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SYNTHESIS AND BIOLOGICAL ACTIVITY OF OXO-7*H*-BENZO[*E*]PERIMIDINE-4-CARBOXYLIC ACID DERIVATIVES AS POTENT, NONPEPTIDE CORTICOTROPIN RELEASING FACTOR (CRF) RECEPTOR ANTAGONISTS

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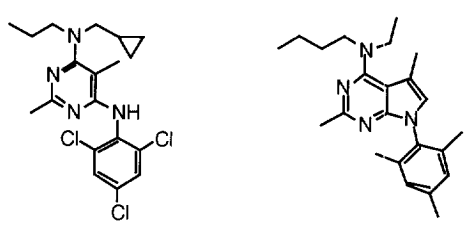
Abstract: A novel series of derivatives of oxo-7*H*-benzo[*e*]perimidine-4-carboxylic acid (**I**) potently displaced radioligand binding of ^{125}I-CRF to both CRF_1 and CRF_2 receptors. The members of this series antagonized CRF-stimulated cAMP formation and CRF-stimulated corticotropin release from rat pituitary in vivo. These are the first nonpeptide antagonists to show activity at both CRF_1 and CRF_2 receptors. © 1999 Elsevier Science Ltd. All rights reserved.

Corticotropin releasing factor (CRF) is a 41-amino acid peptide that mediates the release of corticotropin (ACTH) and proopiomelanocortin products from the anterior pituitary.[1] CRF and CRF-like peptides have been implicated not only as physiological mediators of stress responses, but may be involved in a multitude of other responses including: inflammation, blood pressure regulation, food intake, reproduction, and thermoregulation.[2] CRF and related peptides mediate their effects in the body by binding to G-protein-coupled receptors. To date, two receptor subtypes, each with multiple splice variants, have been cloned.[2] All known CRF receptor subtypes are coupled to the activation of adenylate cyclase through the stimulatory GTP-binding protein, G_s. Until recently, only moderately selective peptide antagonists for CRF receptors existed. Now, several companies have developed high affinity, CRF_1 receptor-selective antagonists (Figure 1).[3,4]

Figure 1. Structures of CRF_1 receptor- selective antagonists[3,4]



We report here the design and synthesis of high affinity CRF receptor antagonists that bind to both the CRF_1 and for the first time, CRF_2 receptors, that may be useful for the treatment of CRF-mediated disorders and for utility in further defining the function of CRF receptor subtypes.

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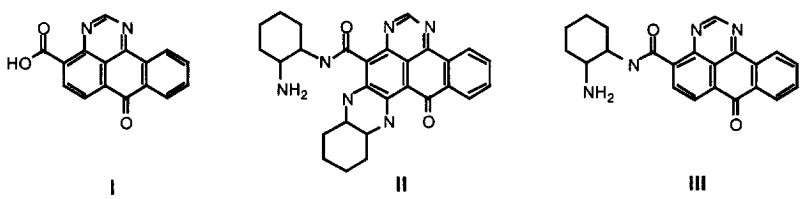
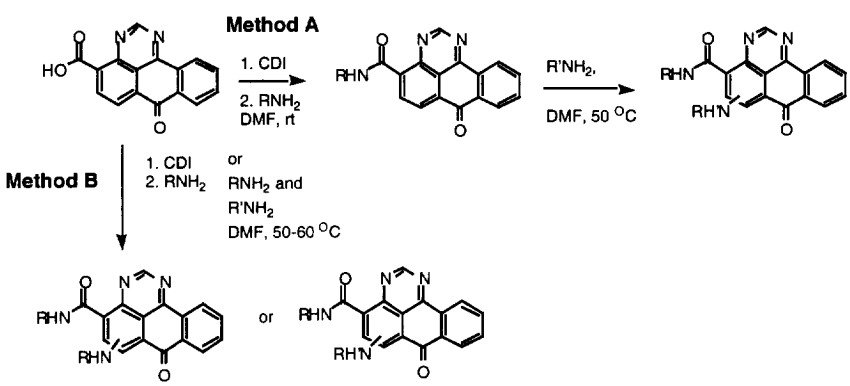
Using proprietary drug-discovery technology, including combinatorial synthesis techniques, we discovered in CRF receptor binding assays, very potent compounds derived from oxo-7*H*-benzo[*e*]perimidine-4-carboxylic acid (**I**, Figure 2). After close investigation of the reaction of **I** with 1,2-cyclohexyldiamine, the active component **II** (Figure 2) was isolated along with the desired amide **III** (Figure 2). A number of compounds with structures similar to that of **II** were synthesized by optimizing the reaction condition to give **II** in reasonable quantity. The mechanism of the reaction was not investigated. A typical experiment involved using either of the methods, **A** or **B**, as shown in Scheme 1.[5,6]

Figure 2



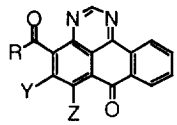
Scheme 1

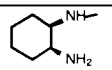
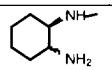
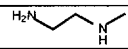
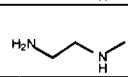
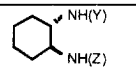
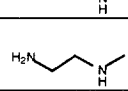
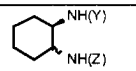
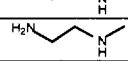
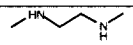
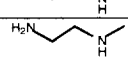
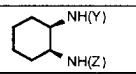
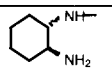
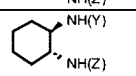
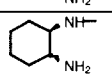
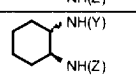


Membranes from 293-EBNA cells stably transfected with cDNA for rat CRF_1 or mouse $CRF_{2\beta}$ receptors were utilized for radioligand binding experiments as described.[5] The organic compounds were dissolved in 100% DMSO and were tested in binding assay buffer containing a final concentration of 1–10% DMSO. The peptides were dissolved in deionized water and were diluted in binding assay buffer. The nonspecific binding was determined in the presence of either 1 μM ovine CRF or 100 nM sauvagine. Table 1 shows the list of compounds made in the series and their activity at both CRF_1 and CRF_2 receptors. K_i values were determined using GraphPad Prism™ software. All hillslopes were not significantly different than unity.

Since CRF mediates its effects on the pituitary by activating adenylate cyclase, the most active compounds were tested in intact cell experiments to determine their ability to block CRF-stimulated cAMP increases.

Table 1. Biological Activity of Oxo-7*H*-benzo[*e*]perimidine-4-carboxylic acid derivatives



Compound	R	Y, Z	CRF_1 K_i (nM)[a]	CRF_2 K_i (nM)[b]
1	OH	H,H	ND	>100,000[c]
2	—NMe_2	H,H	ND	>10000
3[d]		H,H	>10000	>10000
4		H,H	>10000	>10000
5		H,H	>10000	>10000
6			200 ± 50	75 ± 5
7			160 ± 14	45 ± 16
8			2050 ± 350	650 ± 210
9[d]			270 ± 40	830 ± 400
10			110 ± 20	20 ± 4
11			180 ± 40	86 ± 24

ND=Not Determined.

[a]Ki determined from ^{125}I-Tyr^0-ovine CRF binding to recombinant CRF_1 receptors. Values shown are means ± SE for at least three experiments performed in duplicate.

[b]Ki determined from ^{125}I-Tyr^0-ovine CRF binding to recombinant $CRF_{2\beta}$ receptors

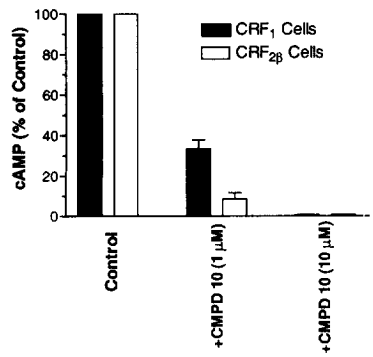
[c]30% inhibition of binding @ 100 μM.

[d]isolated as enatiomeric mixtures

For cAMP determination, approximately two million 293-EBNA cells expressing either CRF_1 or $CRF_{2\beta}$ receptors were incubated in a total volume of 500 μL in triplicate at 37°C in a shaking water bath for 10 min in cAMP generation buffer.[5] Cells were then pre-incubated 25 min with antagonists prior to stimulation for 5 min with half-maximal ovine CRF (1 nM and 10 nM for CRF_1 and $CRF_{2\beta}$ receptors, respectively). The cyclic AMP content of cell pellet extracts was determined with a cAMP RIA kit from New England Nuclear. As a positive control, some cells were incubated with forskolin at a final concentration of 1 μM (not shown). The means ±

SE of three experiments are shown in Figure 3. In 293-EBNA cells stably expressing either recombinant CRF_1 or $CRF_{2\beta}$ receptors, CRF stimulated cAMP formation in a dose-dependent manner (not shown). This effect of CRF in cells containing either the CRF_1 or $CRF_{2\beta}$ receptor subtype was inhibited in a dose-dependent manner by treating the cells with **10** prior to stimulation with CRF. Compound **10** had no effect on basal cAMP formation, suggesting that this compound is not an agonist at these CRF receptor subtypes.

Figure 3. Effect of **10** on ovine CRF-stimulated cAMP formation in cells containing recombinant CRF_1 or $CRF_{2\beta}$ receptors.



In addition, **10** had no effect on forskolin-stimulated cAMP formation (data not shown) or on cells that do not contain CRF receptors, suggesting that the interaction was specific for cells containing CRF receptors and not due to a nonspecific interaction of the compounds with the cells.

For the most potent compound, **10**, a selectivity profile[7] was determined for the compound binding at a number of different receptors. These results are shown in Table 2.

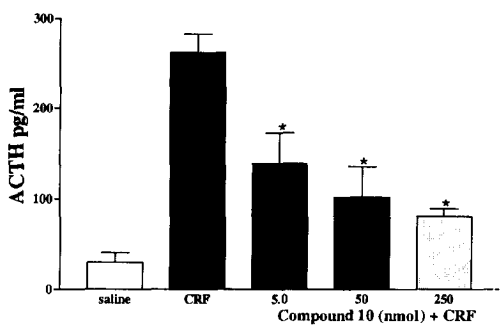
Compound **10** also was tested for efficacy as an antagonist of CRF-stimulated ACTH release in a conscious rat model. Sprague–Dawley rats[8] were instrumented with indwelling femoral venous and arterial catheters to facilitate remote infusions and blood sampling. On the day of the experiment, basal blood samples were drawn from all animals. Thirty minutes following basal sampling, either vehicle (1–10% DMSO/saline) or compound was administered iv. Five minutes later, all animals were given iv injections of CRF (0.05 nmol). This submaximal iv concentration of CRF has been shown to promote ACTH release without affecting blood pressure.[9] Blood samples were drawn 10 min after CRF administration. Plasma samples were analyzed using Allegro Plasma ACTH radioimmunoassay kit (Nichols Institute Diagnostics). The results of these experiments are shown in Figure 4. Values shown in Figure 4 are means ± SE of six animals (5 and 50 nmol doses) and four animals (250 nmol dose). At all doses tested, **10** significantly (*$p < 0.01$ Compound **10** dose vs CRF alone by one-way ANOVA) attenuated CRF-stimulated increases in circulating plasma ACTH in a dose-dependent fashion.

Table 2. Binding selectivity of **10**[7]

Receptor Subtype	K_i (nM)
CRF_1	110
$CRF_{2\beta}$	20
NPY Y_1	4200
Galanin	>10,000
D_2 Dopamine	3200
5-HT_6	>10,000
5-HT_7	1600
Glucagon	>10,000
Glp-1	>10,000

Values shown are means from at least three experiments performed in duplicate.
Standard errors in all assays were less than 25% of the mean values shown.

Figure 4. Inhibition by **10** of CRF-stimulated increases in circulating plasma ACTH in conscious rats.



Compound **10** alone had no effect on basal levels of circulating ACTH nor did the vehicle that was used to administer it (not shown).

Together, these experiments indicate that nonpeptide compounds with high affinity at CRF receptor subtypes and good selectivity for CRF receptors versus other receptors can be produced from this novel series of compounds. These compounds act as antagonists of CRF-stimulated cAMP formation in cells expressing recombinant CRF_1 and $CRF_{2\beta}$ receptors. In a conscious rat model, the most potent of these compounds, **10**, significantly attenuated CRF-mediated increases in circulating plasma ACTH after intravenous administration. These data suggest that these novel compounds and their derivatives may be useful in further elucidating the roles of CRF receptor subtypes and as potential therapeutic agents for the treatment of various neuropathologies attributed to over-stimulation of CRF receptors.

References and Notes

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6. Experimental procedure; Method A: To a solution of 0.276 g (1 mmol) of 7-oxo-7H-benzo[e]perimidine-4-carboxylic acid in 15 mL of dry DMF at 20 °C was added 0.162 g (1 mmol) of 1,1'-carbonyldiimidazole and the reaction mixture was stirred for 60 min. A solution of 1 mmol of the corresponding amine/diamine in 3 mL of dry DMF was added. The reaction was stirred for 18 h and final product was chromatographed on Waters Prep L.C. 4000 System. To a solution of 0.1 mmol of the isolated 7-oxo-7*H*-benzo[*e*]perimidine-4-carboxamide derivative in 10 mL of dry DMF was added a solution of 1 mmol of the corresponding diamine in 2 mL of dry DMF. The reaction was stirred for 6 h at 50 °C and the product chromatographed on Waters Prep L.C. 4000 System. Method B: To a solution of 0.276 g (1 mmol) of 7-oxo-7*H*-benzo[*e*]perimidine-4-carboxylic acid in 15 mL of dry DMF at 20 °C was added 0.162 g (1 mmol) of 1,1'-carbonyldiimidazole and the reaction mixture was stirred for 60 min. A solution of 1 mmol each of the corresponding amine or diamine in 3 mL of dry DMF was added. The reaction was stirred for 18 h at 50 - 60 °C and the mixture of the products was chromatographed on Waters Prep L.C. 4000 System. The products were characterized by ^{1}H NMR and APCIMS. Compound 10; mp 190-193 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ 12.05 (s, 1H), 11.66 (d, J = 8.4 Hz, 1H), 10.88 (s, 1H), 9.12 (s, 1H), 8.89 (d, J = 7.7 Hz, 1H), 8.40 (d, J = 7.7 Hz, 1H), 7.98 (brs, 2H), 7.90-7.95 (m, 1H), 7.82-7.87 (m, 1H), 4.00-4.09 (m, 1H), 3.34-3.42 (m, 1H), 3.10-3.26 (m, 2H), 2.18-2.27 (m, 1H), 1.98-2.10 (m, 3H), 1.73-1.90 (m, 4H), 1.30-1.70 (m, 8H); APCIMS *m/z* 483 (M+1).
7. For NPY Y_1 receptor binding, SK-N-MC cell membranes (25–35 μg/well) were incubated with ^{125}I-PYY (0.02–0.05 nM) and compounds for 1.5 h @ 37 °C in buffer containing 25 mM HEPES, 135 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1%BSA and 0.01% bacitracin. The nonspecific binding was defined by incubation with 30 nM human NPY. The membranes containing recombinant human receptors for Galanin, D_2 dopamine, 5-HT_6 serotonin and 5-HT_7 serotonin were obtained from Receptor Biology, Inc. (Beltsville, MD). Receptor binding assays for these four receptors were performed according to guidelines provided by Receptor Biology, Inc. Glucagon binding was performed by incubating membranes containing recombinant glucagon receptors (0.5–2 μg/well) with ^{125}I-glucagon (0.02–0.04 nM) and compounds for 1 h @ 37 °C in assay buffer containing 25 mM HEPES and 0.1% BSA. Nonspecific binding was determined in the presence of 100 nM glucagon. For Glp-1 binding, membranes containing recombinant Glp-1 receptors (2–5 μg/well) were incubated with ^{125}I-Glp-1 (0.02–0.04 nM) and compounds for 1 h @ 37 °C in assay buffer containing 25 mM HEPES, 0.1% BSA and 0.01% bacitracin. Nonspecific binding was determined in the presence of 1 μM Glp-1. All assays were performed in a final volume of 200 μL with the exception of D_2 dopamine, which was in a final volume of 2 mL.
8. All animals utilized in these studies were housed and cared for in accordance with the NIH guide for the Care and Use of Laboratory Animals. Rats were purchased from Harlan Sprague–Dawley (San Diego, CA). Animals were housed in temperature controlled quarters with a 12-h light 12-h dark cycle and were provided standard rat chow and water *ad libitum*.
9. Fisher, L.; Rivier, C.; Rivier, J.; Brown, M. *Endocrinol.* **1991**, *129*, 1312.