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Perspective

Corticotropin Releasing Factor (CRF) Receptor Modulators: Progress and Opportunities for New Therapeutic Agents

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Introduction

Corticotropin releasing factor (or hormone, CRF or CRH) is a major modulator of the body's responses to stress, and modulation of CRF receptor function has become an important target for drug design. This 41-amino acid peptide presides over a panoply of neuronal, endocrine, and immune processes as the primary regulator of the hypothalamus−pituitary−adrenal (HPA) axis. Extensive preclinical studies on CRF agonists and antagonists as well as limited clinical studies on CRF itself have provided the theoretical foundation for the hypothesis that abnormal CRF secretion or synthesis may underlie the pathologies of a diverse range of neuropsychiatric disorders such as anxiety, depression, obsessive−compulsive disorder, Parkinson's disease, Alzheimer's disease, alcohol withdrawal, and posttraumatic stress disorder. CRF modulation may also provide opportunities for the management of endocrine disorders and immunological-based diseases since regulation of hormonal and immune responses by the HPA axis is well-established. Peptide agonists and antagonists have been available for many years, and they are valuable tools for the investigation of CRF-mediated physiology. Studies with peptide ligands provide overwhelming support for a role for CRF in coordination of the body's responses to stress. Furthermore, considerable progress in the identification of nonpeptide modulators of CRF function has been made in the last 10 years, providing opportunities for definitive clinical studies on treatments for some of the disorders described above. This Perspective reviews the current state of research on CRF. Recent advances in the molecular biology of the CRF receptors and binding protein as well as the quest for selective modulators of CRF function will be presented. The prospects for future drug therapy will also be discussed.

Pharmacological and Anatomic Bases of Stress

The seminal studies of Selye and co-workers sparked the search for the pharmacological and anatomic framework for the body's responses to stress.[1,2] Their formulation of the "general adaptation syndrome" concept established that the neural, endocrine, and immune responses to stress are probably closely integrated in a homeostatic system. Severe or prolonged stress was postulated to damage the plasticity of this system and cause disease. This concept, that dysfunction in neural, endocrine, and immune responses to stress is a cause of disease, did not originate with Selye; it had been stated previously in various forms and has been reviewed at length.[3] His key contribution was the suggestion that a central network exists to coordinate the diverse physiological and behavioral responses to stress. This work, when coupled with the pioneering studies on the neural regulation of pituitary function by Harris,[4] suggested that this integration of responses to stress may be mediated through a specific neuroendocrine architecture.

The pattern of behavioral and physiological responses to stress points to the HPA axis as the central physi-

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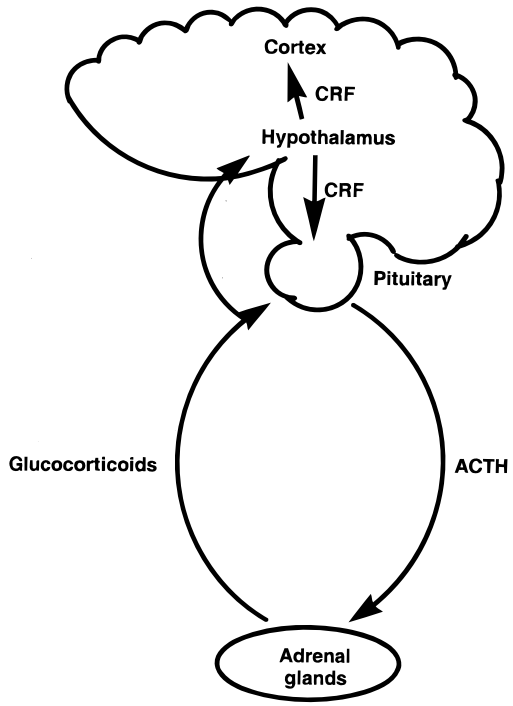


Figure 1. CRF and the HPA axis.

ological network, which coordinates stress responses. Clinical investigations on the causes of depression and anxiety and preclinical studies in laboratory animals on neuroendocrine physiology converged to nominate the HPA axis as the unifying framework for mediating biological responses to stress. Clinicians established that plasma cortisol levels in a large number of depressed patients were elevated relative to normal age- and sex-matched control patients.[5] Early studies showed that alteration of cortisol levels led to parallel changes in behavioral symptomatology (vide infra). Several depressed patients exhibit maladaptive responses to stress. Therefore, cortisol secretion and synthesis became one focus of studies on the physiological mechanism(s) of stress responses as well as depression. The adrenal glands were identified as the locus for cortisol production. In rats, secretions from the pituitary were found to influence the production of the rodent counterpart to cortisol, corticosterone. Adrenocorticotropic hormone (ACTH) was subsequently identified as the humoral factor. The missing link, however, was the chemical factor(s) governing both ACTH and cortisol levels (Figure 1). The pioneering studies of Vale and co-workers on the isolation of ovine CRF provided this link.[6] CRF has been shown to be a potent secretagogue for ACTH, and corticosterone has been shown to exert feedback inhibition on CRF secretion at the levels of the pituitary and the hypothalamus (Figure 1).[7−10] Intracerebroventricular (icv) injection of CRF into rats elicits a constellation of behavioral and physiological alterations similar to those caused by stress.[11−14] Behavioral adaptations to both stress or CRF injection include: rapid redirection of behavior (e.g. "fight or flight"), enhanced vigilance, and immediate suppression of nonessential functions (e.g. feeding or reproduction). Physiological responses to stress or CRF injection maximize the efficiency of energy utilization, such as focused allocation of oxygen and essential nutrients to the central nervous system (CNS), increased cardiovascular and pulmonary tone, and enhanced gluconeogenesis and lipolysis as well as acute immunomodulation.[3,5] CRF has multiple roles in cardiovascular, gastric, immune, and reproductive physiology (vide infra) in acute and chronic stress situations. Furthermore, chronic restraint stress of rodents leads to long-term activation of the HPA axis as marked by increased CRF secretion and CRF receptor down-regulation as well as cognate changes in ACTH and corticosterone levels.[15] Similar effects of stress or intravenous CRF administration have been observed in humans.[16,17] Thus, the clinical and preclinical stories on the effects of stress came together, and the central network, which was envisioned by Selye and co-workers as governing the body's responses to stress, was given a definitive anatomic and physiological foundation.

Table 1. Sequences of Rat, Human, and Ovine CRF

agonist	amino acid sequence
r/h-CRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNR-KLMEII-NH_2
o-CRF	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNR-KLLDIA-NH_2

CRF Molecular Pharmacology and Physiology

A. Isolation, Characterization, and Localization of CRF. CRF was first isolated from ovine hypothalamus in 1981 and characterized as an ACTH secretagogue by Vale and co-workers.[6,10,18] Sequence analysis revealed that this peptide contains 41 amino acids, and the carboxy-terminal amide residue was found to be essential for secretagogue potency (Table 1).[19] CRF stimulates secretion of ACTH in a dose-dependent manner in experiments employing primary cultures of rat pituitary cells (EC_{50} = 50−200 pM)[6] and adenylate cyclase activity in rat brain.[20]

There is substantial sequence homology between CRF and the amphibian peptide sauvagine as well as the telostian peptide urotensin. The three peptides have similar biological properties as hypotensive agents and ACTH secretogogues.[21,22] Recently, a mammalian congener of urotensin, urocortin, has been characterized.[23,24] Data on the distribution of urocortin together with its physiological effects suggested that it may be responsible for some of the biological effects in higher mammals which were originally attributed to CRF.[25] Urocortin may also have a role separate from that of CRF based on its unique distribution in the human CNS.[25]

While CRF was first identified as a secretogogue, investigations into its anatomic distribution suggested this peptide is also a neurotransmitter.[5,7] The paraventricular nucleus of the hypothalamus was first discovered to contain a very high density of CRF-immunoreactive neurons. These neurons are the source of CRF, which modulates release of ACTH from the pituitary.[26] CRF neurons innervate the locus coeruleus with its noradrenergic foci and the central nucleus of the amygdala, which are two brain centers previously shown to be involved in anxiety and stress responses.[26−28] CRF is also found in cells of other hypothalamic nuclei, neocortex, bed nucleus of the stria terminalis, brainstem, and spinal cord.[29] The wide distribution of CRF-containing neurons and their projections argue for an important role for this peptide in multiple brain functions.

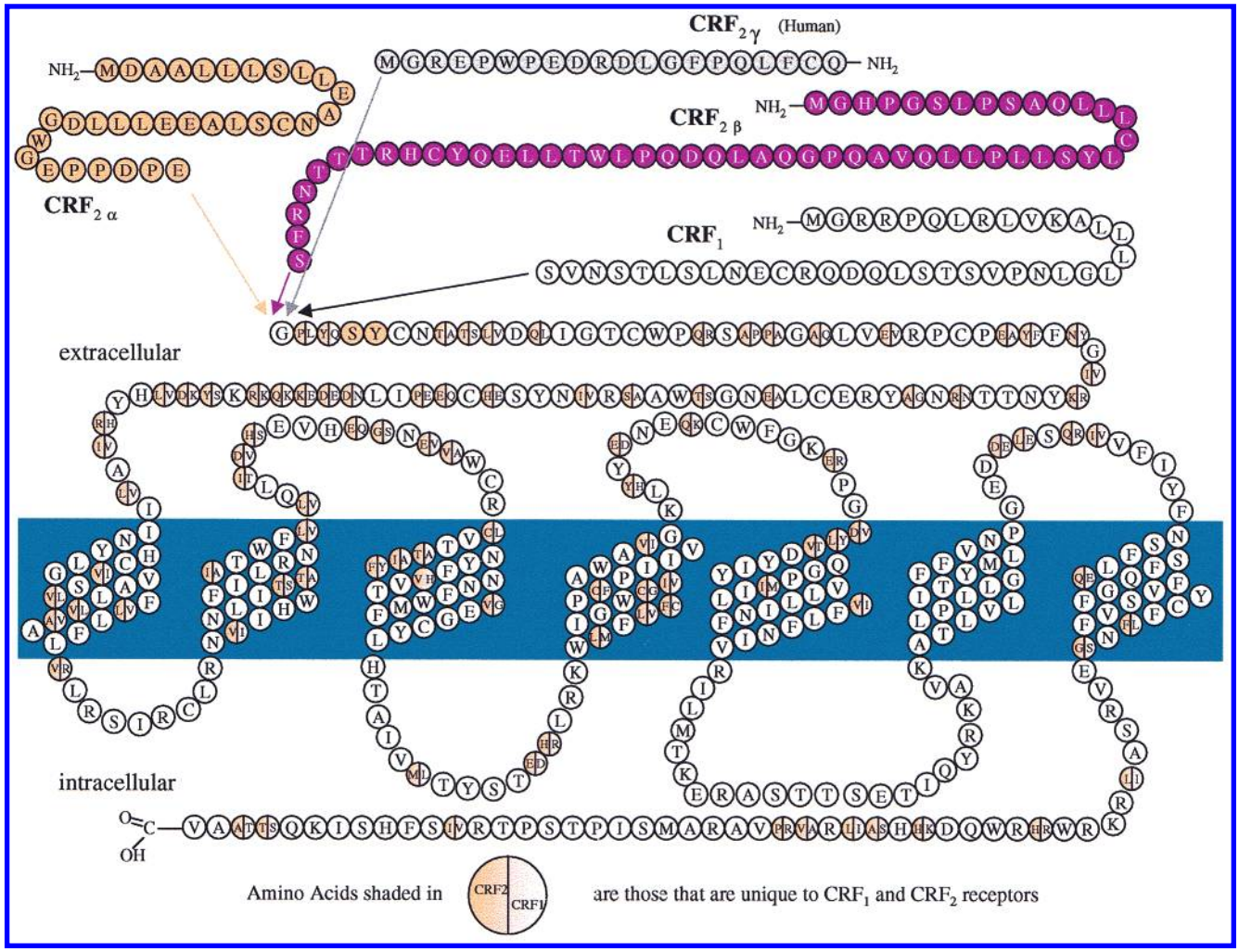


Figure 2. Sequences of rat CRF receptor subtypes.

CRF is found in a number of peripheral tissues using immunocytochemical or in situ hybridization techniques, which suggests additional roles for this peptide.[5,7] It is present in pancreas, stomach, and small intestine, indicating a possible role in digestion and energy storage.[30] CRF is also found in lymphocytes, suggesting a possible involvement in immune function.[31,32] CRF is detected in the placenta,[33] which is the source of the high levels of human plasma CRF during pregnancy, and in the testes,[34] suggesting a reproductive function for CRF. Physiological studies have confirmed these predictions based on anatomical localization (vide infra).

B. CRF Receptors. Shortly after the isolation and characterization of CRF, a specific high-affinity binding site for the peptide was identified in the rat anterior pituitary gland, the CNS, and various peripheral sites.[35] Binding of peptide agonists to this site is modulated by divalent cations and guanyl nucleotides suggesting that the receptor belongs to the seven-transmembrane (7-TM) G protein-coupled family of receptors. CRF was shown to stimulate the accumulation of cyclic adenosine monophosphate (cAMP) in rat pituitary corticotrophs in a dose-dependent manner which was parallel to that of CRF-induced ACTH release in rat pituitary cells. Thus, early evidence pointed to the CRF receptor being coupled to the G_s protein.[36−38]

Subsequent studies demonstrated the presence of CRF receptor binding sites beyond the pituitary in various regions of the CNS as well as in peripheral tissues such as the adrenal medulla, ventral prostate, spleen, liver, kidney, and testes.[30,38−40] The presence of the CRF peptide as well as its receptor in these various organs further supports additional roles for the peptide apart from its central function as a regulator of the HPA axis in response to stress.

Molecular biological experiments led to the discovery of two distinct CRF receptor subtypes designated CRF_1 and CRF_2, which are encoded by distinct genes that are differentially expressed.[41−56] In addition, three splice variants of the CRF_2 receptor have been identified: $CRF_{2\alpha}$, $CRF_{2\beta}$, and $CRF_{2\gamma}$ (see Figure 2). Sequence homology suggests that the CRF receptors belong to the VIP/calcitonin family of G protein-coupled receptors and all subtypes are positively coupled to adenylate cyclase. The CRF_1 receptor contains 415 amino acids; the $CRF_{2\alpha}$, $CRF_{2\beta}$, and $CRF_{2\gamma}$ subtypes include 411−413, 431−438, and 397 amino acids, respectively.

CRF_1 receptors are widely distributed in rat brain, mainly in neocortex and cerebellum. Localization of rat CRF_1 receptors has been studied using in situ hybridization methods.[50] Furthermore, the CRF_1 receptor is the most abundant CRF receptor subtype found in the rodent and primate pituitary.[50,57,58] The localization of

Table 2. Activity of Peptidic Agonists in Agonist-Stimulated Adenylate Cyclase Assay[54,56]

agonist (species)	amino acid sequence	mean adenylate cyclase EC_{50} (nM)[a]	
		h-CRF_1	h-$CRF_{2\alpha}$
urocortin (human)	DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV-NH_2	0.22 ± 0.08	0.32 ± 0.10[b]
sauvagine (frog)	QGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI-NH_2	3.4 ± 1.5	1.4 ± 1.2
r/h-CRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII-NH_2	3.5 ± 1.3	13.2 ± 4.6
urotensin-1 (sucker fish)	NDDPPISIDLTFHLLRNMIEMARIENEREQAGLNRKYLDEV-NH_2	6.3 ± 1.6	1.5 ± 0.8
o-CRF	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA-NH_2	9.7 ± 1.2	61.9 ± 15.8

[a] Standard errors of the mean are reported. See original references for experimental conditions and statistical analyses. [b] Rat receptor data.

CRF_1 receptors defined using in situ hybridization techniques corresponds well with the distribution of rat CRF receptors found by autoradiography studies with [^{125}I-Tyr^0]r/h-CRF.[50−52] Studies on the distribution of CRF_1 receptors in monkeys have yielded similar results.[57,58]

The distribution of CRF_2 receptors has also been studied by multiple methods. In the rodent, $CRF_{2\beta}$ receptors are expressed mainly in the peripheral vasculature and the heart, while $CRF_{2\alpha}$ receptors are found predominantly in subcortical structures in brain such as the septum, amygdala, and hypothalamus using in situ hybridization techniques.[53] Recently, the use of [^{125}I-Tyr^0]sauvagine in combination with a CRF_1-specific small-molecule antagonist in receptor autoradiography, a method designed to specifically study rat CRF_2 sites, confirmed the localization of CRF_2 receptors to areas defined by in situ hybridization techniques.[54,55] In primate tissues, the CRF_2 receptor subtype distribution is different. The $CRF_{2\alpha}$ splice variant predominates and is found in both central and peripheral locations.[41,54] The $CRF_{2\gamma}$ receptor was derived from the human amygdala region.[44] Recent studies have shown the distribution of CRF_2 receptors within the rhesus monkey brain to differ from that found in the rat brain.[59] Neuroanatomic distribution of the CRF_1 and CRF_2 receptor subtypes was assessed by autoradiographic studies with [^{125}I-Tyr^0]sauvagine with or without a selective CRF_1 receptor antagonist, similar to that described above for the rat studies. Moreover, in situ hybridization techniques with radiolabeled human cRNA probes were used to map the localization of the receptor subtype mRNAs. Both CRF_1 and CRF_2 receptors are found in the rhesus monkey pituitary, neocortex, amygdala, and hippocampus. CRF_1, but not CRF_2, receptors are present in locus coeruleus, cerebellar cortex, thalamus, and striatum. CRF_2, but not CRF_1, receptors were found in the choroid plexus and bed nucleus of the stria terminalis. The interspecies differences in receptor subtype distribution may indicate that CRF_2 receptors play a greater role in neuropsychiatric disorders than previously suspected based on the rodent data. Furthermore, the relative roles of CRF_1 and CRF_2 receptors vary with the stage of development of the rat,[60] and this may also be true for higher species.

CRF-related peptide agonists have been found to differ in their activities at CRF_1 and CRF_2 receptors, further confirming the existence of CRF receptor subtypes (Tables 2 and 3).[54−56] In agonist-stimulated adenylate cyclase assays employing the cloned human receptors, the potency rank order at CRF_1 receptors is: human urocortin > sauvagine = rat/human CRF (r/h-CRF) > urotensin-1 > ovine CRF (o-CRF); however, the rank order at $CRF_{2\alpha}$ sites is urotensin-1 = sauvagine > r/h-CRF > o-CRF. In binding studies employing [^{125}I-Tyr^0]sauvagine as the radioligand and the cloned human receptors, the rank order for binding affinity at CRF_1 receptors is: urotensin-1 ≥ human urocortin > r/h-CRF = sauvagine = o-CRF > α-helical CRF_{9-41} (a peptide antagonist); however, at the $CRF_{2\alpha}$ receptors the rank order is: urotensin-1 > sauvagine > human urocortin > α-helical CRF_{9-41} > r/h-CRF ≫ o-CRF. The differences observed using the two methods of receptor activity measurement may relate to the fact that the binding studies were performed under equilibrium conditions while the adenylate cyclase experiments were not. However, differences in second-messenger coupling efficiencies among the peptide agonists cannot be excluded.

Table 3. Binding Affinities of Peptidic Agonists[55]

agonist (species)	mean K_i (nM)[a] vs [^{125}I-Tyr^0]sauvagine	
	h-CRF_1	h-$CRF_{2\alpha}$
urocortin (human)	3.76 ± 0.72	3.60 ± 0.20
sauvagine (frog)	7.30 ± 0.60	2.73 ± 0.83
r/h-CRF	4.43 ± 0.43	12.2 ± 3.63
urotensin-1 (sucker fish)	1.07 ± 0.14	1.26 ± 0.39
o-CRF	7.16 ± 1.41	86.4 ± 25.4

[a] Standard errors of the mean are reported. See original reference for statistical analyses and experimental conditions.

C. CRF-Binding Protein. There is also a CRF-binding protein (CRF-BP), which is present in both the rat and human CNS and in human plasma.[61] One main function of the peripheral binding protein, which binds CRF tightly, is to counteract the hypersecretion of CRF from the placenta during the last trimester of pregnancy; the role of the central binding protein, which is membrane-bound, is the subject of intense investigation. The perception of the binding protein function is changing from that of a sponge, which sops up excess CRF, to an active modulator of CRF receptor function in the CNS, which can control the concentrations of the native ligand at its receptors.[62,63] The CRF-BP is a 322-amino acid peptide, which is subject to three main posttranslational modifications: glycosylation, phosphorylation, and enzymatic cleavage. The CRF-BP gene contains multiple transcription factor binding sites: key transcriptional regulators are NF-kB and steroid hormones. The distribution of the CRF-BP in the rat CNS has been investigated extensively by in situ hydridization and immunochemistry techniques. High expression of the CRF-BP has been found in the rat cortex, amygdala,

and bed nucleus of the stria terminalis as well as the ventral and dorsomedial nuclei of the hypothalamus. Coexpression with CRF and CRF receptors occurs in several regions, most notably in the amygdala and pituitary corticotrophs. The anatomic distribution of the CRF-BP is the main evidence for a possible role for this peptide in cognitive function and stress.[64] Confirmation of this role awaits the testing of selective nonpeptidic CRF-BP−ligand complex inhibitors, which do not also bind to CRF receptors. Interpretation of preliminary reports on the in vivo effects on peptidic binding protein−ligand complex inhibitors on behavior using icv administration is confounded by a failure of these studies to demonstrate clearly penetration from the site of injection to the putative site(s) of action.[64] In contrast, studies on the levels of CRF-BP in the synovial fluid of arthritic patients provide direct evidence for a possible role of CRF-BP and its modulators in immunotherapy.[65]

D. CRF Gene Regulation. CRF gene expression and subsequent secretion are regulated via multiple pathways in a site-specific manner.[66−68] In rats, corticosterone restrains CRF gene expression in the paraventricular nucleus of the hypothalamus but induces such expression in the central nucleus of the amygdala and the lateral bed nucleus of the stria terminalis.[66,69] Central regulation of CRF secretion may be independent of the regulation of peripheral CRF secretion. In the CNS, serotonin is a key regulator of central CRF secretion.[70] In the periphery, serotonin shares this function with cytokines and humoral factors. Thus, it may be possible to modulate the central functions of CRF with minimal perturbation of peripheral systems.

Preclinical Pharmacology and Physiology

A. Animal Studies. Experiments in laboratory animals demonstrate a function for CRF in behavioral responses to stress;[71] icv administration of CRF in rats elicits a freezing response in novel surroundings, amplifies fear-related behaviors, increases conflict responses, and decreases sleep, feeding and sexual behavior. Behaviors, induced by CRF administration or by stress, can be blocked by a peptide antagonist, α-helical CRF_{9-41}. Urocortin, a CRF-related peptide, also suppresses feeding in both fasted and unfasted rats after icv administration in a dose-dependent manner, and its effect on feeding is more potent than that of CRF.[72]

There have been numerous studies supporting a role for CRF in the etiology of anxiety-related disorders.[73−75] CRF produces anxiogenic effects in animals in a variety of behavioral anxiety models as stated above. However, conflicting reports on the anxiogenic effects of its congener, urocortin, have been reported.[72,76] Interactions between benzodiazepine/non-benzodiazepine anxiolytics and CRF have been demonstrated. Studies examining the effects of the CRF receptor antagonist α-helical CRF_{9-41} in a variety of behavioral paradigms have demonstrated that the CRF antagonist produces "anxiolytic-like" effects qualitatively similar to those for the benzodiazepines. Chlordiazepoxide attenuates the "anxiogenic" effects of CRF in both the conflict test and the acoustic startle model in rats; icv injection of CRF into immature rhesus monkeys elicits distress vocalizations comparable to that observed as a result of maternal deprivation.[77]

Stress dramatically produces persistent alterations in behavior and the HPA axis.[78−82] Adverse rearing conditions in macaques elevated CRF concentrations in CSF for long periods of time. Neonatal rats, which have undergone maternal deprivation, have elevated basal and stress-induced levels of plasma ACTH long after the initial periods of stress. Moreover, increases in immunoreactive CRF levels are found in discrete brain regions (median eminence and parabrachial nucleus), and decreases in central CRF receptor expression were observed in these maternally deprived rats.[82] These data demonstrate that long-term stress damages the plasticity of the HPA axis.

B. CRF Transgenic and Knockout Mice. Studies with genetically altered mice have shed additional light on the role of CRF in behavior and neurophysiology.[83] CRF-Tg^+ mice express a chimeric CRF transgene containing a mMT-1 promoter, and they continuously overproduce CRF in the CNS and, consequently, ACTH and corticosterone in plasma.[84,85] Muscle atrophy, thin skin, fat accumulation, and female infertility occur in these animals. The locomotor activity of these animals in a novel environment is suppressed in a manner similar to that observed in animals receiving CRF via icv administration. In the elevated plus maze test, the CRF transgenic mice spend more time in the closed arms of the apparatus, evincing a heightened state of anxiety. These studies indicate that supranormal production of CRF in mice alters behavior and physiology in a manner very similar to that observed for stress.

Molecular biology studies have also produced "knockout" mice which have null mutations for CRF, as well as the CRF_1 and CRF_2 receptor subtypes. CRF-deficient mice have been studied at length.[86−88] Plasma levels of ACTH and corticosterone in homozygous CRF-deficient mice are approximately 25% of those levels in control animals. This result demonstrates the primacy of CRF in HPA axis regulation, but it may also indicate that CRF antagonists will not shut down the HPA axis completely. The zona fasciculata, the region of the adrenal gland responsible for corticosterone production, has reduced size and function in these mutant mice. The effect of ether and restraint stress on plasma corticosterone levels is blunted in mutant mice null for the CRF gene. Offspring of CRF-null mice require exogenous corticosterone for normal lung maturation during fetal development, but after birth, these animals are normal except for the physiological and behavioral effects noted above. CRF_1 receptor knockout mice differ significantly from normal mice in their physiological and behavioral responses to stress.[89] Pituitary cells from homozygous mutant mice null for the CRF_1 receptor do not increase ACTH secretions in the presence of increasing concentrations of CRF in vitro. Furthermore, cAMP accumulation in these cells is reduced relative to cells from control animals. Plasma corticosterone levels in these mutant mice also are depressed relative to those from control animals, and the normal diurnal variation of these levels was absent in the null mice. This effect appears to be due to a reduction in size of the zona fasciculata. Restraint stress of these mutant mice fails to increase plasma ACTH levels, and its effect on plasma corticosterone levels is blunted. Nonetheless, basal secretion of ACTH and corticotrope development is normal for the

CRF_1-null mice. Loss of the CRF_1 receptor is accompanied by increased CRF expression in the paraventricular nucleus of the hypothalamus without alterations in the expression of CRF_2 receptors or arginine−vasopressin, a minor co-regulator of the HPA axis. Finally, CRF_1-deficient mice display reduced anxiety in rodent behavioral tests. These data support a leading role for CRF_1 receptors in anxiety and the development of an operant HPA axis. CRF_2 receptor-deficient mice have also been reported recently.[90] Feeding and behavioral studies with these mice suggest a possible role for this receptor subtype in regulation of appetite and anxiety.

C. Effects of Exogenous CRF Adminstration. Preclinical studies have also shown that CRF has many functions in the CNS and the periphery.[5,71,91,92] Differential analysis of the central and peripheral effects of CRF is complicated by the fact that there are two receptor subtypes, one binding protein, and possibly two endogenous ligands (e.g. CRF and urocortin), which can modulate these effects in concert and most of which are present in the CNS and the periphery. Most investigators have used icv administration of CRF to probe for the central effects of this neuropeptide, but in some instances, intracisternal and direct tissue injections (e.g. amygdala and PVN) have been employed. Leakage of peptides from the ventricles into the general circulation has been suspected in some studies.[71] Transit from the site of injection to the putative sites of action is uncertain in other instances.[64,71] There are limited examples where the effects of iv and icv administration of CRF have been studied in parallel, and the short half-life of CRF in plasma is a confounding variable in these studies.[71] The effects of CRF on the autonomic nervous system have been documented (vide infra), adding another layer of complexity to the study of central and peripheral effects of CRF. There are multiple feedback mechanisms controlling CRF synthesis and secretion (vide supra). The multiple functions are probably integrated by the site-specific control of CRF gene expression and secretion (vide supra).[66−68] In the context of these complexities, the effects of exogenous CRF are summarized below.

The effects of icv adminstration of CRF on peripheral functions have been studied extensively.[93−99] CRF-containing neurons in the brain mediate a range of autonomic responses. In rats, CRF administered via icv injection activates the sympathetic nervous system, elevates concentrations of glucose and catecholamines in plasma, and increases heart rate and blood pressure. CRF (icv) also decreases food intake and gastric acid secretion. In dogs, icv administration of CRF activates the sympathetic nervous system, raises plasma vasopressin, adrenaline, and noradrenaline concentrations, inhibits gastric emptying, and increases mean arterial pressure and heart rate. In contrast, iv administration of CRF in dogs decreases mean arterial pressure but increases heart rate.

CRF plays a significant role in the regulation of energy balance.[100] This neuropeptide and its congener, urocortin, have thermogenic and anorectic effects after icv administration in rodents. CRF exerts its effects on energy regulation in concert with other factors, most notably neuropeptide Y.[100] There is early evidence of feedback mechanisms. Exogenous leptin reduces CRF expression in the paraventricular nucleus of leptin-deficient obese rats. Leptin increases the expression of $CRF_{2\alpha}$ receptors in the normal rat ventromedial hypothalamus, a brain region involved in insulin secretion and the regulation of energy stores.

D. CRF and Reproduction. CRF is an integral component of reproductive physiology. CRF and its receptors have been localized in reproductive tissues: testes, ovary, and placenta.[101−104] The testes have been the most extensively studied to date.[101] Leydig cells secrete CRF, which in turn activate testicular CRF receptors, which are functionally distinct from their central counterparts. Leydig cell CRF receptors are not coupled to G_s or G_i but rather are coupled to a pertussis toxin-insensitive G protein, which appears to be functionally linked to phospholipase C. CRF is a potent negative regulator of luteinizing hormone (LH) effects in Leydig cells. LH stimulates directly the secretion of serotonin, which acts on $5HT_2$ receptors to stimulate the secretion of CRF. This inhibitory loop serves to attenuate androgen production by gonadotropin. CRF in Leydig cells also stimulates β-endorphin secretion in these cells, which in turn inhibits follicle-stimulating hormone (FSH) in the testes through receptors in Sertoli cells. The downstream effects of CRF are reduced steroidogenesis and spermatogenesis. Furthermore, restraint stress in nonhuman primates leads to sperm damage.[105] Preliminary data indicate a possible role for CRF in other reproductive processes. Recently, administration of CRF_1 antagonists in fetal sheep has been shown to delay the onset of parturition.[106]

E. CRF and the Immune System. CRF is also a key player in several immune system functions both in vitro and in vivo.[107] CRF suppresses the rat immune system through indirect effects on glucocorticoid plasma levels and on the sympathetic nervous system after icv injection.[108] Glucocorticoids and catecholamines have been shown to alter immune function via control of key regulatory cytokines such as IL-4, IL-10, IL-12, and IFN-α and of T-helper cell subtype expression.[109−113] Conversely, the cytokines TNFα, IL-1, and IL-6 stimulate CRF and vasopression secretion in the hypothalamus. However, in the periphery, CRF is proinflammatory, where it stimulates the secretion of several cytokines.[107] CRF and its receptors have been localized in immune tissues and sites of inflammation, such as leukocytes, mast cells, macrophages, and synovial fluid.[107,114−121] CRF antagonists, which act selectively at peripheral sites, may therefore be effective antiinflammatory agents.

Clinical Investigations

A. Depression. One of the first disease states, in which the role of CRF was examined, was depression. Numerous studies have been performed in depressed patients to analyze the levels of CRF in cerebrospinal fluid (CSF) and plasma as well as the levels of ACTH and cortisol in plasma. These studies have been reviewed at length[122,123] and will only be summarized here. As noted above, early studies established that many patients suffering from major depressive disorder have elevated concentrations of cortisol in their plasma relative to those from normal volunteers.[124−127] How-

ever, it was discovered subsequently that there are subtypes of depression for which there is no hypercortisolemia.[128] Current estimates are that approximately 50% of depressed patients exhibit elevated plasma cortisol levels.[128,129] In these individuals, plasma glucocorticoid levels and ACTH concentrations are not suppressed by administration of dexamethasone (a synthetic glucocorticoid). Furthermore, there is corresponding increase in CRF levels in CSF.[130,131] Intravenous administration of CRF to patients suffering from major depression results in a blunted effect on ACTH plasma concentrations but a full effect on cortisol levels.[128,131,132] In contrast, normal patients exhibit full responses for both ACTH and cortisol: the plasma levels of both are elevated. These studies clearly illustrate a role for CRF in depression at the level of the hypothalamus or higher centers in the brain. In depressed patients, it has been proposed that chronic CRF hypersecretion and the resulting down-regulation of CRF receptors contribute to the blunted plasma ACTH response.[128] A 4-fold increase in CRF mRNA expression in the paraventricular nucleus (PVN) of the hypothalamus has been observed in postmortem studies on depressed patients, who received antidepressant medication for at least 1 month prior to their deaths.[133] Limited clinical investigations have shown that treatment with tricyclic antidepressant drugs reduce plasma ACTH and cortisol levels to normal values.[134] Other studies suggest a positive correlation between reduction of CRF concentrations in the CSF and reduction in the severity of symptoms with treatment for depression. Administration of fluoxetine, a selective serotonin reuptake inhibitor, to patients with major depressive disorder leads to significant decreases in the concentrations of CRF in the CSF in most instances, which is accompanied by a corresponding amelioration of depressive symptoms.[135] Electroconvulsive shock therapy (ECST) has been reported to induce a decline in urinary free cortisol and a parallel decrease in CSF concentrations of CRF in one female patient after therapy for 16 days.[136] A separate study was performed on nine patients, suffering from major depressive disorder with psychotic features, to measure CRF levels in CSF before and after ECST. These patients were not compromised by other illnesses, cognitive decline, or medication, and they tolerated well a lumbar puncture procedure before and after ECST. Analysis of CSF levels revealed a statistically significant decline in the levels of CRF.[137] Furthermore, there was a positive trend toward improvement in symptoms in the above studies. The above data provide additional support for the hypothesis that CRF is hypersecreted in major depression, but clinical trials with a CRF antagonist are needed to prove this point.

B. Other Neuropsychiatric Disorders. Suggestive data for a role of CRF in other neuropsychiatric disorders have recently been reported. CSF levels of CRF are elevated in patients suffering from anorexia nervosa,[138] obsessive−compulsive disorder,[139] posttraumatic disorder,[140] and alcohol withdrawal,[141] but not panic disorder.[142] In anorexia nervosa, the alterations in CRF concentrations in CSF disappear with weight normalization, but there is a definite correlation between depression scores of these patients and the CSF levels of CRF. Elevated concentrations of CRF in the CSF have also been reported for withdrawing human alcoholics. In panic disorder, a blunted effect of intravenously administered CRF on ACTH levels has been reported, suggesting that pulsatile hypersecretion of CRF may occur in this condition. Studies on degenerative dementia reveal a substantial reduction (∼50%) of cortical and CSF levels of CRF with a corresponding increase in cortical receptor expression.[143] Furthermore, cognitive decline has been correlated with diminished CRF levels. Regiospecific release of CRF might improve cognitive function, and this hypothesis is a basis for the search for CRF binding protein−ligand complex inhibitors (vide infra).[122,143]

C. Immune Diseases. Clinical investigations have supported the proposition that certain immune disorders may involve CRF. Immunohistochemical studies have detected elevated levels of CRF in the joints of patients with rheumatoid arthritis[120] and in the thyroid glands of people suffering from Hashimoto's thyroiditis.[121] Therefore, selective antagonists of peripheral CRF function may provide opportunities for immunotherapy based on these data and the preclinical studies described earlier.

Modulators of CRF Receptor Function

The data and conclusions from the preceding discussion have motivated several researchers to investigate the structure−activity relationships for peptidic and nonpeptidic modulators of CRF function in the hope of designing new therapeutic agents. The published studies will be summarized in this section.

A. Peptidic Ligands. Several reports on modification of CRF have been reported over the years.[144−149] Single amino acid modification studies identified the key regions of the peptide for agonist/antagonist potency as well as receptor binding affinity and provided the basis for assigning its secondary structure. The assignment of the α-helical structure to CRF-related peptides as the preferred conformation in solution was a critical insight, which serves as the foundation for the design of more potent peptide agonists and antagonists.[149] α-Helical CRF_{9-41} was the first peptide antagonist to be widely used in pharmacological and behavioral studies, which helped to define the functions of CRF in stress responses.[18,150,151]

The design of conformationally restricted peptides has led to the discovery of a potent CRF antagonist, astressin. The lactam astressin (cyclo(30−33)-[D-Phe^{12},$Nle^{21,38}$,Glu^{30},Lys^{33}]r/h-CRF_{12-41}) has high affinity for h-CRF_1 receptors (mean K_i = 2 nM) but very low affinity for the CRF-BP.[152] Astressin blocks secretion of ACTH stimulated by r/h-CRF from rat anterior pituitary cells at low concentrations (mean IC_{50} = 1.0 nM vs 374 nM for α-helical CRF_{9-41}). Recently, [^{125}I-Tyr^1]astressin and [^{125}I-Tyr^0]rat urocortin have been characterized as radioligands for cloned human CRF_1, mouse $CRF_{2\alpha}$, and rat $CRF_{2\beta}$ receptors as well as native rat cerebellar and brainstem receptors. The specific binding of the radiolabeled version of astressin is very high and insensitive to guanyl nucleotides, making it an excellent radioligand.[153] Intravenous administration of astressin (30 or 100 μg/kg) decreases ACTH secretion 2-fold in rats after adrenalectomy. Electric shock-

induced ACTH secretion in male rats is also blocked by this peptide. Astressin (12 μg/kg, iv) antagonizes the gastric stasis induced in rats by CRF and urocortin and completely blocks abdominal surgery-induced inhibition of gastric emptying, when it is administered 3 h after surgery.[154] Astressin has no effect on basal gastric emptying, and its efficacy in the gastric model is superior to those of two nonpeptide CRF_1 antagonists (antalarmin and NBI-27914, vide infra). No pharmacokinetic data was presented in this report to confirm exposure.

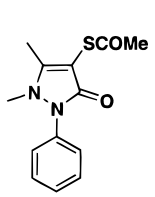
Recent progress has been reported on the design of CRF_2-selective peptidic antagonists.[155] Four sauvagine (Svg) derivatives, [D-Phe^{11},His^{12}]Svg$_{11-40}$ (antisauvagine-30), [D-Leu^{11}]Svg$_{11-40}$, [D-Phe^{11}]Svg$_{11-40}$, and Svg$_{11-40}$, show selective binding affinity for mouse $CRF_{2\beta}$ receptors over rat CRF_1 receptors, each expressed in HEK293 cell homogenates (110-, 80-, 68-, and 54-fold selectivities, respectively). Antisauvagine-30 blocks Svg-stimulated adenylate cyclase activity albeit at a high concentration (1 μM).[155] The 11- and 12-positions of sauvagine and the corresponding positions in related peptides appear to be critical for receptor subtype binding selectivity. The new peptide tools have been useful for demonstrating different roles for CRF_1 and CRF_2 receptors in learning and memory. Rat/human CRF injection in the dorsal hippocampus enhances learning and memory via CRF_1 receptors, but injection in the lateral septum inhibits learning and memory through CRF_2 sites.[156] Direct injection of astressin, a nonselective CRF antagonist, blocks the effects of direct injection of CRF in the dorsal hippocampus on memory and learning in mice, while similar injection of antisauvagine-30 does not.[156] However, injection of antisauvagine-30 blocks the effects of CRF injection into the lateral intermediate septum.

Currently, there are only very limited data for comparison of peptidic and nonpeptidic CRF ligands in animal models of efficacy. Conclusions about the relative roles of peptidic and nonpeptidic ligands in therapy are therefore premature. Additional explorations of the therapeutic utilities of peptidic versus nonpeptidic CRF ligands are necessary. These studies should define and optimize pharmacokinetic parameters to ensure comparable receptor occupancy by nonpeptidic and peptidic ligands in the comparative experiments.

B. Nonpeptidic Ligands. The extensive work on peptidic ligands described above has not yet directly contributed to the discovery of nonpeptidic ligands to date. This may be a result of the reported different binding domains for peptidic and nonpeptidic ligands (vide infra). Future studies may reveal that peptidic and nonpeptidic ligands have complementary roles in modulating CRF function.

The search for selective nonpeptidic antagonists of CRF receptors began at several pharmaceutical companies with empirical screening efforts to discover leads. Most published reports describe only nonpeptidic antagonists for the CRF_1 receptor. There is one published report on combined CRF_1/CRF_2 receptor ligands (vide infra) and an anecdotal report suggesting that CRF_2-selective agents may have been found.[157] The bulk of the structure−activity relationship (SAR) data on nonpeptidic CRF_1 antagonists still lies in the proprietary archives of pharmaceutical companies. However, recent publications permit a preliminary analysis of the CRF_1 receptor antagonist SAR.

B.1. Pyrazolones. The first patent publication on nonpeptidic CRF receptor antagonists[158] described 4-substituted-thiopyrazolone **1** and its disulfide counterpart **2**. These compounds weakly inhibit the binding of [^{125}I-Tyr^0]o-CRF to rat cerebral cortex membrane preparations (IC_{50} > 1 μM) and antagonize CRF-stimulated adenylate cyclase activity in these same membrane homogenates (IC_{50} ≥ 1 μM). Disulfide **2** is the most potent antagonist in the latter assay (binding IC_{50} = 3.3 μM (n = 3) and cyclase IC_{50} = 1.0 μM (n = 2)). However, the preferred compound **1** had weaker affinity and antagonist potency (binding IC_{50} = 13.7 μM (n = 3) and cyclase IC_{50} = 2.9 μM (n = 2)). No subsequent reports have appeared on these compounds from the group at Nova Pharmaceuticals, although the structural motif of an aryl-substituted five-membered heterocycle would appear in subsequently discovered CRF antagonist chemical series.



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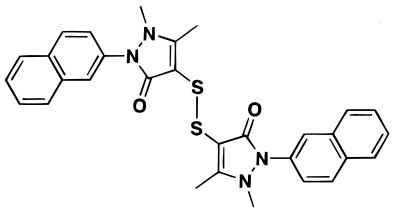

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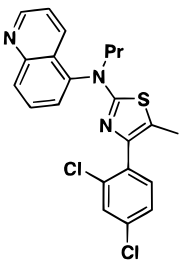
B.2. Thiazoles. The next published CRF receptor antagonist series was 2-(substituted-amino)-5-(aryl or heteroayl)thiazoles such as compounds **3** and **4**.[159−163] Several examples were disclosed by researchers at Sanofi, 50 of which were listed as preferred analogues, in a series of patents. No biological data have been published on these compounds to date. The vast majority of the preferred examples contain 2,4-disubstituted- or 2,4,6-trisubstituted-phenyl moieties, where halo, alkyl, and alkoxy groups predominate in the list of cited substituents but a wide variety of substituted-amino groups are attached to the 2-position of the thiazole ring. Recently, **3** (SR95577A) was presented as an example of this series (binding IC_{50} = 80 nM, species not reported).[164−165]



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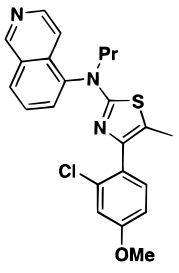

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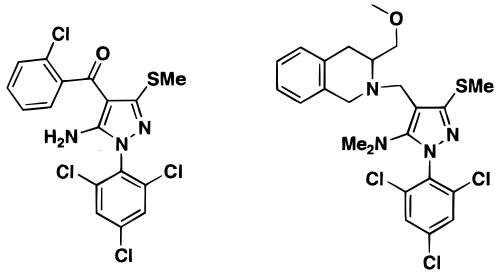
B.3. Pyrazoles. Additional variants on the five-membered heterocyclic cores presented above were reported in four patent applications.[166−169] Starting from an empirical lead **5**,[170] researchers at Pfizer prepared a variety of substituted-pyrazole derivatives, such as analogue **6**, which were claimed to possess CRF antagonist activity. No biological data were presented however for these particular compounds. Researchers at Neuro-

gen reported CRF_1 antagonist structures represented by example **7**, which are regioisomeric to the above Pfizer series. No biological data were presented however.[171]



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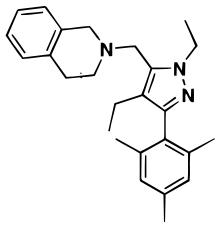

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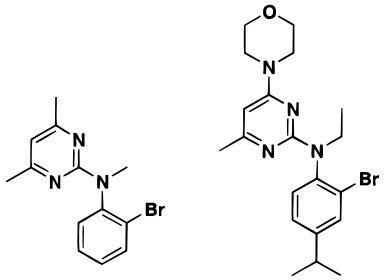
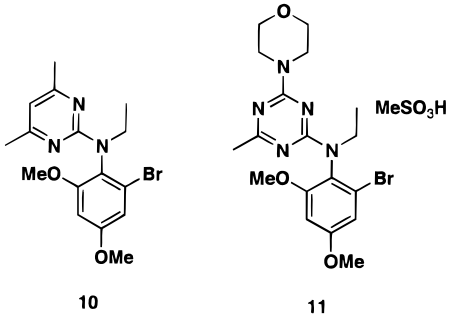
B.4. Anilinopyrimidines and -triazines. Researchers at DuPont discovered 2-anilinopyrimidines and -triazines as CRF receptor antagonists.[172−175] Screening of their chemical library using a rat CRF receptor assay identified 2-anilinopyrimidine **8** as a weak ligand for rat cortical receptors when compared to the peptide antagonist α-helical CRF_{9-41} (K_i = 5700 nM vs 1 nM). Furthermore, analogue **8** weakly blocks CRF-stimulated adenylate cyclase activity in the same tissue homogenate, but it is less potent than α-helical CRF_{9-41} (IC_{50} = 20000 nM vs 250 nM). Systematic SAR studies, using a cloned human CRF_1 receptor assay, defined the structural requirements for optimal binding affinity of the 2-anilinopyrimidines and related 2-anilinotriazines. For the phenyl group, medium-sized lipophilic moieties at the ortho-position, preferably bromine, iodine, or a methylthio group, enhance h-CRF_1 binding affinity. Medium-sized lipophilic groups at the para-position of the phenyl ring, which may or may not form weak hydrogen bonds, contribute to good binding affinity (e.g. isopropyl, methoxy, dimethylamino, or acetyl groups). Small hydrocarbon chains on the central nitrogen are optimal for h-CRF_1 binding affinity. A methyl group is the optimal substituent at the 6-position of the pyrimidine or triazine cores. In contrast, a wide variety of substituents at the 4-position of the heteroaryl rings is tolerated (e.g. example **9**, mean h-CRF_1 K_i = 63 nM). Other groups have reported analogous anilinopyrimidines, and the very limited SAR data provided is consistent with the above summary.[176,177] Anilinopyrimidine **10** (XQ771) and anilinotriazine **11** (SA627) are silent h-CRF_1 antagonists.[178] Rat pharmacokinetic studies were performed on compounds **9**−**11**. Administration of compound **9** by multiple routes (iv, ip, po) affords very low rat plasma levels. For analogue **10** (mean h-CRF_1 K_i = 12 nM), good plasma levels are observed at 30 mg/kg (iv, ip, and po) (mean C_{max} = 1884 nM (ip) and 366 nM (po)), but bioavailability is strongly dependent on the route of administration (26% (ip) vs 4% (po)). Triazine **11** (mean h-CRF_1 K_i = 32 nM) affords good rat plasma levels at 12 mg/kg (iv) and 30 mg/kg (ip, po) (C_{max} = 2698 nM (ip) and 753 nM (po)). Different bioavailabilities were calculated for the two routes of administration, reflecting lower oral absorption (40% (ip) vs 3% (po)). In the dog, compound **11** has a superior pharmacokinetic profile at 5 mg/kg (iv, po) than that observed in the rat: oral biovailability = 20%, C_{max} = 730 nM (po), and $t_{1/2}$ = 6 h (iv). Thus, systematic SAR studies significantly improved receptor binding affinity and pharmacokinetic profiles relative to the earlier leads.



8 **9**



10 **11**

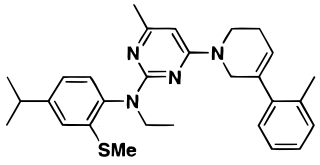
Subsequent SAR studies on anilinopyrimidines led to the discovery that tetrahydropyridinyl analogues have high rat receptor binding affinity and good rodent behavioral efficacy.[179−182] Compounds **12** (CRA0165, IC_{50} = 10 nM), **13** (CRA1000, IC_{50} = 12.7 nM), and **14** (CRA1001, IC_{50} = 22.1 nM) have 4-tetrahydropyridinyl substituents projecting into a region of space, which has previously been shown to tolerate groups with diverse topologies.[172−175] In a CRF-induced anxiety model in rats, the minimal effective doses for **13** and **14** were 0.3 and 1.0 mg/kg (po) for reducing the effects of CRF (1 μg/10 μL, icv) on time spent in the open arms of an elevated plus maze.

B.5. Bicyclic Antagonists. Investigations into the preferred conformers of the 2-anilinopyrimidines and -triazines for h-CRF_1 receptor binding led to the design of more potent h-CRF_1 antagonists with superior pharmacokinetic profiles.[183,184] Variable-temperature NMR studies established that there is restricted rotation about the two bonds connecting the central nitrogen to the heteroaryl and phenyl moieties in the anilinopyrimidines and -triazines. SAR studies had already established that an ortho substituent on the phenyl ring is necessary for optimal binding affinity (vide supra). It was subsequently proposed that this substituent enforces a twisted relationship between the phenyl and heteroaryl rings. X-ray crystallographic studies on **9** and

Table 4. Summary[a] of Pharmacokinetic Parameters for **18** (SC241)[178]

species	dose (mg/kg, route)	C_{max} (nM, po)	T_{max} (h, po)	%F (po)	CL (L/h/kg)	$t_{1/2}$ (h)	$V_{d,ss}$ (L/kg)
rat	1 (iv)				3.0	7.6	
	1 (po)	30		17			
dog	1 (iv)				0.478 (±0.078)	33.2 (±9.7)	15.55 (±3.90)
	1 (po)	307.6 (±131.6)	0.67 (±0.026)	30.7			

[a] See original reference for statistical analyses and experimental conditions. Parenthetical values are the standard deviations for those few instances reported in the original reference. $V_{d,ss}$ = volume of distribution at steady state; CL = clearance; %F = bioavailability; C_{max} = maximal plasma level; T_{max} = time for C_{max}; iv vehicle = EtOH:propylene glycol:PEG400:H_2O = 10:30:30:30; oral vehicle = 0.5% methocel.



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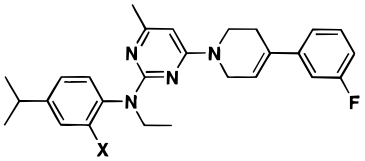
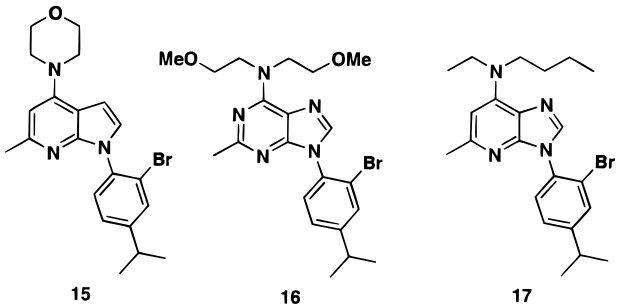
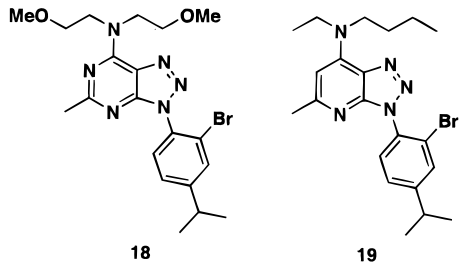


13 X = SMe
14 X = Br

10 established this spatial orientation to be the case for the crystalline conformations. Moreover, the N−C bond for the substituent on the central nitrogen is roughly coplanar with the heteroaryl group in the X-ray structures and in molecular models. While bound conformations may differ substantially from their solution or crystalline counterparts in a variety of biological systems, the latter conformations in this series were very useful for designing high-affinity pyrrolopyridine CRF antagonists, exemplified by analogue **15**. Compound **15** has good affinity for rat cortical CRF receptors (rat mean K_i = 28 nM) but a modest rat pharmacokinetic profile. These studies established clearly a preferred binding mode for the unsymmetrically substituted pyrimidine **9** and triazine **11**.



15 **16** **17**

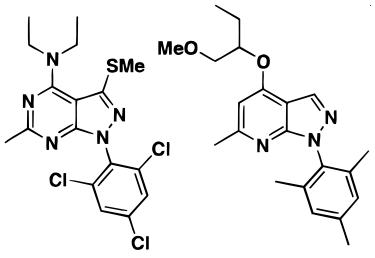


18 **19**

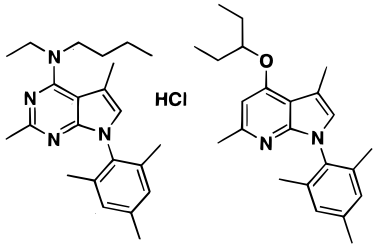
Efforts to optimize the pharmacokinetic profiles of pyrrolopyridines **15** led to the discovery of imidazopyrimidines/pyridines (e.g. **16** and **17**) and triazolopyrimidines/pyridines (**18** and **19**).[185,186] Initial investigations of the SAR in these series were on systems with the 2-bromo-4-isopropylphenyl substituent on the triazole or imidazole ring, since this moiety enhances h-CRF_1 receptor binding affinity in the monocyclic series (vide supra). As in the case of the anilinopyrimidines and -triazines,[175] there is a need for at least one ortho substituent on the phenyl ring for high binding affinity, conferring a twisted orientation between the planes of the phenyl and bicyclic ring systems.[186] The para-position on the phenyl ring favors the presence of an alkyl substituent as long as it does not exceed certain steric requirements as found for the earlier series. Acyclic amino groups on the bicyclic core are found to be superior to their cyclic counterparts in enhancing receptor binding affinity for h-CRF_1 receptors. Furthermore, unsymmetrical acyclic amines were superior to symmetrical ones in the receptor binding assay. Bis-(methoxyethyl)amino compound **18** (SC241) possesses high receptor binding affinity (mean h-CRF_1 K_i = 3.7 nM) and good pharmacokinetic profiles in the rat and dog (Table 4).[186] This compound has good oral bioavailability in the rat; moreover, the plasma concentrations persisted above the K_i level of the compound for 16 h. The steady-state brain-to-plasma ratios were calculated to be 0.6 in the rat, using data from continuous iv infusion studies. Analogue **18** was subsequently administered to dogs at 1 mg/kg (iv, po); the data are summarized in Table 4. Thus, systematic SAR studies, buttressed by molecular modeling and, in a few instances, NMR and X-ray crystallographic experiments, defined a common pharmacophore for CRF_1 receptor antagonists and led to orally bioavailable agents.

Fused derivatives of pyrazole **6** and related pyrroles were also explored by Pfizer researchers.[170,187−191] Pyrazolopyrimidine **20**, pyrazolopyridine **21**, pyrrolopyrimidine **22**, and pyrrolopyridine **23** have been reported to possess CRF antagonist activity using a rat receptor assay employing cortical homogenates. However, no specific biological data were provided in these published patent applications. The vast majority of the preferred examples contain 2,4-disubstituted- or 2,4,6-trisubstituted-phenyl moieties, where halo, alkyl, and alkoxy groups predominate in the list of cited substituents. Substituted amino or substituted alkoxy groups are most frequently cited as substituents on the bicyclic core. Subsequently, several publications on compound **22** (CP154526-1) have appeared. This compound has high receptor binding affinity (mean K_i = 2.7 nM) for human CRF_1 receptors in IMR32 neuroblastoma cells,

and it is effective in some rodent models for anxiety and depression (vide infra).

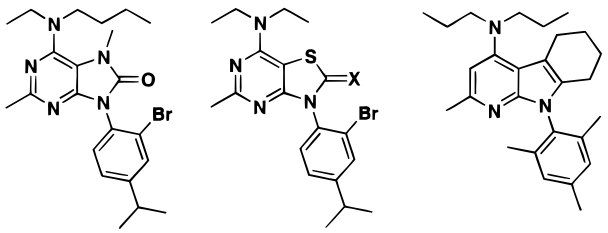


20 **21**

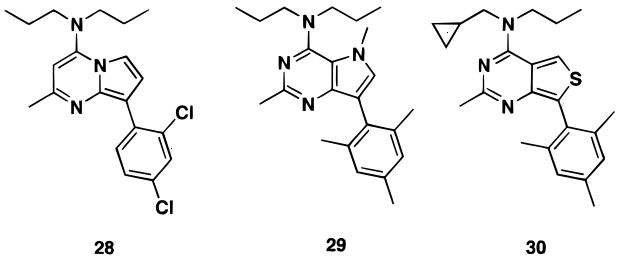


22 **23**

Several variants on the bicyclic structural motif have been reported in the patent and general literature. Purinone[192,193] **24** (h-CRF_1 K_i = 5.0 nM) and thiazolo[4,5-*d*]pyrimidinethiones and -ones[189,190] **25** and **26** (h-CRF_1 K_i = 4.1 and 9.4 nM, respectively) have been described. Fused pyrrolopyridines (e.g. **27**),[191,192] pyrrolopyrimidines (e.g. **28** and **29**),[190,196] thienopyrimidines (e.g. **30** and **31**),[192,193] and imidazotriazines (e.g. **32**)[199]

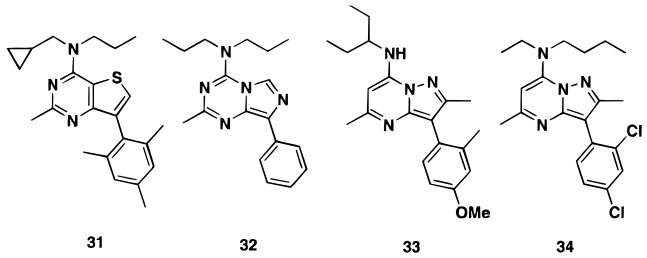


24 **25 X = S** **26 X = O** **27**

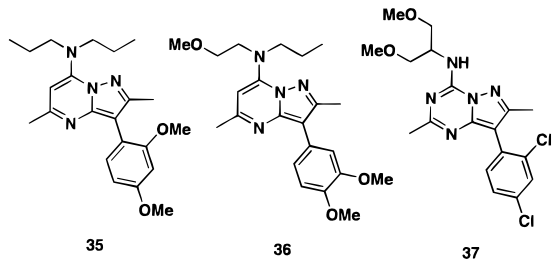


28 **29** **30**

have been disclosed as CRF antagonists without data. Pyrazolo[1,5-*a*]pyrimidines **33** (DMP904), **34** (PD171729), **35** (NBI 30545), and **36**[199−206] and pyrazolo[1,5-*a*]triazine **37** (DMP696)[200,207,208] are potent selective CRF_1 antagonists (mean h-CRF_1 K_i = 1.0, 5.0, 2.8,[202] 13.7,[209] and 1.8 nM, respectively). They are potent antagonists in CRF-stimulated adenylate cyclase assays, and they have good efficacy in rodent behavioral models, suggesting anxiolytic or antidepressant activity (vide infra). Although the core structures may differ for the h-CRF_1 antagonists described above, the peripheral substituents occupy essentially the same regions of space as the previous series.[210]

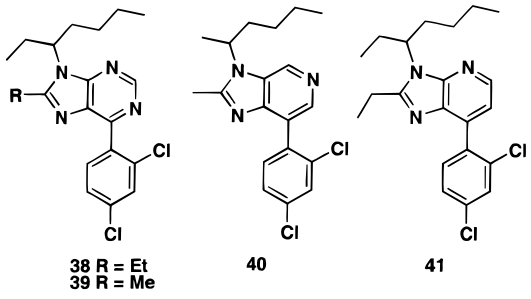


31 **32** **33** **34**

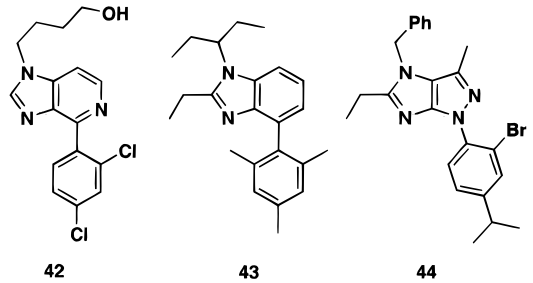


35 **36** **37**

Interposition or replacement of the six- and five-membered rings in the above structures leads to alternative CRF_1 antagonist chemical series. Arylpurines **38** and **39** (NBI 21224, rat CRF K_i = 8.8 nM) have been disclosed as CRF antagonists with limited data.[198,211] By extension, imidazopyridines **40**−**42**[197,211] and benzimidazole **43**[212] have been reported to be CRF antagonists without data. Several 6,6-fused bicyclic core series have been reported to be CRF antagonists in the patent literature without supporting biological data.[213−215] Recently, compounds from a 5,5-fused bicyclic series have been reported to be CRF_1 antagonists.[216,217] Imidazo[4,5-*c*]pyrazole **44** has high binding affinity for the h-CRF_1 receptor (mean K_i = 4 nM).



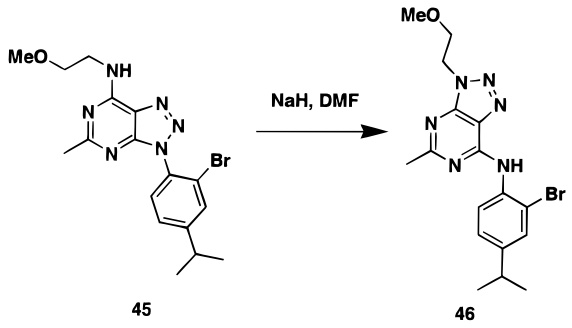
38 R = Et **39 R = Me** **40** **41**



42 **43** **44**

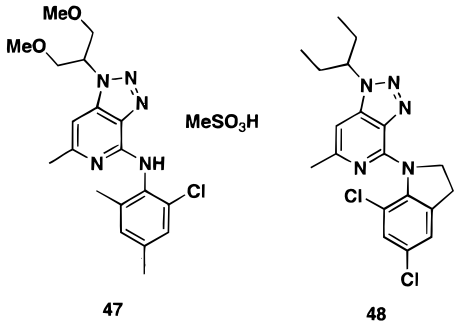
A novel molecular rearrangement (Scheme 1) of triazolopyrimidines related to compound **15** led to the discovery of regioisomeric CRF_1 antagonists (e.g. **46**) and the corresponding triazolopyridine **47** (DMP695)[218−223]

Scheme 1



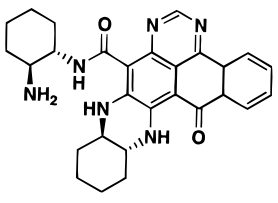
45 46

which have high affinity for h-CRF_1 receptors. Compound **47** (DMP695) is a potent antagonist in vitro and has an excellent pharmacokinetic profile in dogs.[221] This rearranged triazolopyridine has high affinity for h-CRF_1 receptors (K_i = 3.3. nM), and it potently inhibits CRF-mediated increases in cAMP levels using either HEK293 cells expressing h-CRF_1 receptors (IC_{50} = 75 nM vs 100 nM o-CRF) or rat cortical homogenates (IC_{50} = 79 nM vs 100 nM o-CRF). Administration of **47** (1 mg/kg, iv and po) to dogs generates high plasma levels of moderate duration and good oral bioavailability (59%). Conformationally restricted analogues of **47** have been recently reported; example **48** is a high affinity CRF antagonist (h-CRF_1 K_i = 1.3 nM).[224,225]



47 48

B.6. Perimidines. Recently, compound **49** has been reported to have affinity for both CRF_1 and CRF_2 receptors.[226,227] This oxo-7*H*-benzo[*e*]perimidine-4-carboxamide binds both subtypes with moderate affinity (rat CRF_1 K_i = 110 nM, mouse $CRF_{2\beta}$ K_i = 20 nM), and it moderately inhibits CRF-mediated adenylate cyclase activity. This combined antagonist also inhibited CRF-stimulated ACTH release in rats (50% of control value at 10 nmol (iv) vs 0.05 nmol o-CRF (iv)). This polycycle is a marked departure from the structures previously reported as CRF antagonists.



49

B.7. Structure−Activity Summary. The combined SAR for CRF_1 antagonist studies, which were described above, may be summarized as depicted in Figure 3. One basic nitrogen (preferably sp^2-hybridized) is essential

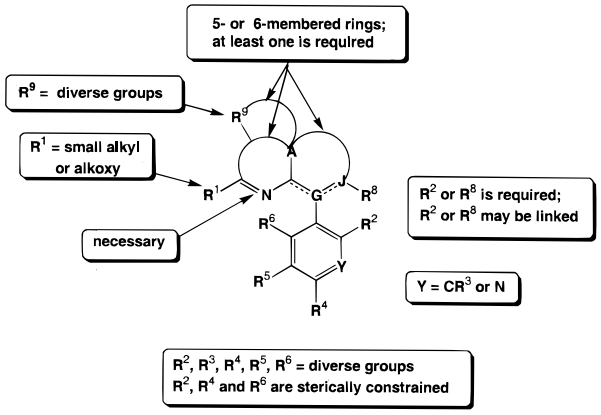
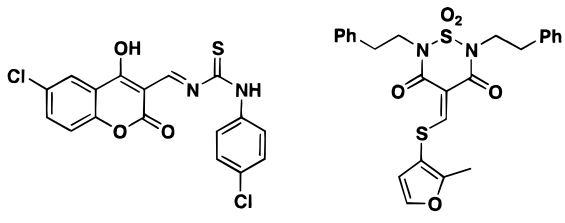


Figure 3. CRF_1 antagonist SAR.

for receptor binding affinity. This nitrogen may be included in a ring, provided a critical hydrogen-bonding interaction between the nitrogen and the receptor is maintained. A twisted conformation between this nitrogen-containing ring and a required aryl/heteroaryl ring containing Y is optimal for receptor binding affinity. Flanking "ortho" substituents on either ring enforce this active conformation. The conformation of these structures may be restricted further by a tether between A and a linker atom J or between R^9 and A. The tethers with their associated atoms may form five- to six-membered rings. At least one of the three possible rings is required for receptor binding affinity provided the peripheral groups are held in the correct spatial orientation. Again the aryl/heteroaryl ring containing Y is preferably not coplanar with the other ring systems. The linker atoms G or J may be carbon or nitrogen based on overlap of the thiazole, pyrazole, and triazolopyrimidine structures **3**, **7**, and **18** described above. Structurally diverse R^9 groups are well-tolerated for receptor binding affinity, while small R^1 groups are required. When J is not part of a ring, J-R^8 may represent a small alkyl group, a small alkenyl group, or hydrogen. R^2 and R^8 may also be linked to each other in some structures.

B.8. CRF-BP-Ligand Complex Inhibitors. Patent applications have recently been published describing small-molecule inhibitors of the CRF-BP−ligand complex.[228,229] Structure **50** is also reported to have moderate efficacy at 1 mg/kg (po) in the rat Morris swim maze test, while compound **51** has been reported without data.



50 51

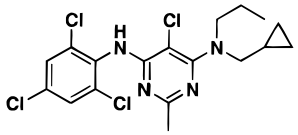
Nonpeptidic CRF_1 Antagonist Pharmacology

The synthesis and characterization of a number of small-molecule CRF_1 antagonists (vide supra) have provided useful tools for the characterization of CRF_1 receptor ligand domains. The small-molecule CRF_1 antagonists are highly specific ligands and have negli-

gible activities at alternate receptors or other potential sites of interaction. Several lines of evidence suggest that these small molecules interact with the CRF_1 receptor in a region other than that which recognizes the peptide agonists. Chimera and mutagenicity studies have suggested that the peptide agonists and the α-helical CRF_{9-41} antagonist bind to the N-terminal extracellular domain and the fourth extracellular cytoplasmic loop of the receptor and that the small-molecule antagonists bind in a pocket of the receptor deep within the plasma membrane.[230−235]

Another piece of evidence for differential sites for peptide and small-molecule ligands is the observation of nonreciprocal binding inhibition for peptide CRF_1 agonists and small-molecule antagonists. While small-molecule CRF antagonists inhibit the binding of peptide agonists to the receptor, the peptide agonists (and the peptide antagonist α-helical CRF_{9-41}) have no effect on the binding of small-molecule antagonists.[232,236] The small molecule **18** (SC241) displaces [^{125}I-Tyr^0]o-CRF, but o-CRF does not affect the binding of [^{3}H]**18** to the receptor. Further studies have shown that **18** increases the off-rate of [^{125}I-Tyr^0]o-CRF from the receptor suggesting that **18** decreases the binding of the peptide to the receptor through an allosteric mechanism.

Compound **52** (NBI 27914) is a CRF_1-selective brain-penetrant ligand.[237] It has high affinity for CRF_1 receptors (K_i = 2 nM) but very low affinity for CRF_2 sites (K_i > 10 000 nM). This ligand inhibits CRF-mediated increases in adenylate cyclase activity and ACTH release from rat anterior pituitary cells (EC_{50} = 150 and 70 nM, respectively). Systemic dosing of **50** affords high CRF_1 receptor occupancy in rats. This compound competes with [^{125}I-Tyr^0]sauvagine for CRF_1 receptors in ex vivo binding studies using brain slices, demonstrating brain penetration for the compound.

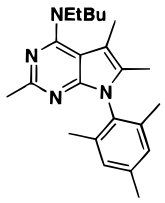


52

The preclinical pharmacology of nonpeptidic modulators of CRF function has buttressed the hypotheses concerning the therapeutic utilities of these agents. Specifically, the evaluation of nonpeptidic CRF_1 antagonists in animal behavioral models has provided a large body of data supporting the use of CRF_1 antagonists in the treatment of neuropsychiatric disorders, especially anxiety and depression.

Pyrrolopyrimidine **22** (CP154526-1) is a potent h-CRF_1 antagonist, which has been reported to be effective in rodent models for anxiety and depression.[238−240] This compound has high affinity for h-CRF_1 receptors (K_i = 2.7 nM, human IMR32 neuroblastoma cells), and it antagonizes stimulation of adenylate cyclase activity by 100 nM o-CRF. No effects on forskolin-stimulated or basal adenylate cyclase activity are observed. Compound **22** antagonizes plasma ACTH secretion (ID_{50} = 13 mg/kg (sc, 30-min pretreatment), which is induced by o-CRF (4 μg/kg (iv)), while the peptide antagonist α-helical CRF_{9-41} (3 mg/kg, iv) causes a 50% reduction when it is co-administered with the agonist. Antagonist **22** also completely reverses the r/h-CRF-enhanced acoustic startle responses of rats at 17.8 mg/kg (ip), while the peptide antagonist D-Phe-CRF_{12-41} (3.2 μg, icv) completely antagonizes the effect when it was co-administered with r/h-CRF. Compound **22** (17.8 mg/kg (ip)) and D-Phe-CRF_{12-41} (3.2 μg, icv) also antagonize the fear-potentiated acoustic startle response in rats. In other models of anxiety, the rat elevated plus maze and the rat conflict paradigms (punished lever pressing and punished drinking), this pyrrolopyrimidine (0.6−20 mg/kg, ip, 30-min pretreatment) had no effect, unlike diazepam (2.5−10 mg/kg, ip, 30-min pretreatment). Therefore, the anxiolytic efficacy of **22** varied with the stressor. In the rat learned helplessness test, a putative model for depression, compound **22** (10−32 mg/kg, ip, 60-min pretest) reversed the effects of exposure to inescapable foot shocks in a dose-dependent manner while imipramine (17.8 and 32 mg/kg, sc) had no significant effect. The rodent behavioral profile of **22** suggests it may have an anxiolytic or antidepressant potential, but oral efficacy data are needed to complete the profile.

Another pyrrolopyrimidine **53** (antalarmin) has been used to probe for the immune, behavioral, and endocrine effects of CRF_1 antagonists.[107,241−243] This high-affinity ligand (r-CRF_1 K_i = 1 nM) displaces radioiodinated CRF binding in the rat pituitary, frontal cortex, and cerebellum tissues, but not in heart tissue. Compound **53** (20 mg/kg, ip) significantly inhibits CRF-stimulated ACTH release and carageenin-induced inflammation. This agent (10 mg/kg, iv) also antagonizes CRF-induced dermal vasodilation and vascular permeability. Antagonist **53** (20 mg/kg, ip) blocks the conditioned fear response caused by inescapable foot shock (100 shocks, 1.6 mA, 5 s/shock). This compound also antagonizes increases in ACTH levels caused by mild (2 shocks) but not severe (5 or 100 shocks) stress. These data support a role for CRF_1 receptors in anxiety and inflammation.



53

Compound **33** (DMP904), a pyrazolo[1,5-*a*]pyrimidine, is a h-CRF_1 antagonist with anxiolytic behavioral efficacy in rats.[206,244] It binds to h-CRF_1 receptors with high affinity (h-CRF_1 K_i = 1.0 nM) and potently blocks h-CRF_1-coupled adenylate cyclase activity in HEK293 cells (IC_{50} = 10 nM vs 10 nM r/h-CRF); α-helical CRF_{9-41} has weaker activity (IC_{50} = 280 nM). Good oral activity in the rat situational anxiety test has been observed for this compound; the minimum effective dose (MED) is 1 mg/kg (po). Maximal efficacy occurs at this dose; a 57% reduction in latency time in the dark compartment at this dose is observed. Chlordiazepoxide causes a 72% reduction in latency at 20 mg/kg (po). The Pfizer compound **22** (CP154526-1, 30 mg/kg (po)) is inactive in this test. Pyrazolopyrimidine **33** does not inhibit open-field locomotor activity at 100 mg/kg (po) in rats, whereas there is a smaller separation between

efficacy and motor side effects for chlordiazepoxide. Administration of this compound (5 mg/kg, iv, po) to dogs generates good plasma exposure (C_{max}(po), $t_{1/2}$(po), and oral bioavailability = 1260 nM, 45 h, and 33%, respectively).

Other pyrazolo[1,5-*a*]pyrimidines have been reported to be potent h-CRF_1 antagonists. Analogue **35** (NBI 30545)[201,202] has high selective affinity for h-CRF_1 receptors (h-CRF_1 K_i = 2.8 nM, h-CRF_2 K_i > 10 000 nM), good antagonist potency in a CRF_1-stimulated adenylate cyclase assay (EC_{50} = 142 nM), and activity in a CRF-induced locomotor activity model in mice and a rat elevated plus maze test at 20 mg/kg (po). Another analogue, **34** (PD171729),[204,205] has been reported to be a potent h-CRF_1 antagonist. This derivative binds h-CRF_1 receptors selectively (K_i = 5 nM), and it inhibits o-CRF-stimulated adenylate cyclase activity in CHO-p5 cells (IC_{50} = 357 nM). Furthermore, the compound attenuates CRF-induced increases in norepinephrine and *m*-hydroxy-*p*-methoxyphenylglycol (MHPG) levels in rat medial prefrontal cortex in a dose-dependent manner (5−20 mg/kg (po)), and it moderates the effects of o-CRF administration on plasma ACTH levels and locomotor activity in rats.

Compound **37** (DMP696), a pyrazolo[1,5-*a*]-1,3,5-triazine, is a h-CRF_1 antagonist with high affinity (K_i = 1.7 nM vs 7.5 nM for α-helical CRF_{9-41}) and potency (h-CRF_1 adenylate cyclase IC_{50} = 82 nM vs 286 nM for α-helical CRF_{9-41}). [207,208,245] This agent has a good pharmacokinetic profile in rats (5 mg/kg, iv, po, n = 4). Selected oral pharmacokinetic parameters are: $t_{1/2}$ = 16.5 h (po), mean C_{max} = 317 nM (po), oral bioavailability = 36.7%. In dogs (1 mg/kg, iv or po), the pharmacokinetic profile was also favorable. Following oral administration, the mean C_{max}, $t_{1/2}$, and bioavailability parameters are 240 nM, 33 h, and 50%, respectively. Compound **37** is active in the rat situational anxiety model (MED = 3 mg/kg (po); maximal efficacy at 10 mg/kg (po)), whereas the reference h-CRF_1 antagonist **22** (CP154526-1) was inactive at 30 mg/kg (po). Overall, the profile of **37** suggests that it may be an anxiolytic agent, which may have reduced motor side effect liability.

Prospects for Future Therapy

The above survey on CRF receptor modulators clearly suggests a broad range of therapeutic utilities for these compounds, particularly in neuropsychiatric disorders. Small-molecule CRF_1-selective receptor antagonists may be potential anxiolytic and antidepressant agents, based on published preclinical data. The available clinical data on CRF function in humans strongly suggest antidepressant potential for CRF antagonists. These agents could possibly fill a clear and urgent need for new therapies for anxiety and depression since a large number of patients do not respond well to existing medications or do not tolerate their side effects.[130] A nonpeptide CRF_1-selective antagonist is reported to be in human clinical trials for depression.[246] The therapeutic potential for CRF_2-selective agents is less clear because of species differences in the distribution of this receptor subtype. CRF_2-selective agonists may be useful in the treatment of obesity on the basis of rodent data, which would fulfill an important unmet medical need given the co-morbidities associated with this disorder.[100] However, the primate data on the localization of CRF_2 receptors in the CNS may suggest additional CNS applications of CRF_2-selective or combined CRF_1−CRF_2 antagonists. These same data may argue against the use of CRF_2-selective agonists for obesity since it raises the possibility of anxiogenic side effects. CRF-BP−ligand complex inhibitors may have potential as palliative treatments for Alzheimer's disease if the rodent data extrapolates to the human situation. The initial clinical trials with CRF_1 receptor antagonists in the next few years will be critical developments in this research area, and if successful, these agents could revolutionize the treatment of anxiety or depression.

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Biographies

Paul J. Gilligan received his Ph.D. degree from Yale University in 1982 under the direction of Frederick Ziegler. He joined the Animal Health Division of Pfizer Inc. in 1981. Subsequently, he joined DuPont Medical Products Division (now DuPont Pharmaceuticals Co.) in 1987. He is currently Director in the Chemical and Physical Sciences Department.

David W. Robertson received his Ph.D. degree from the University of Illinois in 1981 under the direction of J. A. Katzenellenbogen. He joined Eli Lilly Research laboratories in 1981, Ligand Pharmaceuticals Inc. in 1991, and DuPont Pharmaceuticals Co. in 1996. Recently, he has joined Pharmacia & Upjohn where he is Vice-President, Research.

Robert Zaczek received his Ph.D. degree from John Hopkins University in 1988 under the direction of Joseph Coyle. He then performed postdoctoral research at the National Institute of Drug Abuse under the direction of Errol De Souza. Subsequently, he joined the DuPont−Merck Pharmaceuticals Co. (now DuPont Pharmaceuticals Co.) in 1991. He is currently Director in the CNS Diseases Research Department.

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