, %	pEC_{50}	$E_{max} \pm$ SEM, %
CHO	recombinant NK-1	substance P	10.24 (9.86−10.62)	122 ± 15	10.31 (9.94−10.68)	113 ± 11
CHO	recombinant B_2	bradykinin	10.31 (10.25−10.37)	125 ± 2	10.47 (9.90−11.04)	115 ± 11
CHO	recombinant UT	urotensin-II	8.39 (7.50−8.88)	224 ± 21	8.46 (8.19−8.73)	234 ± 19
CHO-α_{qi5}	recombinant MOP	dermorphin	7.98 (7.85−8.11)	186 ± 11	7.96 (7.71−8.21)	186 ± 15
CHO-α_{qi5}	recombinant DOP	DPDPE	8.53 (8.23−8.73)	169 ± 26	8.71 (8.65−8.77)	177 ± 11
CHO-α_{qi5}	recombinant KOP	dynorphin A	8.20 (7.81−8.59)	143 ± 12	8.63 (8.33−8.99)	139 ± 13
CHO-α_{qi5}	recombinant NOP	N/OFQ	9.41 (9.26−9.56)	231 ± 12	9.55 (9.11−9.99)	207 ± 30
A549	native PAR2	SLIGKV-NH_2	4.55 (4.06−5.04)	449 ± 25	4.50 (3.96−5.04)	445 ± 30

[a] Data are the mean ± SEM of three separate experiments performed in duplicate. The chimeric protein α_{qi5} was used to force NOP and classical opioid receptors to couple with the calcium pathway. N/OFQ: nociceptin/orphanin FQ.

insertion of a carbon atom between the ^{t}Bu moiety and the peptide backbone (**11**) caused an important reduction of peptide potency associated with a clear increase in efficacy ([^{t}Bu-D-Gly^5]NPS $\alpha = 0$, $pK_B = 7.06$; [^{t}Bu-D-Ala^5]NPS $\alpha = 0.35$, $pK_B = 6.32$). Similar results were obtained with the isopropyl ([D-Val^5]NPS and [D-Leu^5]NPS[20]). Collectively these findings indicated that a short side chain favors high potency and pure receptor antagonism. In addition, a comparison of the effects of the side chain structures (^{t}Bu and isopropyl) clearly indicates that aliphatic branched moieties are better recognized by the NPSR receptor. The best results are obtained with the ^{t}Bu moiety in which the substitution of a CH_3 with SH does not change the biological activity.

Then [^{t}Bu-D-Gly^5]NPS was further characterized by assessing its antagonist behavior using the classical Schild protocol and its selectivity of action over eight unrelated GPCRs. Figure 1 summarizes the data obtained by determining concentration−response curves to NPS in the presence of increasing concentrations (0.1−10 μM) of [^{t}Bu-D-Gly^5]NPS. This peptide produced a concentration dependent rightward shift of the concentration−response curve to NPS which was associated with a slight but significant depression of NPS maximal effects. From these experiments a pK_B of 6.78 ($CL_{95\%} = 6.33-7.23$) was derived (using the equation described on page 117 of ref 22). This value is close to that obtained in inhibition response curve studies (7.06, Table 1). Thus, these experiments confirmed the pure and potent antagonist properties of [^{t}Bu-D-Gly^5]NPS. However, these results are not compatible with a simple competitive interaction between [^{t}Bu-D-Gly^5]NPS and NPS. Similar results (rightward shift associated with significant depression of agonist maximal effects) were previously reported for [D-Val^5]-NPS,[20]which was tested at room temperature and without the three cycles of mixing. Under these same experimental conditions [^{t}Bu-D-Gly^5]NPS caused a profound depression of NPS maximal effects (down to less than 50%) in a concentration dependent manner (data not shown). This depression of E_{max} was strongly reduced even if not completely eliminated performing the experiments at 37 °C and introducing the three cycles of mixing. Collectively these findings suggest that the depression of NPS E_{max} caused by NPSR antagonists in the calcium mobilization assay may likely come from hemiequilibrium conditions due to lack of stirring[22] rather than from a real insurmountable type of antagonism.

To get information about the selectivity of action of [^{t}Bu-D-Gly^5]NPS, the peptide was evaluated as agonist and antagonist in calcium mobilization experiments performed using eight unrelated human G protein coupled receptors (Table 2). These included the native PAR2 receptors expressed in A549 cells and the recombinant NK-1, B_2, and UT receptors expressed in CHO cells. Moreover, this investigation was extended to nociceptin/orphanin FQ peptide receptor (NOP) and classical opioid receptors forced to couple with the calcium pathway by the chimeric protein α_{qi5}.[23,24] [^{t}Bu-D-Gly^5]NPS up to 10 μM neither stimulated calcium mobilization in these cells nor affected the stimulatory effects elicited by the reference receptor agonists (Table 2). These results suggest that [^{t}Bu-D-Gly^5]NPS behaves as a selective NPSR antagonist. However, the panel of receptors investigated is probably too limited to draw final conclusions about compound selectivity at this time.

Collectively the present findings demonstrated that the peptide [^{t}Bu-D-Gly^5]NPS behaves as a pure, potent ($pK_B \approx 7$), and selective NPSR antagonist. This molecule should be compared to the NPSR antagonists so far available which are the moderate potency ($pK_B \approx 6.5$) peptide antagonists [D-Cys(^{t}Bu)^5]NPS[21] and [D-Val^5]NPS[20] and the highly potent ($pK_B \approx 7.5$) bicyclic piperazine derivative SHA 68.[25] The antagonist properties of these molecules have been already confirmed in in vivo studies[5,20,21,25,26] while the evaluation of [^{t}Bu-D-Gly^5]NPS in vivo actions is under way in our laboratories.

In conclusion, the present study (i) confirmed previous indications that the D relative configuration of amino acid residues at position 5 of NPS promotes antagonist activity, (ii) indicated that the peptide antagonist potency is inversely related to the D-Xaa^5 side chain size, and (iii) demonstrated that the ^{t}Bu (and its sulfhydryl derivative) directly linked to the Cα carbon atom is the best chemical moiety for increasing antagonist potency. [^{t}Bu-D-Gly^5]NPS and [D-Pen^5]NPS identified in the context of the present study represent the most potent NPSR peptide antagonists so far identified.

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Supporting Information Available: Table listing the retention times determined by analytical HPLC analyses using two different chromatographic systems and the calculated and found molecular

weight of the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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