Pharmacological Characterization of a Novel Nonpeptide Antagonist Radioligand, (±)-N-[2-Methyl-4-methoxyphenyl]-1-(1-(methoxymethyl) propyl)-6-methyl-1H-1,2,3-triazolo[4,5-c]pyridin-4-amine ([3H]SN003) for Corticotropin-Releasing Factor₁ Receptors

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ABSTRACT

The in vitro pharmacological profile of a novel small molecule corticotropin-releasing factor 1 (CRF₁) receptor antagonist, (±)-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 ([3H]SN003) are described. SN003 has high affinity and selectivity for CRF₁ receptors expressed in rat cortex, pituitary, and recombinant HEK293EBNA (HEK293e) cells with respective radiolabeled ovine CRF ([125I]oCRF) binding Kᵦ values of 2.5, 7.9, and 6.8 nM. SN003 was shown to be a CRF₁ receptor antagonist inasmuch as it inhibited CRF-induced cAMP accumulation in human CRF₂,HEK293e cells and CRF-stimulated adrenocorticotropin hormone release from rat pituitary cells without agonist activities. Significant decreases in the Bₘₐₓ of [125I]oCRF binding by SN003 suggest that this antagonist is not simply competitive. To further explore the interaction of SN003 with the CRF₁ receptors, [3H]SN003 binding to rat cortex and human CRF₁,HEK293e cell membranes was characterized and shown to be reversible and saturable, with Kᵦ values of 4.8 and 4.6 nM, and Bₘₐₓ values of 0.142 and 7.42 pmol/mg protein, respectively. The association and dissociation rate constants of [3H]SN003 (kₒ, 0.292 nM⁻¹ min⁻1 and kᵦ, 0.992 × 10⁻² min⁻¹) were also assessed using human CRF₁,HEK293e cell membranes, giving an equilibrium dissociation constant of 3.4 nM. Moreover, [3H]SN003 binding displayed a single affinity state and insensitivity to 5'-guanylylimidodiphosphate, consistent with characteristics of antagonist binding. Incomplete inhibition of [3H]SN003 binding by CRF peptides also suggests that SN003 is not simply competitive with CRF at CRF₁ receptors. The distribution of [3H]SN003 binding sites was consistent with expression pattern of CRF₁ receptors in rat brain regions. Small molecule CRF₁ antagonist radioligands like [3H]SN003 should enable a better understanding of small molecule interactions with the CRF₁ 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, im...
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, CRF$_1$ and CRF$_2$. 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 G$_s$ proteins to cAMP signaling, CRF$_1$ and CRF$_2$ receptors differ markedly from each other in pharmacological properties and anatomical distribution (DeSouza et al., 1998; Gilligan et al., 2000; Dautzenberg and Hauger, 2002). CRF$_1$ receptors are widely distributed in the central nervous system. There exist three splice variants of the CRF$_2$ receptor (CRF$_{2a}$, CRF$_{2b}$, and CRF$_{2c}$) with distinct anatomical localization. CRF$_{2a}$ receptors are primarily located in discrete rat brain areas such as lateral septum, and CRF$_{2b}$ receptors are located in rat choroid plexus, heart, lung, and skeletal muscle. CRF$_1$ and CRF$_2$ 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 CRF$_1$ and CRF$_2$ receptors.

Studies of the neuronal circuitry mediating fear and anxiety states (Davis, 1992) suggest that both CRF$_1$ and CRF$_2$ receptors located in differential brain areas may be involved in the regulation of various stress-induced behaviors, albeit the relative importance of CRF$_2$ 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 CRF$_1$ 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, nonpeptidic CRF$_1$ antagonists to evaluate the putative role of CRF$_1$ receptors in psychopathology and to test their potential as novel therapeutic agents. Schulz et al. (1996) were the first to report a pyrazolopyrimidine CRF$_1$ antagonist, CP-154,526, with high affinity for the CRF$_1$ receptor and anxiolytic activity in rats. Additional CRF$_1$ antagonists, such as antalarmin, 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 CRF$_1$ 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 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 ([H]SN003), a small-molecule radioligand for rat and human CRF$_1$ receptors. The binding characteristics of [H]SN003 were profiled in rat cortical and human CRF$_1$ cell membranes, and the specificity and anatomic distribution of [H]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 CRF$_1$ 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 CRF$_1$ 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 CRF$_1$ 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 CRF$_1$ 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 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 ([H]SN003), a small-molecule radioligand for rat and human CRF$_1$ receptors. The binding characteristics of [H]SN003 were profiled in rat cortical and human CRF$_1$ cell membranes, and the specificity and anatomic distribution of [H]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 CRF$_1$ 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 CRF$_1$ receptors.

Fig. 1. Chemical structures of SN003 and other small molecule CRF$_1$ 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.
CRF$_{2-41}$ (α-helical CRF), and [δ-Phe$^{12}$,Nle$^{21-38}$,C$^{37}$-MeLeu$^{37}$]-CRF$_{12-41}$ (δ-Phe-CRF), were purchased from American Peptide Co., Inc. (Sunnyvale, CA), Bachem California, (Torrance, CA), and Peninsula Laboratories (Missenhead, UK). 1,25I]oCRF and 1,25I]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 [H]SN003.** [H]SN003 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. Trifluoroacetic methyl iodide (250 μl, 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). Fractons containing the product were lyophilized to provide radiochemically pure compound 2 ([H]SN003, purity >99%). [H]SN003 was dissolved and stored as an ethanolic solution (1 mCi/ml; specific activity 74 Ci/mmol).

**Cell Culture of HEK293e Cells Expressing Human CRF Receptors.** Full-length human cDNAs for human CRF$_1$ and CRF$_{2a}$ receptors were subcloned into plasmids and transfected into HEK293EBNA (HEK293e) cells (Invitrogen) using lipofectamine (Intravent). 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$_1$ or CRF$_{2a}$ receptors were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C in a humid environment (5% CO$_2$) for 10 days. 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$^7$ 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$_1$ or CRF$_{2a}$ receptors. Fresh tissues or cell pellets were thawed on ice and homogenized in tissue buffer (containing 50 mM HEPES, 10 mM MgCl$_2$, 2 mM EGTA, and 1 mM EDTA) at 4°C. The homogenate was centrifuged at 48,000 rpm for 4 min. Cells were resuspended in 100% stimulation buffer, and the cell pellet (containing approximately 1 × 10$^7$ HEK293e cells) was stored at -80°C until use.

**Equilibrium competition binding experiments.** To determine the nature of inhibition (competitive and noncompetitive) of SN003, homologous isotopic displacement of [125I]oCRF (150 pM) binding by ovine CRF was conducted in membranes prepared from HEK293e cells expressing human CRF$_1$ receptors in the absence and presence of SN003. The K$_i$ and B$_{max}$ values from homologous competition curves were calculated using the nonlinear regression analysis in Prism (1999; GraphPad Software, Inc., San Diego, CA). A Scatchard plot of the homologous binding with [125I]oCRF was generated for visualization of any K$_i$ or B$_{max}$ changes. Specific bind (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.).

**[H]SN003 Binding to Membranes.** Equilibrium binding experiments in cell or tissue homogenates were performed under conditions similar to those described for the [125I]oCRF binding with a few exceptions. GF/B filters were used in the filtration assay. The saturation experiments using [H]SN003 as a radioligand and rat cortex and HEK293e cell homogenates as CRF$_1$ receptor sources were conducted in 12 concentrations of [H]SN003 (0.60–40 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 [H]SN003 K$_0$ 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$_1$ Cells.** Intracellular cAMP levels were measured using the Adenyl cyclase Activation Flash Plate kit purchased from PerkinElmer Life Sciences. This radiomimunoassay-based kit enables direct detection of cAMP generated in live cells in a 96-well format. HEK293e cells expressing CRF$_1$ receptors were grown in the Dulbecco’s modified Eagle’s medium supplement with 10% fetal bovine serum, 1-glutamine (2 mM), and hygromycin (400 μg/ml) at 37°C in a humid environment with 5% CO$_2$. On the assay day, cells were dissociated from flasks and centrifuged down at 1,200 rpm for 4 min. Cells were resuspended in 100% stimulation buffer, counted, and diluted to 0.6 × 10$^5$ cell/ml hCrF (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$_1$ 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 [125I]cAMP (100 μl/well). Assay signal is based on competition of endogenous cAMP and [125I]cAMP for CAMP antibodies coated on the Flash Plate. Radioactivity from binding of [125I]cAMP 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 μg/ml DNase II, and 0.4% (w/v) collagenase type 2) for 3 h at 37°C. Cells were spun down, decanted, and incubated with 0.25% trypsin solution.
linear regression of transformed data. The association rate constant were generated from a slope of the line via the Cheng-Prusoff equation. Saturation data (GraphPad Software, Inc.). 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.). Kinetic studies to determine Kd (obs) (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 (k+1) was generated based on the equation k+1 = (kobs − k−1)/[L], where L is the ligand concentration. The equilibrium dissociation constant derived from kinetic studies was calculated as Kd = k−1/k+1.

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), and dried, and stored at −20°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 [3H]SN003 for 2 h at 23°C. As a comparison, one set of adjacent sections was incubated with 200 pM [125I]sauvagine. At this concentration (200 pM), [125I]sauvagine 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 ([3H]SN003) or for 12 h ([125I]sauvagine), respectively. The screens were then scanned with a Cyclone phosphorimaging scanner, and captured images were analyzed with the OptiQuant 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 [125I]oCRF (200 pM) binding to CRF1 receptors, with maximal inhibition identical to that of α-helical CRF and DMP696. The Kd 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 Kd 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 Kd = 0.6 nM) and pituitary (mean Kd = 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...
(mean $K_i = 5.3 \text{ nM}$) and pituitary (mean $K_i = 8.1 \text{ nM}$) tissues, this difference was not statistically significant ($p = 0.22$), as summarized in Table 1.

Figure 3A shows that SN003 specifically inhibited $[^{125}\text{I}]\text{oCRF}$ binding to human CRF$_1$ receptors expressed in HEK293e cells with high affinity ($K_i = 6.8 \pm 0.8 \text{ nM}$) like DMP696 ($K_i = 2.8 \pm 0.5 \text{ nM}$) and $\alpha$-helical CRF$_{9-41}$ ($K_i = 7.9 \pm 1.5 \text{ nM}$). The specificity of SN003 for the CRF$_1$ receptor was also assessed by examining its ability to compete with $[^{125}\text{I}]\text{sauvagine}$ binding to the human CRF$_2$ receptors. As depicted in Fig. 3B, $\alpha$-helical CRF was a potent inhibitor of $[^{125}\text{I}]\text{sauvagine}$ binding to human CRF$_{2\alpha}$ receptors ($K_i = 11.2 \pm 1.8 \text{ nM}$), whereas SN003 was inactive at the CRF$_{2\alpha}$ receptor up to 10 $\mu\text{M}$ concentrations ($n = 3$). The data indicate that SN003 is highly selective in its affinity for CRF$_1$ receptors, being approximately 1000 times more potent against $[^{125}\text{I}]\text{oCRF}$ binding to human CRF$_1$ ($K_i = 6.8 \text{ nM}$) than against $[^{125}\text{I}]\text{sauvagine}$ binding to human CRF$_{2\alpha}$ ($K_i > 10 \mu\text{M}$).

Homologous competition experiments were conducted to determine SN003 effects on the $K_D$ and $B_{\text{max}}$ values of oCRF binding in the human CRF$_1$-HEK293e cell membranes. The Scatchard plot in Fig. 4 shows that SN003 dramatically decreased the $B_{\text{max}}$ (the x-intercept) to $85.3 \pm 1.9\%$, $67.4 \pm 10.7\%$, and $46.4 \pm 3.0\%$ of the control value without change of $K_D$ (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 CRF$_1$ 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 by response to CRF in a concentration-dependent manner. CRF-induced maximal stimulation of ACTH release (3490–4093 pg/100-$\mu\text{L}$ sample), which is 5- to 9-fold of the basal level (400–520 pg/100-$\mu\text{L}$ sample), was achieved at 10 nM CRF during a 3-h incubation ($n = 4$). The mean $E_{\text{max}} \pm \text{S.E.M.}$ 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 $IC_{50}$ 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 CRF$_1$ receptors. To test potential agonist activity, SN003 (1 and 10 $\mu\text{M}$), 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 CRF$_1$ receptors were examined in HEK293e cells expressing recombinant human CRF$_1$ 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 secretion with vehicle treatment is defined as 0% stimulation. B, the antagonism of CRF (0.3 nM)-stimulated ACTH secretion by SN003. Rat pituitary cells were exposed to CRF (0.3 nM) and/or various concentrations of SN003 for 3 h at 37°C. ACTH levels were normalized to percentage of control (0.3 nM hrCRF alone). The mean ± S.E.M. values were obtained with a statistical analysis of four individual EC_{50} or IC_{50} determinations.
response curves with no significant decrease in CRF efficacy \( (E_{\text{max}}) \) by SN003 (antagonist potency constant \( K_i = 58 \pm 3 \text{ nM} \)). 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 \( (\uparrow E_{\text{max}}) \) in a concentration-dependent manner, and high concentrations of SN003 (1 and 3 \( \mu \text{M} \)) significantly decreased \( E_{\text{max}} \) to 89 \( \pm \) 2\% and 77 \( \pm \) 3\%, respectively, of the control level in the Y-79 cell line \((n = 3); p < 0.01 \text{ by Student’s } t \text{ test}; \ast, p < 0.05 \) was considered as significant. \( \ast \ast, p < 0.01; \ast \ast \ast, p < 0.001 \).

Characterization of \( ^{[3]}\text{H} \text{SN003} \) Binding to Membranes Expressing CRF\(_1\) Receptors. To examine the binding characteristics of \( ^{[3]}\text{H} \text{SN003} \) to CRF\(_1\) receptors, a time course of \( ^{[3]}\text{H} \text{SN003} \) (\( 4.8 \text{ nM} \)) equilibrium binding was performed in rat frontal cortex homogenates at 23\(^\circ\)C. Under these conditions, \( ^{[3]}\text{H} \text{SN003} \) 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 \( k_{\text{obs}} \) \((1.62 \pm 0.3 \times 10^{-2} \text{ min}^{-1}) \) 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), \( ^{[3]}\text{H} \text{SN003} \) 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 \( ^{[3]}\text{H} \text{SN003} \) that was consistent with the characteristic of antagonist binding. These data indicate that the affinity of \( ^{[3]}\text{H} \text{SN003} \) for CRF\(_1\) receptors is independent of the coupling state of receptors. Specific binding of \( ^{[3]}\text{H} \text{SN003} \) 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.

\( ^{[3]}\text{H} \text{SN003} \) association and dissociation studies were performed at a 4.8 nM concentration of the ligand in human CRF\(_1\), HEK293e cell homogenates using 5 to 8 \( \mu \text{g} \) of protein at 23\(^\circ\)C. \( ^{[3]}\text{H} \text{SN003} \) 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 2.42 \( \pm \) 0.6 \( \times \) \( 10^{-2} \) min\(^{-1}\) and a dissociation rate constant \( (k_{-1}) \) of 0.992 \( \pm \) 0.05 \( \times \) \( 10^{-2} \) 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% by Student’s t test; n = 5) of specific [125I]oCRF binding as compared with the control. In contrast to [3H]SN003 binding, the specific binding at time t, B, represents specific binding at time t and B0 represents binding at equilibrium. Kinetic data were fit to mono- and biexponential curves by weighted nonlinear curve-fitting; in all cases the biexponential model did not give a significant improvement over the monoexponential model (p > 0.05; F-test). Nonspecific binding was defined as that occurring in the presence of 5 μM DMP696. Data shown are the mean from three independent experiments performed in triplicate.

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 KI values of small molecules derived from [3H]SN003 binding were slightly lower than that determined from [125I]oCRF binding.
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 [3H]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 [3H]SN003 binding (mean $K_i = 14.1$ nM) as compared with inhibition of [125I]oCRF binding (mean $K_i = 1.4$ nM). The same case was observed with oCRF (Table 2). Third, the peptide antagonists α-helical CRF and D-PheCRF, which lack the N-terminal sequence of CRF, were inactive up to 1 μM concentrations, as shown in Fig. 11 and Table 2. Identical results and inhibition patterns were also seen with rat CRF1 receptors natively expressed in the frontal cortex tissue, such as the one presented in Fig. 11, performed in the recombinant CRF1 HEK293 cell line, with maximal inhibition of [3H]SN003 binding by DMP696 (100%), hrCRF (66%), and α-helical CRF or D-PheCRF (0%), respectively.

To further extend these observations, we determined whether partial inhibition of [3H]SN003 binding is a general characteristic of CRF-related peptide agonists at human CRF1 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 sauavagine in the [3H]SN003 binding assay as described above. The peptide agonists all exhibited incomplete inhibition of specific [3H]SN003 binding to human CRF1 receptors with maximal inhibition ranging from 58 to 84% as compared with small molecule CRF1 antagonists (DMP696, DMP904, and CP-154,526), which led to complete displacement of specific [3H]SN003 binding. Similar to the earlier studies, α-helical CRF and D-PheCRF were inactive up to 1 μM in this experiment. For comparison, binding data for CRF peptides and nonpeptide CRF1 antagonists against [125I]oCRF peptide and [3H]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 [3H]SN003 binding, suggesting that CRF does not interact with the small molecule CRF1 antagonist, SN003, at the CRF1 receptor in a simple competitive manner.

Anatomical Distribution of [3H]SN003 Binding Sites. The distribution of specific [3H]SN003 binding sites in slide-mounted rat brain sections was assessed and compared with that of [125I]sauavagine binding sites. Figure 12A exhibits storage phosphorimages of [3H]SN003 binding in the absence and presence of 1 μM DMP696. Specific [3H]SN003 binding was measured by digitally subtracting nonspecific binding (Fig. 12A, right panel), defined by 1 μM 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. [3H]SN003 binding sites appeared as granular particles with space resolution close to that labeled with [125I]sauavagine but not in lateral septal nucleus and choroid plexus.

Figure 12B shows the distribution pattern of specific

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Table 2
Summary data for peptides and nonpeptide ligands inhibiting [125I]oCRF and [3H]SN003 binding to human CRF1 receptors

<table>
<thead>
<tr>
<th>Compounds</th>
<th>[3H]SN003 Mean $K_i$ (nM)</th>
<th>[3H]SN003 Mean Maximum Inhibition (%)</th>
<th>[125I]oCRF Mean $K_i$ (nM)</th>
<th>[125I]oCRF Mean Maximum Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nM</td>
<td>% control</td>
<td>nM</td>
<td>% control</td>
</tr>
<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>α-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>D-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>

Data shown are the mean (± S.E.M.) from three to nine experiments performed in duplicate. The number of independent experiments performed is shown in parentheses.
the absence (left panel) and presence (right panel) of H9262 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 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**

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

% control

[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.

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Discussion

The present study describes the in vitro pharmacological characteristics of a novel nonpeptide radioligand, \(^{[3]}\text{H}\)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\(_{2\alpha}\) 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 \(^{[3]}\text{H}\)SN003 were also suggested by a single class of \(^{[3]}\text{H}\)SN003 binding sites and insensitivity to guanine nucleotides. The distribution pattern of \(^{[3]}\text{H}\)SN003 binding sites was consistent with CRF\(_1\) receptor mapping in the brain, further supporting the specific labeling of CRF\(_1\) receptors by \(^{[3]}\text{H}\)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 \(^{[3]}\text{H}\)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 valencies 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 CRF1 receptors revealed previously by binding autoradiography using the radioligand [125I]oCRF (De Souza et al., 1985; Aguilera et al., 1987) or by in situ hybridization of CRF1 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 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 CRF1, antagonists and developing high-energy isotope-labeled, small molecule ligands with applications in clinical positron emission tomography/single photon emission computed tomography studies of CRF1 antagonists.

In summary, we describe the in vitro characterization of a high-affinity, selective nonpeptidic antagonist radioligand, [3H]SN003, for CRF1 receptors. Since this radioligand possesses an excellent signal/noise ratio in a recombinant human CRF1 receptor (CRF1) cDNA expression system, it may be useful for high-throughput screening of antagonist modulators. Future studies are needed to evaluate its potential for the in vivo characterization of CRF1 receptors in brain and peripheral tissues.

Acknowledgments

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