

Characterization of (2S,4R)-1-[5-Chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl]-4-hydroxy-N,N-dimethyl-2-pyrrolidine carboxamide (SSR149415), a Selective and Orally Active Vasopressin V_{1b} Receptor Antagonist

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ABSTRACT

(2S,4R)-1-[5-Chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1H-indol-3-yl]-4-hydroxy-N,N-dimethyl-2-pyrrolidine carboxamide (SSR149415), the first selective, nonpeptide vasopressin V_{1b} receptor antagonist yet described, has been characterized in vitro and in vivo. SSR149415 showed competitive nanomolar affinity for animal and human V_{1b} receptors and exhibited much lower affinity for rat and human V_{1a}, V₂, and oxytocin receptors. Moreover, this compound did not interact with a large number of other receptors, enzymes, or ion channels. In vitro, SSR149415 behaved as a full antagonist and potently inhibited arginine vasopressin (AVP)-induced Ca²⁺ increase in Chinese hamster ovary cells expressing rat or human V_{1b} receptors. The in vivo activity of SSR149415 has been studied in several models of elevated corticotropin secretion in conscious rats. SSR149415 inhibited exogenous AVP-induced increase in plasma corticotropin, from

3 mg/kg i.p. and 10 mg/kg p.o. upwards. Similarly, this compound antagonized AVP-potentiated corticotropin release provoked by exogenous corticoliberin at 3 mg/kg p.o. The effect lasted for more than 4 h at 10 mg/kg p.o. showing a long-lasting oral effect. SSR149415 (10 mg/kg p.o.) also blocked corticotropin secretion induced by endogenous AVP increase subsequent to body water loss. Moreover, 10 mg/kg i.p. SSR149415 inhibited plasma corticotropin elevation after restraint-stress in rats by 50%. In the four-plate test, a mouse model of anxiety, SSR149415 (3 mg/kg p.o. upwards) displayed anxiolytic-like activity after acute and 7-day repeated administrations. Thus, SSR149415 is a potent, selective, and orally active V_{1b} receptor antagonist. It represents a unique tool for exploring the functional role of V_{1b} receptors and deserves to be clinically investigated in the field of stress and anxiety.

The neurohypophysial hormone vasopressin (AVP) exerts several central and peripheral actions in mammals, including water and solute excretion by the kidney, platelet aggregation, liver glycogenolysis, uterus, and vascular smooth muscle cell contraction, mitogenesis, aldosterone secretion by the adrenals, clotting factor release, and corticotropin release by the adenohypophysis. Together with oxytocin (OT), another structurally related cyclic nonapeptide, AVP is implicated in interneuronal communication in the CNS and modulates behavioral functions such as feeding, memory,

thermoregulation, control of adaptive behavior, and social and sexual processes (Dreifuss et al., 1991; Barberis et al., 1999). These multiple effects of AVP are based upon a local or systemic release pattern into the organism and occur via interaction with seven transmembrane domain G protein-coupled receptors. So far three AVP receptors, V_{1a}, V_{1b} (or V₃), and V₂ receptors have been cloned and characterized by their primary structure, gene localization, mRNA distribution, pharmacology, and functions. The V₂ receptor is positively coupled to adenylylcyclase and is mostly found in the

ABBREVIATIONS: AVP, arginine vasopressin; OT, oxytocin; CNS, central nervous system; DMSO, dimethyl sulfoxide; RIA, radioimmunoassay; dDAVP, desamino-[D-Arg⁸]-vasopressin; dPal, [deamino-Cys, D-3-(pyridyl)-Ala²-Arg⁸]-vasopressin; dPen, [deamino-penicillamine-O-Me-Tyr², Arg⁸]-vasopressin; fura-2/AM, fura 2-acetoxymethyl ester; BSA, bovine serum albumin; CHO, Chinese hamster ovary; ANOVA, analysis of variance; intracellular Ca²⁺, [Ca²⁺]_i; HPA, hypothalamo-pituitary-adrenal; SR 121463, (1-[4-(N-tert-butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)cyclohexane]indol-2-one, equatorial isomer.

kidney where it mediates the antidiuretic effect of AVP. The ubiquitous V_{1a} receptors mediate the actions of AVP in platelets, vessels, liver, adrenals, uterus, and brain, and like V_{1b} receptors, induce phospholipase C activation and intracellular calcium mobilization (Lolait et al., 1995a; Thibonnier et al., 1998). The recently cloned V_{1b} receptor is mainly involved in the stimulating effect of AVP on corticotropin secretion in the pituitary (De Keyzer et al., 1994; Sugimoto et al., 1994). AVP is a direct corticotropin secretagogue and also synergizes corticotropin-induced corticotropin release in many species, including human (Gillies et al., 1982; Rivier and Vale, 1983; Antoni et al., 1984; Gaillard et al., 1984; Dickstein et al., 1996). Regulation of corticotropin secretion is a critical component in the adaptive organism response to stress or emotional situations. Data have shown that AVP plays a primary role during adaptation to stress (Aguilera and Rabadan-Diehl, 2000). In chronic stress, the expression of AVP in parvocellular neurons of the paraventricular nucleus and its secretion into pituitary portal circulation increases. In addition, stress regulates pituitary V_{1b} receptors, increasing the corticotropin-releasing activity of AVP (De Goeij et al., 1992; Rabadan-Diehl et al., 1995). As demonstrated by *in situ* hybridization and immunohistochemistry, V_{1b} receptor mRNA and protein are widely distributed in the rat CNS, suggesting that not only V_{1a} but also V_{1b} receptors mediate different AVP functions in the rat brain (Lolait et al., 1995b; Vaccari et al., 1998; Hernando et al., 2001). Of note, the presence of V_{1b} receptors has been also reported in several small cell lung cancer tumors (North et al., 1998) and the V_{1b} (V₃) receptor gene is overexpressed in corticotropin-secreting tumors (De Keyzer et al., 1998). An endocrine role of V_{1b} receptors in other organs such as the pancreas and the adrenals in regulating glucagon and insulin release has also been suggested (Lee et al., 1995; Yibchok-anun et al., 1999). To date, due to the lack of selective V_{1b} receptor ligands (agonists/antagonists) and to the absence of orally active V_{1b} receptor antagonists, the V_{1b} receptor is still poorly characterized and the precise role of AVP via central and peripheral V_{1b} receptors remains to be elucidated. Interestingly, to explore the functions of this receptor, a knockout mouse has been generated. Preliminary data showed that these animals display behavioral alterations, e.g., reduced aggression and social memory that could be attributed to the absence of V_{1b} receptors in specific brain structures (Lolait et al., 2000; Hernando et al., 2001).

In the present study we report the biochemical and pharmacological characterization of (2*S*,4*R*)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulfonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-hydroxy-*N,N*-dimethyl-2-pyrrolidine carboxamide, isomer (–) (SSR149415) (Fig. 1), the first

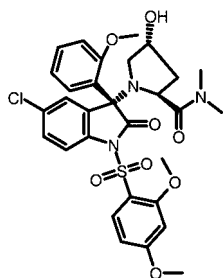


Fig. 1. Chemical structure of SSR149415.

selective, nonpeptide, and orally active V_{1b} receptor antagonist yet described. Because marked species differences exist for AVP/OT receptors in terms of binding affinity and pharmacological properties, SSR149415 was also studied in various animal and human preparations expressing V_{1b} receptors. Inasmuch as V_{1b} receptors control corticotropin release, the *in vivo* activity of SSR149415 has been studied in rats in several models of corticotropin secretion induced by various factors (AVP, AVP plus corticotropin-releasing hormone, body water loss- and restraint-stress). Finally, the anxiolytic-like properties of SSR149415 were investigated in the four-plate test in mice, a well validated model of anxiety. We clearly demonstrate that SSR149415 is a unique tool for exploring the role and the localization of V_{1b} receptors and that this type of drug exhibits a promising therapeutic profile in the field of anxiety and stress-related disorders.

Experimental Procedures

Materials

The nonpeptide molecules, SSR149415 and SR121463 (Serradeil-Le Gal et al., 1996) were synthesized at Sanofi-Synthelabo Recherche, Montpellier and Toulouse, respectively, France. The chemical structures were determined by ¹H and ¹³C NMR, mass spectrometry, and infrared spectroscopy. The purity, measured by high-pressure liquid chromatography, thin layer chromatography, and elemental analysis, was >98%. For *in vitro* experiments the compound was initially dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10⁻² M and then diluted in the appropriate test solvent. AVP, oxytocin, desamino-[D-Arg⁸]-vasopressin (dDAVP), [deamino-Cys, D-3-(pyridyl)-Ala²-Arg⁸]-vasopressin (dPal), [deamino-penicillamine, -O-Me-Tyr², Arg⁸]-vasopressin (dPen), bacitracin, Pluronic F-127, and Cremophor EL were from Sigma Chemical (St. Louis, MO). Corticotropin was purchased from Neosystem (Strasbourg, France). Fura-2/acetoxymethyl ester (fura-2/AM) was from Molecular Probes (Eugene, OR). Bovine serum albumin (BSA) fraction V was obtained from IBF (Paris, France). All cell culture reagents were from Roche Molecular Biochemicals (Meylan, France). Tris, MgSO₄, and DMSO were purchased from Merck-Clevenot (Nogent-sur-Marne, France). All other chemicals were from Prolabo (Nogent-sur-Marne, France). The radioligands [³H]AVP [8-L-arginine, [phenylalanyl-3,4,5-3*H*(*N*)]-vasopressin; 75 Ci/mmol], [³H]SR121463 (47.5 Ci/mmol), and [¹²⁵I]-OT antagonist, [d(CH₂)₅Tyr(Me)₂, Thr⁴, Orn⁸ [¹²⁵I]-Tyr⁹-NH₂] (2000 Ci/mmol), were synthesized by PerkinElmer Life Sciences (Boston, MA).

Biological Material

Male Sprague-Dawley CD rats (150–200 g, except in restraint-stress experiments in which animals weighed between 275 to 300 g) were purchased from Charles River (St. Aubin Les Elbeufs, France). They were used for *in vivo* activity and membrane preparations in *in vitro* binding studies. Male NMRI mice (20 ± 3 g) purchased from R. Janvier (Le Genest, France) were used in the four-plate test. Male homozygous Brattleboro rats with central diabetes insipidus (300–350 g) were from Harlan Bioproducts for Science (Indianapolis, IN). Rats were housed five per cage and mice 20 per cage in climate- and illumination-controlled rooms (lights on 7:00 AM, lights off 7:00 PM). Water and chow were available *ad libitum*. Fed animals were used in the different experiments. All protocols performed have been approved by the Animal Care and Use Committee of Sanofi-Synthelabo Recherche.

Human hypophyses were collected in conformity with French national ethical rules. Hypophyses were obtained within 6 h of death, chilled in cold saline, and immediately frozen in liquid nitrogen. Bovine hypophyses were obtained from a local slaughterhouse. Mam-

mary tissue was taken from 19-day-old Sprague-Dawley pregnant rats and stored in liquid nitrogen until use.

In Vitro Experiments

Cell Culture and Membrane Preparation. Ltk⁻ cells were transfected with the cDNA coding for the human OT receptor. CHO-dhFr⁻ cells (DXB11) were transfected with an expression vector derived from plasmid 7055 containing the cDNA encoding the human V₂, V_{1a}, or V_{1b} receptor. Stably transformed cell lines were isolated as described previously (Serradeil-Le Gal et al., 1996, 2000). They were grown in 10 mM HEPES, pH 7.4, minimal essential medium supplemented with 5% fetal calf serum and 8 g/l sodium bicarbonate and 300 μg/ml geneticin at 37°C in a humidified atmosphere containing 5% CO₂. Wild-type CHO cells were routinely grown in a similar culture medium. Culture medium was removed every other day and cells were subcultured by treatment with 0.05% trypsin, 0.02% EDTA. Membranes from Ltk⁻ cells, transfected with the human oxytocin receptor and from CHO cells expressing the human V_{1a}, V_{1b}, and V₂ receptor, were prepared as in Serradeil-Le Gal et al. (1996). Briefly, cells were harvested, washed twice in phosphate-buffered saline without Ca²⁺ and Mg²⁺, polytron-homogenized in lysis buffer (15 mM Tris-HCl, pH 7.5; 2 mM MgCl₂; 0.3 mM EDTA), and centrifuged at 100g for 5 min at 4°C. Pellets were washed in a buffer A consisting of 50 mM Tris-HCl, pH 7.4; 5 mM MgCl₂ and centrifuged at 44,000g for 20 min at 4°C. Membranes were suspended in a small volume of buffer A and protein contents were determined. Aliquots of membranes were used immediately or stored at -80°C. Rat and human hypophyseal membranes, rat kidney (papilla and inner medulla), liver, and mammary glands were prepared as previously described (Serradeil-Le Gal et al., 1993, 1996). Protein concentration was determined by the method of Bradford (1976) by using bovine serum albumin as a standard.

Binding Studies. Binding of [³H]AVP to rat liver and human, bovine, and rat pituitary, and binding of [¹²⁵I]-OT antagonist to rat mammary glands and to Ltk⁻ cell membranes expressing the human uterine oxytocin receptor, were performed as described in (Serradeil-Le Gal et al., 1996). Binding assays of [³H]SR121463 to rat kidney medullary membranes were conducted according to Serradeil-Le Gal et al. (2000). Binding assays on membranes of CHO cells transfected with the human or rat V_{1b} receptor were performed in an incubation medium containing 50 mM Tris-HCl, pH 7.4; 3 mM MgSO₄; 0.1% BSA; 0.1% bacitracin; [³H]AVP (0.02–30 nM for saturation experiments or 2–3 nM for competition studies); and increasing amounts of the tested compound (SSR149415 and reference peptides). The reaction was started by the addition of membranes (~35 and 5 μg/assay for human and rat V_{1b} CHO membranes, respectively) and incubated at 20°C for 45 min. Saturation binding experiments were performed in membranes of CHO cells transfected with the human V_{1b} receptor in the absence (control) or presence of SSR149415 (0.9, 1.8, 3.7, 7.5, and 15 nM). The reaction was stopped by adding 4 ml of ice-cold buffer followed by filtration through GF/B Whatman glass microfiber filters presoaked in ice-cold buffer. Filters were washed twice with 4 ml of ice-cold buffer and counted for radioactivity by liquid scintillation in Beta Packard 1900 TR. Nonspecific binding was determined in the presence of 1 μM unlabeled AVP.

Binding Data Analysis. The IC₅₀ value was defined as the concentration of inhibitor required to obtain 50% inhibition of the specific binding. Inhibition constant (K_i) values were calculated from the IC₅₀ values by using the Cheng and Prusoff (1973) equation. Data for equilibrium binding [apparent equilibrium dissociation constant (K_d), maximum binding density (B_{max})], and competition experiments [IC₅₀, Hill coefficient (n_H)] were analyzed using an iterative nonlinear regression program (Serradeil-Le Gal et al., 1996).

Intracellular Ca²⁺ ([Ca²⁺]_i) Measurements. Subconfluent CHO cells, cultured in 175-cm² flasks as described above, were collected by trypsinization (0.05% trypsin, 0.02% EDTA) and centrifuged (230g, 5 min). As described in Serradeil-Le Gal et al. (1995), the cells were suspended in culture medium at a final con-

centration of 5 × 10⁶ cells/ml then incubated with 5 μM fura-2/AM and 0.02% Pluronic F-127 at 30°C for 20 min under continuous shaking. At the end of the incubation, the cells were centrifuged (230g, 5 min) and washed with culture medium. The cells were washed twice in Hanks' buffer [137 mM NaCl, 5.4 mM KCl, 0.34 mM Na₂HPO₄, 5.5 mM glucose, 4.2 mM NaHCO₃, 0.8 mM MgSO₄, 10 mM HEPES (0.1 mM EGTA for the first wash only), pH 7.4]. The cells were resuspended in this buffer to a final concentration of 2.7 × 10⁶ cells/ml and kept at 4°C in the dark until use. Calcium transients were measured with an SLM 8000 C spectrofluorometer at 37°C (excitation at 340 and 380 nm, emission at 510 nm). Cytosolic free Ca²⁺ determination was performed as described by Grynkiewicz et al. (1985). Results were expressed as means ± S.E. and analyzed using RS1 software (BBN Domain, Cambridge, MA).

In Vivo Experiments

In Vivo Interaction of SSR149415 with AVP V_{1a} and V₂ Receptors. Potential in vivo interaction of SSR149415 with AVP vascular V_{1a} and renal V₂ receptors was studied in male Sprague-Dawley rats. The effects of SSR149415 (30 mg/kg p.o. in 0.6% methylcellulose) were studied on AVP (40 ng/kg i.v.)-induced hypertension and on spontaneous urine flow rate during a 6-h observation period as previously described (Serradeil-Le Gal et al., 1993, 1996). To detect potential antidiuretic agonist V₂ properties, additional experiments have been performed in vasopressin-deficient Brattleboro rats with SSR149415 (10 mg/kg i.p. in 5% DMSO, 5% Cremophor in saline); urine was collected for the next 24 h for volume and osmolality measurements (Serradeil-Le Gal et al., 1996).

In Vivo Plasma Corticotropin Measurements in Conscious Rats.

Exogenous AVP-induced corticotropin secretion and plasma corticotropin measurements. In a first set of experiments, dose effects were performed with SSR149415 (1–30 mg/kg) administered either 30 min i.p. or 2 h p.o. before an exogenous AVP injection (0.3 μg/kg i.v. in 0.1% bovine serum albumin in distilled water). Ten minutes after the AVP challenge, the vehicle- (5% DMSO, 5% Cremophor in saline) and SSR149415-treated rats were sacrificed by decapitation and trunk blood collected in a 10-mg/ml EDTA solution (1, 1/10; volume/volume dilution). After centrifugation (760g × 10 min; 2–4°C) plasma was collected and stored as aliquots at -20°C until Corticotropin measurements by RIA (DiaSorin S.A., Stillwater, MN) were made. Under similar operating conditions, time course studies were performed with i.p. and oral 10 mg/kg SSR149415 administered 1, 2, 3, 4, and 6 h before the AVP challenge.

Potiation of exogenous corticoliberin by AVP on corticotropin secretion. In preliminary experiments, we studied the dose-effect and the kinetics of corticoliberin-induced corticotropin secretion, alone and in combination with AVP. Exogenous corticoliberin (dissolved in 0.1% acetic acid and 0.1% BSA in distilled water) produced a significant increase in corticotropin plasma levels from the dose of 0.3 μg/kg i.v. and the effect was maximal 30 min after corticoliberin injection. As previously described for AVP, the maximal effect on corticotropin secretion was observed 10 min after administration (Bernardini et al., 1994). By combining doses of corticoliberin (0.1 μg/kg i.v. administered 30 min before sacrifice) and AVP (0.03 μg/kg i.v. administered 10 min before sacrifice), devoid of significant effect on corticotropin secretion when injected alone, we observed a significant synergization of corticoliberin effects by AVP, as reported previously (Rivier and Vale, 1983). In the dose-effect experiments, the animals (11–19/group) were treated either with the vehicle (5% DMSO, 5% Cremophor in saline) or with SSR149415 (1–10 mg/kg) 2 h before the AVP (plus corticoliberin) challenge. Similarly the time course study was performed with oral 10 mg/kg p.o. SSR149415 administered 1, 2, 3, 4, and 6 h before the AVP (plus corticoliberin) challenge. Ten minutes after AVP administration animals were sacrificed and plasma corticotropin measured as described above.

SR121463-Induced Corticotropin Secretion. SR121463 is a selective and orally active V₂ receptor antagonist displaying power-

ful aquarectic properties in several species. In rats, it has been previously observed that high doses of SR121463 (≥ 3 mg/kg p.o.) induced important water loss, leading to endogenous AVP release associated with increased plasma corticotropin, which is maximal 2 h after oral SR121463 administration (Lacour et al., 2000). In this set of experiments, groups of 16 to 19 animals pretreated or not with SSR149415 (1–30 mg/kg p.o.) received 1 h later a high dose of SR121463 (10 mg/kg p.o.). Animals were sacrificed 2 h after SR121463 administration and plasma corticotropin measured as described above. Control animals were administered with SR121463 and SSR149415 vehicles at the corresponding times.

Restraint Stress-Induced Corticotropin Secretion in Rats.

All experiments were conducted between 8:30 AM and 11:00 AM. Rats (8–10/group) received intraperitoneal injection of the vehicle (2 ml/kg; 5% DMSO, 5% Cremophor, 90% saline) or 10 mg/kg SSR149415. Thirty minutes after the injection, rats were placed in transparent Plexiglas restrainers (6 cm in width by 4 cm in height) for 15 min. At the end of the immobilization period, they were placed individually in a cage, carried to an adjacent room, and immediately sacrificed by decapitation. Nonstressed (home-cage) control rats were sacrificed 45 min after their i.p. injection. Blood was collected and corticotropin plasma levels measured as described above.

Four-Plate Test in Mice. The test apparatus is based on the one described by Boissier et al. (1968). The apparatus consists of a cage with a floor composed of four rectangular metal plates connected to a device that can generate electric shocks (1 mA; 0.2 s). After a 15-s latency period, the animal is subjected to an electric shock every time it moves from one plate to another. The number of punished crossings is recorded during a 1-min test period. Experiments were carried out, respectively, 30 or 60 min after i.p. or p.o. injection of SSR149415. In a second experiment, the duration of the anxiolytic-like action of 10 mg/kg p.o. SSR149415 was investigated. Mice were administered with the compound and placed in the apparatus 1, 2, 4, or 6 h later. Each animal was tested once. In a third experiment, possible development of tolerance to the anxiolytic-like activity was investigated after repeated administration of SSR149415. The drug was given orally at the dose of 10 mg/kg, once daily for 7 consecutive days. Animals were tested 1 h after the 7th administration.

In Vivo Data Statistical Analysis. Data, expressed as the mean \pm S.E.M., were analyzed by a single-factor ANOVA or with the nonparametric Kruskal-Wallis test. Subsequent comparisons between treatment groups and control were carried out using Dunnett's or Student's *t* test procedure, or the nonparametric Mann-Whitney *U* test with α adjustment of Holm, respectively, using RS1 software.

Results

Interaction of SSR149415 with Animal and Human AVP V_{1b} Receptors and Selectivity Profile

As shown in Table 1 and Fig. 2A, SSR149415 displays high nanomolar affinity for animal and human V_{1b} receptors. This compound dose dependently antagonized [³H]AVP binding to various membrane preparations from CHO cell lines transfected with the rat and human V_{1b} receptors or from human, rat, and bovine pituitary tissues expressing native V_{1b} receptors. As illustrated in Fig. 2A, SSR149415 had an affinity for human V_{1b} receptors close to that of the natural hormone, AVP (K_i values of 1.54 ± 0.82 and 0.80 ± 0.25 nM, respectively). In contrast, SSR149415 exhibited higher affinity than the nonselective reference agonist (dDAVP, dPal) and antagonist (dPen) V_{1b} peptides (K_i values of 20 ± 8 , 12 ± 5 and 21 ± 6 nM, respectively). K_i values obtained for these peptides are consistent with affinities previously reported for the human V_{1b} receptor (Thibonnier et al., 1997, 1998). Hill coefficients from binding competition experiments were close

TABLE 1

Binding affinity of SSR149415 for vasopressin and oxytocin receptors in animal and human species

Binding assays were performed as described under *Experimental Procedures* using either CHO/Ltk⁻ cells transfected with the corresponding AVP/OT receptor or native tissues constitutively expressing this receptor. Inhibition constants (K_i) were determined from competition experiments and calculated according to the Cheng and Prusoff (1973) equation. Values are mean \pm S.D. of at least three determinations.

	K_i			
	V _{1b}	V _{1a}	V ₂	OT
	<i>nM</i>			
Human				
Hypophysis	4.2 \pm 1.1			
CHO	1.5 \pm 0.8			
CHO		91 \pm 23		
CHO			1412 \pm 314	
Ltk ⁻				174 \pm 35
Rat				
Hypophysis	3.7 \pm 1.3			
CHO	1.3 \pm 0.9			
Liver		1050 \pm 112		
Kidney			2897 \pm 509	
Mammary				270 \pm 39
Bovine				
Hypophysis	2.7 \pm 0.8			

to unity, indicating a single-site competitive model. Moreover, saturation binding experiments were performed in CHO cells transfected with the human V_{1b} receptor in the absence or presence of SSR149415 (0.9, 1.8, 3.7, 7.5, and 15 nM). Scatchard analysis of these data indicated that SSR149415 inhibited [³H]AVP binding in a competitive manner because in the presence of this molecule, the K_d was dose dependently decreased, whereas the B_{max} was not modified (Fig. 2B). The K_i value calculated from Scatchard plots (2.51 ± 0.45 nM) was consistent with the K_i value obtained according to the Cheng and Prusoff (1973) equation in competition experiments (1.54 ± 0.82 nM) (Table 1).

The selectivity of SSR149415 was first assessed for other AVP (V_{1a} and V₂) and OT receptors from rat and human origin. As shown in Table 1, SSR149415 exhibited only a weak affinity for these receptors and displayed a 70, 1000, and 100 higher affinity for human V_{1b} versus V_{1a}, V₂, and OT human receptors, respectively. SSR149415 discriminated between rat and human V_{1a} receptors consistent with previous species differences reported in the field of AVP/OT (Table 1). To complete the functional characterization of SSR149415 at OT and V_{1a} receptors, we have previously studied this compound on Ca²⁺ transients in cells stably transfected with human OT or V_{1a} receptors. In both cell lines, 1 μ M SSR149415 was unable to increase intracellular Ca²⁺ when tested alone and decreased AVP- or OT-induced Ca²⁺ increase, showing a total absence of agonist effect and an antagonist profile at high concentrations.

The high degree of specificity of SSR149415 for the V_{1b} receptor was also demonstrated in several additional assays ($n = 100$). In a variety of binding tests, SSR149415 (10 μ M) did not interact with receptors of nonpeptide (adenosine, adrenergic, angiotensin, benzodiazepin, cannabinoid, dopamine, histamine, acetylcholine, serotonin, glucocorticoid, Ca²⁺, Na⁺ Cl⁻, and K⁺ channels) or peptide ligands (neuropeptide Y, endothelin, neurotensin, bradykinin, galanin, nociceptin, and somatostatin) nor with several enzymes (cyclooxygenases, phosphodiesterases, angiotensin-converting enzyme, protein kinase C, monoamine oxidase, ATPase, and

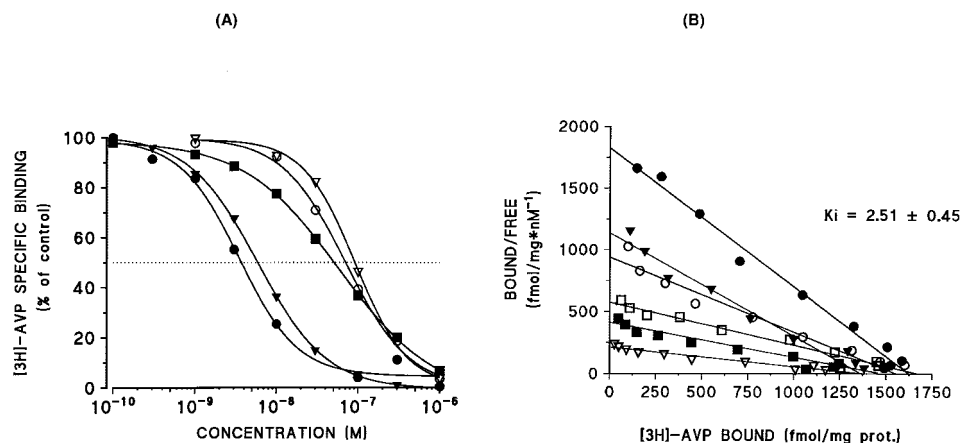


Fig. 2. Effect of SSR149415 on $[^3\text{H}]\text{AVP}$ binding to human AVP V_{1b} receptors expressed in CHO cells. A, inhibition of $[^3\text{H}]\text{AVP}$ -specific binding to human V_{1b} receptors by SSR149415 (\blacktriangledown) and reference peptide compounds: \bullet , AVP; \blacksquare , dDAVP; \circ , dPen; ∇ , dPal. B, Scatchard plots of $[^3\text{H}]\text{AVP}$ binding to CHO V_{1b} membranes without (\bullet) or with 0.9 nM (\blacktriangledown), 1.8 nM (\circ), 3.7 nM (\square), 7.5 nM (\blacksquare), and 15 nM (∇) SSR149415. Binding assays were performed for 45 min at 20°C in the presence of 30 $\mu\text{g}/\text{assay}$ of CHO membranes as described under *Experimental Procedures*. Results represent data from a typical experiment performed in duplicate, which was repeated three times without noticeable change.

acetylcholinesterase). The selectivity profile of SSR149415 was further investigated *in vivo* by studying the effect of this compound on the pressor response to exogenous AVP and on diuresis in rats, to assess a potential interaction with V_{1a} and V_2 receptors, respectively. SSR149415 (up to 30 mg/kg p.o.) neither modified the hypertensive response to exogenous AVP (40 ng/kg i.v.) nor urine excretion volume in conscious rats. Moreover, in vasopressin-deficient Brattleboro rats, a sensitive model used for detecting potential agonist antidiuretic activity, 10 mg/kg i.p. SSR149415 had no effect on urine flow rate (data not shown).

Effect of SSR149415 on AVP-Induced $[\text{Ca}^{2+}]_i$ Increase in CHO Cells Expressing V_{1b} Receptors

To determine the agonist or antagonist profile of SSR149415, we studied the activity of this compound on AVP-induced $[\text{Ca}^{2+}]_i$ increase, in CHO cells transfected with the rat and the human V_{1b} receptors. In these preparations, AVP dose dependently increased $[\text{Ca}^{2+}]_i$ with an EC_{50} value of 0.41 ± 0.21 nM, and 0.44 ± 0.17 nM respectively. As shown in Fig. 3, SSR149415 antagonized in a dose-dependent man-

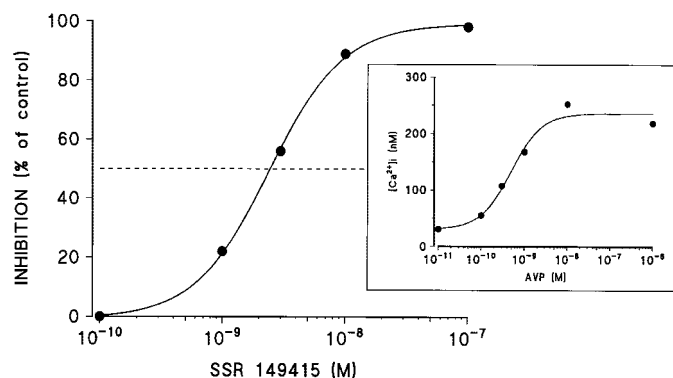


Fig. 3. Inhibition by SSR149415 of AVP-induced Ca^{2+} increase in CHO cells transfected with the human V_{1b} receptor. Dispersed CHO cells ($4\text{--}5 \times 10^5$ cells/ml), preloaded with 2 μM fura-2/AM, were incubated 20 min at 30°C with or without increasing concentrations of SSR149415 before stimulation with 30 nM AVP as described under *Experimental Procedures*. Inset, dose-response curve of AVP. Results represent data from a typical experiment that was repeated five to eight times without noticeable change.

ner 30 nM AVP-evoked $[\text{Ca}^{2+}]_i$ elevation in CHO cells expressing the human V_{1b} receptor, giving a K_i value of 1.26 ± 0.60 nM ($n = 5$). Similar results were obtained in CHO cells transfected with the rat V_{1b} receptors ($K_i = 2.0 \pm 0.6$ nM). When tested alone up to 10^{-5} M, SSR149415 was unable to increase $[\text{Ca}^{2+}]_i$ in these cells, showing a total absence of agonistic effect.

In Vivo Experiments on Corticotropin Secretion in Conscious Rats

Effect of SR149415 on Exogenous AVP-Induced Corticotropin Secretion in Rats. A direct stimulating effect of AVP on corticotropin secretion in corticotroph cells has been extensively described both *in vitro* and *in vivo* (Gillies et al., 1982; Rivier and Vale, 1983; Antoni et al., 1984; Gaillard et al., 1984; Dickstein et al., 1996). We demonstrated that in conscious rats exogenous AVP dose dependently increased corticotropin secretion; the effect was maximal 10 min after AVP injection, in agreement with previous reports (Bernardini et al., 1994). At 0.3 $\mu\text{g}/\text{kg}$ i.v., AVP increased basal corticotropin levels by about 3-fold from 76 ± 11 to 231 ± 26 pg/ml. As shown in Fig. 4, 1 to 30 mg/kg SSR149415 antagonized AVP-induced corticotropin secretion in a dose-dependent manner by both intraperitoneal and oral routes. The inhibition was significant from 10 mg/kg p.o. and 3 mg/kg i.p. upwards. It is important to note that the inhibitory action of SSR149415 lasted significantly for more than 2 h at 10 mg/kg i.p. and up to 4 h at 10 mg/kg p.o. (data not shown). When tested alone, SSR149415 had no effect on basal corticotropin plasma levels up to 30 mg/kg p.o. [76 ± 11 and 76 ± 15 pg/ml corticotropin for vehicle ($n = 20$) and SSR149415-treated ($n = 6$) rats, respectively].

Effect of SSR149415 on AVP-Induced Potentiation of Corticoliberin Effect on Corticotropin Secretion in Rats. The ability of AVP to enhance the action of corticoliberin on corticotropin secretion *in vitro* and *in vivo* is well established and this property is considered as a typical V_{1b} -mediated effect. We developed such a model in conscious rats and observed that 0.03 $\mu\text{g}/\text{kg}$ i.v. AVP synergized with 0.1 $\mu\text{g}/\text{kg}$ i.v. corticoliberin to promote corticotropin release, whereas each dose alone had no significant effect on cortico-

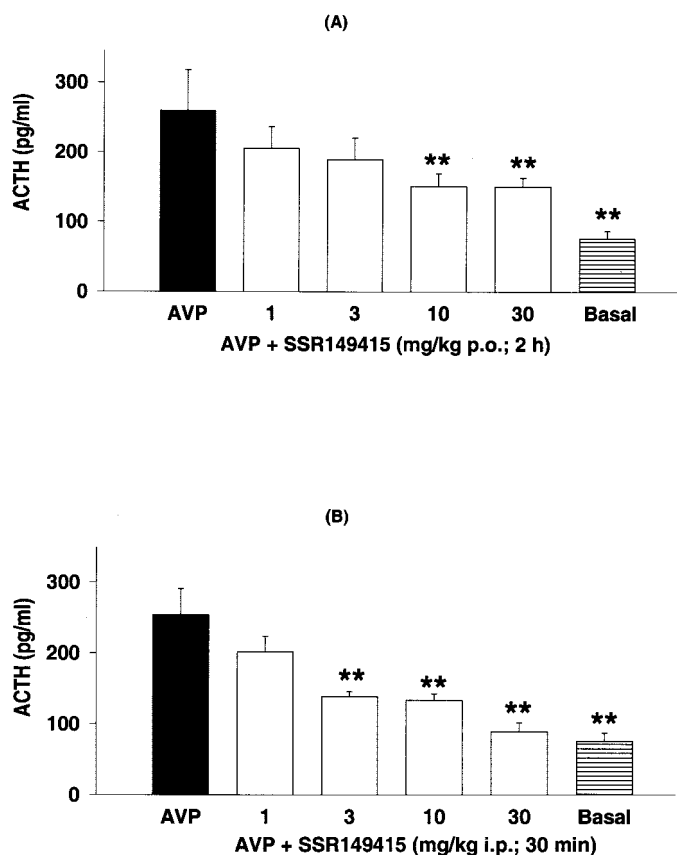


Fig. 4. Effect of oral (A) and i.p. (B) administration of SSR149415 on exogenous AVP-induced ACTH secretion in conscious rats. Conscious rats were administered with i.p. (A) and oral (B) SSR149415 [1 to 30 mg/kg or vehicle (5% DMSO, 5% Cremophor, 90% saline) before stimulation with 0.3 μ g/kg i.v. AVP]. Plasma ACTH levels, measured by RIA 10 min after the AVP challenge, yielded a basal value of 76 ± 11 pg/ml in rats treated with the vehicle ($n = 20$). Values are the mean \pm S.E.M. of six determinations per group. Statistical analysis was performed using a one-way ANOVA followed by a Dunnett's test and the level of significance was taken as $P < 0.05$ for comparison with the AVP-treated control group (*, $P < 0.05$; **, $P < 0.01$). ACTH, corticotropin.

trypsin secretion. Oral administration of SSR149415 (1–30 mg/kg) produced powerful dose-dependent inhibition of the corticotropin increase in response to exogenous AVP plus corticoliberin; the effect was significant from the dose of 3 mg/kg p.o. (Fig. 5A). Complete blockade was achieved at 10 mg/kg. The oral time course of 10 mg/kg SSR149415 showed a fast onset of action, the inhibitory effect of SSR149415 being already maximal at 1 h after administration. The inhibitory effect on corticotropin secretion lasted significantly more than 4 h, demonstrating a long-lasting oral effect in a specific V_{1b}-related model (Fig. 5B).

Effect of SSR149415 on Endogenous AVP-Induced Corticotropin Secretion in Rats. In rats, high doses (≥ 3 mg/kg) of SR121463, a selective V₂ receptor antagonist with powerful aquaretic properties, induce a strong and rapid water loss resulting in endogenous AVP secretion to avoid body dehydration and significantly increased plasma corticotropin levels (Lacour et al., 2000). As shown in Fig. 6, 10 mg/kg p.o. SR121463 induced a strong elevation (about 6-fold) in plasma corticotropin in conscious rats (46 ± 6 – 288 ± 24 pg/ml corticotropin, $n = 16$). In animals pretreated with 1 to 30 mg/kg SSR149415 a dose-dependent

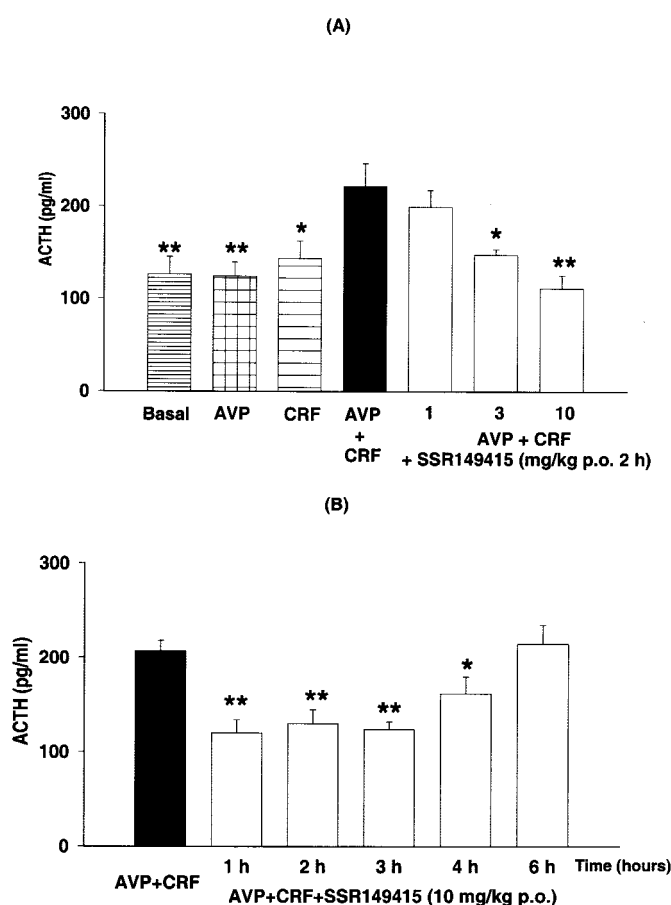


Fig. 5. Effect of oral SSR149415 on the potentiation of exogenous CRF by AVP on ACTH secretion in conscious rats. Dose-effect (A) and time course (B) studies. A, 1 to 10 mg/kg SSR149415 was administered by gavage 2 h before 0.1 μ g/kg i.v. CRF and 0.03 μ g/kg i.v. AVP injection. B, time course effect of SSR149415 was studied at 10 mg/kg p.o. on plasma ACTH secretion. Values are the means \pm S.E.M. of 7 to 10 animals per group for the time course and 11 to 19 animals for the dose effect. Statistical analysis was performed using a one-way ANOVA followed by a Dunnett's test or by using the nonparametric Kruskal-Wallis test. The level of significance was taken as $P < 0.05$ for comparison with the AVP + CRF-treated control group (*, $P < 0.05$; **, $P < 0.01$). We observed that 0.03 μ g/kg i.v. AVP synergized with 0.1 μ g/kg i.v. CRF to promote ACTH release, whereas each dose alone had no significant effect on ACTH secretion. CRF, corticoliberin; ACTH, corticotropin.

inhibition in plasma corticotropin secretion was observed, significant from the dose of 10 mg/kg p.o.

Effect of SSR149415 on Restraint Stress-Induced Corticotropin Secretion in Rats. As previously observed, various physical stresses are able to induce corticotropin secretion. The stress-induced release of corticotropin is believed to involve the activation of several humoral and neural pathways, including that mediated by AVP (Rivier and Vale, 1983; Linton et al., 1985). As shown in Table 2, in rats submitted to an immobilization period of 15 min there was a significant increase (more than 5-fold) in plasma corticotropin levels ($P < 0.01$ versus control). Pretreatment with SSR149415 at 10 mg/kg i.p., 30 min before the stress period caused a 50% inhibition of plasma corticotropin elevation in comparison with stressed animals treated with the corresponding vehicle.

Effect of SSR149415 in Four-Plate Test in Mice. The anxiolytic properties of SSR149415 were studied in the four-plate test, a model of anxiety based on unconditioned fear. It

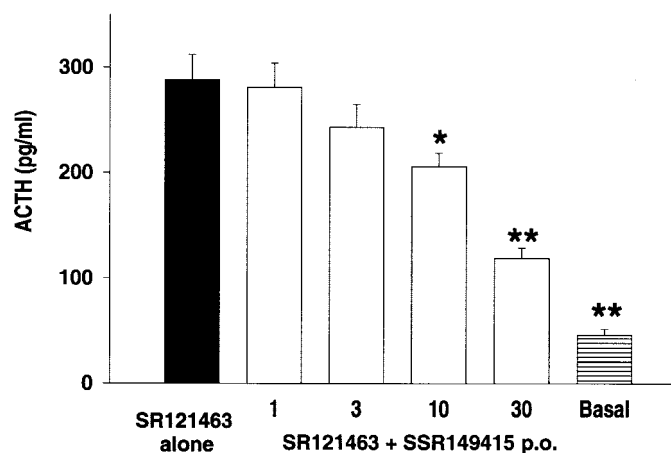


Fig. 6. Effect of SSR149415 on SR121463-induced plasma ACTH increase in conscious rats. Animals received first a high dose (10 mg/kg p.o.) of the aquaretic V_2 receptor antagonist SR121463. One hour later, 1 to 30 mg/kg SSR149415 was administered orally and plasma ACTH measured 1 h after the SSR149415 treatment. Values are the means \pm S.E.M. of 16 to 19 animals per group. Statistical analysis was performed using a one-way ANOVA followed by a Dunnett's test and the level of significance was taken as $P < 0.05$ for comparison with the SR121463-treated control group (*, $P < 0.05$; **, $P < 0.01$). ACTH, corticotropin.

TABLE 2
Effect of SSR149415 on restraint-stress-induced corticotropin secretion in conscious rats

Control animals treated with the vehicle are submitted (stress control, $n = 8$ animals) or not (control, $n = 10$ animals) to a restraint test of 15 min of immobilization as described under *Experimental Procedures*. Thirty minutes after injection with SSR149415 (10 mg/kg i.p.), another group of nine animals was submitted to the same immobilization period. The animals were sacrificed and plasma corticotropin levels were measured by RIA. Data are mean \pm S.E.M. Statistical significance was assessed by an ANOVA on transformed logarithmic data followed by Dunnett's test.

Test	Dose mg/kg i.p.	Corticotropin pg/ml
Control	0	61.9 \pm 8.0
Stress control	0	329.4 \pm 33.1*
SSR149415 + stress	10	192.1 \pm 28.7**

* $p < 0.01$ versus control.

** $p < 0.01$ versus stress control.

is sensitive to the action of classical (i.e., benzodiazepines) and atypical (i.e., 5-hydroxytryptamine reuptake inhibitors and metabotropic glutamate receptor ligands) anxiolytics (Bourin et al., 1992; Tatarczynska et al., 2001). In the acute experiments, i.p. and p.o. SSR149415 increased the number of punished crossings showing marked anxiolytic effects (Fig. 7). Post hoc analysis revealed that these effects reached statistical significance with SSR149415 from 3 mg/kg p.o. and i.p. Moreover, when SSR149415 was given repeatedly at 10 mg/kg p.o. for 7 days, the anxiolytic-like activity was still significantly maintained ($P < 0.01$). Finally, the oral time course of the anxiolytic-like action of SSR149415 performed at 10 mg/kg indicated that effects lasted for more than 4 h (Fig. 7).

Discussion

AVP is an important physiological regulator of the hypothalamo-pituitary-adrenal (HPA) axis, whose stimulation is a major component of the mammalian adaptative responses to stress (Aguilera and Rabadan-Diehl, 2000). AVP stimulates and potentiates corticosterone-induced corticotropin secretion by activating a specific anterior pituitary receptor,

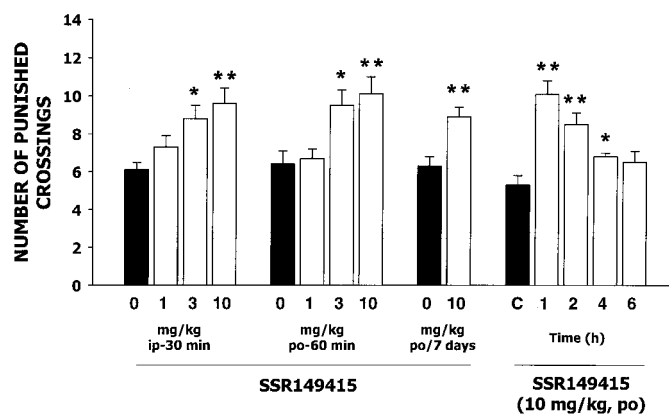


Fig. 7. Effects of acute and 7-day repeated treatments with SSR149415 in the four-plate test in mice, and time course of the acute effects. Data represent mean \pm S.E.M. *, $P < 0.05$; **, $P < 0.01$ (Dunnett's or Student's t test, or Kruskal-Wallis test). $n = 10$ to 15 animals.

recently cloned from mouse, rat, and human, and designated as V_{1b} or V_3 receptor (De Keyser et al., 1994; Sugimoto et al., 1994; Ventura et al., 1999). Indeed, molecular cloning of this receptor has provided key information concerning the cDNA expression in rat and human tissues, the localization of the V_{1b} protein in the rat CNS and binding/signal transduction characterization due to stable expression of this cloned receptor in mammalian cells (Lolait et al., 1995b; Thibonnier et al., 1997; Vaccari et al., 1998). However, the precise functional role of the V_{1b} receptor is still obscure due to the lack of selective V_{1b} receptor ligands and orally active molecules, which are crucial tools for investigating the central and peripheral functions or pathological disorders associated with this receptor.

The present study reports the biochemical and pharmacological characterization of SSR149415, the first selective, nonpeptide V_{1b} receptor antagonist described so far, with potent oral antagonist effects on corticotropin secretion and anxiolytic-like properties in rodents. This new compound (Fig. 1), belonging to an original chemical series, shows high affinity and marked selectivity for AVP V_{1b} receptors from animal and human origin, expressed in CHO cells or from native pituitary tissues (Table 1). Because species differences are very common in the field of AVP/OT receptors (Pettibone et al., 1992; Serradeil-Le Gal et al., 1993), it is important to emphasize that SSR149415 exhibits a similar nanomolar affinity for V_{1b} receptors in the different species studied. In binding studies with [3 H]AVP as a ligand, SSR149415 behaves as a fully competitive antagonist at human V_{1b} receptors (Fig. 2B) and displayed a K_i value close to that obtained for the natural hormone, AVP, and about 10-fold higher than the (V_{1a} , V_{1b}) peptide antagonist dPen, used as a reference tool up to now.

The highly selective V_{1b} profile of SSR149415 is an important characteristic of this molecule. First, in vitro, this compound has low affinity for the three other AVP/OT-related receptors both from rat and human origin (Table 1). Second, at the highest doses used to block the V_{1b} receptors in vivo (10 mg/kg i.p. and 30 mg/kg p.o.), SSR149415 does not modify the V_{1a} vascular response to AVP in conscious rats nor the urine flow rate controlled by renal V_2 receptors, both in normally hydrated and vasopressin-deficient Brattleboro rats. Third, the total lack of interaction of 10 μ M SSR149415

with a large number of receptors, ion channels, or enzymes has been evidenced. Thus, this molecule constitutes a unique ligand for targeting specifically V_{1b} receptors. One could also expect a particularly safe profile judging from this highly selective pattern of action. Indeed, *in vivo* pharmacological studies performed using SSR149415 with acute and repeated treatments in mice and rats and described herein have confirmed the good tolerability of this drug.

The V_{1b} receptor antagonistic properties of SSR149415 have been demonstrated *in vitro* and *in vivo*. By hypophyseal V_{1b} receptor activation, AVP directly stimulates corticotropin secretion and powerfully synergizes with corticoliberin in releasing corticotropin. This latter action has been reported as a typical V_{1b}-mediated phenomenon *in vitro* and *in vivo*. Earlier cellular events upstream of corticotropin release, provoked by occupancy of corticotroph V_{1b} receptors by AVP, include activation of phospholipase C, protein kinase C, and the mobilization of intracellular free Ca²⁺, mainly via G_{q/11} G protein recruitment. In CHO cells transfected with the human V_{1b} receptor other intracellular pathways have also been described (e.g., cAMP production, stimulation of DNA synthesis), clearly depending on the level of the V_{1b} receptor expression (Thibonnier et al., 1997, 1998). In our hands, SSR149415 behaves as a potent antagonist of AVP-induced [Ca²⁺]_i increase in CHO cells expressing either rat or human V_{1b} receptors. The K_i value obtained around 1 nM is consistent with the nanomolar affinity found in binding studies by using the same cellular preparations. Of note, SSR149415 was devoid of any agonist effect *per se*. *In vivo*, pharmacology performed measuring corticotropin secretion induced by various stimulants such as hormones and physical stress confirmed the full antagonist profile of SSR149415. In all these situations, SSR149415 antagonized corticotropin secretion, which constitutes a critical response of the organism to stress in emotional situations. The direct corticotropin response to exogenous stimuli, such as AVP, AVP plus corticoliberin, and to endogenous AVP increase subsequent to important body water loss or physical stress, was dose dependently inhibited by SSR149415 from the oral doses of 3 or 10 mg/kg, according to the model. It is important to note that SSR149415 demonstrated higher efficacy on the potentiation of corticoliberin effect by AVP, a mechanism described as a typical V_{1b}-mediated effect. In this latter model, significant inhibition of corticotropin secretion was observed from the oral dose of 3 mg/kg, total blockade occurred at 10 mg/kg *p.o.*, and this effect lasted for more than 4 h. It is worth noting that corticotropin levels are highly increased (at least 6-fold versus basal values) after body water loss/dehydration induced by a V₂ receptor antagonist, which could explain a somewhat lower efficacy of SSR149415 in this extreme situation. The lower corticotropin increase observed after the injection of exogenous 0.3 μg/kg *i.v.* AVP than after the elevation of endogenous AVP induced by SR121463 could be explained by the fact that the strong dehydration induced by the high dose of the aquaretic compound (10 mg/kg *p.o.*) provokes central AVP secretion, which rapidly activates nearby anterior pituitary V_{1b} receptors. Conversely, intravenous exogenous AVP could be subject to plasmatic degradation during its blood transport and lower concentrations of AVP could be available at pituitary V_{1b} receptors.

SSR149415 also antagonized the effects of restraint stress in rats as measured by the significant decrease in cortico-

tropin secretion induced by the immobilization period (50% at 10 mg/kg *i.p.*). Thus, the regulation of corticotropin plasma levels, and consequently of the HPA axis in these situations, are largely mediated by V_{1b} receptors and SSR149415 offers a new tool to control emotional or physical stress. Indeed, several neuroendocrine studies strongly suggest that dysregulation of the HPA system plays a causal role in the development and the course of diseases such as generalized anxiety, depression, and addiction. In addition, many clinical conditions are accompanied by an exaggerated response to stress (Holsboer, 1999). One can speculate that all such situations are potential indications for the use of V_{1b} receptor antagonists since these disorders have been associated with excessive HPA activity in both humans and animals. We demonstrated that SSR149415 displayed anxiolytic-like activity in the four-plate test in mice a well validated model of anxiety sensitive to the action of various classes of anxiolytics such as benzodiazepines, metabotropic glutamate receptor ligands, and 5-hydroxytryptamine uptake inhibitors (Bourin et al., 1992; Klodzinska et al., 1999; Hascoet et al., 2000; Tatarczynska et al., 2001). In this test, SSR149415 induces a marked, dose-dependent increase in punished responding, an effect that is indicative of an anxiolytic-like activity. Moreover, these effects are not accompanied by undesirable side effects such as sedation, decrease in spontaneous locomotor activity, or motor coordination disturbance (data not shown). Two additional experiments performed in the four-plate test showed that the anxiolytic-like activity of 10 mg/kg SSR149415 lasted for more than 4 h and was still present after repeated administration of the drug for 7 days, indicating first, a long-lasting oral effect for SSR149415, and second, no development of tolerance to the anxiolytic-like activity of the drug. Interestingly, a recent report has described the first mice carrying a null mutation of the V_{1b} receptors (Lolait et al., 2000; Hernando et al., 2001). In keeping with the anxiolytic-like properties of SSR149415, knockout mice displayed behavioral alterations such as reduced aggression, confirming the role of V_{1b} receptors in anxiety. Moreover, the V_{1b} receptor protein has a surprisingly wide distribution in the rat brain as recently shown by immunohistochemistry. In particular V_{1b} receptor immunoreactivity was observed in the hypothalamus, amygdala, cerebellum, and in areas close to circumventricular organs devoid of a blood-brain barrier (Hernando et al., 2001). This localization in key brain structures associated with specific central functions also strongly supports a role of central V_{1b} receptors in learning, memory, and various emotional and behavioral situations. Extensive studies are ongoing in our laboratory to further characterize the CNS pharmacological profile of SSR149415 in various animal models.

In conclusion, SSR149415 is a potent, selective, and orally effective V_{1b} receptor antagonist. It is a unique tool for exploring the role of V_{1b} receptors and deserves to be further investigated in various CNS disorders. This class of drug exhibits a promising therapeutic profile in the field of stress, anxiety, and depression.

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