British Journal of Pharmacology (2000) 129, 1700–1706

www.nature.com/bjp

Characterization of a novel nonpeptide vasopressin V_2-agonist, OPC-51803, in cells transfected human vasopressin receptor subtypes

*,[1,3]Shigeki Nakamura, [1]Yoshitaka Yamamura, [1]Shuji Itoh, [1]Takahiro Hirano, [1]Kenji Tsujimae, [1]Masashi Aoyama, [1]Kazumi Kondo, [1]Hidenori Ogawa, [1]Tomoichi Shinohara, [1]Keizo Kan, [1]Yoshihisa Tanada, [1]Shuji Teramoto, [1]Takumi Sumida, [1]Sunao Nakayama, [1]Kazuo Sekiguchi, [3]Toshimi Kambe, [2]Gozoh Tsujimoto, [1]Toyoki Mori & [1]Michiaki Tominaga

[1]Second Tokushima Institute of New Drug Research, Otsuka Pharmaceutical Co. Ltd., 463-10, Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan and [2]Department of Molecular, Cell Pharmacology, National Children's Medical Research Center, 3-35-31 Taishido, Setagaya-ku, Tokyo 154-8509, Japan

1 We discovered the first nonpeptide arginine-vasopressin (AVP) V_2-receptor agonist, OPC-51803. Pharmacological properties of OPC-51803 were elucidated using HeLa cells expressing human AVP receptor subtypes (V_2, V_{1a} and V_{1b}) and compared with those of 1-desamino-8-D-arginine vasopressin (dDAVP), a peptide V_2-receptor agonist.

2 OPC-51803 and dDAVP displaced [^{3}H]-AVP binding to human V_2- and V_{1a}-receptors with K_i values of 91.9 ± 10.8 nM ($n=6$) and 3.12 ± 0.38 nM ($n=6$) for V_2-receptors, and 819 ± 39 nM ($n=6$) and 41.5 ± 9.9 nM ($n=6$) for V_{1a}-receptors, indicating that OPC-51803 was about nine times more selective for V_2-receptors, similar to the selectivity of dDAVP. OPC-51803 scarcely displaced [^{3}H]-AVP binding to human V_{1b}-receptors even at 10^{-4} M, while dDAVP showed potent affinity to human V_{1b}-receptors with the K_i value of 13.7 ± 3.2 nM ($n=4$).

3 OPC-51803 concentration-dependently increased cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) production in HeLa cells expressing human V_2-receptors with an EC_{50} value of 189 ± 14 nM ($n=6$). The concentration-response curve for cyclic AMP production induced by OPC-51803 was shifted to the right in the presence of a V_2-antagonist, OPC-31260.

4 At 10^{-5} M, OPC-51803 did not increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in HeLa cells expressing human V_{1a}-receptors. On the other hand, dDAVP increased $[Ca^{2+}]_i$ in HeLa cells expressing human V_{1a}- and V_{1b}-receptors in a concentration-dependent fashion.

5 From these results, OPC-51803 has been confirmed to be the first nonpeptide agonist for human AVP V_2-receptors without agonistic activities for V_{1a}- and V_{1b}-receptors. OPC-51803 may be useful for the treatment of AVP-deficient pathophysiological states and as a tool for AVP researches.
British Journal of Pharmacology (2000) **129,** 1700–1706

Keywords: OPC-51803; vasopressin; nonpeptide V_2-agonist; receptor binding; cyclic AMP

Abbreviations: ACTH, adrenocorticotropic hormone; AVP, arginine vasopressin; BSA, bovine serum albumin; EC_{50}, concentration exerting 50% effectiveness of maximal response; IC_{50}, concentration exerting 50% inhibition; cyclic AMP, cyclic adenosine 3′, 5′-monophosphate; dDAVP, 1-desamino-8-D-arginine vasopressin; DMSO, dimethylsulphoxide; K_D, dissociation constant; DMEM, Dulbecco's minimum Eagle's medium; EGTA, ethylene glycol-bis (β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid; FBS, foetal bovine serum; Fura-2-AM, fura-2 acetoxymethyl ester; G418, geneticin disulphate; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulphonic acid; K_i, inhibition constant; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; IBMX, 3-isobutyl-1-methylxanthine; B_{max}, number of binding sites; PBS, phosphate-buffered saline; Triton X-100, polyethylene glycol mono-*p*-isooctylphenyl ether; SDS, sodium dodecyl sulphate

Introduction

Vasopressin (AVP) is a neurohypophysial hormone which is synthesized in the hypothalamus and released from the pituitary. AVP plays an important role in the regulation of body fluid homeostasis. Physiological actions of AVP are exerted through seven transmembrane G-protein-coupled receptors (Laszlo *et al*., 1991; Liu & Wess, 1996). V_{1a}-receptors are the most widespread and are coupled with $G_{q/11}$; they are responsible for vasoconstriction, platelet aggregation, glycogenolysis and so on, and V_{1b}-receptors are also coupled with $G_{q/11}$ and are responsible for adrenocorticotropic hormone (ACTH) secretion, *via* stimulation of inositol phosphate turnover. V_2-receptors are coupled to G_s, and responsible for promotion of water reabsorption in the collecting ducts of the kidney *via* stimulation of cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) production.

In the 1990's, we reported orally effective nonpeptide antagonists of AVP receptors, V_{1a}-selective OPC-21268 (Ogawa *et al*., 1993; Yamamura *et al*., 1991), V_2-selective OPC-31260 (Ogawa *et al*., 1996; Yamamura *et al*., 1992) and OPC-41061 (Kondo *et al*., 1999; Yamamura *et al*., 1998). Then

*Author for correspondence at: First Institute of New Drug Research, Otsuka Pharmaceutical Co. Ltd., 463-10, Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan
E-mail: s_nakamura@research.otsuka.co.jp
[3]Current address: First Institute of New Drug Research, Otsuka Pharmaceutical Co. Ltd., 463-10, Kagasuno, Kawauchi-cho, Tokushima 771-0192, Japan

several nonpeptide antagonists of AVP receptors, such as V_{1a}-selective SR-49059 (Serradeil-Le Gal *et al*., 1993), V_2-selective SR-121463A (Serradeil-Le Gal *et al*., 1996), and VPA-985 (Albright *et al*., 1998), and nonselective YM-087 (Tahara *et al*., 1997) followed. These nonpeptide antagonists could overcome the problem of intrinsic agonistic activity associated with peptide vasopressin antagonists. Since peptide antagonists have an intrinsic agonistic activity, they acted as an agonist when tested in human. Though it has been difficult to develop antagonists without an agonistic activity for human AVP V_2-receptors by peptide analogues, nonpeptide compounds succeeded in acting as an antagonist in human (Ohnishi *et al*., 1993; 1995). The approach for developing the nonpeptide ligands for peptide hormone receptors may enable us to produce functionally separate ligands, agonist and antagonist, and elucidate the mechanisms of receptor-ligand interactions.

Recently, nonpeptide agonists have been targeted for the seven transmembrane G-protein-coupled receptors as well as antagonists. In fact, nonpeptide agonists have been reported in the angiotensin system (Kivlighn *et al*., 1995; Perlman *et al*., 1995) and the cholecystokinin system (Aquino *et al*., 1996; Willson *et al*., 1996). During our development of nonpeptide antagonists for AVP receptors, some compounds were also found to possess antidiuretic effects in rats. Since molecular cloning of human AVP receptor subtypes was identified (Birnbaumer *et al*., 1992; de Keyzer *et al*., 1994; Hirasawa *et al*., 1994; Lolait *et al*., 1992; Sugimoto *et al*., 1994; Thibonnier *et al*., 1994), we designed the stable expression of each human AVP receptor in HeLa cells (Yamamura *et al*., 1998) and attempted to develop functionally acting, human V_2-receptor agonists. After the lead optimization of congeners of OPC-31260 and OPC-41061, OPC-51803 was selected as the first nonpeptide AVP V_2-receptor agonist. In this study, we used cells stably expressing each human AVP receptor subtype (V_2, V_{1a}, and V_{1b}) and tried to characterize the binding and functional properties of OPC-51803, (5*R*)-2-[1-(2-chloro-4-(1-pyrrolidinyl) benzoyl) -2,3,4,5- tetrahydro-1*H*-1- benzazepin-5-yl] -*N*-isopropylacetamide (Figure 1) and compared it with dDAVP, a standard peptide AVP V_2-receptor agonist.

Methods

Materials

OPC-51803 and OPC-31260 were synthesized by the Tokushima Second Factory, Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). AVP was purchased from Peptide Institute Inc. (Osaka, Japan), [^{3}H]-AVP from NEN^{TM} Lifescience Products, Inc. (Boston, MA, U.S.A), dDAVP, [deamino-Pen^1, O-Me-Tyr^2, Arg^8]-vasopressin, penicillin, streptomycin, 3-isobutyl-1-methylxanthine (IBMX), ethylene glycol-bis (*β*-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), bovine serum albumin (BSA), $MnCl_2$, and trypsin inhibitor from Sigma (St. Louis, MO, U.S.A), lipofectamine, foetal bovine serum (FBS), and trypsin-EDTA from GIBCO BRL (Rockville, MD, U.S.A), Dulbecco's minimum Eagle's medium (DMEM) from Nissui Pharmaceutical Co. (Tokyo, Japan), phosphate-buffered salts from Takara Shuzo Co., Ltd. (Kusatsu, Japan), 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES), Fura-2 and Fura-2 acetoxymethyl ester (Fura-2-AM) from Dojindo Laboratories (Kumamoto, Japan), polyethylene glycol mono-*p*-isooctylphenyl ether (Triton X-100) from Nacalai Tesque (Kyoto, Japan), and geneticin disulphate (G418) and other chemicals from Wako Pure Chemicals (Osaka, Japan).

Preparation of HeLa cells expressing human AVP V_2-, V_{1a}-, and V_{1b}-receptors

Cloned cDNAs for AVP receptors were subcloned into pBluescript KS II(+) (Stratagene, La Jolla, CA, U.S.A). Nucleotide sequence analysis was performed using an ABI 373A DNA Sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A) and the obtained clones were confirmed to be identical to the previously reported human AVP-receptor cDNAs. These cDNA fragments were ligated in SRα promoter-based mammalian expression vectors psvk3 or pME18S and resulting constructs were used for transfection.

HeLa cells, human endocervical carcinoma cell line, grown in 5% CO_2 at 37°C, were plated at a density of 1×10^5 cells in 10-cm plate with 10 ml of DMEM supplemented containing 10% FBS and penicillin/streptomycin, and allowed to attach overnight. Each expression vector for vasopressin receptors and pSV2neo vector (Clontech, Palo Alto, CA, U.S.A.), which contained a neomycin resistance gene for selection of stable transformants, were co-transfected into HeLa cells by the lipofectamine method (Hawley-Nelson *et al*., 1993). After overnight transfection, cells were fed with fresh medium, allowed to grow for 2–3 days, adjusted to a density of 1×10^3 cells per plate, and incubated for an additional 24 h. Cells were continually selected for 3–4 weeks in a medium containing 400–800 μg ml^{-1} of the antibiotic G418. Single colonies were then isolated, expanded, and harvested for radioligand binding assays to measure the expression of receptors. Cells were maintained in the medium containing 200 μg ml^{-1} of G418. The medium was changed every 3 days, and the cells were subcultured after trypsinization.

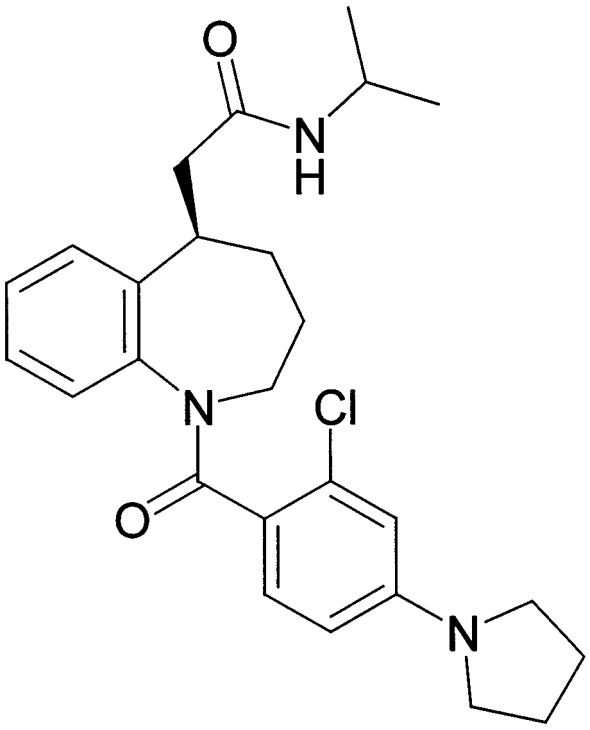

Figure 1 Structure of OPC-51803.

Radioligand binding assay to HeLa cells expressing human AVP receptors

The experiments were performed after each cell reached confluence in 12-well (V_{1a} and V_{1b}) or 24-well (V_2) dishes. After washing three times with ice-cold phosphate-buffered saline (PBS), the cells were incubated with [^{3}H]-AVP with or without unlabelled AVP (final concentration of 1 μM) to determine the total binding or the nonspecific binding, respectively in DMEM containing 0.3% BSA brought to a pH of 7.4 with 10 mM HEPES-NaOH. For the competition experiments, OPC-51803 dissolved in dimethylsulphoxide (DMSO), AVP and dDAVP dissolved in saline were diluted with DMEM medium and added to each well at several appropriate concentrations. After incubation at 4°C for 2 h, the reaction was stopped by removing the buffer, and the cells were rinsed three times with ice-cold PBS. The cells were lysed with 500 μl (12-well) or 250 μl (24-well) of 0.1 N NaOH containing 0.1% sodium dodecyl sulphate (SDS), transferred into scintillation vials, and mixed with 5 ml of Aquazol II (Packard, Tokyo, Japan). Radioactivity was detected using a liquid scintillation counter (LSC-1050, Aloka, Tokyo, Japan). The cells in some wells were lysed in 0.1 N NaOH and protein contents were determined by the dye method (Bradford, 1976).

Cyclic AMP production in HeLa cells expressing V_2-receptors

After reaching confluence in 24-well dishes, the cells were washed twice with ice-cold PBS and incubated with the DMEM containing 0.3% BSA and 1 mM IBMX brought to a pH of 7.4 with 10 mM HEPES-NaOH. After the addition of certain concentrations of OPC-51803, AVP, or dDAVP, the incubation was carried out at 37°C for 10 min. For the inhibition experiments, OPC-51803 was added following OPC-31260, a nonpeptide V_2 antagonist, at 10^{-7} M and 10^{-6} M. The reaction was stopped by aspiration of the medium and the cells were rinsed twice with ice-cold PBS. Cyclic AMP was extracted from the cells by adding 250 μl of 0.1 N HCl and stored at −20°C until cyclic AMP determination using a radioimmunoassay kit (Yamasa, Tokyo, Japan).

Intracellular Ca^{2+} concentration in HeLa cells expressing V_{1a}- or V_{1b}- receptors

The harvested cells were suspended in Ca^{2+}-loading buffer (in mM: NaCl 140, KCl 4, $MgCl_2$ 1, $CaCl_2$ 1.25, Na_2HPO_4 1, glucose 11, BSA 0.2%, and HEPES 5, brought to a pH of 7.4 with NaOH) and incubated at 37°C for 30 min with Fura-2-AM at 5 μM under protection from light. After the incubation, the cells were washed twice and resuspended with Ca^{2+}-loading buffer.

The fluorescence was monitored with fluorescence spectrophotometer (F-2000, Hitachi, Tokyo, Japan) by excitation at 340 and 380 nm and emission at 510 nm. Maximum and minimum fluorescence levels were measured using 0.1% Triton X-100 and 3 mM EGTA, respectively. Autofluorescence of the cells was measured by applying 5 mM $MnCl_2$. Actual $[Ca^{2+}]_i$ was calculated using the equation of Grynkiewicz *et al*. (1985) with a K_D for Fura-2 of 224 nM. OPC-51803 dissolved in DMSO and AVP and dDAVP dissolved in PBS were applied cumulatively, and concentration-dependent curves were obtained. For the inhibition experiments, [deamino-Pen^1, O-Me-Tyr^2, Arg^8]-vasopressin (a peptide V_1-antagonist) at 10^{-7} M and 10^{-6} M dissolved in PBS, were pretreated with the cells before adding test compounds. The test compounds were confirmed not to interfere with the Fura-2-Ca^{2+} fluorescence at used concentrations.

Data analysis

All data are expressed as the mean ± s.e.mean. In saturation binding experiments, the dissociation constant (K_D) and the number of binding sites (B_{max}) were determined by Scatchard analysis (Scatchard, 1949). The IC_{50} of OPC-51803, AVP, and dDAVP were determined by displacement curves of [^{3}H]-AVP binding. The inhibition constant (K_i) was calculated from the equation of $K_i = IC_{50}/(1 + [L]/K_D)$, where IC_{50} is the concentration exerting 50% inhibition and [L] is the concentration of a radiolabelled ligand (Cheng & Prusoff, 1973). Regarding the activity of each compound on cyclic AMP production and $[Ca^{2+}]_i$, EC_{50} was determined as that concentration which produces 50% of the maximal response.

Results

Binding affinities of OPC-51803, AVP, and dDAVP for human AVP receptors

HeLa cells expressing human AVP receptors (V_2-, V_{1a}-, or V_{1b}-subtype) showed constant K_D and B_{max} throughout repeated cultures (V_2: passage 9–18, V_{1a}: passage 8–14, and V_{1b}: passage 10–20). These K_D and B_{max} values are listed in Table 1. The stimulation by AVP increased cyclic AMP production in cells expressing V_2-receptors and $[Ca^{2+}]_i$ in cells expressing V_{1a}- and V_{1b}-receptors, indicating that these transfected HeLa cells acted functionally throughout the experiments.

Unlabelled AVP displaced [^{3}H]-AVP binding for all three receptor subtypes with similar K_i values (Figure 2). These K_i values are also comparable to K_D values of [^{3}H]-AVP (Table 2). OPC-51803 displaced [^{3}H]-AVP binding to human V_2- and V_{1a}-receptors in a concentration-dependent manner, although it scarcely displaced [^{3}H]-AVP binding to human V_{1b}-receptors even at 10^{-4} M (Figure 2). The K_i value of OPC-51803 for V_2-receptors was 91.9 ± 10.8 nM ($n = 6$), and that for V_{1a}-receptors was 819 ± 39 M ($n = 6$), which showed about nine times more selectivity for V_2-receptors than V_{1a}-receptors. While dDAVP is well-known as a selective V_2-agonist, it displaced [^{3}H]-AVP binding not only to V_2-receptors but also to V_{1a}- and V_{1b}-receptors. These results are consistent with previous observations (Chini *et al*., 1995; Pettibone *et al*., 1992; Saito *et al*., 1997). Receptor selectivity of dDAVP was about 13 times less potent for V_{1a}-receptors and four times less potent for V_{1b}-receptors than for V_2-receptors.

Table 1 Dissociation constants (K_D) for AVP and numbers of AVP receptors (B_{max}) in HeLa cells expressing human AVP V_2-, V_{1a}-, and V_{1b}-receptors

	HeLa cells transfected each human AVP receptor gene		
	V_2-receptors	*V_{1a}-receptors*	*V_{1b}-receptors*
K_D (nM)	3.22 ± 0.11	2.20 ± 0.09	2.43 ± 0.25
B_{max}(fmol mg^{-1} protein)	3354 ± 564	446 ± 63	98 ± 9

Values are expressed as the mean ± s.e.mean of six or seven experiments performed in duplicate.

Cyclic AMP production in HeLa cells expressing V_2-receptors

To confirm the functional properties of OPC-51803, cyclic AMP production was examined using intact cells expressing V_2-receptors. OPC-51803 concentration-dependently increased cyclic AMP production in HeLa cells expressing human V_2-receptors at OPC-51803 concentrations of 10^{-9}–10^{-5} M (Figure 3a). At 10^{-5} M, cyclic AMP production of 6.70 ± 0.45 pmol well^{-1} was seen and the production was 35 times higher than the basal value (0.19 ± 0.01 pmol well^{-1}). The EC_{50} value of OPC-51803 on cyclic AMP production

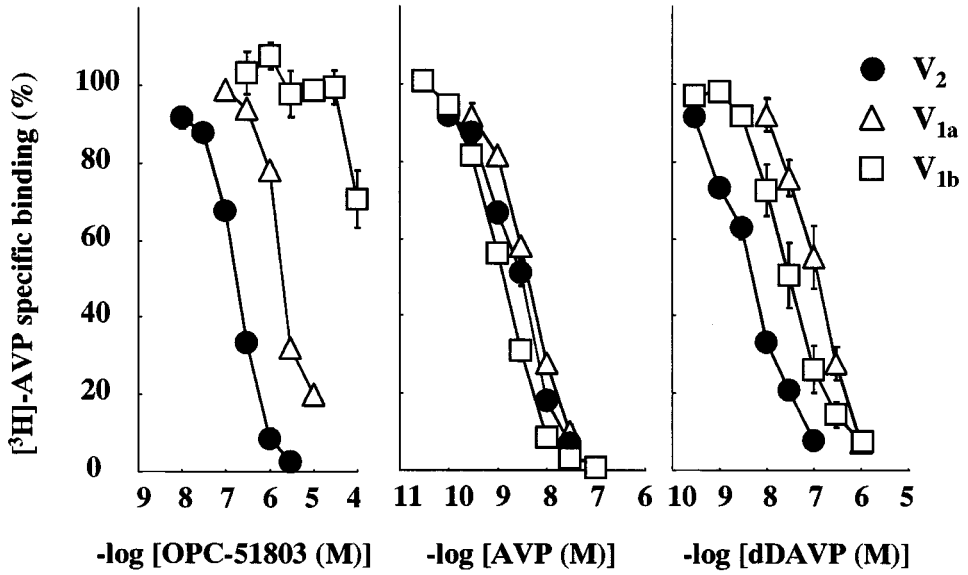

Figure 2 Displacements of OPC-51803, AVP, and dDAVP on [^{3}H]-AVP binding to human V_2-, V_{1a}-, and V_{1b}-receptors in HeLa cells. Values are expressed as the mean ± s.e.mean of 4–6 experiments performed in duplicate.

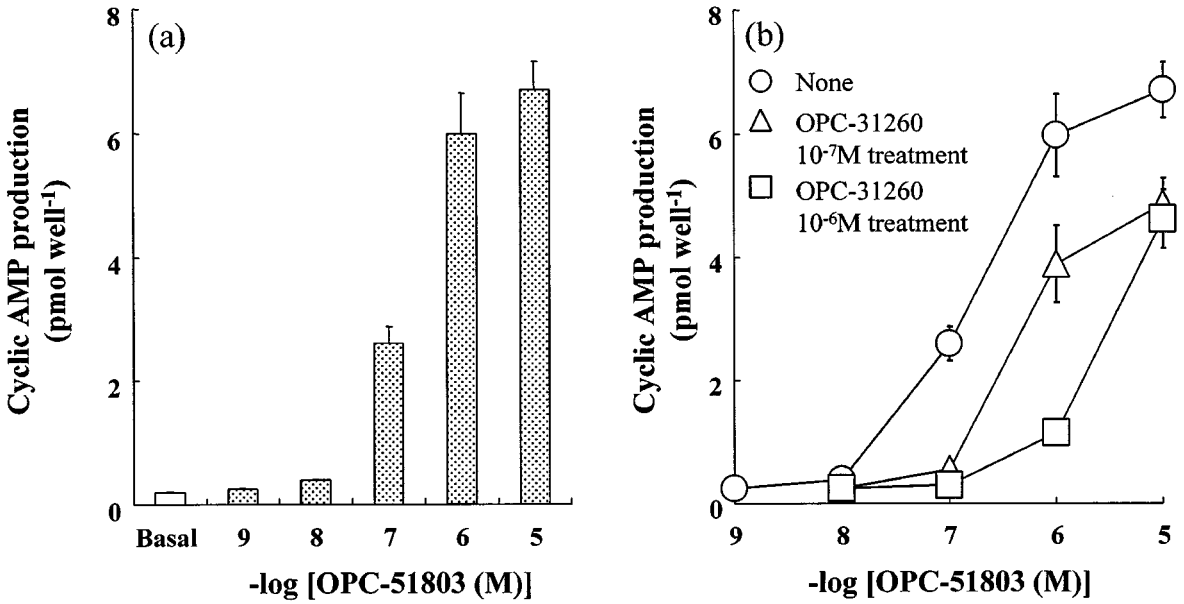

Figure 3 (a) OPC-51803-induced increases in cyclic AMP production and (b) the inhibitory effect of OPC-31260 in HeLa cells expressing human V_2-receptors. Values are expressed as the mean ± s.e.mean of five experiments performed in triplicate.

Table 2 Inhibition constant (K_i) and effective concentration (EC_{50}) of OPC-51803, AVP, and dDAVP in human HeLa cells expressing V_2-, V_{1a}-, and V_{1b}-receptors

	V_2-receptors		*V_{1a}-receptors*		*V_{1b}-receptors*	
	K_i (nM)	*EC_{50}* (nM)	K_i (nM)	*EC_{50}* (nM)	K_i (nM)	*EC_{50}* (nM)
OPC-51803	91.9 ± 10.8	189 ± 14	819 ± 39	NE	NE	ND
AVP	1.44 ± 0.23	0.56 ± 0.10	1.51 ± 0.11	0.86 ± 0.17	0.46 ± 0.08	3.00 ± 0.50
dDAVP	3.12 ± 0.38	4.77 ± 1.43	41.5 ± 9.9	28.9 ± 2.6	13.7 ± 3.2	25.4 ± 2.5

Values are expressed as the mean ± s.e.mean of 4–6 experiments. NE; no effect, ND; not determined.

was 189±14 nM (n=5, Table 2). OPC-31260, a selective V_2-receptor antagonist, did not influence basal cyclic AMP production (data not shown). In the presence of OPC-31260 at 10^{-7} M and 10^{-6} M, the concentration-response curve of OPC-51803 was shifted to the right (Figure 3b), which suggests that the OPC-51803-induced increases in cyclic AMP are mediated through activation of V_2-receptors. AVP and dDAVP also enhanced cyclic AMP production in HeLa cells expressing human V_2-receptors with EC_{50} values of 0.56±0.10 nM and 4.77±1.43 nM (n=5 each, Table 2) and the maximal increases in cyclic AMP were higher than that by OPC-51803 (Figure 4).

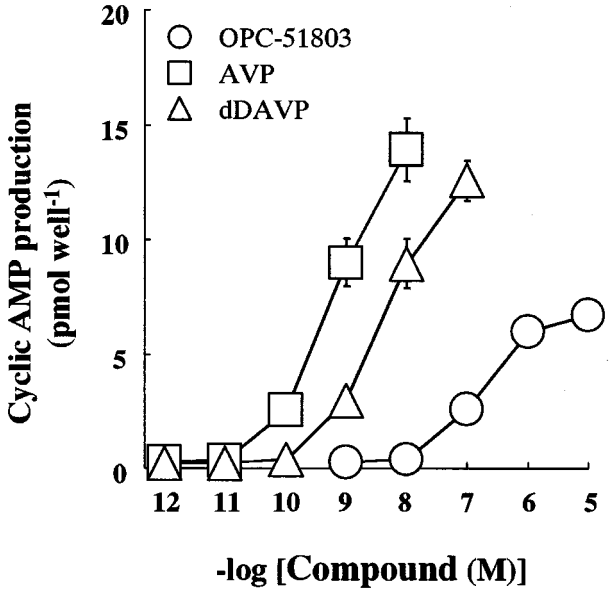

Figure 4 Comparison of OPC-51803 with AVP and dDAVP on cyclic AMP production in HeLa cells expressing human V_2-receptors. Values are expressed as the mean±s.e.mean of five experiments performed in triplicate.

Intracellular Ca^{2+} concentration in human HeLa cells expressing V_{1a}- and V_{1b}-receptors

We further examined agonistic action on human V_{1a}- and V_{1b}-receptors by measuring the change in $[Ca^{2+}]_i$, using the Fura-2 fluorescence method (Figure 5 and Table 2). AVP at concentrations of 10^{-10}–10^{-7} M increased $[Ca^{2+}]_i$ from 104±9 to 530±15 nM in HeLa cells expressing V_{1a}-receptors (n=5). dDAVP at 10^{-9}–10^{-6} M also raised $[Ca^{2+}]_i$ from 115±12 to 403±45 nM in these cells (n=5). Concentration-response curves of AVP and dDAVP for $[Ca^{2+}]_i$ were shifted to the right in the presence of a peptide V_{1a}-antagonist at 10^{-7} and 10^{-6} M (data not shown). On the other hand, OPC-51803 at 10^{-6} and 10^{-5} M did not increase $[Ca^{2+}]_i$ in V_{1a}-receptor-expressing HeLa cells (Figure 5). Rather, the pretreatment of OPC-51803 at 10^{-5} M inhibited the AVP-induced increase in $[Ca^{2+}]_i$ apparently.

In HeLa cells expressing human V_{1b}-receptors, AVP (n=6) and dDAVP (n=6) at concentrations of 10^{-9}–10^{-6} M increased $[Ca^{2+}]_i$ from 126±9 to 355±17 nM and from 123±8 to 282±18 nM, respectively. As OPC-51803 showed little affinity for V_{1b}-receptors and directly suppressed the Fura-2-Ca^{2+} fluorescence at 10^{-4} M, we did not evaluate the effect of OPC-51803 on intracellular Ca^{2+} concentration in HeLa cells expressing human V_{1b}-receptors.

Discussion

In the present study, we evaluated the affinity and agonistic activity of OPC-51803, a first nonpeptide AVP V_2 agonist, for human V_2-, V_{1a}-, and V_{1b}-receptors expressed in HeLa cells. OPC-51803 bound to human V_2- and V_{1a}-receptors. It increased cyclic AMP production through V_2-receptors

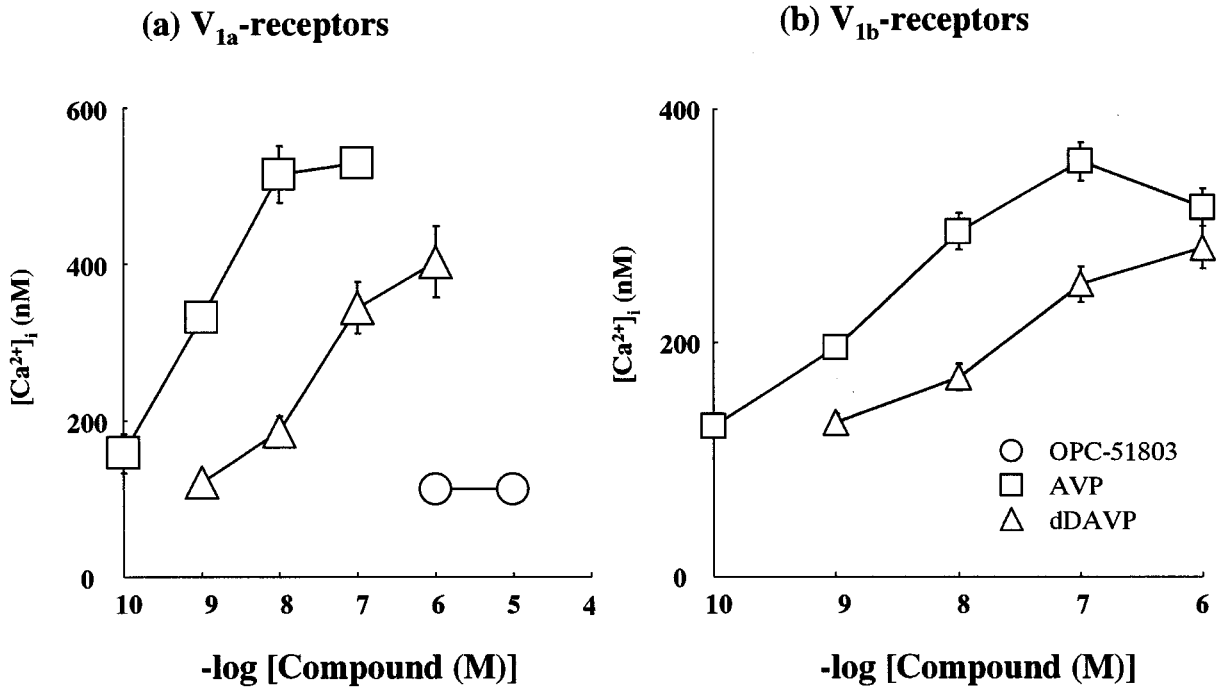

Figure 5 Effect of OPC-51803, AVP, and dDAVP on $[Ca^{2+}]_i$ in HeLa cells expressing human (a) V_{1a}- and (b) V_{1b}-receptors. Values are expressed as the mean±s.e.mean of five or six experiments. The experiment of OPC-51803 on agonistic activity in HeLa cells expressing V_{1b}-receptors was not performed.

though, it did not increase $[Ca^{2+}]_i$ through V_{1a}-receptors. On the other hand, it inhibited the AVP-induced increase in $[Ca^{2+}]_i$ at higher concentrations. These results suggest that OPC-51803 may act rather as an antagonist for V_{1a}-receptors than as an agonist. As to V_{1b}-receptors, OPC-51803 inhibited only 30% of [^{3}H]-AVP binding even at 10^{-4} M, indicating that it has little affinity for V_{1b}-receptors. Therefore, we did not further investigate its pharmacological properties for V_{1b}-receptors, as it seems not to play a physiological role through V_{1b}-receptors. These results indicate that OPC-51803 is a selective V_2-receptor agonist without agonistic potencies for V_{1a}- and V_{1b}-receptors.

The cyclic AMP production by OPC-51803 was increased concentration-dependently in HeLa cells expressing V_2-receptors. The EC_{50} value of the cyclic AMP production is comparable to the K_i value of the binding study as well as those of AVP and dDAVP. However, the maximal increase in cyclic AMP by OPC-51803 did not reach the maximal response seen with AVP or dDAVP. To confirm this partial agonism, we examined the inhibitory effect of OPC-51803 on the cyclic AMP production by AVP. OPC-51803 at concentrations of 10^{-9}–10^{-5} M slightly but concentration-dependently suppressed cyclic AMP production induced by a submaximal dose (10^{-9} M) of AVP (data not shown). These results may suggest that OPC-51803 acts as a partial agonist to V_2-receptors.

Among other nonpeptide agonists, it was reported that a nonpeptide angiotensin II agonist, L-162,313, showed a partial activity in *in vitro* experiments, but in *in vivo* experiments, it increased blood pressure to the same degree as angiotensin II (Kivlighn *et al*., 1995). In preliminary experiments, we employed AVP-deficient Brattleboro rats in which diabetes insipidus is the predominant characteristic. Orally administered OPC-51803 decreased urine volume and increased urine osmolality in six animals (Hirano *et al*., 1998). It is likely that the mechanism for this action is similar to that of dDAVP.

dDAVP is well-known as a strong and selective V_2-receptor agonist and has long been utilized as a standard reagent for studies involving V_2-receptors. However, since molecular cloning of human AVP receptors was achieved and the functional analysis system using their clones expressing in cells was utilized, species differences and more precise receptor selectivity of dDAVP were elucidated (Pettibone *et al*., 1992; Saito *et al*., 1997). dDAVP inhibited [^{3}H]-AVP binding not only for human V_2-receptors but also for V_{1a}- and V_{1b}-receptors, and with similar affinities. In our present study, similar results were obtained whereby the affinities of dDAVP for human AVP receptors was 13 times less potent for V_{1a}-receptors and four times less potent for V_{1b}-receptors compared to V_2-receptors. More interestingly, dDAVP stimulated intracellular signalling through V_{1a}- and V_{1b}-receptors as well as through V_2-receptors. It was also recently reported that dDAVP has an agonistic activity for human V_{1b}-receptors (Saito *et al*., 1997); dDAVP dose-dependently stimulated inositol turnover in COS-1 cells expressing human V_{1b}-receptors. Our results in this study are consistent with the finding that dDAVP increased intracellular Ca^{2+} concentration in HeLa cells expressing human V_{1b}-receptor with the similar EC_{50} value of AVP.

As to V_{1a}-receptors, there are some differences between their and our results. We observed a concentration-dependent increase in $[Ca^{2+}]_i$ with an EC_{50} of 28.9 ± 2.6 nM, while they have seen a slight increase in inositol phosphates. dDAVP-induced increase in $[Ca^{2+}]_i$ in HeLa cells expressing human V_{1a}-receptors was inhibited by the pretreatment of peptide V_{1a}-antagonist (data not shown). The reason for this difference is not clear and there are few reports that dDAVP shows V_{1a}-agonistic action for human V_{1a}-receptors. In rats, dDAVP shows vasoconstrictor activity *via* V_{1a}-receptors, although it is less potent than antidiuretic activity (Sawyer *et al*., 1974a,b). It is also reported that MAP kinases were activated by dDAVP in rat 3Y1 fibroblasts through V_{1a}-receptors (Nishioka *et al*., 1995). These results suggest that dDAVP may have some agonistic properties for V_{1a}-receptors.

Considering the clinical implications, dDAVP (Desmopressin^{TM}) is currently used for the treatment of patients with central diabetes insipidus and primary nocturnal enuresis, which are AVP-deficient states. From recent studies and our results, dDAVP is not a highly selective V_2-agonist; it stimulates strongly V_{1b}-receptors and possibly V_{1a}-receptors. In contrast, OPC-51803 does not stimulate either V_{1a}-receptors or V_{1b}-receptors and shows highly selective V_2-receptors agonism. Although the physiological significance of these actions of dDAVP through V_{1a}- and V_{1b}-receptors is unclear, the specificity of OPC-51803 for V_2-receptors may be desirable for the treatment of conditions involving water metabolism disorders. On the other hand, although dDAVP has antidiuretic activity when administered by the oral route, its bioavailability is less than 1% in humans and the large individual variability in the amounts or times of treatments was seen because of poor bioavailability. OPC-51803 is absorbed well and shows oral effectiveness *in vivo*. Therefore, it might offer new therapeutic advantages in the treatment of water metabolism disorders.

In conclusion, we found a novel nonpeptide human AVP V_2-agonist, OPC-51803. This drug is expected to be an oral effective and practical one for patients with low circulating levels of AVP.

The authors express sincere thanks to Dr Y. Yabuuchi for encouraging the project, to Drs A. Ozaki and A. Schwartz for valuable comments and continuing encouragement, to Mr P. Randolph for his excellent assistance in the preparation of this manuscript, and to Ms M. Murakami for her excellent technical assistance.

References

ALBRIGHT, J.D., REICH, M.F., DELOS SANTOS, E.G., DUSZA, J.P., SUM, F.W., VENKATESAN, A.M., COUPET, J., CHAN, P.S., RU, X., MAZANDARANI, H. & BAILEY, T. (1998). 5-Fluoro-2-methyl-N-[4-(5H-pyrrolo[2,1-c]-[1,4]benzodiazepin-10(11H)- ylcarbonyl)-3-chlorophenyl]benzamide (VPA-985): an orally active arginine vasopressin antagonist with selectivity for V_2 receptors. *J. Med. Chem.,* **41,** 2442–2444.

AQUINO, C.J., ARMOUR, D.R., BERMAN, J.M., BIRKEMO, L.S., CARR, R.A., CROOM, D.K., DEZUBE, M., DOUGHERTY, JR R.W., ERVIN, G.N., GRIZZLE, M.K., HEAD, J.E., HIRST, G.C., JAMES, M.K., JOHNSON, M.F., MILLER, L.J., QUEEN, K.L., RIMELE, T.J., SMITH, D.N. & SUGG, E.E. (1996). Discovery of 1,5-benzodiazepines with peripheral cholecystokinin (CCK-A) receptor agonist activity. 1. Optimization of the agonist 'trigger'. *J. Med. Chem.,* **39,** 562–569.

BIRNBAUMER, M., SEIBOLD, A., GILBERT, S., ISHIDO, M., BARBERIS, C., ANTARAMIAN, A., BRABET, P. & ROSENTHAL, W. (1992). Molecular cloning of the receptor for human antidiuretic hormone. *Nature,* **357,** 333–335.

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.

CHENG, Y. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_1) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.,* **22,** 3099–3108.

CHINI, B., MOUILLAC, B., ALA, Y., BALESTRE, M.N., TRUMPP-KALLMEYER, S., HOFLACK, J., ELANDS, J., HIBERT, M., MANNING, M., JARD, S. & BARBERIS, C. (1995). Tyr115 is the key residue for determining agonist selectivity in the V_{1a} vasopressin receptor. *EMBO J.,* **14,** 2176 – 2182.

DE KEYZER, Y., AUZAN, C., LENNE, F., BELDJORD, C., THIBONNIER, M., BERTAGNA, X. & CLAUSER, E. (1994). Cloning and characterization of the human V_3 pituitary vasopressin receptor. *FEBS Lett.,* **356,** 215 – 220.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J. Biol. Chem.,* **260,** 3440 – 3450.

HAWLEY-NELSON, P., CICCARONE, V., GEBEYEHU, G., JESSEE, J. & FELGNER, P.L. (1993). Lipofectamine^{TM} reagent: A New, higher efficiency polycationic liposome transfection reagent. *FOCUS,* **15,** 73 – 79.

HIRANO, T., NAKAMURA, S., SUMIDA, T. & MORI, T. (1998). Antidiuretic effects of OPC-51803, a nonpeptide vasopressin V_2-receptor agonist, administered orally to Brattleboro rats and normal rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **358,** (Suppl. 2): R746. (Abstract).

HIRASAWA, A., SHIBATA, K., KOTOSAI, K. & TSUJIMOTO, G. (1994). Cloning, functional expression and tissue distribution of human cDNA for the vascular-type vasopressin receptor. *Biochem. Biophys. Res. Commun.,* **203,** 72 – 79.

KIVLIGHN, S.D., HUCKLE, W.R., ZINGARO, G.J., RIVERO, R.A., LOTTI, V.J., CHANG, R.S., SCHORN, T.W., KEVIN, N., JOHNSON, JR., R.G., GREENLEE, W.J. & SIEGL, P.K.S. (1995). Discovery of L-162,313: a nonpeptide that mimics the biological actions of angiotensin II. *Am. J. Physiol.,* **268,** R820 – R823.

KONDO, K., OGAWA, H., YAMASHITA, H., MIYAMOTO, H., TANAKA, M., NAKAYA, K., KITANO, K., YAMAMURA, Y., NAKAMURA, S., ONOGAWA, T., MORI, T. & TOMINAGA, M. (1999). 7-Chloro -5- hydroxy -1- [2 - methyl -4- (2-methylbenzoylamino)benzoyl]- 2,3 , 4, 5-tetrahydro-1H-1 - benzazepine (OPC-41061): a potent, orally active nonpeptide arginine vasopressin V_2 receptor antagonist. *Bioorg. Med. Chem.,* **7,** 1743 – 1754.

LASZLO, F.A., LASZLO, JR., F. & DE WIED, D. (1991). Pharmacology and clinical perspectives of vasopressin antagonists. *Pharmacol. Rev.,* **43,** 73 – 108.

LIU, J. & WESS, J. (1996). Different single receptor domains determine the distinct G protein coupling profiles of members of the vasopressin receptor family. *J. Biol. Chem.,* **271,** 8772 – 8778.

LOLAIT, S.J., O'CARROLL, A.M., MCBRIDE, 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.

NISHIOKA, N., HIRAI, S., MIZUNO, K., OSADA, S., SUZUKI, A., KOSAKA, K. & OHNO, S. (1995). Wortmannin inhibits the activation of MAP kinase following vasopressin V_1 receptor stimulation. *FEBS Lett.,* **377,** 393 – 398.

OGAWA, H., YAMAMURA, Y., MIYAMOTO, H., KONDO, K., YAMASHITA, H., NAKAYA, K., CHIHARA, T., MORI, T., TOMINAGA, M. & YABUUCHI, Y. (1993). Orally active, nonpeptide vasopressin V_1 antagonists. A novel series of 1-(1-substituted 4-piperidyl)-3,4-dihdyro-2(1H)-quinolinone. *J. Med. Chem.,* **36,** 2011 – 2017.

OGAWA, H., YAMASHITA, H., KONDO, K., YAMAMURA, Y., MIYAMOTO, H., KAN, K., KITANO, K., TANAKA, M., NAKAYA, K., NAKAMURA, S., MORI, T., TOMINAGA, M. & YABUUCHI, Y. (1996). Orally active, nonpeptide vasopressin V_2 receptor antagonists: a novel series of 1-[4-(benzoylamino)benzoyl]-2,3,4,5-tetrahydro-1H-benzazepines and related compounds. *J. Med. Chem.,* **39,** 3547 – 3555.

OHNISHI, A., ORITA, Y., OKAHARA, R., FUJIHARA, H., INOUE, T., YAMAMURA, Y., YABUUCHI, Y. & TANAKA, T. (1993). Potent aquaretic agent. A novel nonpeptide selective vasopressin 2 antagonist (OPC-31260) in men. *J. Clin. Invest.,* **92,** 2653 – 2659.

OHNISHI, A., ORITA, Y., TAKAGI, N., FUJITA, T., TOYOKI, T., IHARA, Y., YAMAMURA, Y., INOUE, T. & TANAKA, T. (1995). Aquaretic effect of a potent, orally active, nonpeptide V_2 antagonist in men [published erratum appears in J. Pharmacol. Exp. Ther. 1995 Sep;274(3):1573]. *J. Pharmacol. Exp. Ther.,* **272,** 546 – 551.

PERLMAN, S., SCHAMBYE, H.T., RIVERO, R.A., GREENLEE, W.J., HJORTH, S.A. & SCHWARTZ, T.W. (1995). Non-peptide angiotensin agonist. Functional and molecular interaction with the AT_1 receptor. *J. Biol. Chem.,* **270,** 1493 – 1496.

PETTIBONE, D.J., KISHEL, M.T., WOYDEN, C.J., CLINESCHMIDT, B.V., BOCK, M.G., FREIDINGER, R.M., VEBER, D.F. & WILLIAMS, P.D. (1992). Radioligand binding studies reveal marked species differences in the vasopressin V_1 receptor of rat, rhesus and human tissues. *Life Sci.,* **50,** 1953 – 1958.

SAITO, M., TAHARA, A. & SUGIMOTO, T. (1997). 1-desamino-8-D-arginine vasopressin (DDAVP) as an agonist on V_{1b} vasopressin receptor. *Biochem. Pharmacol.,* **53,** 1711 – 1717.

SAWYER, W.H., ACOSTA, M., BALASPIRI, L., JUDD, J. & MANNING, M. (1974a). Structural changes in the arginine vasopressin molecule that enhance antidiuretic activity and specificity. *Endocrinology,* **94,** 1106 – 1115.

SAWYER, W.H., ACOSTA, M. & MANNING, M. (1974b). Structural changes in the arginine vasopressin molecule that prolong its antidiuretic action. *Endocrinology,* **95,** 140 – 149.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. NY. Acad. Sci.,* **51,** 660 – 672.

SERRADEIL-LE GAL, C., LACOUR, C., VALETTE, G., GARCIA, G., FOULON, L., GALINDO, G., BANKIR, L., POUZET, B., GUILLON, G., BARBERIS, C., CHICOT, D., JARD, S., VILAIN, P., GARCIA, C., MARTY, E., RAUFASTE, 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., GARCIA, C., LACOUR, C., GUIRAUDOU, P., CHRISTOPHE, B., VILLANOVA, G., NISATO, 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.

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.

TAHARA, A., TOMURA, Y., WADA, K.I., KUSAYAMA, T., TSUKADA, J., TAKANASHI, M., YATSU, T., UCHIDA, W. & TANAKA, A. (1997). Pharmacological profile of YM087, a novel potent nonpeptide vasopressin V_{1A} and V_2 receptor antagonist, in vitro and in vivo. *J. Pharmacol. Exp. Ther.,* **282,** 301 – 308.

THIBONNIER, M., AUZAN, C., MADHUN, Z., WILKINS, P., BERTI-MATTERA, L. & CLAUSER, E. (1994). Molecular cloning, sequencing, and functional expression of a cDNA encoding the human V_{1a} vasopressin receptor. *J. Biol. Chem.,* **269,** 3304 – 3310.

WILLSON, T.M., HENKE, B.R., MOMTAHEN, T.M., MYERS, P.L., SUGG, E.E., UNWALLA, R.J., CROOM, D.K., DOUGHERTY, R.W., GRIZZLE, M.K., JOHNSON, M.F., QUEEN, K.L., RIMELE, T.J., YINGLING, J.D. & JAMES, M.K. (1996). 3-[2-(N-phenylacetamide)]-1,5-benzodiazepines: orally active, binding selective CCK-A agonists. *J. Med. Chem.,* **39,** 3030 – 3034.

YAMAMURA, Y., NAKAMURA, S., ITOH, S., HIRANO, T., ONOGAWA, T., YAMASHITA, T., YAMADA, Y., TSUJIMAE, K., AOYAMA, M., KOTOSAI, K., OGAWA, H., YAMASHITA, H., KONDO, K., TOMINAGA, M., TSUJIMOTO, G. & MORI, T. (1998). OPC-41061, a highly potent human vasopressin V_2-receptor antagonist: pharmacological profile and aquaretic effect by single and multiple oral dosing in rats. *J. Pharmacol. Exp. Ther.,* **287,** 860 – 867.

YAMAMURA, Y., OGAWA, H., CHIHARA, T., KONDO, K., ONOGAWA, T., NAKAMURA, S., MORI, T., TOMINAGA, M. & YABUUCHI, Y. (1991). OPC-21268, an orally effective, nonpeptide vasopressin V_1 receptor antagonist. *Science,* **252,** 572 – 574.

YAMAMURA, Y., OGAWA, H., YAMASHITA, H., CHIHARA, T., MIYAMOTO, H., NAKAMURA, S., ONOGAWA, T., YAMASHITA, T., HOSOKAWA, T., MORI, T., TOMINAGA, M. & YABUUCHI, Y. (1992). Characterization of a novel aquaretic agent, OPC-31260, as an orally effective, nonpeptide vasopressin V_2 receptor antagonist. *Br. J. Pharmacol.,* **105,** 787 – 791.

(Received October 4, 1999
Accepted January 10, 2000)