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4-(2-Chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine Hydrochloride (SSR125543A): A Potent and Selective Corticotrophin-Releasing Factor$_1$ Receptor Antagonist. I. Biochemical and Pharmacological Characterization

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ABSTRACT

4-(2-Chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine hydrochloride (SSR125543A), a new 2-aminothiazole derivative, shows nanomolar affinity for human cloned or native corticotrophin-releasing factor $(CRF)_1$ receptors (pK_i values of 8.73 and 9.08, respectively), and a 1000-fold selectivity for CRF_1 versus $CRF_{2\alpha}$ receptor and CRF binding protein. SSR125543A antagonizes CRF-induced stimulation of cAMP synthesis in human retinoblastoma Y 79 cells (IC_{50} = 3.0 ± 0.4 nM) and adrenocorticotropin hormone (ACTH) secretion in mouse pituitary tumor AtT-20 cells. SSR125543A is devoid of agonist activity in these models. Its brain penetration was demonstrated in rats by using an ex vivo [^{125}I-Tyr^0] ovine CRF binding assay. SSR125543A displaced radioligand binding to the CRF_1 receptor in the brain with an ID_{50} of 6.5 mg/kg p.o. (duration of action >24 h). SSR125543A also inhibited the increase in plasma ACTH levels elicited in rats by i.v. CRF (4 μg/kg) injection (ID_{50} = 1, 5, or 5 mg/kg i.v., i.p., and p.o., respectively); this effect lasted for more than 6 h when the drug was given orally at a dose of 30 mg/kg. SSR125543A (10 mg/kg p.o.) reduced by 73% the increase in plasma ACTH levels elicited by a 15-min restraint stress in rats. Moreover, SSR125543A (20 mg/kg i.p.) also antagonized the increase of hippocampal acetylcholine release induced by i.c.v. injection of 1 μg of CRF in rats. Finally, SSR125543A reduced forepaw treading induced by i.c.v. injection of 1 μg of CRF in gerbils (ID_{50} = ~10 mg/kg p.o.). Altogether, these data indicate that SSR125543A is a potent, selective, and orally active CRF_1 receptor antagonist.

Corticotrophin-releasing factor (CRF) is the prime coordinator of the neuroendocrine and behavioral responses to stress (Owens and Nemeroff, 1991). This 41-amino acid peptide is the major hypothalamic factor responsible for the stimulation of corticotrophin (ACTH) secretion from the anterior pituitary, which in turn induces synthesis and release of glucocorticoids from the adrenal cortex (Vale et al., 1981). The highest density of CRF-containing cell bodies is found in the medial paraventricular nucleus of the hypothalamus, a brain region that projects to the median eminence (Sawchenko and Swanson, 1991). CRF-containing neurons are also found in extrahypothalamic areas, e.g., limbic structures (Gray and Bingaman, 1996), suggesting that CRF may also play a neurotransmitter role, mediating both stress response and affective behavior (Arborelius et al., 1999). Because CRF hypersecretion associated with overactivation of the hypothalamo-pituitary-adrenal (HPA) axis has been implicated in depression and anxiety, the discovery of nonpeptide molecules that selectively inhibit CRF activity is of major clinical interest (Holsboer, 1999).

ABBREVIATIONS: CRF, corticotropin-releasing factor; ACTH, adrenocorticotropin hormone; HPA, hypothalomo-pituitary-adrenal axis; CRF-BP, corticotropin-releasing factor-binding protein; DMSO, dimethyl sulfoxide; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; ANOVA, analysis of variance; ACh, acetylcholine; R-121919, 3-[6-(dimethylamino)-4-methyl-pyrid-3-yl]-2,5-dimethyl-*N*,*N*-dipropyl-pyrazolo[2,3-*a*]pyrimi-

The functional effects of CRF are mediated via the activation of two receptor subtypes, CRF_1 and CRF_2, that are 70% homologous in their amino acid sequences but appear pharmacologically and anatomically distinct. Both receptor subtypes are members of the G protein-coupled receptor superfamily positively coupled to adenylate cyclase. CRF_1 is the predominant receptor within the pituitary, cerebellum, and neocortex. Two CRF_2 isoforms exist: the $CRF_{2\alpha}$, which is expressed in limbic regions, e.g., lateral septum and dorsal raphe nucleus; and the $CRF_{2\beta}$, more abundant in the periphery (Chalmers et al., 1995). Moreover, a CRF binding protein (CRF-BP) binds native rat/human CRF with higher affinity than CRF receptors (Behan et al., 1995). CRF-BP is expressed in the brain of numerous species, where it might regulate CRF-mediated neurotransmission.

A second CRF receptor endogenous agonist, urocortin, has been described (45% homology with CRF) and binds to CRF_2 receptors with a 10-fold higher affinity than CRF. Urocortin mRNA expression is prominent in the Edinger-Westphal nucleus, which does not contain CRF mRNA and is colocalized with the $CRF_{2\alpha}$ receptor mRNA in the rat lateral septum and dorsal raphe nucleus (Vaughan et al., 1995). Recently, urocortin II, which possesses only 26% homology with CRF, has been cloned and found to be a selective agonist at CRF_2 receptors (Reyes et al., 2001).

The hypothesis that CRF plays a role in the pathophysiology of affective disorders has been put forward on the basis of experimental behavioral data, and is consistent with the contribution of CRF system alterations to the etiology of psychiatric disorders exacerbated or precipitated by stress. Thus, high levels of cerebrospinal fluid CRF and an increased number of CRF immunoreactive neurons in the hypothalamic paraventricular nucleus have been measured in patients with depressive disorders (Nemeroff et al., 1984). After electroconvulsive therapy or antidepressant treatment, HPA axis and CRF function normalize, suggesting that CRF overactivity may be a marker for human depression (Nemeroff et al., 1991). Moreover, intra-amygdala injection of antisense oligonucleotides directed against the CRF_1 and CRF_2 receptor mRNA in the rat and knock out of the CRF_1 receptor gene in mice have been associated with reduced levels of anxiety and lower anxiogenic responses to i.v. CRF injections (Liebsch et al., 1995; Heinrichs et al., 1997; Smagin and Dunn, 2000). Furthermore, CRF_1 receptor antagonists have demonstrated anxiogenic effects in rodents (Gutman et al., 2000).

A number of synthetic CRF_1 receptor antagonists have been identified (Gutman et al., 2000), e.g., butylethyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-yl]-amine (CP-154,526), 5-chloro-*N*-cyclopropylmethyl-2-methyl-*N*-propyl-*N*′-(2,4,6-trichlorophenyl)-pyrimidin-4,6-diamine (NBI 27914), 4-(3-pentylamino)-2,7-dimethyl-8-(2-methyl-4-methoxyphenyl)-pyrazolo-[1,5-*a*]pyrimidine (DMP904), 2-[(*N*-(2-methylthio-4-isopropylphenyl)-*N*-ethylamino]-4-[4-(3-fluorophenyl)-1,2,3,6-tetrahydropyridin-1-yl)-6-methylpyrimidine (CRA 1000), and R-121919 (formerly NBI 30775). However, it is of interest to note that these molecules have close structural similarities, including pyrrolo-, pyrazolo-, and other substituted pyrimidine moieties. The more recent compounds offer better solubility and central nervous system penetration than their predecessors. For example, R-121919 (K_i value of 3

possess pharmacological activity in experimental models of anxiety after oral administration in the 3- to 30-mg/kg range (Gutman et al., 2000). Beneficial effects of this compound have been observed in an open clinical trial performed in depressed patients, supporting the view that CRF_1 receptor antagonism could be of therapeutic value in the treatment of depression. However R-121919's development has been stopped because of hepatic toxicity (Zobel et al., 2000). In the present study, we report on the characterization of a new CRF_1 receptor antagonist, 4-(2-chloro-4-methoxy-5-methylphenyl)-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-amine hydrochloride (SSR125543A), obtained by the optimization of a lead compound discovered by random screening of several thousand chemicals. This compound belongs to the novel 2-aminothiazole chemical family (Fig. 1).

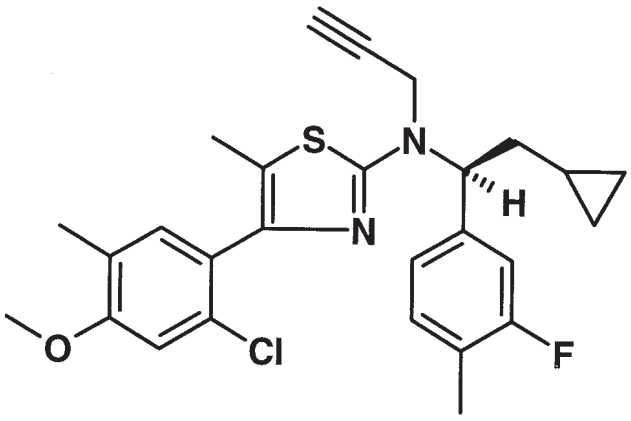
Experimental Procedures

Animals

Male Sprague-Dawley CD rats and female OF1 mice purchased from Iffa Credo (L'Arbresele, France), and male Mongolian gerbils from Janvier (Le Genest St. Isle, France) were housed in a controlled temperature and light-dark environment with water and chow available ad libitum before the experiments. All experimental procedures were approved by the Animal Care and Use Committee of Sanofi-Synthelabo Recherche and were carried out in accordance with French legislation.

Materials

SSR125543A (Fig. 1) and antalarmin were synthesized by Sanofi-Synthelabo Recherche (Toulouse, France). Both compounds were solubilized in pure DMSO for the in vitro assays and in 5% DMSO and 5% Cremophor EL in saline when administered to mouse, rat, and gerbil. Rat/human CRF, ovine CRF, [D-Phe^{11},His^{12}]Svg$_{(11-40)}$ (antisauvagine-30), and rat/human $CRF_{(6-33)}$ from Neosystem (Strasbourg, France) were solubilized in 0.1% acetic acid solution containing 1 mg/ml serum bovine albumin. [^{125}I-Tyr^0] ovine CRF, [^{125}I-Tyr^0] rat/human CRF, and [^{125}I-Tyr^0] sauvagine were purchased from PerkinElmer Life Sciences (Boston, MA). Cell culture media, antibiotics, and fetal calf serum were obtained from Invitro-



, HCl

Fig. 1. Chemical structure of the selective CRF_1 receptor antagonist

gen (Cergy Pontoise, France). All other chemicals were from commercial sources.

Cell Cultures

CHO cells stably transfected with the human CRF_1 receptor ($hCRF_1$-CHO cells) or with the human $CRF_{2\alpha}$ receptor ($hCRF_{2\alpha}$-CHO cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum, 300 μg/ml L-glutamine, nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.17 μg/ml amphothericine. Y 79 cells purchased from American Type Culture Collection (Rockville, MD) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum and 300 μg/ml L-glutamine.

AtT-20 cells purchased from American Type Culture Collection were cultured in Dulbecco's modified Eagle's medium containing only in supplement 10% fetal calf serum, 300 μg/ml L-glutamine, HEPES, and sodium pyruvate. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2 except AtT-20, which was incubated with 15% CO_2.

Preparation of Cell Membrane Homogenates

Cells were cultured to confluence and the flasks were washed with 10 ml of phosphate-buffered saline (PBS) medium and filled with an equal volume of PBS medium. Cells ($hCRF_1$-CHO, $hCRF_{2\alpha}$-CHO, and AtT-20) were detached from the flask with a cell scraper. Y 79 cells were cultured in suspension. After centrifugation at 800*g* for 5 min, the cell pellet was homogenized at 4°C by using a Polytron (setting 6, 2 × 20 s) in 50 mM Tris-HCl pH 7.4, 2 mM EDTA buffer for $hCRF_1$-CHO and $hCRF_{2\alpha}$-CHO cells. Homogenization was performed in 50 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 2 mM EDTA, 0.1% serum bovine albumin, 8 mg/l aprotinin, and 0.5 mg/ml soybean trypsin inhibitor for Y 79 cells. After centrifugation at 40,000*g* for 20 min at 4°C, the pellet was homogenized at 4°C by using a Polytron in binding buffer (see below). Aliquots obtained from the membrane suspension were stored in liquid nitrogen.

Preparation of Brain Membrane Homogenates

Because the in vivo pharmacological profile of the compound was to be characterized in rodents, the inhibitory effects of SSR125543A on [^{125}I-Tyr^0] ovine CRF binding to rat, mouse, and gerbil brain were assessed.

Mouse, rat, and gerbil were sacrificed by decapitation and brains were rapidly removed and homogenized at 4°C by using a Polytron (setting 4, 30 s) in 50 mM Tris-HCl pH 7.4, 2 mM EDTA buffer. After centrifugation at 40,000*g* for 20 min at 4°C, the 0.5-mg/ml pellet was homogenized at 4°C by using a Polytron in binding buffer (see below). Aliquots obtained from the membrane suspension were stored were stored at −80°C.

CRF_1 Receptor Binding Assay

[^{125}I-Tyr^0] ovine CRF binding was performed with $hCRF_1$-CHO cell membranes, Y 79 cell membranes, or rodent brain membrane homogenates in the presence of 25 pM radiolabeled CRF in 50 mM Tris-HCl pH 7.2, 10 mM $MgCl_2$, 2 mM EDTA, 0.1% serum bovine albumin, 8 mg/l aprotinin, and 0.5 mg/ml soybean trypsin inhibitor under a final volume of 400 μl. Nonspecific binding was determined in the presence of 1 μM rat/human CRF. Agonists and antagonists were added in 1% DMSO (final concentration). After incubation at 20°C for 2 h, the incubation mixture was filtered on Whatman GF/B filters presoaked in 0.5% bovine serum albumin solution for 2 h. The filters were washed twice with ice-cold Tris-HCl pH 7.2 buffer and the radioactivity was determined with a gamma scintillation counter (LKB 1261 multi gamma; EG G Instruments, Evry, France). Specific binding was determined as the difference between total and nonspecific binding. IC_{50} values were determined using a nonlinear least-square regression analysis (Munson and Rodbard, 1980) with RS/1 (BBN Software Product Corporation, Cambridge, MA) and an internal computerized interactive procedure.

CRF_2 Receptor Binding Assay

[^{125}I-Tyr^0] sauvagine binding was performed using a similar protocol as with [^{125}I-Tyr^0] ovine CRF binding. In this case, $hCRF_{2\alpha}$-CHO cell membranes were used at the concentration of 2.5 μg of protein/tube in presence of 20 pM radiolabeled sauvagine, under a final volume of 250 μl. Nonspecific binding was determined in presence of 1 μM unlabeled sauvagine.

CRF-BP Binding Assay

Displacement of CRF from CRF-BP was measured by a detergent phase separation assay. Recombinant human CRF-BP was incubated at 20°C for 2 h with 30 pM [^{125}I-Tyr^0] rat/human CRF in 0.02% Nonidet-40 phosphate-buffered saline, pH 7.4. Bound and free CRF were then separated by the addition of Triton X-114 (octylphenoxypolyethoxyethanol) buffer stirring and incubation 20 min at 37°C. Free CRF segregates to the detergent phase at the bottom of the tube, and the CRF/CRF-BP complex remains in the aqueous phase. The amount of radioactivity in an aliquot of the aqueous phase was determined with a gamma scintillation counter (LKB 1261 multi gamma; EG G Instruments). Values were expressed as the mean ± S.E.M. of at least three determinations performed in triplicate. Specific binding was determined as in CRF_1 binding assays.

Measurement of Intracellular cAMP Synthesis in Y 79 Cells

CRF-induced cAMP synthesis in human retinoblastoma Y 79 cells was assessed as described by Hauger et al. (1997). In the present article, two types of experiments were performed on Y 79 cells. In the first experiment, Y 79 cells were incubated for 15 min at 37°C under stirring in presence of 10 nM rat/human CRF with increasing concentrations of SSR125543A in 1 mM isobutylmethyl xanthine supplemented RPMI buffer, pH 7.2. The intracellular cAMP content was measured after lysing the cells by 0.5% ice-cold Triton X-100 by using a cAMP ^{125}I scintillation proximity assay kit (Amersham Biosciences plc, Little Chalfont, Buckinghamshire, UK). IC_{50} values were determined using a nonlinear least-square regression analysis (Munson and Rodbard, 1980) with RS/1 (BBN Software Product Corporation) and an internal computerized interactive procedure.

In the second experiment, Y 79 cells were incubated for 15 min at 37°C under stirring with increasing concentrations of rat/human CRF alone or in presence of three concentrations of SSR125543A. Intracellular cAMP synthesis was expressed as the percentage of maximal release after subtraction of basal release. Values were expressed as the mean of at least three determinations performed in duplicate.

Measurement of ACTH Secretion by AtT-20 Cells

CRF-induced ACTH secretion in mouse pituitary AtT-20 cells was previously described by Litvin et al. (1984). A subclone of AtT-20/D16v cells was used in this study. Cells were seeded in 12-well plates and cultured overnight in their growth medium. They were incubated for 120 min at 37°C with 2 ml of basal medium, alone or with increasing concentrations of rat/human CRF in the presence or absence of three concentrations of SSR125543A. ACTH release was measured on supernatant samples by using a radioimmunoassay (Diasorin, Stillwater, MN). Values were expressed as mean values of three determinations performed in triplicate.

Ex Vivo Binding Assay in Rats

SSR125543A or the corresponding vehicle was administered p.o. or i.v. to rats (three per group) at various doses (dose-effect studies) and times (time course studies) before rat decapitation and organ (brain and pituitary) removal. Tissues were homogenized in 10 ml of

diluted (1/20) with the same incubation buffer and submitted to a [^{125}I-Tyr^0] ovine CRF binding assay procedure as previously described. To determine the relative population of CRF_1 binding sites present in crude homogenates from rat brain and pituitary, binding studies were performed in vitro, on naïve brain and pituitary tissue. Competition curves were determined for ovine CRF, antisauvagine-30, and rat/human $CRF_{(6-33)}$, two peptides selective for CRF_2 receptor and CRF-BP, respectively (Behan et al., 1995; Ruhmann et al., 1998). In the ex vivo binding assay, nonspecific binding was defined with 100 nM antalarmin. Values were expressed as the mean percentage of specific binding ± S.E.M. Statistical differences between drug- and vehicle-treated groups were assessed by a Student's *t* test.

CRF-Induced ACTH Secretion in Rats

Animals were habituated to the experimental procedure 1 day before the experiment. SSR125543A or its vehicle was administered p.o. or i.v. to rats (3–7/group) at various doses (dose-effect studies) and times (time course studies) before intravenous injection of 4 μg/kg rat/human CRF. Thirty minutes later, animals were sacrificed by decapitation and trunk blood samples were collected in a 1 mg/ml EDTA solution for the determination of ACTH plasma levels by radioimmunoassay (Diasorin). Results were expressed as the mean values ± S.E.M. Statistical differences between drug- and vehicle-treated groups were assessed by a Student's *t* test. The median inhibitory doses (ID_{50}) with 95% confidence limits were determined by fitting of the dose-response curve to the four-parameter logistic model according to Ratkowsky and Reedy (1986). The adjustment was performed by nonlinear regression by using the Levenberg-Marquardt algorithm in the RS/1 software.

Restraint Stress-Induced ACTH Secretion in Rats

One hour after oral administration of SSR125543A or its vehicle, rats were placed into hemicylindrical Plexiglas enclosures (6 cm in width and 4 cm in height) for 15 min. After this stress period, the animals were placed back in their cages, carried to an adjacent room and immediately sacrificed. Nonstressed control animals remained in their cage for 15 min before sacrifice. Blood was collected in a 1 mg/ml EDTA solution for the determination of ACTH plasma levels by radioimmunoassay (Diasorin). Values were expressed as the mean ACTH levels ± S.E.M. Statistical differences between drug- and vehicle-treated groups were assessed by a single factor ANOVA or by the nonparametric Kruskall-Wallis test followed by Dunnett's *t* test or by the Mann-Whitney *U* test with α adjustment of Holm on RS1/software, respectively.

CRF-Induced Hippocampal Acetylcholine Release

Surgery and Microdialysis. Rats were anesthetized with urethane (1.4 g/kg i.p.) and then placed in a stereotaxic frame. A microdialysis probe (CMA-12, length 2 or 3 mm and outer diameter 0.5 mm; Carnegie Medicine AB, Stockholm, Sweden) was stereotaxically implanted in the dorsal hippocampus. The coordinates were 3.5 mm posterior to bregma, 2 mm lateral to the midline, and 3.8 mm down from the dural surface for the hippocampus (Paxinos and Watson, 1986). For i.c.v. injection of CRF, ejection pipettes were implanted into the left lateral ventricle at the following coordinates: 0.8 mm posterior to bregma, 1.5 mm lateral to the midline, and 3.4 mm down from the dural surface. The ejection of CRF (1 μg/2 μl/90 s) was performed by applying air pressure with a 1-ml syringe connected to the nontapered side of the pipette by Tygon tubing. The probes were perfused with a gassed Ringer's solution containing 125 mM NaCl, 3 mM KCl, 1.3 mM $CaCl_2$, 1.0 mM $MgCl_2$, 23 mM $NaHCO_3$, and 1.5 mM KH_2PO_4, pH 7.4, at a rate of 2 μl/min by using a microinjection pump (CMA-100; Carnegie Medicine AB). To reduce acetylcholine degradation in the dialysate, 1 μM neostigmine was added to the Ringer's solution perfused in the hippocampal probe. Microdialysis sampling started 90 min after the probe was placed in the hippocampus. Antalarmin, and vehicle were given intraperitoneally (5 ml/kg of body weight) 180 and 30 min before peptide application.

The time course of the CRF effects was analyzed by ANOVA with repeated measures and Dunnett's *t* test was used for individual time comparisons. The antagonism of the CRF effect was evaluated by comparing the area under the curve during the 120 min after peptide injection. A statistical analysis was carried out by using the Student's *t* test.

Assay of Acetylcholine (ACh). ACh levels were measured in 30-min dialysate samples (50 μl) by using a high-performance liquid chromatography system (Waters, Milford, MA) as previously described by Steinberg et al. (1995) except for the electrochemical detection system (Coulochem II; ESA, Chelmsford, MA). Briefly, the analytical system for ACh included a trapping precolumn and immobilized enzyme reactor (BAS.MF-6151). The mobile phase, 35 mM phosphate buffer, pH 8.5, supplemented with the antibacterial reagent Kathon (5 ml/l; BAS DF-2150), was pumped at a flow rate of 0.8 ml/min and replaced with a fresh preparation every 3 days. The enzyme postcolumn reactor converted ACh to hydrogen peroxide that was electrochemically detected using a platinum electrode (ESA P/N 55-0183) set at 0.3 V. The chromatographic column and enzyme reactor were kept at 35°C. The detection sensitivity was 0.2 pmol/50 μl.

CRF-Induced Forepaw Treading in Gerbils

This test was based on the observation that i.c.v. injection of CRF (1 μg/2 μl) produces forepaw treading ("piano playing") in gerbils, an effect that is prevented by treatment with the CRF_1 receptor antagonist R-121919 (Owens and Nemeroff, 1999). Gerbils were placed individually in small transparent plastic cages for 30 min. They were then pretreated with SSR125543A p.o. or antalarmin i.p. CRF (1 μg) was injected i.c.v. (free-hand method; Jung et al., 1996) 15 min (antalarmin) or 60 min (SSR125543A) later. In each experiment, a control group was injected i.c.v. with the vehicle. Forepaw treading was measured by an observer unaware of the drug treatment, for 1 min every 15 min over a 2-h period (8 min in total cumulative times). The cumulative forepaw treading time was calculated for each gerbil and then expressed as the mean and S.E.M. Comparisons between control and treated groups were performed using the Kruskall-Wallis test, followed by Mann-Whitney *U* test with α adjustment of Holm.

Results

Affinity of SSR125543A for CRF_1 Receptors

SSR125543A inhibited the specific binding of [^{125}I-Tyr^0] ovine CRF to human CRF_1 receptors expressed in CHO cells

TABLE 1
Affinity of SSR125543A for CRF receptor subtypes and for CRF_1 receptors of various species
Values of pK_i and Hill coefficients are the means ± S.E.M. of three experiments performed in triplicate.

Binding Assay	Results	
CRF_1 binding: [^{125}I-Tyr^0]ovine CRF ligand	pK_i	*n*
hCRF$_1$-CHO cells	8.73 ± 0.15	1.25 ± 0.10
Y 79 cells	9.08 ± 0.20	0.97 ± 0.17
Rat brain	8.77 ± 0.23	0.89 ± 0.12
Mouse brain	8.90 ± 0.10	1.16 ± 0.13
Gerbil brain	9.00 ± 0.00	0.75 ± 0.11
$CRF_{2\alpha}$ binding: [^{125}I-Tyr^0] ovine sauvagine ligand	Inhibition at 10 μM	
hCRF$_{2\alpha}$-CHO cells	0%	
CRF-BP binding: [^{125}I-Tyr^0] rat/human CRF ligand	Inhibition at 10 μM	
hCRF-BP	0%	
rCRF-BP	0%	

with a pK_i value of 8.73 ± 0.15 (mean ± S.E.M.; Table 1), which was comparable to that of antalarmin and higher than that of the natural ligand rat/human CRF (pK_i = 8.70 and 8.22, respectively). It also recognized with high affinity the native CRF_1 receptors present on the human retinoblastoma cell line Y 79 (pK_i = 9.08 ± 0.20). At 10 μM, SSR125543A did not interact with the human $CRF_{2\alpha}$ receptor expressed in CHO cells, or human and rat recombinant CRF-BP (Table 1). Binding studies performed with [^{125}I-Tyr^0] ovine CRF on membrane preparations obtained from rodent brains (rat, mouse, and gerbil) did not reveal species differences in affinity because the respective pK_i values of 8.77 ± 0.23, 8.90 ± 0.10, and 9.00 ± 0.00 were very close to the pK_i for the human CRF_1 receptor (Table 1). The high selectivity of SSR125543A for the CRF_1 receptor was demonstrated by its lack of activity (inhibition lower than 50%) at 1 or 10 μM in 125 assays performed by Panlabs and Cerep (receptors, transporters, enzymes, and ion channels) (Table 2).

CRF_1 Receptor Antagonism by SSR125543A: In Vitro Studies

When rat/human CRF was applied to Y 79 cells, which express constitutively CRF_1 receptors, the intracellular cAMP production was increased by ~7-fold over basal levels, with an EC_{50} value of 4.0 ± 0.9 nM (mean ± S.E.M). SSR125543A did not modify the basal level of cAMP but fully blocked the CRF (10 nM) response with an IC_{50} value of 3.0 ± 0.4 nM (mean ± S.E.M., n = 3; Fig. 2A). Under similar experimental conditions, the IC_{50} for antalarmin was 0.8 ± 0.1 nM (mean ± S.E.M., n = 3; data not shown). Increasing concentrations of SSR125543A produced a rightward shift of the rat/human CRF concentration-response curve (Fig. 2B), without modifying the maximal cAMP production obtained with rat/human CRF alone. EC_{50} values for CRF were 2.5 nM (2.1–2.9), 8.3 (5.5–12.4), 55.6 (42.5–73.0), and 92.2 (55.7–144.9) (means and confidence limits) in the presence or absence of 3, 30, and 100 nM SSR125543A, respectively.

When rat/human CRF was applied to mouse pituitary AtT-20 cells, which express CRF_1 receptors, ACTH secretion was stimulated by ~3-fold over basal levels. SSR125543A did not modify basal secretion of ACTH but antagonized the ACTH secretion induced by increasing concentrations of rat/human CRF. Increasing concentrations of SSR125543A also produced a rightward shift of the rat/human CRF dose-response curve and a concentration-dependent inhibition of the maximal ACTH secretion elicited by rat/human CRF alone (Fig. 3). In the course of three experiments, EC_{50} values for CRF were 1.6 (1.4–1.9), 11.9 (10.0–14.1), 49.8 (34.2–74.3), and 128.1 (104.6–160.0) (means and confidence limits) in the presence or absence of 3, 30, and 100 nM SSR125543A, respectively.

Ex Vivo Binding Assay

The nonspecific binding obtained in the presence of 1 μM rat/human CRF in the ex vivo [^{125}I-Tyr^0] ovine CRF binding assay averaged 20%. To determine the real proportion of CRF_1 binding sites in this model, competition studies were performed with ovine CRF, antalarmin, SSR125543A, antisauvagine-30, and rat/human $CFR_{(6-33)}$ on crude brain and pituitary homogenates prepared from untreated rats. As shown in Fig. 4A and Table 3, the binding of radiolabeled ovine CRF could be separated in ovine CRF with a high-affinity curve, suggesting two populations of 50 and 30% of the total binding sites and respective pK_i values of 8.35 and 6.23. Antalarmin and SSR125543A displaced only the first population of sites in a monophasic manner and similar pK_i values of 8.80 and 8.89. Antisauvagine-30 competed weakly with iodinated ovine CRF with a shallow monophasic curve (pK_i = 6.77, n_H = 0.67). In contrast, the selective CRF-BP ligand rat/human $CRF_{(6-33)}$ inhibited only 30% of the total binding that represents the non-CRF_1 component. Its low affinity for ovine CRF compared with rat/human CRF explains the weak pK_i (6.43) measured in this study. Unlike crude brain membranes, the specific binding of [^{125}I-Tyr^0] ovine CRF to crude rat pituitary homogenates represented 90% of the total binding and was completely displaced by 100 nM antalarmin. Taking into account the high selectivity of antalarmin, CRF_1 specific binding to crude tissue homogenates was considered as the maximal displacement measured in the presence of 100 nM antalarmin.

The blockade of brain and pituitary CRF_1 receptors was evaluated in binding studies performed on crude tissue homogenates prepared from rats treated with SSR125543A (ex vivo binding assay). No specific CRF_1 binding could be measured after a 2-h oral treatment at the dose of 30 mg/kg, whereas at 4 h postadministration, the binding was still reduced by 76 ± 2% (Fig. 4C). The presence of SSR125543A at the pituitary level was also demonstrated in the same experiment, by a decrease of 67 ± 1% in binding 1 h after SSR125543A oral administration that reached 78 ± 1% at 2 and 4 h (Fig. 4C). Another experiment performed under similar conditions demonstrated that ligand binding inhibition was still present 24 h after SSR125543A treatment, with 62 ± 14% inhibition in the brain and 80 ± 2% in the pituitary (mean and S.E.M., n = 3). Dose-effect study performed 2 h after oral treatment revealed a dose-dependent inhibition of brain CRF_1 receptor binding with an ID_{50} of 6.5 (3.2–11.8) mg/kg (mean and confidence limits; Fig. 4B). SSR125543A also reached both brain and pituitary after i.p. injection, ID_{50} determined 2 h post-treatment being 11.7 (6.0–23.0) mg/kg, slightly higher than after oral administration (data not shown).

CRF_1 Receptor Antagonism by SSR125543A: In Vivo Studies

CRF-Induced ACTH Secretion in Rats. In conscious rats, the plasma level of ACTH determined by radioimmunoassay was 34 ± 4 pg/ml (mean ± S.E.M., n = 12). Oral administration of 30 mg/kg SSR125543A, 2 h before blood sampling significantly diminished the ACTH level (18 ± 2 pg/ml, $p < 0.01$, n = 5). CRF (4 μg/kg i.v.) injection, 30 min before blood sampling, induced a more than 10-fold stimulation (269 ± 20 pg/ml) of the ACTH secretion. When administered orally at the dose of 30 mg/kg, SSR125543A inhibited the increase in ACTH secretion induced by CRF injection with significant effects from 1 to 6 h (Fig. 5A). Dose-effect studies performed after oral administration of SSR125543A, 2 h before the CRF injection, yielded ID_{50} values of 4.9 (3.0–8.6) mg/kg (Fig. 5B) (means and confident limits). After i.v. injection of 3 mg/kg SSR125543A, the maximal inhibition of CRF-induced ACTH secretion was observed at 5 min postinjection. The dose-effect study performed at the same

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