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The influence of 1-aminocyclopentane-1-carboxylic acid at position 2 or 3 of AVP and its analogues on their pharmacological properties

WIOLETA KOWALCZYK,[a]* ADAM PRAHL,[a] OLGA DAWIDOWSKA,[a] IZABELA DERDOWSKA,[a] DARIUSZ SOBOLEWSKI,[a] BIANCA HARTRODT,[b] KLAUS NEUBERT,[b] JIŘINA SLANINOVÁ[c] and BERNARD LAMMEK[a]

[a] Department of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland
[b] Institute of Biochemistry, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Straβe 3, 06120 Halle (Saale), Germany
[c] Department of Biological Chemistry, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of Czech Republic, Flemingovo Square 2, Prague 6, CZ-16610, Czech Republic

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Abstract: This study describes the synthesis and some pharmacological properties of eight new analogues of arginine vasopressin (AVP) substituted at position 2 or 3 with cycloleucine (1-aminocyclopentane-1-carboxylic acid, Apc). All new peptides were tested for their pressor, antidiuretic and uterotonic *in vitro* potency. The Apc^3 modification resulted in an almost complete loss of potency in all three tests, which is interpreted as a loss of interaction with all three neurohypophyseal hormone receptors. On the other hand, the Apc^2 modification resulted in compounds having differently modified activities (high antidiuretic potency, low and graded pressor activity and either no activity or low oxytocin antagonizing activity in the uterotonic *in vitro* test) thus selectively altering the interaction with the receptors similar to that of 1-aminocyclohexane-1-carboxylic acid (Acc). The results obtained may be helpful for designing new analogues of arginine vasopressin. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: analogues; arginine vasopressin; conformational constraints; cycloleucine

INTRODUCTION

The neurohypophyseal hormone arginine vasopressin (AVP) acts predominantly on vascular smooth muscles and increases water re-absorption in the kidney. Its effects are mediated by three types of cell surface receptors (V_{1a}, V_{1b} and V_2) that are distinguished by their affinities for various vasopressin analogues and by the second messenger system they activate [1,2,3].

Since 1954, when du Vigneaud and co-workers isolated, sequenced and synthesized AVP [4], many structural analogues of this hormone have been synthesized and pharmacologically characterized which has resulted in a considerable amount of information concerning the structure–activity relationship [1,5–7].

Positions 2 and 3 in the AVP molecule play a significant role in its activity. The L-phenylalanine residue at position 3 is responsible for recognition and binding to the receptor, while the L-tyrosine residue at position 2 seems to take part in initiating the pressor response [8]. Investigation of the role of position 3 in AVP by substituting it with aliphatic, aromatic, conformationally restricted, polar or charged amino acids suggests that this position is tolerant to structural modifications with aliphatic amino acids [9].

It is also well known that deamination of position 1 usually leads to an increase in antidiuretic activity of the AVP analogues [10], while substitution of the D-arginine residue at position 8 yields analogues with a dramatically decreased pressor agonism [11].

During the past decade conformationally constrained analogues of bioactive peptides have awakened growing interest in studies of their structure–activity relationships. Restrictions can be imposed either through the formation of cyclic structures within a peptide backbone, or through reduction of peptide flexibility by introducing amino acids with limited conformational freedom that has an impact on specific orientations of the peptide backbone and the side chains.

Recently residues were replaced at position 2 or 3 of AVP and some of its agonistic and antagonistic analogues with an aliphatic moiety, 1-aminocyclohexane-1-carboxylic acid (Acc) [12,13]. The Acc^2 modification has been shown to selectively modulate the activities of the analogues, as [Acc^2]AVP was found to be a potent antidiuretic agonist with moderate pressor agonistic activity and weak oxytocin antagonizing properties in the uterotonic *in vitro* test. Other analogues tested ([Acc^2, Val^4]AVP, [Mpa^1, Acc^2]AVP, [Mpa^1, Acc^2,D-Arg^8]VP and [Mpa^1, Acc^2, Val^4,D-Arg^8]VP) were highly potent antidiuretic compounds with different activities

Abbreviations: Acc, 1-aminocyclohexane-1-carboxylic acid; Apc, 1-aminocyclopentane-1-carboxylic acid; Cpa, 1-mercaptocyclohexane-acetic acid; MBHA, *p*-methylbenzhydrylamine; Mpa, 3-mercaptopropionic acid; otherwise as in *J. Peptide Sci.* **9**:1–8 (2003).

*Correspondence to: Wioleta Kowalczyk, Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland; e-mail: wiola@chem.univ.gda.pl

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in pressor and oxytocin tests. On the other hand, the Acc^3 substitution led to analogues which failed to exhibit any of the activities studied with the exception of the [Mpa^1, Acc^3, Val^4,D-Arg^8]VP analogue which exhibited antidiuretic activity matching that of AVP. Unlike AVP, it was selective and it was inactive in the pressor and uterotonic tests.

Considering all this, it was decided to replace the residues at position 2 or 3 of AVP and some of its agonistic and antagonistic analogues with 1-aminocyclopentane-1-carboxylic acid (Apc). The Apc, like Acc, should reduce the flexibility of the peptides by imposing steric constriction into the peptide chain limiting conformational freedom. However, Apc has the side chain ring reduced by one CH_2 group and thus the side chain is less bulky and also less flexible.

This modification is an example of the $C^{\alpha} \leftrightarrow C^{\alpha}$ cyclization whereby a dialkylated glycine residue is converted into a cyclic side chain (1-aminocycloalkane-1-carboxylic acid) [14]. In this case the ring consists of five atoms constituting the 1-aminocyclopentane-1-carboxylic acid residue.

The structures of new analogues are as follows:

X-Y-Z-M-Asn-Cys-Pro-R-Gly-NH_2

where:

X = Cys, Y = **Apc**, Z = Phe, M = Gln, R = Arg (I)

X = Cys, Y = **Apc**, Z = Phe, M = Gln, R = **D–Arg** (II)

X = Cys, Y = **Apc**, Z = Phe, M = **Val**, R = Arg (III)

X = **Mpa**, Y = **Apc**, Z = Phe, M = Gln, R = Arg (IV)

X = **Mpa**, Y = **Apc**, Z = Phe, M = Gln, R = **D–Arg** (V)

X = Cys, Y = TyrZ = **Apc**, M = Gln, R = Arg (VI)

X = **Mpa**, Y = Tyr, Z = **Apc**, M = Gln, R = Arg (VII)

X = **Cpa**, Y = Tyr, Z = **Apc**, M = Gln, R = Arg (VIII)

EXPERIMENTAL PROCEDURES

Thin-layer chromatography (TLC) was carried out on silica plates (Merck), and spots were visualized with iodine or ninhydrin. The solvent system used was 1-butanol/acetic acid/water/ethyl acetate (1 : 1 : 1 : 1, v/v). High-performance liquid chromatography (HPLC) was carried out on a Waters (analytical and preparative) chromatograph equipped with a UV detector ($\lambda = 226$ nm). The purity of the peptides was determined on a Waters C_{18} column (5 μm, 100 Å; 150 × 3.9 mm). The following solvent systems were used: [A] 0.1% aqueous trifluoroacetic acid (TFA), [B] acetonitrile : 0.1% aqueous TFA (80 : 20 v/v). Linear gradient from 10% to 60% of [B] was applied for all peptides for 30 min at a flow rate of 1 ml/min. Preparative HPLC was carried out using a Kromasil C_8 column (5 μm, 25 × 250 mm) in a gradient running from 10% to 50% of [B] for 120 min at a flow rate of 10 ml/min. FAB/MS of the peptides were recorded on a MALDI TOF mass spectrometer.

Mpa(Trt) was obtained as described for Cys(Trt) [15] using 3-mercaptopropionic acid instead of L-cysteine hydrochloride. Cpa(Mob) was synthesized using a procedure described in the literature [16].

All amino acid derivatives were purchased from NovaBiochem, except Boc-Apc and Fmoc-Apc, which were provided by Neosystem.

Peptide Synthesis

All peptides were obtained by solid-phase synthesis. The syntheses of analogues **I–VII** were performed on a *Symphony* synthesizer (Protein Technologies, Inc.) using Fmoc-chemistry on polystyrene resin (Fmoc-Gly **T**enta**G**el S RAM, capacity 0.22 mmol/g) and peptide **VIII** was synthesized manually using Boc-chemistry on a methoxybenzhydryl resin (MBHA resin, Senn Chemicals AG, 1% DVB, 200–400 mesh, 0.67 mmol/g) according to standard procedures, using *in situ* neutralization [17].

Mixtures of protected amino acid : TBTU : HOBt : DIEA (1 : 1 : 1 : 2) in DMF or protected amino acid : HATU : HOAt : DIEA (1 : 1 : 1 : 2) in DMF or in a mixture of DMF : NMP (1 : 1 v/v) containing 1% Triton were used for coupling. The completeness of each coupling reaction during manual synthesis was monitored by the Kaiser test [18] or chloroanil test [19]. Re-coupling was performed when the test was positive. With peptides **VI, V, VII** and **VIII** Mpa(Trt) or Cpa(Mob) were used during the final coupling step.

The Fmoc deprotection was accomplished using a 20% solution of piperidine in DMF. A solution of TFA : H_2O : TIS : PhOH (92.5 : 2.5 : 2.5 : 2.5, v/v) was used for the cleavage of peptides from the **T**enta**G**el resin (3 h). Solutions of the cleaved peptides were filtered and evaporated *in vacuo* to a volume of ca. 1 ml. Then the peptides were precipitated with diethyl ether to afford crude products.

The Boc deprotection was accomplished using a 33% solution of TFA in DCM. The protected acylpeptidyl resin was treated with 10 ml of liquid hydrogen fluoride (HF) containing 1 ml of anisole at −70 °C and stirred for 60 min at 0 °C [20]. After removal of HF and anisole *in vacuo*, the mixture was washed successively with anhydrous diethyl ether and acetic acid and the solution was diluted with methanol.

The resulting dithiols were oxidatively cyclized with 0.1 M I_2 in methanol using the standard procedure. The solvents were evaporated under reduced pressure and the residue was dissolved in water and lyophilized. The crude products were desalted on a Sephadex G-15 column, and eluted with aqueous acetic acid (30%) at a flow rate of 3 ml/h. After freeze-drying, the fractions comprising the major peak were purified by RP-HPLC. The peptides were eluted as single peaks. The purity and identity of each peptide was determined by HPLC and FAB mass spectroscopy (molecular ion).

Biological Evaluation

Wistar rats were used in all experiments. Female rats were oestrogenized 48 h before the experiment. The uterotonic test was carried out *in vitro* in the absence of magnesium ions [21,22]. The vasopressor test was performed using

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phenoxybenzamine-treated male rats [23]. Synthetic oxytocin was used as a standard in uterotonic tests, and synthetic arginine vasopressin in the pressor test. Dose-response (single administration) or cumulative dose-response (measurements without washing steps between the administration of enhanced doses) curves were constructed in the case of pressor and uterotonic *in vitro* test, respectively. In the case of antagonists, the dose-response curves of standard oxytocin or vasopressin were constructed in the presence or absence of the tested compounds. For agonists the activity was expressed in IU/mg, for antagonists as pA_2. The values reported are averages of three to five separate experiments.

Tests to assess the antidiuretic or diuretic properties were conducted on conscious male rats in two variations of the modified Burn test [24,25]. In the standard manner with hydrated rats, the animals having fasted for 16 h were weighed and then given tap water through a stomach catheter. The water load was 4% of the body weight. Immediately after the water load, the tested substances (or physiological saline as control) were administered subcutaneously at doses of 0.001–100 nmol/kg. The rats were then placed in individual metabolic cages, and their urine was collected over a 5 h period. The time $t_{1/2}$ in which the rats excreted half the water load was determined and then plotted against the dose. As the dose response curves were not parallel, such doses were chosen for comparison of compounds yielding $t_{1/2}$ equal to 200 min and the so-called threshold doses yielding $t_{1/2}$ equal to 60 min (equal to the value of $t_{1/2}$ obtained with the physiological solution). On each day of the experiment, 21 rats divided into five groups of four or five animals were used to which different doses and compounds were administered; each dose being tested in two or three independent experiments (different days, different rats). To test for diuretic effects with nonhydrated rats, no water load was given to the fasting animals. As standards synthetic AVP and dDAVP were used. For details see reference [26]. The results were thus expressed in IU/mg in comparison with AVP (the value 465 IU/mg was taken for AVP for both $t_{1/2}$ 60 min and $t_{1/2}$ 200 min) and in percent of activity when compared with dDAVP (100% for both $t_{1/2}$ 60 min and $t_{1/2}$ 200 min).

RESULTS

Eight new analogues of AVP (**I–VIII**) were synthesized by Fmoc or Boc strategy and characterized. Their physicochemical properties are presented in Table 1. The values of the molecular ions were as expected.

The results of pharmacological evaluation of the new analogues **I–VIII**, together with relevant values for AVP and some related peptides, are shown in Table 2. The activities of the analogues were determined in the rat uterotonic *in vitro* test in the absence of magnesium ions, in the rat pressor test, and in the antidiuretic assay on conscious rats as described in the Methods section. It is difficult to compare the data of the antidiuretic activity of new analogues with the earlier published ones as different testing procedures were used. In order to compare the effects of compounds with a different regression line of the dose-response curve, doses of AVP or dDAVP were compared with the new analogues that gave the same antidiuretic response, i.e. the dose that caused rats to excrete half the water load ($t_{1/2}$) in 60 and 200 min. For AVP the activity was arbitrarily taken to be 465 IU/mg for both responses, for dDAVP as 100%.

All the analogues modified at position 2 with Apc exhibited antidiuretic activity higher than AVP (**I–V**). Analogues **I** and **IV** showed moderate pressor agonism. The analogues exhibited moderate (**IV**), weak (**I, III, V**) or no (**II**) antioxytocic activity in the uterus *in vitro* test. Compound **II** turned out to be the most selective of the peptides tested.

Peptides with Apc at position 3 showed only very weak antidiuretic activity, and no activity over the range of concentrations tested in the other two tests.

DISCUSSION

The present work is a continuation of our detailed study aimed at clarifying the impact of steric restrictions in the *N*-terminal part of the AVP molecule on pharmacological properties. Previously it was reported that 1-aminocyclohexane-1-carboxylic acid residue at position 2 or 3 of AVP and some of its agonistic and antagonistic analogues influenced significantly the interaction with V_{1a}, V_2 and oxytocic receptors as expressed by modulated pharmacological activities [12,13], see also the Introduction and Table 2.

Table 1 Physicochemical Properties of Peptides **I**–**VIII**

Analogue		HPLC t_r	Formula	MW	$[M + H]^+$
[Apc^2]AVP	**I**	12.07	$C_{43}H_{67}N_{15}O_{11}S_2$	1032.2	1032.1
[Apc^2,D-Arg^8]VP	**II**	12.29	$C_{43}H_{67}N_{15}O_{11}S_2$	1032.2	1032.1
[Apc^2,Val^4]AVP	**III**	14.41	$C_{43}H_{68}N_{14}O_{11}S_2$	1003.2	1003.3
[Mpa^1,Apc^2]AVP	**IV**	15.29	$C_{43}H_{66}N_{14}O_{11}S_2$	1017.2	1017.4
[Mpa^1,Apc^2,D-Arg^8]VP	**V**	15.62	$C_{43}H_{66}N_{14}O_{11}S_2$	1017.2	1017.5
[Apc^3]AVP	**VI**	7.25	$C_{43}H_{67}N_{15}O_{12}S_2$	1048.2	1048.3
[Mpa^1,Apc^3]AVP	**VII**	10.64	$C_{43}H_{66}N_{14}O_{12}S_2$	1033.2	1033.3
[Cpa^1,Apc^3]AVP	**VIII**	15.41	$C_{48}H_{73}N_{14}O_{12}S_2$	1101.3	1101.2

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Table 2 Pharmacological Properties of New Analogues of AVP (IU/mg or pA_2)

Analogue		Activity			
		Oxytocic Uterus *in vitro* test no Mg^{2+}	Pressor IU/mg or pA_2	Antidiuretic[b]	
				IU/mg	% of dDAVP
AVP[a]		17	412	465	10 (0.2)
[Val^4]AVP[a]		—	32	738[e]	
[D-Arg^8]VP[a]		0.4	4.1	114–257[e]	23.2 (1.7)
[Mpa^1]AVP[a]		27–63	346–370	1300–1745[e]	73.9 (97.3)
[Cpa^1]AVP[a]		$pA_2 = 8.15$	$pA_2 = 8.35$	0.033[e]	
[Mpa^1,D-Arg^8]VP[a]		1.5–5.1	~0.39	800–50 000[e]	100 (100)
[Acc^2]AVP[c]		$pA_2 \sim 5.6$	56.6	750–900 (~9300)	~15 (4)
[Acc^2,D-Arg^8]VP[c]		$pA_2 \sim 5.7$	$pA_2 \sim 5.8$	750–900 (~9300)	~15 (4)
[Acc^2,Val^4]AVP[d]		$pA_2 = 6.9$	0.9 ± 0.2	~2300 (23 000)	~50 (10)
[Mpa^1,Acc^2]AVP[d]		$pA_2 = 6.1$ and 0.7 IU/mg	17.2 ± 0.8	~4500 (50 000)	~100 (20)
[Mpa^1,Acc^2,D-Arg^8]VP[d]		$pA_2 = 6.5$ and 0.3 IU/mg	$pA_2 = 5.70$	~4500 (50 000)	~100 (20)
[Apc^2]AVP	**I**	$pA_2 = 6.0$ and 0.2 IU/mg	13.4 ± 3.8	~1800 (1800)	50 (1.4)
[Apc^2,D-Arg^8]VP	**II**	0	0	~1000 (9000)	~25 (5)
[Apc^2,Val^4]AVP	**III**	$pA_2 = 6.6$	0.20 ± 0.02	~1000 (9000)	~25 (5)
[Mpa^1,Apc^2]AVP	**IV**	$pA_2 = 7.1$	15.5 ± 3.5	~1000 (9000)	~25 (5)
[Mpa^1,Apc^2,D-Arg^8]VP	**V**	$pA_2 = 6.5$	0	~1000 (9000)	~25 (5)
[Apc^3]AVP	**VI**	0	0	<0.5	<0.1
[Mpa^1,Apc^3]AVP	**VII**	0	0	~4.5 (45)	<0.1
[Cpa^1,Apc^3]AVP	**VIII**	0	0	<0.5	<0.1

[a] Values taken from [7].
[b] The activities obtained by comparing doses of AVP and the analogues or dDAVP and the analogues resulting in an antidiuresis time of $t_{1/2} = 60$ min; in parentheses, the activities obtained by comparing doses of AVP and the analogues or dDAVP and the analogues resulting in an antidiuresis time of $t_{1/2} = 200$ min.
[c] Values taken from [12].
[d] Values taken from [13].
[e] The antidiuretic activity from antidiuretic test on anaesthetized rats.

This paper describes a series of analogues substituted at position 2 or 3 with a compact amino acid, 1-aminocyclopentane-1-carboxylic acid (Apc), which has the side chain ring shortened for one CH_2 group in comparison with the previously described Acc, thus being more rigid and less bulky. Both the Apc and Acc modifications changed the character of a fragment of the molecule from aromatic to aliphatic and reduced the conformational freedom of the relevant part of the peptides as the residues are sterically constrained.

The results presented in Table 2 show that while in the case of analogues **I** and **II** the antidiuretic potency was comparable to their Acc counterparts, that of the remaining analogues was lower. Both the agonistic and antagonistic pressor activities were either comparable or decreased compared with those of the Acc analogues. The same applies to the activity in the uterotonic test. The combination of Apc^2 substitution with inversion of the Arg^8 amino acid configuration resulted in analogue **II** which was devoid of the pressor and uterotonic activities and exhibited similar antidiuretic activity as [Acc^2,D-Arg^8]VP. As the antidiuretic tests reflect the interaction with V_2 receptor, this analogue is a highly selective V_2 agonist. This finding is in good agreement with previous knowledge as the only difference between peptides **I** and **II** is inversion of the Arg residue at position 8.

The combination of Apc^2 substitution with Val^4 modification (analogue **III**), did not affect the activity in uterotonic test *in vitro*, however, resulted in about a twofold decrease in the antidiuretic activity and diminished pressor activity (from 0.9 IU/mg to 0.2 IU/mg). In comparison to Acc^2 analogue it displayed weaker antidiuretic potency.

Deamination of compounds **I** and **II** (analogues **IV** and **V)** had virtually no influence on the pressor or antidiuretic activities while unexpectedly it increased antioxytocic activity of both analogues (**IV** and **V**).

The Apc^3-modified analogues exhibited drastic reduction of the antidiuretic, pressor and antioxytocic potencies. None of the analogues displayed significant biological activity. It was also the case with the Acc^3 modification as previously demonstrated [12,13]. Thus the slight reduction in bulkiness and increase in rigidity in the cyclic side chain moiety at position 3 did not improve any one of the activities.

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CONCLUSION

In summary, the replacement of 1-aminocyclohexane-1-carboxylic acid (Acc) by 1-aminocyclopentane-1-carboxylic acid (Apc) did not result in any surprising change in potency of the analogues. Reduction of the cyclic side chain moiety from cyclohexane to cyclopentane at position 2 led either to retention or a slight decrease in the activities. One of the new analogues, namely [Apc^2,D-Arg^8]VP (**II**), is the most selective of the Acc^2 and Apc^2 analogues reported here and in the literature [12,13]. Introduction of a cyclic aliphatic residue into position 3 of AVP and some of its analogues resulted in almost inactive compounds. These results provide very useful information on the structure–activity relationships and open up new possibilities for designing potent and selective antidiuretic compounds.

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