Title

Characterization of Multisubstituted Corticotropin Releasing Factor (CRF) Peptide Antagonists (Astressins)

Journal

Journal of Medicinal Chemistry, 59(3)

ISSN

0022-2623

Authors

Erchegyi, Judit
Wang, Lixin
Gulyas, Jozsef
et al.

Publication Date

2016-02-11

DOI

10.1021/acs.jmedchem.5b00926

Peer reviewed

HHS Public Access

Author manuscript

J Med Chem. Author manuscript; available in PMC 2018 May 18.

Published in final edited form as:

J Med Chem. 2016 February 11; 59(3): 854–866. doi:10.1021/acs.jmedchem.5b00926.

Characterization of Multisubstituted Corticotropin Releasing Factor (CRF) Peptide Antagonists (Astressins)

Judit Erchegyi[†], **Lixin Wang**[‡], **Jozsef Gulyas**[†], **Manoj Samant**[†], **Marilyn H. Perrin**[†], **Kathy Lewis**[†], **Charleen Miller**[†], **Joan Vaughan**[†], **Cynthia Donaldson**[†], **Wolfgang Fischer**[†], **William Low**[†], **Seiichi Yakabi**[‡], **Hiroshi Karasawa**[‡], **Yvette Taché**[‡], **Catherine Rivier**[†], and **Jean Rivier**[*,†]

[†]The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, 10010 N. Torrey Pines Road, La Jolla, California 92037, United States

[‡]Department of Medicine, CURE/Digestive Diseases Center, Digestive Diseases Division, University of California at Los Angeles, and Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, California 90073, United States

Abstract

CRF mediates numerous stress-related endocrine, autonomic, metabolic, and behavioral responses. We present the synthesis and chemical and biological properties of astressin B analogues {cyclo(30–33)[D-Phe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{27,40},Glu^{30},Lys^{33}]-acetyl-h/r-CRF$_{(9–41)}$}. Out of 37 novel peptides, 17 (**2**, **4**, **6**–**8**, **10**, **11**, **16**, **17**, **27**, **29**, **30**, **32**–**36**) and 16 (**3**, **5**, **9**, **12**–**15**, **18**, **19**, **22**–**26**, **28**, **31**) had k_i to CRF receptors in the high picomolar and low nanomole ranges, respectively. Peptides **1**, **2**, and **11** inhibited h/rCRF and urocortin 1-induced cAMP release from AtT20 and A7r5 cells. When Astressin C **2** was administered to adrenalectomized rats at 1.0 mg subcutaneously, it inhibited ACTH release for >7 d. Additional rat data based on the inhibitory effect of (**2**) on h/rCRF-induced stimulation of colonic secretory motor activity and urocortin 2-induced delayed gastric emptying also indicate a safe and long-lasting antagonistic effect. The overall properties of selected analogues may fulfill the criteria expected from clinical candidates.

Graphical abstract

[*]**Corresponding Author:** Phone: 858-453-4100, extension 1350. Fax: 858-552-1546. jrivier@salk.edu.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.5b00926. Experimental section covering all experimental protocols (PDF)

Author Contributions

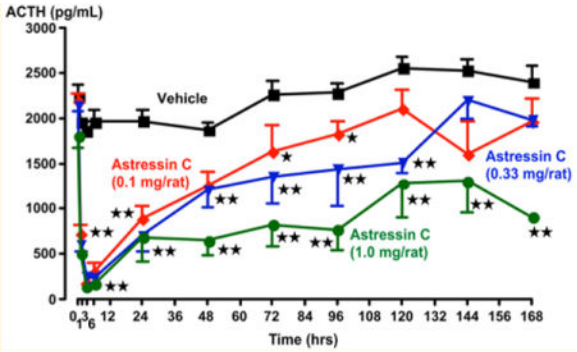
The manuscript was written through contributions of all authors. J.E., J.G., M.S., and C.M. synthesized and characterized the peptides; M.H.P., K.L., J.V., C.M., and C.D. carried out the in vitro assays; W.L. and W.F. carried out the mass spectrometric analysis; S.Y. and H.K. carried out the in vivo assays at UCLA; Y.T. and L.W. supervised and L.W., H.K., and S.Y. carried out the in vivo gastrointestinal studies at UCLA; C.R. supervised and carried out the in vivo ACTH assays at Salk; and J.R., J.E., C.R., and Y.T. contributed to the writing of the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): J.R. is Founder and President of Sentia Medical Sciences Inc.

ADDITIONAL NOTE

The abbreviations for the common amino acids are in accordance with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (*Eur. J. Biochem.* **1984**, *138*, 9–37). The symbols represent the L-isomer except when indicated otherwise.



INTRODUCTION

Since the discovery of the corticotropin releasing factor (CRF), a 41 amino acid-containing neuropeptide,[1–4] our laboratory has developed three generations of CRF antagonists.[5] The first one, α-helical-CRF$_{(9–41)}$,[6] showed marked differences in its ability to antagonize the actions of CRF in three in vivo bioassay systems.[7] Later, we found that the introduction of Nle residues at positions 21 and 38 of CRF (in lieu of Met) generated analogues that were significantly more potent than α-helical-CRF $_{(9–41)}$.[8] One such antagonist, [D-Phe^{12},Nle^{21,38}]-h/rCRF$_{(12–41)}$, was used as our assay standard for many years and is ~18 times more potent than α-helical-CRF$_{(9–41)}$ at inhibiting the release of adrenocorticotropin (ACTH) by rat pituitary cells in culture and in adrenalectomized rats.[8] This analogue was tested widely, yet was still relatively inefficient and short acting at inhibiting ACTH secretion, which limited its use for chronic treatments in animal models or for clinical investigations. The introduction of a lactam bridge that spanned the side chains of Glu^{30}, Lys^{33} yielded the third generation of antagonists, based on astressin (Ast) (cyclo (30–33)[D-Phe^{12},Nle^{21,38},Glu^{30},Lys^{33}]-h/rCRF$_{(12–41)}$). This antagonist was 32 times more potent than the linear [D-Phe^{12},Nle^{21,38}]-h/rCRF$_{(12–41)}$ at inhibiting CRF-stimulated ACTH release by cultured rat anterior pituitary cells.[8] Other in vivo studies showed that the intracerebroventricular (icv) injection of Ast dose-dependently blunted the stimulation of fecal pellet output induced in conscious rats as indicative of colonic propulsive motor activation.[9] However, Ast was still short-acting in vivo. Chemical modifications such as elongation of the peptide chain at the N-terminus (from 12–41 to 9–41), acetylation of the N-terminus, and methylation of α-carbons, in addition to the introduction of the Glu^{30}-Lys^{33} lactam bridge, resulted in a much improved nonselective, long-acting antagonist Ac-Asp-Leu-Thr-D-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Nle-Ala-Arg-Ala-Glu-Gln-C$^{\alpha}$MeLeu-Ala-Gln-Glu-Ala-His-Lys-Asn-Arg-Lys-Leu-Nle-Glu-C$^{\alpha}$MeLeu-Ile-NH$_2$ (AstB, **1**).[10,11] As a result, AstB has been used by a number of investigators as a CRF receptor antagonist to study the biological actions of the CRF signaling system.[12–19] Under basal conditions, AstB produced no obvious side effects in rats, pigs, or monkeys while inhibiting cardinal effects of stress on endocrine, gastrointestinal, and behavioral function.[10–13] Indeed, the subcutaneous injection of very high doses (10 mg/kg, sc) of a number of antagonists based on this series did not induce any detectable adverse symptoms and was accompanied by survival of all of the animals (C. Rivier and J. Rivier, unpublished results). As we were studying the effects of these third generation CRF antagonists, we repeatedly observed that

AstB showed differing profiles of activity, compared with non-peptide CRF receptor (CRF-R) antagonists.[11] We therefore believe that the development of long-acting peptidic CRF-R1/CRF-R2 antagonists that act in the periphery (as opposed to in the brain) is of significant importance in the context of chronic stress and future translation to the clinic. Whereas similarities between non-peptide and peptide CRF antagonists are few, if any at all, one has to consider that with four different ligands (CRF, urocortin (Ucn 1), urocortin 2 (Ucn 2), and urocortin 3 (Ucn 3) to two receptors (CRF-R1 and CRF-R2) and two main sites of action one on either side of the blood–brain barrier (BBB) (i.e., in the periphery and within the BBB), the number of permutations is not easily manageable. Presently, there is only one synthetic non-peptide family of small CRF R1-selective ligands that may or may not cross the BBB, be toxic, or be orally active. Peptide ligands on the other hand are not supposed to cross the BBB and/or be orally active. They have multiple sites of action and can be CRF-R1-selective, CRF-R2-selective, or CRF-R1/2-nonselective. Peptide CRF ligands are easily soluble in either aqueous or oily solvents, acceptable for intravenous (iv), subcutaneous (sc), and intramuscular (im) delivery where they form a slow release depot. Overall, peptide CRF ligands are valuable research tools and most likely good potential drug candidates. Predicting which of these ligands will be best for any particular indication will have to be demonstrated experimentally until enough examples are described. At this time, the nonselective CRF-R1/2 antagonist *AstB* is alone in its ability to induce hair regrowth in aging CRF-overexpressing mice when CRF-R2-selective astressin$_2$ B (Ast2B),[19] minoxidil, a commercial product used for hair regrowth, 2,4-pyrimidinediamine, 6-(1-piperidinyl)-, 3-oxide or antalarmin (*N*-butyl-*N*-ethyl-2,5,6-trimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolol[2,3-*d*]pyrimidin-4-amine hydrochloride, a CRF-R1-selective small molecule, all give similar spotty results.

Here, we present pertinent preclinical data that include the synthesis, purification, chemical characterization (HPLC, MS, solubility), and biological properties, including binding selectivity, their ability to inhibit ACTH release in adrenalectomized (ADX) rats, their effect on the intracellular cAMP accumulation stimulated by h/rCRF or rat rUcn1 in CRF-R1 and CRF-R2 expression cell lines, as well as their in vivo effect on hCRF-induced stimulation of colonic secretory motor activity and on human hUcn 2-induced delayed gastric emptying.

RESULTS AND DISCUSSION

1. Synthesis and Physicochemical Characterization of the Peptides (See Supporting Information)

All analogues shown in Tables 1 and 2 were synthesized either manually or automatically on a methylbenzhydrylamine (MBHA) resin using the Boc strategy with orthogonal protection of the side chains of the glutamic acid (OFm) and the lysine (Fmoc) residues to be cyclized. DIC/HOBt reagents in NMP mediated the main chain assembly. The couplings of the C$^{\alpha}$-Me amino acids themselves and to Aib, C$^{\alpha}$MeLeu, C$^{\alpha}$MeVal, and C$^{\alpha}$MePhe were always repeated. After coupling Boc-Glu(OFm) at position 22, the OFm and Fmoc protecting groups of Glu^{22} and Lys^{25}, respectively, were removed by treating the resin with 20% piperidine/NMP (2 × 10 min). Using the coupling reagent PyBop and DIPEA base for 2 h mediated the lactam-bridge formation. The synthesis was then continued. The deprotected

N-terminus was acetylated with an excess of acetic anhydride in DCM for 15 min. The substitution of the N-terminal acetyl group with acyl groups in AstD (**11**) resulted in **32**–**38** (Table 2) with molecular weights ranging from ~3800 to ~4100. They were coupled with DIC activation or the corresponding anhydride. The Dnp side chain-protecting group of His was removed with the treatment of 20% thiophenol in NMP for 2 h. The peptide resins were treated with anhydrous HF to liberate the fully deprotected crude peptides, which were purified using reverse-phase HPLC and three different buffer systems in the following order: TEAP pH 2.25, TEAP pH 4.5, or TEAP pH 6.5, and 0.1% TFA. Using RP-HPLC supports for preparative purification consisting of radial compressed cartridges (often packed in the laboratory) and orthogonal solvent systems yielded highly purified compounds that are in general greater than 95% pure by quantitative analytical HPLC using UV detection at 214 nm. Analysis using CE and CZE (taking advantage of possible ionic and hydrophobic interactions) is yet another sensitive (UV detection at 214 nm) complementary methodology. See Tables 1 and 2. The purified final products were isolated as the trifluoroacetate (TFA) salts and characterized using analytical RP-HPLC, CE, CZE, and mass spectrometry (MALDI and ESI-MS). In analogues **20** and **21**, the disulfide bridge was formed in 75% acetic acid solution stirring the peptide with iodine for 40 min. The measured masses obtained were in agreement with those calculated for the protonated molecule ions. Solubility studies of selected peptides were carried out in DMSO (20%) and 5% D-mannitol in H_2O (80%), which was the vehicle used in the in vivo assays, at a peptide concentration of 10 mg/mL. Results are summarized in Tables 1 and 2. Among all of the selected analogues, only **15**, **36**, and **37** were insoluble and **17** formed a gel at the given concentration after 2 h.

2. In Vitro Biological Characterization and SAR Studies Based on Receptor Binding Assay

The compounds were tested for their ability to bind CRF-R1 and CRF-R2β. The K_i values are given in Tables 1 and 2.

AstB (**1**) has a His residue at position 24. Substitution of His^{24} by Aib (AstC, **2**), NMe-D-Ala (**3**), Ala (**4**), D-Ala (**5**), D-Aph(Cbm) (**6**), C$^{\alpha}$MeLeu (**7**), and D-C$^{\alpha}$MeLeu (**8**) did not influence the binding affinity of the resulting analogues for CRF-R1 receptor, and all of them showed only modestly increased (~2- to 3-fold) binding affinity for CRF-R2β in the 0.3–0.5 nM range. One should note that **6**–**8**, **14**, and **35** are not as pure as desired. We have found that in adequately controlled experiments, most repeat biological tests with a purer material yielded in vivo potencies and in vitro binding affinities that are not significantly different from those obtained with analogs that are >80% pure. Noteworthy is the high affinity of these analogues (K_i < 1.0 nM) and the remarkable reproducibility (n = 2 or 3) of three different assays. This is not surprising in view of the intrinsic errors associated with weighings, number of successive dilutions, and animal responses. The replacement of C$^{\alpha}$MeLeu at positions 19 and 32 in **1** with D-Ala resulted in **9**, which was less potent than AstB (**1**) at both receptors. On the other hand, the replacement of C$^{\alpha}$MeLeu at positions 19 and 32 in **1** with Aib resulted in **10**, which showed similar binding affinity to AstB at both receptors. The introduction of three Aib residues at positions 19, 24, and 32 in AstB was well tolerated in AstD (**11**) when compared to **1** with respect to receptor binding affinity and selectivity. Only a slight loss of binding affinity at CRF-R1 and some improved binding

affinity at CRF-R2β could be observed. The D-Ala replacements of C$^{\alpha}$MeLeu^{19,32} resulted in **12**, an analogue less potent than **1**. Since C$^{\alpha}$-methylation in a peptide can favor a bioactive conformation while also preventing degradation and/or elimination resulting in significant extension of duration of action, we substituted C$^{\alpha}$MeLeu^{19,32} with other C$^{\alpha}$-methylamino acids like C$^{\alpha}$MePhe and/or C$^{\alpha}$MeVal and used Aib^{24} in the sequence. The binding affinities of **1**, and **13**–**17** were similar at both receptors. On the other hand, the solubility of these peptides was different. Peptide **15** was insoluble, and **17** formed a gel in the given conditions (Table 1). The substitution of D-Glu^{12} for Glu^{12} in **18** showed no difference regarding binding affinity and selectivity. Previously, we successfully applied Aph(Hor) combined with DAph(Cbm) in the sequence of gonadotropin releasing hormone (GnRH)[21] and somatostatin[22] antagonists. This building block contains urea/carbamoyl functionalities that might increase intra- and intermolecular hydrogen bonding opportunities for structural stabilization and peptide/receptor interactions, respectively. These substitutions result in analogues with increased hydrophilicity. The substitution of Aph(L-Hor) for Ala^{23} and D-Aph(Cbm) for His^{24} in AstB resulted in **19** with decreased binding affinity at both receptors compared with that of AstB. Replacing the lactam bridge with a disulfide bridge (**20**, **21**) led to decreased binding affinity at both receptors (~40-fold) and did not induce selectivity. We propose that in addition to the biologically unfavorable difference in steric hindrance of the disulfide bridge versus a lactam bridge (**20**, **21**), the lactam (γ carboxyl of Glu (*i*) to the epsilon amino (*i* + 3) of Lys bridge (unlike the ε amino of Lys (*i*) to the γ carboxyl (*i* + 3) of Glu) offers biologically favorable structural stability by aligning backbone and side chains dipole moments.[23] Phe, 1-Nal, 2-Nal, β-(2-thienyl)-Ala, and β-(3-thienyl)-Ala substitutions at position 30 in **1** resulted in **22**–**26** that were as potent as AstB. Ala and D-Ala substitutions at positions 17, 21, and 31 had little effect on binding affinity and did not induce selectivity (**27**–**30**). We also synthesized **31**, which is the elongated version of a short CRF-R ligand published by Yamada et al.[24] We found that **31** showed high binding affinity at both receptors. We have shown that Aib can substitute both C$^{\alpha}$MeLeu residues in AstC (**2**) without noticeable change in the binding affinity and receptor selectivity. EC_{50} is 0.18 nM vs 0.61 nM at CRF-R1 and 0.36 nM vs 0.38 nM at CRF-R2β, for AstC and AstD, respectively (Table 1). Substitution of the acetyl group at the N-terminus of AstD with other acyl groups that differed in the number of the carbon atoms (**32**–**38**) did not significantly affect the binding affinity, yet it contributed to a graded ability to dissolve and remain in solution all the way to gelling or needing additional DMSO for solubilization (see Table 2). Acylation with 21-amino-4,7,10,13,16,19-hexaoxaheneicosanoic acid resulted in **38** with very good solubility properties. The solubility properties of additional analogues were in agreement with their RP-HPLC retention times on a C_{18} column. The most soluble, **38**, eluted at 14.7 min after injection, and the least soluble, **37**, eluted at 41.8 min.

Recently, we published receptor-binding assays using [^{125}ITyr^{0}-Glu^{1}]-PD-Svg as the radioligand.[20,25] PD-sauvagine is secreted by the skin of the Mexican leaf frog *Pachymedusa dacnicolor*[20,25] referred to hereinafter as PD-Svg. When compared with the original radioligand [^{125}ITyr^{0}-Glu^{1},Nle^{17}]-PS-Svg,[20,25] from PS-sauvagine secreted from the skin of the frog *Phyllomedusa sauvageii*,[26] the binding was of high affinity and saturable for both radioligands. For PD-Svg tracer, the K_d values were 0.42 nM and 0.19 nM, and the corresponding numbers of binding sites were 6.5 pmol/mg and 13 pmol/mg protein for CRF-

R1 and CRF-R2β receptors, respectively. For PS-Svg tracer, the K_d values were 0.43 nM and 0.40 nM and the corresponding numbers of binding sites were 0.93 pmol/mg and 6.2 pmol/mg for CRF-R1 and CRF-R2β receptors, respectively. Thus, while the affinities of the two analogues for both CRF receptors were comparable, the PD-Svg radioligand detected a greater number of sites than its PS-Svg counterpart.[25] To validate these observations and demonstrate the superiority of the PD-Svg radioligand, several analogues were tested for their receptor binding affinities using both PS-Svg and PD-Svg. PD-Svg data are identified by an asterisk * in Tables 1 and 2.

3. Characterization of Antagonistic Properties Based on the Inhibition of cAMP Accumulation Induced by Human CRF and Rat Urocortin 1 (rUcn1)

To confirm the antagonistic properties of selected peptides **1**, **2**, **11**, and **35**, intracellular cAMP was measured from mouse pituitary tumor cells, AtT-20, which express endogenous CRF-R1 receptors, or rat aortic smooth muscle cells, A7r5, which express endogenous CRF-R2β receptors after hCRF or rUcn1 stimulation. Figure 1 shows that these analogues indeed are antagonists, since they all inhibit the hCRF- (Figure 1A) or rUcn1- (Figure 1B) stimulated intracellular cAMP release from AtT-20 or A7r5 cells, respectively.

4. In Vivo Biological Characterization Based on ACTH Release

Our aim to design even longer-acting CRF antagonists than AstB (**1**) was achieved by replacing His^{24} with Aib, resulting in AstC (**2**). Figures 2 and 3 show that AstC (**2**) is twice as long-acting as **1** in blocking ACTH release in ADX rats regardless of the solvent used for administration (peanut oil in Figure 2 and D-mannitol in Figure 3). Compounds **1** and **2** were administered sc, and plasma levels of ACTH immunoreactivity were measured at varying times after administration (Figure 2). Inhibition of basal ACTH release with **2** lasted over 6 h. Doubling the dose of **2** produced a significant inhibition of ACTH secretion for almost 48 h (Figure 3), which is far longer than any CRF receptor antagonist tested so far. AstD (**11**), in which both the expensive resolved C^{α}MeLeu residues were substituted with Aib, caused a relatively rapid inhibition of basal ACTH release, with maximal inhibition observed within an hour of administration. This inhibition was more rapid than that seen with either **1** or **2** and lasted for at least 3 h (Figure 3). These studies indicate that **1**, **2**, and **11** are potent CRF antagonists. AstC (**2)** is more effective and longer acting than AstB (**1)** or AstD (**11)**. Acylated derivatives of AstD were synthesized and tested in vivo (Figure 4) to gain an appreciation of hydrophilicity and increased doses (125 μg/rat) when compared to 50 μg/rat used in Figure 2. Increased duration of action was most noticeable for AstC.

A dose response for AstC is shown in Figure 5. We assigned seven ADX rats (~250 g each) to each treatment group at the start of the experiment. Three to four animals of each group randomly lost their catheter, and blood could not be collected. However, there was no observable toxicity of the peptide at any time.

The peptide was administered in 50 μL of 10% DMSO in 5% D-mannitol. It was first dissolved at a concentration of >100-fold active dose in DMSO, then diluted with 5% D-mannitol. All three concentrations of AstC (2, 6.6, and 20 mg/mL) resulted in a stable clear solution, thereby suggesting that this peptide can be formulated to achieve protracted action.

Suppression of ACTH secretion was immediate and profound (~90%) for 3–6 h; then, at the highest dose tested, it stabilized at about 50% over more than 1 week. A clear dose response was observed with duration of action increasing in parallel with increasing doses. We concluded that AstC is relatively stable to degradation and elimination. The peptide is likely to make a local depot at the site of administration or to accumulate in other organs acting as reservoirs. The kinetics of dissolution results in a pharmacologically active concentration of the peptide over a prolonged period (greater than 7 days at the highest dose). It should also be noted that because even low levels of ACTH are able to maintain full corticosteroid release, the 50% inhibition of ACTH release is likely to be of no deleterious consequence with respect to the inhibition of corticosterone secretion. However, such a level of inhibition may be sufficient to restore a homeostatic state in stress-related pathologies. The observation that three doses of the peptide gave the same degree of ACTH inhibition in the first 24 h also suggests that an initial burst of peptide is released in the circulation prior to depot formation or organ accumulation. Standard errors of mean (SEMs) were remarkably consistent throughout the assay, including on day 7. We therefore conclude that even though only a limited number of animals maintained patent iv cannulae over the entire course of the experiments and could therefore yield ACTH data at all times tested (four rats for control and high dose treatments, three and five rats for the intermediate doses at day 6, and three rats per group at day 7), data at the latest time points of the assays remain valid. Of note, the error bar for the high dose on day 7 is within the size of the dot. Whereas the ACTH levels after low and intermediate doses of AstC or AstD returned to baseline (Figures 2, 3, and 4), there is no ACTH rebound as often seen in other peptide systems. This is noteworthy, as a rebound could have undesired pharmacological consequences. Another unusual observation (see Figures 3 and 4) is a significant inhibition of ACTH at time 48 h with no inhibition at 24 h for AstD and hexanoyl-AstD (**35**). It is as if a second pool of AstD is being released about 24 h after the original injection.

5. In Vivo Biological Characterization Based on the Effect on h/rCRF-Induced Stimulation of Colonic Secretory Motor Activity in Rats

5.1. Dose-Related Antagonist Effect of AstB and C Given sc 12 h before ip CRF-Induced Stimulation of Colonic Function—

In sc vehicle pretreated rats (−12 h), CRF (10 μg/kg, ip) increased pellet output (number/h) reaching 9.1 ± 1.2/h (Veh-CRF) compared with 2.6 ± 0.8/h in control group (sc + ip vehicles) (Figure 6A). The diarrhea score was 2.6 ± 0.4, and watery feces occurred in 10/12 rats at 1 h after ip CRF while the vehicle group had no diarrhea (0 ± 0, n = 8 rats) (Figure 6B). The sc pretreatment 12 h earlier with AstC at either 100 or 200 μg/kg similarly abolished the colonic stimulatory action of ip CRF. The number of pellets/h was decreased to 2.5 ± 0.8/h and 2.0 ± 0.6/h and the diarrhea score to 0.5 ± 0.3 (2/6 rats) and 0.2 ± 0.2 (1/6 rats), respectively (all $p < 0.05$ vs vehicle + CRF and nonsignificant vs vehicle + vehicle). AstB, tested under the same conditions, also reduced the defecation at 100 μg/kg (6.3 ± 1.2 pellets/h) and the diarrhea score (1.2 ± 0.5; 4/6 rats) from that of sc vehicle + ip CRF (Figure 6A and Figure 6B); however, AstC showed a significantly greater antagonist effect on defecation than AstB (Figure 6A). At 200 μg/kg, AstB significantly reduced the number of pellets/h and diarrhea score to 3.7 ± 1.2 and 0.8 ± 0.5 (2/6 rats) (Figure 6A and Figure 6B). There was a significant

difference between AstC and B antagonistic action in ip CRF-induced stimulation of defecation test (two-way ANOVA, $F_{1,20} = 8.39$, $p < 0.01$) but not in the diarrhea score.

Compared to sc vehicle, AstB or AstC (200 μg/kg, sc) injected sc did not alter the cumulative fecal output and food intake during the 12 h subsequent period (Table 3) indicative of a lack of effect on basal colonic motor function and ingestive behavior.

5.2. Effect of AstB and AstC Given sc 24 h before ip CRF-Induced Stimulation of Colonic Function—To further assess the duration of action of AstB and AstC injected sc at the maximal effective dose (200 μg/kg), the pretreatment was performed 24 h before injecting ip CRF. CRF injected ip stimulated similar defecations (pellets/h) in sc vehicle and AstB pretreated rats 24 h before (11.5 ± 1.4 and 9.7 ± 2.2, respectively, vs 2.0 ± 0.9 in sc vehicle + ip vehicle, $P < 0.05$) (Figure 6C). By contrast, AstC given 24 h before ip CRF still partially suppressed the defecation as shown by values (6.7 ± 1.5 pellets/h) which were no longer significant compared to sc vehicle + ip vehicle or sc vehicle + ip CRF (Figure 6C). In vehicle-pretreated rats 24 h before ip CRF, the diarrhea score reached 3.2 ± 0.5 (6/6 rats) compared to no diarrhea (0/6 rats) ($P < 0.05$) and the response in AstB or AstC pretreated was no longer significant compared to sc vehicle + ip vehicle or sc vehicle + ip CRF (diarrhea score 1.5 ± 0.7 and 1.7 ± 0.7 in 3/6 and 4/6 rats, respectively) (Figure 6D).

Compared to sc vehicle, AstB and AstC injected sc did not alter the cumulative fecal output and food intake during the 24 h periods following the sc injection (Table 3).

5.3. Effects of Other AstB Analogues Given sc 12 h before ip CRF-Induced Stimulation of Colonic Function—Peptides, in which C^{α}MeLeu was substituted with other C^{α}Me-amino acids such as C^{α}MePhe and C^{α}MeVal, were tested at the optimal regimen (200 μg/kg sc, 12 h before ip CRF). Compound **8** or **17** induced a significant reduction of the defecation from 7.2 ± 0.9 in ip CRF to 3.6 ± 1.3 or 3.8 ± 1.3 pellets/h, respectively ($p < 0.05$), while the other peptides, AstD (**11**), **13**, and **15**, tested under the same conditions did not significantly decrease the number of feces compared to that of sc vehicle + ip CRF (Figure 7A). By contrast, only peptide **17** had a significant effect to reduce diarrhea score to 0.3 ± 0.3 compared to 1.9 ± 0.3 in ip CRF. For all the other peptides, the diarrhea score values did not reach significance compared with the vehicle group (Figure 7B).

6. In Vivo Biological Characterization Based on Human Urocortin 2-Induced Delay of Gastric Emptying in Rats

6.1. Antagonist Effect of AstB and AstC Given 12 h before ip hUcn 2-Induced Delay of Gastric Emptying—Measured by plasma levels of acetaminophen at 20, 40, and 60 min after orogastric administration of acetaminophen, basal gastric emptying was inhibited by the ip injection of hUcn 2 (CRF-R2-selective agonist) at 30 μg/kg (5.1 ± 1.6 vs 15.3 ± 4.1, 5.5 ± 1.4 vs 26.9 ± 4.7, and 7.4 ± 2.0 vs 39.4 ± 3.0 mg/dL compared to controls treated with vehicle, $p < 0.01$; Figure 8 and data not shown). Pretreatment 12 h before ip hUcn 2 with AstB or AstC (200 μg/kg) resulted in similar prevention of the delayed gastric emptying (Figure 8).

The ip injection of CRF induced defecation and diarrhea and that of hUcn 2 delayed gastric emptying of a non-nutrient solution in rats and are consistent with previous reports.[27–30] The colonic stimulatory response to ip CRF has been well established to be mediated by activating colonic myenteric cholinergic neurons and serotonin release through CRF interaction with CRF-R1 located on myenteric neurons and enterochromaffin cells,[31–33] and the inhibitory effect of ip hUcn 2 on the gastric transit is CRF-R2-mediated.[34] The present study showed that AstB and the newly developed AstC given sc at 200 μg/kg as a pretreatment 12 h before blocked both ip CRF-induced stimulation of propulsive colonic motor function and diarrhea and ip hUcn 2-induced delayed gastric emptying in rats. These data indicate that AstC exerts an antagonistic action in vivo on both CRF-R1 and CRF-R2 as established for AstB.[10] Of relevance was the potency of AstC as shown by the significant difference to antagonize ip CRF-induced defecation/diarrhea between AstC and AstB when both peptides were injected at 100 μg/kg 12 h before ip CRF treatment. AstC induced full blockade of ip CRF actions while AstB required 200 μg/kg. In addition, AstB was no longer active after a 24 h pretreatment while there was a partial effect of AstC at the 200 μg/kg dose. In a previous report, AstB injected sc at 100 μg/kg in DMSO/oil blocked the CRF-R1-mediated elevated ACTH secretion in adrenalectomized rats for over 24 h.[10] The present study provides the first evidence of the long duration of action of peptide antagonists against peripheral CRF-related gut alterations. We can rule out that the prevention of defecation in response to ip CRF by sc AstB or AstC pretreatment is due to prior alterations of food intake or defecation that will impact on gut content to be expelled by ip CRF thereafter. The data showed that neither food intake nor defecation was influenced by peptide pretreatment for the 12 or 24 h period after sc AstB or AstC injection and before that of the CRF injection. This indicates that these CRF-R1/CRF-R2 antagonists did not influence basal functions as previously reported.[35] The potency of AstC is also supported by the lack of significant effect of several other peptides tested under the same conditions, although among them, **17** injected sc at 200 μg/kg 12 h before that of ip CRF was able to antagonize partially both the defecation and diarrhea. Collectively, these in vivo data indicate that AstC provides a valuable new tool with long duration of action to investigate the role of CRF pathways under conditions of chronic stress.

To summarize, a great majority of the analogues reported here exhibit high affinity binding in the 10^{-10} M ranges for CRF-R1/CRF-R2. Overall, these studies exemplify how minor substitutions may have dramatic effects on biological properties.[21] In this instance, it is suspected that the two C^{α}MeLeu side chains are making contact with corresponding hydrophobic pockets in the receptor while the Aib residue at position 24 further stabilizes the 30–33 cycle (CRF numbering) and additionally that the formation of unstable amyloid fibrils is responsible for the long duration of action of AstB, AstC, and AstD.[36] We present here new analogues of AstB that are longer acting and less onerous to make. We show the set of physicochemical and biological properties required and sufficient for subsequent clinical evaluation. These peptides demonstrate the CRF-R1/R2 antagonistic nature as shown by the inhibition of cAMP accumulation in vitro, the blocking of stimulated ACTH release in ADX rats, the decrease in the number of stimulated fecal pellet output and diarrhea score in intact rats, induced by peripheral injection of h/rCRF, and the ability to block the ip hUcn 2-induced delayed gastric emptying in rats.[27,37]

CONCLUSION

Thirty-three years of efforts in CRF research led to remarkable insights in mechanism of action, distribution of receptors, and their clinical potential. The development of synthetic super agonists and antagonists that are safe, long acting, and receptor selective or not paves the way to drug candidacy. The present contribution describes the stepwise optimization of high affinity CRFR1/2 antagonists that are safe, potent, stable, easy to formulate in aqueous as well as in oil that exhibit long duration of action after sc or im (ip in rats) administration. Considering that CRF family members are acknowledged to oversee and regulate all "anatomy systems" inclusive of the cardiovascular, digestive, endocrine, female and male reproductive, immune, lymphatic, muscular, nervous, respiratory, skeletal, and urinary systems, it is expected that any deviation from a homeostatic state will manifest itself in a pathological condition involving at least one or the other systems. Consequently, by counteracting these effects, CRF antagonists may provide therapeutic benefits in chronic stress-dependent illnesses of which there are greater than 100.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank D. Kirby for the synthesis of **11**, Y. Haas for technical assistance, and D. Doan for manuscript preparation. J.R. is the Dr. Frederik Paulsen Chair in Neurosciences. This work was supported by NIH Grants P01 DK026741-34 (J.R., J.E., M.H.P., J.G., M.S., K.L., C.M., J.V., W.F., W.L.), DK041301 (Animal Core, Y.T., L.W.), R01 DK057238 (Y.T.), and VA Career Scientist Award (Y.T.).

ABBREVIATIONS

Ac	acetyl
AA	amino acid
ACTH	adrenocorticotropin hormone
Adx	adrenalectomized
ANOVA	analysis of variance
Aph(Cbm)	carbamoyl-4-aminophenylalanine
Aph-(Hor)	hydroorothylaminophenylalanine
Ast	astressin
Boc	*tert*-butoxycarbonyl
Bzl	benzyl
2-Br-Z	2-bromobenzyloxycarbonyl
2-Cl-Z	2-chlorobenzyloxycarbonyl

cAMP	cyclic adenosine monophosphate
CE	capillary electrophoresis
Cha	cyclohexylalanine
CRF	corticotropin-releasing factor
h	human
CRF-R1	corticotropin-releasing factor receptor 1
CRF-R2	corticotropin-releasing factor receptor 2
hCys	homocysteine
DCM	dichloromethane
DIC	*N*,*N*′-diisopropylcarbodiimide
DMF	dimethylformamide
DIPEA	*N*,*N*-diisopropylethylamine
DMSO	dimethyl sulfoxide
Dnp	dinitrophenyl
ECD	extracellular domain
EC_{50}	half maximal effective concentration
Fmoc	9-fluorenylmethoxycarbonyl
HATU	(2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
cHex	cyclohexyl ester
HOBt	1-hydroxybenzotriazole human urocortin 2 (hUcn 2)
ic	intracisternal
icv	intracerebroventricular
ip	intraperitoneal
iv	intravenous
MBHA	4-methylbenzhydrylamine
Mob	*p*-methoxybenzyl
1Nal	3-(1-naphthyl)alanine
2Nal	3-(2-naphthyl)alanine

Nle	norleucine
NMP	*N*-methylpyrrolidinone
OFm	*O*-fluorenylmethyl
PyBop	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate rat urocortin 1 (rUcn 1)
RIA	radioimmunoassay
RP-HPLC	reversed-phase high-performance liquid chromatography
SAR	structure–activity relationship
sc	subcutaneous
TEA	triethylamine
TEAP	triethylammonium phosphate
TFA	trifluoroacetic acid
Tos	tosyl
Ucn	urocortin
Xan	*N*′-xanthyl

References

1. Vale W, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates the secretion of corticotropin and β-endorphin. Science. 1981; 213:1394–1397. [PubMed: 6267699]
2. Spiess J, Rivier J, Rivier C, Vale W. Primary structure of corticotropin-releasing factor from ovine hypothalamus. Proc Natl Acad Sci USA. 1981; 78:6517–6521. [PubMed: 6273874]
3. Shibahara S, Morimoto Y, Furutani Y, Notake M, Takahashi H, Shimizu S, Horikawa S, Numa S. Isolation and sequence analysis of the human corticotropin-releasing factor precursor gene. EMBO J. 1983; 2(5):775–779. [PubMed: 6605851]
4. Rivier J, Spiess J, Vale W. Characterization of rat hypothalamic corticotropin-releasing factor. Proc Natl Acad Sci USA. 1983; 80:4851–4855. [PubMed: 6603620]
5. Rivier JE, Rivier C. Corticotropin-releasing factor peptide antagonists: design, characterization and potential clinical relevance. Front Neuroendocrinol. 2014; 35:161–170. [PubMed: 24269930]
6. Rivier J, Rivier C, Vale W. Synthetic competitive antagonists of corticotropin releasing factor: Effect on ACTH secretion in the rat. Science. 1984; 224:889–891. [PubMed: 6326264]
7. Fisher L, Rivier C, Rivier J, Brown M. Differential antagonist activity of a-helical CRF(9-41) in three bioassay systems. Endocrinology. 1991; 129:1312–1316. [PubMed: 1651845]
8. Hernandez JF, Kornreich W, Rivier C, Miranda A, Yamamoto G, Andrews J, Taché Y, Vale W, Rivier J. Synthesis and relative potencies of new constrained CRF antagonists. J Med Chem. 1993; 36:2860–2867. [PubMed: 8411001]
9. Martínez V, Rivier J, Wang L, Taché Y. Central injection of a new corticotropin-releasing factor (CRF) antagonist, astressin, blocks CRF-and stress-related alterations of gastric and colonic motor function. J Pharmacol Exp Ther. 1997; 280:754–760. [PubMed: 9023288]

10. Rivier JE, Kirby DA, Lahrichi SL, Corrigan A, Vale WW, Rivier CL. Constrained corticotropin releasing factor antagonists (astressin analogues) with long duration of action in the rat. J Med Chem. 1999; 42:3175–3182. [PubMed: 10447963]
11. Broadbear JH, Winger G, Rivier JE, Rice KC, Woods JH. Corticotropin-releasing hormone antagonists, astressin B and antalarmin: differing profiles of activity in rhesus monkeys. Neuropsychopharmacology. 2004; 29:1112–1121. [PubMed: 14997174]
12. Xiao E, Xia-Zhang L, Vulliemoz N, Rivier J, Ferin M. Astressin B, a corticotropin-releasing hormone receptor antagonist, accelerates the return to normal luteal function after an inflammatory-like stress challenge in the rhesus monkey. Endocrinology. 2007; 148:841–848. [PubMed: 17082255]
13. Vulliemoz NR, Xiao E, Xia-Zhang L, Rivier J, Ferin M. Astressin B, a nonselective corticotropin-releasing hormone receptor antagonist, prevents the inhibitory effect of ghrelin on luteinizing hormone pulse frequency in the ovariectomized rhesus monkey. Endocrinology. 2008; 149:869–874. [PubMed: 18063681]
14. Martinez V, Rivier J, Taché Y. Peripheral injection of a new corticotropin-releasing factor (CRF) antagonist, astressin, blocks peripheral CRF- and abdominal surgery-induced delayed gastric emptying in rats. J Pharmacol Exp Ther. 1999; 290:629–634. [PubMed: 10411571]
15. Moeser AJ, Klok CV, Ryan KA, Wooten JG, Little D, Cook VL, Blikslager AT. Stress signaling pathways activated by weaning mediate intestinal dysfunction in the pig. Am J Physiol: Gastrointest Liver Physiol. 2007; 292:G173–G181. [PubMed: 16901995]
16. Overman EL, Rivier JE, Moeser AJ. CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha. PLoS One. 2012; 7:e39935. [PubMed: 22768175]
17. Ji G, Neugebauer V. Differential effects of CRF1 and CRF2 receptor antagonists on pain-related sensitization of neurons in the central nucleus of the amygdala. J Neurophysiol. 2007; 97:3893–3904. [PubMed: 17392412]
18. Larauche M, Gourcerol G, Wang L, Pambukchian K, Brunnhuber S, Adelson DW, Rivier J, Million M, Taché YF. Cortagine, a CRF1 agonist, induces stress-like alterations of colonic function and visceral hypersensitivity in rodents primarily through peripheral pathways. Am J Physiol Gastrointest Liver Physiol. 2009; 297:G215–G227. [PubMed: 19407218]
19. Wang L, Million M, Rivier J, Rivier C, Craft N, Stenzel-Poore MP, Taché Y. CRF receptor antagonist astressin-B reverses and prevents alopecia in CRF over-expressing mice. PLoS One. 2011; 6:e16377. [PubMed: 21359208]
20. Zhou Y, Jiang Y, Wang R, Bai B, Zhou M, Chen T, Cai J, Wang L, Shaw C. PD-sauvagine: a novel sauvagine/corticotropin releasing factor analogue from the skin secretion of the Mexican giant leaf frog, Pachymedusa dacnicolor. Amino Acids. 2012; 43:1147–1156. [PubMed: 22134582]
21. Jiang G, Stalewski J, Galyean R, Dykert J, Schteingart C, Broqua P, Aebi A, Aubert ML, Semple G, Robson P, Akinsanya K, Haigh R, Riviere P, Trojnar J, Junien JL, Rivier JE. GnRH antagonists: a new generation of long acting analogues incorporating p-ureido-phenylalanines at positions 5 and 6. J Med Chem. 2001; 44:453–467. [PubMed: 11462984]
22. Cescato R, Erchegyi J, Waser B, Piccand V, Maecke HR, Rivier JE, Reubi JC. Design and in vitro characterization of highly sst2-selective somatostatin antagonists suitable for radiotargeting. J Med Chem. 2008; 51:4030–4037. [PubMed: 18543899]
23. Miranda A, Lahrichi SL, Gulyas J, Koerber SC, Craig AG, Corrigan A, Rivier C, Vale WW, Rivier JE. Constrained corticotropin releasing factor (CRF) antagonists with i-(i+3) Glu— Lys bridges. J Med Chem. 1997; 40:3651–3658. [PubMed: 9357532]
24. Yamada Y, Mizutani K, Mizusawa Y, Hantani Y, Tanaka M, Tanaka Y, Tomimoto M, Sugawara M, Imai N, Yamada H, Okajima N, Haruta J. New class of corticotropin-releasing factor (CRF) antagonists: small peptides having high binding affinity for CRF receptor. J Med Chem. 2004; 47:1075–1078. [PubMed: 14971886]
25. Perrin MH, Tan LA, Vaughan JM, Lewis KA, Donaldson CJ, Miller C, Erchegyi J, Rivier JE, Sawchenko PE. Characterization of a Pachymedusa dacnicolor-Sauvagine analog as a new high-affinity radioligand for corticotropin-releasing factor receptor studies. J Pharmacol Exp Ther. 2015; 353:307–317. [PubMed: 25736419]

26. Montecucchi PC, Henschen A. Amino acid composition and sequence analysis of sauvagine, a new active peptide from skin of *Phyllomedusa sauvagei*. Int J Pept Protein Res. 1981; 18:113–120. [PubMed: 7309372]
27. Million M, Maillot C, Saunders P, Rivier J, Vale W, Taché Y. Human urocortin II, a new CRF-related peptide, displays selective CRF(2)-mediated action on gastric transit in rats. Am J Physiol Gastrointest Liver Physiol. 2002; 282:G34–G40. [PubMed: 11751155]
28. Maillot C, Million M, Wei JY, Gauthier A, Taché Y. Peripheral corticotropin-releasing factor and stress-stimulated colonic motor activity involve type 1 receptor in rats. Gastroenterology. 2000; 119:1569–1579. [PubMed: 11113078]
29. Million M, Grigoriadis DE, Sullivan S, Crowe PD, McRoberts JA, Zhou H, Saunders PR, Maillot C, Mayer EA, Taché Y. A novel water-soluble selective CRF1 receptor antagonist, NBI 35965, blunts stress-induced visceral hyperalgesia and colonic motor function in rats. Brain Res. 2003; 985:32–42. [PubMed: 12957366]
30. Million M, Zhao JF, Luckey A, Czimmer J, Maynard GD, Kehne J, Hoffman DC, Taché Y. The newly developed CRF1-receptor antagonists, NGD 98-2 and NGD 9002, suppress acute stress-induced stimulation of colonic motor function and visceral hypersensitivity in rats. PLoS One. 2013; 8:e73749. [PubMed: 24040053]
31. Wu SV, Yuan P-Q, Lai J, Wong K, Chen MC, Ohning GV, Taché Y. Activation of Type 1 CRH receptor isoforms induces serotonin release from human carcinoid BON-1N cells: an enterochromaffin cell model. Endocrinology. 2011; 152:126–137. [PubMed: 21123435]
32. Yuan P, Million M, Wu SV, Rivier J, Taché Y. Peripheral corticotropin releasing factor (CRF) and a novel CRF1 receptor agonist, stressin1-A activate CRF1 receptor expressing cholinergic and nitrergic myenteric neurons selectively in the colon of conscious rats. Neurogastroenterol Motil. 2007; 19:923–936. [PubMed: 17973638]
33. Movassat J, Calderari S, Fernández E, Martín MA, Escrivá F, Plachot C, Gangnerau MN, Serradas P, Alvarez C, Portha B. Type 2 diabetes—a matter of failing beta-cell neogenesis? Clues from the GK rat model. Diabetes, Obes Metab. 2007; 9:187–195. [PubMed: 17919193]
34. Taché Y, Bonaz B. Corticotropin-releasing factor receptors and stress-related alterations of gut motor function. J Clin Invest. 2007; 117:33–40. [PubMed: 17200704]
35. Stengel A, Taché Y. Corticotropin-releasing factor signaling and visceral response to stress. Exp Biol Med (London, UK). 2010; 235:1168–1178.
36. Maji SK, Schubert D, Rivier C, Lee S, Rivier JE, Riek R. Amyloid as a depot for the formulation of long-acting drugs. PLoS Biol. 2008; 6:e17. [PubMed: 18254658]
37. Saunders PR, Maillot C, Million M, Taché Y. Peripheral corticotropin-releasing factor induces diarrhea in rats: role of CRF1 receptor in fecal watery excretion. Eur J Pharmacol. 2002; 435:231–235. [PubMed: 11821031]
38. Rivier J, Gulyas J, Kirby D, Low W, Perrin MH, Kunitake K, DiGruccio M, Vaughan J, Reubi JC, Waser B, Koerber SC, Martinez V, Wang L, Taché Y, Vale W. Potent and long-acting corticotropin releasing factor (CRF) receptor 2 selective peptide competitive antagonists. J Med Chem. 2002; 45:4737–4747. [PubMed: 12361401]

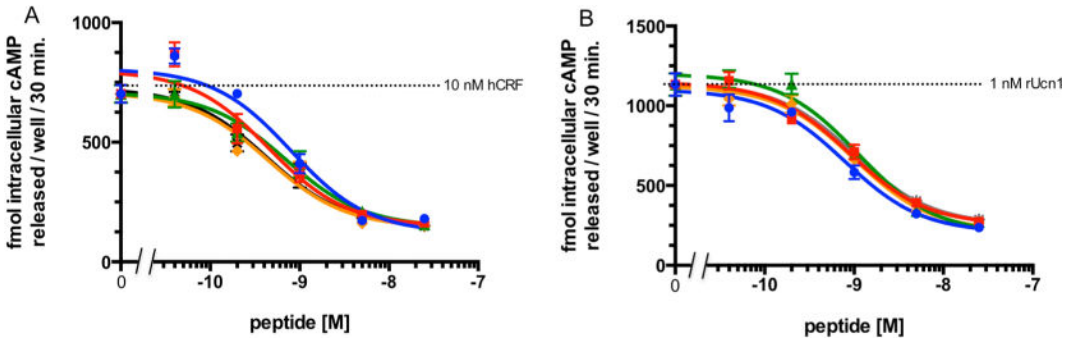


Figure 1.

(A) AstB and its analogues show similar potency as the nonselective antagonist Ast[8] at inhibiting the intracellular cAMP accumulation stimulated by 10 nM hCRF in AtT-20 cells expressing CRF-R1 receptors. EC_{50} (nM) value for the reference peptide Ast (black stars) was ~0.42 (0.14–1.3), for AstB (**1**) (blue circles) ~0.80 (0.11–5.7), for AstC (**2**) (red squares) 0.45 (0.05–3.9), for AstD (**11**) (green triangles) ~0.70 (0.28–1.8), and for hexanoyl-AstD (**35**) (orange diamonds) ~0.44 (0.08–2.5). (B) AstB and its analogues show similar potency as the CRF-R2β-selective antagonist Ast2B[38] at inhibiting the intracellular cAMP accumulation stimulated by 1 nM rUcn1 in A7r5 cells expressing CRF-R2β receptors. EC_{50} (nM) value for the reference peptide Ast2B (gray stars) was ~1.0 (0.88–1.1), for AstB (**1**) ~1.0 (0.42–2.4), for AstC (**2**) ~0.63 (0.19–2.1), for AstD (**11**) ~0.91 (0.4–2.1), and for hexanoyl-AstD (**35**) ~0.77 (0.21–2.9).

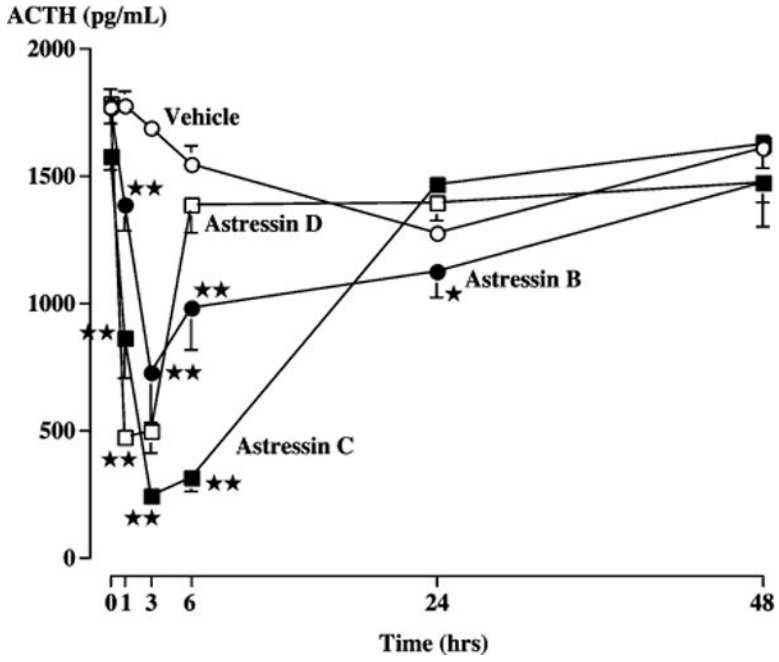


Figure 2.

In vivo ability of astressins [● B (**1**), ■ C (**2**), □ D (**11**)] to reduce ACTH release. For these studies, adrenalectomized rats (200–250 g, $n \geq 5$) were injected subcutaneously with astressins (50 μg/rat in 250 μL of 2.3% DMSO in peanut oil) or vehicle [○] (2.3% DMSO and peanut oil). Blood collection was performed at 0, 1, 3, 6, 24, and 48 h after peptide or vehicle administration, and ACTH immunoreactivity was measured by RIA. Values represent the mean ± SEM ACTH levels at the different time points: (*) $P < 0.05$ and (**) $P < 0.01$ vs vehicle. CRF antagonists were dissolved in DMSO first and then peanut oil was added or vehicle (5% DMSO and peanut oil).

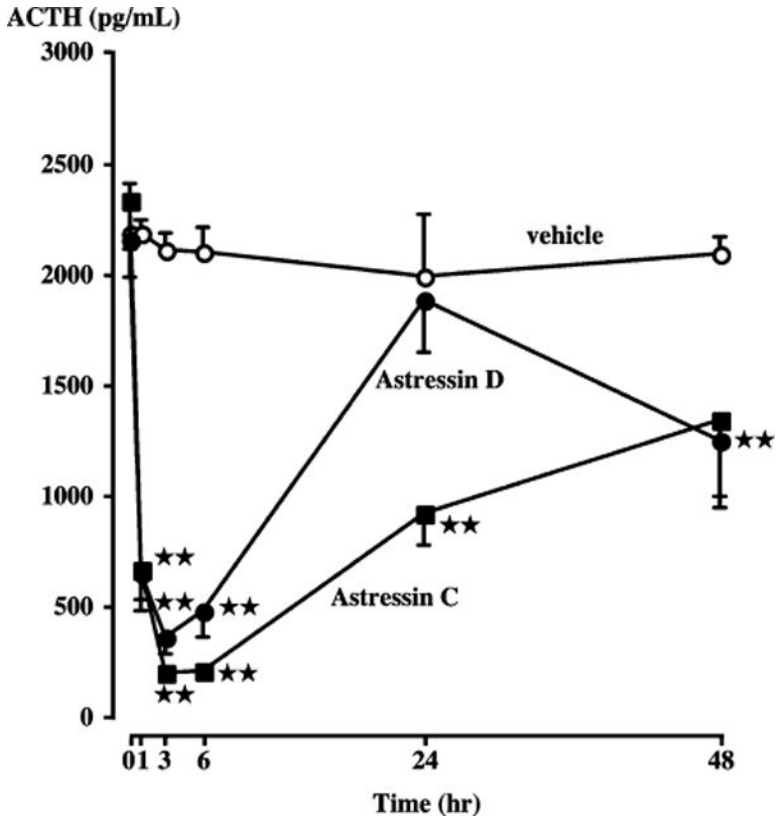


Figure 3.

Prolonged inhibition of ACTH release by astressins C (■) and D (●). Adrenalectomized rats (200–250 g, $n \geq 5$) were injected subcutaneously with AstC or AstD (100 μg/rat in vehicle) or vehicle (○) (200 μL of 15% DMSO in 5% D-mannitol), and subsequent effect on ACTH release was measured. Blood samples were taken at 1, 3, 6, 24, and 48 h after administration, and ACTH immunoreactivity was measured by RIA. Values represent the mean ± SEM ACTH levels at the different time points: (**) $P < 0.01$ vs vehicle.

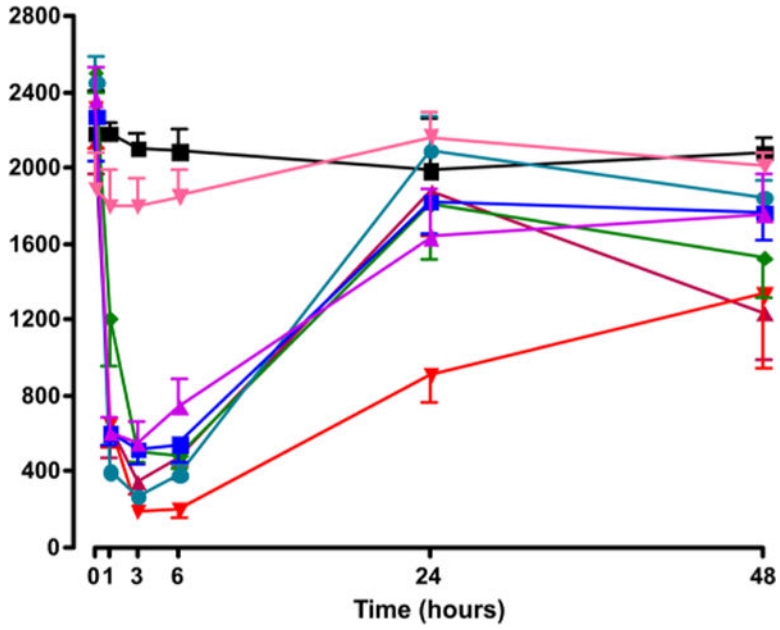


Figure 4.

In vivo ability of AstD (**11**) and its analogues (**32**–**35**, **38**) to reduce ACTH release and their duration of action compared with AstC (**2**). For these studies, adrenalectomized rats (200–250 g, $n \geq 5$) were injected subcutaneously with the analogues (500 μg/kg in about 200 μL of 5% DMSO in peanut oil): vehicle (■); AstC (**2**) (orange down-triangle); AstD (**11**) (red up-triangle); propionyl-AstD (**32**) (green circle); butyroyl-AstD (**33**) (blue box); valeroyl-AstD (**34**) (purple up-trianlge); hexanoyl-AstD (**35**) (green tilted-square); 21-aminohexao AstD (pink down-triangle). See caption of Figure 1 for details.

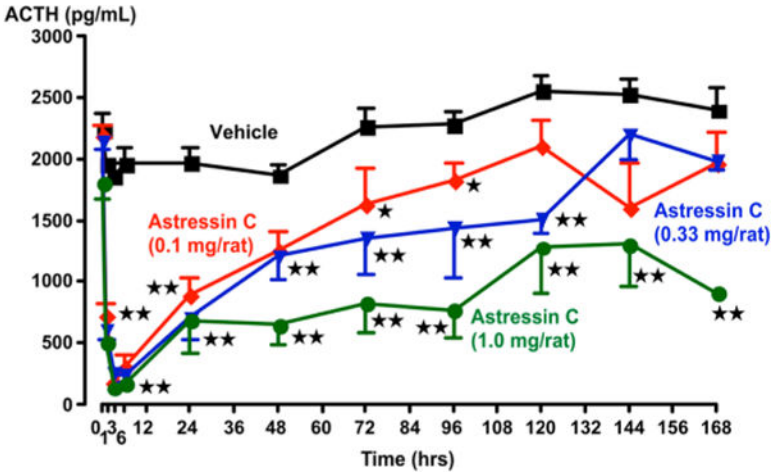


Figure 5.

Plasma ACTH levels in adrenalectomized rats injected with the vehicle or different doses of AstC (**2**): 0.1 mg/rat (orange tilted-square); 0.33 mg/rat (blue down-triangle); 1.0 mg/rat (green circle); vehicle (■); (*) $P < 0.05$ and (**) $P < 0.01$ vs vehicle.

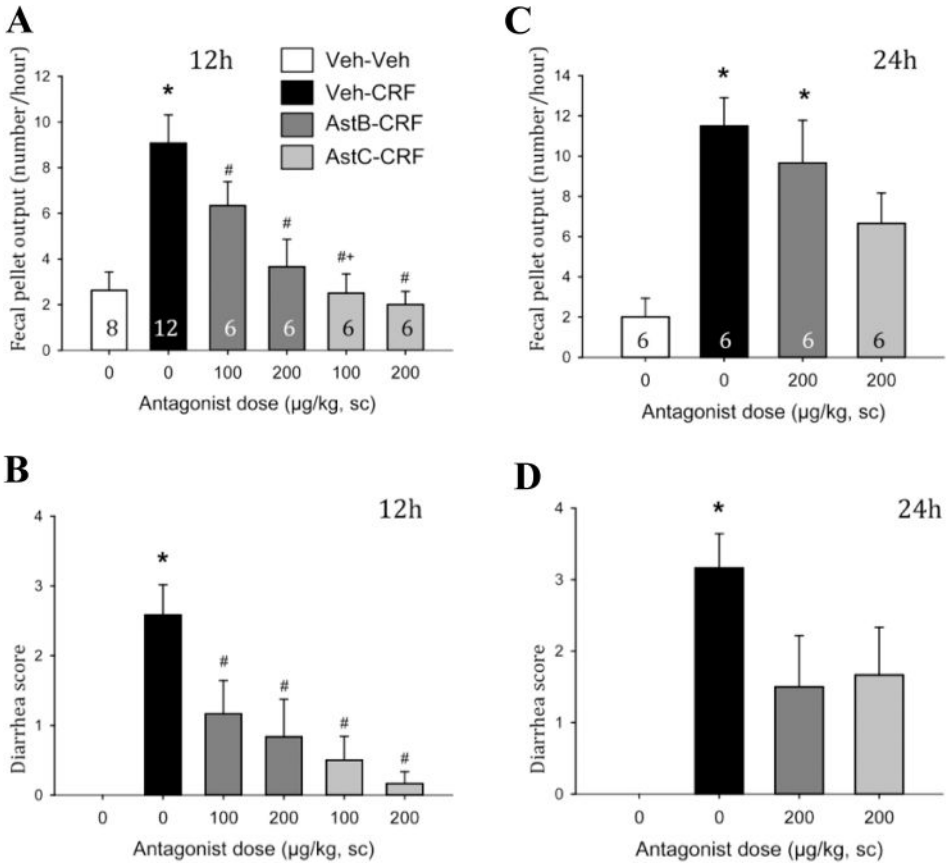


Figure 6.

Antagonistic action of AstB (**1**) and AstC (**2**) given subcutaneously against intraperitoneal CRF-induced stimulation of defecation and occurrence of diarrhea in rats: dose-related (A, B) and time course (C, D). Rats were injected sc with vehicle, AstB or AstC (100 or 200 μg/kg) 12 h before (A, B) or with vehicle, AstB or AstC (200 μg/kg) 24 h before (C, D) the ip injection of saline or CRF (10 μg/kg). The defecation and diarrhea were monitored for 1 h after the ip injection. Each column is the mean ± SEM of number of rats indicated at the bottom: (*) $p < 0.05$ vs Veh-Veh; (#) $p < 0.05$ vs Veh-CRF; (+) vs AstB-CRF; Veh = vehicle; AstB = AstB; AstC = AstC.

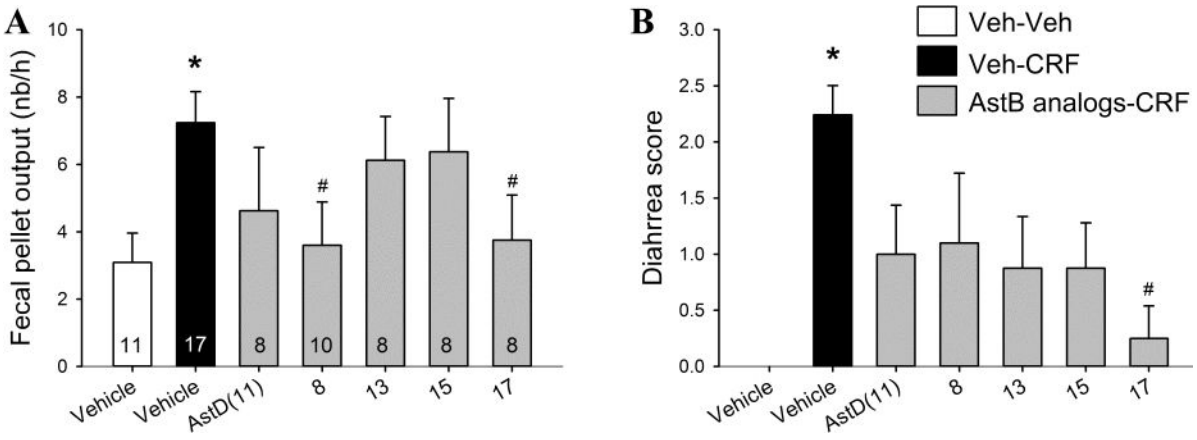


Figure 7.

Effect of AstD (**11**) and other AstB analogues on ip CRF-induced stimulation of defecation (A) and occurrence of diarrhea (B). Rats were injected sc with vehicle, AstD (**11**), **8**, **13**, **15**, and **17** (200 μg/kg) and 12 h later ip with saline or CRF (10 μg/kg). The defecation and diarrhea were monitored for 1 h after the ip injection. Each column is the mean ± SEM of number of rats indicated: (*) $p < 0.05$ vs Veh-Veh and (#) $p < 0.05$ vs Veh-CRF. Veh = vehicle.

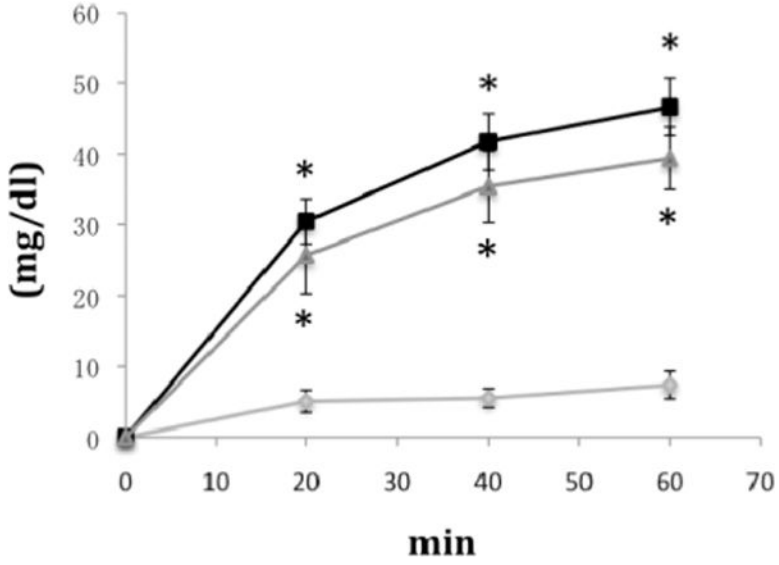


Figure 8.

Antagonistic action of AstB (**1**) and AstC (**2**) injected sc against the ip hUcn 2-induced delay of gastric emptying in rats. Overnight fasted rats were injected sc with vehicle (◆), AstB (■), or AstC (▲) (200 μg/kg) and 12 h later injected ip with vehicle or hUcn 2 (30 μg/kg). Ten minutes later, rats received an oral gavage with acetaminophen in 1.5% methylcellulose and blood was collected at 20, 40, and 60 min to assess gastric emptying. Data are the mean ± of five rats in each group: (*) $p < 0.05$ vs veh + hUcn 2.

Table 1

Physicochemical Characteristics and Binding Affinities of AstB Analogues

ID	AstB analogues		HPLC	CZE	$[M + H]_{calc}$	$[M + H]_{obs}$	hCRF-R1 K_i (nM)	mCRF-R2 K_i (nM)	solubility studies in DMSO (20%) and 5% D-mannitol in H_2O (80%), peptide concentration 10 mg/mL		
									5 min	2 h	24 h
1	Cyclo(30–33)[D-Phe^{12}, Nle^{21,38},C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ Ac-DLTfHLLREVLEXARAEQZAQEAHKNRKLXEZI-NH$_2$ f = D-Phe; X = Nle; Z = C$^{\alpha}$MeLeu	AstB	97	97	3961.25	3961.25	0.31 (0.12–0.78) (n = 6), 0.18* (0.13–0.24)	0.94 (0.51–1.72) (n = 5), 0.36* (0.27–0.48)	Soluble	Soluble	Soluble
2	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, Aib^{32}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aib^{24}]-AstB	AstC	96	97	3909.24	3909.17	0.16 (0.04–0.63) (n = 5), 0.48* (0.38–0.61)	0.35 (0.26–0.48) (n = 5), 0.95* (0.73–1.23)	Soluble	Soluble	Soluble
3	Cyclo(30–33) [D-Phe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{27,40},NMe-D-Ala^{32},Glu^{30},Lys^{33}]-Acetyl-h/r–CRF$_{(9–41)}$ [NMe-D-Ala^{24}]-AstB		94	96	3909.24	3909.7	1.15 (0.92–1.46) (n = 2)	0.81 (0.66–0.99) (n = 2)			
4	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, Ala^{32}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Ala^{24}]-AstB		96	95	3895.23	3895.08	0.53 (0.11–2.67) (n = 3)	0.55 (0.31–0.95) (n = 3)			
5	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, D-Ala^{32}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [D-Ala^{24}]-AstB		99	98	3895.23	3895.26	0.79 (0.42–1.50) (n = 1), 0.35* (0.19–0.64)	0.17 (0.08–0.38) (n = 1), 1.07* (0.89–1.27)			
6	Cyclo(30–33)[D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, D-Aph (Cbm)^{32}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [D-Aph(Cbm)^{24}]-AstB		78	85	4029.28	4030.17	0.22 (0.15–0.33) (n = 3)	0.44 (0.25–0.76) (n = 3)			
7	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38},C$^{\alpha}$MeLeu^{27,32,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [C$^{\alpha}$MeLeu^{24}]-AstB		90	77	3951.29	3951.33	0.30 (0.20–0.51) (n = 3)	0.60 (0.40–0.90) (n = 3)	Soluble	Soluble	Soluble
8	Cyclo(30–33) [D-Phe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{27,40},D-C$^{\alpha}$MeLeu^{32},Glu^{30},Lys^{33}]-Acetyl-h/-r-CRF$_{(9–41)}$ [D-C$^{\alpha}$MeLeu^{24}]-AstB		84	75	3951.29	3951.59	0.29 (0.07–1.11) (n = 3)	0.46 (0.27–0.79) (n = 3)	Soluble	Soluble	Soluble
9	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, D-Ala^{27,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [D-Ala^{19}, D-Ala^{32}]-AstB		97	97	3849.12	3849.14	8.05 (4.6–11.2) (n = 2)	4.03 (3.65–4.43) (n = 2)			
10	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, Aib^{27,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aib^{19}, Aib^{32}]-AstB		91	96	3877.16	3877.20	0.42 (0.14–1.3) (n = 2)	0.62 (0.25–1.52) (n = 2)			
11	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, Aib^{27,32,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aib^{19}, Aib^{24}, Aib^{32}]-AstB	AstD	98	96	3825.15	3824.98	0.61 (0.28–1.35) (n = 5)	0.38 (0.27–0.54) (n = 5)	Soluble	Soluble	Soluble
12	Cyclo(30–33)		99	99	3739.12	3739.11	4.58 (2.31–9.10) (n = 2)	2.18 (1.92–2.47) (n = 2)			

ID	AstB analogues	HPLC	CZE	$[M + H]_{calc}$	$[M + H]_{obs}$	hCRF-R1 K_i (nM)	mCRF-R2 K_i (nM)	solubility studies in DMSO (20%) and 5% D-mannitol in H_2O (80%), peptide concentration 10 mg/mL		
								5 min	2 h	24 h
	[D-Phe^{12}, Nle^{21,38}, D-Ala^{27,40}, Aib^{32}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [D-Ala^{19}, Aib^{24}, D-Ala^{32}]-AstB									
13	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MePhe^{27}, Aib^{32}, Glu^{30}, Lys^{33}, Aib^{40}]-Acetyl-h/r-CRF$_{(9–41)}$ [C$^{\alpha}$MePhe^{19}, Aib^{24,32}]-AstB	94	99	3901.18	3901.08	0.49 (0.23–1.06) (n = 3)	0.86 (0.7–1.1) (n = 2)	Soluble	Soluble	Soluble
14	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, Aib^{27,32}, Glu^{30}, Lys^{33}, C$^{\alpha}$MePhe^{40}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aib^{19}, Aib^{24}, C$^{\alpha}$MePhe^{32}]-AstB	80	84	3901.18	3901.18	0.91 (0.20–4.70) (n = 2)	0.89 (0.50–1.60) (n = 2)	Soluble	Soluble	Soluble
15	Cyclo(30–33)[D-Phe^{12}, Nle^{21,38}, Phe (Me)^{27,40}, Aib^{32}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [C$^{\alpha}$MePhe^{19}, Aib^{24}, C$^{\alpha}$MePhe^{32}]-AstB	97	92	3977.21	3977.76	0.79 (0.24–2.60) (n = 3), 0.46* (0.31–0.69)	1.23 (1.00–1.50) (n = 2)	Insoluble	Insoluble	Insoluble
16	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, Aib^{27,32}, Glu^{30}, Lys^{33}, C$^{\alpha}$MeVal^{40}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aib^{19}, Aib^{24}, C$^{\alpha}$MeVal^{32}]-AstB	90	97	3853.18	3853.05	0.70 (0.38–1.26) (n = 2)	0.40 (0.32–0.50) (n = 3)	Soluble	Soluble	Soluble
17	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeVal^{27,40}, Aib^{32}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [C$^{\alpha}$MeVal^{19}, Aib^{24}, C$^{\alpha}$MeVal^{32}]-AstB	91	95	3881.22	3881.19	0.68 (0.36–1.29) (n = 2)	0.62 (0.31–1.25) (n = 3)	Soluble	Gel	Gel
18	Cyclo(30–33) [D-Phe^{12}, D-Glu^{20}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [D-Glu^{12}]-AstB	97	98	3961.24	3960.72	0.45 (0.31–0.64) (n = 3)	0.92 (0.73–1.16) (n = 3)			
19	Cyclo(30–33)[D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Aph (Hor)^{31}, D-Aph(Cbm)^{32}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aph(Hor)^{23}, D-Aph(Cbm)^{24}]-AstB	94	95	4260.34	4260.38	5.94* (4.38–8.05)	2.03 (1.49–2.77)			
20	Cyclo(30–33) [D-Phe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{27,40},hCys^{30,33},Aib^{32}]-Acetyl-h/r-CRF$_{(9–41)}$ [hCys^{22,25}, Aib^{24}]-AstB	96	99	3902.15	3902.43	48.6 (31.8–74.4) (n = 2)	46.18 (35.96–59.28) (n = 2)			
21	Cyclo(30–33)[D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, hCys^{30,33}, Aph (Hor)^{31}, D-Aph(Cbm)^{32}]-Acetyl-h/r-CRF$_{(9–41)}$ [hCys^{22,25}, Aph(Hor)^{23}, D-Aph(Cbm)^{24}]-AstB	94	96	4253.25	4253.30	48.98 (12.23–196.12) (n = 3)	40.72 (27.18–61.01) (n = 2)			
22	Cyclo(30–33) [D-Phe^{12}, Nle^{21}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}, Phe^{38}]-Acetyl-h/r-CRF$_{(9–41)}$ [Phe^{30}]-AstB	96	99	3995.24	3995.37	0.34 (0.21–0.55) (n = 3)	2.02 (0.75–5.49) (n = 2)			
23	Cyclo(30–33) [D-Phe^{12}, Nle^{21}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}, 1Nal^{38}]-Acetyl-h/r-CRF$_{(9–41)}$ [1Nal^{30}]-AstB	97	96	4045.25	4045.15	0.50 (0.50–0.50) (n = 2)	4.02 (1.49–10.81) (n = 3)			
24	Cyclo(30–33) [D-Phe^{12}, Nle^{21}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}, 2Nal^{38}]-Acetyl-h/r-CRF$_{(9–41)}$ [2Nal^{30}]-AstB	92	97	4045.25	4045.36	0.49 (0.40–0.60) (n = 3)	0.99 (0.69–1.42) (n = 3)			

ID	AstB analogues	HPLC	CZE	$[M + H]_{calc}$	$[M + H]_{obs}$	hCRF-R1 K_i (nM)	mCRF-R2 K_i (nM)	solubility studies in DMSO (20%) and 5% D-mannitol in H_2O (80%), peptide concentration 10 mg/mL		
								5 min	2 h	24 h
25	Cyclo(30–33)[D-Phe^{12}, Nle^{21}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}, βAla (2-thienyl)^{38}]-Acetyl-h/r-CRF$_{(9–41)}$ [β(2-thienyl)-Ala^{30}]-AstB	97	97	4001.19	4001.19	0.24 (0.18–0.33) (n = 2)	2.0 (1.86–2.14) (n = 2)			
26	Cyclo(30–33)[D-Phe^{12}, Nle^{21}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}, βAla (3-thienyl)^{38}]-Acetyl-h/r-CRF$_{(9–41)}$ [β-(3-thienyl)-Ala^{30}]-AstB	98	97	4001.19	4001.19	0.25 (0.09–0.73) (n = 3)	2.39 (1.73–3.30) (n = 2)			
27	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, Ala^{25}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Ala^{17}]-AstB	96	97	3903.25	3903.04	0.20 (0.05–0.8) (n = 4)	0.17 (0.09–0.33) (n = 4)			
28	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, DAla^{25}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [D-Ala^{17}]-AstB	99	98	3903.24	3903.27	1.07 (0.74–1.54) (n = 3)	0.55 (0.44–0.70) (n = 3)			
29	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, Ala^{29}, Glu^{30}, Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Ala^{21}]-AstB	95	96	3904.23	3904.35	0.69 (0.56–0.85)(n = 2)	0.79 (0.47–1.33) (n = 3)			
30	Cyclo(30–33) [D-Phe^{12}, Nle^{21,38}, C$^{\alpha}$MeLeu^{27,40}, Glu^{30}, Lys^{33}, Ala^{39}]-Acetyl-h/r-CRF$_{(9–41)}$ [Ala^{31}]-AstB	99	97	3903.25	3903.38	0.43 (0.25–0.75) (n = 4)	0.22 (0.14–0.34) (n = 4)			
31	Cyclo(30–33) [D-Phe^{12},Nle^{21},Aib^{27},Glu^{30},D-Ala^{31},Glu^{32},Lys^{33},Cha^{38},Asp^{39},C$^{\alpha}$MeLe-u^{40}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aib^{19},D-Ala^{23}, Glu^{24}, Cha^{30},Asp^{31}]-AstB	96	99	3937.20	3937.21	1.13 (0.72–1.78) (n = 3)	0.33 (0.26–0.40) (n = 2)			

Table 2

Physicochemical Characteristics and Binding Affinities of AstD Analogues with Different Acylating Reagents at the N-Terminus

ID	AstD analogues	HPLC[a] t_R min	HPLC	CZE	$[M + H]_{calc}$	$[M + H]_{obs}$	CRF-R1 K_i (nM)	CRF-R2 K_i (nM)	solubility studies in DMSO (20%) and 5% D-mannitol in H_2O (80%), peptide concentration 10 mg/mL		
									5 min	2 h	24 h
11	Cyclo(30–33)[D-Phe^{12},Nle^{21,38},Aib^{27,32,40}, Glu^{30},Lys^{33}]-Acetyl-h/r-CRF$_{(9–41)}$ [Aib^{19}, Aib^{24}, Aib^{32}]-AstB AstD	15.53	98	96	3825.15	3824.98	0.61 (0.28–1.35) (n = 5)	0.38 (0.27–0.54) (n = 5)	Soluble	Soluble	Soluble
32	Propionyl-AstD	17.12	96	99	3839.14	3839.19	0.69 (0.57–0.83)	0.37 (0.32–0.41), 0.32[a] (0.27–0.38)	Soluble	Soluble	Soluble
33	Butyroyl-AstD	18.60	96	94	3853.14	3853.02	0.74 (0.58–0.94)	0.35 (0.28–0.43), 0.29[a] (0.24–0.36)	Soluble	Soluble	Soluble
34	Valeroyl-AstD	20.36	95	98	3867.14	3867.20	0.68 (0.50–0.89), 0.59[a] (0.51–0.68)	0.32 (0.28–0.38)	Slightly soluble	Soluble	Soluble
35	Hexanoyl-AstD	22.54	84	99	3881.20	3881.89	0.2 (0.05–0.85) (n = 2), 0.28[a] (0.13–0.66) (n = 2)	0.37 (0.34–0.39) (n = 2), 0.26[a] (0.21–0.32)	Insoluble	Soluble	Soluble
36	Decanoyl-AstD	31.17	88	91	3937.89	3937.10	0.64 (0.26–1.56) (n = 3), 0.52[a] (0.45–0.59)	0.39 (0.31–0.49)	Insoluble	Slightly soluble	Slightly soluble
37	Tetradecanoyl-AstD	41.83	92	96	3993.89	3994.29	0.81 (0.60–1.10), 2.17[a] (1.78–2.65)	2.16 (1.91–2.45)	Insoluble	Insoluble	Slightly soluble
38	21-amino-4,7,10,13,16,19-hexaoxa heneicosanoyl-AstD	14.66	84	88	4117.38	4118.55	3.00 (2.36–3.82), 3.5[a] (2.4–5.1)	2.11 (1.37–3.23), 1.07[a] (0.81–1.42)	Very soluble	Very soluble	Very soluble

[a]PD-Svg radioligand: [^{125}ITyr^{0}-Glu^{1}]-PD-Svg YEGTSLDLTFDLLRHNLEIAKQEALKKQAAKNRLLLDTI-NH$_2$.[25] This new peptide differs substantially from its PS-Svg counterpart in primary structure and was found to be more potent in bioassays.

Table 3

AstB and AstC Effect on 12 and 24 h Basal Fecal Pellet Output and Food Intake in Nonfasted Rats[a]

time (h)	treatment	fecal output (pellets)	food intake (g)
12	vehicle	33.4 ± 1.2	17.8 ± 0.8
	AstB	37.8 ± 2.4	19.0 ± 0.7
	AstC	35.3 ± 1.9	16.3 ± 1.5
24	vehicle	47.3 ± 3.4	22.9 ± 0.9
	AstB	54.5 ± 2.9	22.8 ± 0.5
	AstC	53.3 ± 4.4	23.7 ± 0.8

[a]AstB and AstC were injected sc at 200 μg/kg (n = 6/group).