Structure-activity relationship of 1-desamino-8-D-arginine vasopressin as an antiproliferative agent on human vasopressin V2 receptor-expressing cancer cells

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Abstract. The synthetic nonapeptide 1-desamino-8-D-arginine vasopressin (dDAVP) can reduce tumor cell growth through agonist action on the vasopressin V2 receptor. A structure-antiproliferative activity relationship analysis of dDAVP was performed using the alanine scanning technique on the aggressive MDA-MB-231 human breast carcinoma cell line. The results from this analysis demonstrated that the amino acids located at the loop of dDAVP are important for the antiproliferative activity of dDAVP, highlighting the key role of the N-terminal region of the peptide in the interaction with the tumor cell surface receptor. The findings from this study present novel strategies for designing improved compounds with enhanced stability for cancer therapy.

Introduction

Desmopressin (1-desamino-8-D-arginine vasopressin, also known as dDAVP) is a synthetic derivative of the arginine vasopressin hormone, used clinically in the treatment of water imbalance and certain hemostatic disorders. Vasopressin is a cyclic nonapeptide with a disulfide bridge between residues cysteine[1] (Cys^1) and Cys^6, and a tail comprising residues seven to nine. Vasopressin has been extensively studied and modified in order to enhance its specificity and half-life for designing agonists and antagonists as potential therapeutic agents (1,2). Vasopressin mediates its action through three known receptors: $V1_a$, found in the vasculature, which mediates the pressor activity via the phospholipase C pathway; $V1_b$ in the anterior pituitary, which mediates adrenocorticotropic hormone release via the same pathway; and V2 in the renal collecting ducts, which mediates antidiuretic action via the adenylate cyclase pathway (2).

dDAVP contains two modifications with respect to natural vasopressin, including deamination of Cys^1, which improves the half-life and enhances the antidiuretic activity, and an L-arginine (L-Arg) to D-Arg substitution at position 8, which abolishes the vasopressor $V1_a$ receptor-dependent activity. The biological activity of dDAVP is thus selectively mediated through its interaction with V2 receptors in the kidneys, as well as in the microvasculature, inducing the secretion of coagulation factors. The presence of vasopressin V2 receptors has been reported in different cell lines and tumors, including breast cancer (3). In a previous study we demonstrated modest but significant antiproliferative effects of dDAVP on an experimental mammary carcinoma by agonist-V2 receptor interaction *in vivo* (4). Furthermore, perioperative administration of dDAVP during cancer surgery has been found to reduce the metastatic progression associated with the cytostatic and hemostatic effects of the compound (reviewed in 5).

The alanine-scanning technique (Ala-Scan) involves the sequential substitution of each amino acid in a lead peptide with an Ala residue in order to identify the amino acids that are critical for biological function (6). Ala is used in this technique since it is considered to be the most neutral amino acid, it has a simple side-chain, it is not highly hydrophobic and it has no charge. These features enable the structure-activity

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relationship of the peptide of interest to be determined (7). Ala-Scan has been used to investigate the structure-activity relationship of a number of potential therapeutic molecules and has been utilized to design analogs with enhanced activity and/or selectivity (8-10).

The aim of the present study was to identify key positions involved in the antiproliferative activity of dDAVP, using Ala-Scan analysis of the synthetic peptide. The identification of key residues involved in the antiproliferative activity of dDAVP may contribute to the rational design of improved antitumor compounds. Additionally, proline^{7} (Pro^{7}) was substituted with a hydroxyproline (Hyp) residue in order to analyze the effect of a polar amino acid near the positive charge of the peptide tail (Arg^{8}).

Materials and methods

Chemicals. Rink amide resin, fluorenylmethyloxycarbonyl (Fmoc)-amino acids and coupling reagents were obtained from Iris Biotech GmbH (Marktredwitz, Germany). Solvents for the peptide synthesis and purification were obtained from Tedia Company Inc. (Fairfield, OH, USA).

Analog design: dDAVP Ala-Scan. Positions 2-5 and 7-9 were sequentially substituted with Ala. Positions 1 and 6, involved in the disulfide-bridge formation, were not Ala-substituted in order to maintain the cyclic feature of the analogs (Table I). Additionally, the amino acid at position 7 was substituted by a Hyp residue (Table II).

Peptide synthesis. Peptides were synthesized in solid phase, using N^{α}-Fmoc protection, following the 'tea-bag' strategy as previously described by Houghten (11). Amide resin was used as the solid support in order to obtain the amide peptides. Cyclization of the peptides was performed on solid phase, taking advantage of the pseudo-dilution phenomenon, which favors intramolecular bridge formation (12). Briefly, (Trt)-3-mercaptopropionic acid and (Trt)-Cys were deprotected in a trifluoroacetic acid (TFA), dichloromethane and triisopropylsilane (TIS) (2:95:3) solution for 1 h at room temperature, following oxidation with 10 eq I_2 in N,N dimethylformamide for 30 min at room temperature. The peptides were then deprotected and cleaved from the resin using a TFA, H_2O and TIS (95:2.5:2.5) solution for 3 h at room temperature.

Peptide purification and quantification. Peptides were purified using reversed-phase high-performance liquid chromatography on an Ultrasphere ODS C-18 column (Beckman Instruments, Palo Alto, CA, USA) using a linear gradient of 10-40% acetonitrile in water containing 0.05% TFA for 30 min. Peptides were quantified using a commercial dDAVP standard from BCN Peptides (San Quinti de Mediona, Barcelona, Spain).

Peptide characterization. Peptides were identified using electrospray ionization-mass spectrometry in a LCQ-Duo (ion trap) mass spectrometer (Thermo Fisher Scientific, Inc., San José, CA, USA). Samples were introduced from a Surveyor pump (Thermo Fisher Scientific, Inc.) in a 40-μl/min solvent flow. Peptide analysis was performed by full scan spectra covering the mass range of 200-2,000 amu.

Theoretical molecular weights were calculated using the ProtParam tool from the Expasy server (available at http://www.expasy.org/tools/protparam.html).

Cell lines and culture conditions. The aggressive MDA-MB-231 human breast carcinoma cell line [American Type Culture Collection (ATCC) cat. no. HTB-26] was grown in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 80 μg/ml gentamycin, in a monolayer culture at 37˚C in a humidified atmosphere of 5% CO_2. The hormone-dependent MCF-7 cell line (ATCC cat. no. HTB-22), a well-differentiated breast carcinoma, was also used as a reference of V2 receptor-positive cells.

Vasopressin receptor expression. The expression of V2 receptor was investigated using reverse transcription-polymerase chain reaction (RT-PCR). Primers corresponding to the mRNA sequences (GenBank accession no. NM000054.4; gi:225903383) were as follows: 867-886, forward 5'-GTG GCC AAG ACT GTG AGG AT-3' and 1,356-1,375 reverse, 5'-ATA CAG CTG GGG ATG TGG AG-3'.

In vitro growth assays. The antiproliferative effects of dDAVP and the peptide analogs were analyzed against rapidly growing breast cancer cells. A range of concentrations between 100 nM and 1.5 μM was used with a three-day exposure of log-phase growing cells. MDA-MB-231 cells were seeded on 96-well plates (2.5-5x10^3 cells/well) in DMEM plus 10% FBS. After 24 h, peptides were added and the cells were cultured for a further 72 h, prior to being analyzed using the MTT assay. Additionally, the cytostatic effects of dDAVP were verified at low cell density using the colony formation assay. Cells were plated at 0.8-1.2x10^3 cells/well in 24-well plates and grown for seven days in complete medium in the presence of varying concentrations of dDAVP. Cultures were then fixed with formalin and stained with crystal violet, and colonies were counted. The 50% inhibitory concentration (IC50) was determined by plotting the percentage of cell colonies versus drug concentration.

Results

dDAVP analogs and their effect on proliferation and colony formation. The features of the synthetic peptides are summarized in Table I and II. The expression of vasopressin V2 receptor in the aggressive MDA-MB breast carcinoma cell line-231 was first verified. As shown in Fig. 1A, the expected fragment of 508 bp was observed in the cells using RT-PCR. Well-differentiated MCF-7 cells also expressed the receptor, as previously reported (3). The antiproliferative effects of dDAVP on log-phase growing cells were then investigated. Following exposure to dDAVP for 72 h, a modest but significant inhibition of cell growth was observed at doses of ≥100 nM (Fig. 1B). Higher doses (1 or 1.5 μM) reduced proliferation in breast cancer cell cultures by ~20%. By contrast, dDAVP had a stronger effect on colony formation at low cell density (Fig. 1C), with IC50 values of 1.44 μM (R^2=0.96; P=0.0033) and 0.97 μM (R^2=0.98; P=0.0012) against MDA-MB-231 and MCF-7 cells, respectively.

Table I. dDAVP Ala-Scan: Name, sequence and physicochemical features of dDAVP and its analogs.

Peptide	Sequence	Retention time (min)	MW in Da, experimental (theoretical)
dDAVP	MpaYFQNCPrG-NH_2	24.7	1068.45 (1068.24)
[Ala^2] dDAVP	Mpa<u>A</u>FQNCPrG-NH_2	20.3	976.40 (976.14)
[Ala^3] dDAVP	MpaY<u>A</u>QNCPrG-NH_2	15.5	992.41 (992.14)
[Ala^4] dDAVP	MpaYF<u>A</u>NCPrG-NH_2	26.3	1011.54 (1011.18)
[Ala^5] dDAVP	MpaYFQ<u>A</u>CPrG-NH_2	28.1	1025.45 (1025.21)
[Ala^7] dDAVP	MpaYFQNC<u>A</u>rG-NH_2	23.7	1042.58 (1042.20)
[Ala^8] dDAVP	MpaYFQNCP<u>A</u>G-NH_2	24.9	983.40 (983.13)
[Ala^9] dDAVP	MpaYFQNCPr<u>A</u>-NH_2	25.3	1082.49 (1082.26)

The underlined letter indicates the position at which an amino acid has been substituted with A. A, alanine (Ala); Y, tyrosine (Tyr); F, phenylalanine (Phe); Q, glutamine (Gln); N, asparagine (Asn); C, cysteine (Cys); P, proline (Pro); r, D-arginine (D-Arg); G, glycine (Gly); dDAVP, 1-desamino-8-D-arginine vasopressin; Mpa, 3-mercaptopropionic acid; MW, molecular weight.

Table II. dDAVP Hyp analog: Name, sequence and physicochemical features of [Hyp^7] dDAVP.

Name	Sequence	Retention time (min)	MW in Da, experimental (theoretical)
dDAVP	MpaYFQNCPrG-NH_2	24.7	1068.45 (1068.24)
[Hyp^7] dDAVP	MpaYFQNC<u>Hyp</u>rG-NH_2	22.7	1084.55 (1084.24)

The underlined letter indicates the position at which an amino acid has been substituted with Hyp. Y, tyrosine (Tyr); F, phenylalanine (Phe); Q, glutamine (Gln); N, asparagine (Asn); C, cysteine (Cys); P, proline (Pro); Hyp, hydroxyproline; r, D-arginine (D-Arg); G, glycine (Gly); dDAVP, 1-desamino-8-D-arginine vasopressin; Mpa, 3-mercaptopropionic acid; MW, molecular weight.

Identification of key residues in dDAVP. The structure antiproliferative activity relationship of dDAVP was performed on the aggressive MDA-MB-231 cell line. The peptides concentration was 1 μM, which exerts a significant antiproliferative effect of dDAVP on log-phase growing cells. The results from the Ala-Scan demonstrated the importance of the amino acids located at the loop of dDAVP for antiproliferative activity. The activity was reduced by up to 60% when amino acids 2-5 were substituted (Fig. 2). A similar profile was observed at lower doses of dDAVP (250 nM) in the colony formation assay (data not shown). The cytostatic effect on log-phase growing cells was conserved when amino acids in positions 7 and 8 were substituted and partially reduced when the substitution occurred at position 9 (Fig. 2). Similarly, a polar substitution at position 7 by Hyp had no effect on the antiproliferative activity of the resultant analog (data not shown).

As mentioned, the results from the Ala-Scan demonstrated the importance of the amino acids located at the loop of dDAVP for the antiproliferative activity (Fig. 2). The antiproliferative activity was reduced by 30-60% when amino acids 2-5 were substituted, while the activity was conserved when the amino acids Pro^7 and D-Arg^8 were substituted. The activity was also reduced 30% when $glycine^9$ was substituted. Consistent with these results, Table III shows the classification of the residues of dDAVP into three groups according to the reduction in antiproliferative activity of the resultant analog.

Discussion

The structure-antiproliferative activity relationship of dDAVP was assessed in the present study using Ala-Scan on MDA-MB-231 breast carcinoma cells. The analysis highlighted the important role of the amino acids located in the peptide loop (Table III), and suggested that amino acids 2, 3 and 5 were crucial for the agonistic interaction of dDAVP with the vasopressin V2 receptor, as proposed in a previous molecular modeling study (13). This interaction led to the antitumor effects on V2 receptor-expressing breast cancer cells. Substitution of residues 4 and 9 only partially reduced the antiproliferative activity of dDAVP. By contrast, the antiproliferative activity of the peptide was unaffected by the substitution of residues 7 and 8, located at the tail of the peptide, and the same was observed when residue 7 was substituted by a Hyp residue.

In a recent study, we reported that the analog 4-valine-5-glutamine-dDAVP (known as [Val^4Gln^5]dDAVP) was a potent agonist for the vasopressin V2 receptor in MCF-7 cell cultures (14). Glutamine^4 (Gln^4) is located in the loop of the dDAVP, and is the best candidate to be substituted by a hydrophobic residue. As hypothesized by Manning *et al* (1), enhancing hydrophobicity at position 4 improves the interaction of vasopressin analogs with the V2 receptor; thus, a Gln by valine (Val) substitution was introduced. In a separate study,

Table III. Classification of dDAVP residues according to their role in the antiproliferative activity of the compound.

Group	Residues	Antiproliferative activity reduction (%)	Feature
I	2, 3, 5	50-60	Crucial for antiproliferative activity
II	4, 9	30	Tolerant to substitution
III	7, 8	0-5	Unrelated to antiproliferative activity

dDAVP, 1-desamino-8-D-arginine vasopressin.

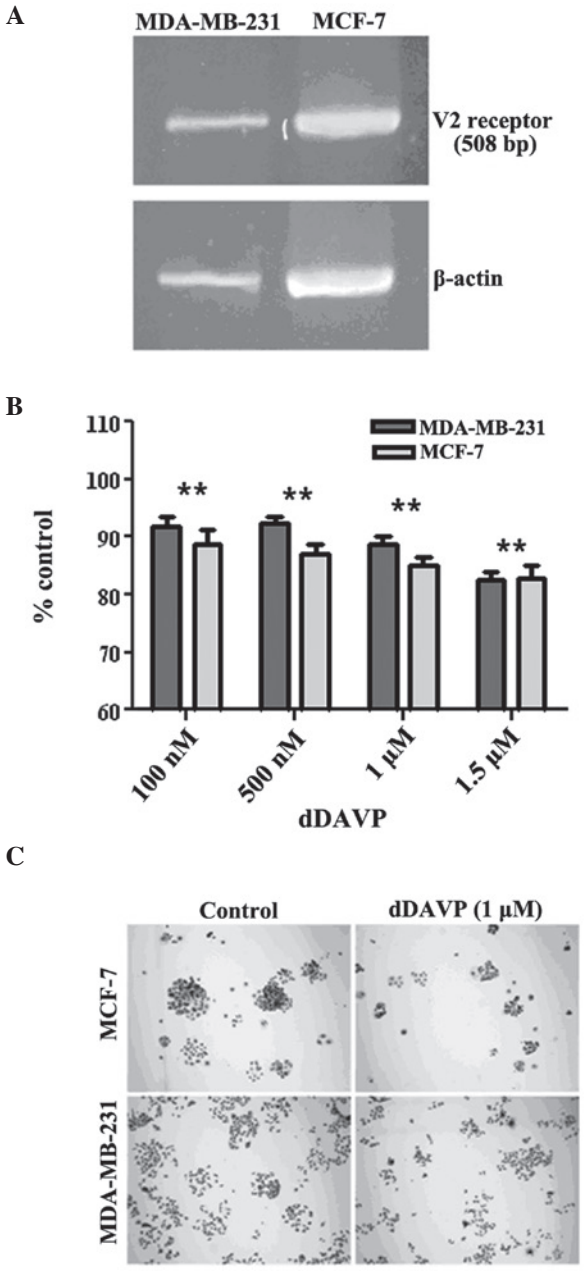

Figure 1. Cytostatic activity of dDAVP in vasopressin V2 receptor-expressing human breast cancer cell lines. (A) Reverse transcription-polymerase chain reaction for V2 receptor expression. β-actin was used as an internal control for gene expression. (B) Effect of 72-h dDAVP exposure on log-phase growing cells. Data are presented as the mean ± standard error. **P<0.01, versus control; analysis of variance plus Dunnett's test. Results are representative of at least three independent experiments. (C) Effect of seven-day dDAVP treatment on the colony formation at low cell density (magnification, x40). dDAVP, 1-desamino-8-D-arginine vasopressin.

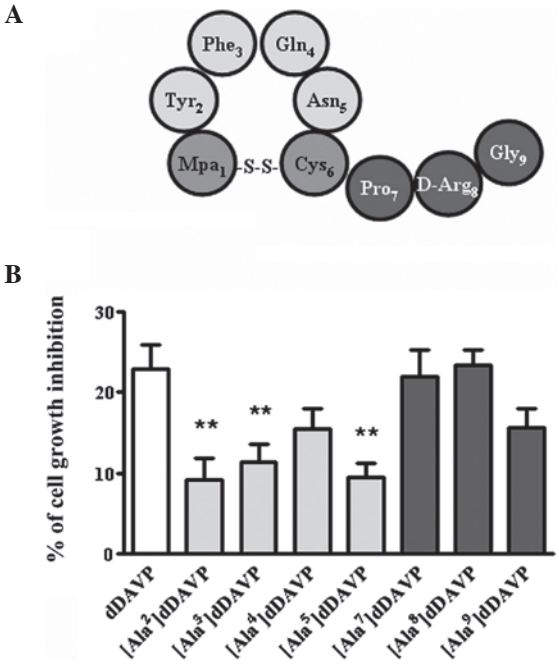

Figure 2. Ala-Scan analysis of dDAVP in MDA-MB-231 breast cancer cells. (A) Schematic representation of dDAVP. (B) Growth inhibition following a 72-h exposure to dDAVP on log-phase growing cells at a peptide concentration of 1 μM. Data are presented as the mean ± standard error. **P<0.01, versus dDAVP; analysis of variance plus Dunnett's test. Mpa, 3-mercaptopropionic acid; Tyr, tyrosine; Phe, phenylalanine; Gln, glutamine; Asn, asparagine; Cys, cysteine; Pro, proline; D-Arg, D-arginine; Gly, glycine; dDAVP, 1-desamino-8-D-arginine vasopressin.

Manning *et al* (2) also found that [Val^4]dDAVP has a 10-fold higher affinity for the human V2 receptor than dDAVP (2). In order to improve the stability of the analog, we therefore introduced a conservative substitution at position 5, replacing asparagine with Gln, based on its distinctive susceptibility to the deamidation process, which resulted in the cytostatic analog [Val^{4}Gln^5]dDAVP (14).

The results from the present study are in accordance with those from previous studies with regard to the key role of the N-terminal region of the molecule (loop) in the physiological activities of the neurohypophyseal hormone vasopressin (1,2,15). In the present study, it was demonstrated that there is a close relationship between the loop of dDAVP and its antiproliferative activity, as assayed on MDA-MB-231 cells. This knowledge may aid the development of novel strategies for the design of dDAVP analogs with improved antitumor properties. V2 receptor expression has been found in a number

of types of human cancer, including breast (16) gastrointestinal (17) and small cell lung (18) cancer. Furthermore, it is known that dDAVP exerts a specific effect on V2 receptors present in microvascular endothelial cells, and therefore induces a rapid release of multimeric forms of von Willebrand factors *in vivo*. Such hemostatic factors have a protective role against tumor cell dissemination, by causing the death of metastatic cells early after their arrest at the target organ (19,20).

The key roles of the loop of dDAVP and its improved analog [Val^4Gln^5]dDAVP warrant further investigation, including the synthesis and analysis of the cyclic fragments of both peptides (residues 1-6) to determine the minimal active sequences. These active sequences may be introduced in highly stable scaffolds, such as cyclotides, which are cyclic and knotted peptides with extreme thermal, chemical and enzymatic stability (21,22). This hypothesis is likely to be the starting point to encourage the design of cytostatic peptide compounds with oral bioavailability.

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