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Pharmacological characterization of F-180: a selective human V_{1a} vasopressin receptor agonist of high affinity

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1 The pharmacological properties of F-180, a vasopressin (VP) structural analogue, were determined on CHO cells expressing the different human vasopressin and oxytocin (OT) receptor subtypes. Binding experiments revealed that F-180 exhibited a high affinity for the human V_{1a} receptor subtype (K_i = 11 nM) and was selective for this receptor subtype.

2 Functional studies performed on CHO cells expressing human V_{1a} receptors indicate that similarly to AVP, F-180 can stimulate the accumulation of inositol phosphate. The activation constant (K_{act}) for both F-180 and AVP was 1.7 nM. F-180 was also an agonist for the human V_2 and V_{1b} receptor subtypes and an antagonist for the human OT receptor.

3 Since marked species pharmacological differences for vasopressin receptors have been described, we studied the properties of F-180 on various mammalian species. F-180 showed high affinity and good selectivity for human and bovine V_{1a} receptors, but weak affinity and non selective properties for rat V_{1a} receptors.

4 To assess the functional properties of F-180 on a native biological model, we performed studies on primary cultures of cells from bovine zona fasciculata (ZF). As AVP, F-180 stimulated inositol phosphate accumulation and cortisol secretion with similar efficiency.

5 In conclusion, we demonstrate that F-180 is the first selective V_{1a} agonist described for human and bovine vasopressin receptors. Therefore F-180 can be used as a powerful pharmacological tool to characterize the actions of vasopressin that are mediated by V_{1a} receptor subtypes.
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Keywords: F-180; vasopressin; agonist; V_{1a} receptors

Abbreviations: ACTH, adrenocorticotropic hormone; AVP, arginine[8] vasopressin; B_{max}, maximal binding capacity; BSA, bovine serum albumin; CHO cells, Chinese hamster ovary cells; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediamine tetra-acetic acid; HO-LVA, linear vasopressin antagonist; IC50, analogue concentration leading to 50% of the maximal inhibition; IPs, total inositol phosphates; K_{act}, activation constant; K_i, inhibitory dissociation constant; K_{inact}, inhibition constant for antagonist; OT, oxytocin; OTA, oxytocin antagonist; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; VP, vasopressin; VT, vasotocin; ZF, zona fasciculata

Introduction

In mammals, the neurohypophyseal peptide arginine vasopressin (AVP) exerts three major physiological actions: (i) regulation of water reabsorption in the kidney (antidiuretic activity), (ii) modulation of vascular tone (pressic activity) and (iii) stimulation of ACTH secretion by the anterior pituitary (for review see Jard, 1998). These effects are triggered by three distinct receptor isoforms called: V_2, V_{1a} and V_{1b}. Other physiological effects of AVP have been described in various organs such as: liver, brain, adrenal gland, heart, pancreas, and uterus. Yet, all of them involve the activation of one of the classical VP receptors isoforms previously described (for review see Barberis *et al*., 1999).

The characterization of these VP receptor subtypes has been made possible by the availability of numerous VP receptor radioligands as well as unlabelled VP structural analogues (for review see Barberis *et al*., 1999). This pharmacological classification was confirmed later by the cloning of three distinct genes encoding for V_2, V_{1a} and V_{1b} receptors (Lolait *et al*., 1992; Morel *et al*., 1992; Sugimoto *et al*., 1994).

For many years, the design of specific agonists and antagonists that could recognize specifically VP receptor subtypes, constituted an active research field (for review see Chan *et al*., 2000). The design of such selective compounds would be important for the development of useful tools with potential therapeutic interest. Several specific antagonists of the human V_2 receptor (hV_2-R) have been described previously: OPC-31260 (Yamamura *et al*., 1992) and SR-121463A (Serradeil-Le Gal *et al*., 1996). These two orally bioavailable non-peptide analogues exhibit high selectivity for the hV_2-R but only SR-121463A presents range affinity in the nanomolar for this receptor. The non-peptide agonist of the hV_2-R, OPC-51803 (Nakamura *et al*., 1998), is more specific for hV_2-R than dDAVP (desmopressin) (Zaoral *et al*., 1967) which presents a mixed V_2/V_{1b} pharmacological profile (Saito *et al*., 1997; Butlen *et al*., 1978) and is used in the treatment of human central *diabetes insipidus*. Concerning V_{1b} analogues,

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Schwartz and coworkers described the potent selective rat V_{1b} receptor agonist 1-deamino[D-3-(pyridyl) Ala^2]AVP (d[D-3-Pal]VP) (Schwartz *et al*., 1991). However, this compound could not differenciate between rat and human VP receptors (Barberis *et al*., 1999; and Guillon, unpublished results). By contrast, SR-149415, a non-peptidic V_{1b} antagonist with affinity in the nanomolar range and high selectivity for human V_{1b} receptor has been recently described (Serradeil-Le Gal *et al*., 2001). Specific antagonists of the human V_{1a} receptor (hV_{1a}-R) have also been designed. One of the most potent V_{1a} receptor antagonist in rat and human species is $d(CH_2)_5$[Tyr $(Me)^2$]AVP that was synthetized by M. Manning (Kruszynski *et al*., 1980). This compound exhibits high affinity and selectivity for hV_{1a} vasopressin receptors. However, $d(CH_2)_5$[$Tyr(Me)^2$]AVP also shows relatively high affinity for human OT receptor (hOT-R), and therefore it cannot be as a therapeutical agent (Gavras *et al*., 1984). More recently various bioavailable non-peptide antagonists of the hV_{1a} receptors were synthesized. SR-49059, the most potent and selective V_{1a} antagonist yet described *in vitro* and *in vivo* in relevant models (Serradeil-Le Gal *et al*., 1993), has entered clinical trials for hypertension and congestive heart failure, and represents an important new pharmacological tool. Huguenin (1964) described a vasotocin (VT) analogue ([Phe^2, Orn^8]VT) with high pressor activity and low antidiuretic potency in rat. Yet, this compound presents a mixed V_{1a}/OT pharmacological profile for human receptors (Barberis *et al*., 1999). On the other hand, another structural analogue of [Phe^2, Orn^8]VT, F-180, was designed (Aurell *et al*., 1990). Preliminary tests performed in rat indicated that F-180 is a potent vasopressor analogue with a negligible antidiuretic activity (Bernadich *et al*., 1998). Such results would indicate that F-180 might be a potent selective V_{1a} agonist.

To answer this question, we studied the pharmacological properties of F-180 on different biological models expressing vasopressin and oxytocin receptors. In the present report, we demonstrate that F-180 is an agonist with high affinity and selectivity for human and bovine V_{1a} receptors. Thus, F-180 may be an important vasopressin structural analogue to identify V_{1a} receptor in mammalian tissues.

Methods

Chemicals

Most standard chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), Boehringer Mannheim (Mannheim, Germany), or Merck & Co., Inc. (Darmstadt,. Germany), unless otherwise indicated. [^{3}H]-AVP (80 Ci mmol^{-1}), [8,^{3}H]-adenine (24 Ci mmol^{-1}) and *myo*-[2-^{3}H]-inositol (10–20 Ci mmol^{-1}) were from Du-Pont New England Nuclear (Boston, MA, U.S.A.).

[Arg^8]vasopressin and oxytocin were supplied by Bachem (Bubendorf, Switzerland). F-180 was synthesized by Ferring Research Institute Inc. (San Diego, CA, U.S.A.). For structure and synthesis see Aurell *et al*. (1990). SR-49059 was a generous gift from C. Serradeil-Le Gal (Sanofi Synthelabo, France). All vasopressin analogues were initially disolved in dimethyl sulfoxide (DMSO) at 1 mM and diluted to the desired concentration with the corresponding buffer. The final concentration of DMSO in the assay buffer never exceeded 1%, a concentration which did not statistically affect radioligand specific binding (data not shown).

GF/C glass fibre filters used were obtained from Whatman (Whatman International Ltd., Maidstone, U.K.). All other chemicals were of the highest reagent grade available. The origins of other chemicals used for this study are given in the text.

Synthesis and radioiodination of vasopressin structural analogues

Vasopressin analogues to be radiolabelled were obtained by solid phase synthesis by M. Manning (Toledo, U.S.A.). They were radioiodinated on the phenolic substituent by means of the oxidant 1,3,4,6-tetrachloro-3α,6α-diphenyl-glycouril (iodogen, Pierce Chem. Co.) in the presence of 1 mCi [^{125}I]-Na (IMS 30, Amersham, Bucks, U.K.), as previously described by Elands *et al*. (1988) and purified by HPLC. The pharmacological properties of each radiolabelled AVP analogues used in this study have been previously published. Thus [^{125}I]-(HO)-Phenylacetyl^1-D-Tyr$(Me)^2$-Phe^3-Gln^4-Asn^5-Arg^6-Pro^7-Arg^8-NH_2 ([^{125}I]-HO-LVA) is a V_{1a} antagonist of high affinity (Barberis *et al*., 1995). [^{125}I]-$d(CH_2)_5$[$Tyr(Me)^2$,Thr^4,Tyr-$NH_2{}^9$]OVT ([^{125}I]-OTA) is an OT antagonist with high affinity (Elands *et al*., 1988).

Cell culture and membrane preparation

CHO (Chinese hamster ovary) cells stably expressing hV_{1a}-R, hV_{1b}-R, hV_2-R and hOT-R were maintained in culture in Petri dishes in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) supplemented with 10% foetal calf serum, 4 mM L-glutamine, 500 units ml^{-1} penicillin and streptomycin, 0.25 μg ml^{-1} amphotericin B in an atmosphere of 95% air and 5% CO_2 at 37°C. Depending on the experiment to be conducted, cells were treated overnight with 5 mM sodium butyrate to increase receptor expression (Kassis *et al*., 1984). Membranes from CHO cells were prepared according to Cotte *et al*., 1998. Briefly, cells were harvested, washed twice in PBS without Ca^{2+} and Mg^{2+}, polytron-homogenized in lysis buffer (15 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$, 0.3 mM EDTA) and centrifuged at $100 \times g$ for 5 min at 4°C. Supernatants were recovered and centrifuged at $44{,}000 \times g$ for 20 min at 4°C. Pellets were washed in buffer A (50 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$) and centrifuged at $44{,}000 \times g$ for 20 min at 4°C. Membranes were suspended in a small volume of buffer A, frozen and stored at −80°C until use. Protein concentration was measured by the method of Bradford (1976) with the Bio–Rad protein assay kit and using bovine serum albumin (BSA) as a standard.

Animals and biological material

Bovine tissue samples from liver, kidney and pituitary gland were collected from 11–13 month old male calves immediately after death at a local slaughter-house, placed in ice-cold Locke solution and transported immediately to the laboratory. Adult Wistar rats were killed by cervical dislocation, decapitated and liver, kidney and pituitary gland were rapidly removed and placed in ice-cold homogenization buffer. All manipulations were performed according to the recommenda-

tions of the French Ethical Committee and under the supervision of an authorized investigator. All tissues were cleaned from adhering fat, dissected out, and used immediately for membrane preparation or rapidly frozen by immersion in liquid nitrogen and stored at −80°C until use.

Membrane preparation from bovine and rat tissues

Plasma membranes were isolated as previously described by Maeda *et al*. (1983) with some minor modifications. Briefly, the different tissues were minced and homogenized in 3 volumes 20 mM Tris, pH 7.4 containing 0.25 M sucrose, 0.5 mM EDTA, and 0.1 mM phenylmethylsulphonyl fluoride (PMSF) (homogenization buffer) using a Polytron homogenizer (IKA Ultra-Turrax T25) three times at 9500 r.p.m. for 15 s. The homogenate was centrifuged for 5 min at $1500 \times g$ and the pellet was resuspended in homogenization buffer, layered on a discontinuous sucrose gradient (41% W v^{-1}) and run for 65 min at $95{,}000 \times g$. The interfacial plasma membrane fraction was diluted with : 20 mM Tris, pH 7.9, 1.5 mM EDTA, 140 mM NaCl and centrifuged for 30 min at $95{,}000 \times g$. During all the membrane preparation procedures, the temperature was kept at 4°C. Membranes were suspended in a small volume of homegenization buffer and protein contents were determined. Aliquots of purified plasma membranes were used immediately or frozen in liquid nitrogen and stored at −80°C until use. The purity of this fraction has been previously determined with marker proteins (Trueba *et al*., 1991).

Binding studies

The pelleted membrane fractions were resuspended in 50 mM Tris-HCl pH 7.4 containing 2 mM $MgCl_2$, and 0.1 mM PMSF. Aliquots of membrane suspension (5–10 μg protein) were incubated in the appropiate incubation medium (50 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 1 mg ml^{-1} bovine serum albumin (BSA), 0.01 mg ml^{-1} leupeptine and 0.1 mM PMSF) with increasing amounts of [^{125}I]-HO-LVA (hV_{1a}-R), [^{3}H]-AVP (hV_{1b}-R and hV_2-R) or [^{125}I]-OTA (hOT-R), or, for competition studies, with 25–30 pM [^{125}I]-HO-LVA, 5–10 nM [^{3}H]-AVP or 100 pM [^{125}I]-OTA, and varying concentrations of unlabelled peptides in a final volume of 200 μl. The reaction was allowed to proceed at 37°C for 60 min for [^{125}I]-HO-LVA and [^{125}I]-OTA and 45 min for [^{3}H]-AVP. Non-specific binding was determined in the presence of 10 μM AVP. Bound and free radioactivity were subsequently separated by vacuum-filtration over Whatman GF/C filters, pre-soaked (for at least 2 h) either in 20 mg ml^{-1} BSA ([^{3}H]-AVP, [^{125}I]-OTA) or in 0.5% polyethylenimine ([^{125}I]-HO-LVA). Filters were then washed four times with 3 ml filtration buffer (5 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$,). When [^{3}H]-AVP was used, filters were dried and counted for radioactivity in a Packard Tri-Carb 2100 TR liquid scintillation analyser. When [^{125}I]-radioligands were used, the radioactivity retained on the filters was directly quantified in a Packard Cobra^{TM}II auto-gamma counter with 75% efficiency.

Preparation and isolation of adrenocortical cells

Bovine zona fasciculata (ZF) cell primary culture was performed as previously described (Bird *et al*., 1990). Briefly, adrenal glands were cleaned and cut into flat sections. Thin tissue slices were obtained using a Stadie-Riggs tissue slicer from Thomas Scientific (St. Laurent, Canada). The first slice consisting in the adrenal capsule and mainly the zona glomerulosa, was eliminated. Only the second slice was finely chopped into 1–2-mm pieces and washed twice in HBS medium. Cell dissociation was achieved after 1- or 2-incubation periods of 60 min at 37°C each in DMEM containing a collagenase mixture (1 mg ml^{-1}) plus deoxyribonuclease (25 μg ml^{-1}). Cells were then dissociated by gentle aspiration before being filtered and centrifuged for 10 min at $100 \times g$. The resulting cell pellet was resuspended in DMEM supplemented with 10% foetal calf serum, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. ZF cells were plated at a density of about $6–8 \times 10^5$ in 35-mm tissue-culture dishes and were grown in a humidified atmosphere of 95% air–5% CO_2 at 37°C. The culture medium was changed 24 h after seeding, and cells were used 3 days after.

Steroid secretion measurements by RIA

The steroid secretion of ZF primary cultures was measured as previously described (Tremblay *et al*., 1991). In brief, after 3 days in culture cells were washed and incubated for 2 h at 37°C in a free serum medium (DMEM 0%). Cells were again washed and incubated for 15 min in HBS supplemented with $NaHCO_3$ 75 mg ml^{-1}, bacitracin 0.1 mg ml^{-1}, Trypsin inhibitor 0.1 mg ml^{-1}, LiCl 10 mM, glucose 1 mg ml^{-1} and BSA 0.1 mg ml^{-1} (assay medium). The agonist or corresponding vehicle (control) were then added, and the incubation was continued for 2 h at 37°C in a humidified atmosphere with 5% CO_2 in air. The incubation was stopped by transferring the assay medium to eppendorf tubes and freezing at −20°C until steroid measurements were performed. Cortisol concentration levels were determinated by radioimmunoassay (RIA), as previously described (Cymeryng *et al*., 1998).

Inositol phosphate assays

Inositol phosphate (IPs) accumulation was determined as previously described (Grazzini *et al*., 1999). Briefly, CHO cells expressing the different human vasopressin/oxytocin receptor subtypes were plated and grown in 12-well dishes for 48 h in Dulbecco's modified Eagle's medium. Cells were then incubated for another 24 h period in a serum-free, inositol-free medium (Life Technologies, Inc.) supplemented with 1 μCi ml^{-1} *myo*-[2-^{3}H]-inositol. Cells were then washed twice with PBS medium, equilibrated at 37°C in PBS for 30 min, and then incubated for 15 min in PBS supplemented with 10 mM LiCl. CHO cells were stimulated for 15 min with increasing concentrations of AVP, OT or F-180. After stopping the reaction with 5% (v v^{-1}) perchloric acid, total IPs ($IP_1 + IP_2 + IP_3$) were extracted and purified on anion exchange chromatography column (Dowex AG1 × 8, formate form, 200–400 mesh; Bio-Rad). For each sample, a fraction containing total IPs was collected and counted. The same experimental procedure was used for ZF cell primary culture except that cells were labelled at least 2 days in DMEM medium containing 10% foetal calf serum and 3 μCi ml^{-1} *myo*-[2-^{3}H]-inositol.

Adenylate cyclase assays

CHO cells expressing hV_2-R were grown as described above, and the production of cyclic AMP assessed after 2 days in culture as previously described (Côté *et al*., 1999). Cells were plated in 12-well plates and [^{3}H]-adenine was added to the medium (3 μCi ml^{-1}) for the last day of culture. The medium was aspirated and replaced with Dulbecco's modified Eagle's medium supplemented with 5.5 mM 3-isobutyl-methyl-xanthine and 0.1% BSA with the vehicle alone or various concentration of agonists for 10 min at 37°C. In experiments with antagonists, these were added 10 min before agonist addition. After a further 10 min incubation period, the reaction was terminated by addition of 1 ml of 5% trichloroacetic acid. Unlabelled ATP and cyclic AMP were added to the acid extracts at the concentration of 0.5 mM. Relative intracellular cyclic AMP levels were determined by measuring the formation of [^{3}H]-cyclic AMP from the prelabelled adenine nucleotide pool. Labelled cyclic AMP was separated by sequential chromatography on Dowex and alumina columns. Radioactivity present in the cyclic AMP fractions was expressed as per cent of the sum of radio-activity recovered in the cyclic AMP fraction and radio-activity which was not retained by the Dowex column which mainly corresponded to labelled ATP.

Data analysis

The radioligand binding data were analysed by GraphPad Prism™ (GraphPad Software, Inc., San Diego, CA, U.S.A.). The dissociation constant (K_D) and the maximal binding capacity (B_{max}) were deduced from Scatchard experiments. The inhibitory dissociation constants (K_i) for unlabelled AVP analogues were calculated from binding competition experiments according to the Cheng & Prusoff (1973) $K_i = IC_{50}(1+[L]/K_D)$, where IC_{50} is the concentration of unlabelled analogue leading to half maximal inhibition of specific binding, [L] the concentration of the radioligand present in the assay and K_D its affinity for the AVP receptor studied. K_{act} values, the concentrations of AVP analogue leading to half maximal stimulation of second messenger accumulations, were calculated from functional studies using GraphPad PRISM. K_{inact}, the inhibition constants for antagonists were calculated from IC_{50} (the concentration of antagonist leading to half maximal inhibition of agonist induced second messenger accumulation) using the following equation : $K_{inact} = IC_{50}\ (1+[H]/K_{act})$, where [H] is the concentration of agonist used in the assay and K_{act} its activation constant.

Results are expressed as the mean ± s.e.mean of at least three distinct experiments performed in triplicate unless noted.

Results

Binding properties of F-180 for vasopressin/oxytocin receptors

To determine the affinity (K_i) of F-180 for the different human VP/OT receptor subtypes, we used a plasma membrane preparation from CHO cells stably transfected with DNA encoding the three VP receptor subtypes and the OT receptor. As illustrated in Figure 1, whatever the CHO membrane preparations used, AVP, OT and F-180 completely displaced specific radioligand binding in a dose-dependent manner. The K_i for each of these peptides were calculated from the competition experiments, and are summarized in Table 1. These data validate the binding assays used in this study, since the affinity of the natural peptides AVP and OT for their respective receptors were in the nanomolar range as previously described (for review see Barberis *et al*., 1999). More interestingly, our results indicate that F-180 exhibits high affinity for the hV_{1a}-R subtype ($K_i = 11$ mM) and is selective for this receptor isoform because its affinity for the hV_2-R, hV_{1b}-R and hOT-R was at least 44 fold higher.

Since both affinity and selectivity for a given vasopressin analogue of VP receptors may vary among the species (Pettibone *et al*., 1992), we decided to measure the K_i of F-180 for rat and bovine VP receptors. We used liver, kidney and pituitary membrane preparations for these experiments because, in mammals, these tissues are known to naturally express V_{1a}, V_2 and V_{1b} vasopressin receptors, respectively (for review see Jard, 1998). The values summarized in Table 1 show that F-180 exhibits a similar pharmacological profile for human and bovine VP receptors. For these two species, this compound resulted to be selective for the V_{1a} receptor subtype in human and bovine species and presents high affinity for this receptor isoform. Yet, the K_i for F-180 is slightly higher than that measured for AVP. By contrast, F-180 was found to be non-selective for rat V_{1a} VP receptor and exhibits a low affinity for this receptor subtype ($K_i = 478$ nM).

Functional properties of F-180 on CHO cells expressing human vasopressin receptors

To determine the agonist or antagonist properties of F-180, we examined the activity of this compound on phosphoinositide and cyclic AMP metabolism in CHO cells expressing the hV_{1a}-R, hV_{1b}-R, hOT-R, and hV_2-R, respectively.

As illustrated in Figure 2A, F-180 behaves like a V_{1a} agonist. It stimulated IPs accumulation in a dose-dependent manner with a maximal efficiency that was slightly lower than that observed for AVP (see Table 2). The concentration of F-180 which was able to cause half maximum stimulation ($K_{act} = 1.7$ nM) was also similar to that determined for AVP ($K_{act} = 1.3$ nM). As shown in Figure 2B, F-180 was a specific agonist of h-V_{1a} receptor, since its stimulatory effects on inositol phosphate accumulation was completely abolished by SR-49059, a specific antagonist of h-V_{1a} receptor ($K_i = 3$ nM). F-180 also stimulated IPs accumulation in CHO cells expressing hV_{1b}-R, yet its K_{act} was 460 fold higher than that measured in CHO cells expressing hV_{1a}-R (see Figure 3A and Table 2). Despite this high K_{act} value, in good agreement with its K_i for hV_{1b}-R, F-180 maximally stimulated IPs accumulation as AVP did. By contrast, F-180, up to 1 μM, was not able to stimulate IPs accumulation in CHO cells expressing hOT-R (Figure 3B). F-180 might be considered to be a full antagonist of hOT receptor, as it fully inhibited OT-stimulated IPs accumulation. The inactivation constant (K_{inact}) of F-180 was similar to its K_i, as determined from binding experiments (Figure 3C and Table 2).

Parallel experiments were carried out to determine the functional properties of F-180 on hV_2-R. As seen in Figure 4, addition of F-180 resulted in a dose-dependent accumulation

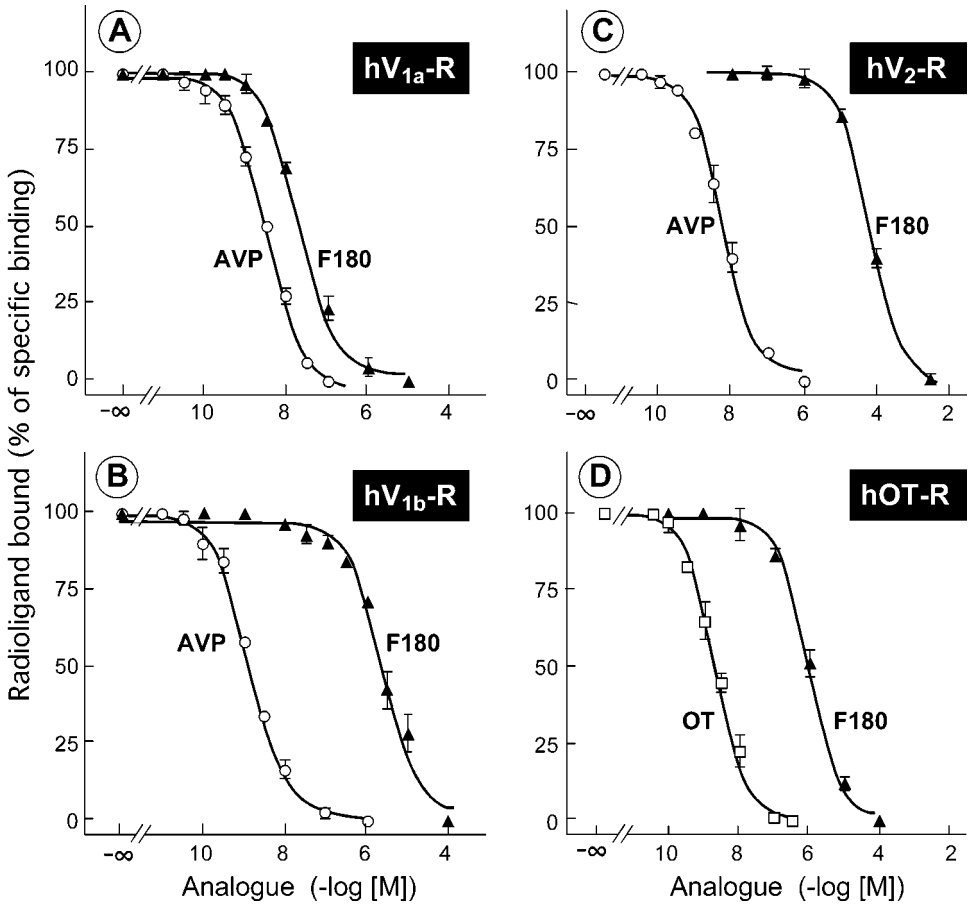

Figure 1 Binding properties of AVP and F-180 to human AVP/OT receptors. Competition experiments using unlabelled AVP, OT or F-180 were performed using membrane preparations from CHO cells stably transfected with DNA encoding the human wild-type V_{1a}-R, V_{1b}-R, V_2-R and OT-R, and [^{125}I]-HO-LVA (A), [^{3}H]-AVP (B and C) or [^{125}I]-OTA (D) as radioligands. Non-specific binding experiments were determined either in the presence of 1 μM AVP (A, B and C) or 1 μM OT (D). Data are expressed as per cent of specific binding measured in each condition in the absence of unlabelled competitor. Values are given as the mean$\pm$s.e.mean of three independent assays performed in triplicate.

Table 1 Affinities of AVP/OT and F-180 for vasopressin/oxytocin receptors from different mammalian species

		K_i (nM)			
Species	*peptide*	*V_{1a}-R*	*V_{1b}-R*	*V_2-R*	*OT-R*
Human	AVP	1.0$\pm$0.1	0.68$\pm$0.01	3.3$\pm$0.6	1.6$\pm$0.5
	F-180	11.7$\pm$0.2	2100$\pm$800	>10000	520$\pm$70
	OT	146$\pm$20	1160$\pm$90	1540$\pm$260	0.8$\pm$0.2
Bovine	AVP	10$\pm$3	10$\pm$0.5	4.0$\pm$0.9	n.d.
	F-180	56$\pm$13	6400$\pm$1900	>10000	n.d.
Rat	AVP	0.8$\pm$0.1	0.9$\pm$0.1	0.32$\pm$0.08	n.d.
	F-180	480$\pm$30	750$\pm$190	2000$\pm$300	n.d.

Binding experiments were performed with the appropriate radioligand on plasma membranes from CHO cells stably transfected with DNA encoding for each subtype of human VP or OT receptors, or from rat or bovine liver, anterior pituitary and kidney tissues, as described in Methods. Inhibition constants (K_i) were determined from competition experiments as illustrated in Figure 1. Values are the mean$\pm$s.e.mean of 4–6 independent determinations each performed in triplicate. n.d.: not determined.

of cyclic AMP. Yet, no saturation could be observed due to the very low affinity of F-180 for hV_2-R.

Functional properties of F-180 on ZF cells primary culture

To evaluate the functional activity of F-180 on a native model known to express V_{1a} vasopressin receptor, we decided to measure IPs accumulation and cortisol secretion in bovine ZF cells maintained in culture (Bird *et al*., 1990).

Both AVP and F-180 stimulated IPs accumulation in a dose-dependent manner in these cells (Figure 5A). Maximal stimulations, as compared to control, were 223%$\pm$5 and 181%$\pm$4, and the corresponding K_{act} values were 0.15$\pm$0.04 nM and 0.41$\pm$0.03 nM for AVP and F-180 respectively. Similarly, both peptides also induced cortisol

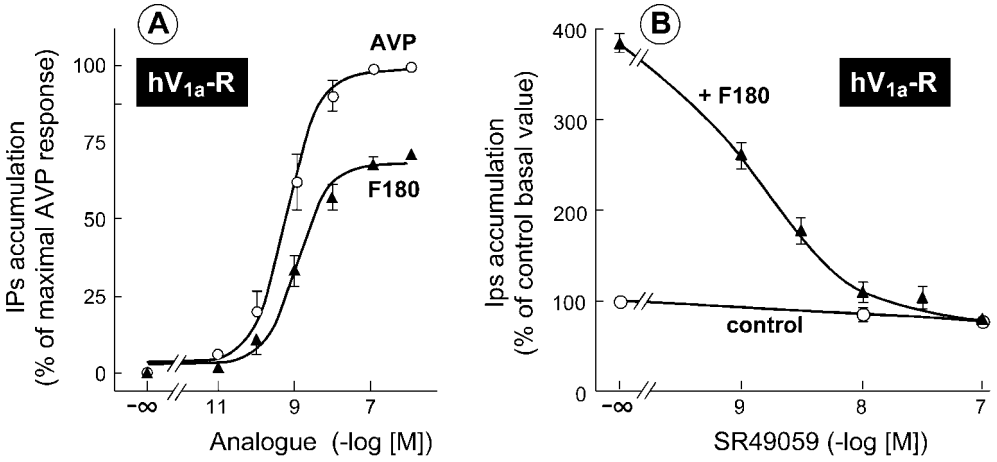

Figure 2 Functional properties of F-180 on a CHO cell line expressing human V_{1a} receptors. (A) *myo*-[2, ^{3}H]-inositol prelabelled CHO cells stably transfected with DNA encoding the hV_{1a}-R were preincubated for 15 min at 37°C with 10 mM LiCl and further stimulated for 15 min with or without (control) increasing concentrations of AVP or F-180. Total IPs accumulated were measured as described in Methods and were expressed as percentage of maximal AVP response. (B) *myo*-[2, ^{3}H]-inositol prelabelled cells were preincubated for 15 min at 37°C with 10 mM LiCl and increasing concentrations of SR-49059 or vehicle (control). Then, 3 nM F-180 was added in the incubation medium and the reaction allowed to proceed for another 15 min period. IPs accumulation was measured and expressed as percentage of IPs accumulated under F-180 stimulation. Values are given as the mean ± s.e.mean of two or three independent triplicate experiments.

Table 2 Functional properties of AVP/OT and F-180 in CHO cells stably transfected with DNA encoding the human VP/OT receptors

	K_{act}, K_{inact} (nM)			
	IPs accumulation			*cyclicAMP accumulation*
Peptide	hV_{1a}-*R*	hV_{1b}-*R*	*hOT-R*	hV_2-*R*
AVP	1.3 ± 0.6	2.0 ± 0.6	nd	0.9 ± 0.1
OT	nd	nd	0.65 ± 0.13	nd
	Agonist	Agonist	Antagonist	Agonist
F-180	K_{act} = 1.7 ± 0.8 (71% ± 1)	K_{act} = 790 ± 160 (100% ± 1)	K_{inact} = 390 ± 50 (1% ± 1)	K_{act} > 10000 (n.d.)

CHO cells stably expressing the different human VP or OT receptors were incubated with or without (control) increasing amounts of AVP, OT or F-180. Inositol phosphate (CHO cells expressing hV_{1a}-R, hV_{1b}-R, hOT-R) or cyclic AMP accumulation (CHO cells expressing hV_2-R) were measured as described in Methods. Activation and inactivation constants (K_{act}, K_{inact}) were calculated as previously described from the concentration dose response curves performed for each compound (see Figures 2, 3 and 4). Values are the mean ± s.e.mean of three independent experiments each performed in triplicate. For F-180, values in parentheses represent the maximal stimulation of second messenger accumulation expressed as percentage of maximal AVP or OT responses. n.d.: not determined.

secretion (Figure 5B). These effects were saturable and dose dependent (K_{act} values were 0.4 ± 0.03 and 1.4 ± 0.3 pM and maximal cortisol secretion expressed as per cent of basal value were 790% ± 60 and 550% ± 40 for AVP and F-180 respectively). As found for the human V_{1a} receptor, F-180 was a partial V_{1a} agonist of bovine V_{1a} receptor. Its maximal effect represented 80 and 70% of maximal AVP effects on IPs accumulation and cortisol secretion, respectively. Moreover, a preincubation with the V_{1a} antagonist, SR-49059 (100 nM), caused an inhibition of 92% ± 3 in the IPs accumulation induced by the agonist F-180 (100 nM) on ZF cells in primary culture. In these experimental conditions, the rate of cortisol secretion induced by F-180 also returned to basal level in presence of the antagonist SR-49059 (data not shown).

Discussion

Up to date, very few selective agonist of nanomolar affinity for VP receptor isoforms have been described. In this study, we characterized the pharmacological properties of F-180, a selective V_{1a} agonist for human and bovine VP receptors.

Binding experiments performed on plasma membranes from CHO cells expressing the human VP and OT receptors demonstrated that F-180 is a selective analogue of human V_{1a} receptor. Its affinity for this receptor subtype was at least 44 fold higher than that measured for the human V_{1b}, V_2, or OT receptors (see Table 1). A similar selective pharmacological profile was also observed for bovine VP receptors. Yet, the affinity of F-180 for bovine V_{1a}-R was found to be five times lower. This loss of affinity was still more pronounced for the rat V_{1a}-R (K_i = 450 nM). Moreover, for this species, F-180 was found to be non-selective (see Table 1). These data supported the existence of a strong species variability typical of vasopressin analogues (Pettibone *et al*., 1992).

Functional studies performed on CHO cell lines transfected with human V_{1a} receptors demonstrated that F-180, like AVP, stimulated IPs accumulation in a dose-response manner (Figure 2). Its K_{act} was 6 fold lower than its affinity (K_i) deduced from binding experiments, suggesting a tight

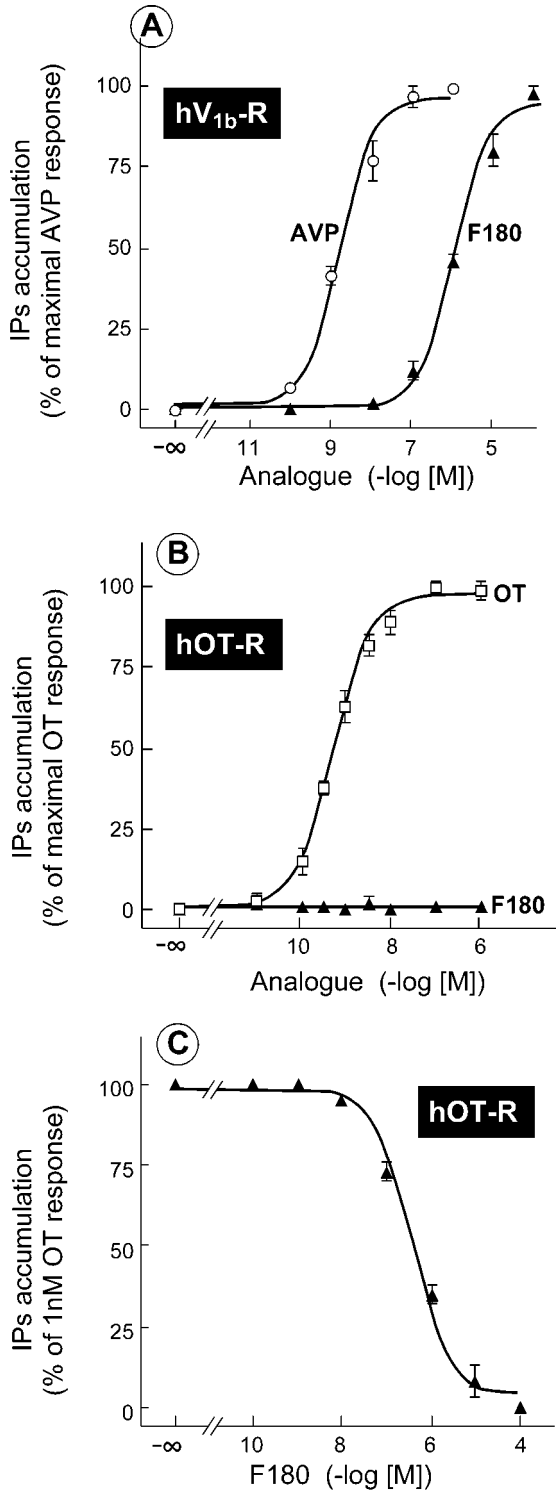

Figure 3 Functional properties of F-180 on CHO cell lines expressing human V_{1b}, and OT receptors: *myo*-[2, ^{3}H]-inositol prelabelled CHO cells were stably transfected with DNA encoding the hV_{1b} or hOT receptors. (A and B) Cells were preincubated for 15 min at 37°C with 10 mM LiCl and further stimulated with or without (control) increasing concentrations of AVP, OT or F-180. Total IPs accumulated were measured as described in Methods and expressed as percentage of maximal AVP or OT responses. (C) *myo*-[2, ^{3}H]-inositol prelabelled cells expressing hOT-R were preincubated for 15 min at 37°C with 10 mM LiCl and increasing concentrations of F-180 or vehicle (control). Then, 1 nM OT was added in the incubation medium and the reaction allowed to proceed for another 15 min period. IPs accumulation were measured and expressed as percentage of IPs accumulated under OT stimulation. Each point represents the mean ± s.e.mean of three independent assays performed in triplicate.

coupling between the human V_{1a} receptor, the α_q/α_{11} G protein and the phopholipase Cβ as previously observed for the human V_{1a} receptors expressed in human adrenocortical cells (Grazzini *et al*., 1999). The specificity of F-180 for human and bovine V_{1a} receptors was further evidenced by the use of SR-49059. This specific V_{1a} antagonist for both V_{1a} human and bovine receptor (Serradeil-Le Gal *et al*., 1993; Andrés, unpublished results) was able to fully inhibit the agonistic effect of F-180 both on CHO cells expressing human V_{1a} receptor (Figure 2) and bovine ZF cells in primary culture.

F-180 was also shown to be an agonist of human V_{1b} and V_2 receptors. By contrast, it behaves like a full antagonist of human OT receptors. Similar data were also obtained using bovine adrenocortical cells in primary culture naturally expressing the V_{1a} receptor (Bird *et al*., 1990). In this homologous model, F-180 both stimulates inositol phosphate production and cortisol secretion as AVP does. Yet, as found for CHO cell lines which expressed the human V_{1a} receptor, F-180 behaves like a partial agonist (Figures 2 and 5). This property was also observed in rat where F-180 was shown to be a potent vasopressor analogue, although four times less potent than AVP (Bernadich *et al*., 1998). This reduced activity of F-180 as compared to the natural hormone is compensated *in vivo* by an enhanced stability, probably due to deamination of the N-terminal residue of F-180. Such modification reduces its sensitivity to endogenous proteases and may explain the prolonged pressor activity of F-180 previously observed in rat (Aurell *et al*., 1990).

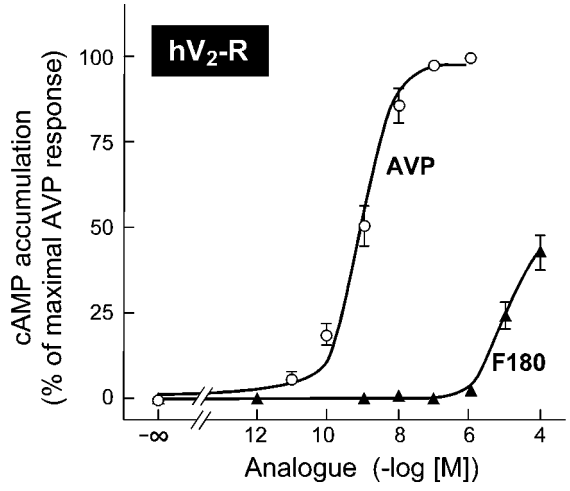

Figure 4 Functional properties of F-180 on a CHO cell line expressing human V_2 receptors. [^{3}H]-adenine prelabelled CHO cells stably transfected with DNA encoding hV_2-R were incubated with or without (control) increasing concentrations of AVP or F-180. Cyclic AMP which accumulated was measured as described in Methods and expressed as percentage of maximal AVP response. Values are the mean ± s.e.mean of 3–5 independent experiments performed in triplicate.

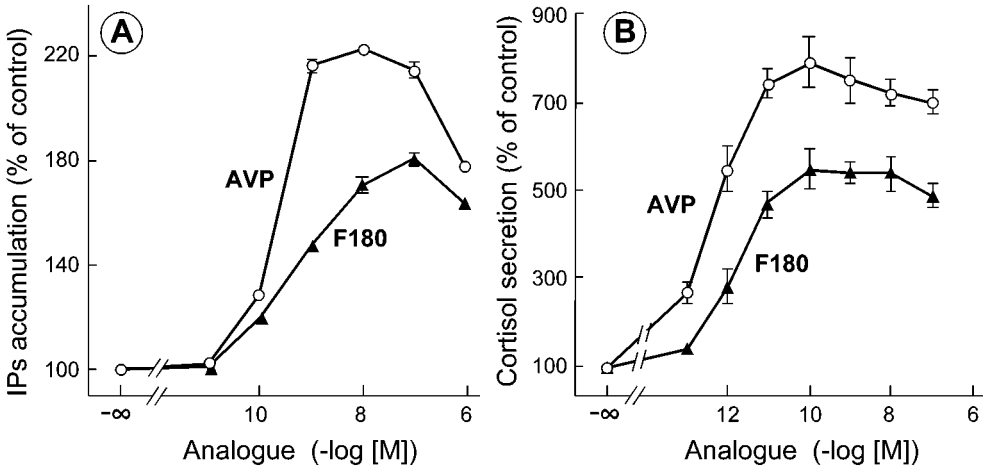

Figure 5 Effects of AVP and F-180 on inositol phosphate metabolism and steroid secretion in bovine adrenocortical cells. (A) *myo*-[2, ^{3}H]-inositol prelabelled zona fasciculata (ZF) cells in primary culture were preincubated for 15 min at 37°C in HBS/LiCl medium and further stimulated for 15 min without (control) or with increasing concentrations of AVP or F-180. Total IPs accumulations were determined as indicated in Methods and expressed as percentage of control. Results are the mean ± s.e.mean of three independent experiments, each performed in triplicate. (B) For steroid secretion measurements, ZF cells in primary culture were washed and incubated at 37°C for 2 h in HBS medium with or without (control) various concentrations of AVP or F-180 as indicated. At the end of the incubation period, the incubation medium was removed and assayed for steroid content, as described in Methods. Each value is the mean ± s.e.mean of three–five independent experiments, each performed in triplicate. Basal cortisol secretion was 24 ± 3 ng/10^6 cells.

In conclusion, our data show that F-180 is the first potent and selective agonist for human and bovine V_{1a} receptors described so far. The potential therapeutical use of a specific V_{1a} agonist is probably limited since the major physiological effect of AVP in man involves water reabsorption and, to a lesser extent, blood pressure control (for review see Johnston, 1985). Yet, this compound may constitute a very useful pharmacological tool to elucidate new physiological effects of vasopressin acting on V_{1a} vasopressin receptor subtype.

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References

AURELL, C.J., BENGTSSON, B., EKHOLM, K., KASPRZYKOWSKA, R., NILSSON, A., PERSSON, R., TROJNAR, J., ABBE, M. & MELIN, P. (1990). Development of vasopressor specific vasotocin analogues with prolonged effects. In *Peptides*. ed. Giralt, E. & Andreu, D. pp. 671–673. Leiden: Escom Science Publishers.

BARBERIS, C., BALESTRE, M-N., JARD, S., TRIBOLLET, E., ARSENIJEVIC, Y., DREIFUSS, J.J., BANKOWSKI, K., MANNING, M., CHAN, W.Y., SCHLOSSER, S.S., HOLSBOER, F. & ELANDS, J. (1995). Characterization of a novel linear radioiodinated vasopressin antagonist: an excellent radioligand for vasopressin V_{1a} receptors. *Neuroendocrinology,* **62,** 135–146.

BARBERIS, C., MORIN, D., DURROUX, T., MOUILLAC, B., GUILLON, G., SEYER, R., HIBERT, M., TRIBOLLET, E. & MANNING, M. (1999). Molecular pharmacology of AVP and OT receptors and therapeutic potential. *Drug News Perspect.,* **12,** 279–292.

BERNADICH, C., BANDI, J.C., MELIN, P. & BOSCH, J. (1998). Effects of F-180, a new selective vasoconstrictor peptide, compared with terlipressin and vasopressin on systemic and splanchnic hemodynamis in a rat model of portal hypertension. *Hepatology,* **27,** 351–356.

BIRD, I.M., NICOL, M., WILLIAMS, B.C. & WALKER, S.W. (1990). Vasopressin stimulates cortisol secretion and phosphoinositide catabolism in cultured bovine adrenal fasciculata/reticularis cells. *J. Mol. Endocrinol.,* **5,** 109–116.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

BUTLEN, D., GUILLON, G., RAJERISON, R. & JARD, S. (1978). Structural requirements for activation of vasopressin sensitive adenylate cyclase, hormone binding and antidiuretic actions. *Mol. Pharmacol.,* **14,** 1006–1017.

CHAN, W.Y., WO, N.C., STOEV, S.T., CHENG, L.L. & MANNING, M. (2000). Discovery and design of novel and selective vasopressin and oxytocin agonists and antagonists: the role of bioassays. *Exp. Physiol.,* **85,** 7S–18S.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099–3108.

CÔTÉ, M., PAYET, M.D., ROUSSEAU, E., GUILLON, G. & GALLO-PAYET, N. (1999). Comparative involvement of cyclic nucleotide phosphodiesterases and adenylyl cyclase on adrenocorticotropin-induced increase of cyclic adenosine monophosphate in rat and human glomerulosa cells. *Endocrinology,* **140,** 3594–3601.

COTTE, N., BALESTRE, M.N., PHALIPOU, S., HIBERT, M., MANNING, M., BARBERIS, C. & MOUILLAC, B. (1998). Identification of residues responsible for the selective binding of peptide antagonists and agonists in the V_2 vasopressin receptor. *J. Biol. Chem.,* **273,** 29462–29468.

CYMERYNG, C.B., DADA, L.A. & PODESTA, E.J. (1998). Effect of nitric oxide on rat adrenal zona fasciculata steroidogenesis. *J. Endocrinol.,* **158,** 197–203.

ELANDS, J., BARBERIS, C., JARD, S., TRIBOLLET, E., DREIFUSS, J.J., BANKOWSKI, K., MANNING, M. & SAWYER, W.H. (1988). ^{125}I-labelled d(CH_2)$_5$[Tyr(Me)^2,Thr^4,Tyr-NH_2^{9}]OVT: a selective oxytocin receptor ligand. *Eur. J. Pharmacol.,* **147,** 197–207.

GAVRAS, H., RIBEIRO, A.B., KOHLMANN, O., SARAGOCA, M., MULINARI, R.A., RAMOS, O. & GAVRAS, I. (1984). Effects of a specific inhibitor of the vascular action of vasopressin in humans. *Hypertension,* **6,** I156–I160.

GRAZZINI, E., BRETON, C., DERICK, S., ANDRÉS, M., RAUFASTE, D., RICKWAERT, F., BOCCARA, G., COLSON, P., GUÉRINEAU, N., SERRADEIL-LE GAL, C. & GUILLON, G. (1999). Vasopressin receptors in human adrenal medulla and pheochromocytoma. *J. Clin. Endocrinol. Metab.,* **84,** 2195–2203.

HUGUENIN, R.L. (1964). Synthése de la Phe^2-Orn^8-vasopressine et de la Phe^2-Orn^8-ocytocine, deux analogues de la vasopressine doués d'une activité pressorique sélective. *Helv. Chim. Acta,* **47,** 1934–1941.

JARD, S. (1998). Vasopressin receptors: a molecular historical survey. In *Vasopressin and oxytocin. Molecular cellular clinical advance*. ed. Zingg, H.H., Bourque, C.W. & Bichet, D. pp. 1–13. New York: Plenum Press.

JOHNSTON, C.I. (1985). Vasopressin in circulatory control and hypertension. *J. Hypertension,* **3,** 557–269.

KASSIS, S., HENNEBERRY, R.C. & FISHMAN, P.H. (1984). Induction of catecholamine-responsive adenylate cyclase in HeLa cells by sodium butyrate. Evidence for a more efficient stimulatory regulatory component. *J. Biol. Chem.,* **259,** 4910–4916.

KRUSZYNSKI, M., LAMMEK, B., MANNING, M., SETO, J., HALDAR, J. & SAWYER, W.H. (1980). [1-(β-Mercapto-β,β-cyclopentamethylene propionic acid), 2-(O-methyl)tyrosine]arginine-vasopressin and [1-(β-mercapto-β,β-cyclopentamethylene propionic acid)]-arginine-vasopressin, two highly potent antagonists of the vasopressor response to arginine-vasopressin. *J. Med. Chem.,* **23,** 364–368.

LOLAIT, S.J., O'CARROLL, A.M., MC BRIDE, O.W., KONIG, M., MOREL, A. & BROWNSTEIN, M.J. (1992). Cloning and characterization of a vasopressin V_2 receptor and possible link to nephrogenic diabetes insipidus. *Nature,* **357,** 336–339.

MAEDA, T., BALAKRISHNAN, K. & MEHDI, S.Q. (1983). A simple and rapid method for the preparation of plasma membranes. *Biochim. Biophys. Acta.,* **73,** 115–120.

MOREL, A., O'CARROLL, A.M., BROWNSTEIN, M.J. & LOLAIT, S.J. (1992). Molecular cloning and expression of a rat V_{1a} arginine vasopressin receptor. *Nature,* **356,** 523–526.

NAKAMURA, S., YAMAMURA, Y., ITOH, S., KONDO, K., SUMIDA, T., TERAMOTO, S., TSUJIMOTO, G. & MORI, T. (1998). Characterization of OPC-51803, a novel nonpeptide vasopressin V_2-receptor agonist. *Naunyn Schmiederbergs Arch. Pharmacol.,* **358,** R-551.

PETTIBONE, D.J., KISHEL, T.M., WOYDEN, C.J., CLINESCHMIDT, B.V., BOCK, M.G., FREIDENGER, R.M., VEBER, D.F. & WILLIAMS, P.D. (1992). Radioligand binding studies reveal marked species differences in the vasopressin V1 receptor of rat, rhesus and human tissues. *Life Sci.,* **50,** 1953–1958.

SAITO, M., TAHARA, A. & SUGIMOTO, T. (1997). 1-deamino-8-D arginin vasopressin as an agonist on V1b receptor. *Biochem. Pharmacol.,* **53,** 1711–1717.

SCHWARTZ, J., DERDOWSKA, I., SOBOCINSKA, M. & KUPRYSZEWSK, I.G. (1991). A potent new synthetic analog of vasopressin with relative agonist specificity for the pituitary. *Endocrinology,* **129,** 1107–1109.

SERRADEIL-LE GAL, C., LACOUR, C., VALETTE, G., GARCÍA, G., FOULON, L., GALINDO, G., BANKIR, L., POUZET, B., GUILLON, G., BARBERIS, C., CHICOT, D., JARD, S., VILAIN, P., GARCÍA, C., MARTY, E., RAFAUSTE, D., BROSSARD, G., NISATO, D., MAFFRAND, J.P. & LE FUR, G. (1996). Characterization of SR 121463A, a highly potent and selective, orally active vasopressin V_2 receptor antagonist. *J. Clin. Invest.,* **98,** 2729–2738.

SERRADEIL-LE GAL, C., WAGNON, J., GARCÍA, C., LACOUR, C., GUIRAUDOU, P., CHRISTOPHE, B., VILLANOVA, G., NISTATO, D., MAFFRAND, J.P., LE FUR, G., GUILLON, G., CANTAU, B., BARBERIS, C., TRUEBA, M., ALA, Y. & JARD, S. (1993). Biochemical and pharmacological properties of SR 49059, a new potent, nonpeptide antagonist of rat and human vasopressin V_{1a} receptors. *J. Clin. Invest.,* **92,** 224–231.

SERRADEIL-LE GAL, C., WAGNON, J., VALETTE, G., GARCÍA, C., PASCAL, M., MAFFRAND, J.P. & LE FUR, G. (2001). Nonpeptide vasopressin receptor antagonists: development of selective and orally active V1a, V2 and V1b ligands. Abstracts of the World Congress on Neurohypophysial hormones. Bordeaux (France), September 8–12. pp. 40–41.

SUGIMOTO, T., SAITO, M., MOCHIZUKI, S., WATANABE, Y., HASHIMOTO, S. & KAWASHIMA, H. (1994). Molecular cloning and functional expression of a cDNA encoding the human V_{1b} vasopressin receptor. *J. Biol. Chem.,* **269,** 27088–27092.

TREMBLAY, E., PAYET, M.D. & GALLO-PAYET, N. (1991). Effects of ACTH and angiotensin II on cytosolic calcium in cultured adrenal glomerulosa cells. Role of cAMP production in the ACTH effect. *Cell Calcium,* **12,** 655–673.

TRUEBA, M., IBARROLA, I., OGIZA, K., MARINO, A. & MACARULLA, J.M. (1991). Specific binding sites for corticosterone in isolated cells and plasma membrane from rat liver. *J. Membr. Biol.,* **120,** 115–124.

YAMAMURA, Y., OGAWA, H., YAMASHITA, H., CHICHARA, T., MIYAMOTO, H., NAKAMURA, S., ONOGAWA, T., YAMASHITA, T., HOSOKAWA, T., MORI, T., TOMINAGA, T. & YABUUCHI, Y. (1992). Characterization of a novel aquaretic agent, OPC-31260, as an orally effective, non-peptide vasopressin V_2 receptor antagonist. *Br. J. Pharmacol.,* **105,** 787–91.

ZAORAL, M., KOLC, J. & SORM, F. (1967). Amino acids and peptides. LXXI. Synthesis of 1-deamino-8-D-γ-aminobutyrine-vasopressin, 1-deamino-8-D-lysine-vasopressin, and 1-deamino-8-D-arginine-vasopressin. *Coll. Czech. Chem. Comm.,* **32,** 1250–1257.

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