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Letters

New Class of Corticotropin-Releasing Factor (CRF) Antagonists: Small Peptides Having High Binding Affinity for CRF Receptor

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Abstract: The discovery of small and potent peptide antagonists of the corticotropin-releasing factor (CRF) receptor is described. Through the structure−activity relationship studies of 12-amino acid peptide corresponding to the C-terminal residues of astressin, we assumed that a particular surface of the α-helix was important for binding to the receptor. The small peptide containing D-Ala^{31} and cyclohexylalanine^{38} on that surface was as potent as astressin in binding to the CRF receptor and showed significant ACTH suppression when administered to rats.

Corticotropin-releasing factor (CRF) was initially characterized as a 41-amino acid peptide that regulates humoral response to stress by stimulating secretion of adrenocorticotropic hormone (ACTH).[1,2] A large number of preclinical and clinical findings reported so far support the hypothesis that CRF accounts for a wide range of stress-related disorders such as anxiety, depression, eating disorders, gastrointestinal maladies, irritable bowel syndrome, and postoperative stress.[3−5] Because of a variety of pathological conditions induced by CRF, many potent non-peptide antagonists of CRF have been prepared as a drug target; however, a clinically useful antagonist has not been reported yet.[6]

Peptide ligands designed on the basis of CRF sequence were developed a decade ago. The peptide agonists and antagonists of CRF receptors played a significant role in the investigation of CRF-mediated physiology. Astressin (cyclo(30−33)-[D-Phe^{12},Nle^{21,38},-Glu^{30},Lys^{33}]h-CRF(12−41)), discovered by Rivier et al., is well-known as a potent peptidic CRF antagonist (K_i = 2 nM for type 1 receptor of human CRF (CRFR1)).[7] From earlier studies, CRF-related peptides have been supposed to assume an α-helical structure upon binding to their receptors.[8] Rivier et al. have concluded that the structural restriction by lactam ring in astressin would likely elicit stabilization of α-helical conformation and consequent high affinity for CRF receptor. After discovery of astressin, a number of its analogues consisting of 30 amino acids have been reported,[9−11] whereas no peptidic CRF antagonist smaller than astressin has

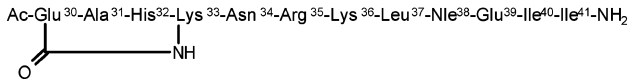

Figure 1. Sequence of peptide **1**.

Table 1. Binding Affinities of Peptides for CRFR1[a]

	peptide	K_i (nM)
astressin		2.0
1		50.0
2	[Dap^{31}]peptide-**1**	>1000
3	[Asp^{31}]peptide-**1**	>1000
4	[Leu^{34}]peptide-**1**	>1000
5	[Dab^{34}]peptide-**1**	>1000
6	[Asp^{34}]peptide-**1**	>1000
7	[Gln^{34}]peptide-**1**	>1000
8	[Ala^{35}]peptide-**1**	70.9
9	[Lys^{35}]peptide-**1**	45.1
10	[Gly^{35}]peptide-**1**	>1000
11	[Glu^{32},Asp^{39}]peptide-**1**	36.7
12	[Glu^{32}]peptide-**1**	130
13	[Lys^{32}]peptide-**1**	44.8
14	[Leu^{32}]peptide-**1**	48.9
15	[Asp^{32}]peptide-**1**	>1000
16	[Phe^{32}]peptide-**1**	>1000
17	[Glu^{32},Cha^{38},Asp^{39}]peptide-**1**	5.5
18	[Glu^{32},Phe^{38},Asp^{39}]peptide-**1**	81.0
19	[Aib^{31},Glu^{32},Cha^{38},Asp^{39}]peptide-**1**	3.0
20	[D-Ala^{31},Glu^{32},Cha^{38},Asp^{39}]peptide-**1**	3.1

[a] Dap: (2*S*)-2,3-diaminopropionic acid. Dab: (2*S*)-2,4-diaminobutyric acid. Cha: cyclohexylalanine. Aib: 2-aminoisobutyric acid.

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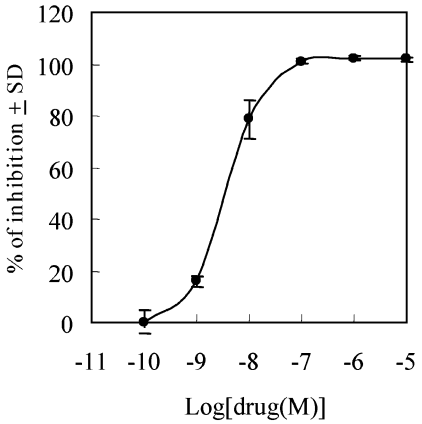

Figure 2. Competition by peptide **20** for [^{125}I]Tyr^{0}-rat/human CRF binding to CRFR1. A concentration−response curve for the inhibition of CRF binding to CRFR1 by **20** is represented as the mean of three determinations, each done in duplicate.

been known for a long time. Recently, the Solvay Pharmaceuticals group reported small peptide CRF antagonists that were about half the size of astressin in their patent application (WO01/29086), although the activities of these peptides were not clarified.

We were interested in exploring a small peptide of less than 30 residues as a new class of CRF antagonists. Small peptides would have some advantages in comparison with larger peptides from the viewpoints of stability, permeability, and synthetic cost. Furthermore, small peptide antagonists of CRF would be useful tools to investigate the mode of binding between CRF and its receptor. In this report, the design and synthesis of novel small peptidic CRF antagonists consisting of 12 amino acids are described, and the structure−activity relationships are discussed as well.

To evaluate the influence of shortening astressin at the N-terminus on binding potency to CRF receptor, we first synthesized a peptide of 12 residues, cyclo(30−33)-[Glu^{30},Lys^{33},Nle^{38}]Ac-hCRF(30−41) (**1**) (Figure 1), which is one of the claimed peptides in the Solvay's patent. In the CRFR1 binding assay,[12] peptide **1** showed interesting affinity (K_i = 50 nM), although it was less potent than astressin. From this result, we concentrated our efforts on investigating which position in peptide **1** is essential to receptor binding. Table 1 shows the peptides **1**−**20** that we prepared and their K_i values in the CRFR1 binding assay.[12] Figure 2 shows a concentration−response curve for the inhibition of CRF binding to CRFR1 by **20** as an example of the CRFR1 binding assay.

At first, we directed our attention toward the three amino acids Ala^{31}, Asn^{34}, and Arg^{35} because these residues were conserved in all of the CRF-like peptides. These residues in peptide **1** were replaced by other amino acids to evaluate their roles in the receptor binding.

The replacements of Ala^{31} in **1** by (2*S*)-2,3-diaminopropionic acid (Dap) (**2**) and Asp (**3**) greatly decreased the affinities for CRFR1. Similar significant reductions of the affinities were observed in the replacements of Asn^{34} by Leu (**4**), (2*S*)-2,4-diaminobutyric acid (Dab) (**5**), Asp (**6**), and Gln (**7**). On the other hand, the replacements of Arg^{35} in **1** afforded interesting results. Peptides **8** and **9** replaced by Ala and Lys, respectively, at

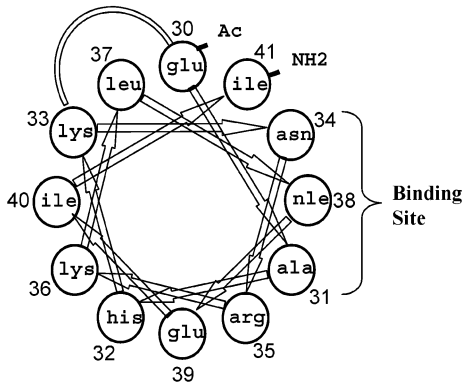

Figure 3. Helical wheel diagrams of peptide **1**. The view is from the N-terminus.

position 35 showed almost the same affinities as **1**, while peptide **10** replaced by Gly was less potent than **1**. To discuss these structure−activity relationship results, we built a putative model describing the relative orientation of the α-helix of peptide **1** as shown in Figure 3. In this model, both Ala^{31} and Asn^{34} are aligned on the same side of the helical cylinder surface, while Arg^{35} is situated on the other side, which faces a different direction from the surface containing Ala^{31} and Asn^{34}. Accordingly, we speculated that a particular surface of helix, on which Ala^{31} and Asn^{34} exist, might play an important role in the interaction with CRF receptor.

To verify our speculation, we further synthesized some peptides substituted by various amino acids at positions 32, 36, 39, and 40 of peptide **1**. These positions are on a surface different from that containing Ala^{31} and Asn^{34} in the helix model. As we had speculated, the modifications at these positions did not afford the remarkable improvements of affinities, although peptide **11** possessing Glu^{32} and Asp^{39} was slightly more potent than **1**. During the course of these peptide modifications, we found interesting structure−activity relationships at position 32. The replacements of His^{32} by Glu (**12**), Lys (**13**), and Leu (**14**) scarcely affected the affinities for CRFR1, while the replacements by Asp (**15**) and Phe (**16**) greatly reduced the affinities. The similar results were obtained in the substitutions at position 35 as described above. In the helix model of peptide **1**, His^{32} is located on the same side of the helical cylinder surface as Arg^{35} is. According to the study by Williams et al. concerning the frequency of the amino acid in different types of secondary structure of protein, it follows that His, Glu, Lys, Ala, and Leu prefer the α-helix compared with Asp, Phe, and Gly.[13] Therefore, the results obtained in the substitutions at positions 32 and 35 indicate that these positions are not important for direct interaction with the receptor but for stabilization of α-helical bioactive conformation of the peptides.

From above results, we hypothesized that the particular surface containing Ala^{31} and Asn^{34} on the helical cylinder faced the CRF receptor in their binding. On the basis of our hypothesis, we next turned our attention to position 38 because Nle^{38} of peptide **1** is also located on the same side as Ala^{31} and Asn^{34} are in the helix model. On the assumption that Nle^{38} contributes to the interaction with the receptor, it is predicted that a hydrophobic character of the side chain of Nle is likely important for binding to the receptor. Thus, we attempted to replace Nle^{38} in peptide **11** by other

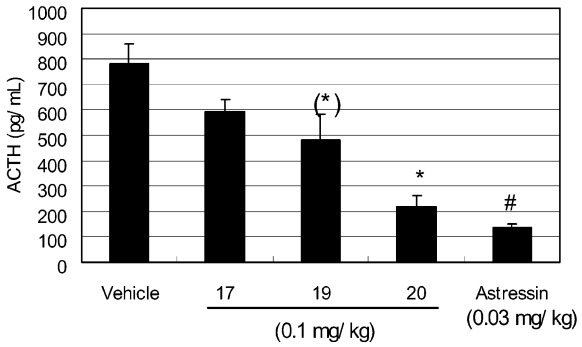

Figure 4. Inhibition of ACTH secretion by peptides **17**, **19**, **20** (0.1 mg/kg, iv) and astressin (0.03 mg/kg, iv) in rat CLP model. Concentration of plasma ACTH after 10 min from the administration of peptide is presented as the mean ± SEM (n = 6). Statistical analyses were performed by Dunnett's test ($^{(*)}$ $p < 0.05$; * $p < 0.01$) or by the t test ($^{\#}p < 0.01$).

hydrophobic amino acids. Surprisingly, the replacement of Nle[38] by cyclohexylalanine (Cha) (**17**) induced drastic elevation of the affinity for CRFR1, demonstrating that the hydrophobic side chain at position 38 is essential to the receptor binding. The K_i value of peptide **17** was 10-fold higher than that of **1** and close to that of astressin. On the other hand, the replacement by Phe (**18**) did not increase the affinity. Compared with the butyl group of Nle or the benzyl group of Phe, the cyclohexylmethyl group of Cha is more bulky and has presumably a suitable size to maximize the hydrophobic interaction between the peptide and the receptor.

The foregoing findings of the structure−activity relationships support our hypothesis that the particular surface of the peptide helix, which comprises the residues at positions 31, 34 and 38, deeply participates in the binding to the CRF receptor.

To evaluate an action of potent peptide **17** as a CRF antagonist, the inhibition of ACTH secretion by **17** in rat, performed by an operation of cecal ligation and puncture (rat CLP model), was examined.[14] After administration of **17** (0.1 mg/kg, iv) to the rat CLP model, the plasma ACTH level was measured.[15,16] As shown in Figure 4, it was found that peptide **17** had no significant effect on ACTH secretion. This result is probably due to the low stability of **17** by enzymatic degradation in rats. Introduction of unnatural amino acid is an effective means to improve the resistance of peptide to peptidases. For example, Takahata et al. have reported that the N-terminal degradation rate of peptide by aminopeptidase M is much slower when D-amino acid is introduced at the second position from the N-terminus.[17] To increase the stability of **17** against enzymatic degradation, we replaced Ala[31] of **17** by unnatural amino acids, 2-aminoisobutyric acid (Aib) (**19**) and D-Ala (**20**). Both **19** and **20** were equipotent with **17** in the CRFR1 binding assay so that they were administered (0.1 mg/kg, iv) to the rat CLP model. As a result, peptide **20** containing D-Ala[31] showed a significant inhibition of ACTH secretion, although the inhibitory effect by **20** (0.1 mg/kg, iv) was somewhat weaker than that by astressin (0.03 mg/kg, iv) (Figure 4). It is noteworthy that the potency in vivo was improved by introducing only one unnatural amino acid into the peptide.

In summary, peptides consisting of 12 amino acids were synthesized to explore a small peptide antagonist having high affinity for CRF receptor. Through this study, we found that the particular surface of the α-helix, which comprised the residues at positions 31, 34, and 38, deeply participated in the binding to CRF receptor. This information led to the discovery that the introduction of Cha to position 38 greatly increased the affinity for the receptor. Furthermore, it was found that the introduction of D-Ala to position 31 improved the potency in vivo. Peptide **20** possessing D-Ala[31] and Cha[38] showed the same affinity as that of astressin in the CRFR1 binding assay and significantly inhibited ACTH secretion when administered to the rat CLP model. This peptide is the first potent CRF antagonist of small size of less than 30 residues. We expect **20** to be not only a useful tool for biological studies but also a novel therapeutic agent for CRF-mediated disorders.

Supporting Information Available: Experimental details for the synthesis of peptide **20** and LC/MS data for peptides **1**−**20**. This material is available free of charge via the Internet at http://pubs.acs.org..

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(12) Affinity of the peptides for human CRF1 receptor (CRFR1) was determined by binding studies using membrane preparations of HeLa cells, expressing the human CRFR1 (transfected with a plasmid containing genes coding for human CRFR1) and [^{125}I]-Tyr^{0}-rat/human CRF as the ligand. Separation of bound and free ligand is performed using scintillation proximity assay (SPA) beads (American Life Sciences; RPNQ0001) with wheatgerm aggulutinin (WGA), according to previously reported methods (Higelin, J.; et al. *Neuropharmacology* **2001**, *40*, 114−122). Briefly, SPA beads precoupled with membrane preparations of HeLa cells expressing human CRFR1 were incubated for 180

min at 22 °C with 0.2 nM [^{125}I]Tyr^{0}-rat/human CRF in the absence or presence of the compounds in 200 μL of assay buffer [50 mM Tris-HCl (pH 7.7), 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 0.1% BSA, 0.1% bacitracin, 0.05% FCS, and 5% horse serum]. Following incubation, radioactivity on the beads is measured in a scintillation counter (Topcount, Packard) using a scintillation cocktail (Microscinti-20, Packard). Specific radioligand binding to the receptors is defined as the difference between total binding and nonspecific binding determined in the presence of an excess of rat/human CRF. All IC_{50} values were converted to inhibitory constants (K_i) using the Cheng−Prusoff equation (Cheng, Y. C.; Prusoff, W. H. Relationship between the inhibition constant, Ki, and the concentration of inhibition which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099).

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(15) Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Afterward, an intravenous injection of peptide followed by treatment of cecal ligation and puncture (CLP) was performed. After 10 min, rats were decapitated, and the blood samples were assayed for ACTH with a commercially available kit (Mitsubishi Kagaku Medical, Inc.).

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