Journal of Peptide Science, Vol. 1, 236–240 (1995)

Conformational Rigidity *versus* Flexibility in a Novel Peptidic Neurokinin A Receptor Antagonist

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Received 16 November 1994
Accepted 23 December 1994

Abstract: Neurokinin A receptor antagonists have been proposed as a new class of drugs for several applications in humans (asthma, intestinal motility, etc.). The rational design, synthesis, structural characterization and biological activity evaluation of a new potent, highly selective, long-lasting, peptide-based receptor antagonist are reported. The structure–activity relationship indicates that the conformational rigidity determines potency, specificity and especially the long life of the molecule in the living body. MEN10627 is the prototype of a new class of cyclic, peptide-based, neurokinin A receptor antagonists and it is a suitable candidate for clinical testing in humans.

Keywords: Tachykinins; NK-2 antagonist; cyclic peptide; conformation

Abbreviations

NKA, neurokinin A; Tks, tachykinins; Dap, 2,3-diamino propionic acid; *t*-Boc, *tert*-butoxycarbonyl; PAM, phenylacetamidomethyl; PyBop, benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluoro-phosphate; RP-HPLC, reverse-phase high-performance liquid chromatography; GPI, guinea pig ileum; RPA, rabbit pulmonary artery; RVD, rat vas deferens; GPG, guinea pig gallbladder; HT, hamster trachea; RPV, rat portal vein.

Peptides are molecules of great potential interest for their wide variety of biological actions, extreme potency and very low toxicity. However, their limited half-life in the blood stream, mainly because of proteolytic degradation, has brought several laboratories to abandon their possible therapeutical applications in favour of the development of peptidomimetics. Structure–activity studies are conducted on natural peptides to obtain small and potent lead compounds as templates for the rational design of peptidomimetics. Crucial to this issue is the conformational flexibility of short peptides in solution; the three-dimensional structure of a bioactive peptide, when interacting with its specific receptor, is generally unknown or somehow different from the most abundant conformational family of the isolated molecule in solution. The use of molecular tools which should be able, when appropriately positioned in a bioactive peptide sequence, to freeze the molecular conformation, may provide appropriate correlation between three-dimensional structure and biological responses and may therefore allow the bioactive conformer to be envisaged.

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CCC 1075-2617/95/040236-05

We recently applied this approach to the development of a highly potent and conformationally constrained NKA receptor antagonist. NKA is a neuropeptide of the Tks family that is widely distributed in the central and peripheral nervous systems, where it produces several biological effects. NKA acts through preferential interaction with the NK-2 receptors, and NKA receptor antagonists have been proposed as a new class of drugs for several applications in humans (asthma, intestinal motility, etc.) [1]. At the beginning of our study for the development of a NKA receptor antagonist, some information was available on the biological activity of NKA analogues acting either as agonists or antagonists, but very little was known on the structural requirements for their interaction with NK-2 receptors. Recently, a report appeared in the literature [2] on a constrained NKA antagonist, L659,877, or *cyclo*-(Met^1-Gln^2-Trp^3-Phe^4-Gly^5-Leu^6), which displays good antagonist activity and selectivity for the NK-2 receptors. We have therefore attempted to determine the bioactive conformation of L659,877 by comparison with known structures of other cyclic hexapeptides [3]. The predicted bioactive conformation of L659,877 is reported in Figure 1. It contains, as other cyclic hexapeptides [3], two β-turns. The first is centred around the Trp-Phe segment and the second around the Leu-Met residues. The glycine and glutamine residues are in an extended conformation. With the aim of reproducing this model structure in a more rigid molecule, we started from the hypothesis that two consecutive β-amino acids, when incorporated in cyclo-tetrapeptides of a 14-membered ring size, are able to force the remaining pair of α-amino acids to adopt a β-turned structure [4]. The model we achieved corresponds to two cyclic tetrapeptides

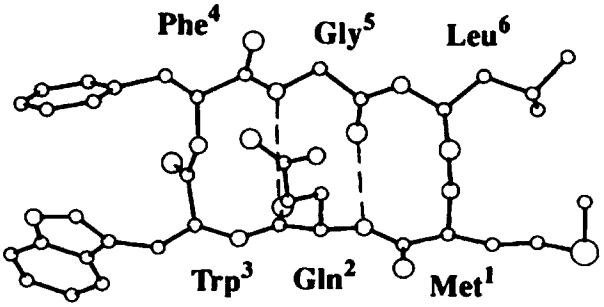

Figure 1 Schematic model for the hypothetical bioactive conformation of L659,877.

fused together, each containing a β-turned structure. MEN10627, namely *cyclo*(Met^1-Asp^2-Trp^3-Phe^4-Dap^5-Leu^6)*cyclo*(2β-5β), contains the amino acids Asp^2 and Dap^5 which allow ring closures and fusion through their β functional groups.

Structural analysis in solution, using NMR spectroscopy, and in the crystal state, using the X-ray diffraction technique, fully confirmed the hypothetical model (details will be published elsewhere). Figure 2 shows the molecular structure as found in the crystal state. The overall shape of the molecule is compact, almost flat and rectangular, with the four hydrophobic side-chains pointing outwards from the four corners. All peptide and amide bonds are *trans*. The Trp-Phe segment is located at the corner of a type I β-turn, with the aromatic side chains at close contact. The Leu-Met segment corresponds to a chain reversal typical of a type II β-turn. These turned structures are stabilized by intramolecular hydrogen bonds. $C'_2{=}O_2{\leftarrow}H{-}N_5$ and $C'_5{=}O_5{\leftarrow}H{-}N_2$. The Asp^2 and Dap^5 residues are in an extended conformation and their relative orientation is typical of hydrogen-bonded residues in an anti-parallel β-sheet arrangement. The cross-linking groups, corresponding to the Asp and Dap side chains, further stabilize the overall molecular structure and the β-turn types by two additional hydrogen bonds, β-$C'_2{=}O_2{\leftarrow}H{-}N_4$ and $C'_6{=}O_6{\leftarrow}\beta{-}H{-}N_5$. This structure was almost identical in acetonitrile solution, as ascertained by NMR spectroscopy, and displays an unusual conformational rigidity, as indicated by trajectory analysis of molecular dynamic simulations *in vacuo* (unpublished material).

The *in vitro* biological activity of MEN10627 was determined on the following bioassay: GPI for the NK-1 receptor, RPA, RVD, GPG and HT for the NK-2 receptors, and RPV for the NK-3 receptor. Conditions for the bioassays were as described previously [5]. In Table I the activity of MEN10627 is compared with that of the monocyclic antagonist L659,877. MEN10627 exerts a potent and competitive antagonism toward NK-2 receptor-mediated responses in all preparations examined and displays a high degree of selectivity for the NK-2 receptors, as shown by the poor interaction with NK-1 and NK-3 receptors. MEN10627 is the most potent NKA receptor antagonist thus far described in the HT. As compared to L659,877, the modifications inserted in MEN10627 led to an increase in absolute potency at NK-2 receptors, without significantly modifying the affinity for NK-1 or NK-3 receptors. The *in vivo* activity of MEN10627 was investigated in urethane-anesthetized rats for its ability to inhibit contraction of the

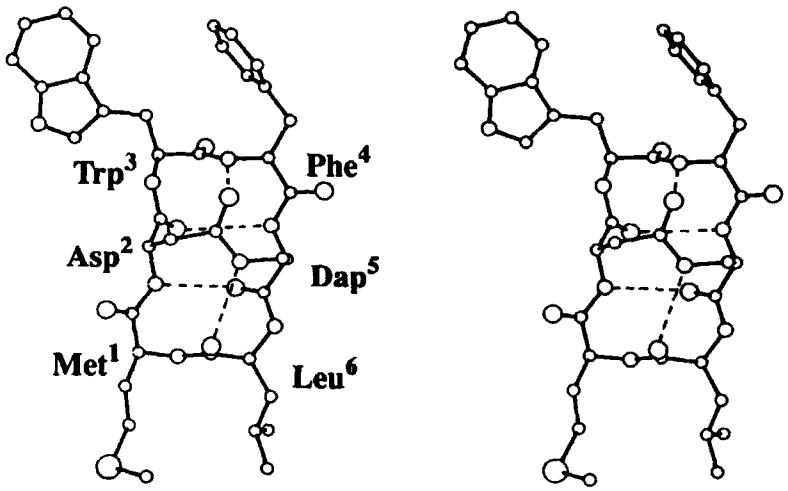

Figure 2 Stereo-view of MEN10627 X-ray molecular structure. Intramolecular hydrogen bonds are indicated as broken lines.

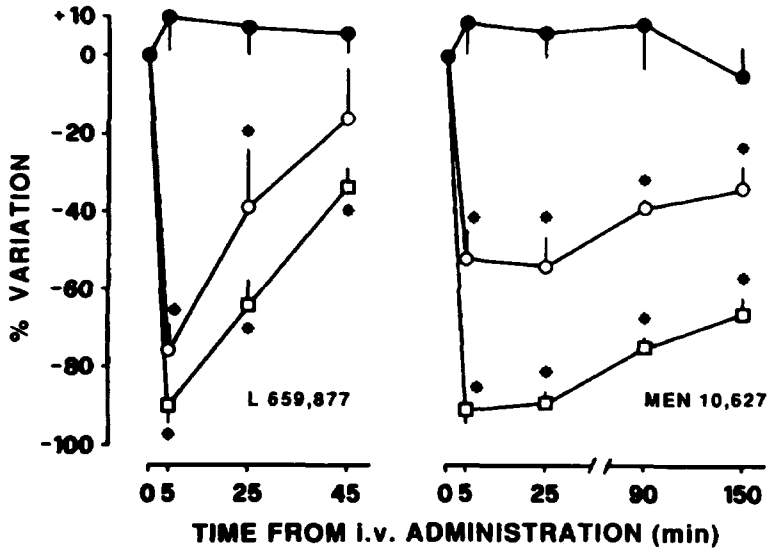

Figure 3 Inhibitory effect time course of L659,877 (left panel) and MEN10627 (right panel) on the urinary bladder contraction produced by the i.v. administration of Tks NKA receptor selective agonist [β-Ala^8] NKA(4–10) in urethane-anesthetized rats. After having recorded at least two reproducible responses to the agonist, L659,877 (100 nmol kg^{-1}, ○; 300 nmol kg^{-1}, □) or MEN10627 (10 nmol kg^{-1}, ○; 30 nmol kg^{-1}, □) were administered intravenously. Changes in the response to the agonist were determined up to 150 min from the antagonist's administration. In both panels, changes in the response to the agonists are shown as the control response percentage variation determined in each animal. ● Represents the variation in the response to the agonist in rats receiving the vehicle used to dissolve L659,877 and MEN10627 (dimethylsulfoxide). Each value is mean ± s.e.m. of 4–6 experiments. * Indicates $P < 0.05$ as compared to vehicle. Details of the method are described in reference [6].

Table 1 Comparison of MEN10627 and L659,877 *in vitro* Biological Activity on Various Isolated Smooth Muscle Preparations, Expressed as pK_B values ± SE of the Mean ($n = 9$–12) (pK_B Corresponds to the Apparent Affinity Constant Negative Logarithm)

Antagonist	GPI	RPA	HT	RVD	GPG	RPV
MEN10627	6.1 ± 0.1	8.1 ± 0.05	10.1 ± 0.1	8.7 ± 0.1	7.9 ± 0.1	inactive[a]
L659,877	5.6 ± 0.1	6.7 ± 0.1	7.9 ± 0.1	7.9 ± 0.1	6.3 ± 0.1	5.4 ± 0.1

[a] Inactive at 1 mM.

urinary bladder following i.v. administration of the NK-2 selective agonist [β-Ala^8]NKA(4–10) (1 nmol/kg i.v.); experimental conditions were as reported previously [6]. MEN10627 inhibited in a dose-dependent manner the response to the NK-2 agonist. A comparison between the inhibitory effect of MEN10627 and L659,877 in *in vivo* experiments is reported in Figure 3. The bicyclic compound shows an activity comparable to that of the monocyclic peptide at 10-fold lower *in vivo* doses. Furthermore, the inhibitory effect is maintained even after 2 h from administration. Resistance to proteolytic degradation of MEN10627 was evaluated in the human, guinea pig and rat plasma. The product appeared stable through an incubation period of 8 h [7].

MEN10627 is the prototype of a new class of polycyclic peptide-based Tks receptor antagonists. Owing to its high potency an long-lasting activity *in vivo*, MEN10627 and its analogues are suitable candidates for clinical testing in humans.

The high potency of MEN10627 can be attributed to its conformational rigidity. Limited unfavourable entropy loss occurs when MEN10627 interacts with its specific receptor, as compared with other more flexible NKA antagonists (L659,877) [8–10].

Also the high selectivity of MEN10627 can be attributed to the conformational rigidity of the molecule. It is reasonable to believe that MEN10627 would not be able to adopt the bioactive conformation assumed by NKA or by L659,877 when interacting with the NK-1 or NK-3 receptors.

We also believe that the long-lasting activity is related to its conformational rigidity. Our interpretation of this unexpected long-lasting activity is that the molecular conformation is such that it cannot fit well into any protease active site and cannot even be adapted because of its rigidity.

In conclusion, the present work demonstrates that it is still of great interest to pursue the development of peptides as drugs provided it is possible to design and to synthesize a peptide analogue with a well-determined, rigid three-dimensional structure corresponding to the bioactive conformation. The conformational rigidity determines potency, specificity and especially resistance to proteolytic degradation in the living body.

EXPERIMENTAL PART

MEN10627 was synthesized by the solid phase method, using *t*-Boc chemistry on a Boc-Leu-OCH_2-Pam resin. The side chains of Asp and Dap residues were protected as fluorenylmethyl ester and fluorenylmethoxycarbonyl derivative, respectively. Treatment with piperidine and cyclization, using PyBop [11, 12], was performed, before coupling Boc-Met-OH. After completion of peptide assembling on the resin and removal (with the low–high HF procedure) [13] of the monocyclic peptide, the second cyclization step was performed in diluted dimethylformamide solution using PyBop. The overall yield of the RP-HPLC purified bicyclic peptide was 27%. The purity of the product was ascertained by analytical RP-HPLC, on a Vydac C_{18} column (4.6 × 150 mm; 5 μm), eluted with H_2O/0.1% TFA (A) and CH_3CN/0.1% TFA (B). A linear gradient from 20 to 80% of (B) over 25 min at flow rate of 1 ml/min was employed (R_t: 18.1 min). Fast atom bombardment mass spectroscopy gave a molecular ion peak $[M-H]^+$ of 761 a.m.u., as expected.

Acknowledgements

We wish to thank Dr Attilio Crea for measurements on peptide degradation in plasma.

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