

Selectivity of d[Cha⁴]AVP and SSR149415 at human vasopressin and oxytocin receptors: evidence that SSR149415 is a mixed vasopressin V_{1b}/oxytocin receptor antagonist

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1 A possible role of arginine vasopressin (AVP) V_{1b} receptor subtype in stress-related disorders has been recently highlighted by the discovery of the agonist [1-deamino-4-cyclohexylalanine] AVP (d[Cha⁴]AVP) and the antagonist SSR149415. Both compounds have been proposed to target specifically V_{1b} receptors, since the reported affinities for the related V_{1a}, V₂ and oxytocin receptors are in the micromolar or submicromolar range. In the present study, we further investigated the binding affinities of d[Cha⁴]AVP and SSR149415 at recombinant human vasopressin V_{1b} (hV_{1b}) and oxytocin (hOT) receptors expressed in Chinese hamster ovary (CHO) cells and functional properties of both compounds at hV_{1b}, hV_{1a}, hV₂ and hOT receptors.

2 d[Cha⁴]AVP bound to hV_{1b} receptors and hOT receptors with pK_i values of 9.68 ± 0.06 and 7.68 ± 0.09, respectively. SSR149415 showed pK_i values of 9.34 ± 0.06 at hV_{1b} and 8.82 ± 0.16 at hOT receptors.

3 d[Cha⁴]AVP stimulated [Ca²⁺]_i increase in hV_{1b}-CHO cells with a pEC₅₀ value of 10.05 ± 0.15. It showed pEC₅₀ values of 6.53 ± 0.17 and 5.92 ± 0.02 at hV_{1a} and hV₂ receptors, respectively, and behaved as a weak antagonist at hOT receptors (pK_B = 6.31 ± 0.12). SSR149415 inhibited the agonist-induced [Ca²⁺]_i increase with pK_B values of 9.19 ± 0.07 in hV_{1b}-CHO and 8.72 ± 0.15 in hOT-CHO cells. A functional pK_i value of 7.23 ± 0.10 was found for SSR149415 at hV_{1a} receptors, whereas it did not inhibit 20 nM AVP response at hV₂ receptors up to 3 μM.

4 Data obtained confirmed the high potency and selectivity of d[Cha⁴]AVP at hV_{1b} receptors, but revealed that SSR149415, in addition to the high potency at hV_{1b} receptors, displays a significant antagonism at hOT receptors.

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Abbreviations: ACTH, adrenocorticotropin hormone; AT, 3-amino-1,2,4-triazole; AVP, [arginine⁸] vasopressin; B_{max}, receptor density; BSA, bovine serum albumin; [Ca²⁺]_i, intracellular calcium; CHO, Chinese hamster ovary; CNS, central nervous system; d[Cha⁴]AVP, [1-deamino-4-cyclohexylalanine] arginine vasopressin; DMSO, dimethyl sulphoxide; FDGlu, fluorescein-*d*-B-glucopyranoside; FLIPR, fluorescence imaging plate reader; GPCR, G-protein-coupled receptors; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid; HPA, hypothalamic-pituitary-adrenal; IC₅₀, half-maximal inhibitory concentration; IP, inositol phosphate; L-371,257, (1-{1-[4-[(*N*-acetyl-4-piperidinyl)oxy]-2-methoxybenzoyl]piperidin-4-yl}-4*H*-3,1-benzoxazin-2(1*H*)-one); OT, oxytocin; PBS, phosphate-buffered saline; SR49059, ((2*S*)-1-((2*R*, 3*S*)-5-chloro-3-(2-chloro-phenyl)-1-(3,4-dimethoxybenzene-sulphonyl)-3-hydroxy-2,3-dihydro-1*H*-indole-2-carbonyl)pyrrolidine-2-carboxamide); SSR149415, ((2*S*,4*R*)-1-[5-chloro-1-[(2,4-dimethoxyphenyl)sulphonyl]-3-(2-methoxy-phenyl)-2-oxo-2,3-dihydro-1*H*-indol-3-yl]-4-hydroxy-*N,N*-dimethyl-2-pyrrolidine carboxamide)

Introduction

Arginine vasopressin (AVP) is a cyclic hypothalamic non-peptide that exhibits many physiological effects, such as facilitation of water reabsorption by the kidney, contraction of the smooth muscle in arterioles (Jard *et al.*, 1987), stimulation of hepatic glycolysis, gluconeogenesis, esterification and oxidation of free fatty acids (Hems & Whitton, 1973), platelet

aggregation (Haslam & Rosson, 1972) and adrenocorticotropin hormone (ACTH) secretion (Antoni, 1993).

AVP effects are mediated by at least three different 7-transmembrane G-protein-coupled receptor (GPCR) subtypes that have been defined on the basis of their tissue distribution and pharmacology. The V_{1a} receptor subtype is mainly distributed in the vascular wall, central nervous system (CNS), liver, kidney and platelets (Hirasawa *et al.*, 1994). V_{1b} receptors are predominantly found in the anterior pituitary

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but they are also significantly expressed in CNS areas such as hippocampus, frontal, piriform and cingulate cortex, caudate putamen, medial habenula, central amygdala and hypothalamus (Lolait *et al.*, 1995; Hernando *et al.*, 2001; Griebel *et al.*, 2002; Stemmelin *et al.*, 2005).

The V₂ receptor subtype is distributed almost exclusively in the kidney (Jard, 1998). Activation of V_{1a} and V_{1b} receptors stimulates phosphatidylinositol (inositol phosphate (IP)) hydrolysis and mobilises intracellular calcium, whereas V₂ receptors are positively coupled to adenylyl cyclase (Thibonnier *et al.*, 1998a). The neurohypophyseal hormone oxytocin (OT), which differs from AVP by only two amino acids, induces uterine contraction and milk ejection through a GPCR linked to IP hydrolysis and calcium mobilisation. OT and AVP receptors are highly homologous and AVP binds to the OT receptor with appreciable affinity, causing agonist-like effects (Thibonnier *et al.*, 1998b).

The wide range of physiological effects mediated by AVP and OT makes the potential use of selective pharmacological agents extremely valuable for the treatment of human diseases. These receptors have been proposed as potential targets for the treatment of debilitating disorders such as congestive heart failure, arterial hypertension, dysmenorrhoea (V_{1a} receptors), hyponatremia, water-retaining diseases, ocular hypertension (V₂ receptors) and preterm labour (OT receptors) (Paranjape & Thibonnier, 2001; Thornton *et al.*, 2001). Intense efforts in this field have resulted in the discovery of selective V_{1a} receptor antagonists such as OPC-21268 and SR49059 (Yamamura *et al.*, 1991; Serradeil-Le Gal *et al.*, 1993), selective V₂ receptor antagonists such as SR121463 and OPC-31260 (Yamamura *et al.*, 1992; Serradeil-Le Gal, 2001) and selective OT receptor antagonists such as L-366,509 and L-371,257 (Evans *et al.*, 1992; Williams *et al.*, 1995).

More recently, further research in this area led to the identification of d[Cha⁴]AVP (Derick *et al.*, 2002) and SSR149415 (Serradeil-Le Gal *et al.*, 2002) as high-affinity and selective agents at V_{1b} receptors with respect to other AVP/OT receptor subtypes. d[Cha⁴]AVP is a peptido mimetic V_{1b} agonist *in vitro* and its administration in rats stimulates ACTH and corticosterone release without showing vasopressor or antidiuretic responses (Derick *et al.*, 2002). SSR149415 has been shown to be a potent V_{1b} antagonist *in vitro* and has been demonstrated to inhibit AVP-induced ACTH release *in vivo* (Serradeil-Le Gal *et al.*, 2002). Interestingly, SSR149415 also showed anxiolytic and antidepressant-like properties in preclinical models after systemic administration (Griebel *et al.*, 2002).

The selectivity of both SSR149415 and d[Cha⁴]AVP at V_{1b} receptors was established on the basis of binding affinity at recombinant and native AVP and OT receptors, whereas functional studies were carried out extensively only at human vasopressin V_{1b} (hV_{1b}) receptors (Serradeil-Le Gal *et al.*, 2002).

In this study, a functional characterisation of d[Cha⁴]AVP and SSR149415 at human recombinant V_{1b}, V_{1a}, V₂ and OT receptors was carried out. Our results confirmed high potency and selectivity of d[Cha⁴]AVP at hV_{1b} receptors and revealed that the compound exhibits weak antagonist properties at hOT receptors. SSR149415 was confirmed to be a potent antagonist at hV_{1b} receptors, but, in contrast with published data (Serradeil-Le Gal *et al.*, 2002), the compound showed a significant antagonism at hOT receptors. Functional data were supported by radioligand binding studies, in which SSR149415 showed nanomolar affinity at hOT receptors.

Methods

Materials

SSR149415, d[Cha⁴]AVP, SR49059 and L-371,257 were synthesised at Medicinal Chemistry department, Glaxo-SmithKline (Verona, Italy). [³H]AVP (2.22 TBq mmol⁻¹) and [³H]OT (1.2 TBq mmol⁻¹) were purchased from Perkin-Elmer Life Sciences (Monza, Italy). AVP, OT, Bacitracin, Leupeptin, Pefabloc, Pepstatin A, Probenecid, bovine serum albumin (BSA) and 3-amino-1,2,4-triazole (AT) were purchased from Sigma (Milan, Italy). (d(CH₂)₅ ¹Tyr(Me)², Thr⁴, Orn⁸, Tyr-NH₂⁹)-vasotocin was purchased from Bachem (Wehil am Rhein, Germany). Fluo-4 and Fluorescein-*d*-B-glucopyranoside (FDGlu) were purchased from Molecular Probes (Paisley, U.K.). All drugs to be tested were diluted primarily in dimethyl sulphoxide (DMSO) and further diluted in the assay buffer to give a final DMSO concentration not exceeding 1%.

Cell culture

Chinese hamster ovary (CHO) cells were stably transfected with hV_{1b}, hV_{1a} or hOT receptors using pCIN1 vectors (Rees *et al.*, 1996). Transfected cells were selected by antibiotic (G418/geneticin) resistance. Individual clones have been functionally validated by FLIPR. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented fetal calf serum and, 5 mM glutamine, in an atmosphere of 95% air and 5% CO₂ at 37°C.

Membrane preparation

When confluent, cells were harvested in phosphate-buffered saline (PBS) containing 5 mM EDTA and centrifuged at 913 × *g* for 8 min at 4°C. Cells were then resuspended in 10 volumes of HEPES (50 mM) buffer (pH 7.4), containing Leupeptin (0.1 mM), Bacitracin (40 µg ml⁻¹), EDTA (1 mM), Pefabloc (1 mM) and Pepstatin A (2 µM) and homogenised using a Polytron. The suspension was centrifuged at 48,000 × *g* for 20 min at 4°C. The final pellet was resuspended in 10 volumes of the same buffer and rehomogenised. Suspensions of membrane were then frozen at -80°C until required. Protein concentration was determined by the Bio-Rad Protein assay using BSA as internal standard.

Binding experiments

Binding assays were carried out in 96 deep-well plates (Whatman). In saturation experiments, increasing concentrations of [³H]AVP (2 pM–5 nM) and [³H]OT (4 pM–10 nM) were incubated with 14 µg well⁻¹ of hV_{1b} and 16 µg well⁻¹ of hOT receptor preparations, respectively, for 60 min at room temperature in a final volume of 400 µl of 50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂ and 0.1% BSA (binding buffer). Nonspecific binding was determined by the presence of 1 µM AVP (hV_{1b} receptors) or 1 µM OT (hOT receptors). In competition experiments, increasing concentrations of displacing compounds were incubated as above in the presence of 1 nM [³H]AVP (hV_{1b} receptors) or 1 nM [³H]OT (hOT receptors).

Reactions were stopped by rapid filtration through GF/C filterplates (Packard) presoaked in ice-cold binding buffer

using a cell harvester. Filters were washed three times with 1.5 ml of ice-cold 0.9% w v⁻¹ NaCl, and radioactivity was counted in a microplate scintillation counter (Top Count, Packard). In each independent experiment, every concentration of displacer was tested in duplicate.

Intracellular Ca²⁺ measurements in hV_{1b}, hOT and hV_{1a}-CHO cells

hV_{1b}-CHO and hOT-CHO cells were seeded into black-walled, clear bottom 96-well plates at a density of 50,000 cells per well and cultured overnight. Cells were then incubated for the labelling in the culture medium containing the fluorescent calcium indicator Fluo-4 AM (2 μM), the organic anion transport blocker Probenecid (5 mM) and HEPES (20 mM) for 30 min in a humidified atmosphere of 5% CO₂. After washing with Hanks' balanced salts solution (HBSS) containing 20 mM HEPES and 2.5 mM Probenecid (wash buffer), cells were incubated for 15 min at 37°C in wash buffer containing 0.02% BSA (assay buffer) either in the absence (control) or presence of antagonists. The plates were then placed into an FLIPR (Molecular Devices, Sunnyvale, CA, U.S.A.) to monitor cell fluorescence (λ_{ex} = 488 nm, λ_{em} = 510–570 nm) before and after the addition of different concentrations of agonists in assay buffer.

FLIPR experiments were carried out by using a laser setting of 1.0 W and a 0.4 s CCD (charge-coupled device) camera shutter speed.

FLIPR assays in hV_{1a}-CHO cells were carried out as above in 384-well format by plating 10,000 cells per well. The ability of test compounds to increase [Ca²⁺]_i or to inhibit 1 nM AVP-induced response was evaluated.

hV₂ receptor yeast reporter assay

hV₂ receptors and yeast chimeric G-α subunits containing the 5' C-terminal amino acids of human G-αs were transfected in a modified *Saccharomyces cerevisiae* strain following the method described by Brown *et al.* (2000).

Receptor activation was determined by measuring the induction of exoglucanase activity under the control of the *HIS3* inducible reporter gene. Agonist stimulation of the pheromone mating pathway leads to expression of *HIS3* and production of exoglucanase, an enzyme that cleaves fluorescein-di-β-glucopyranoside (FDGlu) to yield fluorescein (which is directly proportional to cell number).

Yeast cells were grown overnight to exponential phase in synthetic complete growth media (21 mg l⁻¹ L-arginine, 102 mg l⁻¹ L-aspartic acid, 102 mg l⁻¹ L-glutamic acid, 31 mg l⁻¹ L-lysine HCL, 21 mg l⁻¹ L-methionine, 51 mg l⁻¹ L-phenylalanine, 384 mg l⁻¹ L-serine, 205 mg l⁻¹ L-threonine, 31 mg l⁻¹ L-tyrosine, 153 mg l⁻¹ L-valine, pH 5.5, 6.7 g l⁻¹ yeast nitrogen base without amino acids, 20 g l⁻¹ glucose and 0.024 g l⁻¹ histidine,) at 30°C with constant shaking at 200 r.p.m. Cells were diluted to 0.02 OD units at 600 nm in synthetic complete assay media (synthetic complete growth media pH 7.0, minus histidine, supplemented with 3.6 g l⁻¹ NaH₂PO₄·2H₂O, 3.7 g l⁻¹ NaH₂PO₄ anhydrous, 10 μM FDGlu, 5 mM 3-AT). Assays were completed in 384-well black-walled, microtitre plates (NUNC, U.K.) containing compound and yeast cells in 50 μl synthetic complete assay media. The plates were incubated at 30°C for 24 h and fluorescence read at an

excitation wavelength of 485 nm and an emission wavelength of 535 nm. For antagonist studies, 20 nM AVP was added to plates containing antagonist and yeast cells in synthetic complete assay media.

Data analysis

Binding assays Radioligand binding data were analysed by nonlinear regression analysis using GraphPad Prism 4.0 (GraphPad Software, CA, U.S.A.). Determination of K_D and receptor density (B_{max}) of [³H]AVP and [³H]OT at respective receptors was made by elaborating saturation experiments using one-site binding (hyperbola) equation. Curve fitting from competition binding experiments was determined by using one site competition equation after checking with *F*-test (*P* < 0.05) that Hill slopes in the four parameter logistic equation were not statistically different from 1.0. Half-maximal inhibitory concentration (IC₅₀) values were converted to K_i using the Cheng–Prusoff equation (Cheng & Prusoff, 1973). Results are expressed as mean pK_i ± s.e.m.

Functional assays In FLIPR experiments, functional responses were measured as fluorescence intensity (FI) produced after receptor stimulation minus basal FI. Agonist EC₅₀ values were determined by fitting the concentration–response curves with a four-parameter logistic equation in GraphPad Prism. The potency of antagonists at hV_{1b} and hOT receptors was determined by using Schild analysis (Arunlakshana & Schild, 1959). Alternatively, Gaddum equation (pK_B = log(DR–1) – log[antagonist], where DR is the ratio between the EC₅₀ value of the agonist in the presence of the antagonist and the EC₅₀ of agonist alone), was used to estimate an apparent pK_B value of antagonists.

The antagonism at hV_{1a} receptors was expressed as functional (*f*) pK_i by using the adapted Cheng–Prusoff equation K_i = IC₅₀/(1 + [L])/EC₅₀, where IC₅₀ is the concentration of antagonist required for 50% inhibition of the maximum response, [L] is the concentration of AVP and EC₅₀ is the AVP concentration giving the 50% of maximal response. Results are expressed as mean ± s.e.m. All experiments were independently repeated at least three times in duplicate.

Results

Saturation binding experiments

Saturation experiments on membranes prepared from hV_{1b}-CHO and hOT-CHO cells showed that [³H]AVP and [³H]OT binding at respective receptors is saturable (Figure 1a and b). Scatchard analysis gave linear plots consistent with the presence of a single class of high-affinity binding sites. The negative log of the apparent dissociation constant (pK_d) was 9.33 ± 0.06 (*n* = 5) for [³H]AVP at hV_{1b} receptors and 9.40 ± 0.03 (*n* = 3) for [³H]OT at hOT receptors. The calculated maximum binding capacity (B_{max}) was 2177 ± 147 and 506 ± 25 fmol mg⁻¹ protein for hV_{1b} and hOT receptors, respectively.

Competition binding experiments

Compounds tested in competition binding experiments both at hV_{1b} and hOT receptors are listed in Table 1. The V_{1b} agonist d[Cha⁴]AVP showed pK_i values of 9.68 ± 0.06 and 7.68 ± 0.09 at hV_{1b} and hOT receptors, respectively (Figure 2a and b). SSR149415 showed pK_i values of 9.34 ± 0.06 at hV_{1b} (Figure 2a) and 8.82 ± 0.16 at hOT receptors (Figure 2b).

Intracellular Ca²⁺ increase

Addition of AVP and OT at hV_{1b}-CHO and hOT-CHO cells, respectively, resulted in an increase in [Ca²⁺]_i in a concentration-dependent manner (AVP pEC₅₀ = 10.50 ± 0.07 (n = 19), OT pEC₅₀ = 8.93 ± 0.09 (n = 16); Figure 3a and b). The V_{1b}

agonist d[Cha⁴]AVP stimulated the [Ca²⁺]_i increase in hV_{1b}-CHO cells with a pEC₅₀ value of 10.05 ± 0.15 (Figure 3a, Table 2). When added to hOT-CHO cells, d[Cha⁴]AVP did not show any agonist-like response up to 10 μM concentration, but showed a weak antagonism, since it inhibited the OT response with an apparent pK_B value of 6.31 ± 0.12 (Figure 3b). SSR149415 (1–100 nM) showed a competitive antagonism at hV_{1b} receptors (Schild's slope = 0.98 ± 0.02) with a pK_B value of 9.15 ± 0.10, calculated according to Schild's analysis (Figure 4). The OT antagonist L-371,257 showed nanomolar potency at hOT receptors, with an apparent pK_B of 8.19 ± 0.13 (n = 3) when tested at 10 nM concentration. At higher concentrations (100 nM, 1 μM), L-371,257 produced an unsurmountable antagonism, since it caused depression of the maximal OT responses along with rightward shifts of the agonist concentration–response curves, thus precluding Schild's analysis (Figure 5a). L-371,257 did not produce any inhibition of AVP response at hV_{1b} receptors up to 1 μM concentration (n = 3, data not shown). When tested at hOT

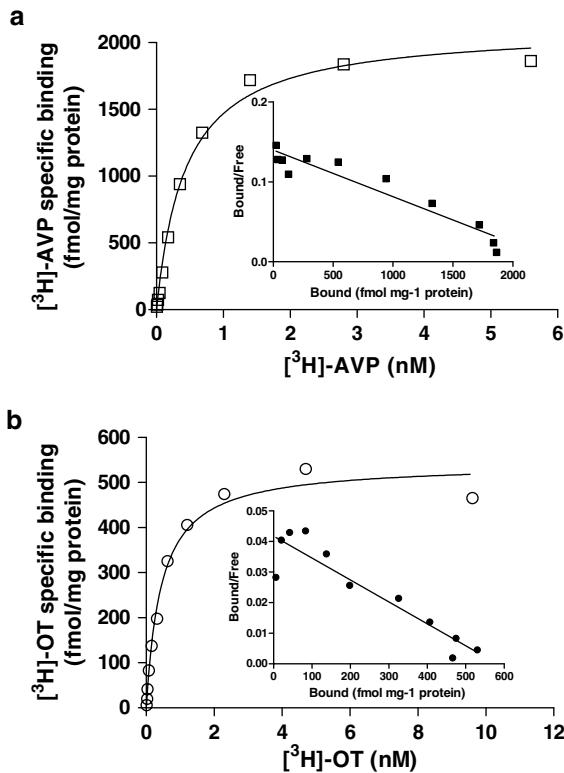


Figure 1 Saturation experiments on [³H]AVP (a) and [³H]OT (b) binding to receptors on plasma membranes prepared from CHO cells transfected with human V_{1b} and OT receptors, respectively. Inset: Scatchard linear transformation of the data. Data shown are representative of specific binding curves from five (hV_{1b}) and three (hOT) independent experiments performed in duplicate.

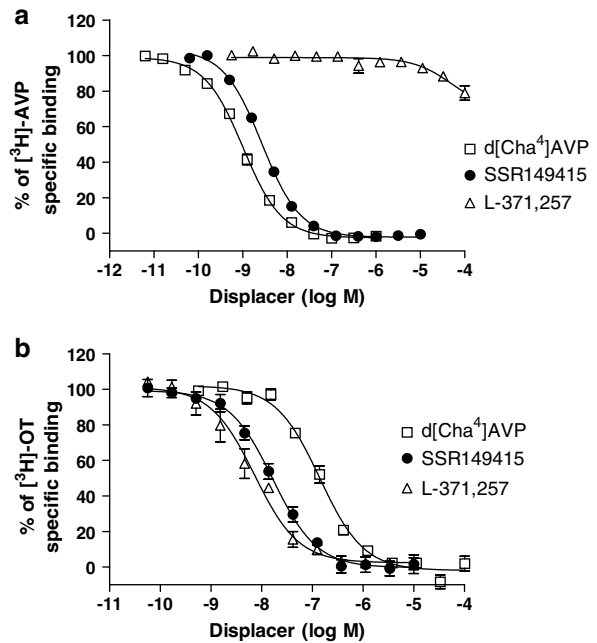


Figure 2 Inhibition of [³H]AVP binding at hV_{1b} receptors (a) and [³H]OT binding at hOT receptors (b) by d[Cha⁴]AVP, SSR149415 and L-371,257. Results are expressed as % of specific radioligand binding against the log concentration of each compound and values are mean ± s.e.m. The data were taken from three independent experiments in which d[Cha⁴]AVP, SSR149415 and L-371,257 were tested in the same assay.

Table 1 Binding pK_i values of different AVP and OT receptor agonists and antagonists at hV_{1b} and hOT receptors

	hV _{1b}	hOT	V _{1b} /OT Selectivity ratio
AVP	9.38 ± 0.03 (n = 4)	9.26 ± 0.13 (n = 3)	1.3
OT	6.97 ± 0.13 (n = 3)	9.61 ± 0.03 (n = 3)	0.002
SR49059	6.34 ± 0.22 (n = 3)	7.00 ± 0.06 (n = 3)	0.2
(d(CH ₂) ₅ Tyr(Me) ² , Thr ⁴ , Orn ⁸ , Tyr-NH ₂ ⁹)-vasotocin	< 6 (n = 3)	9.99 ± 0.08 (n = 3)	< 0.001
L-371,257	< 6 (n = 3)	8.83 ± 0.04 (n = 3)	< 0.001
d[Cha ⁴]AVP	9.68 ± 0.06 (n = 5)	7.68 ± 0.09 (n = 4)	100
SSR149415	9.34 ± 0.06 (n = 8)	8.82 ± 0.16 (n = 5)	3.2

Data are expressed as mean ± s.e.m. of n independent experiments performed in duplicate.

receptors, SSR149415 (10 nM–1 μ M) did not induce [Ca²⁺]_i response, but inhibited the OT response in an apparent unsurmountable way with an apparent pK_B value of 8.72 ± 0.15, as calculated at 10 nM concentration (Figure 5b). At hV_{1a} receptors, AVP showed a pEC₅₀ of 9.87 ± 0.06 (*n* = 8), whereas d[Cha⁴]AVP displayed weak agonist properties (pEC₅₀ = 6.53 ± 0.17; Table 2). SSR149415 potency in inhibiting AVP response was in the submicromolar range (fpK_i = 7.23 ± 0.14; Table 2). In the same assay, the V_{1a} antagonist SR49059 showed an fpK_i of 9.95 ± 0.06 (*n* = 4, data not shown).

hV₂ receptor assay in yeast

At hV₂ receptors, AVP showed a pEC₅₀ of 8.53 ± 0.06 (*n* = 6), whereas d[Cha⁴]AVP displayed weak agonist properties (pEC₅₀ = 5.92 ± 0.02, *n* = 3). SSR149415 did not inhibit 20 nM AVP response up to 3 μ M concentration (*n* = 3).

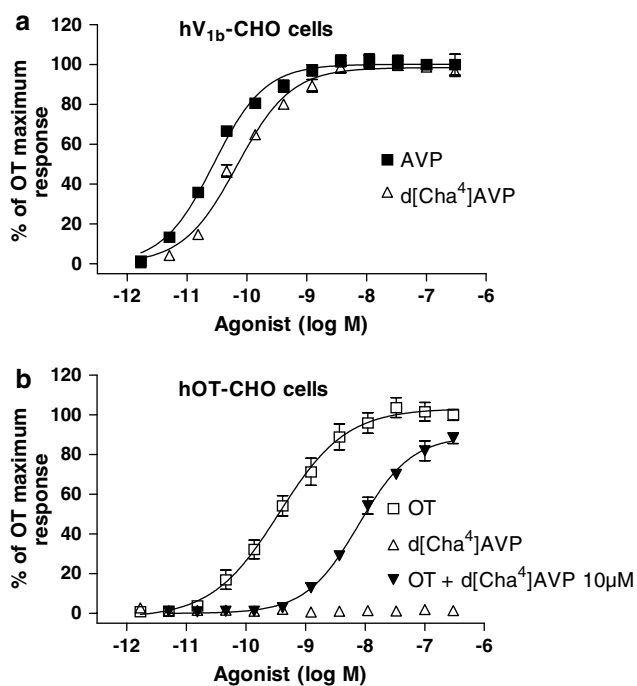


Figure 3 Increase in [Ca²⁺]_i concentrations after agonist stimulation as determined in FLIPR experiments. (a) Concentration–response curves of AVP and d[Cha⁴]AVP in hV_{1b}-CHO cells. (b) Concentration–response curves of OT in the absence or presence of 10 μ M d[Cha⁴]AVP cells. d[Cha⁴]AVP alone did not stimulate any [Ca²⁺]_i increase up to 10 μ M concentration in hOT-CHO cells. Curves depicted are the mean fit of three independent experiments performed in duplicate.

Discussion

The absence of selective pharmacological agents at V_{1b} receptors had severely limited the investigation of the physiological role of these receptors, until the recent discovery of d[Cha⁴]AVP as the first selective peptido mimetic agonist (Derick *et al.*, 2002), and SSR149415 as the first selective nonpeptide antagonist (Serradeil-Le Gal *et al.*, 2002) at V_{1b} receptors.

Since only binding data at V_{1a}, V₂ and OT receptors have been produced from the above studies for SSR149415 and d[Cha⁴]AVP, in this work we carried out an extensive functional characterisation of the two compounds at human recombinant AVP and OT receptors to complete their *in vitro* pharmacological profile. Binding data at hV_{1b} and hOT receptors were also provided to establish a correlation with functional experiments.

In agreement with data reported in the literature (Derick *et al.*, 2002; Cheng *et al.*, 2004), our study confirmed that

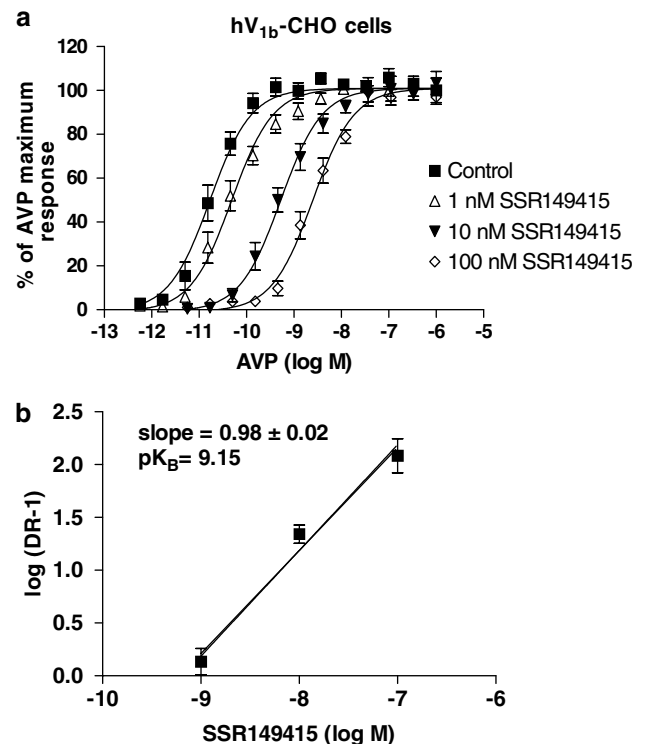


Figure 4 Inhibition of AVP-induced [Ca²⁺]_i increase in hV_{1b}-CHO cells by SSR149415. (a) Concentration–response curves for AVP in the absence or presence of 1, 10 and 100 nM SSR149415. Curves depicted are the mean fit of three independent experiments performed in triplicate. (b) Schild analysis of the antagonism produced by SSR149415. Data are expressed as mean ± s.e.m.

Table 2 Functional activities of d[Cha⁴]AVP and SSR149415 at human recombinant vasopressin and oxytocin receptors

	hV _{1b}	[Ca ²⁺] _i measurement hV _{1a}	hOT	Yeast growth assay hV ₂
d[Cha ⁴]AVP pEC ₅₀ (pK _B *)	10.05 ± 0.15 (<i>n</i> = 3)	6.53 ± 0.17 (<i>n</i> = 4)	6.31 ± 0.12* (<i>n</i> = 3)	5.92 ± 0.02 (<i>n</i> = 3)
SSR149415 pK _B /fpK _i	9.19 ± 0.07 (<i>n</i> = 6)	7.23 ± 0.10 (<i>n</i> = 7)	8.72 ± 0.15 (<i>n</i> = 8)	Inactive up to 3 μ M (<i>n</i> = 3)

* = pK_B.

Data are expressed as mean ± s.e.m. of *n* independent experiments performed in duplicate.

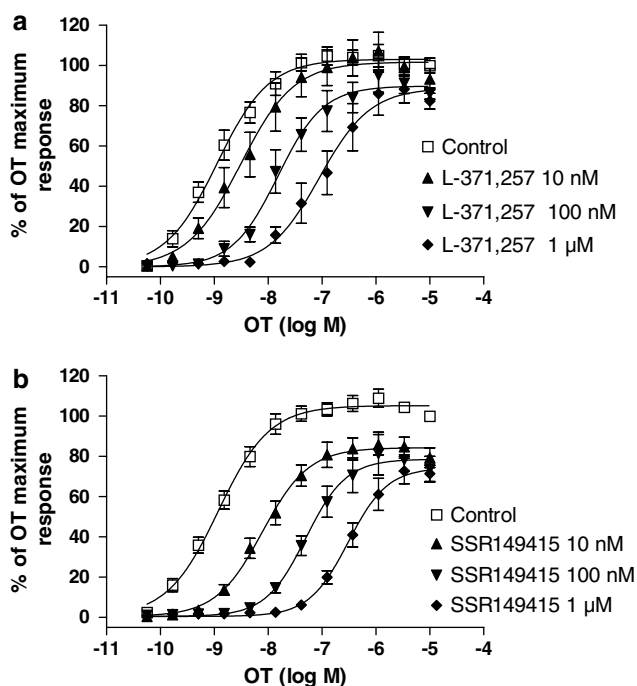


Figure 5 Inhibition of OT-induced $[Ca^{2+}]_i$ increase in hOT-CHO cells by L-371,257A and SSR149415. Concentration–response curves for OT in the absence or presence of 10 nM, 100 nM and 1 μ M L-371,257 (a), or 10 nM, 100 nM and 1 μ M SSR149415 (b). Curves depicted are the mean fit of three independent experiments performed in duplicate.

d[Cha⁴]AVP is a potent agonist at hV_{1b} receptors with more than 100-fold selectivity with respect to the other AVP/OT receptor subtypes. Interestingly, in FLIPR functional experiments, d[Cha⁴]AVP did not stimulate $[Ca^{2+}]_i$ increase but displayed weak antagonist properties at hOT receptors. The potency calculated for d[Cha⁴]AVP in functional experiments is about 10-fold lower compared to the affinity calculated here in [³H]OT binding experiments. The discrepancy between binding and functional data can be due to the particular chemico–physical properties of this peptido-mimetic compound. As measured by liquid chromatography/mass spectrometry (LC/MS), d[Cha⁴]AVP content in the buffer used in functional assays was about three-fold lower compared to that measured in binding assay buffer (data not shown), suggesting a poor solubility or a nonspecific binding of the compound to plastic surfaces in the functional assay conditions. Hence, it can be hypothesised that data obtained in the functional assay may underestimate the actual potency of d[Cha⁴]AVP. However, both binding and functional data show that d[Cha⁴]AVP is at least 100-fold selective at hV_{1b} versus hOT receptors, in agreement with data published by Derick *et al.* (2002).

In functional experiments, SSR149415 inhibited in a competitive manner the AVP response at hV_{1b} receptors with a nanomolar potency. Consistent results were obtained in [³H]AVP binding experiments, where SSR149415 showed an affinity value in agreement with functional potency, and with data previously reported by Serradeil-Le Gal *et al.* (2002). SSR149415 was confirmed to be a weak antagonist at human V_{1a} receptor with potency in the submicromolar range, whereas it did not significantly inhibit AVP response at V₂

receptors up to 3 μ M concentration. However, in functional assays at hOT receptors, SSR149415 inhibited the $[Ca^{2+}]_i$ increase induced by OT with high potency, comparable to that of L-371,257, a high-affinity ligand and potent antagonist at both human and rat OT receptors (Williams *et al.*, 1995; 1999).

SSR149415 and L-371,257 displayed an unsurmountable antagonism versus OT at hOT receptors. In FLIPR assay, receptors are preincubated with the antagonist before their challenge with the agonist. Under these conditions, agonists can only produce full receptor stimulation if the antagonist in question is both competitive and sufficiently rapidly dissociating to make accessible the entire receptor population at the time at which the maximal response is measured. Hence, the apparent noncompetitive antagonism displayed by SSR149415 and L-371,257 may be attributable to a lack of equilibrium between OT and antagonists with hOT receptors within the short duration of rapid transient effects of OT on intracellular calcium. A similar observation was reported by Miller *et al.* (1999) about the apparent noncompetitive antagonism at H(1)-histamine receptors found in FLIPR experiments by known competitive antagonists. This hypothesis is supported by the finding that L-371,257 has been shown to be a competitive OT receptor antagonist in the isolated uterus of rats (Pettibone *et al.*, 1995).

In agreement with functional experiments, in [³H]OT binding experiments, SSR149415 and L-371,257 showed similar pK_i values at hOT receptors. Serradeil-Le Gal *et al.* (2002) reported that in binding assays performed on human recombinant receptors, SSR149415 shows 100-fold higher affinity at V_{1b} with respect to OT receptors. We report herein a low selectivity profile for SSR149415. Even though SSR149415 showed about three-fold higher affinity and potency at hV_{1b} compared to hOT receptors, the activity at the latter receptors is in the nanomolar range.

One possible explanation for the difference in SSR149415 affinity observed in the two studies could be attributed to the radioligand chosen in the OT binding assays. In the work of Serradeil-Le Gal *et al.* (2002), the OT antagonist [d(CH₂)₅Tyr(Me)₂, Thr⁴, Orn⁸ [¹²⁵I]Tyr⁹-NH₂]vasotocin was utilised, whereas in the present study, we used the endogenous agonist [³H]OT. It has been demonstrated that the OT antagonist (d(CH₂)₅¹Tyr(Me)², Thr⁴, Orn⁸, Tyr-NH₂)⁹-vasotocin binds to transmembrane regions different from those participating in OT binding at the porcine OT receptor (Postina *et al.*, 1996), even though the observation that this antagonist exhibits a subnanomolar affinity versus [³H]OT indicates a mutually exclusive binding. It could be hypothesised that SSR149415 shows different affinities versus [³H]OT and the radiolabelled antagonist, resulting in an apparent lower affinity of SSR149415 at OT receptors. However, an unequivocal interpretation of the functional data obtained with SSR149415 at hOT receptors is hampered by the lack of similar data in the literature.

A possible role of AVP and V_{1b} receptors in mood disorders is supported by an increasing body of evidence. In particular, it has been proposed that the HPA axis hyperactivity observed in prolonged stressful states and in patients affected by major depression could be maintained by increased secretion of AVP (Aguilera & Rabadan-Diehl, 2000; Scott & Dinan, 2002). The anxiolytic and antidepressant profile shown by SSR149415 in preclinical models after systemic administration (Griebel *et al.*, 2002; 2003) suggests that the behavioural effects are not only

related to the antagonism at V_{1b} receptors located in the pituitary, but also to the blockade of central V_{1b} receptors (Griebel *et al.*, 2002; Stemmelin *et al.*, 2005). Furthermore, a reduction in aggressive behaviour after systemic administration of SSR149415 in hamsters has been reported (Blanchard *et al.*, 2005) confirming the findings in V_{1b} knockout mice (Wersinger *et al.*, 2002). The evidence that SSR149415 is not highly selective with respect to OT receptors could question whether the anxiolytic- and antidepressant-like effects observed *in vivo* after SSR149415 administration (Griebel *et al.*, 2002) are mediated only by antagonism at V_{1b} receptors. The effect of OT receptors on anxiety-related behaviour is controversial. A large body of evidence supports the idea that stimulation of OT receptors inhibits HPA axis activation (Neumann *et al.*, 2000) and that OT mediates anxiolytic-like effects after central infusion in rats (Bale *et al.*, 2001). Furthermore, an increase in anxiety-related behaviour has been observed in female OT-deficient mice (Mantella *et al.*, 2003; Amico *et al.*, 2004). Studies on the behavioural effects after systemic administration of OT receptor antagonists are missing, even though a decrease of immobility time in the

forced swimming test has been recently reported, after administration into amygdala of an OT receptor antagonist in rat (Ebner *et al.*, 2005), a possible indication of antidepressant-like effects. Altogether, these studies do not elucidate if the effects of SSR149415 are exclusively mediated by antagonism at V_{1b} receptors. The observation of possible behavioural effects induced by central administration of the selective V_{1b} receptor agonist d[Cha⁴]AVP could help in understanding the role of V_{1b} receptors located in the CNS. Further studies are warranted to clarify the implications of our results obtained on human recombinant receptors. Even though a significant improvement in the comprehension of the functional role of V_{1b} receptors has been made possible by the discovery of d[Cha⁴]AVP and SSR149415, there is still the need of additional pharmacological tools at these receptors to provide an unequivocal demonstration of the therapeutic potential of these agents.

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