Characterization of SR 121463A, a Highly Potent and Selective, Orally Active Vasopressin V2 Receptor Antagonist


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Abstract

SR 121463A, a potent and selective, orally active, nonpeptide vasopressin V2 receptor antagonist, has been characterized in several in vitro and in vivo models. This compound displayed highly competitive and selective affinity for V2 receptors in rat, bovine and human kidney (0.6 ≤ Ki [nM] ≤ 4.1). In this latter preparation, SR 121463A potently antagonized arginine vasopressin (AVP)-stimulated adenyl cyclase activity (Ki = 0.26±0.04 nM) without any intrinsic agonistic effect. In autoradiographic experiments performed in rat kidney sections, SR 121463A displaced [3H]AVP labeling especially in the medullary-papillary region and confirmed that it is a suitable tool for mapping V2 receptors. In comparison, the nonpeptide V2 antagonist, OPC-31260, showed much lower affinity for animal and human renal V2 receptors and lower efficacy to inhibit vasopressin-stimulated adenyl cyclase (Ki in the 10 nanomolar range). Moreover, OPC-31260 exhibited a poor V2 selectivity profile and can be considered as a V2/V1a ligand. In normally hydrated conscious rats, SR 121463A induced powerful aquarexia after intravenous (0.003–0.3 mg/kg) or oral (0.03–10 mg/kg) administration. The effect was dose-dependent and lasted about 6 hours at the dose of 3 mg/kg p.o. OPC-31260 had a similar aquaretic profile but with markedly lower oral efficacy. The action of SR 121463A was pure aquaretic with no changes in urine Na+ and K+ excretions unlike that of known diuretic agents such as furosemide or hydrochlorothiazide. In addition, no anti-diuretic properties have been detected with SR 121463A in vasopressin-deficient Brattleboro rats. Thus, SR 121463A is the most potent and selective, orally active V2 antagonist yet described and could be a powerful tool for exploring V2 receptors and the therapeutical usefulness of V2 blocker aquaretic agents in water-retaining diseases. (J. Clin. Invest. 1996. 98:2729–2738.) Key words: SR 121463A • vasopressin • nonpeptide antagonist • V2 receptor • aquaretic

Introduction

The importance of arginine vasopressin (AVP) in the regulation of blood pressure and volume and in the control of fluid and electrolyte balance is well established. AVP plays a major role as an antidiuretic hormone regulating water and solute excretion by the kidney through specific interaction with the renal V2 receptors present all along the collecting duct from cortex to papilla in the mammalian nephron (1, 2).

So far, three AVP receptors subtypes, V1a, V1b, and V2 have been identified based upon their primary structure (3–10), their coupling mechanisms, their tissular distributions and their pharmacological properties (for review see 11, 12). The V2 receptor belongs to the seven transmembrane G protein-coupled receptor superfamily and is positively coupled to a Gs/adenyl cyclase system. This V2 receptor has been cloned in different species including rat, pig, bovine and human (7–10). Moreover, several constitutive AVP V2 receptor gene mutations have now been identified as the molecular basis for the lack of urine concentration in Nephrogenic Diabetes Insipidus (13, 14).

Receptor-specific AVP V2 antagonists, so-called “aquaretic agents,” able to block the action of AVP in the collecting duct cells and thus to promote specifically water excretion, could be of high therapeutic value for the treatment of several water-retaining disorders such as SIADH (Syndrome of Inappropriate Antiurietic Hormone secretion), liver cirrhosis, certain stages of congestive heart failure and hypertension, nephrotic syndrome (15–18). In most of these diseases an abnormal increase of circulating AVP plasma level, activating renal V2 receptors, seems to be the key event in water retention and subsequent hypotonic hyponatremia (18–20). Thus, for these pathologies, there is great clinical interest in the development of potent V2 receptor antagonists to provide specific water diuretic/aquaretic compounds devoid of the well-known side effects of classical diuretic or saluretic agents on the solute excretion (urine Na+ and/or K+ loss).

Although several potent peptide vasopressin V2 receptor antagonists have been synthesized, the evaluation of their therapeutic utility has been severely hampered by their lack of oral bioavailability, species differences and especially by their agonist antidiuretic effects when tested in man (21). Recent years have marked a turning point with the design of the first nonpeptide, orally effective AVP V1a and V2 receptor antago-

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1. Abbreviations used in this paper: AVP, arginine vasopressin; cAMP, cyclic adenosine monophosphate; DI, diabetes insipidus; OT, oxytocin.
nists (22–24). In this field, Yamamura et al. reported an orally effective V₂ compound, OPC-31260, exerting aquareysis in several animal models and in man without agonistic activity (25, 26).

In this study, we describe the structure and the pharmacological properties of SR 121463A, (1-[4-(N-tert-butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)cyclohexane]-indol-2-one, fumarate), a novel, highly potent and V₂-selective nonpeptide AVP receptor antagonist (see Fig. 1). In all the biological tests used, a close comparison was performed between the effects of the two nonpeptide molecules, SR 121463A and OPC-31260, belonging to different chemical series.

In addition, despite the close structural similarities between AVP V₂ receptors from different species (7–10), marked differences between their pharmacological properties have been described (21). For these reasons, special attention was paid to the evaluation of SR 121463A in several human tissues and in predictive pharmacological models in order to design a compound devoid of partial agonist effects and with the expected aquaretic activity in man.

**Methods**

**Materials**

The newly described nonpeptide molecule, SR 121463A, (1-[4-(N-tert-butylcarbamoyl)-2-methoxybenzene sulfonyl]-5-ethoxy-3-spiro-[4-(2-morpholinoethoxy)cyclohexane]-indol-2-one, fumarate; equatorial isomer), (Fig. 1), was synthesized in Sanofi Recherche, Toulouse, France.) and belongs to an original chemical series of potent and selective V₂ receptor antagonists. The only other published nonpeptide V₂ receptor antagonist, OPC-31260, (±)(5-dimethylamino-1-[4-(2-methylbenzylamino)benzyl]-2,3,4,5-tetrahydro-1H-benzazepine) (24), was synthesized in Sanofi Recherche, Montpellier, France, and used as a reference in the different biological tests (Fig. 1). The structures of SR 121463A and OPC-31260 were determined by 'H and ¹³C NMR and infrared spectroscopy. The molecular weights, determined by mass spectrometry, are 736.6 and 427.5 for SR 121463A and OPC-31260, respectively. Melting points of 172 and 207.8°C, respectively, were obtained. The purity, measured by high pressure liquid chromatography, thin layer chromatography and elemental analysis, was >98%. The analytical parameters reported above for OPC-31260 are identical to those initially described for this molecule (27).

For in vitro experiments, SR 121463A and OPC-31260 were dissolved in DMSO (10⁻² M) and then diluted in the appropriate test solvent. SR 121463A was dissolved in saline and DMS for intravenous and intraperitoneal administration in rats, respectively. For oral treatment, all compounds used were administered in 0.6% methylcellulose solution.

AVP, polybrene, PMSF, OT, and bacitracin were from Sigma Chemical Co. (L’Isle d’Abeau, France). BSA type V was obtained from IBF (Villeneuve La Garenne, Paris, France). DME and PBS were from Boehringer Mannheim (Meylan, France). All other cell culture reagents were from GIBCO (Life Technologies, Gibco BRL, France). EDTA, Tris and DMSO were purchased from Merck (Darmstadt, Germany). Human pituitary glands were collected from deceased persons within 6 h after death and immediately stored in liquid nitrogen. Human pituitary glands were collected from deceased persons within 6 h after death and immediately stored in liquid nitrogen. Crude plasma membranes were prepared from the frozen tissue before each experiment. Bovine kidneys were obtained from a local slaughterhouse. Mammary tissue was taken from 19-d-old Sprague-Dawley pregnant rats and stored in liquid nitrogen until used. Male Sprague-Dawley rats, 250–350 g, (Iffa-Credo, Lyon, France) were used for in vitro binding studies and for in vivo activity measurements. Two series of male homozygous Brattleboro rats with central Diabetes Insipidus (DI) weighing 300–350 g, bred in house (INSERM Unité 90, Hôpital Necker, Paris, France) or bought from Harlan Sprague-Dawley (Indianapolis, IN), were used for evaluation of potential agonist antidiuretic activities. All protocols performed in Sanofi Recherche have been approved by the Animal Care and Use Committee of Sanofi Recherche.

**Biological material**

Human tissue samples from adrenals, kidneys, and pituitaries were collected in conformity with the French national ethical rules. Healthy human adrenals were obtained from human donors after brain death. Adrenals were chilled in cold saline and dissected. Crude plasma membranes were prepared within 3–5 h and stored in liquid nitrogen. Human kidneys were obtained from nephrectomy for renal carcinoma. The tissue was chilled into cold saline and dissected 2–8 h after excision. Only the non pathological part of the kidney was used to prepare crude plasma membranes which were stored in liquid nitrogen. Human pituitary glands were collected from deceased persons within 6 h after death and immediately stored in liquid nitrogen. Crude plasma membranes were prepared from the frozen tissue before each experiment. Bovine kidneys were obtained from a local slaughterhouse. Mammary tissue was taken from 19-d-old Sprague-Dawley pregnant rats and stored in liquid nitrogen until used. Male Sprague-Dawley rats, 250–350 g, (Iffa-Credo, Lyon, France) were used for in vitro binding studies and for in vivo activity measurements. Two series of male homozygous Brattleboro rats with central Diabetes Insipidus (DI) weighing 300–350 g, bred in house (INSERM Unité 90, Hôpital Necker, Paris, France) or bought from Harlan Sprague-Dawley (Indianapolis, IN), were used for evaluation of potential agonist antidiuretic activities. All protocols performed in Sanofi Recherche have been approved by the Animal Care and Use Committee of Sanofi Recherche.

**In vitro experiments**

**Membrane preparations.** Human hypophysal membranes were prepared as previously described in (23). Membrane preparations from human kidneys or adrenal cortex were obtained according to Guillout et al. (29) and (30), respectively. Membranes from Ltk⁻ cells, trans-
ected with the DNA encoding for the human oxytocin receptor, were prepared as in (31). Briefly, 72 h after transfection, cells were harvested, washed twice in PBS without Ca\(^2+\) and Mg\(^2+\), polytron-homogenized in lysis buffer (15 mM Tris-HCl pH 7.5; 2 mM MgCl\(_2\); 0.3 mM EDTA), and centrifuged at 100 g for 5 min at 4°C. Pellets were washed in a buffer A consisting of 50 mM Tris-HCl, pH 7.4; 5 mM MgCl\(_2\); and centrifuged at 44,000 g 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.

Membranes from rat and bovine kidney (papilla and inner medulla), rat liver and rat mammary glands, were prepared according to Stassen et al. (32), Pripic et al. (33) and Serradeil-Le Gal et al. (23), respectively.

Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard (34).

**Binding assays**

Binding affinity constants of SR 121463A and OPC-31260 for the different AVP/OT receptors investigated were deduced from competition experiments using the appropriate radiolabeled ligands: [\(^{3}H\)]AVP (bovine and rat kidney, rat liver and human pituitary); [\(^{125}\)I]linear AVP antagonist (adrenals); [\(^{125}\)I]OT antagonist (rat mammary glands and Ltk− cells expressing the uterine oxytocin receptor). Experiments were performed as described earlier (23). Binding assays of [\(^{3}H\)]AVP to rat kidney medullary membranes were conducted according to the method of Yamamura et al. (24).

For competition experiments, increasing concentrations of SR 121463A or OPC-31260 were incubated with membranes and the corresponding ligand under the above-specified conditions. Saturation binding experiments using [\(^{3}H\)]AVP (from 0.08 to 20 nM) as a ligand were performed in bovine kidney in the absence (control) or presence of SR 121463A (0.25, 0.50, 1, 2, and 4 nM).

**Binding data analysis**

The IC\(_50\) 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 IC\(_50\) values using the Cheng and Prusoff equation (34). Data for equilibrium binding (K\(_D\), B\(_{max}\)), competition experiments (IC\(_50\), nHill), and kinetic constants (K\(_m\), K\(_i\)) were analyzed using an iterative non-linear regression program (35). IC\(_50\) values of SR 121463A and OPC-31260 were compared using Student’s t test. Values of P < 0.05 were taken as significant.

**Autoradiography**

Male Sprague-Dawley rats (250–300 g) were killed by decapitation and the kidneys were rapidly removed and frozen in isopentane at −45°C. Serial sections (15 μm) were cut in a cryostat microtome and thaw-mounted onto chrome alum gelatin-coated glass slides (gelatin 1%, chrome-alum 0.05%). Sections were stored at −80°C until use. Slide-mounted sections (3–4 sections/slide), brought to room temperature, were preincubated for 15 min in the binding buffer (50 mM Tris-HCl, pH 8.1, 2 mM MgCl\(_2\), 1 mM EDTA, 0.1% bovine serum albumin and bacitracin). Incubation was carried out for 1 h at room temperature in the incubating medium in the presence of 2 nM [\(^{3}H\)]AVP. Nonspecific binding was determined by incubating additional slides under the same conditions and in the presence of 1 μM unlabeled AVP. After incubation, the sections were washed three times for 10 min each in ice-cold buffer, dipped briefly in distilled water, and dried under a stream of cold air. Labeled sections were placed on a phosphor-imaging plate (Fuji) for 4 d and further analyzed with a Bio-Image Analyser (BAS 2000, Fuji) as described in (37).

**Adenyl cyclase assay.** Adenyl cyclase activity was measured as previously described (29) by the rate of conversion of [\(^{32}\)P]ATP into cAMP. The incubation medium contained: 10 mM sodium azide; 0.1 mM ouabain; 1 mM cAMP; 0.25 mM ATP; 0.17 μCi/ml [\(^{3}H\)]cAMP; 20 mM creatine phosphate; 1 mg/ml bovine serum albumin and various amounts of the nonpeptide analogues. Membrane proteins, 10–30 μg per assay, were preincubated for 15 min at 30°C and the reaction was initiated by adding ATP 0.25 mM plus 1 μCi of [\(^{32}\)P]ATP with or without 10 nM AVP. The membranes were incubated for an additional 10 min period and the reaction stopped by adding 500 μl of a solution containing: 100 mM Tris-HCl, pH 8.0; 80 mM sodium dodecylsulfate; 1 mM ATP and 1 mM cAMP. Labeled cAMP was separated and counted by liquid scintillation spectrometry as previously described in (29). All values were corrected for cAMP recovery estimated from the recovery of the [\(^{3}H\)]cAMP added to the incubation medium and for a blank value determined in the absence of membranes. Adenyl cyclase activities were expressed as pmol cAMP accumulated/10 min per mg protein. All determinations were performed in duplicate.

**In vivo experiments**

Properties of SR 121463A in normally hydrated conscious rats. Male Sprague Dawley rats weighing 290–320 g were kept in an air-conditioned room at 22±2°C and fed with a standard rat diet (AO4, UAR Epinay sur Orge, France) with water provided ad libitum. In a first set of experiments, SR 121463A was administered intravenously at doses varying from 0.003 to 0.3 mg/kg (i.e. 0.004 to 0.4 μmol/kg) body weight (n = 6) to normally hydrated conscious rats. SR 121463A was injected (1 ml/kg) in 0.9% NaCl through a catheter placed in a jugular vein, 48 h before the experiment. The animals were then housed individually in metabolic cages with water and food ad libitum. Urine was collected for 4 h. In a second set of experiments, SR 121463A, 0.03–10 mg/kg (0.04–14 μmol/kg, n = 7), OPC-31260, 10 mg/kg (23 μmol/kg, n = 8), furosemide, 30 mg/kg (91 μmol/kg, n = 8), hydrochlorothiazide, 30 mg/kg (101 μmol/kg, n = 8) or vehicle (methylcellulose 0.6%, n = 20) were administered orally to rats by gavage (3 ml/kg). After treatment, the rats were placed individually in metabolic cages with food and water ad libitum. Urine was collected throughout the 24-h period after treatment. In the time-course study, the aquaretic effect was measured by collecting urine at 2 h intervals for 6 h and then from 6 to 24 h in control (methylcellulose 0.6%, n = 20) and treated groups (SR 121463A, 0.03–10 mg/kg p.o., and OPC-31260, 10 mg/kg). The effects of the drugs tested on urine osmolality, and urine Na+ and K+ excretions were tested on a 24-h urine collection period after drug administration. Urine osmolality was measured with a freezing point depression osmometer (model Fisk OS 110, Elvetect, Montpellier, France) and urinary sodium and potassium concentrations with a flame photometer (IL 943, Instrument Laboratories, Marseille, France).

Statistical significance of the results was analyzed by one-way analysis of variance on independent measurements followed by Dunnett or Kruskal-Wallis’ test. A two-way analysis of variance followed by Dunnett’s test was also used when appropriate.

**Activity of SR 121463A in vasopressin-deficient Brattleboro rats in comparison with the V1 peptide antagonist SK&F 101926**

The activity of SR 121463A was compared with that of the reference AVP V1 peptide, SK&F 101926, in an experimental vasopressin-deficient rat strain. Male Brattleboro rats (300–350 g), exhibiting hereditary DI (38), were used in these studies. They were placed in metabolic cages and received food and tap water ad libitum throughout the experiment. After at least seven days of adaptation to the metabolic cages, rats received an i.p. injection of drug or vehicle (100 μl rat) at ~ 9:00 a.m. and urine was collected for the next 24 h. Urine volume was measured gravimetrically, and urine osmolality measured with a freezing point osmometer (Roehling, Berlin, Germany). SR 121463A was dissolved in dimethylformamide and administered at a dose of 10 mg/kg. The vasopressin analogue, SK&F 101926, was dissolved in 0.9% NaCl and administered at a dose of 0.01 mg/kg. Control rats of each series received the same volume of dimethylformamide or 0.9% NaCl, respectively. For each drug, control and treated groups were compared by Student’s t test.
Table I. Comparative Affinities of SR 121463A and OPC-31260 for Vasopression and Oxytocin Receptors in Animal and Human Species

<table>
<thead>
<tr>
<th></th>
<th>Kᵢ (nM)</th>
<th>V₂</th>
<th>V₁α</th>
<th>V₁b</th>
<th>OT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kidney</td>
<td>Bovine</td>
<td>Human</td>
<td>Rat</td>
<td>Human</td>
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<tr>
<td>SR 121463A</td>
<td>1.42±0.98</td>
<td>0.64±0.14</td>
<td>4.1±0.8</td>
<td>10,600±6,000</td>
<td>460±120</td>
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<tr>
<td>OPC-31260</td>
<td>21.7±9.1</td>
<td>10.9±0.8</td>
<td>25.4±8.6</td>
<td>748±254</td>
<td>260±30</td>
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</tbody>
</table>

Binding assays were performed as described in Methods. Inhibition constants (Kᵢ) were determined from competition experiments and calculated according to the equation of Cheng and Prusoff (35). Values are the mean±SD of at least three determinations. V₂ IC₅₀ values of SR 121463A and OPC-31260 were compared using Student’s t test. Values of P < 0.05 were taken as significant.

Results

Interaction of SR 121463A with animal and human AVP V₂ receptors, and in vitro selectivity profile. As shown in Table I, SR 121463A displayed a high affinity for AVP V₂ receptors from several species including man. This nonpeptide compound dose-dependently inhibited [³²P]cAMP specific binding to kidney medullo-papillary membranes from rat, bovine and human origin with a Hill coefficient close to the unity, compatible with a single site competitive model as illustrated in human kidney membranes (Fig. 2 A).

Moreover, saturation binding experiments performed in a bovine kidney preparation in the absence or presence of SR 121463A (0.25, 0.50, 1, 2, and 4 nM) confirmed that this compound interacted competitively with renal AVP V₂ receptors. Indeed, in the presence of this molecule, the apparent dissociation constant (Kᵢ) was dose-dependently decreased, whereas the maximal binding capacity (Bₘₐₓ) was not significantly modified (Fig. 3). The Kᵢ value calculated from Scatchard plots (0.78±0.15 nM) was consistent with that obtained according to the Cheng and Prusoff equation in competition experiments performed in bovine kidney preparations (Kᵢ = 0.87 nM).

In terms of selectivity, the highly V₂-specific profile of SR 121463A was firstly evidenced for other AVP receptor subtypes (V₁α, V₁b) and for the closely related oxytocin (OT) receptor from both animal and human origin. As shown in Table I, SR 121463A exhibited only weak affinities for other AVP/OT receptors and interacted with at least a 100 fold lower potency with V₁α, V₁b, and OT receptors than with V₂. Second, the high degree of specificity of SR 121463A for the V₂ receptor was further demonstrated in several additional binding assays (n = 50) showing that SR 121463A (1 μM) was unable to bind to a variety of receptors of nonpeptide (histamine, adrenergic, dopamine, serotonin, adenosine, L-type calcium channel, benzodiazepine) or peptide ligands (angiotensin II, endothelin, neuropeptide Y, cholecystokinin, CRF, neurotensin) (not shown).

The comparison with the nonpeptide compound, OPC-
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31260, tested under similar experimental conditions, showed that this molecule displayed significantly lower affinity (6 to 17-fold) than SR 121463A for AVP V2 receptors in the different species tested (Table I, Fig. 2A). The affinity found here for OPC-31260 both in rat kidney and liver membranes is in agreement with the original published values (24). In addition, OPC-31260 showed only a relatively weak specificity for V2 receptors since this molecule also exhibited significant affinity for AVP V1a receptors especially in human tissues (Table I).

The selectivity index of SR 121463A and OPC-31260 for the human V2 receptor versus the human V1a, V1b, and OT receptors clearly evidenced a better specificity for SR 121463A (> 100) (Table II) showing that OPC-31260 should rather be considered as a V2/V1a compound, whereas SR 121463A is the first potent and highly selective nonpeptide V2 ligand yet described.

**Autoradiographic localization of AVP V2 receptors in the rat kidney using SR 121463A.** Autoradiographic experiments, using [3H]AVP as a ligand, provided the mapping and localization of AVP receptors in the rat kidney. As illustrated in Fig. 4A, [3H]AVP intensively labeled the medullo-papillary region of the kidney and, to a lesser extent, the cortex, as previously described (39, 40). SR 121463A (100 nM) displaced most of [3H]AVP labeling in the medullo-papillary area (Fig. 4C). The remaining amounts of labeling observed, particularly in the region of the medulla, in the pelvic wall and in the cortex could be due to [3H]AVP binding to V1a receptors also present in this organ (37, 40). At the same 100 nM concentration, OPC-31260 appears to be less potent than SR 121463A in displacing [3H]AVP labeling in rat kidney sections (Fig. 4D), as expected according to the affinity in rat kidney membranes observed for OPC-31260, almost 20-fold lower than for SR 121463A (Table II).

<table>
<thead>
<tr>
<th>Selectivity Profile of the Two Nonpeptide AVP V2 Receptor Antagonists, SR 121463A and OPC-31260, for Human Vasopressin and Oxytocin Receptors</th>
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<tbody>
<tr>
<td>Selectivity index</td>
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<tr>
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</tr>
<tr>
<td>SR 121463A</td>
</tr>
<tr>
<td>OPC-31260</td>
</tr>
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</table>

Inhibition constants ($K_i$) used in the calculation of these ratios are given in Table I.

![Figure 3. Scatchard plots of [3H]AVP binding to bovine kidney membranes without (●) or with 0.25 (○), 0.5 (▲), 1 (○), 2 (■), and 4 (□) nM SR 121463A. Results represent data from a typical experiment. All experiments were performed in duplicate.](image)

![Figure 4. Autoradiograms of [3H]AVP binding to rat kidney sections in the absence (A) or presence of 1 μM AVP (B), 0.1 μM SR 121463A (C), or 0.1 μM OPC-31260 (D). The nonspecific binding (B) was obtained by incubating alternate sections in the presence of 1 μM unlabeled AVP. The scale in A represents 5 mm.](image)
Thus, SR 121463A represents a selective V₂ probe for studying the in situ localization of V₂ receptors.

Effect of SR 121463A on adenylyl cyclase activity in human kidney membranes. To determine the agonist or antagonist properties of SR 121463A, we examined the activity of this compound on the AVP-induced adenylyl cyclase activity in human kidney membranes. In these preparations, AVP maximally stimulated cAMP production by 3.6±0.3-fold with a Kᵣ of 2.5 nM (data not shown) in good agreement with previous results (29). Data shown in Fig. 2 B demonstrate that both SR 121463A and OPC-31260 dose-dependently inhibited AVP (10 nM)-induced adenylyl cyclase stimulation yielding respective Kᵣ values of 0.26±0.04 and 17.6±8.3 nM (n = 3). These results clearly evidenced a higher potency of SR 121463A versus OPC-31260 in inhibiting AVP-stimulated adenylyl cyclase, as expected from the binding affinities found for these two compounds for the human kidney V₂ receptors (Fig. 2 A). Moreover, neither SR 121463A nor OPC-31260 were able to stimulate the basal adenylyl cyclase activity in human kidney membranes in concentrations up to 100 μM, showing the total absence of agonistic properties (not shown).

In vivo activity of SR 121463A in normal conscious rats. In normally hydrated conscious rats, oral administration of SR 121463A, 0.03–10 mg/kg (0.04–14 μmol/kg), increased urine excretion and decreased urine osmolality dose-dependently (Fig. 5). This effect was significant from 0.03 mg/kg on urine osmolality and from 0.1 mg/kg and upwards for urine volume (P < 0.05). The time-course showed a rapid effect on urine flow rate. The maximal effect was reached during the first 2 hour-period after the administration of the different doses of SR 121463A (Fig. 6). The higher doses (3 and 10 mg/kg) had effects lasting into the 6–24 h sampling period as shown by a urine flow rate higher than that occurring in the vehicle-treated group. Under identical experimental conditions, OPC-31260, 10 mg/kg p.o. on the drop in urine flow rate which was exactly parallel to the oral dose-response curve (Fig. 7). From these plots, a ratio of 5 between intravenous and oral efficacies could be deduced (0.1 mg/kg [0.14 μmol/kg] i.v. induced similar effect to 0.5 mg/kg [6.8 μmol/kg] p.o.) demonstrating good oral bioavailability for SR 121463A.

Intravenous administration of SR 121463A from 0.003 to 0.3 mg/kg, (0.004 –0.4 μmol/kg), induced a dose-dependent increase in urine flow rate which was exactly parallel to the oral dose-response curve (Fig. 7). From these plots, a ratio of 5 between intravenous and oral efficacies could be deduced (0.1 mg/kg [0.14 μmol/kg] i.v. induced similar effect to 0.5 mg/kg [6.8 μmol/kg] p.o.) demonstrating good oral bioavailability for SR 121463A.

Table III further summarizes the effects of SR 121463A on urine flow rate, osmolality and Na⁺/K⁺ excretion in comparison with those of traditional diuretic compounds such as furosemide and hydrochlorothiazide. These agents were used at oral doses eliciting similar effects on urine volume excretion (0.3 mg/kg i.e. 0.4μmol/kg for SR 121463A, 30 mg/kg for furo-
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...compound could also be detected in the vasopressin-deficient Brattleboro rat strain because these rats have higher sensitivity to antidiuretic agonists than AVP-repleted rats. These findings suggest that this experimental model allows the assessment of the partial agonistic activities of putative aquaretic agents. We studied the influence of SR 121463A in comparison with SK&F 101926 on urine excretion and osmolality in these rats (Fig. 8). As expected, control Brattleboro rats, exhibiting hereditary central DI, were characterized by a high urine flow rate (~200 ml/24 h) and low urine osmolality (~170 mOsm/kg H2O). As shown in Fig. 8, SR 121463A (10 mg/kg i.p.) induced significant aggravation of these parameters by further enhancing urine flow rate by 77% (354±42 ml/24 h) and lowering urinary osmolality by 54% (92±8 mOsm/kg H2O) (P < 0.01). In contrast, SK&F 101926 induced antidiuretic effects in this model by lowering urine volume and increasing urine osmolality by 60% each, as previously described. It is important to note that further experiments, performed with increasing concentrations of SR 121463A, have shown that the effects observed on urine volume and osmolality in DI rats are dose-dependent and are detectable with doses as low as 0.01 mg kg i.p. (data not shown). In conclusion, SR 121463A appears to be devoid of any antidiuretic properties even when administered at a high dose in Brattleboro rats. Moreover, this molecule is able to enhance diuresis in this animal model.

Table III. Effects of SR 121463A, Furosemide and Hydrochlorothiazide p.o. on Urine Volume and Osmolality, and on Na⁺ and K⁺ Urinary Excretion in Normally Hydrated Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Urine Volume (ml/24 h)</th>
<th>Urine Osmolality (mOsm/kg H2O)</th>
<th>Na⁺ (μmol/24 h)</th>
<th>K⁺ (μmol/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.1±0.7</td>
<td>1,308±73</td>
<td>912±63</td>
<td>1,464±95</td>
</tr>
<tr>
<td>SR 121463A 0.3 mg/kg</td>
<td>9.8±1.2*</td>
<td>1,392±70*</td>
<td>1,026±100</td>
<td>1,410±87</td>
</tr>
<tr>
<td>Furosemide 30 mg/kg</td>
<td>17.8±3*</td>
<td>524±67*</td>
<td>1,276±69*</td>
<td>1,680±109</td>
</tr>
<tr>
<td>Hydrochlorothiazide 30 mg/kg</td>
<td>16.1±1.3*</td>
<td>613±37*</td>
<td>1,727±150*</td>
<td>2,047±168*</td>
</tr>
</tbody>
</table>

Control were treated with 0.6% methylcellulose as described in the Methods section. Groups of 7–8 animals were treated with oral SR 121463A (0.3 mg/kg, i.e., 0.4 μmol/kg), furosemide (30 mg/kg, i.e., 91 μmol/kg) or hydrochlorothiazide (30 mg/kg, i.e., 101 μmol/kg) and urines were collected for 24 h after drug or vehicle administration. Statistical significance was assessed by Dunnett or Kruskal-Wallis test: *P < 0.05.

Discussion
Specific blockade of renal AVP V₂ receptors seems to be a relevant approach for generating selective aquaretic agents for the treatment of water-retaining disorders (15–20). Therefore, intensive work and active investigations have been performed to design selective ligands for AVP receptors (22–24, 43). The present study describes the biochemical and pharmacological profile of SR 121463A, a newly potent and selective nonpeptide AVP V₂ receptor antagonist with powerful oral aquaretic properties in the rat.

This new molecule (Fig. 1), belonging to an original chemical series, shows high affinity and marked selectivity for AVP V₂ receptors from animal and human origin (Table I). In binding experiments, SR 121463A inhibits [3H]AVP labeling to rat, bovine and human kidney membranes with nanomolar and even subnanomolar potency (Kᵢ values of 1.42, 0.64, and 4.1 nM, respectively). As demonstrated in bovine kidney preparations, SR 121463A is a full competitive inhibitor (Fig. 3). Since...
we (23, 44), and others (29, 45, 46), have previously demonstrated marked species differences in the in vitro affinity and potency of several nonpeptide AVP/OT receptor antagonists, especially when tested in human tissues, it is of importance to underline that SR 121463A also potently interacts with human renal V2 receptors. Moreover, the high affinity of SR 121463A found in this latter preparation is consistent with the powerful antagonist effect of this compound in inhibiting AVP-stimulated adenylyl cyclase (Ki value of 0.26 nM) without any agonistic response (Fig. 2).

Another striking finding is the high degree of selectivity of SR 121463A for V2 receptors as evidenced in several binding tests in vitro. Firstly, this compound has very low affinity for AVP V1a, V1b, and for the related oxytocin receptors (selectivity index at least > 100; Table II) and secondly, SR 121463A does not interact with more than 50 receptors of other nonpeptide or peptide ligands. It is noteworthy that despite the low affinity of SR 121463A for V1a receptors, this molecule is able to discriminate rat liver and human adrenal V1a receptors (Table I).

When the properties of OPC-31260, the only other published nonpeptide V2 receptor antagonist, and SR 121463A are compared under similar experimental conditions, OPC-31260 is significantly less potent (Ki > 10 nM versus Ki in the nanomolar range for SR 121463) in inhibiting [3H]AVP binding to rat, bovine and human kidney membranes and in antagonizing AVP-stimulated adenylyl cyclase in human kidney plasma membranes in vitro (Table I and Fig. 2). Moreover, SR 121463A is much more selective towards the V2 AVP receptor subtype than OPC-31260 (Table II). Indeed, despite a well-conserved affinity of OPC-31260 for human V2 receptors, this compound exhibits a poor V2 selectivity profile especially noticeable on human AVP and OT receptors (Tables I and II). These data are consistent with previous results showing a significant interaction of OPC-31260 with human V1a receptors, both in binding (44) and functional studies. In vitro, OPC-31260 effectively antagonizes AVP-induced contractions in human isolated internal mammary and coronary arteries (47, 48), known to express the AVP V1a receptor subtype. Thus, this molecule could be considered as a V2/V1a antagonist compound whereas SR 121463A is a pure V2 ligand.

In addition, autoradiographic experiments performed in rat kidney sections, using [3H]AVP as the ligand and SR 121463A, show an intense localization of V2 binding sites specifically in the medullo-papillary region of the kidney in agreement with previous reports using mRNA V2 receptor in situ hybridization and autoradiographic techniques (39, 40). Indeed, SR 121463A is the most V2 selective ligand so far described. Thanks to its high stability, it may represent a good pharmacological tool for mapping V2 receptors in the organism and for the search of extrarenal V2 sites (49) and/or potential V2 subtypes. In that respect, a V2 receptor subtype has been suggested in rat limbic brain areas (50) and V2 receptor mRNA has also been detected in rat hippocampus (51).

The pharmacological profile of SR 121463A described herein deserves to be underlined and demonstrates that this molecule is a potent fully aquaretic drug in several rat experimental models in vivo. After intravenous and oral administration in normally hydrated rats, SR 121463A enhanced the urine flow rate and decreased urine osmolality in a dose-dependent manner showing that SR 121463A is able to counteract the antidiuretic effect of endogenous vasopressin. The comparison between intravenous and oral responses shows that SR 121463A is well absorbed (oral route only 5 times less potent than i.v. route) and has good bioavailability. The diuretic effect, and the subsequent drop in urine osmolality, at 0.3 mg/kg (0.4 μmol/kg) i.p., are almost equipotent to those of oral OPC-31260, 10 mg/kg (23 μmol/kg), and furosemide 30 mg/kg (91 μmol/kg) or hydrochlorothiazide 30 mg/kg (91 μmol/kg). Moreover, the comparison of SR 121463A with these two classical diuretics on urine parameters clearly evidences different pharmacological profiles due to the different intrinsic mechanisms of action of these drugs (Table III). Furosemide and hydrochlorothiazide elicit a well-known effect on Na+ and/or K+ excretion, associated with water excretion, whereas SR 121463A has a pure aquaretic effect. In fact, the interaction of AVP V2 antagonists with the renal V2 receptors has been shown to prevent AVP-induced insertion of specific water channels (recently cloned aquaporin 2, AQP2) into the luminal membrane.
and thus, to specifically block water reabsorption in collecting duct cells (52, 53). This original mechanism of action highlights the interest of such molecules in several diseases in which water retention is closely associated with hypotonic hyponatremia.

Another important question raised with AVP V2 receptor antagonists is partial agonist antidiuretic activity, in some of the species investigated. The problem encountered by SK&F researchers with peptide V2 antagonists illustrates the difficulty of designing effective aquecic agents in man. One of these peptides, SK&F 101926, was identified as a potent aquecic agent in rats, dogs and squirrel monkeys and was the most potent AVP V2 analogue in inhibiting AVP-stimulated adenyl cyclase in vitro in all these species. But this promising molecule turned out to be a potent antidiuretic agent in man during clinical trials. Interestingly, SK&F 101926 disclosed significant agonist effects in Brattleboro rats when injected at a dose which induced marked diuresis in normal rats (21, 41, 42). Therefore, despite the potent inhibitory effect of SR 121463A on AVP-induced marked diuresis in normal rats (21, 41, 42), the investigation of SR 121463A in the vasopressin-deficient rat revealed surprising effects in all species investigated. The problem encountered by SK&F investigators in the vasopressin-deficient Brattleboro rat strain which seems to be a predictive and sensitive pharmacological model for detecting antidiuretic activity. Experiments performed in Brattleboro rats reveal that SR 121463A is devoid of any V2 agonistic effect, even when administered at high doses (10 mg/kg i.p.). Moreover, in this experimental model, this selective V2 antagonist compound was able to induce a further increase in urine flow rate and a decrease in urine osmolality, suggesting that some V2-mediated antidiuretic action is present in the kidney of Brattleboro rats. This antidiuretic influence could be due to the possible secretion of small amounts of vasopressin by peripheral organs such as the adrenals and testes where immunoreactive AVP has been detected (54–56). Alternatively, oxytocin, which is present at enhanced plasmatic concentrations in Brattleboro rats, may be responsible for the residual antidiuretic activity in those animals (57). Oxytocin has indeed been shown to enable Brattleboro rats to raise their urine osmolality, an effect which can be explained by the fact that oxytocin interacts with V2 receptors and increases water permeability in the rat collecting duct (58).

In conclusion, the nonpeptide AVP V2 receptor antagonist, SR 121463A, can be considered as the most potent and selective V2 ligand in both animal and human preparations described so far. SR 121463A is also the most powerful aquecic agent in rats yet described, with long-lasting oral activity and lack of agonistic properties in vitro and in vivo. Therefore, this molecule is a suitable tool for exploring the pathophysiological role of V2 receptors and the therapeutic usefulness of V2 blocker aquecic agents in water-retaining diseases. Thus, these results highlight the promise of obtaining with SR 121463A a full aquecic drug devoid of agonist effects and suitable for clinical use.

Acknowledgments

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References


