Research Article

Journal of PeptideScience

Received: 4 March 2009 Revised: 25 July 2009 Accepted: 28 August 2009 Published online in Wiley Interscience: 18 November 2009

(www.interscience.com) DOI 10.1002/psc.1189

Novel analogues of arginine vasopressin containing α-2-indanylglycine enantiomers in position 2

Anna Kwiatkowska,[a]* Małgorzta Śleszyńska,[a] Izabela Derdowska,[a] Adam Prahl,[a] Dariusz Sobolewski,[a] Lenka Borovičková,[b] Jiřina Slaninová[b] and Bernard Lammek[a]

Continuing our efforts to obtain potent and selective analogues of AVP we synthesized and pharmacologically evaluated ten new compounds modified at position 2 with α-2-indanylglycine or its D-enantiomer (Igl or D-Igl, respectively). All the peptides were tested for pressor, antidiuretic, and *in vitro* uterotonic activities. We also determined the binding affinity of these compounds to human OT receptor. The Igl^2 substitution resulted in a significant change of the pharmacological profile of the peptides. The new analogues were moderate or potent OT antagonists (pA_2 values ranging from 7.19 to 7.98) and practically did not interact with V_{1a} and V_2 receptors. It is worth emphasizing that these new peptides were exceptionally selective. On the other hand, the D-Igl^2 substituted counterparts turned out to be weak antagonists of the pressor response to AVP and displayed no antidiuretic activity. Some of the results were unexpected, e.g. dual activity in the rat uterotonic test *in vitro*: the D-Igl peptides showed a strong antioxytocic potency (pA_2 values ranging from 7.70 to 8.20) at low concentrations and full agonism at high concentrations. The results provided useful information about the SAR of AVP analogues. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: arginine vasopressin (AVP) analogues; antidiuretic hormone; α-2-indanylglycine; conformational restriction; affinity to OT receptor

Introduction

AVP, otherwise known as antidiuretic hormone, is a cyclic nonapeptide (Figure 1) with multiple functions. The well-known peripheral roles of AVP are the regulation of water balance, the control of blood pressure, and the release of ACTH. In addition to these actions, AVP is involved in several CNS-related processes, including the regulation of memory, body temperature, social behaviour, stress, and reproduction [1–3].

AVP acts both as a hormone and as a neurotransmitter or neuromodulator. It exerts its effects upon binding to three receptor subtypes termed: V_{1A}, V_{1B} (V_3), and V_2 [4–6]. Furthermore, AVP can interact to some extent with the OT receptor [5]. The AVP and OT receptor subtypes show a high degree of homology. This, along with the similar structure of both hormones, may be the reason for the overlap of pharmacological profiles of OT and AVP. All these receptors belong to a large family of G protein-coupled receptors (GPCRs) with seven transmembrane spanning domains [6].

Numerous studies have been carried out on the SAR of AVP during the last four decades, trying to cast light on the mechanism of its action [7–9]. It has been demonstrated that conformation of the *N*-terminal part of AVP analogues is crucial for their pharmacological activity [8,9]. It is well known that antidiuretic properties of the analogues may be enhanced by deamination of position 1 [10]. For example, 1-deamino-AVP and 1-deamino-8-D-arginine vasopressin (also known as Desmopressin, dDAVP) are analogues of AVP with enhanced and prolonged antidiuretic activity. In addition, dDAVP is selective and suitable for the treatment of diabetes insipidus [11]. On the other hand, replacement of Tyr^2 with bulky or sterically restricted substituents is generally favorable for generation of effective antagonistic analogues. This hypothesis is also supported by our already longer than 12-years research focused on the design of potent and selective analogues of AVP. In 2004, we described the synthesis and some pharmacological properties of four new analogues having β-(1-naphthyl)-L-alanine (L-1-Nal) or its D-enantiomer in position 2 [12]. All of the new peptides were potent OT antagonists. Additionally, one of them, namely [L-1-Nal^2, Val^4] AVP, was exceptionally selective, as it practically did not interact with V_{1a} and V_2 receptors. Recently, advancing in this direction, we have replaced the residue in position 2 of AVP with 3,3-diphenyl-L-alanine (Dip) or its D-enantiomer (D-Dip) [13]. Both the L- and

* *Correspondence to: Anna Kwiatkowska, Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland. E-mail: aniak@chem.univ.gda.pl*

a *Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland*

b *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Flemingovo sq. 2, 166-10 Prague, Czech Republic*

Abbreviations used: *Aic, 2-aminoindane-2-carboxylic acid; AVP, arginine vasopressin; Dip, 3,3-diphenyl-L-alanine; D-Dip, 3,3-diphenyl-D-alanine; DMTMM, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mob, 4-methoxybenzyl; NMM, N-methyl-morpholine; NMP, 1-methyl-2-pyrrolidone; L-1-Nal, β-(1-naphthyl)-L-alanine; D-1-Nal, β-(1-naphthyl)-D-alanine; OT, oxytocin.*

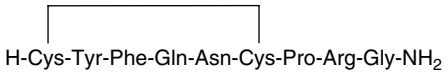

Figure 1. Amino acid sequence of arginine vasopressin.

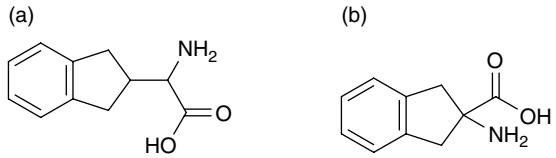

Figure 2. (a) α-2-indanylglycine (Igl). (b) 2-aminoindane-2-carboxylic acid (Aic).

D-Dip modifications were sufficient to dramatically change the properties of the analogues: the antidiuretic activity was preserved and prolonged, the uterotonic activity was transformed into antioxytocic one and the effect on blood pressure was eliminated. Moreover, four compounds, [Mpa^1, D-Dip^2]AVP, [Mpa^1, D-Dip^2, Val^4]AVP, [Mpa^1, D-Dip^2, D-Arg^8]VP, and [Mpa^1, D-Dip^2, Val^4, D-Arg^8]VP, were exceptionally potent antidiuretic agents with significantly prolonged activities. Our approach also included the 2-aminoindane-2-carboxylic acid (Aic) modification in position 2 or 3 [14]. One peptide of this series, namely [Mpa^1, Aic^2, Val^4, D-Arg^8]VP, had antidiuretic activity similar to that of dDAVP, thus being one of the most potent antidiuretic peptides reported to date.

These observations backed up by our earlier investigations prompted us to examine the influence of other sterically restricted moieties placed in position 2 on pharmacological properties of the resulting peptides.

We decided to check how substitution of position 2 with bulky α-2-indanylglycine (Igl) enantiomers (L- and D-, see Figure 2a) would affect biological potency of the analogues. The α-2-indanylglycine was earlier successfully applied for the synthesis of potent and totally enzyme-resistant bradykinin antagonists in Stewart's laboratory [15]. This modification resembles previously used Aic (Figure 2b); however, the α-carbon is not incorporated into the ring. It makes the molecule less compact. Peptides modified with this residue are conformationally less restricted than their Aic^2 counterparts. This modification should also enhance the resistance of the resulting peptides to enzymes. We have designed, synthesized, and determined some pharmacological properties of ten new analogues of AVP where the above mentioned modification was combined with Mpa^1, and/or Val^4 and/or D-Arg^8 substitutions. The structure of the new peptides is shown in Table 1.

Materials and Methods

General

TLC was carried out on silica plates (Merck) and the spots were visualized with iodine. The solvent system used was butan-1-ol/acetic acid/water/ethyl acetate (1 : 1 : 1 : 1, v/v). HPLC was carried out on a Waters (analytical and preparative) chromatograph equipped with a UV detector (λ = 226 nm). The following solvent systems were used: [A] 0.1% aqueous TFA and [B] acetonitrile/0.1% aqueous TFA (80 : 20 v/v). Preparative HPLC was carried out using Waters C_{18} column (15 µm, 100 ´Å, 7.8 × 300 mm) in gradients running from 20 to 50% [B] for 90 min for analogues **I, VI, VIII**, and **X**, from 25 to 55% [B] for 90 min for analogues **II–V** and **VII**, and from 15 to 45% [B] for 80 min for analogue **IX,** all at a flow rate of 2.5 ml/min. The mass spectra of the peptides were recorded on a MALDI TOF mass spectrometer (Biflex III Bruker, λ = 337 nm). The purity of the peptides was determined on a Vydac C_{18} column (5 µm, 4.6 × 250 mm) or Hypersil ODS C_{18} column (5 µm, 4.6 × 250 mm). A linear gradient from 20 to 80% of [B] was applied for 20 min or 30 min at a flow rate of 1 ml/min.

All the amino acid derivatives were purchased from NovaBiochem, except for Fmoc-Igl and Fmoc-D-Igl, which were provided by ChemImpex Int., Inc. Mpa (Trt) was obtained as described for Cys(Trt) [16] using 3-mercaptopropionic acid instead of L-cysteine hydrochloride.

Peptide synthesis

All the peptides were obtained manually by solid-phase method, i.e. by stepwise coupling of Fmoc-amino acids to the growing peptide chain on a polystyrene resin (TentaGel S Ram-resin, capacity 0.23 mmol/g) on a 150 µmol scale. Fmoc-protected amino acids were used with the Trt and the 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl group (Pbf) as side

Table 1. Physicochemical properties of peptides **I–X**

Analogue		Formula	HPLC T_R (min)[a]	MW [M + H$^+$]	
[Igl^2]AVP	**I**	$C_{48}H_{67}N_{15}O_{11}S_2$	12.84	1093.4	1094.1
[D-Igl^2]AVP	**II**	$C_{48}H_{67}N_{15}O_{11}S_2$	22.44[b,c]	1093.4	1094.3
[Mpa^1, Igl^2]AVP	**III**	$C_{48}H_{66}N_{14}O_{11}S_2$	15.23	1078.4	1079.0
[Mpa^1, D-Igl^2]AVP	**IV**	$C_{48}H_{66}N_{14}O_{11}S_2$	21.97[b,c]	1078.4	1079.4
[Igl^2, Val^4]AVP	**V**	$C_{48}H_{68}N_{14}O_{10}S_2$	14.78	1064.4	1065.0
[D-Igl^2, Val^4]AVP	**VI**	$C_{48}H_{68}N_{14}O_{10}S_2$	17.11[b]	1064.4	1065.1
[Mpa^1, Igl^2, Val^4]AVP	**VII**	$C_{48}H_{67}N_{13}O_{10}S_2$	16.90	1049.4	1050.1
[Mpa^1, D-Igl^2, Val^4]AVP	**VIII**	$C_{48}H_{67}N_{13}O_{10}S_2$	19.53[b]	1049.4	1050.3
[Igl^2, D-Arg^8]VP	**IX**	$C_{48}H_{67}N_{15}O_{11}S_2$	15.81[b]	1093.4	1095.0
[Mpa^1, Igl^2, D-Arg^8]VP	**X**	$C_{48}H_{66}N_{14}O_{11}S_2$	19.21[b]	1078.4	1079.2

[a] Linear gradient from 20 to 80% of [B] for 20 min, Vydac C_{18} column.
[b] Linear gradient from 20 to 80% [B] for 30 min, Vydac C_{18} column.
[c] Linear gradient from 20 to 80% [B] for 30 min, Hypersil ODS C_{18} column.
The following solvent systems were used: [A] 0.1 aqueous trifluoroacetic acid (TFA); [B] acetonitrile: 0.1% aqueous TFA (80 : 20 v/v).

chain protecting groups for Asn, Gln, Cys, and Arg, respectively. Full protected peptide resins were synthesized according to standard procedures [17] involving (i) deprotection steps using a 20% solution of piperidine in DMF, 5 and 10 min. The couplings in a mixture of DMF/1-methyl-2-pyrrolidone (NMP) (1 : 1 v/v) containing 1% Triton were carried out using TBTU, HOBt in the presence of NMM. In most cases, the amino acids were coupled at a threefold excess. The couplings after Fmoc-Igl, Fmoc-d-Igl were mediated by HATU, HOAt in the presence of NMM in the mixture of DMF/NMP (1 : 1 v/v) containing 1% Triton or DMTMM in the presence of DIPEA in the NMP. The completeness of each coupling reaction was monitored by the Kaiser [18] or chloranil test [19]. Recoupling was performed when the test was positive. After completion of the synthesis, the protected nonapeptidyl resins were treated with TFA : H_2O : TIS : PhOH (92.5 : 2.5 : 2.5 : 2.5) and stirred for 3 h. Solutions of the cleaved peptides were filtered and evaporated *in vacuo* to a volume of about 1 ml. Then the peptides were precipitated with diethyl ether to afford crude products. The resulting dithiols were oxidatively cyclized with a 0.1 m I_2 in methanol using a standard procedure [16]. The solvents were evaporated under reduced pressure and the resulting materials were dissolved in water, frozen, and lyophilized to give the final crude oxidized products. 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 as described above. The purity of the peptides was controlled using analytical HPLC. MALDI TOF mass spectroscopy (molecular ion) was used to confirm the identity of the pure products.

Biological Evaluation

Wistar rats were used in all experiments. Handling of the experimental animals was done under supervision of the Ethics Committee of the Academy of Sciences according to §23 of the law of the Czech Republic no. 246/1992.

The uterotonic test was carried out *in vitro* on the strips of rat uterus in the absence of magnesium ions [20,21]. Rats in induced estrus by the injection of estrogen 48 h before the experiments were used and the height of the single isometric contraction of a uterine strip was measured. In principle, cumulative dosing was applied in the experiments, i.e. doses of standard (in the presence or absence of analogues) or of the analogue were added successively to the uterus in the organ bath in doubling concentrations and at 1 min intervals without the fluid being changed until the maximal response was obtained. Synthetic OT was used as standard. Each analogue was tested on uteri excised from 4 to 5 different rats.

The pressor activity was determined on phenoxybenzamine treated rats [22]. In short, male rats (220–260 g) were anesthetized using urethane and their vena femoralis and arteria carotis were canulated for drug administration and blood pressure determination, respectively. Then phenoxybenzamine was administered in two doses. After pressure stabilization, usually after 30 min, bolus doses of the standard or tested substance were administered in random order. When antagonistic activity was expected, a standard dose of vasopressin was administered 1 min after the administration of the test substance. Each analogue was tested on 3–4 animals. A more detailed description of these protocols can be found in Ref. 23.

With agonists, the activity was expressed in IU/mg, with antagonists as pA_2. The pA_2 values represent the negative logarithm to the base 10 of the average molar concentration of an antagonist which reduces the response to 2 *x* units of the agonist to the response to *x* units of the agonist. The volume of distribution in the *in vivo* experiments is arbitrarily taken as 67 ml/kg. The values reported are averages of 3 to 5 separate experiments. The responses to standard doses of OT or vasopressin were stable for several hours, without problems with tachyphylaxis. For details see Ref. 23.

Tests to assess the antidiuretic property were conducted on conscious male rats in a 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 the compound's potency 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 5 groups of 4 or 5 animals each were administered different doses of different compounds; each dose being tested in 2 or 3 independent experiments (different days, different rats). As standards, synthetic AVP and dDAVP were used. For details see Ref. 23. The results were thus expressed in IU/mg in comparison to AVP (the value 465 IU/mg was taken for AVP for both $t_{1/2}$ 60 min and $t_{1/2}$ 200 min)

Binding affinities to the human OT receptor were determined as described in Refs 26,27, using tritiated OT from NEN Life Science, Boston, MA, USA. In brief, a membrane fraction from HEK OTR cells was incubated with [^{3}H]OT (2 nM) and various concentrations of peptides (0.1–10 000 nM) for 30 min at 35 °C. The total volume of the reaction mixture was 0.25 ml. Incubation medium consisted in HEPES (50 mM, pH 7.6), $MnCl_2$ (10 mM), and BSA (1 mg/ml). The reaction was terminated by quick filtration on a Brandel cell harvester. OT was used as a control and for determination of nonspecific binding. Binding affinities were expressed as K_i values calculated according to the expression $K_i = IC_{50}/[(c_{3HOT}/K_{dOT})+1]$, where K_{dOT} is taken as 1.8 nM [28].

Results

The ten new analogues of AVP (**I**–**X**) were obtained as crude products in about 70–85% yields. After HPLC purification, their purity was higher than 98% as determined by analytical HPLC. The MALDI TOF mass spectrometry confirmed that the purified peptides were the desired products. Physicochemical data of the new peptides are presented in Table 1.

Pharmacological data of the new analogues together with those of AVP and some related peptides are summarized in Table 2.

Peptides having Igl in position 2 were moderate (**I, IX**) or high (**III, V, VII, X**) oxytocic antagonists in the uterus *in vitro* test. Analogues with D-Igl at position 2 (**II, IV, VI, VIII**) exhibited dual activity. At low concentrations, they were strong antioxytocic agents, whereas at high concentrations they were full agonists.

Regarding the pressor activity, all the new analogues modified at position 2 with Igl (**I, III, V, VII, IX, X**) were devoid of any activity, while their counterparts with D-Igl were weak antagonists of AVP.

Table 2. Pharmacological properties of the new AVP analogues

Analogue		Activity			
		Oxytocic uterus *in vitro* test no Mg^{2+}	Pressor[c] IU/mg or pA_2	Antidiuretic IU/mg 60 min (200 min)	Affinity to human OTR K_i (nM)
AVP[a]	–	17	412	465	–
[Val^4]AVP[a]	–	–	32	738[d]	–
[D-Arg^8]VP[a]	–	0.4	4.1	114–257[d]	–
[Mpa^1]AVP[a]	–	27–63	346–370	1300–1745[d]	–
[Mpa^1, D-Arg^8]VP[a]	–	1.5–5.1	~0.39	800 – 50 000[d]	–
[Aic^2]AVP[b]	–	$pA_2 = 7.27 \pm 0.22$	9.4 ± 2.8	~450 (45 000)	740
[Igl^2]AVP	**I**	$pA_2 = 7.19 \pm 0.10$	0	[e]	1005 ± 282
[D-Igl^2]AVP	**II**	$pA_2 \sim 8.20$ and agonist ~1.1 IU/mg	$pA_2 \sim 5.7$	[e]	209 ± 59
[Mpa^1, Aic^2]AVP[b]		$pA_2 = 7.49 \pm 0.16$	5.3 ± 2.5	~450 (45 000)	215
[Mpa^1, Igl^2]AVP	**III**	$pA_2 = 7.94 \pm 0.22$	0	[e]	202 ± 16
[Mpa^1, D-Igl^2]AVP	**IV**	$pA_2 \sim 8.20$ and agonist ~5.5 IU/mg	$pA_2 \sim 5.7$	[e]	35 ± 59
[Aic^2, Val^4]AVP[b]		$pA_2 = 7.93 \pm 0.17$	0	~450 (45 000)	1800
[Igl^2, Val^4]AVP	**V**	$pA_2 = 7.68 \pm 0.16$	0	[e]	745 ± 175
[D-Igl^2, Val^4]AVP	**VI**	$pA_2 \sim 7.88$ and agonist ~0.9 IU/mg	$pA_2 \sim 5.7$	[e]	138 ± 38
[Mpa^1, Aic^2, Val^4]AVP[b]		$pA_2 = 8.06 \pm 0.11$	$pA_2 = 6.25 \pm 0.12$	~4500 (450 000)	1550
[Mpa^1, Igl^2, Val^4]AVP	**VII**	$pA_2 = 7.75 \pm 0.10$	0	[e]	658 ± 57
[Mpa^1, D-Igl^2, Val^4]AVP	**VIII**	$pA_2 \sim 7.70$ and agonist ~0.8 IU/mg	$pA_2 \sim 7.0$	[e]	70 ± 30
[Igl^2, D-Arg^8]VP	**IX**	$pA_2 = 7.40 \pm 0.15$	0	~0.45 (~20)	540 ± 10
[Mpa^1, Igl^2, D-Arg^8]VP	**X**	$pA_2 = 7.98 \pm 0.24$	0	~4.5 (~4.5)	188 ± 22

[a] Values taken from Ref. 8.
[b] Values taken from Ref. 14.
[c] 0 means no activity up to the dose of 0.15 mg/kg of experimental animal.
[d] Values taken from Ref. 23.
[e] Negligible antidiuretic activity; about 1000 times lower than AVP at the threshold level (60 min) and about ten times or more lower at the effect level (200 min); steeper dose response curve.

All the new compounds exhibited only negligible antidiuretic activity, about 1000 times lower than that of AVP at the threshold level (60 min) and about ten times or more lower at the effect level of 200 min. Analogue **X,** where Igl^2 modification was combined with Mpa^1 and D-Arg^8 substitutions, showed about 1% of vasopressin antidiuretic activity at both activity levels.

Binding properties of new analogues to human OT receptors are also summarized in Table 2.

Discussion

One of the main topics of our research is investigation of the role of Tyr^2 of AVP and its analogues in pharmacological properties.

This paper describes the synthesis and pharmacological evaluation of ten new analogues (**I**–**X**) designed by substitution of position 2 of AVP and of some of its analogues (having different combinations of changes at position 1, 4, and 8) with Igl enantiomers. The results presented in Table 2 show that the Igl^2 and D-Igl^2 peptides have strikingly different pharmacological properties as compared to that of the parent hormone.

The antidiuretic potency of all the new analogues is negligible with exception of peptides **IX** and **X**, where Igl modification is combined with D-Arg substitution and deamination, which are nevertheless only weak agonists. This finding is extremely interesting especially when compared with antidiuretic activity of Aic^2 analogues. The Aic^2 peptides, which were considered as models of the new compounds **I**–**VI**, exhibited potent and long lasting antidiuretic effect. Aic differs from Igl only by lacking one –CH– group (Figure 2a and b); however, the group is in its crucial position, which thus results in a more compact structure and a decreased conformational freedom of the analogues. Both noncoded amino acids have a bulky planar side chain, however in the case of Igl in comparison to Aic, the α-carbon is not incorporated into the ring which results in a higher flexibility of the side chain.

In regard to the pressor test, all the analogues having L-enantiomer at position 2 (**I, III, V, VII, IX, X**) were devoid of the pressor potency, while their counterparts with D-Igl were moderately (**VIII**) or weakly (**II, IV, VI**) potent antagonists of AVP. The data presented in Table 2 show that the combination of D-Igl^2 and deamination of position 1 and Val^4 substitution results in an increase of the pressor antagonism (peptide **VIII**). Also in the case of the pressor activity, the structural difference between Aic and Igl plays an important role as the [Aic^2]AVP and deamino [Aic^2]AVP peptides are pressor agonists, while those containing Igl have no pressor activity.

As far as the oxytocic test is concerned, single substitution of Tyr in the AVP molecule with Igl, resulted in a moderately potent and selective antagonist of OT (compound **I**). Deamination, Val^4 substitution, a change of the configuration of Arg at position 8, or a combination of these changes significantly increased antioxytocic potency of the resulting analogues (**III, V, VII, IX, X**). The most potent antioxytocic peptides were obtained either by combination of Igl^2 modification with deamination of position 1 (analogue **III**, $pA_2 = 7.94$) or by deamination and the change of configuration of Arg at position 8 (analogue **X**, $pA_2 = 8.00$). [Mpa^1, Igl^2]AVP is thus a very potent and selective OT antagonist, while its Aic^2 counterpart is medium potent and not a selective blocker of OT

receptors. Analogues having D-Igl in position 2 are also exceptional as all of them show dual activity in this test – they showed high antioxytocic potency (pA_2 values ranging from 7.70 to 8.20) at low concentrations and full agonism at high concentrations. This phenomenon is difficult to explain.

The data of pharmacological tests on rats were supplemented by determination of the affinities of the analogues to the human OT receptors stably expressed on the HEK cells [27] using tritiated OT. The data are given in Table 2. The most potent oxytocic antagonist of the pure antagonist (compounds **I, III, V, VII, IX, X**), [Mpa^1, Igl^2, D-Arg^8]VP (pA_2 = 8.00), showed also the highest affinity to the OT receptors (affinity constant 188 nM). However, the affinity of compounds with D-Igl, which exhibited dual uterotonic activity displayed even higher binding affinity (values ranging from 35 to 209 nM). The substances with D-Igl have mostly by one order of magnitude higher K_i than their Igl containing counterparts. The high binding to human OTR points to the fact that the analogues should be active not only in rats but also in humans. The question is however whether they would be antagonists or agonists. The binding data to human OTR are not sufficient to explain the dual activity in the rat uterotonic test. More light for the explanation of this paradox might be shed by binding data using vasopressin ligands and vasopressin receptors. We plan to carry out such investigation in the future.

These, in our opinion interesting findings, demonstrate the usefulness of our approach to the SAR study and for the design of new highly active and selective analogues of AVP with desired pharmacological properties. These results are even more interesting when we recall our previous findings claiming that the presence of other aromatic amino acid residues in position 2 (3,3-diphenylalanine enantiomers, L-1-Nal enantiomers, 2-aminoindane-2-carboxylic acid [12–14]) has strong influence on pharmacological properties of the analogues, and when combined with additional substitution of positions 1, 4, and 8, may give peptides with favorable antidiuretic or antioxytocic activities. One of the examples is [Mpa^1, D-Dip^2]AVP which is an exceptionally potent and selective antidiuretic agent with significantly prolonged activity. On the other hand, the results presented in this paper are very similar to those obtained by modification of position 2 of AVP by L-1-Nal enantiomers. Analogues modified with L- or β-(1-naphthyl)-D-alanine (D-1-Nal) were potent or moderately potent OT antagonists *in vitro*, and displayed either no or low antidiuretic activity while their pressor potency was removed or had become converted into moderate antagonistic [12,29]. It is clear that introduction of Igl or Nal enantiomers into position 2 of AVP forces peptide backbone and side chains to adopt specific orientations that resulted in compounds having high antioxytocin potency. These results are encouraging to perform the structural analysis and molecular modelling of these peptides in order to explain similar pharmacological profile. Moreover, comparison of Aic^2 and Igl^2 substituted analogues of AVP has once more demonstrated that a slight difference in the structure could cause significant changes in biological activity. Our new Igl^2-substituted peptides proved that the presence of a less sterically restricted but bulky and aromatic amino acid residue may result in highly active and selective antioxytocic agents. It should be emphasized that one of the new peptides, [Mpa^1, Igl^2]AVP, has a very high antioxytocic potency. In the recent years, there has been increasing interest in OT antagonists, as it is thought that OT is involved in the initiation of the term and preterm labour. In 1995, Manning described a series of moderately potent and fairly selective antagonists of OT [30]. More recently, Flouret *et al*. designed novel bicyclic analogues derived from potent OT antagonists [31]. Other studies described the synthesis and biological activity of OT analogues containing conformationally restricted residues in position 7 [32]. All these studies resulted in several potent and selective antiuterotonic agents. From this point of view our finding that only a single modification of position 2 of AVP with Igl is sufficient to obtain selective and potent antagonists of OT is important.

On the basis of these results, we are undertaking further SAR studies on our best Igl^2 modified AVP analogues using NMR, CD, and theoretical molecular modelling methods. These methods may contribute to the explanation of the relations between the backbone structure and the functional group orientation and biological activity.

Acknowledgement

Partial funding for this work was provided by Polish State Committee for Scientific Research under the grant No. 0230/B/H03/2008/35 and 2385/B/HO3/2008/34 and by research project No. Z4055905 of the Academy of Sciences of the Czech Republic.

References

1 Barberis C, Tribollet E. Vasopressin and oxytocin receptors in the central nervous system. *Crit. Rev. Neurobiol.* 1996; **10**: 119–154.
2 Engelmann M, Wotjak CT, Neumann I, Ludwig M, Landgraf R. Behavioral consequences of intracerebral vasopressin and oxytocin: focus on learning and memory. *Neurosci. Biobehav. Rev.* 1996; **20**: 341–358.
3 Ring RH. The central vasopressinergic system: examining the opportunities for psychiatric drug development. *Curr. Pharm. Des.* 2005; **11**: 205–225.
4 Jard S, Barberis C, Audigier S, Tribollet E. Neurohypophyseal hormone receptor systems in brain and periphery. *Prog. Brain Res.* 1987; **72**: 173–187.
5 Gimpl G, Fahrenholz F. The oxytocin receptor system: structure, function, and regulation. *Physiol. Rev.* 2001; **81**: 629–683.
6 Barberis C, Morin D, Durroux T, Mouillac B, Guillon G, Seyer R, Hibert M, Tribollet E, Manning M. Molecular pharmacology of vasopressin and oxytocin receptors and therapeutic potential. *Drug News Perspect.* 1999; **12**: 279–292.
7 Manning M, Sawyer WH. Discovery, development, and some uses of vasopressin and oxytocin antagonists. *J. Lab. Clin. Med.* 1989; **114**: 617–632.
8 Lebl M, Jošt K, Brtnik F. *Tables of Analogs in Handbook of Neurohypophyseal Hormone Analogs*, vol. 2, part 2, CRC Press: Boca Raton, FL, 1987; 127–267.
9 Manning M, Bankowski K, Sawyer WH. Selective agonists and antagonists of vasopressin. In *Vasopressin, Principles and Properties*, Gash DM, Boer GJ (eds). Plenum Press: New York, 1987; 335–368.
10 Manning M, Grzonka Z, Sawyer WH. Synthesis of posterior pituitary hormones and hormone analogues. In *The Pituitary*, Beardwell C, Robinson G (eds). Butterworth: Kent, 1981; 265–296.
11 Andersson KE, Arner B. Effects of DDAVP, a synthetic analogue of vasopressin, in patients with cranial diabetes insipidus. *Acta Med. Scand.* 1972; **192**: 21–27.
12 Derdowska I, Prahl A, Kowalczyk W, Janecki M, Melhem S, Trzeciak HI, Lammek B. Influence of enantiomers of 1-naphthylalanine in position 2 of VAVP and DVAVP on their pharmacological properties. *Eur. J. Med. Chem.* 2005; **40**: 63–68.
13 Kwiatkowska A, Sobolewski D, Prahl A, Borovicková L, Slaninová J, Lammek B. Arginine vasopressin and its analogues – the influence of position 2 modification with 3,3-diphenylalanine enantiomers. Highly potent V_2 agonists. *Eur. J. Med. Chem.* 2009; **44**: 2862–2867.
14 Kowalczyk W, Sobolewski D, Prahl A, Derdowska I, Borovicková L, Slaninová J, Lammek B. The effects of N-terminal part modification of arginine vasopressin analogues with 2-aminoindane-2-carboxylic acid: a highly potent V2 agonist. *J. Med. Chem.* 2007; **50**: 2926–2929.

15 Gera L, Stewart JM. A new class of bradykinin antagonists containing indanylglycine. *Immunopharmacol* 1996; **33**: 174–177.
16 Stewart JM. *Solid Phase Peptide Synthesis*. Pierce Chemical Corporation: Rockford, 1984; 87.
17 Bodanszky M, Bodanszky A. *The Practice of Peptide Synthesis*. Springer-Verlag: Berlin, 1984; 83.
18 Kaiser E, Colescott RL, Bossinger CD, Cook PI. Color test for detection of free terminal amino groups in the solid-phase synthesis of peptides. *Anal. Biochem.* 1970; **34**: 595–598.
19 Christensen TC. A chloroanil color test for monitoring coupling completeness in solid-phase peptide synthesis. In *Peptides – Structure and Biological Function*, Gross E, Meienhofer J, (eds). Pierce Chemical Corporation: Rockford, 1979; 385.
20 Holton P. A modification of the method of Dale and Laidlaw for standardization of posterior pituitary extract. *Br. J. Pharmacol.* 1948; **3**: 328–334.
21 Rudinger J, Krejci I. Dose-response relations for some synthetic analogues of oxytocin, and the mode of action of oxytocin on the isolated uterus. *Experientia* 1962; **18**: 585–588.
22 Dekanski J. The quantitative assay of vasopressin. *Br. J. Pharmacol.* 1952; **7**: 567–572.
23 Slaninová J. Fundamental Biological Evaluation. In *Handbook of Neurohypophyseal Hormone analogs*, Vol. 1, Part 2, Jošt K, Lebl M, Brtník F (eds). CRC Press: Boca Raton, 1987; 83–107.
24 Burn HJ, Finney DJ, Goodwin LG. *Biological Standardization*, 2nd edn, Oxford University Press: London, 1950; 187.
25 Vávra I, Machová A, Krejčí I. Antidiuretic action of 1-deamino-8-D-arginine vasopressin in unanesthetized rats. *J. Pharmacol. Exp. Ther.* 1974; **188**: 241–247.
26 Fahrenholz F, Boer R, Crause P, Fritzsch G, Grzonka Z. Interactions of vasopressin agonists and antagonists with membrane receptors. *Eur. J. Pharmacol.* 1984; **100**: 47–58.
27 Gimpl G, Burger K, Fahrenholz F. Cholesterol as modulator of receptor function. *Biochemistry* 1997; **36**: 10959–10974.
28 Soloff MS, Swartz TL. Characterization of a proposed oxytocin receptor in the uterus of the rat and sow. *J. Biol. Chem.* 1974; **249**: 1376–1381.
29 Sobocińska M, Łempicka E, Konieczna E, Derdowska I, Lammek B, Melhem S, Kozik W, Janecka J, Janecki M, Trzeciak HI. Analogues of arginine vasopressin modified in position 2 or 3 with naphthylalanine: selective antagonists of oxytocin *in vitro*. *J. Pharm. Pharmacol.* 2000; **52**: 1105–1112.
30 Manning M, Miteva K, Pancheva S, Stoev S, Wo NC, Chan WY. Design and synthesis of highly selective *in vitro* and *in vivo* uterine receptor antagonists of oxytocin: comparisons with Atosiban. *Int. J. Pep. Protein Res.* 1995; **46**: 244–252.
31 Flouret G, Chaloin O, Borovickova L, Slaninova J. Design of novel bicyclic analogues derived from a potent oxytocin antagonist. *J. Peptide Sci.* 2006; **12**: 412–419.
32 Fragiadaki M, Magafa V, Borovickova L, Slaninova J, Cordopatis P. Synthesis and biological activity of oxytocin analogues containing conformationally-restricted residues in position 7. *Eur. J. Med. Chem.* 2007; **42**: 799–806.

