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NEURONORM IS A POTENT AND WATER SOLUBLE NEUROKININ A RECEPTOR ANTAGONIST

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Abstract – We report here the synthesis and preliminary pharmacological characterization of a novel water soluble Neurokinin A receptor antagonist named *Neuronorm*. The synthesis was achieved in high yield by a combination of classical peptide synthesis methodologies, in both solution and solid phase. The pharmacological properties as neurokinin A receptor antagonist were assessed in *in vitro* experiments on rat vas deferens and guinea pig trachea, and were compared to those of MEN10627.

Tachykinins (TKs) are a family of peptides widely distributed in the central and peripheral nervous system of amphibians and mammals, where they produce several important biological effects, such as changes in vascular tone and permeability, effects on smooth muscle contractility and mast cell degranulation.[1] Among all the TKs known so far, substance P (SP) was the first to be discovered[2] and purified.[3] Two other mammalian TKs, termed neurokinin A (NKA) and neurokinin B (NKB), were subsequently isolated in the 1980s.[4] SP, NKA and NKB preferentially interact in the mammalian tissues with the NK-1, NK-2 and NK-3 receptor, respectively.[1a]

A dense network of sensory nerve fibers is present in the respiratory tract from the nasal mucosa to the smallest bronchi that may release TKs, such as substance P and neurokinin A, thus leading to a series of responses collectively referred to as lung neurogenic inflammation.[5] Moreover, TKs have been implicated in non-adrenergic non-cholinergic (NANC) bronchoconstriction, neurogenic mucosal plasma extravasation and mucus hypersecretion in the airways.[6]

NKA receptor antagonists may represent a new class of agents, useful in inflammatory diseases of airways such as asthma, and in others diseases, in which smooth muscle contraction caused by TKs plays a pathogenic role (*e.g.* exaggerated intestinal motility). In the last few years, several non-peptide[7] or peptide-based[8] NKA receptor antagonists have been discovered; some of them display good antagonist activity and selectivity for the NK-2 receptor and they may be useful in therapeutic applications. We rationally designed, synthesized and characterized few years ago a highly constrained molecule, MEN 10627, corresponding to two 14-membered bicyclic peptides fused together in a bicyclic structure.[9] MEN10627 is, to the best of our knowledge, the most

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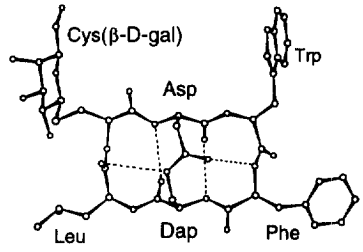
potent, highly selective and long lasting, peptide-based NK-2 antagonist known to date.[10] Its unique biological and pharmacological features are closely related to the conformational rigidity of its bicyclic structure.[9,11] Despite its potency, specificity of action and long lasting activity *in vivo*, MEN10627 resulted of limited solubility in water (15 μg/ml), and it was impossible to obtain an acceptable pharmaceutical composition for administration in humans. We have thus attempted to develop water-soluble NK-2 antagonist with a biological and pharmacological profile comparable to that of MEN10627. This appeared to be a quite challenging goal to be achieved, because a high potency of NK-2 antagonists seemed to be closely related to a high hydrophobicity of the antagonist.[12] We succeeded with *Neuronorm*, namely cyclo[Cys^1(β-D-gal)-Asp^2-Trp^3-Phe^4-Dap^5-Leu^6]cyclo(2β-5β) (see Figure 1) (β-D-gal: β-D-galactopyranosyl; Dap: 2, 3 di-amino propionic acid). It is a water-soluble compound (1.8 mg/ml) and it displays an *in vitro* potency comparable to MEN10627 as NK-2 antagonist.

Figure 1: Schematic representation of the structure of Neuronorm

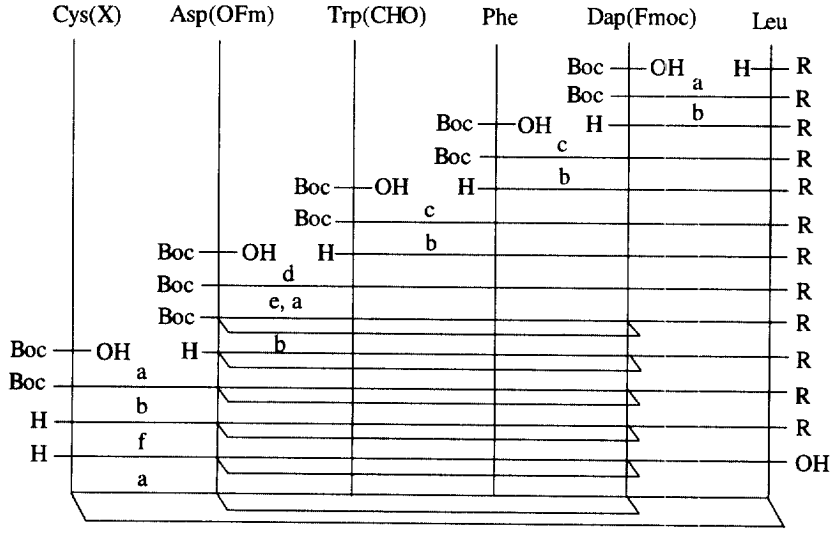


The bicyclic peptide Neuronorm was synthesized by using both solution and solid phase peptide methodologies (see Figure 2). Some slight modifications were introduced into the classical N-α-*t*-Boc (*tert*-butyloxy carbonyl) protocol. The side chains of Dap and Asp were protected as Fmoc (fluoren-9-ylmethoxycarbonyl) and OFm (fluorenyl methyl ester) derivatives, respectively. These side chain protecting groups are stable to the TFA (trifluoroacetic acid) treatment employed for removal the N-α-*t*-Boc group; moreover they can easily be removed with piperidine, without cause of any side-reaction or loss of peptide from the solid support. This behavior allows to perform the Asp-Dap side chain to side chain cyclization on the solid support. After the complete assembly of peptide chain, the mono-cyclic peptide was cleaved from the resin by the low-high HF strategy.[13] The second cyclization step was performed in diluted DMF (dimethylformamide) solution using pyBop (benzotriazol-1-yl-oxy(tris-pyrrolidinyl)phosphonium hexafluorophosphate) as activating agent.[14] Finally, the O-acetyl groups were removed by CH_3ONa treatment. The overall yield of the HPLC purified bicyclic peptide was 20 %.

Table 1 summarizes the results obtained with Neuronorm and MEN10627 in the rat vas deferens (RVD) and in the guinea pig trachea (GPT).[15] In RVD Neuronorm antagonized the effect of β-Ala^8-NKA[4-10], a selective

NK-2 agonist, like MEN10627. In fact, both Neuronorm and MEN10627 showed the same pA_2 in these preparations (8.25 ± 0.04 and 8.21 ± 0.04, respectively). In GPT both the peptides were able to shift to the right the concentration-effect curve to β-Ala^8-NKA[4-10]. In these preparations MEN10627 was slightly more effective than Neuronorm (pA_2 values: 7.63 ± 0.3 and 7.10 ± 0.05, respectively).

Figure 2: Synthetic strategy of Neuronorm



a: pyBop/DIEA in DMF; *b*: TFA in CH_2Cl_2; *c*: 0.5 M dicyclohexylcarbodiimide in CH_2Cl_2; *d*: 0.5 M dicyclohexylcarbodiimide in CH_2Cl_2 and 0.5 M 1-hydroxybenzotriazole in DMF; *e*: 20 % piperidine in DMF; *f*: HF/dimethyl sulfide/*p*-cresol/*p*-thiocresol 2.5/6.5/0.5/0.5 (0 °C, 4 h); HF/*p*-thiocresol 9.5/0.5 (0 °C, 0.5 h); *R*: -OCH_2-PAM Resin; X: tetra-O-acetyl-β-D-gal.

Neuronorm contains quite unique structural features:

1. *Neuronorm is a hexapeptide with a bicyclic structure* obtained by a combined backbone to backbone and side chain to side chain cyclization. This gives rise to an inflexible structure[9,11] that can be used to place in the appropriate fixed positions and orientations the necessary pharmacophores. It is expected that a rigid conformation in a peptide, when the bioactive conformation is finely tuned, may have a beneficial effect on potency and selectivity of action. In addition, the conformational rigidity may also determine a considerable resistance to proteolytic degradation,[9,10] and thus a long lasting activity can be predicted for Neuronorm.
2. *Neuronorm is a glicopeptide.* The β-D-galactosyl moiety provides sufficiently high water solubility to Neuronorm. The water solubilty seemed to be an essential requisite to prepare an acceptable pharmacological formulation. In addition, the β-D-galactosyl moiety is unable to modify the orientation of the pharmacophores, in relation to MEN10627. It is also known that bioavailability can significantly be increased by the insertion of a sugar unit[16] and thus an increased activity *in vivo* is expected.

3. *Neuronorm contains a tioacetale bond* connecting the β-D-galactosyl moiety to the peptide scaffold. The higher chemical stability of S-glycosylated amino acids was proven by Baran and Drabarek.[17]

Table 1: Antagonist Activity of Neuronorm and MEN10627 at NK-2 Receptor in Two Different Preparations. β-Ala^8-NKA[4-10] was used as agonist. Each value is the mean ± S.E.M. of five preparations.

		RVD		GPT	
	[Antagonist] (nM)	**Emax (%)**	**pA_2**	**Emax (%)**	**pA_2**
Control		100		100	
β-Ala^8-NKA[4-10] and Neuronorm	10	96 ± 1.2	8.25 ± 0.04	98 ± 0.1	7.10 ± 0.5
	30	99 ± 0.9		93 ± 1.0	
	100	92 ± 2.9		92 ± 1.9	
β-Ala^8-NKA[4-10] and MEN10627	10	95 ± 1.9	8.21 ± 0.04	93 ± 0.9	7.63 ± 0.3
	30	96 ± 1.2		90 ± 0.7	
	100	94 ± 1.1		98 ± 0.8	

The preliminary data on the pharmacological activity of Neuronorm, a novel glico-peptide NK-2 antagonist, in various smooth muscle preparations showed that this compound is capable of blocking NK-2 receptors. The antagonism was competitive in RVD and in GPT. No reduction of the maximal responses to agonist tested was observed in these preparations. Neuronorm was more effective in RVD than in GPT.

Further investigations are in due course to test the selectivity of Neuronorm toward the NK-1 and NK-3 receptors. *In vivo* experiments are being performed to better characterize the pharmacological profile of Neuronorm and to assess its usefulness for the treatment of several diseases, including asthma.

Structural analysis of Neuronorm in solution by ^{1}H NMR spectroscopy, presently under progress, confirms the desired structure for Neuronorm (see figure 1) and the β-linkage of the sugar unit.

Experimental Procedures - Fmoc-Cys(tetra-O-acetyl-β-D-gal)-OH was synthesized starting from (Fmoc-Cys-OH)$_2$ and using a Lewis acid as promoter for the glycosylation reaction, as reported by Eloffson *et al.*[18]

Neuronorm was synthesized by the solid phase method, on an Applied BioSystems Model 430A automatic peptide synthesizer. The classical protocols of the *t*-Boc chemistry were used. The peptide chain was assembled on *t*-Boc-Leu-OCH$_2$-Pam Resin (Pam: phenylacetamido-methyl) by consecutive addition of the appropriate N-α-*t*-Boc protected amino acid. The side chain protecting groups were: Asp(OFm), Trp(CHO) and Dap(Fmoc). Before coupling the Fmoc-Cys(tetra-*O*-acetyl-β-D-gal)-OH, the side chains of Asp and Dap were deblocked with piperidine in DMF (20 % v/v; 3 + 7 min) and the linear precursor was cyclized by treatment with pyBop (3 equivalents) and DIEA (diisopropylethylamine, 6 equivalents) in DMF, overnight. The coupling of Fmoc-Cys(tetra-*O*-acetyl-gal)-OH was achieved by *in situ* activation with pyBop:[19] 3 equivalents amino acid, 3

equivalents pyBop and 6 equivalents DIEA in DMF. After deprotection of the N-terminal Fmoc protecting group with piperidine in DMF, the monocyclic peptide was cleaved from the resin by the low/high HF procedure.[19] After purification by preparative RP-HPLC, the second cyclization step was performed in diluted DMF solution (1 mM), using pyBop as activating reagent.[14] Finally, the acetyl groups were removed from the β-D-galactopyranose by treatment (2 h at room temperature) of the peptide (C: 1.5 mM in dry CH_3OH) with one equivalent of CH_3ONa, which was freshly prepared as a 0.2 M solution by dissolving Na in dry CH_3OH. The final purification by RP-HPLC afforded the desired bicyclic peptide in 20 % yield. Fast atom bombardment mass spectrometry gave the expected molecular weight ($[M-H]^+$ 895 amu).

The activity of Neuronorm has been assessed in *in vitro* experiments on smooth muscle preparations expressing the NK-2 receptors,[15] like RVD and GPT. The effect of Neuronorm has been compared to the NK-2 selective antagonist MEN10627 using several *in vitro* bioassays. β-Ala^8-NKA[4-10] was used as NK-2 agonist. All the experiments were performed on tissues incubated with atropine (1 μM) and thiorphan (1 mM).

The RVD was suspended vertically under 1 g tension in 10 ml organ baths. Drugs changes in smooth muscle tone as well as electrically stimulated contractile response were studied.[15b] Rectangular single pulse of 1 msec duration, 60 V submaximal voltage at a frequency of 6 min^{-1} were applied by dual impedence stimulator (Hugo Sachs Elektronik 215/II). After the equilibrium period (90 min), tissue were electrically stimulated and cumulative concentration-response curves to β-Ala^8-NKA[4-10] (0.1 to 100 nM) were obtained. Moreover, cumulative concentration-response curves for β-Ala^8-NKA[4-10] were obtained previous Neuronorm (10 nM to 100 nM for 30 min) or MEN10627 (10 nM to 100 nM for 15 min) incubation.

The GPT tissues were equilibrated for 90 min in Krebs solution. Acethylcholine (100 μM) was added to the organ baths, to assess tissue responsiveness. Tissues were incubated with 3 μM indomethacine, prior to agonist affinity determination. β-Ala^8-NKA[4-10] (0.1 to 100 nM), concentration-response curves were obtained before and after incubation with Neuronorm (10 to 100 nM) or MEN10627 (10 to 100 nM) for 30 and 15 min, respectively. Only one agonist concentration-response curve was generated for each tissue.

Agonist potency was expressed as EC_{50} (agonist concentration needed to reduce 50% of the maximal response) and pD_2 values (negative logarithm of EC_{50}). Schild plot analysis was performed for each antagonist in various preparations. When the results were compatible with competitive antagonism (slope of Schild not significantly different from unity), pA_2 values were calculated according to Tallarida.[20]

All data in the text are means ± S.E.M. of five preparations. Statistical analysis was performed by means of the Student's test for paired and unpaired data or by means of analysis of variance when applicable.

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