Pergamon

Bioorganic & Medicinal Chemistry Letters 8 (1998) 1153–1156

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A NOVEL SUPER-POTENT NEUROKININ A RECEPTOR ANTAGONIST CONTAINING DEHYDROALANINE

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Received 22 January 1998; accepted 2 April 1998

Abstract – We report here the synthesis and preliminary pharmacological characterization of a novel Neurokinin A receptor antagonist. This molecule contains a dehydroalanine residue. It displays a high conformational rigidity and possesses very high activity. Its pharmacological properties as a neurokinin A receptor antagonist were assessed in *in vitro* experiments on rat vas deferens and were compared to those of Neuronorm and MEN10627.

Neurokinin A (NKA) is a member of a neuropeptide family, the tachykinins (TKs), which share a common pentapeptide sequence (Phe-Xaa-Gly-Leu-Met-NK2: Xaa: variable amino acid) and are widely distributed in the central and peripheral nervous system of mammals, including humans.[1] NKA plays an important role in a variety of physiological and pathophysiological processes, such as smooth muscle contraction in the airways, intestine and genitourinary tract,[2] pain processing[3-5] and anxiety.[6] NKA exerts these biological effects by preferentially activating the NK-2 receptor; however NKA also interacts with lower affinity with the NK-1 and NK-3 receptors, which are better activated by the other two mammalian TKs, substance P and Neurokinin B, respectively.[7-9] Because NKA can be involved in several pathologies, NK-2 receptor antagonists are of great interest. In fact, the development of selective antagonists for the NK-2 receptor may lead to a new class of therapeutic agents for treatment of a number of human diseases.

Conformationally constrained peptides seem to be good candidates in developing receptor antagonists with enhanced potency, selectivity and enzymatic stability. The first example of a selective NK-2 receptor antagonist was the cyclic hexa-peptide L659,877, developed by McKnight *et al.*[10] Structural characterization in solution by NMR spectroscopy and Molecular Dynamic calculations showed for L659,877 several conformational families to be allowed, despite its cyclic structure.[11-14]

With the aim of obtaining more active and selective NK-2 receptor antagonists, we undertook the design of highly constrained hexa-peptides; the compounds that we achieved are characterized by a bicyclic structure

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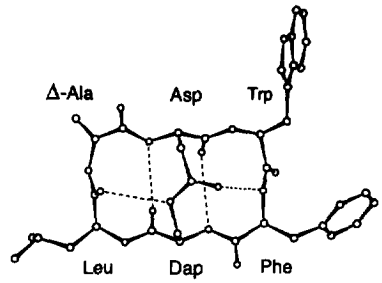
0960-894X/98/$19.00
PII: S0960-894X(98)00178-4

obtained by a combined backbone to backbone and side chain to side chain cyclization.[15-18] The design, synthesis, structural and pharmacological characterization of the most active compounds, namely MEN10627[15,16] and Neuronorm,[18] have recently been reported. MEN10627 is highly hydrophobic with the following sequence: cyclo[Met^{1}Asp^2-Trp^3-Phe^4-Dap^5-Leu^6]cyclo(2β-5β) (Dap: 2,3-diamino propionic acid). To the best of our knowledge, it is among the most potent, selective and long lasting, peptide-based NK-2 antagonists known to date.[19] Neuronorm, (cyclo[Cys^1(β-D-gal)-Asp^2-Trp^3-Phe^4-Dap^5-Leu^6]cyclo(2β-5β); β-D-gal = β-D-galactopyranosyl), is a water soluble analog in which the hydrophobic Met^1 residue has been replaced with the hydrophilic residue Cys^1(β-D-gal). This single amino acid substitution is sufficient to provide a water solubility of 1.8 mg/ml for Neuronorm, higher than that found for MEN10627, which is 15 μg/ml. The NK-2 receptor antagonism of Neuronorm is comparable to that of MEN10627, as determined in *in vitro* experiments on several pharmacological preparations, not withstanding the increased hydrophilicity.[20]

During the last step of the synthesis of Neuronorm, a treatment with methanolic sodium methoxide was performed to convert the protected cyclo[Cys^1(tetra-O-acetyl-β-D-gal)-Asp^2-Trp^3-Phe^4-Dap^5-Leu^6]cyclo(2β-5β) to the unprotected Neuronorm (cyclo[Cys^1(β-D-gal)-Asp^2-Trp^3-Phe^4-Dap^5-Leu^6]cyclo(2β-5β)).[18] Under mild conditions (CH_3ONa concentration = 1.5 mM and reaction time > 2 h) no side-reaction was observed. In contrast, when more drastic conditions were employed (CH_3ONa concentration > 5 mM and reaction time > 5 h) the base catalyzed β-elimination occurred. In fact, a side-product was isolated by RP-HPLC and characterized by fast atom bombardment (FAB) mass spectrometry and ^{1}H NMR spectroscopy.[21] It was found to correspond to cyclo[Δ-Ala^1-Asp^2-Trp^3-Phe^4-Dap^5-Leu^6]cyclo(2β-5β) (Δ-Ala = dehydroalanine; see figure 1). The acronym used in the text is Δ-Ala^1-Neuronorm.

Figure 1: Schematic representation of the structure of Δ-Ala^1-Neuronorm



The activity of Δ-Ala^1-Neuronorm was assessed in *in vitro experiments* on smooth muscle preparation expressing the tachykinin NK-2 receptors,[8,22] such as rat vas deferens (RVD). The effect of Δ-Ala^1-Neuronorm was compared to the NK-2 selective antagonists, Neuronorm and MEN10627. β-Ala^8-NKA[4-10] was used as NK-2 agonist. Table 1 summarizes the results obtained with Δ-Ala^1-Neuronorm, Neuronorm, and MEN10627 in RVD.[23] Δ-Ala^1-Neuronorm antagonized the effect of β-Ala^8-NKA[4-10] similarly to Neuronorm and

MEN10627. All the peptides were able to shift to the right the concentration-effect curve to β-Ala^8-NKA[4-10]. Δ-Ala^1-Neuronorm was more potent than both Neuronorm and MEN10627. A pA_2 of 9.65 ± 0.02 was determined, while both Neuronorm and MEN10627 showed about the same pA_2 in these preparations (8.25 ± 0.04 and 8.21 ± 0.04, respectively). The preliminary data on the pharmacological activity of Δ-Ala^1-Neuronorm, in RVD smooth muscle preparations showed that this compound is capable of competitively antagonizing NK-2 receptors. No reduction of the maximal responses to the agonist tested was observed in this preparation.

Table 1: Antagonist Activity of Δ-Ala^1-Neuronorm, Neuronorm and MEN10627 at NK-2 receptor in RVD preparations. β-Ala^8-NKA[4-10] was used as agonist. Each value is the mean ± S.E.M. of five preparations.[24]

	Control	β-Ala^8-NKA[4-10] and Δ-Ala^1-Neuronorm			β-Ala^8-NKA[4-10] and Neuronorm			β-Ala^8-NKA[4-10] and MEN10627		
[Antag] (nM)		0.3	1	3	10	30	100	10	30	100
Emax (%)	100	97±2	98±1	95±3	96±1.2	99±1	92±3	95±2	96±1	94±1
pA_2		9.65±0.02			8.25±0.04			8.21±0.04		

Further investigations are in due course to test the selectivity of Neuronorm and D-Ala1-Neuronorm toward the NK-1 and NK-3 receptors. It represents a new lead for the development of a new drug for the treatment of several diseases, including asthma. Structural analysis of D-Ala1-Neuronorm in solution by 1H NMR spectroscopy is presently under progress and will be published elsewhere. The structural analysis confirmed the structure for D-Ala1-Neuronorm to be similar to that of MEN10627 (see figure 1).

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21. The CH_3ONa/CH_3OH treatment used to remove the acetyl groups from the carbohydrate moiety was accidentally prolonged overnight. A 5 mM CH_3ONa solution, freshly prepared as a 0.2 M solution by dissolving Na in dry CH_3OH, was used The purification of the reaction mixture by RP-HPLC afforded the side-product in 7 % yield, based on the initial resin substitution. FAB-Mass spectrometry gave a molecular ion peak $[M-H]^+$ of 699 amu, corresponding to Δ-Ala^1-Neuronorm.
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23. The tissues were excised from albino Wistar rats (300-320 g). The experimental conditions were as previously reported.[18] After the equilibrium period (90 min), tissue were electrically stimulated[22]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 incubation of Δ-Ala^1-Neuronorm (0.3 nM to 3 nM for 30 min), Neuronorm (10 nM to 100 nM for 30 min) or MEN10627 (10 nM to 100 nM for 15 min).
24. 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.[25]
All data in the text are means ± S.E.M. of five preparations. Statistical analysis was performed by means of the Student's t test for paired and unpaired data or by means of an analysis of variance when applicable.
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