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Review

# The "homeostasis hormone" and its $CRF_1$ receptor. From structure to function

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## INTRODUCTION

The maintenance of homeostasis is a basic prerequisite for life, ensuring the stability of our body in response to external or internal stimuli (stressful stimuli). The maintenance of homeostasis requires alterations in the function of the endocrine system, as well as several other adaptive responses, involving changes in the behavior and the function of the central nervous system (CNS), immune, cardiovascular and other systems. A distorted regulation of the adaptive responses to various stressful stimuli may affect our physiologic functions, thus rendering us vulnerable to various disorders, such as depression and anxiety.

Corticotropin releasing hormone (CRH), or corticotropin releasing factor (CRF), a hypothalamic hormone, plays a key role in the maintenance of homeostasis.[1] CRF is secreted by the paraventricular nucleus of the hypothalamus in response to stress and is transported via the portal vein to the anterior lobe of the pituitary gland where it causes the release of corticotropin (ACTH). Subsequently, the ACTH is transported by the blood to the adrenals, where it stimulates the release of glucocorticoids (Figure 1).[1,2] In addition to the regulation of the hypothalamic-pituitary-adrenal axis (HPA), CRF plays an important role in stress as well as in many physiological and pathophysiological processes by being involved in the control of the CNS[3-9] as well as the cardiovascular, gastrointestinal, behavioral, immune and reproductive systems.[10-26]

**Key words:** CRF-peptides, CRF-receptors, Non-peptide antagonists, Physiological/pathophysiological role, Structure

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## PEPTIDE AND NON-PEPTIDE CRF ANALOGS

### *The CRF family*

CRF, which was first isolated from ovine hypothalamus (oCRF), belongs to a family of structurally related, highly homologous peptides (CRF-peptides) from several species, such as rat (h/rCRF), human (h/rCRF), goat, cow, pig and xenopus CRF.[1,27-33] In addition to CRF-peptides, the CRF family includes peptides from different species such as sauvagine, urotensin and urocortins, which are closely related to CRF (CRF-like peptides). Sauvagine (SVG) and urotensin (URO) have been characterized from the frog *Phyllomedusa sauvagei* and the sucker fish *Catostomus commersoni,* respectively, whereas urocortin (Ucn), urocortin II (UcnII) and urocortin III (UcnII) have been charac-

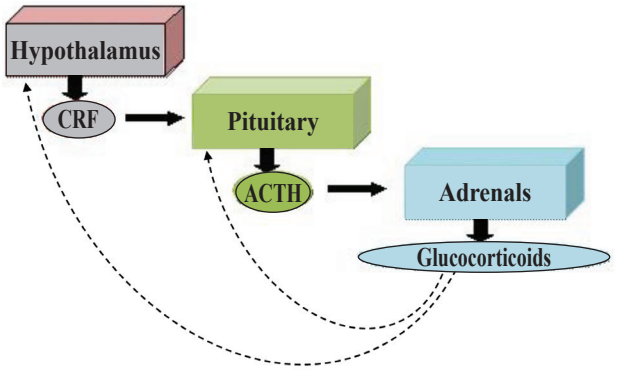



**Figure 1.** Schematic illustration of the hypothalamic-pituitary-adrenal axis (HPA). CRF is secreted by the paraventricular nucleus of the hypothalamus and is transported through the portal veins to the anterior lobe of the pituitary gland where it binds to CRF receptors (CRF-R) and causes the release of corticotropin (ACTH). Subsequently, the ACTH is transported through the blood to the adrenals, where it stimulates the release of glucocorticoids. Glucocorticoids exert a negative feedback control on both the hypothalamus and anterior pituitary (dashed arrows). In addition to the pituitary, CRF receptors (CRF-R) are also located in the hypothalamus and adrenals.

terized in mammals.[34-37] In addition to natural ligands, numerous peptide and non-peptide CRF analogs have been synthesized and are described below.

### *Structure and function of CRF family peptides*

CRF, SVG and URO are peptides containing an amino-terminal (1-7 residues) and a carboxyl-terminal (36-41 residues) region connected to each other via an internal segment (8-32) of 25 amino acids.[38] This internal segment has a high chance of adopting an alpha-helical structure, which is the preferred conformation of CRF and its related peptides in hydrophobic or amphiphilic environments, whereas it is destabilized in aqueous environments.[38,39] The ability of CRF family peptides to adopt an alpha-helical conformation in hydrophobic or amphiphilic environments led to the hypothesis that their binding to specific CRF receptors located in the amphiphilic environment of the cell membrane alters the conformation of peptides to a biologically active alpha-helical form.[38-40] Indeed, replacement of several residues of oCRF with alpha-helical preferred ones resulted in an increase of the biological potency of the peptide.[41] Similarly, Beyermann et al (2000) have suggested that the alpha helicity of an internal region connecting the amino-terminal (1-21 residues) and carboxyl-terminal (33-40 residues) regions of CRF and UCN is critical for peptide function.[42] Peptide analogs with internal regions constructed with highly flexible linkers such as those composed by ε-aminocaproic acid residues had lower potencies than UCN and CRF.[42] In contrast, analogs with linkers rich in alanines (alpha helical promoting amino acids) were equipotent to CRF.[42,43] Similarly, connection of the amino-terminal and the carboxyl-terminal regions of UCN with a linker consisting exclusively of the negatively charged Glu and the positively charged Lys, which were arranged in such way that helix stabilization could occur by salt bridge formation between side chains at positions i and i4, resulted in an analog equipotent to UCN.[42]

Like the alpha-helical structure, the amino-terminal (first 21 residues) and carboxyl-terminal (last 8 residues) regions of CRF family peptides also play an important role in biological activity. Peptides having only one of these regions were biologically unimportant.[42,44] Furthermore, alanine substitution for several residues in the carboxyl-terminal region of CRF, removal of the last two amino acids of this region or replacement of the amidated carboxyl-terminal end of CRF with a free acid resulted in a significant to a complete loss of peptide biopotency.[1,45] Similar to the carboxyl-terminal region, modifications of the amino-terminal region of CRF family peptides seriously affected their biological activities. In particular, removal of the first 8 amino-terminal residues of oCRF resulted in the biologically inactive analog, oCRF (9-41), whereas alanine substitution for almost all of the amino acids in this region resulted in a significant to a complete loss of CRF potency to stimulate ACTH release.[41,45]

Removal of the first 8 amino-terminal residues from oCRF, thus creating the oCRF (9-41), abolished its ability to stimulate ACTH release, without largely affecting the binding capacity of peptide, as measured by its ability to antagonize the effects of CRF.[41] Additional modifications of oCRF (9-41) by replacing some of its residues with alpha helical preferred ones created the first CRF antagonist, alpha-helical-CRF (9-41).[41] Similarly, removal of the first 11 amino-terminal residues from r/hCRF, and modifications of the truncated peptide (r/hCRF (12-41)) created the antagonist cyclo(30-33)[D-Phe$^{12}$, Nle$^{21,28}$, Glu$^{30}$, Lys$^{33}$] h/rCRF (12-41).[46] This antagonist, termed astressin,

had negligible intrinsic activity and it was 100 times more potent than the alpha-helical CRF (9-41).[46] More recently, new CRF antagonists and agonists have been developed, which are described below.

### *Non-peptide CRF antagonists*

Several non-peptide CRF antagonists were developed as new leads in drug discovery to treat various stress-related disorders like depression, anxiety and addictive disorders.[1] Most non-peptide CRF antagonists are substituted five-membered rings or bicyclic and tricyclic rings and are discussed in detail below.

## CRF RECEPTORS

CRF and its analogs exert their actions by interacting with two types of plasma membrane receptors, type 1 ($CRF_1$) and type 2 ($CRF_2$), which belong to the secretin-like family B of G protein-coupled receptors (GPCRs).[47,48] In addition to $CRF_1$ and $CRF_2$, a third type of CRF receptor ($CRF_3$) has been characterized from the catfish and it is expressed in the pituitary gland, urophysis and brain.[49] Furthermore, $CRF_1$ and $CRF_2$ receptors have been shown to be expressed as several functional splice variants ($CRF_{1\alpha}$-$CRF_{1m}$, $CRF_{2\alpha}$, $CRF_{2\beta}$ and $CRF_{2\gamma}$) and were extensively reviewed by Hillhouse and Grammatopoulos.[50] Among the splice variants of $CRF_1$ receptor, $CRF_{1\alpha}$ (which will be mentioned in this manuscript as $CRF_1$ for simplicity) is the main functional variant that mediates the actions of CRF family peptides, whereas the other $CRF_1$ variants have impaired functional properties.[50] In contrast to $CRF_1$, the splice variants of $CRF_2$ did not significantly differ pharmacologically from each other.[51,52]

The $CRF_1$ and $CRF_2$ receptors bind the CRF family peptides with different affinities. In particular, h/rCRF and oCRF bind to $CRF_1$ with higher affinities than to $CRF_2$, with oCRF being the most $CRF_1$-selective peptide among the natural peptides of the CRF family. The affinity of oCRF for $CRF_1$ receptor is 180-fold higher than that for $CRF_2$. Similarly, the CRF non-peptide analogs (chemically described below) bind selectively to $CRF_1$ receptor and antagonize the actions of CRF. In marked contrast, UcnII and UcnIII are the $CRF_2$-selective natural peptides of the CRF family. On the other hand, UCN and SVG bind to $CRF_1$ and $CRF_2$ receptors with similar affinities. Similarly, the difference in the binding affinities of the synthetic analogs, astressin and alpha-helical CRF (9-41) for $CRF_1$ and $CRF_2$, is only 4-10-fold. Recently, a variety of modifications of CRF family peptides created CRF receptor subtype-selective ligands such as the $CRF_2$-selective antagonists, cyclo(31-34) [DPhe(11), His(12), C(alpha) MeLeu(13,39), Nle(17), Glu(31), Lys(34)] Ac SVG ((8-40)) (or astressin2-B), [DPhe11, His12]Svg(11-40), (or antiSVG-30), and [D-Phe11, His12, Nle17] Svg(11-40), (or K41498) and the $CRF_1$-selective agonists (cyclo(31-34)[DPhe12, Nle21,38, Glu31, Lys34]-Ac-hCRF(4-41) (or stressin1-A) and the chimeric peptide ([Glu(21), Ala(40)][Svg(1-12)]-[human/ratCRF(14-30)]-[Svg(30-40)]) (or cortagine).[53-57]

The binding of CRF family peptides to their receptors results in the activation of several heterotrimeric ($\alpha\beta\gamma$) G-proteins having different $G\alpha$ subunits, such as $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha q}$, and $G_{\alpha z}$.[50] Activation of $G\alpha$ subunits by CRF receptors results in their dissociation from the $G\beta\gamma$ heterodimers and the subsequent regulation of several signaling pathways by the activated $G\alpha$ subunits and the $G\beta\gamma$ dimers as well. Thus, the $CRF_1$ receptor has been shown to activate through $G_{\alpha s}$ the adenylate cyclase, thus resulting in the accumulation of intracellular cAMP and the subsequent activation of protein kinase A (PKA) in various cell lines and tissues.[58-63] In CHO cells expressing the $CRF_1$ receptor, stimulation of the $G_{\alpha s}$-cAMP-PKA pathway has been shown to stimulate the MAPK kinase, MEK1, which in turn activates the extracellular signal regulated kinase 1/2 (ERK1/2).[63] In the locus coeruleus, however, the $CRF_1$-mediated stimulation of cAMP has been shown to activate ERK in a PKA-independent manner.[64] In addition to cAMP-dependent pathways, the activated $CRF_1$ is able to inhibit the proliferation of corticotropic tumour (AtT-20) cells via a cAMP independent pathway.[65] Furthermore, following peptide binding, $CRF_1$ and the $CRF_2$ could alter the intracellular $Ca^{2+}$ signaling through $G_{\alpha q}$-mediated or $G_{\beta\gamma}$-mediated stimulation of phospholipace C (PLC).[63,66] Moreover, the CRF receptor-mediated activation of $G_{\beta\gamma}$-subunits has been shown to stimulate the phosphatidylinositol 3-kinase, which, through the production of PI(3, 4, 5) P3, activates the PLC, thus resulting in the mobilization of $Ca^{2+}$.[63] The $CRF_1$-mediated activation of PI3-K and PLC pathways, as well as mobilization of intracellular calcium stores, has been shown to activate ERK1/2.[63,67,68]

In addition to ERK1/2, $CRF_1$ and $CRF_2$ are able to activate another functionally important kinase, the p38 mitogen protein kinase (p38 MAPK).[67,69,70]

Even more interestingly, previous experiments have demonstrated that activation of $CRF_1$ receptor by CRF in several brain regions resulted in the activation of ERK1/2 in only a few of them which are related to external environmental information processing and behavioral aspects of stress, thus suggesting a specific involvement of this pathway in mediating behavioral adaptation to stress.[71] In addition to the brain, the regulation of ERK1/2 activity by CRF receptors has also been identified in other tissues, such as the myometrium.[72]

## STRUCTURE AND FUNCTION OF CRF RECEPTORS

CRF receptors, like all GPCRs, consist of 7 alpha helical membrane-spanning segments (TMs), an extracellular amino-terminal domain (N-domain) and an intracellular carboxyl-terminal tail (C-domain). The TMs of CRF receptors are connected with each other with three extracellular loops (ELs) and three intracellular loops (ILs) (Figure 2).[47,73-76] Experimental findings from numerous studies suggest that the C-domain and the ILs of CRF receptors interact with various G-proteins, thus playing an important role in receptor-mediated signaling.[47,60,77-82] In contrast to ILs which interact with the G-proteins, the ELs and the N-domain of the CRF receptors may, it has been suggested, interact with the CRF family peptides. Substitution of the N-domain of $CRF_1$ with the corresponding one of GH-releasing hormone receptor abolished the binding of the radiolabelled astressin and UCN.[83] In contrast, the reverse chimeric receptor retained UCN and astressin binding, albeit with a reduced affinity, thus implying the participation of this region in peptide binding.[83] Similarly, a soluble form of the N-domain of $CRF_1$ as well as a chimera created by replacing the extracellular region of activin receptor (a single membrane-spanning segment receptor) with the N-domain of $CRF_1$ have been shown to bind to UCN and astressin with affinities that are lower than wild type receptor but still biologically considerable.[84-86]

Structure-function studies have established that the amino acids, important for ligand binding, in the

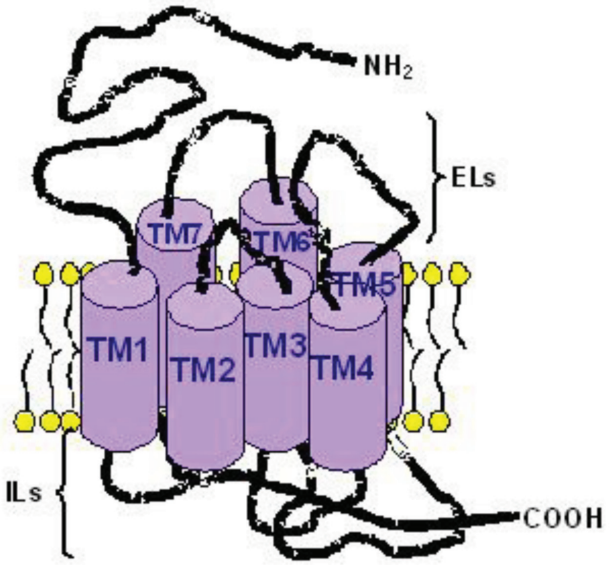



**Figure 2.** Schematic illustration of $CRF_1$ receptor. Hydropathy analysis of the cloned CRF receptors revealed the presence of seven hydrophobic regions (TM1-TM7) characteristic of the membrane-spanning segments of G-protein coupled receptors (GPCRs), which are depicted as cylinders and are connected to each other with three extracellular (ELs) and three intracellular loops (ILs).[47] These loops as well as the amino-terminal extracellular domain (N-domain) and the carboxy-terminal intracellular tail (C-domain) are depicted as lines.

N-domain of $CRF_1$ are located between residues 43-50 and 76-84 of the receptor.[87,88] The precise interactions of the N-domain of CRF receptors with various peptides have been determined by crystallography and NMR studies using soluble forms of this receptor's segment. In particular, the crystal structure of the N-domain of the $CRF_1$ receptor complex with CRF revealed that the peptide adopts a relatively straight, continuous alpha-helix and docks into a hydrophobic surface composed of the β1-β2 hairpin loop, loop 2, Tyr99, Pro69 and Cys68-Cys102 disulfide of the receptor's N-domain.[89] This crystallographic study has also proposed that the CRF binds with its amino-terminal residues 1-25 pointing toward the ELs and TMs of the receptor, whereas the carboxyl-terminal amino acids such as Leu37, Met38 and the C-terminal amide group (of residue 41) interact with the N-domain of $CRF_1$.[89] Interestingly, NMR studies have revealed the important part played by the corresponding residues of astressin in peptide binding to a soluble form of $CRF_{2\beta}$, despite the fact that the mode of binding of

astressin has been proposed as being different than that of CRF.[89,90]

In contrast to several peptide residues that play a common role in the binding of different ligands, other peptide residues might be responsible for $CRF_1$ or $CRF_2$ selectivity. In particular, a crystallographic study on the N-domain of $CRF_1$ proposed that the selective binding of CRF to $CRF_1$ over UcnII and UcnIII may be due to interactions that place Arg35 of CRF between Glu39 of peptide and Glu104 of receptor.[89] The Arg35 of CRF is replaced with an Ala in UcnII and UcnIII, which is more compatible than Arg35 with the hydrophobic Pro of $CRF_2$ that corresponds to Glu104 of $CRF_1$, thus explaining the $CRF_2$-selectivity of UcnII and UcnIII.[89]

The N-domain of $CRF_1$ receptor also contains three disulfide bridges between Cys30 and Cys54, Cys44 and Cys87 and Cys68 and Cys102, which play a crucial role in maintaining the receptor in its functional conformation.[86,89,91] Reduction of these bonds with DTT or mutation of Cys which participate in these bonds to Ser or Ala significantly decreased CRF binding.[92] The disulfide arrangement in the N-domain of $CRF_{2\beta}$ is identical to that of the corresponding region of $CRF_1$, but different than that in the N-domain of $CRF_{2\alpha}$, which has four Cys linked to each other with disulfide bonds and one free Cys.[85,86,89,93]

In addition to the N-domain of CRF receptors, their extracellular loops as well as the extracellular portions of their TMs have been revealed as important for peptide binding. In particular, upon sauvagine binding to $CRF_1$, residues 17 and 16 were shown to be located in close proximity to residue 117 in the extracellular portion of the TM1 and residue 257 in the second extracellular loop (EL2) of the receptor, respectively.[94,95] The latter finding is in agreement with the results of an alanine mutagenesis study, which has suggested that Trp259 and Phe260 in the EL2 of $CRF_1$ receptor most likely interact with ligands, and specifically with the amino-terminal residues 8-10 of SVG and the corresponding ones of CRF.[96] Furthermore, this study has proposed that the interaction between the amino-terminal region of CRF family peptides and Trp259 and Phe260 of $CRF_1$ seems to be critical for receptor activation and the subsequent appearance of a biological effect.[96]

In addition to EL2, the first extracellular loop (EL1) of $CRF_1$ has been demonstrated as playing a part in peptide binding, given that the amino-terminally located residues 17 and 22 of UCN analogs have been shown in a recent study to be located in close proximity to residues Trp170-Glu179 in the EL1 of $CRF_1$.[97] These results are in agreement with the experimental findings of a previous structure-function study, which suggested that residues 174–178 of EL1 are implicated in peptide binding.[98,99] In addition to the binding sites, EL1 and EL2 contain two Cys, which are highly conserved among GPCRs and connect these regions of receptor with a disulfide bond that plays a very important role in receptor function.[92] Like EL1 and EL2, EL3 of CRF receptors also plays a role in peptide binding since it was demonstrated that Ala substitution for Tyr346, Phe347 and Asn348 in the EL3 of $CRF_1$ significantly reduced the binding affinity of CRF.[100]

The binding of CRF family peptides to CRF receptors has been proposed as being represented by a two-domain model, in which an initial interaction of the carboxyl-terminal region of peptides with the N-domain of receptor serves to dock the amino-terminal residues of peptides into a receptor's domain (J-domain) formed by the extracellular loops and the upper portions of TMs.[101,102]

## THE MEMBRANE-SPANNING SEGMENTS

In contrast to the extracellular regions of CRF receptors which are important for the binding of the bulky peptides, the receptor TMs have been proposed as playing a role in the binding of small non-peptide CRF antagonists. Specifically, His199 and Met276 in the third (TM3) and the fifth (TM5) membrane-spanning segments of $CRF_1$ have been suggested as being involved in the binding of the non-peptide antagonist, NBI 27914 (structure shown under compound 16c), because mutation of these residues to the corresponding ones of $CRF_2$ significantly reduced NBI 27914 affinity for $CRF_1$.[98,103] Despite the fact that Met276 has been demonstrated as playing an indirect role in ligand binding, experimental findings from a structure-function study suggested that this residue is still very important for the interaction of $CRF_1$ with non-peptide antagonists, most likely by

positioning their heterocyclic core in the vicinity of Met276.[103] The involvement of CRF receptor TMs in ligand binding is further supported by the fact that the corresponding regions of family A GPCRs bind the non-peptide small molecules, such as catecholamines or acetylcholine.[104-107]

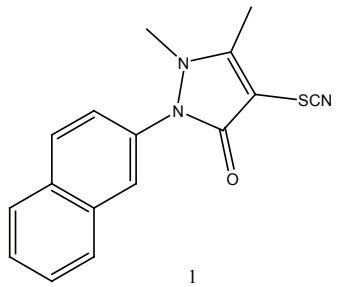
Although the TMs of $CRF_1$ have been proposed as playing a role in the binding of small non-peptide CRF analogs, the exact interactions have not yet been determined due to the lack of significant structural information about these regions of CRF receptors and all family B GPCRs as well, in contrast to the TMs of family A GPCRs, which have been structurally characterized in many crystallographic, biophysical and biochemical studies. In addition, the development of accurate molecular models of family B GPCRs, which would provide structural information concerning their TMs, is very difficult because these receptors display very little sequence similarity to those of family A receptors.[108-110] Only recently, a study using the substituted cysteine accessibility method (SCAM) provided structural information about the TMs of $CRF_1$ and suggested that, similarly to family A GPCRs, the TMs of this receptor form a water-accessible crevice, the binding-site crevice, which extends from the extracellular surface of $CRF_1$ into the plane of the membrane and that the contact sites of small non-peptide CRF analogs must be located on the surface of this crevice.[111] Specifically, this study has shown that the endogenous Cys211 (in TM3), Cys233 (in TM4) and Cys364 (in TM7) are located on the surface of the binding-site crevice of $CRF_1$.[111] Subsequently, Gkountelias et al. mapped the TM residues that form the surface of the binding-site crevice of $CRF_1$ receptor by applying SCAM and starting from the extracellular portion of TM3.[112] The results of this study have suggested that Thr192, Ala193, Tyr195 and Asn196 of TM3 are located on the water-accessible surface of the binding-site crevice of $CRF_1$ and that the pattern of accessibility is consistent with an alpha-helical conformation for this region of TM3.[112]

## NON-PEPTIDE $CRF_1$-SELECTIVE ANTAGONISTS

Several non-peptide CRF antagonists were developed as new leads in drug discovery to treat various stress-related disorders like depression, anxiety and addictive disorders. Most non-peptide CRF antagonists discovered to date are substituted five-membered rings or bicyclic and tricyclic rings and bind selectively to $CRF_1$. The non-peptide CRF antagonists offer advantages over the peptide congeners in terms of stability, ease of preparation, ease of further modification to enhance the pharmacokinetic profile and better brain penetrability.

### *1. Substituted Pyrazolones:*

Nova Pharmaceuticals reported the synthesis of 4-substituted thiopyrazolones and its disulfide congener.[113] These derivatives inhibited the binding of [$^{125}$I]Tyr-oCRF to rat cortical membranes as well as the CRF-mediated stimulation of adenylate cyclase. Among the reported compounds, compound **1** [Binding $IC_{50}$= 3.8μM & cyclase inhibition 3.6μM] and the disulfide derivatives **2** [Binding $IC_{50}$= 2.2μM & cyclase inhibition 1.1μM] and **3** [Binding $IC_{50}$= 3.3μM & cyclase inhibition 1.0μM] were potent.

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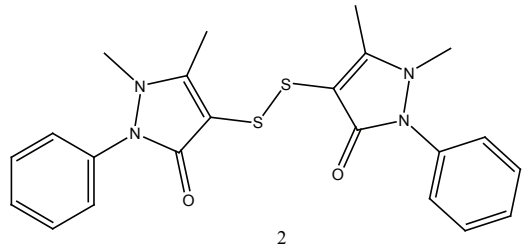


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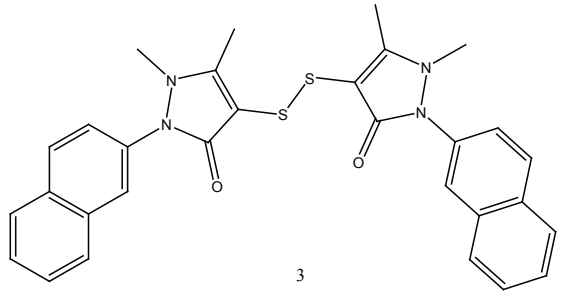


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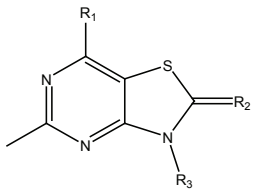
### *2. Thiazolo[4,5-d]-pyrimidines:*

A research study performed by DuPont Pharmaceuticals reported various thiazolo[4,5-d]pyrimidines

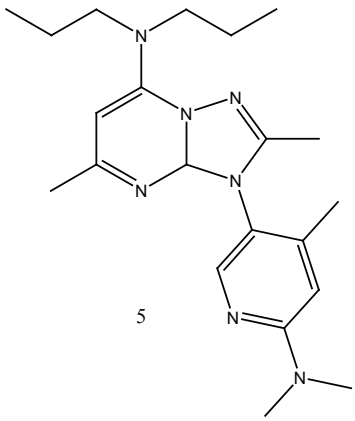
having the general structure **4**.[114] Substitution of noncyclic diamino groups in $R_1$ position was found to increase the binding affinity of CRF for the human $CRF_1$ receptor compared to the cyclic amino group (e.g. morpholine). The thiazolones (X = O) were also equipotent in binding to $CRF_1$ receptors compared to the precursor thiazolothiones (X = S).



| | | | |
|---|---|---|---|
| 4a, $R_1$= N(Et)$_2$ | $R_2$= S | $R_3$= 2-Br$^{-4}$-isopropyphenyl | *Ki*= 4.1 nM |
| 4b, $R_1$= N(Et)$_2$ | $R_2$= O | $R_3$= 2-Br$^{-4}$-isopropyphenyl | *Ki*= 9.4 nM |
| 4c, $R_1$= N(Pr)$CH_2$-cPr | $R_2$= S | $R_3$= 2-Br$^{-4}$-isopropyphenyl | *Ki*= 12.6 nM |
| 4d, $R_1$= N(Pr)$CH_2$-cPr | $R_2$= O | $R_3$= 2-Br$^{-4}$-isopropyphenyl | *Ki*= 4.1 nM |
| 4e, $R_1$= N($CH_2CH_2OCH_3$)$_2$ | $R_2$= S | $R_3$= 2,4,6-trimethylphenyl | *Ki*= 8.6 nM |
| 4e, $R_1$= N($CH_2CH_2OCH_3$)$_2$ | $R_2$= O | $R_3$= 2,4,6-trimethylphenyl | *Ki*= 5.8 nM |

### *3. Quinolines and Isoquinolines:*

In search of novel $CRF_1$ antagonists with water solubility, Chen et al. prepared compound **5** with high p$K_a$ (p$K_a$ = 7.1).[115] However, clinical trials of this compound have been discontinued due to its hepatotoxicity.



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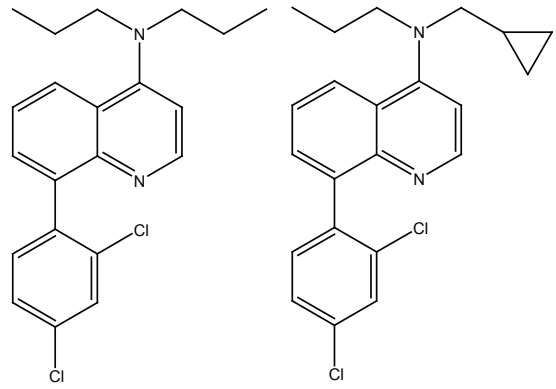
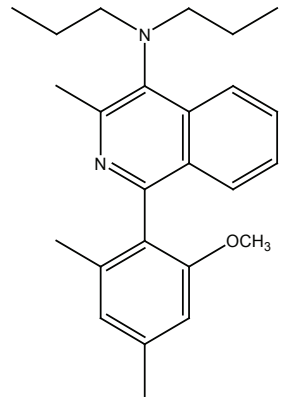
Furthermore, 4-substituted 8-aryl-2-methylquinolines with general structure **6** have recently been synthesized. Since the p$K_a$ of the 4-amino quinolone was about 9.08, it has been speculated that these compounds should be largely charged at physiological pH (7.4) thus increasing water solubility. Presence of the dipropylamino and *N*-cyclopropane-methyl-*N*-propylamino group greatly enhanced activity compared to the smaller diethylamino group. Indeed, non-polar groups of the aromatic ring enhance activity.



6a, $K_i$ = 0.9 nM

6b, $K_i$ = 0.5 nM

By topological modification of previously reported high-affinity CRF antagonists, Yoon et al. prepared 1-aryl-4-aminoalkylisoquinolines and tested their affinities in competition binding studies in IMR-32 human neuroblastoma cells and using $^{125}$I-Sauvagine as a radioligand. [116] Compounds with mono-substituted aryl groups were found to have lower affinity than those with di-substitution at both the *ortho* and *para* positions with at least one methoxy group at one *ortho* position. Compounds having a dipropylamino group at 4-position (-N-$Pr_2$) showed enhanced affinity, as seen in compound **7**.

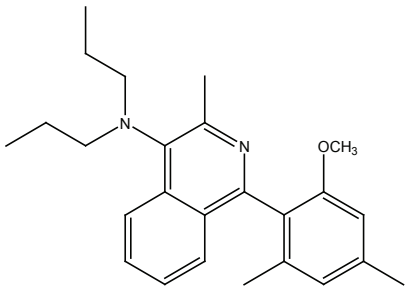


7, $K_i$ = 6 nM

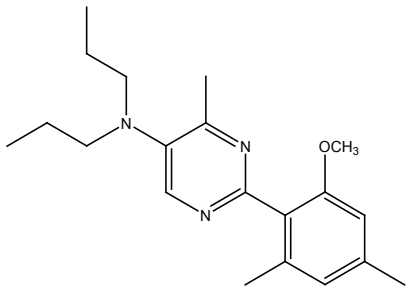
### *4. Pyrimidine derivatives:*

High lipophilicity, poor water solubility and long half-life of many potent $CRF_1$ receptor antagonists make them unattractive for clinical development and hinder further development. Yoon et al. developed less lipophilic and more water soluble aryl pyrimidine derivatives in order to improve their pharmacokinetic profile.[117] Further introduction of a small alkoxy

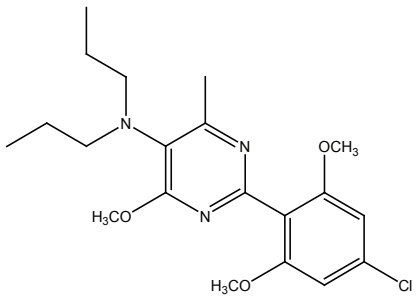
group at the available un-substituted positions in the pyrimidine ring enhanced the affinity. Further modifications of the pyrimidine 2-aryl group yielded 2-(2,4,6-tri-substituted) compounds, the most potent of which was the 2,4-dimethoxy-6-chloro derivative **8**.



$K_i$ = 6 nM
cLogP = 7.4

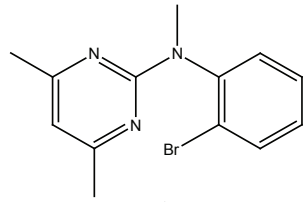


cLogP = 7.4

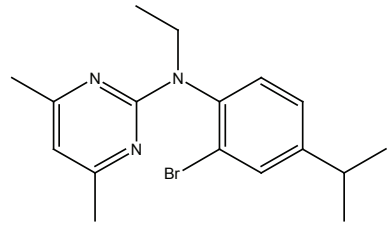


8, *K*i =2 nM
cLogP = 5.9

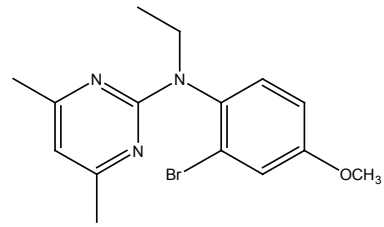
Screening of a library of compound by DuPont Pharmaceuticals revealed that compound **9** inhibited [$^{125}$I] Tyr-oCRH binding in rat frontal cortex homogenates.[118] Initial structure-activity relationship studies (SAR) of this lead compound resulted in the synthesis of compound **10,** which was subjected to further optimization. Replacement of the bromo group with methyl, trifluoromethyl or thiomethyl resulted in compounds having comparable affinities to **10**, while removal of any substituent at that position greatly reduced receptor binding affinity.



9



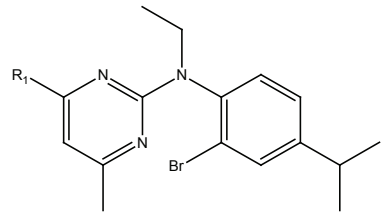
10



11 *K*i =35 nM

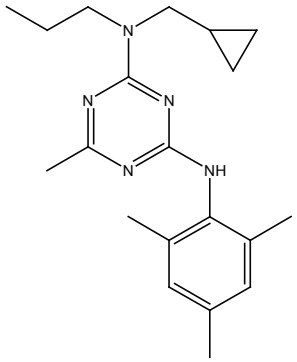
Substitution of the 4-position isopropyl group with larger groups (e.g. butyl, t-butyl) resulted in further decreased receptor binding than groups which have approximately the same size or smaller than isopropyl (e.g. methoxy) as in compound **11**.

In addition, it was noticed that substitution of the 4- or 6-positions of the pyrimidine ring with groups larger than methyl decreased receptor binding activity. Retaining the methyl at 6-position while having different selected substituents at 4-position ($R_1$) of the pyridine ring yielded many compounds **12a**, **12b**, **12c** and **12d** with enhanced activity.

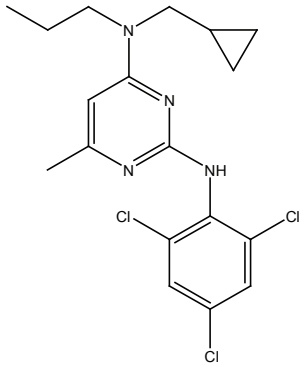


12a, $R_1$ = 3=pentyl $K_i$ = 2 nM
12b, $R_1$ = $N(Pr)_2$ $K_i$ = 9 nM
12c, $R_1$ = N(Pr)(*c*-$C_3H_5$) $K_i$ = 5 nM
12d, $R_1$ = N(Pr)($CH_2$-*c*-$C_3$H5) $K_i$ = 9 nM

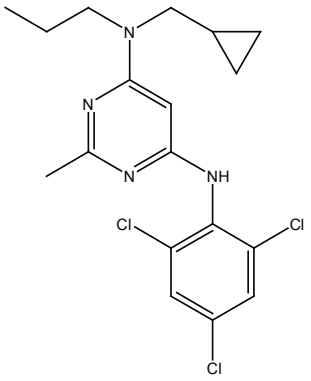
Removal of the 1- or 5-nitrogen in the triazene compound **13** [119] resulted in active pyrimdines **14** and **15** according to Chen et al.[120] However, removal of the 3-nitrogen of the triazine resulted in complete loss of activity.



13, $K_i$ =57 nM

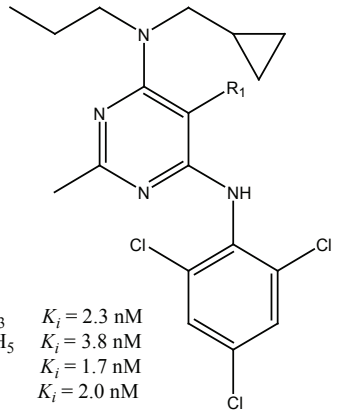


14, $K_i$ = 70 nM



15, $K_i$ = 30 nM

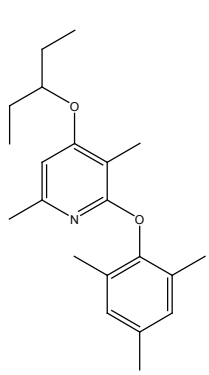
A pharmacophore model has been proposed where the aniline group was predicted to be orthogonal to and below the pyrimidine ring. Addition of small groups (e.g. methyl, ethyl, chloro and bromo) resulted in compounds **16a**, **16b**, **16c** (NBI 27914) and **16d** with nanomolar range receptor binding affinity.



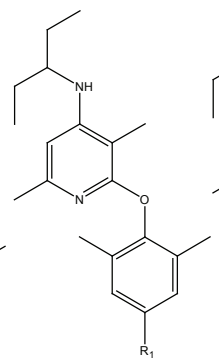
16a, $R_1$= $CH_3$ $K_i$ = 2.3 nM
16b, $R_1$= $C_2H_5$ $K_i$ = 3.8 nM
16c, $R_1$= Cl $K_i$ = 1.7 nM
16d, $R_1$= Br $K_i$ = 2.0 nM

## *5. Pyridine derivatives:*

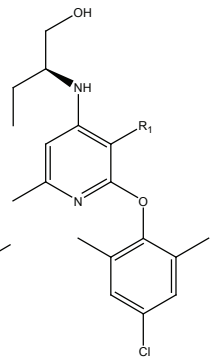
Several promising pyridines were developed at Pfizer. Compound **17** showed increased binding affinity but poor pharmacokinetic profile. To improve the pharmacokinetic properties, the oxygen atom in the alkoxy or aryloxy groups have been replaced by a nitrogen atom to increase basicity and thus water solubility.[121] Thus replacing the alkoxy group with alkylamino function resulted in compound **18a**, which had higher basicity and increased aqueous solubility in simulated gastrointestinal fluid. Further structural optimization has been achieved by replacing the methyl group at the para-position of the 2-phenoxy ring with chloro or bromo atoms, thus yielding the compounds **18b** and **18c**. Further structural modifications resulted in compound **19a** and **19b** with increased polarity and decreased binding.



17



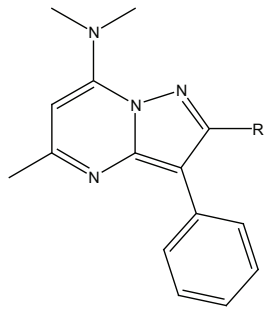
18a, $R_1$= $CH_3$ $K_i$ = 5.1 nM
18b, $R_1$= Cl $K_i$ = 5.1 nM
18c, $R_1$= Br $K_i$ = 5.6 nM



19a, $R_1$= $CH_3$ $K_i$ = 6.4 nM
19b, $R_1$= Cl $K_i$ = 31 nM

## *6. Pyrazolo[1,2-b]pyrimidines:*

Several pyrazolo[1,2-b]pyrimdines were prepared as potential CRF receptor antagonists. Compound **20** displayed promising activity.[122]



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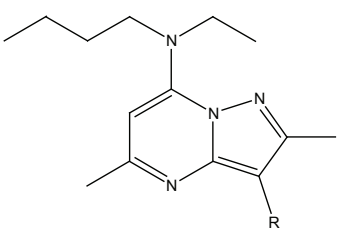
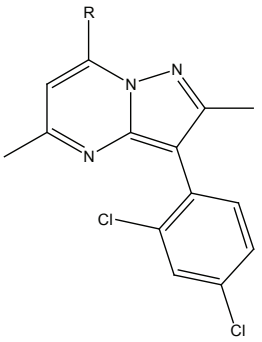
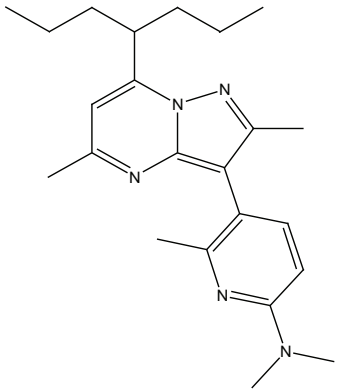


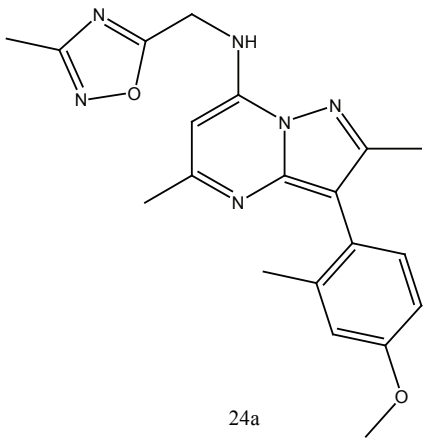
21a, R=phenyl $K_i$= 511 nM
21b, R=2-chlorophenyl $K_i$= 15 nM
21c, R=2,4-dichlorophenyl $K_i$= 5 nM
21d, R=2,4,6-trimethylphenyl $K_i$= 93 nM



22a, R=N(*n*-Pr)$CH_2$(*c*-Pr) $K_i$= 3.2 nM
22b, R=N(Et)($CH_2CH_2OCH_3$) $K_i$= 11 nM



23



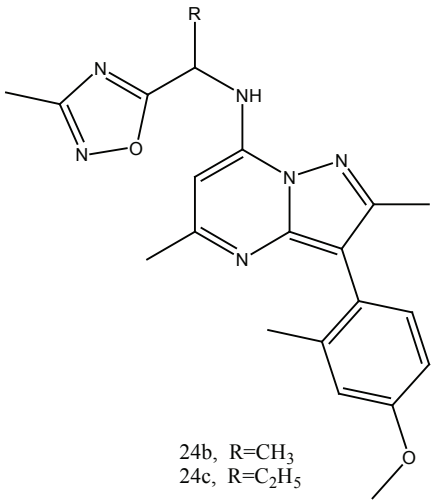
24a

SAR studies of this class demonstrated that replacement of the phenyl group (**21a**, $K_i$ = 511 nM) with 2-Cl phenyl resulted in enhanced binding affinity (**21b,** $K_i$ = 15 nM). Similarly, substitution with 2,4-dichlorophenyl created the most potent derivative of the series (**21c** with $K_i$ = 5 nM). However, unlike most similar non-peptide CRF antagonists, the 2,4,6-trimethylphenyl derivative displayed 17-fold reduction of binding affinity (**21d**, $K_i$= 93 nM).

As expected, SAR studies also revealed that the presence of non-cyclic dialkyl amino groups with small groups had better activity than their cyclic counterparts (e.g. compounds **22a** and **22b**).

Compound **23** showed excellent potential in depression and anxiety tests; however, it caused reversible increase in hepatic enzymes, which hindered its further development.[123,124]

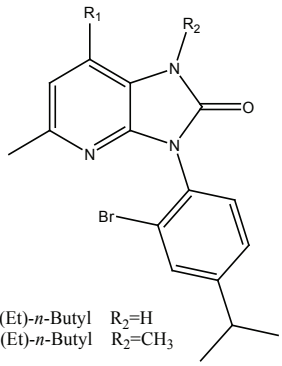
Attempts to lower the lipophilicity by substitution of N-alkyl side chain with heterocycles, while retaining the CRF antagonistic activity, were sought.[125] Several 1,2,4-oxadiazolyl derivatives were prepared, among them compound **24a** (p$K_i$=7.2±0.1) and **24b** (p$K_i$=7.6±0.1), which exhibited an improved metabolic stability. The best combination of metabolic stability and CRF binding affinity was observed in compound **24c** (p$K_i$=8.1±0.1).



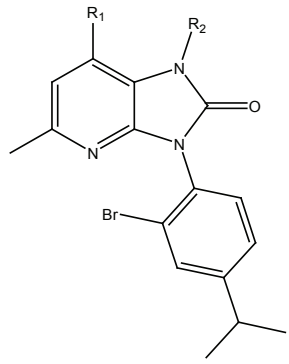
24b, R=$CH_3$
24c, R=$C_2H_5$

### *7. Purines:*

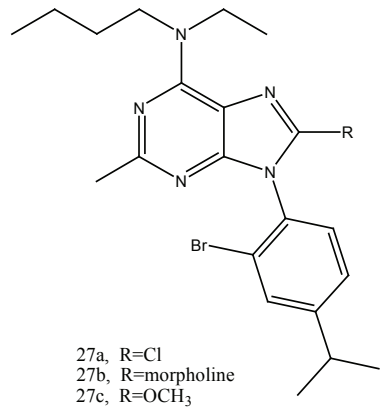
Many substituted purin-8-analogs have emerged as a new class of CRF antagonists.[126] Based on the previous SAR work, the 2-bromo-4-isopropylphenyl derivatives were selected as lead compounds. Compound **25a** had weak CRF binding affinity ($K_i$=890 nM), in contrast to its *N*-methyl derivative **25b**, which had a high binding affinity ($K_i$= 5 nM). Further modification with steric bulky groups resulted in decreased binding. The dialkylamine substituted derivatives had higher affinities than mono-substituted compounds (compounds **26a**, **26b**, **26c** and **26d**).



25a, $R_1$=N(Et)-*n*-Butyl $R_2$=H
25b, $R_1$=N(Et)-*n*-Butyl $R_2$=$CH_3$



26a, $R_1$=$N(Pr)_2$ $R_2$=$CH_3$ $K_i$= 7.6 nM
26b, $R_1$=N(Pr)$CH_2$-*c*-Pr $R_2$=$CH_3$ $K_i$= 5.7 nM
26c, $R_1$=N(H)(Et)$CH_2OCH_3$ $R_2$=$CH_3$ $K_i$= 42.8 nM
26d, $R_1$=N(H)$CH_2CH_2OCH_3$ $R_2$=H $K_i$= 1179 nM



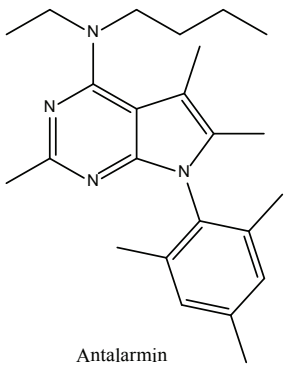
27a, R=Cl
27b, R=morpholine
27c, R=$OCH_3$

The replacement of the oxo group with a chloro function created a derivative with excellent binding affinity (**27a**, $K_i$=1.5 nM). Similarly, the replacement with small alkoxy groups (e.g. methoxy) resulted in the high affinity derivative **27c** ($K_i$=1.5 nM). In contrast, the binding affinity was dramatically reduced by the replacement of chloro function with morpholine (**27b**, $K_i$=345 nM). Larger-size groups resulted in loss of affinity.

## NON-PEPTIDE CRF RECEPTOR ANTAGONISTS IN CLINICAL TRIALS

Several non-peptide CRF receptor antagonists, including antalarmin, are currently being studied in clinical trials. Antalarmin blocks the $CRF_1$ receptor and, consequently, reduces the release of ACTH in response to chronic stress.[127] Antalarmin reduces the behavioral response to stressful stimuli.[128] It should be mentioned that several newer non-peptide CRF antagonists are currently under development,[129] tar-

geting specific brain regions with the aim of ameliorating the health consequences of chronic stress and for use in the clinical management of anxiety and depression.[130-132]



Antalarmin

Promising results have also been observed using antalarmin as a potential treatment for CRF-induced hypertension. Central administration of CRF results in endocrinological, cardiovascular and behavioral effects that suggest stress or anxiety. Among these is a marked pressor response. Antalarmin was found to antagonize the pressor effect induced by central CRF.[133]

Similar promising results for antalarmin and other $CRF_1$ antagonists were also observed in the area of drug addiction disorders. Evaluation of antalarmin effects on cocaine dependence in cocaine-addicted monkeys showed a reduction of its use. Similarly, antalarmin tested on cocaine-addicted rats prevented dose escalation, suggesting that it might modulate the cocaine addictive effects over time. Antalarmin also displayed positive effects in reducing withdrawal symptoms from chronic opioid use and significantly reduced self-administration of ethanol in ethanol-addicted rodents. [134-137]

Antalarmin also showed anti-inflammatory effects and has been suggested as having potential uses in the treatment of inflammatory conditions such as arthritis[138] as well as stress-induced gastrointestinal ulcers[139] and irritable bowel syndrome.[140,141]

Chronic blockade of $CRF_1$ with systemic antalarmin significantly ameliorated rat adjuvant-induced arthritis, reducing the severity of inflammation in peripheral joints, this evidenced by clinical and histopathology results, and weight loss associated with disease onset. Antalarmin neither induced nor exacerbated arthritis expression in rats, despite suppression of levels of adjuvant-induced corticosterone, the major anti-inflammatory glucocorticoid in rats. Systemic blockade of $CRF_1$ appeared to predominantly block peripheral pro-inflammatory effects of immune CRF rather than the systemic glucocorticoid mediated antiinflammatory effects of hypothalamic CRF. Results indicate that chronic treatment with a $CRF_1$ antagonist attenuates progressive inflammation-induced degeneration of synovia, cartilage and bone in arthritic joints, suggesting that antalarmin may have therapeutic potential in treatment of human autoimmune and inflammatory disorders. [138]

Upon exposure to prolonged stress, rats develop gastric ulceration, enhanced colon motility with depletion of its mucin content and signs of physiological and behavioral arousal. When antidepressants (fluoxetine and bupropion), anxiolytics (diazepam and buspirone) or antalarmin were evaluated for their potential to modify these responses, fluoxetine, bupropion, diazepam and antalarmin all suppressed stress-induced gastric ulceration in male Sprague-Dawley rats exposed to four hours of plain immobilization. Antalarmin was found to produce the most pronounced anti-ulcer effect and additionally suppressed the stress-induced colonic hypermotility, mucin depletion, autonomic hyperarousal and struggling behavior. Non-peptide $CRF_1$ antagonists may therefore be of value as prophylactics against stress ulcer in the critically ill and as therapeutics for other related gastrointestinal disorders such as peptic ulcer disease and irritable bowel syndrome. [139]

The characterization of neuroendocrine-regulating CRF family peptides, in conjunction with the cloning and pharmacological characterization of the two major CRF receptor subtypes ($CRF_1$ and $CRF_2$) and the development of selective CRF receptor antagonists, provided new insights to explain the mechanisms of stress and the potential involvement of the CRF system in different pathophysiological conditions, including gastrointestinal disorders, mainly irritable bowel syndrome (IBS), and psychopathologies such as anxiety/depression. Compelling pre-clinical data demonstrated that central CRF administration mimics acute stress-induced colonic responses and enhances colorectal distension-induced visceral pain in rats through $CRF_1$ receptors. Similarly, peripheral CRF

reduced the pain threshold to colonic distension and increased colonic motility in both humans and rodents. These observations mimic the manifestations of IBS characterized by abdominal bloating and discomfort and altered bowel habits. $CRF_1$ pathways have been implicated in the development of anxiety/depression and these psychopathologies, together with stressful life events, have high co-morbidity with IBS and are considered significant components of the disease. $CRF_1$ receptors have been suggested as a target to treat IBS. Peripherally acting $CRF_1$ antagonists might directly improve IBS symptoms, as related to motility, secretion and immune response. On the other hand, central actions will be beneficial for the prevention of the psychopathologies that co-exist with IBS and as a way to modulate the central processing of stress- and visceral pain-related signals.[140,141]

## SUMMARY

CRF plays a key role in the maintenance of homeostasis by regulating the hypothalamic-pituitary-adrenal axis, functioning as a neurotransmitter within the central nervous system and being involved in the control of the cardiovascular, gastrointestinal, behavioral, immune and reproductive systems. CRF exerts its actions by interacting with the $CRF_1$ and $CRF_2$ receptors, which belong to family B of G-protein coupled receptors. Considerable progress has been made in the determination of the structure and function of peptide and non-peptide CRF analogs, thus advancing to some extent the development of new compounds, including non-peptide $CRF_1$-selective antagonists. The non-peptide CRF analogs have significantly contributed to the determination of the role of CRF and its receptors in several physiological and pathophysiological conditions. Progress in elucidating the interactions of CRF ligands with their receptors will further advance the design and synthesis of new CRF analogs with potential clinical applications.

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