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Surprising Pharmacological Activity of Analogues Designed by Substitution of Position 3 in Arginine Vasopressin (AVP) and 8-D-Arginine Vasopressin with L-2-Naphthylalanine

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Abstract

In an attempt to develop more active and selective analogues of arginine vasopressin (AVP), two peptides have been designed, synthesized and tested for vasopressor (V_1-receptors) and antidiuretic (V_2-receptors) activities. We also estimated the uterotonic and anti-uterotonic activities of these compounds in-vitro.

The first peptide, [(L-2-Nal)^3] AVP is a highly active V_2-agonist. The second analogue, [(L-2-Nal)^3, (D-Arg)^8]VP is among the most potent antagonists of the vasopressor response to AVP. Moreover, it is the first V_1-antagonist devoid of anti-uterotonic activity.

High antipressor potency of the second peptide was achieved without modification of position 1.

Agonists and antagonists of arginine vasopressin (AVP) have been widely used in biochemical, pharmacological, and physiological studies aimed at clarifying the importance of endogenous vasopressin in a variety of physiological and pathophysiological conditions. Moreover, antagonists of the antidiuretic responses to AVP (antagonists of V_2 receptors) have potential as therapeutic agents for the treatment of hyponatraemia secondary to the syndrome of inappropriate secretion of antidiuretic hormone. It is also worth pointing out that one of the agonists of V_2 receptors, namely 1-desamino-8-D-arginine vasopressin (dDAVP) is now the drug of choice for the treatment of diabetes insipidus. The importance of AVP analogues has prompted many scientists to synthesize thousands of new peptides to find a relationship between the structure and function, aimed at the development of analogues with selective enhancement of one of the several activities they possess, or with the prolongation of a chosen activity, or analogues as specific inhibitors of a chosen biological effect. However, despite this effort, the design of peptides that are truly selective for V_1 (pressor effect), V_2 and other AVP receptors still remains a real challenge. To date, many fairly selective V_2 agonists have been synthesized, but all of them showed either weak pressor agonistic activity or were inhibitors of the pressor effect (Manning et al 1987; Thibonnier 1990). With respect to the V_1 receptors the situation is worse, no equally selective agonist is yet available (Manning et al 1987; Thibonnier 1990). As regards antagonists of AVP, it is evident that none of the available peptides is truly specific for V_1 or V_2 receptors. Thus, although the V_1 antagonists are highly specific for V_1 receptors and do not block V_2 receptors in-vitro, they can nonetheless also block uterine-type oxytocin receptors (Manning et al 1987). All the V_2 antagonists block V_1 and uterine-type oxytocin receptors with varying degrees of effectiveness (Manning et al 1987). It is believed that an L-phenylalanine residue in position 3 of AVP is mainly involved in recognition of the hormone and its binding to the receptors (Hlavacek 1987). So far, most of the analogues modified in this position were modified by the introduction of aromatic amino acid residues (Manning et al 1987). As a continuation of our effort in attempting to recognize the role of Phe in AVP or its analogues better, we designed and synthesized two new peptides modified with L-2-naphthylalanine; [(L-2-naphthylalanine)^3]AVP, [(L-2-Nal)^3]AVP (I) and [(L-2-naphthylalanine)^3, (D-arginine)^8]AVP, [(L-2-Nal)^3, (D-Arg)^8]AVP (II). First we decided to substitute only one position in the AVP molecule in order to learn how single substitution of position 3 with L-^{2}-Nal would influence the pharmacological properties of the resulting analogue I. In the next peptide we linked the above modification with the inversion of configuration of arginine in position 8. The synthesized analogues have the following general structure:

Cys-Tyr-(L-2-Nal)-Gln-Asn-Cys-Pro-X-Gly-NH_2

X = Arg, I
X = D-Arg, II

Materials and Methods

Peptide synthesis

The protected peptide precursors, Z-Cys(Bzl)-Tyr(Bzl)-(L-2-Nal)-Gln-Asn-Cys(Bzl)-Pro-Arg(Tos)-Gly-NH_2 (Ia) and Z-Cys(Bzl)-Tyr(Bzl)-(L-2-Nal)-Gln-Asn-Cys(Bzl)-Pro-D-Arg(Tos)-Gly-NH_2 (IIa) required for the synthesis of both peptides (I and II) were prepared by the solid-phase method of peptide synthesis entirely on resin (Manning 1968; Lammek et al 1988). Each protected peptide was

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synthesized by stepwise coupling of BOC-amino acids to the growing peptide chain on a chloromethylated Merrifield resin. *N*-Benzyloxycarbonyl-*S*-benzyl-L-cysteine was used in the final coupling steps. After completion of the synthesis, the protected nonapeptidyl resins were ammonolysed in methanol (Manning 1968). Following evaporation of the solvent, the products were extracted into hot dimethylformamide (DMF), precipitated with boiling water and left overnight at room temperature (20°C). The peptides were collected by filtration, washed with water and dried in-vacuo over P_2O_5. The products were further purified by dissolving in DMF and reprecipitating with $MeOH/Et_2O$ (1:3). Precursors were deblocked with sodium in liquid ammonia (Manning et al 1970) and the resulting disulphydryl compounds were oxidatively cyclized with I_2 in methanol (0·1 M) to give analogues I and II (Lammek et al 1989). The resulting materials were desalted on Sephadex G-15 (50% acetic acid) and purified on Sephadex LH-20 (30% acetic acid). Finally, both analogues were purified by HPLC. The peptides were chromatographed on an HPLC Ultrasphere ODS column (10 × 250 mm) with a linear gradient of from 10 to 60% of (2), [(1) 0·1% trifluoroacetic acid/TFA/, (2) acetonitrile-0·1% TFA (80:20 v/v)] for 40 min, $\lambda = 226$ nm, flow rate 2 mL min^{-1}. The purity and identity of each peptide was ascertained by thin-layer chromatography in two different solvent systems, by HPLC, by fast atom bombardment (FAB) mass spectrometry (molecular ion) and by amino acid analysis.

Bioassay methods

The vasopressor and antidiuretic activities of AVP analogues were tested on male Wistar rats, 270–310 g, bred at the Central Experimental Animal Farm of the Silesian Academy of Medicine.

Vasopressor activity. Vasopressor assays of AVP analogues were performed in-vivo according to the method of Dekanski (1952) as described by Stürmer (1968). The rats were each given 1·75 g kg^{-1} urethane intraperitoneally. After blood pressure stabilization evoked by phenoxybenzamine in two or three repeated doses (each amounting to 1 mg kg^{-1}, i.v.), peptides were injected intravenously into rats. Systolic blood pressure measurements were taken by a pressure transducer fixed into the carotid artery.

Antidiuretic activity. Antidiuretic activity of peptides was estimated in-vivo, in rats under ethanol anaesthesia according to Stürmer's modification (Stürmer 1968) of the method of Sawyer (1958). The animals were given 14% ethanol through a stomach tube. Peptides were injected into the carotid vein in a total volume of ~ 0.3 mL (including wash) at 40-min intervals. After laparotomy, a polyethylene cannula was introduced into the urinary bladder and ligated. The urine flow was collected directly from the urinary bladder in subsequent 10-min periods following analogue injection and measured by pipette connected to a vacuum system. Water balance was kept constant for each rat by rehydrating the 0·35% sodium chloride through a stomach tube in a volume equal to the excreted urine, adding an arbitrary excess to compensate for extrarenal water loss. Antidiuretic response was defined as:

$$(V_p/V_o) \times 100 \qquad (1)$$

where V_o is the urine volume collected in the 10-min period preceding the injection of AVP or analogue and V_p is the volume of urine collected over 10 min starting immediately after injection. Agonistic activity was expressed in international units per milligram of peptide through the comparison of regression lines for AVP and related peptide (Feder et al 1991).

Antagonistic activity of AVP analogues was measured by the method of Schild (1947) and described according to Manning & Sawyer (1985) as effective dose ED50 and pA_2 value. The ED50 is the dose of an antagonist which reduces the response to 2 × units of agonist to the response evoked by 1 × unit of agonist. Each peptide was administered in two doses: high, which reduced the response to 2 × units of agonist to less than the response to 1 × unit of agonist, and low, which did not fully reduce the response obtained by 2 × units of agonist. ED50 values were estimated by interpolation on a logarithmic scale between two doses of antagonist and expressed in nmol kg^{-1} body weight. pA_2 values estimated in-vivo represent the negative logarithms (to the base 10) of effective doses divided by an assumed volume of distribution in rats (67 mL kg^{-1}) (Manning & Sawyer 1985).

AVP was used as a standard agonist in all assays. Its pressor and antidiuretic activity was accepted as 369 units and 323 units mg^{-1}, respectively (Manning et al 1981).

Uterotonic and antiuterotonic activity

Antiuterotonic and uterotonic activity were assayed on rat isolated uterus by the procedure of Holton (1948). Briefly, virgin female Wistar rats, 170–220 g, had proestrus-oestrus artificially induced 48 h earlier by subcutaneous injection of stilboestrol dipropionate (100 μg/rat in 0·1 mL oily solution, Jelfa S.A.). Oestrus stages were determined by vaginal smears made 4–6 h before each experiment; the uterine horn was removed 2 cm close to the ovarian end (under urethane anaesthesia 1·75 g kg^{-1} i.p.), rid of fat and immediately suspended in a 20-mL bathing chamber (Hugo Sachs Elektronik, Germany). The bathing fluid was van Dyke-Hastings solution, in Munsick modification, supplemented with Mg^{++} (Munsick 1960). The solution had the following composition (mM): NaCl 114·0; KCl 6·2; $NaHCO_3$ 30·0; NaH_2PO_4 1·0; $CaCl_2$ 1·0; $MgCl_2$ 0·5 and glucose 2·8. The reservoir and the bath were gassed with a mixture of 95% oxygen and 5% carbon dioxide. The temperature of the bath solution was 31·5°C.

Contractions were measured isometrically after 0·5–1 h stabilization with a force displacement transducer (K-30, Type 351; two-channel bridge amplifier, type 301, Hugo Sachs Elektronik, Germany) and recorded by multi-pen recorder (R-50, Model 83, Rikadenki Kogyo Co., Japan). Initial tension of 1–2 g was applied. The sensitivity of the uterus to synthetic oxytocin was tested and a standard dose producing a response between 40 and 60% of maximal response, was chosen (Jost et al 1974). Under our experimental conditions, the standard doses were between 1 and 3 m units in an organ bath (20 mL). Inhibition was then studied as follows. An inhibitor was added 20–60 s before the administration of synthetic oxytocin, to produce 50% inhibition of the response to the standard dose of oxytocin. Antiuterotonic responses were defined according to the following equation (Slaninova 1987):

$$pA_2 = -\log \frac{Ci}{Coxt_p/Coxt_a - 1} \qquad (2)$$

where Ci denotes the concentration of the inhibitor, $Coxt_a$ is the concentration of oxytocin causing half-maximal response in the absence of the inhibitor, and $Coxt_p$ is the concentration of oxytocin producing half-maximal response in the presence of the inhibitor.

The pA_2 values were determined using groups of at least 12 animals for each peptide, and were expressed as a mean ± s.d.

In the case of weak uterotonic action the contraction for all horns was expressed by concentration of agonist (M) which produced the first minimal response. Synthetic oxytocin was used as a standard agonist and its activity was accepted at 454 units mg^{-1} (Manning et al 1981).

All data were statistically evaluated by Student's *t*-test (Tallarida & Murray 1987).

Results

Some pharmacological properties of new analogues I and II together with those of AVP and some related peptides are presented in Table 1. It should be noted at the outset that none of the new analogues exhibited anti-antidiuretic activity.

The substantial difference between antidiuretic activities (see Table 1) obtained in two laboratories (Manning et al 1976; Zaoral 1985) for dDAVP, which is a very potent V_2-agonist and the drug of choice for the treatment of diabetes insipidus, points to the necessity for caution in the determination and expression of activities of the highly potent V_2-agonists. It appears that for highly active antidiuretic agonists, the dose-response curve of which is not parallel with that of standard, the activity increases with the dose (Zaoral 1985), and cannot be expressed by a single number. With all these in mind we decided to synthesize and assay (according to Vavra et al 1974; Zaoral 1985), [1-(3-mercaptopropionic acid), 8-D-arginine]vasopressin (dDAVP) to compare its activity with that of analogues synthesized in our laboratory. Results obtained for dDAVP by us were similar to those of others (Table 1). Evaluation of antidiuretic activity of [(L-2-Nal)^3]AVP according to Sawyer (1958) showed very high agonistic activity of our analogue (Fig. 1).

As the next step we decided to use the method of Vavra et al (1974) and Zaoral (1985) for our peptide, as we plan to compare its activity with that of dDAVP. However, our analogue did not exhibit antidiuretic activity in this test. We assume that dDAVP, having 3-mercaptopropionic acid instead of Cys at the *N*-terminus and D-Arg instead of Arg at position 8 as compared with AVP molecule, has higher enzymatic stability than our peptide, which differs from AVP only in the presence at position 3 of L-2-Nal instead of Phe.

Summing up, peptide I designed by the substitution of position 3 in the AVP molecule with L-2-naphthylalanine showed very high antidiuretic agonistic activity, which, combined with substantially decreased (as compared with AVP) pressor agonistic and low antioxytocic activities gives it a good degree of selectivity.

Analogue II obtained by the combination of (L-2-Nal)^3 modification and inversion of configuration of Arg^8, is a potent pressor antagonist with a pA_2 value of 8·65. It is thus one of the most potent V_1 antagonists reported to date. This peptide with very low V_2 agonistic potency and only negligible agonistic oxytocic activity in-vitro, is the first example of a potent V_1-antagonist devoid of anti-oxytocic activity.

Discussion

As a continuation of our approach to synthesized analogues of AVP with enhanced potency or selectivity we decided to check how substitution of position 3 of this hormone with L-2-naphthylalanine (L-2-Nal) would influence pharmacological properties of the resulting peptide I. We assumed that although phenylalanine and L-2-Nal which occupy this position of AVP and our analogue, respectively, have a similar chemical character, the hindering effect near the peptide bond caused by a bulky naphthyl moiety may have a significant impact on the bioactive conformation of the peptide molecule and thus influence its interaction with V_1 and V_2 receptors. As regards pressor activity, it is interesting that the modification discussed decreased it substantially. With respect to antidiuretic properties, it appears that our analogue exhibits higher antidiuretic activity than dDAVP, which is a drug of choice for the treatment of diabetes insipidus. It should also be noted that dDAVP was designed by a combination of two changes i.e. deamination (to increase activity) and substitution of position 8 with D-Arg (to improve selectivity). Summing up, it appears that

Table 1. Pharmacological activities of the new vasopressin analogues and some reference compounds.

Compound	Vasopressor (units mg^{-1})	Anti-vasopressor ED50 (nmol kg^{-1}) pA_2	Antidiuretic (units mg^{-1})	Uterotonic (M)	Anti-uterotonic pA_2
AVP[a]	369 ± 6	—	323 ± 16	25·5 ± 0·6	—
[Mpa^1,(D-Arg)^8]VP[b]	0·39 ± 0·02	—	1200 ± 12[b] ~ 5 × 10^4[c]	—	—
[(L-2-Nal)^3]AVP (I)	56 ± 12·7 (8)	—	(4·6 ± 0·16) × 10^6 (16)	—	6·14 ± 0·24 (12)
[(D-Arg)^8]VP[b]	1·08 ± 0·03	—	257 ± 35	1·02 ± 0·08	—
[(L-2-Nal)^3,(D-Arg)^8] VP (II)	—	0·03 ± 0·01 8·65 ± 0·13 (8)	3·79 ± 1·31 (8)	weak* (12)	—

Results are expressed as mean ± s.d. *Inactive up to 2·9 × 10^{-6} M. Values in parentheses indicated number of rats tested. [a] Manning et al (1981). [b] Manning et al (1976), [c] Zaoral (1985).

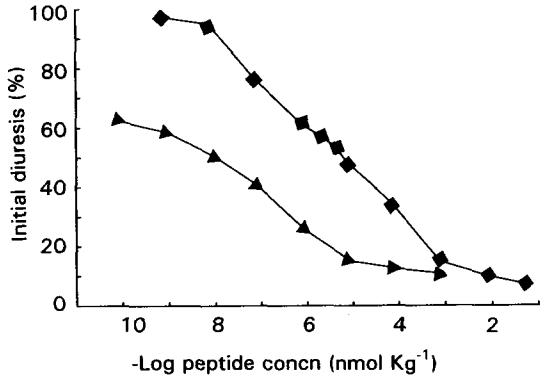

FIG 1. Log dose-response curves of [(L-2-Nal)^3]AVP (▲) and arginine vasopressin (♦). Mean for n = 6–8 rats.

in view of our discovery, deamination of position 1 must no longer be considered to be the most effective single modification which leads to enhanced V_1-agonistic activity. Moreover, since peptide I has only moderate pressor and weak antioxytocic activities, it appears that the modification proposed is also effective in enhancing selectivity. To our surprise, the second substitution introduced into peptide I, i.e. inversion of configuration of Arg^8, which is known to be effective in enhancing the antidiuretic/pressor activity ratio, resulted in an analogue with high antipressor activity. This peptide with a pA_2 value of 8·65 is among the most potent V_1-antagonists reported to date. However, what makes this analogue extremely interesting is its high selectivity. Previous efforts to produce effective antagonists of the pressor response, which do not block oxytocic receptors have yielded disappointing results; all known V_1-antagonist possess varying degrees of oxytocic antagonism (Manning et al 1987; Thibonnier 1990). Our compound with very low V_2-agonistic potency and only negligible oxytocic activity in-vitro, is the first example of a potent V_1 antagonist devoid of antioxytocic activity.

Summing up, D-Arg^8 substitution which should improve antidiuretic selectivity of peptide I, converted it into a potent and selective pressor antagonist. This finding points to the need for caution in extrapolating the effects of structural changes from one peptide to another in the design of analogues. Moreover, it is worth emphasizing that although the substitution of 1-mercaptocyclohexaneacetic acid (or similar) at position 1 is a modification that has been shown to be essential for substantial pressor antagonism of cyclic analogues of AVP, our compound II was designed by changes in position 3 and 8.

Therefore besides providing new information on the role of amino acid residues occupying position 3 of AVP analogues for biological activity, these studies yielded two novel, highly active and selective analogues, which may have potential as pharmacological tools in the study of the action of endogenous AVP or as useful therapeutic agents.

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