Pharmacological Characterization of a Novel Nonpeptide Antagonist Radioligand, \((\pm)\)-N-[2-Methyl-4-methoxyphenyl]-1-(1-(methoxymethyl) propyl)-6-methyl-1H-1,2,3-triazolo[4,5-c]pyridin-4-amine ([\(^3\)H]SN003) for Corticotropin-Releasing Factor\(_1\) Receptors

GE ZHANG, NING HUANG, YU-WEN LI, XIAOPIONG QI, ANNE P. MARSHALL, XIAO-XIN YAN, GERALDINE HILL, CYNTHIA ROMINGER, SHIMOGA R. PRAKASH, RAJAGOPAL BAKTHAVATCHALAM, DAVID H. ROMINGER, PAUL J. GILLIGAN, and ROBERT ZACZEK


Received October 29, 2002; accepted January 03, 2003

ABSTRACT

The in vitro pharmacological profile of a novel small molecule corticotropin-releasing factor 1 (CRF\(_1\)) receptor antagonist, \((\pm)\)-N-[2-methyl-4-methoxyphenyl]-1-(1-(methoxymethyl)propyl)-6-methyl-1H-1,2,3-triazolo[4,5-c]pyridin-4-amine (SN003), and the characteristics of its radioligand ([\(^3\)H]SN003) are described. SN003 has high affinity and selectivity for CRF\(_1\) receptors expressed in rat cortex, pituitary, and recombinant HEK293EBNA (HEK293e) cells with respective radiolabeled ovine CRF ([\(^{125}\)I]oCRF) binding \(K_d\) values of 2.5, 7.9, and 6.8 nM. SN003 was shown to be a CRF\(_1\) receptor antagonist inasmuch as it inhibited CRF-induced cAMP accumulation in human CRF, HEK293e cells and CRF-stimulated adrenocorticotropin hormone release from rat pituitary cells without agonist activities. Significant decreases in the \(B_{\text{max}}\) of [\(^{125}\)I]oCRF binding by SN003 suggest that this antagonist is not simply competitive. To further explore the interaction of SN003 with the CRF\(_1\) receptors, [\(^{3}\)H]SN003 binding to rat cortex and human CRF, HEK293e cell membranes was characterized and shown to be reversible and saturable, with \(K_d\) values of 4.8 and 4.6 nM, and \(B_{\text{max}}\) values of 0.142 and 7.42 pmol/mg protein, respectively. The association and dissociation rate constants of [\(^{3}\)H]SN003 \((k_{1}, 0.292 \text{ nM}^{-1} \text{ min}^{-1} \text{ and } k_{-1}, 0.992 \times 10^{-2} \text{ min}^{-1})\) were also assessed using human CRF, HEK293e cell membranes, giving an equilibrium dissociation constant of 3.4 nM. Moreover, [\(^{3}\)H]SN003 binding displayed a single affinity state and insensitivity to 5'-guanylylimidodiphosphate, consistent with characteristics of antagonist binding. Incomplete inhibition of [\(^{3}\)H]SN003 binding by CRF peptides also suggests that SN003 is not simply competitive with CRF at CRF\(_1\) receptors. The distribution of [\(^{3}\)H]SN003 binding sites was consistent with the expression pattern of CRF\(_1\) receptors in rat brain regions. Small molecule CRF\(_1\) antagonist radioligands like [\(^{3}\)H]SN003 should enable a better understanding of small molecule interactions with the CRF\(_1\) receptor.

Corticotropin-releasing factor (CRF) was first isolated from ovine hypothalamus (Vale et al., 1981) and identified as a key secretagogue for ACTH release from the anterior pituitary. In the past 10 years, considerable progress has been made in understanding the physiological and potential pathological roles of the CRF system. In addition to its endocrine role in the regulation of the hypothalamic-pituitary-adrenal axis in response to stress, CRF appears to be implicated in a variety of other central and peripheral functions including arousal, anxiety-like behaviors, learning and memory, feeding, insensitivity to 5'-guanylylimidodiphosphate; HEK, human embryonic kidney; PBS, phosphate-buffered saline; DMP696, 4-(1,3-dimethoxyprop-2-ylamino)-2,7-dimethyl-8-(2,4-dichlorophenyl)-pyrazolo[1,5-a]-1,3,5-triazine; DMP904, 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo[1,5-a]-pyrimidine; R-121919, 3-[6-dimethylamino]-4-methyl-pyrid-3-yl]-2,5-dimethyl-N,N-dipropyl-pyrazolo[2,3-alpyrimidin-7-amine.
mune, and autonomic functions (Owens and Nemeroff, 1991; De Souza and Grigoriadis, 1998; Gilligan et al., 2000; Dautzenberg and Hauger 2002).

The entire spectrum of CRF peptide effects is mediated through two known receptor subtypes, CRF₁ and CRF₂. Both receptor subtypes belong to the class B family of G protein-coupled receptors that include receptors for secretin and parathyroid hormone, among others (review Dautzenberg and Hauger, 2002). Despite their high degree of sequence homology and their common coupling through G₄ proteins to cAMP signaling, CRF₁ and CRF₂ receptors differ markedly from each other in pharmacological properties and anatomic distribution (DeSouza et al., 1998; Gilligan et al., 2000; Dautzenberg and Hauger, 2002). CRF₁ receptors are widely distributed in the central nervous system. There exist three splice variants of the CRF₂ receptor (CRF₂α, CRF₂β, and CRF₂γ) with distinct anatomic localization. CRF₂α receptors are primarily located in discrete rat brain areas such as lateral septum, and CRF₂β receptors are located in rat choroid plexus, heart, lung, and skeletal muscle. CRF₁ and CRF₂ receptors are activated by several related peptides identified from various species. These include CRF, sauvagine, urotensin, and the urocortins, including recently identified urocortin II and III (Lewis et al., 2001; Reyes et al., 2001), which display differential affinity for CRF₁ and CRF₂ receptors.

Studies of the neuronal circuitry mediating fear and anxiety states (Davis, 1992) suggest that both CRF₁ and CRF₂ receptors located in differential brain areas may be involved in the regulation of various stress-induced behaviors, albeit the relative importance of CRF₂ receptors is less clear (Lewis et al., 2001; Reyes et al., 2001; Bakshi et al., 2002). Clinical findings support the hypothesis that dysfunction of the CRF system is involved in certain neuropsychiatric disorders such as anxiety and depression (Gilligan et al., 2000; Keck and Holsboer, 2001). Numerous animal studies using CRF ligands and genetically altered mice provide strong evidence for the role of CRF₁ receptors in the coordination of the behavioral response to stress and in stress-related psychiatric disorders (Gilligan et al., 2000; Bakshi et al., 2002; Dautzenberg and Hauger, 2002).

In recent years there has been much emphasis on developing orally active, nonpeptide CRF₁ antagonists to evaluate the putative role of CRF₁ receptors in psychopathology and to test their potential as novel therapeutic agents. Schulz et al. (1996) were the first to report a pyrazolopyrimidine CRF₁ antagonist, CP-154,526, with high affinity for the CRF₁ receptor and anxiolytic activity in rats. Additional CRF₁ antagonists, such as antalarmin, NBI 27914, DMP696, R121919, and SSR125543A, also exhibit CRF₁ antagonist properties in vitro and in vivo (He et al., 2000; McCarthy et al., 1999; Habib et al., 2000; Griebel et al., 2002; Gully et al., 2002; Heinrichs et al., 2002; Maciag et al., 2002; McElroy et al., 2002). Although a variety of the iodine-125-labeled peptides such as [¹²⁵I]ovine CRF have been extensively employed in previous studies of CRF₁ receptors, the use of a small molecule CRF₁ antagonist radioligand as a tool would permit investigation of interactions between CRF and small molecule antagonists at CRF₁ receptors and allow direct mapping of the small molecule binding sites in discrete brain regions.

The aim of the present studies is to describe the in vitro pharmacological properties of tritiated (±)-N-[2-methyl-4-methoxyphenyl]-1-(1-(methoxymethyl)propyl)-6-methyl-1H-1,2,3-triazolo[4,5-c]pyridin-4-amine ([³H]SN003), a small-molecule radioligand for rat and human CRF₁ receptors. The binding characteristics of [³H]SN003 were profiled in rat cortical and human CRF₁ cell membranes, and the specificity and anatomic distribution of [³H]SN003 binding sites in rat brain were illustrated by brain section phosphorimaging. The in vitro pharmacological profile of the unlabeled SN003 ligand was also studied. Parts of these studies were previously presented in abstract form (Li et al., 2001; Zhang et al., 2001). This is the first report identifying a small molecule antagonist radioligand specifically labeling CRF₁ receptors in brain tissues and slices. This nonpeptide radioligand as a tool provides an opportunity to further understand the interactions of CRF and small molecule antagonists with CRF₁ receptors.

**Materials and Methods**

**Animals.** Male Sprague-Dawley rats weighing 250 to 350 g were obtained from Charles River Laboratories, Inc. (Wilmington, MA). They were housed two per cage in a room with controlled illumination, humidity, and temperature. Food and water were provided ad libitum. All animal studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Protocols were approved by the Committee on Animal Care and Use of the Bristol-Myers Squibb Company.

**Materials.** SN003 was obtained by directed synthesis efforts (Bakthavatchalam et al., 1997) as were other small-molecule CRF₁ antagonists, DMP696 (He et al., 2000) and DMP904 (Gilligan et al., 2000). SN003, DMP696, DMP904, and CP-154,526 were synthesized by the Department of Chemical and Physical Sciences, Bristol-Myers Squibb Company. The chemical structures of the small molecule CRF₁ receptor antagonists are shown in Fig. 1. CRF-related peptides, human/rat CRF (hrCRF), ovine CRF (oCRF), sauvagine, urocortin I (human), urocortin I (rat), α-helical

---

![Chemical structures of SN003 and other small molecule CRF₁ receptor antagonists. A. SN003 ([±]-N-[2-methyl-4-methoxyphenyl]-1-(1-(methoxymethyl)propyl)-6-methyl-1H-1,2,3-triazolo[4,5-c]pyridin-4-amine); B. DMP696, DMP904, and CP-154,526.](image)
CRF<sub>α</sub>-41 (α-helical CRF), and [α-Phe<sup>12</sup>,Nle<sup>21,38</sup>,C<sup>α</sup>-MeLeu<sup>37</sup>]-CRF<sub>12-41</sub> (α-PheCRF), were purchased from American Peptide Co., Inc. (Sunnyvale, CA), Bachem California (Torrance, CA), and Peninsula Laboratories (Merseyside, UK). [<sup>125</sup>I]oCRF and [<sup>3H</sup>]sauvagine (specific activities, 2200 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Gpp(NH)p (5′-guanylylimidodiphosphate) and other standard reagents were purchased from Sigma-Aldrich (St. Louis, MO) and Invitrogen (Carlsbad, CA).

**Synthesis of [HISN003.** [HISN003 was labeled by the radioligand synthesis facility in the drug metabolism group of the Bristol-Myers Squibb Company (former DuPont Pharmaceuticals Company).

Compound 1 (ST613) was prepared from Compound 2a (SN003) by demethylation with sodium thiomethoxide in dimethylformamide (Fig. 1A). To a solution of compound 1 in dimethylformamide, potassium carbonate was added and the mixture was stirred. Tritium-labeled methyl iodide (250 μCi, 80 Ci/mmol) in toluene was transferred to this reaction mixture, followed by rinsing, extraction, and separation by chromatography on a reversed-phase column (Vydac Protein-peptide C18 Semiprep column). Fractions containing the product were lyophilized to provide radiochemically pure compound 2 ([HISN003, purity >99%). [HISN003 was dissolved and stored as an ethanolic solution (1mCi/ml; specific activity 74 Ci/mmol).

**Cell Culture of HEK293e Cells Expressing Human CRF Receptors.** Full-length human cDNAs for human CRF<sub>1</sub> and CRF<sub>2a</sub> receptors were subcloned into plasmids and transfected into HEK293EBNA (HEK293e) cells (Invitrogen) using lipofectamine (Invitrogen). The details of the plasmid construct were described in previous studies (Horlick et al., 1997; Kostich et al., 1998). HEK293e cells stably expressing human CRF<sub>1</sub> or CRF<sub>2a</sub> receptors were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C in a humid environment (5% CO<sub>2</sub>) for 10 days. The cells were then adapted to spinner culture for bulk processing. Cells were harvested, washed in phosphate-buffered saline (PBS), and counted; and the cell pellet (containing approximately 1 × 10<sup>8</sup> HEK293e cells) was stored at −80°C until use.

**Peptide Radioligand Binding to Homogenates.** For binding assays, the total particulate fraction of rat frontal cortex and pituitary tissues or cell pellets was prepared as a crude membrane source expressing CRF<sub>1</sub> or CRF<sub>2a</sub> receptors. Frozen tissues or cell pellets were thawed on ice and homogenized in tissue buffer (50 mM Tris, 0.25 mg/mL sonication buffer containing 10% (w/v) fetal bovine serum at 37°C in a humid environment (5% CO<sub>2</sub>) for 10 days. The membrane homogenate to 150 μl of assay buffer containing the radioactivity (5 μCi/μl) was stored at −80°C until use.

**ACTH Release Assays in Rat Primary Pituitary Cultures.** Primary pituitary culture was established as described previously (Vale et al., 1972). Pituitaries were harvested from 20 to 25 rats and washed in PBS four to six times. Pituitary cells were dissociated in collagenase buffer [1× PBS containing 25 mM HEPES, 0.2% glucose, 0.4% (w/v) bovine serum albumin, 80 μM/ml DNase II, and 0.4% (w/v) collagenase type II] for 3 h at 37°C. Cells were spun down, decanted, and incubated with 0.25% trypsin solution for 15 min at 37°C. The cell suspension was filtered through a 100 μm nylon mesh to remove cell debris, and the filtrate was centrifuged at 2,000 g for 5 min. The pellet was resuspended in hormone-free Dulbecco’s modified Eagle’s medium, and the cell suspension was added to 5 μl of assay buffer containing the radioligand for 1 h at 37°C. The medium was aspirated and the cells were washed twice with 500 μl ofwash buffer (PBS, pH 7.0, containing 0.01% Triton X-100). After the filter was dried, scintillation cocktail was added and the plate was counted in a 96-well PerkinElmer Unifilter harvester followed by three washes with 0.3 ml of cold wash buffer (PBS, pH 7.0, containing 0.01% Triton X-100). After the filter was dried, scintillation cocktail was added and the plate was counted in a 96-well PerkinElmer Top Counter. The CRF<sub>1</sub> competition binding to membranes from rat frontal cortex and pituitary was performed similarly except for the radioligand concentration of [HISN003 (200 pM) used in the binding.

To determine the nature of interaction (competitive and noncompetitive) of SN003, homologous displacement of [HISN003 (150 pM) binding by ovine CRF was conducted in membranes prepared from HEK293e cells expressing human CRF<sub>1</sub> receptors in the absence and presence of SN003. The K<sub>D</sub> and B<sub>max</sub> values from homologous competition curves were calculated using nonlinear regression analysis in Prism (1999; GraphPad Software, Inc., San Diego, CA). A Scatchard plot of the homologous binding with [HISN003 was generated for visualization of any K<sub>D</sub> or B<sub>max</sub> changes. Specific bound (femtomoles per milligram of protein) and Bound/Free ligand data were transformed. These transformations were performed according to similar procedures described in Prism (1999; GraphPad Software, Inc.).

**[HISN003 Binding to Membranes.** Equilibrium binding experiments in cell or tissue homogenates were performed under conditions similar to those described for the [HISN003 binding with a few exceptions. G/F/B filters were used in the filtration assay. The saturation experiments using [HISN003 as a radioligand and rat cortex and HEK293e cell homogenates as CRF<sub>1</sub> receptors were conducted in 12 concentrations of [HISN003 (0.5–40 nM) in triplicate at 23°C for 2 h. The nonspecific binding was defined in the presence of 5 μM DMP696. Association and dissociation assays were performed at the [HISN003 K<sub>D</sub> concentration of 4.8 nM at 23°C. After 2 h of incubation, when association equilibrium was reached, dissociation reactions were initiated by addition of 5 μM DMP696 and continued for an additional 3 h.

**cAMP Assays in Recombinant Human CRF<sub>1</sub> Cells.** Intracellular cAMP levels were measured using the Adenyl Cyclase Activation Flash Plate kit purchased from PerkinElmer Life Sciences. This radioimmunoassay-based kit enables direct detection of cAMP generated in live cells in a 96-well format. HEK293e cells expressing CRF<sub>1</sub> receptors were grown in the Dulbecco’s modified Eagle’s medium supplement with 10% fetal bovine serum, L-glutamine (2 mM), and hygromycin (400 μg/ml) at 37°C in a humid environment with 5% CO<sub>2</sub>. On the assay day, cells were dissociated from flasks and centrifuged down at 1,200 rpm for 4 min. Cells were resuspended in 100 μM stimulation buffer, counted, and diluted to 0.6 × 10<sup>5</sup> cell/ml. hrCRF (1 nM) in the absence and presence of SN003 in PBS containing 10% stimulation buffer (50 μl) was added to the assay plate. Drug treatment was initiated by adding HEK293e cells expressing CRF<sub>1</sub> receptors (50,000 cells/50 μl/well) to the Flash Plate and incubated for 15 min at 37°C in a final volume of 100 μl. Intracellular cAMP was released from cells through cell lysis resulting from adding detection buffer containing [HISN003 (100 μl/well). After signal was based on competition of endogenous cAMP and [HISN003 for cAMP antibodies coated on the Flash Plate. Radioactivity from binding of [HISN003 to the plate was assessed 2 h later by a 96-well PerkinElmer Top Counter.

**ACTH Release Assays in Rat Primary Pituitary Cultures.** Primary pituitary culture was established as described previously (Vale et al., 1972). Pituitaries were harvested from 20 to 25 rats and washed in PBS four to six times. Pituitary cells were dissociated in collagenase buffer [1× PBS containing 25 mM HEPES, 0.2% glucose, 0.4% (w/v) bovine serum albumin, 80 μM/ml DNase II, and 0.4% (w/v) collagenase type II] for 3 h at 37°C. Cells were spun down, decanted, and incubated with 0.25% trypsin solution for 15 min at 37°C.
[PBS containing 0.25% (w/v) trypsin, 0.4% (w/v) bovine serum albumin, 0.2% (w/v) glucose] for 5 to 10 min at 37°C. Cells were briefly centrifuged and resuspended in M199 culture medium (Invitrogen) containing 10% fetal calf serum, minimal essential medium vitamins, streptomycin/penicillin, insulin/transferrin/ selenium, fibroblast growth factor, and trace elements. Cells (300,000/ml) were added to a 48-well plate (Costar) and grown in culture (37°C; 5% CO₂) for 4 days. On the assay day, medium was removed and replaced with M199 incubation medium identical to that used above except for exclusion of serum and addition of 0.1% (w/v) ovalbumin and 1 μg/ml aprotinin. The hrCRF-induced ACTH release was conducted at 37°C for 3 h. With pretreatment of various concentrations of SN003 or vehicle (control) to cells for 15 min, the antagonist assay of SN003 was performed by adding hrCRF (0.3 nM) and coincubation at 37°C for 3 h. After the incubation period, medium was removed, frozen, and stored at −20°C until assayed for ACTH measurement. ACTH levels were determined using a radioimmunoassay kit purchased from Diasorin Inc. (Stillwater, MN).

**Data Analyses.** The concentrations of compounds to inhibit 50% of radioligand binding (IC₅₀) for CRF₁ and CRF₂a receptors were calculated by fitting data through a competition equation in the iterative nonlinear regression curve-fitting program Prism (GraphPad Software, Inc.). Kᵢ values (equilibrium dissociation constant) for inhibitors in competition experiments were calculated according to the Cheng-Prusoff equation. Saturation data were fit through hyperbolic equations to estimate apparent equilibrium constant (Kᵢ) and the maximal number of binding sites (Bₘₐₓ) using Prism (GraphPad Software, Inc.). Kinetic studies to determine kₐss (observed association rate constant) and k₋₁ (dissociation rate constant) were generated from a slope of the line via linear regression of transformed data. The association rate constant (kₐss) was generated based on the equation kₐss = (kₛobs − k₋₁)/[L], where L is the ligand concentration. The equilibrium dissociation constant derived from kinetic studies was calculated as Kᵢ = k₋₁/kₐss.

For cell-based functional studies, the potency for agonists (EC₅₀) and for antagonists (IC₅₀) was estimated by fitting raw data through the sigmoidal dose-response equation using the iterative nonlinear regression curve-fitting programs in Prism (GraphPad Software, Inc.).

**Brain Section Ligand Binding and Storage Phosphorimaging.** Rats were decapitated, and the brain and pituitary were immediately collected, embedded in M-1 embedding matrix (Thermo Shandon, Pittsburgh, PA), and frozen in iso-pentane chilled with dry ice. Twenty-micrometer coronal sections were cut on a Cryostat, thaw-mounted on superfrost slides (VWR, West Chester, PA), dried, and stored at −70°C until use. Before in vitro binding, sections were brought to 23°C and preincubated for 30 min in assay solution containing 50 mM HEPES, 10 mM MgCl₂, 2 mM EGTA, 100 kallikrein-inactivating units/ml aprotinin, 0.1 M bacitracin, and 0.1% ovalbumin (pH 7.2). Sections were then incubated in the same solution containing 4 to 10 nM [³²P]SN003 for 2 h at 23°C. As a comparison, one set of adjacent sections was incubated with 200 pM [¹²⁵]IovCRF. At this concentration (200 pM), [¹²⁵]IovCRF binds to both CRF₁ and CRF₂ receptors (Rominger et al., 1998). Nonspecific binding was defined by inclusion of 1 μM DMP696. After incubation, sections were rinsed in PBS with 0.01% Triton X-100 for 10 min and subsequently dried under a stream of cold air. Slides of the sections were then placed in cassettes against storage phosphorimaging screens (PerkinElmer Life Sciences) for 1 to 4 weeks ([³²P]SN003) or for 12 h ([¹²⁵]IovCRF) respectively. The screens were then scanned with a Cyclone phosphorimaging scanner, and captured images were analyzed with the OptiQuant acquisition and analysis system (PerkinElmer Life Sciences).

**Results**

**Binding Affinity of SN003 for CRF₁ and CRF₂ Receptors.** The chemical structure of SN003 is shown in Fig. 1A. The binding affinity of SN003 for CRF₁ receptors was determined by competition binding experiments using membranes prepared from HEK293e cells expressing human recombinant CRF₁ receptors, and rat cortex and pituitary membranes containing native CRF₁ receptors. Like the CRF peptide antagonist α-helical CRF and the small molecule CRF₁ antagonist DMP696 (Fig. 2), SN003 potently and completely inhibited [¹²⁵]IocRF (200 pM) binding to CRF₁ receptors, with maximal inhibition identical to that of α-helical CRF and DMP696. The Kᵢ value of SN003 for rat CRF₁ receptors detected in pituitary membranes was 3.4 ± 0.5-fold of that determined in cortical membranes in paired experiments (p = 0.001), as shown in Table 1. A similar shift was seen for DMP696 in pituitary and cortical tissues with a Kᵢ ratio (pituitary/cortex) of 2.5 ± 0.6-fold (p = 0.017). In contrast, the peptide agonist ovine CRF exhibited an equal affinity for rat CRF₁ receptors in cortical (mean Kᵢ = 0.6 nM) and pituitary (mean Kᵢ = 0.7 nM) tissues (p = 0.38; paired Student’s t test). Although the peptide antagonist α-helical CRF appeared to have a small difference in potency between rat cortex and:

![Graph showing competition of [¹²⁵]IocRF binding to rat cortical and pituitary membranes by SN003, DMP696, α-helical CRF, and hrCRF. The assays were performed in duplicate in final concentration of [¹²⁵]IocRF (200 pM) and 12 concentrations of displacers. Nonspecific binding was defined by 1 μM DMP696. The data are plotted and the respective curves are expanded from the parameter estimates derived from fitting a competition equation to the data by nonlinear regression analysis using Prism software.](image-url)
Table 1
Potency of compounds inhibiting [125I]oCRF binding to rat CRF1 receptors

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Pituitary Mean Kᵢ (nM)</th>
<th>Cortex Mean Kᵢ (nM)</th>
<th>Pituitary/Cortex Ratio</th>
<th>Significance (p Values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN003</td>
<td>7.9 ± 0.8</td>
<td>2.5 ± 0.4</td>
<td>3.4 ± 0.5</td>
<td>*0.001(6)</td>
</tr>
<tr>
<td>DMP696</td>
<td>2.6 ± 0.5</td>
<td>1.0 ± 0.1</td>
<td>2.5 ± 0.6</td>
<td>*0.017(6)</td>
</tr>
<tr>
<td>α-helical CRF</td>
<td>8.1 ± 2.3</td>
<td>5.3 ± 1.9</td>
<td>3.3 ± 2.8</td>
<td>0.222(9)</td>
</tr>
<tr>
<td>hrCRF</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.388(6)</td>
</tr>
</tbody>
</table>

(mean Kᵢ = 5.3 nM) and pituitary (mean Kᵢ = 8.1 nM) tissues, this difference was not statistically significant (p = 0.22), as summarized in Table 1.

Figure 3A shows that SN003 specifically inhibited [125I]oCRF binding to human CRF₁ receptors expressed in HEK293e cells with high affinity (Kᵢ = 6.8 ± 0.8 nM) like DMP696 (Kᵢ = 2.8 ± 0.5 nM) and α-helical CRF9–41 (Kᵢ = 7.9 ± 1.5 nM). The specificity of SN003 for the CRF₁ receptor was also assessed by examining its ability to compete with [125I]sauvagine binding to the human CRF₂α receptors. As depicted in Fig. 3B, α-helical CRF was a potent inhibitor of [125I]sauvagine binding to human CRF₂α receptors (Kᵢ = 11.2 ± 1.8 nM), whereas SN003 was inactive at the CRF₂α receptor up to 10 μM concentrations (n = 3). The data indicate that SN003 is highly selective in its affinity for CRF₁ receptors, being approximately 1000 times more potent against [125I]oCRF binding to human CRF₁ (Kᵢ = 6.8 nM) than against [125I]sauvagine binding to human CRF₂α (Kᵢ > 10 μM).

Homologous competition experiments were conducted to determine SN003 effects on the Kᵢ and Bₘₐₓ values of oCRF binding in the human CRF₁,HEK293e cell membranes. The Scatchard plot in Fig. 4 shows that SN003 dramatically decreased the Bₘₐₓ (the x-intercept) to 85.3 ± 1.9% of 67.4 ± 10.7%, and 46.4 ± 3.0% of the control value without change of Kᵢ (1/slope) values of ovine CRF at concentrations of 3, 10, and 30 nM, respectively, suggesting that SN003 is not simply competitive.

Inhibition of CRF-Mediated cAMP Accumulation and ACTH Release by SN003. To evaluate functional activities of SN003 in vitro, SN003 was assessed for inhibition of CRF-mediated responses in rat pituitary and human CRF₁, HEK293e cells. In cell-based functional assays, the antagonist properties of SN003 in CRF-stimulated ACTH secretion in cultured rat pituitary cells were examined. In parallel, evaluation of the agonist concentration-response was included in each experiment (Fig. 5A). Basal secretion of ACTH was markedly enhanced in response to CRF in a concentration-dependent manner. CRF-induced maximal stimulation of ACTH release (3490–4993 pg/100-μl sample), which is 5- to 9-fold of the basal level (400–520 pg/100-μl sample), was achieved at 10 μM CRF during a 3-h incubation (n = 4). The mean EC₅₀ ± S.E.M. of CRF for stimulation of pituitary ACTH secretion was calculated as 0.30 ± 0.05 nM (n = 4). As shown in Fig. 5B, SN003 dose dependently suppressed CRF (0.3 nM)-induced ACTH release with an IC₅₀ value of 241.5 ± 48.4 nM (n = 4) and completely abolished CRF-stimulated ACTH secretion at higher concentrations, suggesting full antagonist properties at pituitary CRF₁ receptors. To test potential agonist activity, SN003 (1 and 10 μM), exposed alone to pituitary cells for 3 h, did not alter basal secretion of ACTH from cells (data not shown), indicating a lack of partial or inverse agonist activities.

The antagonist properties of SN003 for human CRF₁ receptors were examined in HEK293e cells expressing recombinant human CRF₁ receptors (Fig. 6). CRF (1 nM) elicited 60.2 ± 3.9% stimulation of cAMP production as normalized to the maximal stimulation (100%) obtained by
30 nM CRF. As displayed in Fig. 6, SN003 significantly decreased the control CRF response from 60.2 ± 3.9 to 26.4 ± 4.9% and 12.9 ± 2.8% in a dose-dependent manner at concentrations of 100 nM and 300 nM, respectively. As observed in the case of ACTH release, SN003 showed no effect on basal cAMP production in the absence of CRF stimulation. The Schild analysis of SN003 antagonism of CRF-induced cAMP responses was also performed in this recombinant cell line that highly expresses human CRF₁ receptors, showing rightward shifts of CRF concentration-
response curves with no significant decrease in CRF efficacy ($E_{\text{max}}$) by SN003 (antagonist potency constant $K_0 = 58 \pm 3 \text{nM}$). This may be due to the large number of spare receptors expressed in this system. Even after eliminating a large percentage of receptors by SN003 via a noncompetitive mode of inhibition, a small portion of receptors remains adequate for CRF to achieve the maximal responses. Therefore, Schild analysis was performed in human retinoblastoma Y-79 cells (Hauger et al., 1997), which natively express a low level of human CRF$_1$ receptors. SN003 reduced CRF potency ($\uparrow EC_{50}$) in a concentration-dependent manner, and high concentrations of SN003 (1 and 3 $\mu$M) significantly decreased $E_{\text{max}}$ to 89 $\pm$ 2% and 77 $\pm$ 3%, respectively, of the control level in the Y-79 cell line ($n = 3; p < 0.01$ by Student’s $t$ test).

Characterization of $[^3H]$SN003 Binding to Membranes Expressing CRF$_1$ Receptors. To examine the binding characteristics of $[^3H]$SN003 to CRF$_1$ receptors, a time course of $[^3H]$SN003 (~4.8 nM) equilibrium binding was performed in rat frontal cortex homogenates at 23°C. Under these conditions, $[^3H]$SN003 binding was time-dependent (Fig. 7), and its association reaction (>99%) was completed by 120 min. Nonspecific binding was increased slightly within 5 min and remained constant, representing approximately 50% of total binding. The mean $\pm$ S.E.M. value for $k_{\text{obs}}$ (1.62 $\pm$ 0.3 $\times$ 10$^{-2}$ min$^{-1}$) and the corresponding $t_{1/2}$ of 43 min was determined based on linear regression analysis of data from three independent experiments. Subsequent saturation experiments were conducted using a 120-min incubation time.

As presented in the saturation analysis (Fig. 8), $[^3H]$SN003 bound saturably, specifically with high affinity to an apparently homogeneous population of recognition sites, with a $B_{\text{max}}$ of 142 $\pm$ 14 fmol/mg protein and $K_D$ of 4.8 $\pm$ 0.2 nM for CRF$_1$ receptors expressed in rat frontal cortex (Fig. 8A), and a $B_{\text{max}}$ of 7.42 $\pm$ 0.70 pmol/mg protein and a $K_D$ of 4.6 $\pm$ 0.5 nM for recombinant human CRF$_1$ receptors expressed in HEK293e cells (Fig. 8B), respectively. In comparison, expression of CRF$_1$ receptors in the recombinant CRF$_1$ HEK293 cell line is remarkably higher, by 50-fold, than that in the rat frontal cortex, but with identical affinity of this radioligand for rat and human CRF$_1$ receptors. The transformation of saturation data generated a monophasic Scatchard plot (Fig. 8, insets), suggesting a single affinity binding of $[^3H]$SN003 that was consistent with the characteristic of antagonist binding. These data indicate that the affinity of $[^3H]$SN003 for CRF$_1$ receptors is independent of the coupling state of receptors. Specific binding of $[^3H]$SN003 to rat frontal cortex and CRF$_1$ cell homogenates was approximately 50% and 90%, respectively, of total binding at the radioligand concentration equal to its $K_D$ value.

$[^3H]$SN003 association and dissociation studies were performed at a 4.8 nM concentration of the ligand in human CRF$_1$ HEK293 cell homogenates using 5 to 8 $\mu$g of protein at 23°C. $[^3H]$SN003 binding kinetics were monophasic (Fig. 9, insets), with mean $t_{1/2}$ values of 27.2 $\pm$ 9.2 min for association and 60.7 $\pm$ 8.8 min for dissociation obtained from three experiments. As kinetic constants derived from linear regression (Fig. 9, insets), the slope yielded an observed association rate constant ($k_{\text{obs}}$) of 4.22 $\pm$ 0.70 $\times$ 10$^{-2}$ min$^{-1}$ and a dissociation rate constant ($k_{-1}$) of 0.592 $\pm$ 0.20 $\times$ 10$^{-2}$ min$^{-1}$, respectively. The association rate constant ($k_{+1}$) of 0.292 nM$^{-1}$ min$^{-1}$ was calculated for SN003 using the equation described under Materials and Methods. The equilibrium dissociation constant ($K_D$) of 3.4 nM was calculated from kinetic studies, which is comparable with the $K_D$ of 4.8 nM derived from saturation analysis.
To further distinguish antagonist and agonist behaviors in binding experiments, the effect of a nonhydrolyzable GTP analog, Gpp(NH)p (50 μM), on specific binding of [3H]SN003 (5 nM) and [125I]oCRF (150 pM) was examined. As depicted in Fig. 10, addition of Gpp(NH)p in reaction mixtures resulted in a marked decrease (55 ± 1%) of specific [125I]oCRF binding as compared with the control (p = 0.0001 by Student’s t test; n = 5). In contrast to [125I]oCRF binding, the [3H]SN003 binding was not affected by inclusion of 50 μM Gpp(NH)p (p = 0.85 by Student’s t test; n = 4). The reduction of [125I]oCRF binding is consistent with the elimination of high-affinity sites of agonist binding via uncoupling of G proteins to CRF1 receptors in the presence of Gpp(NH)p. In contrast, [3H]SN003 binding was insensitive to guanine nucleotides, demonstrating characteristics of antagonist binding.

In initial [3H]SN003 competition experiments performed in human CRF1 HEK293e cell homogenates, hrCRF was used as a competitor and DMP696 was included as the reference compound. It was of particular interest that hrCRF was able to inhibit [3H]SN003 binding, but the maximal reduction of binding was less compared with that achieved by DMP696. To further characterize the reciprocal interactions between CRF-related peptides and the small molecule antagonist at CRF1 receptors, the displacement of [3H]SN003 binding by CRF peptides including two peptide antagonists and another small molecule antagonist, DMP904, was assessed. Several key observations were made from these studies. First, maximal inhibitions provided by DMP696 and DMP904 did not vary substantially between inhibitions of the small molecule [3H]SN003 and peptide [125I]oCRF binding, although K_i values of small molecules derived from [3H]SN003 binding were slightly lower than that determined from [125I]oCRF bound.
ing. As shown in Fig. 1, these small molecules share a somewhat structural similarity in contrast to the structure difference between small molecules and peptides. Second, hrCRF led to partial inhibition of [$^{3}$H]SN003 binding with a mean maximum suppression of 67 ± 3% when compared with the level of 100% inhibition by DMP696 in the experiments ($p < 0.001$ by Student’s $t$ test). In addition, hrCRF was 10-fold less potent ($p < 0.01$ by Student’s $t$ test) in competition with [$^{3}$H]SN003 binding (mean $K_i = 14.1$ nM) as compared with inhibition of [$^{125}$I]oCRF binding (mean $K_i = 1.4$ nM). The same case was observed with oCRF (Table 2). Third, the peptide antagonists $\alpha$-helical CRF and $\delta$-PheCRF, which lack the N-terminal sequence of CRF, were inactive up to 1 M concentrations, as shown in Fig. 11 and Table 2. Identical results and inhibition patterns were also seen with rat CRF$\alpha$ receptors natively expressed in the frontal cortex tissue, such as the one presented in Fig. 11, performed in the recombinant CRF$\alpha$ HEK293 cell line, with maximal inhibition of [$^{3}$H]SN003 binding by DMP696 (100%), hrCRF (66%), and $\alpha$-helical CRF or $\delta$-PheCRF (0%)

To further extend these observations, we determined whether partial inhibition of [$^{3}$H]SN003 binding is a general characteristic of CRF-related peptide agonists at human CRF$\alpha$ receptors expressed in HEK293e cells. Twelve-point ($10^{-12}$-$10^{-6}$ M) inhibition curves were generated using rat and human urocortin I, hrCRF, ovine CRF, and sauvagine in each cell line, with maximal inhibition ranging from 58 to 84% as compared with small molecule CRF$\alpha$ antagonists (DMP696, DMP904, and CP-154,526), which led to complete displacement of specific [$^{3}$H]SN003 binding. Similar to the earlier studies, $\alpha$-helical CRF and $\delta$-PheCRF were inactive up to 1 M in this experiment. For comparison, binding data for CRF peptides and nonpeptide CRF$\alpha$ antagonists against [$^{125}$I]oCRF peptide and [$^{3}$H]SN003 binding, respectively, were summarized in Table 2. Unlike small molecules, an increase in endogenous peptide concentrations did not eventually provide a complete displacement of specific [$^{3}$H]SN003 binding, suggesting that CRF does not interact with the small molecule CRF$\alpha$ antagonist, SN003, at the CRF$\alpha$ receptor in a simple competitive manner.

**Anatomical Distribution of [$^{3}$H]SN003 Binding Sites.** The distribution of specific [$^{3}$H]SN003 binding sites in slide-mounted rat brain sections was assessed and compared with that of [$^{125}$I]sauvagine binding sites. Figure 12A exhibits storage phosphorimages of [$^{3}$H]SN003 binding in the absence and presence of 1 M DMP696. Specific [$^{3}$H]SN003 binding was measured by digitally subtracting nonspecific binding (Fig. 12A, right panel), defined by 1 M DMP696, from total binding (Fig. 12A, left panel). In the cortex and other brain regions, in which there were higher levels of binding, specific binding accounted for 50 to 60% of the total binding. [$^{3}$H]SN003 binding sites appeared as granular particles with space resolution close to that labeled with [$^{125}$I]sauvagine but not in lateral septal nucleus and choroid plexus.

Figure 12B shows the distribution pattern of specific

**Table 2**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>[$^{3}$H]SN003 Mean $K_i$ (nM)</th>
<th>[$^{3}$H]SN003 Mean Maximum Inhibition</th>
<th>[$^{125}$I]oCRF Mean $K_i$ (nM)</th>
<th>[$^{125}$I]oCRF Mean Maximum Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMP696 (Control)</td>
<td>1.8 ± 0.2</td>
<td>100 ± 1(8)</td>
<td>2.8 ± 0.5</td>
<td>100 ± 2(6)</td>
</tr>
<tr>
<td>SN003</td>
<td>4.9 ± 0.5</td>
<td>100 ± 1(8)</td>
<td>6.8 ± 0.8</td>
<td>101 ± 1(0)</td>
</tr>
<tr>
<td>DMP904</td>
<td>0.4 ± 0.1</td>
<td>100 ± 2(6)</td>
<td>1.0 ± 0.1</td>
<td>100 ± 1(4)</td>
</tr>
<tr>
<td>CP-154,526</td>
<td>0.4 ± 0.1</td>
<td>100 ± 2(3)</td>
<td>1.2 ± 0.5</td>
<td>99 ± 2(3)</td>
</tr>
<tr>
<td>hrCRF</td>
<td>14.1 ± 1.9</td>
<td>67 ± 3(9)</td>
<td>1.4 ± 0.2</td>
<td>99 ± 3(3)</td>
</tr>
<tr>
<td>oCRF</td>
<td>23.1 ± 6</td>
<td>58 ± 8(3)</td>
<td>3.3 ± 0.3</td>
<td>100 ± 1(4)</td>
</tr>
<tr>
<td>$\alpha$-Helical CRF</td>
<td>&gt;1000</td>
<td>3 ± 1(7)</td>
<td>7.9 ± 1.5</td>
<td>99 ± 7(6)</td>
</tr>
<tr>
<td>$\delta$-PheCRF</td>
<td>&gt;1000</td>
<td>2 ± 2(7)</td>
<td>22.4 ± 3.8</td>
<td>96 ± 8(3)</td>
</tr>
</tbody>
</table>
the absence (left panel) and presence (right panel) of 1/2H9262 expressed in HEK293e cell membranes by small molecule CRF1 antagonists and CRF peptides. Nonspecific binding was defined by 1 μM DMP696. Shown is a representative measurement of six to nine independent experiments performed in duplicate.

Fig. 11. Competition of [3H]SN003 binding to human CRF1 receptors and CRF peptides. Nonspecific binding was defined by 1 μM DMP696. Shown is a representative measurement of six to nine independent experiments performed in duplicate.

Fig. 12. Storage phosphate images showing [3H]SN003 binding sites in the rat brain. A, [3H]SN003 binding to coronal sections of the rat brain in the absence (left panel) and presence (right panel) of 1 μM DMP696. B, distribution pattern of [3H]SN003 binding sites in the brain slice section. Olf, olfactory bulb; FC, frontal cortex; CP, caudate-putamen; Pi, pituitary; PC, parietal cortex; Hi, hippocampus, Th, thalamus; PAG, periaqueductal gray; Ceb, cerebellum. Scale bar = 2 mm.

[3H]SN003 binding sites in the rat brain. Abundant [3H]SN003 binding was found throughout the cortical regions and was especially intense in the prefrontal, somatosensory, cingulate, and entorhinal cortex. High levels of binding were also apparent in the olfactory bulb and cerebellar cortex, which is consistent with previous findings that CRF1 receptors highly expressed in these areas, and their functional role and relevance, are not well understood. In the neocortex, the binding density was clearly higher in lamina IV than in other laminae. Among subcortical regions, moderate to high densities were seen in the amygdala, especially its basolateral portion, the nucleus accumbens, and the hippocampus. Fairly uniform, moderate densities of [3H]SN003 binding sites were present in the caudate-putamen and thalamus. [3H]SN003 binding density was low in the hypothalamus but high in the anterior portion of the pituitary. In the brainstem, low to moderate levels of binding sites were seen in the superior and inferior colliculi, the periaqueductal gray, and the dorsal raphe nucleus.

We compared the binding pattern and density of [3H]SN003 with those of [125I]sauvagine in the brain. In the lateral septal nucleus and the choroid plexus there were dense levels of [125I]sauvagine, but not [3H]SN003, binding sites, which were not displaceable by DMP696, indicating that unlike [125I]sauvagine, [3H]SN003 did not bind to CRF2 receptors, which are predominant in these regions (Primus et al., 1997; Rominger et al., 1998). We further compared the relative density of [3H]SN003 with [125I]sauvagine, as summarized in Table 3. The percentage of the relative density in each brain region was calculated by normalizing the specific binding value with reference to that in the neocortex, where binding density was defined as 100%. In most brain regions, the percentage of the relative binding density of [3H]SN003 was similar to that revealed by [125I]sauvagine. The higher percentage of [125I]sauvagine binding in the hypothalamus reflects a higher density of CRF2 receptors existing in the hypothalamic ventromedial nucleus receptors. Another apparent difference was that in the caudate-putamen, hippocampus, and thalamus, there was moderately higher binding density for [3H]SN003 compared with [125I]sauvagine. Specific CRF receptor sites labeled by [125I]oCRF in these brain regions have been reported previously by De Souza et al. (1985). Interestingly, the density and pattern of specific [125I]oCRF binding in the regions (see Figs. 8 and 9 in De Souza et al., 1985) were essentially the same as those labeled by [3H]SN003 in this study.

Table 3
Percentage of binding density of [3H]SN003 and [125I]sauvagine in the rat brain regions.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>[3H]SN003</th>
<th>[125I]Sauvagine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neocortex (control)</td>
<td>100 ± 4</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>105 ± 9</td>
<td>115 ± 7</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>25 ± 2</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Amygdala</td>
<td>57 ± 8</td>
<td>59 ± 3</td>
</tr>
<tr>
<td>Striatum</td>
<td>32 ± 6</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>Thalamus</td>
<td>29 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>9 ± 2</td>
<td>22 ± 4</td>
</tr>
<tr>
<td>Pituitary</td>
<td>101 ± 12</td>
<td>138 ± 8</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>93 ± 7</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>115 ± 8</td>
<td>86 ± 6</td>
</tr>
</tbody>
</table>

Percentage of binding density in each brain region was calculated by normalizing the specific density value with reference to that in the neocortex (control), which was defined as 100%. Data presented are the mean ± S.E.M. values from three independent experiments using three rat brains. Sets of the adjacent sections were incubated with different radioligands in the absence and presence of 1 μM DMP696.
The present study describes the in vitro pharmacological characteristics of a novel nonpeptide radioligand, \[^{3}H\]SN003, for the rat and human CRF\(_1\) receptors. SN003 is a high-affinity and selective ligand for rat and human CRF\(_1\) receptors having more than 1000-fold selectivity over human CRF\(_{2a}\) receptors. SN003 in vitro functions as a CRF\(_1\) receptor antagonist against CRF-mediated responses. Equilibrium binding studies revealed that specific binding of tritium-labeled SN003 to CRF\(_1\) receptors was saturable, reversible, and of high affinity. Antagonist characteristics of \[^{3}H\]SN003 were also suggested by a single class of \[^{3}H\]SN003 binding sites and insensitivity to guanine nucleotides. The distribution pattern of \[^{3}H\]SN003 binding sites was consistent with CRF\(_1\) receptor mapping in the brain, further supporting the specific labeling of CRF\(_1\) receptors by \[^{3}H\]SN003.

Both SN003 and DMP696 had 2- to 3-fold higher affinities (lower \(K_i\) values) for rat CRF\(_1\) receptors in the frontal cortex than in the pituitary and in recombinant CRF\(_1\) receptors expressed in HEK293e cells. The lower potency of small molecules against CRF binding in the pituitary and the recombinant cell line may be in part attributable to the higher density of CRF\(_1\) receptors in the membranes from these receptor sources. On the other hand, the disparity in inhibition of CRF binding observed for small molecules in these two rat tissues may reflect a differential nature of CRF\(_1\) receptors expressed in various cell types. The differential effects of the small molecule antagonists on CRF binding in rat pituitary and cortical tissues could be the result of differing post-translational modification or accessory proteins of CRF\(_1\) receptors from various cell types (Grigoriadis and De Souza, 1988). Differential glycosylation of CRF\(_1\) receptor proteins in anterior pituitary and brain tissues during post-translational modification (Grigoriadis and De Souza, 1989) has been demonstrated. Additional experiments are required to determine whether differential post-translational modifications of CRF\(_1\) receptors have functional consequences.

In functional assays of CRF-mediated ACTH release and cAMP accumulation, SN003 completely antagonized CRF effects without partial or inverse agonist properties. We noted that the functional potency of SN003 as determined in the ACTH assay was approximately 10-fold lower than its binding affinity at rat pituitary CRF\(_1\) receptors. This weaker potency in functional assays is also seen with other GPCR antagonists and, more specifically, CRF\(_1\) antagonists (Bymaster and Falcone, 2000; Heinrichs et al., 2002). The discrepancy between binding and functional potencies of SN003 is not well understood. It could result, in part, from different assay conditions in the functional and binding assays, in particular, the use of intact cells versus membrane preparations and physiological buffer versus hypotonic buffer (Bymaster and Falcone, 2000). Besides assay conditions, differential properties of small molecule CRF antagonists such as their binding affinity and coupling, as well as competitive versus noncompetitive nature, may also have an impact on the potency of the difference between binding and functional activity of CRF ligands in the hypothalamic-pituitary-adrenal axis.

The present work suggests that SN003 is not simply competitive with CRF at the CRF\(_1\) receptor. The maximal number of binding sites for CRF is significantly reduced by SN003 in a concentration-dependent manner, indicative of a noncompetitive interaction. Incomplete reciprocal binding inhibition of peptide agonists and the small molecule antagonist also suggests that the interaction is not simply competitive at CRF\(_1\) receptors; at least SN003 may provide mixed competitive and/or noncompetitive inhibition. Partial inhibition could result from allosteric modulation and noncompetitive inhibition by small molecules or accessibility of peptides to the small molecule binding domains. The underlying mechanisms need to be explored in a future investigation.

These data indicate that the recognition sites for small molecule antagonists and peptide agonists are not mutually exclusive on CRF\(_1\) receptors. In general, the peptide agonist binding domain of class B receptors of the GPCR family, such as CRF\(_1\) receptors, is predominantly formed from the large extracellular N-terminal domain and portions of the extracellular loops of the receptors. Small molecule CRF\(_1\) antagonists appear to bind to transmembrane sites in a pocket formed by helices III to VII (Liaw et al., 1997). It is possible that, depending on the affinity state of the receptor, limited overlap of binding valencies may result in a low-affinity interaction of the N-terminal sequence of CRF with the small molecule binding site. The fact that CRF peptide antagonists without N-terminal amino acids fail to inhibit \[^{3}H\]SN003 binding suggests that it is the N-terminus of CRF that interacts with the small molecule recognition sites of the receptor, possibly sharing binding valences in the transmembrane region. The findings of previous studies demonstrating that the N-terminus of CRF is important for receptor activation (Vale et al., 1981; De Souza, 1987; Beyermann et al., 1996) may be relevant to the present findings in which the N-terminus of CRF was also crucial for CRF inhibition of small molecule binding.

Further studies employing radiolabeled CRF peptides and small molecule antagonists will greatly aid understanding of molecular aspects of CRF\(_1\) receptor signaling and small molecule antagonism. Additionally, this information may have implications for other G protein-coupled receptors with endogenous peptide agonists. This does appear to be the case for the neurokinin-1 and -2 receptors, since partial inhibition and low potencies of endogenous peptides, substance P and neurokinin A, in displacement of small molecule antagonist radioligand binding (Rosenkilde et al., 1994) have been observed with those receptors. Considered together, the data suggest that the interactions between small molecules are more competitive than that between the small molecule and peptide, and binding domains for peptides and small molecule antagonists are not mutually exclusive.

Two nonpeptide CRF\(_1\) antagonists, CP-154,526 and an antalarmin analog, have recently been radiolabeled (Tian et al., 2001; Keller et al., 2002). The tritium-labeled CP-154,526 was used for assessing pharmacokinetics and blood-brain barrier penetration, and the antalarmin analog was developed as a positron emission tomography ligand. However, in these studies there are no descriptions of radioligand binding profiles in either tissues or cells expressing CRF\(_1\) receptors. Tritiated SN003 is the first nonpeptidic CRF\(_1\) antagonist radioligand shown to be capable of de-
testing specific binding in rat brain tissues. The binding pattern of $[^3H]SN003$ in the brain was consistent with the distribution of CRF$_1$ receptors revealed previously by binding autoradiography using the radioligand $[^3H]OICRF$ (De Souza et al., 1985; Aguiler a et al., 1987) or by in situ hybridization of CRF$_1$ mRNA (Potter et al., 1994). The sensitivity of $[^3H]SN003$ binding was moderate considering that it was 50 to 60% specific over total binding in both the homogenized membrane and slide-mounted brain section binding assays. The moderate sensitivity was, perhaps, caused by several factors, including the "sticky" lipophilic property of SN003 and the low energy status of the labeled isotope tritium. In addition, storage phosphorimaging used in the autoradiography study may have compromised the sensitivity in terms of space resolution. However, this technique proved to be very effective for acquiring images marked with tritium-labeled ligands such as $[^3H]SN003$ in as little as 7 days, compared with longer exposure times required for autoradiographic films. These studies may provide a foundation for studying in vivo binding of CRF$_1$ antagonists and developing high-energy isotope-labeled, small molecule ligands with applications in clinical positron emission tomography/single photon emission computed tomography studies of CRF$_1$ antagonists.

In summary, we describe the in vitro characterization of a high-affinity, selective nonpeptide antagonist radioligand, $[^3H]SN003$, for CRF$_1$ receptors. Since this radioligand possesses an excellent signal/noise ratio in a recombinant human CRF$_1$ cell line and distinct features compared with peptide ligands, it provides a useful tool to understand small molecule antagonism of CRF at CRF$_1$ receptors. This information may prove helpful in drug design and development of small molecule CRF$_1$ antagonists for treatment of affective disorders and stress-related diseases.

Acknowledgments

We would like to thank Dr. Rebecca Taub for review and helpful suggestions on the manuscript.

References


Address correspondence to: Dr. Ge Zhang, The Bristol-Myers Squibb Company, Building 21/Room 2344A, 311 Pennington-Rocky Hill Rd., Hopewell, NJ 08524. E-mail: ge.zhang@bms.com