Research Article

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Future Medicinal Chemistry

Search of vasopressin analogs with antiproliferative activity on small-cell lung cancer: drug design based on two different approaches

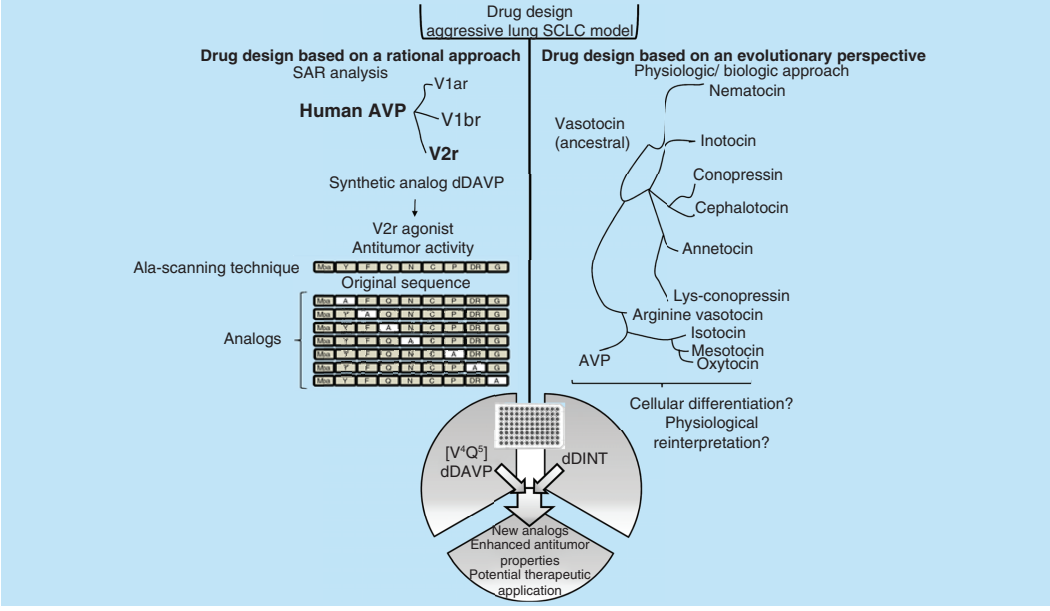
Marina Pifano[1], Juan Garona[1], Natasha T Sobol[1], Marina Albertó[1], Daniel F Alonso[1] & Giselle V Ripoll*,[1]

[1]Laboratory of Molecular Oncology, Quilmes National University, Buenos Aires, 1876, Argentina

* Author for correspondence: Tel.: +54 114 365 7100; gripoll@gmail.com

Aim: Development of compounds with therapeutic application requires the interaction of different disciplines. Several tumors express vasopressin (AVP; arginine vasopressin) receptors with contrasting effects depending on receptor subtype. Desmopressin (dDAVP) is an AVP-selective analog with antiproliferative properties. In this work, an evolutionary approach and a rational strategy were applied in order to design novel AVP analogs. **Results:** We designed two novel analogs; dDInotocin (dDINT, insect analog), and [V^4Q^5]dDAVP, and demonstrated the importance of the dDAVP conformational loop for its antiproliferative activity. [V^4Q^5] dDAVP showed major cytostatic effect on lung cancer cells than dDAVP and its cytostatic effect was abolished by V2R blockade. **Conclusion:** Combination of these strategies could provide the basis for future studies for the development of improved compounds with potential therapeutic applications.

Graphical abstract:



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The diversity in nature has long been and still is one of the biggest sources of pharmacological lead compounds. A great number of natural peptides are present in all organisms from microbes to man. Evolutionary pressures have provided an optimized and sophisticated collection of disulfide-rich peptides that have been produced in a combinatorial manner over millions of years. Many natural peptidic products often display biological activity against unrelated biological targets. Among these peptides we find a very large number of agonists and antagonists, which act on functionally diverse targets as ion channels, transporters and G-protein-coupled receptor super/family (GPCRs) [1]. Knowledge of these products and their evolutionary history can enrich the physiological interpretations and the understanding of the mechanisms of action of peptides, representing a strategy for the search of new lead drug design [2].

In addition to the evolutionary approach there are other compound search strategies. Some methods are based on the screening of compound libraries, in which the search strategy is based exclusively on chance. In contrast other approaches involve rational drug design, where there is knowledge of the target at a molecular, functional and structure level, and each element in the design of the drug is substantiated. The alanine-scanning technique (Ala-Scan) is widely used for the study of structure–activity relationship (SAR) of many potential therapeutic molecules and for designing analogs with improved activity and/or selectivity [3,4].

Vasopressin (arginine vasopressin; AVP) is a multifunctional and highly conserved nonapeptidic hormone synthesized primarily in the hypothalamus. AVP is related evolutionarily and structurally with the oxytocin hormone (OT) and these nonapeptides are thought to have originated from the ancestral hormone vasotocin (VT). All VT/AVP/OT-like peptides present in many different species of vertebrates and invertebrates, share a similar structure: a disulfide bridge between the conserved residues Cys1 and Cys6 forming a cycle, and a C-terminal tail comprising residues 7–9. They are products derived from larger precursors and characterized by a high degree of interspecies conservation, existing VT/AVP/OT-like peptides in the entire animal kingdom with multiple physiologic activities [5]. Dating back >600 million years in evolution [6,7], AVP/OT peptide ligands and their receptors comprise one of the oldest and best-studied peptide GPCR signaling systems. In the field of comparative endocrinology and neurophysiology, it has been an interesting model system. GPCRs constitute the largest known protein family of cell surface receptors regulating a wide spectrum of cellular processes by signal transduction. They have been adapted to bind to a large variety of ligands; the range of endogenous agonists comprises lipids, sugars, volatile organic compounds, amino acids, organic acids and peptides among others [8].

The main effect of AVP in humans is of endocrine character, but it also exerts autocrine and paracrine activities when is secreted locally by normal and pathological tissues [9]. The effects of AVP are mediated by three different GPCRs: the AVP receptors type 1 (V1r or V1AR), type 2 (V2R) and type 3 (V3R or V1BR) [10,11]. V2R are expressed in the collecting ducts of the kidney and in alveolar vascular endothelium epithelial cells, regulating hydrosaline balance and hemostasis, respectively [12]. Although these three receptor subtypes are thought to represent all AVP receptors, a protein named VACM-1 (AVP-activated calcium-mobilizing protein) has been identified as a putative AVP receptor of rabbit medullary kidney cells [13]. There is wide evidence of the expression of AVP and their receptors (V1R and V2R) in several types of cancer such as breast [14,15], pancreatic, colorectal, gastrointestinal [16,17] and small-cell lung cancer (SCLC) [18]. Additionally, it was reported that SCLC and breast cancer cells can express a putative human VACM-1 receptor (HVACM) [18–20]. Despite the fact that V1R and V3R are associated with the stimulation of cellular proliferation, V2R are related to cytostatic effects [15], and VCAM protein seems to inhibit cellular growth by a mechanism that involves MAPK and p53 signaling pathways [21].

With the aim of developing potential therapeutic agents AVP has been extensively studied and modified resulting in novel derivatives with agonist or antagonist activity. From these studies emerged the well-known desmopressin (dDAVP), a synthetic peptide analog of AVP that acts as a selective agonist for the V2R. dDAVP has two modifications when compared with natural AVP, including deamination of Cys1 and an L-Arg ((S)-2-amino-5-guanidinopentanoic acid) by D-arg ((R)-2-amino-5-guanidinopentanoic acid) substitution at position 8. The deamination improves the half-life and enhances the antidiuretic activity, while the substitution at position 8 abolishes the V1R-dependent vasopressor activity. The biological activity of dDAVP is thus selectively mediated through its interaction with V2R-stimulating water absorption in the kidney and release of hemostatic factors from microvasculature [22]. Given its effects, dDAVP is used for the treatment of water imbalance, hemostatic disorders and surgeries with high risk of bleeding [23].

In our laboratory, we reported that dDAVP inhibits the proliferation and migration in breast, lung and prostate cancer cells [24,25] and drastically impairs metastatic spread of aggressive breast [26] and colon cancer [27]. We also demonstrated that perioperative dDAVP treatment dramatically reduces lymph node and lung metastasis in a mouse model of mammary tumor manipulation and surgical excision [26]. Given its antitumor effect and hemostatic properties, dDAVP appears as a promising lead compound for the development of novel peptide analogs with enhanced anticancer activity. In the search of new analogs we have previously synthesized and studied a wide panel of derivatized peptides, including different tetrapeptides (YFQN, YFAQ and YFVQ), new cyclic variants ([A^4Q^5] dDAVP, [V^4Q^5] dDAVP), conformational isomers ([v^4Q^5] dDAVP), etc. This screening resulted not only in promising analogs with antiproliferative activity but also in interesting findings regarding peptide minimal sequence requirements and key structural features for biological activity. Differences in effects between tetrapeptides and parental molecules, particularly at high concentrations, may be due to a loss of the electrostatic interaction between the extracellular portion of V2R and the loop present in the parental molecules, but absent in the tetrapeptides [24,28].

Interestingly, in some cases, a transformation from non-small-cell lung cancer (nSCLC) to SCLC occurs and this neuroendocrine (NE) differentiation is associated with poor treatment response and drug resistance [29]. Nowadays targeted therapies for well-differentiated and slow-growing NE tumors, like NE gastroenteropancreatic, are interferon, somatostatin analogs, VEGF and mTOR inhibitors, while chemotherapy is reserved for poorly differentiated and progressive tumors as SCLC, showing the need of novel therapeutic approaches for this type of tumor [30].

Given its characteristics and the few therapeutic options available, SCLC is a valuable and clinically relevant model of recurrent and drug-resistant disease.

Thus, the aim of this work was to develop and compare novel AVP analogs with enhanced antiproliferative activity in lung cancer obtained by two different methodological approaches for drug design. The first method consisted in exploring bioactive peptides from natural sources focusing on evolutionary processes, paying special attention to the physiological activity. The second approach consisted in conducting SAR studies to identify key amino-acidic positions in the lead compound for its biological activity by the Ala-Scan technique on parental compound dDAVP using cytostatic potency as activity criteria in highly aggressive SCLC cells with NE features.

Materials & methods

Experimental cell lines model

Tumor cell lines were obtained from the American Type Culture Collection. Human aggressive variant with NE features SCLC NCI-H82, (HTB-175) and nSCLC NCI-H125 (CRL-5801) cell lines were grown in RPMI-1640 (Gibco, MD, USA) medium. Human breast carcinoma MCF-7 (HTB-22) cell line was grown in DMEM (Gibco). Cell lines were supplemented with 10% fetal bovine serum and antibiotic (2 mg ml^{-1} glutamine and 80 μg ml^{-1} gentamicin), at 37°C in a humidified atmosphere of 5% CO_2.

Peptide compounds

Drug design based on an evolutionary perspective

We performed a bibliographic review of the molecular evolution of the VT/AVP/OT to gain an insight of the mechanism of receptor–ligand in animals and to design a novel analog with a physiological criterion of biologic activity related to biological key events occurring in cancer. The analog was developed combining the natural peptide with the corresponding changes at positions 1 and 8 present in the first-generation peptide dDAVP. The design analog was: 1-deamino-2-leucine-3-isoleucine-4-thronine-8-D-AVP (dDInotocin; dDINT; MpaLITNCPrG*) and was purchased from American Peptide Company, Inc. (CA, USA; a member of Bachem group). Certificate of analysis: 958.1 atomic mass unit (amu); electrospray exhibits correct MW (MS spectrum); peptide purity 98.4%.

Drug design based on a rational approach by Ala-Scan technique

In order to maintain the cycle analogs positions 2, 3, 4, 5, 7, 8 and 9 were Ala-substituted in dDAVP. Positions 1 and 6 were not Ala-substituted (Supplementary Table). Ala-scanned peptides were synthesized and purified as described elsewhere [31]. Peptide analysis was performed by full scan (200–2000 amu). Theoretical MWs were calculated with the Protparam tool from the Expasyserver [32] as described elsewhere [31]. The resulting peptide was 1-deamino-4-valine-5-glutamine-8-D-AVP (known as [V^4Q^5] dDAVP; MpaYFVQCPrG*) and was purchased

from American Peptide Company, Inc. Certificate of analysis: 1054.2 amu; electrospray exhibits correct MW (MS spectrum); peptide purity 98.4%.

Expression of V2R

We evaluated the V2R expression using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA of NCI-H82, NCI-H125 and MCF-7 was purified from 1×10^6 cells with Trizol. cDNA was obtained with SuperScript III first-Strand (Thermo Fisher Scientific, Inc., CA, USA) according to the manufacturer's protocol. The primers used were: for V2R:5-CTGGCCAAGGACACTTCATC-3 and 5-GAAGGCAGCTGAGCTTC-3; for glyceraldehyde 3-phosphate dehydrogenase: 5-CATGGGTGTGAACCATGAGA-3 and 5-CAGTGATGGCATGGACTGTG-3.

qRT-PCR was performed under the conditions described by our group [25]. Threshold cycle (Ct) values were normalized for housekeeping gene expression levels, glyceraldehyde 3-phosphate dehydrogenase, and normalized to MCF-7 cells (control samples). The analysis was performed as detailed in Pifano *et al.* [25].

V2R expression in NCI-H125, MCF-7 and NCI-H82 was confirmed by immunofluorescence as described previously [33]. Samples were examined using a TE-2000 fluorescence microscope (Nikon, Inc., Tokyo, Japan). Cultures of MCF-7 human breast carcinoma cells were used as a positive control of V2R expression and NCI-H15 as a negative control.

Cell proliferation assay

Effect on cellular proliferation of AVP ([Arg^8]-Vasopressin acetate salt; Sigma-Aldrich, Buenos Aires, Argentina) and analogs was measured on rapidly growing tumor cells using the cell proliferation assay of MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]; Promega, WI, USA). Briefly, cells were plated in 96-well flat bottom plates at a density of 5×10^3 cells/well in 200 µl of RPMI-1640 supplemented with 10% fetal bovine serum, and then treated for 72 h with AVP, dDAVP, Ala-substituted analogs and new analogs, using peptide concentrations (100 and 1000 nM) with a significant antiproliferative effect or vehicle. MTS reagent (20 µl) was added to each well and the plate was incubated at 37°C for 2 h. The absorbance was measured at 490 nm. The optical density of control cells was considered as 100% viability.

Blockage of cytostatic effect by the V2R antagonist tolvaptan & silencing of V2R on NCI-H82

To study the selectivity of [V^4Q^5] dDAVP to V2R a chemical blockade of targeted receptor was achieved by incubation with selective and competitive V2R antagonist tolvaptan (1500 nM; Sigma-Aldrich). We also interfered with the expression of the target receptor using V2R siRNA as described by Pifano *et al.* (2017). Sequences of V2R siRNA used were detailed in Pifano *et al.* [25]. The silencing was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) following manufacturer's instructions. On day 5, after two rounds of transfection, 5000 cells were treated with several concentrations of [V^4Q^5] dDAVP for 72 h. Total RNA of NCI-H82 was purified from 1×10^6 cells and a qRT-PCR was performed.

Statistical analysis

Prism 6 software was used to evaluate statistic significance of the results (GraphPad, Inc., CA, USA). The data are presented as mean values ± standard error of the mean or mean value ± CI. Shapiro–Wilk normality test and Bartlett's test were used to evaluate the normal distribution and the homoscedasticity of data, respectively. To compare more than two experimental groups one-way ANOVA (followed by Tukey's post-test) or two-way ANOVA (followed by mean 95% CI comparison or Tukey's pos-test) were performed. Results correspond to at least three independent experiments (n = 6). Statistically significant differences were considered as $p < 0.05$.

Results & discussion

Validation of molecular target V2R in aggressive lung cancer NCI-H82 cells

Aggressive SCLC tumors with an extremely fast expansion and rate of growth are adequate for studying novel therapeutic compounds. Most SCLC cells are derived from metastatic sites or pleural effusions, representing a drug-resistant disease [34]. The expression of different AVP receptors on several types of cancer, highlights the multifaceted role of AVP, and its interesting pathophysiological implications [11]. V2R is associated with antiproliferative signaling, involving activation of adenylate cyclase followed by intracellular cyclic AMP elevation [35].

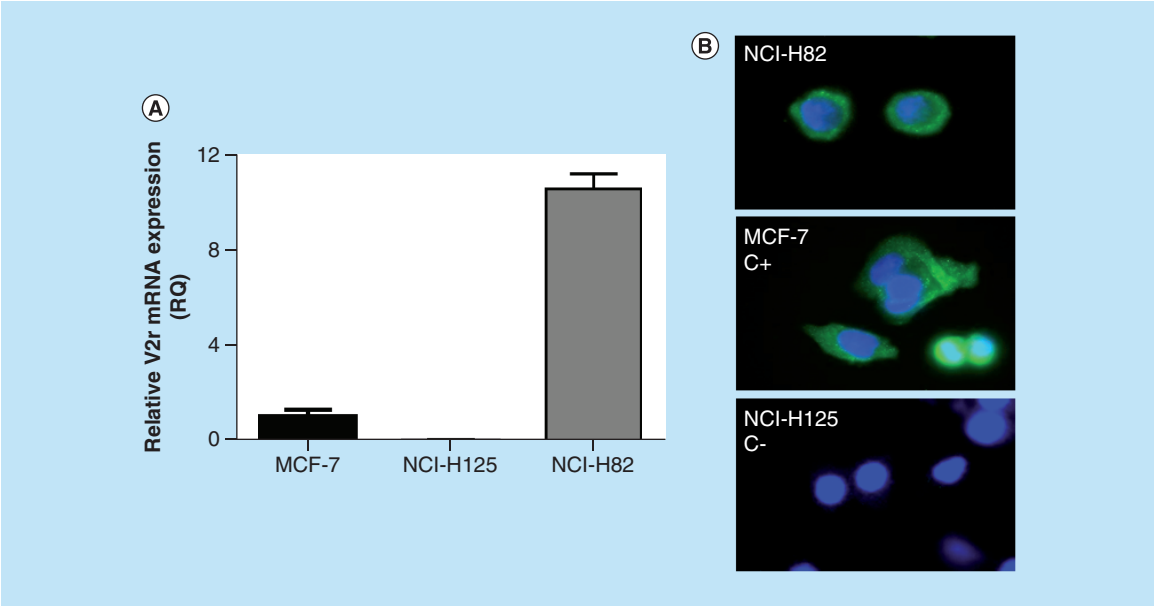

Figure 1. Expression of V2R in the NCI-H82 lung cancer cell model. (A) qRT-PCR: Ct values for V2R were normalized for glyceraldehyde 3-phosphate dehydrogenase expression levels and expressed in relation to MCF-7 (control samples). Results are expressed as mean ± standard error of the mean. **(B)**. Immunofluorescence, V2R expression was detected using a specific anti-V2R primary antibody and a secondary antibody with fluorescein (FITC)-conjugated goat anti-rabbit IgG. NCI-H125 human non-small-cell lung cancer cells were used as a negative control and MCF-7 human breast carcinoma cells as a positive control for the receptor. Original magnification × 1000.

In order to evaluate the expression level of V2R in NCI-H82 cells, a qRT-PCR was conducted. Results showed that NCI-H82 cells express high levels of V2R. MCF-7, a breast cancer cell line that displays all AVP membrane receptors, including V2R, was used as positive control. As shown in Figure 1A, NCI-H82 expresses ten-times more V2R mRNA in comparison to MCF-7. Receptor expression in NCI-H82 was confirmed by immunofluorescence detection (Figure 1B). Human nSCLC cell line, NCI-H125 was used as a negative control [25].

Evaluation of proliferative activity of nonselective neuropeptide AVP & V2R selective analog dDAVP on NCI-H82 cells

In order to evaluate the impact of agonistic intervention on different AVP receptors on cancer cell behavior we tested the effect of the natural ligand AVP and the synthetic analog dDAVP on log-phase growing lung cancer cells. Our results show, by an MTS metabolism assay, that after 72 h exposure, AVP, that has the capacity to bind to all AVP receptors subtypes, significantly increased proliferation by 14%. Although Fay *et al.* described that could be a disruption in V1AR activation cascade in NCI-H82 cells evidenced by the absence of AVP-induced calcium mobilization our findings report a biological effect using a much higher concentration than the one used by the group of Fay [36]. The concentrations used in this work (100 and 1000 nM) were chosen because these showed previously antiproliferative effect; these concentrations were reported in a number of publications both *in vitro* and *in vivo* assays [24–27]. Mitogenic effects of natural ligand AVP were greater at low peptide concentrations (Figure 2). These results indicate that the natural peptide could act as a growth factor in these malignant cells under the conditions described here enhancing transcription, cell proliferation and survival [37]. Interestingly, V2R-selective agonist dDAVP significantly reduced proliferation by 23% at a 1000 nM concentration (Figure 2). These results are in accordance with our previous results and the ones reported by others where the V2R agonist analog dDAVP displays antitumor properties in breast and colorectal preclinical cancer models [24,27]. This compound seems to disrupt cooperative interactions between tumor and endothelial cells during tumor progression, inhibiting angiogenesis and metastasis spread [27,28,33,38].

Due to its interesting antitumor effect in animal studies [39,40] and its known hemostatic properties [12], a prospective, open-label Phase II clinical trial evaluating the perioperative use of dDAVP in breast cancer patients (NCT01606072) [41] was carried out obtaining satisfactory results regarding safety, decrease of bleeding and

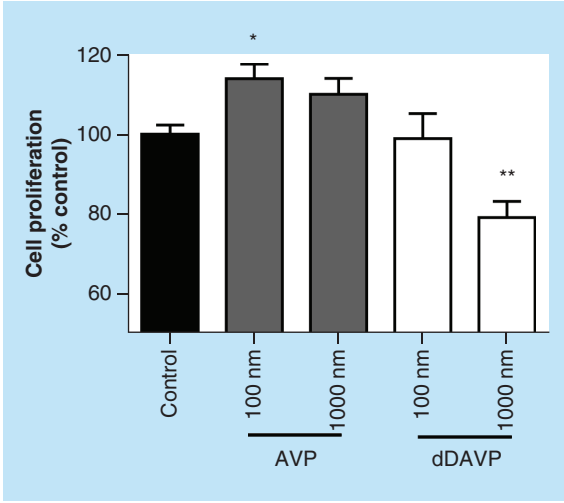

Figure 2. Effect of nonselective peptide arginine vasopressin and V2R-selective analog desmopressin on cellular proliferation on aggressive lung cancer cells. Effects of arginine vasopressin and desmopressin (100 and 1000 nM) on log-phase growing NCI-H82 cells were measured using the MTS assay. Control cells were considered as 100% viability. Results are expressed as mean ± standard error of the mean. One-way ANOVA followed by Tukey's comparison post hoc. *$p < 0.05$, **$p < 0.01$ (corresponding to each analog concentration vs its control).

preliminary anticancer effects (reduction of circulating tumor cells [CTC]) at 24 h post surgery). Taken these data into account, compounds like dDAVP, which specifically activate receptors involved in the regulation of key biological events in tumor cells, are much appreciated as lead compounds for the development of new antitumor derivatized peptides.

Drug design based on an evolutionary perspective

The physiological complexity, evolutionary conservation and, above all, the therapeutic relevance of VT/AVP/OT receptors make this family of receptors one of the most studied GPCRs [8]. The VT/AVP/OT neuropeptidic system is one of the oldest and evolutionary conserved. AVP-related neuropeptides have been found in a wide number of organisms, originating before the split of proto- and deuterostomia [42] and are thought to have evolved via gene duplication from the ancestral VT peptide some 600–700 million years ago. VT-like peptide hormones have been found in invertebrates (molluscs, annelids, arthropods and nematodes), nonmammalian vertebrates and mammals, including the *homo sapiens* [33]. The VT/OT/AVP peptide family share high sequence similarity, with six residues forming a cycle by a disulfide bond between the cysteines residues 1 and 6, and a C-terminal flexible tail with three residues, a highly conserved Pro at position 7 and a glycine amide at position 9 (CXXXXCPXG*). Positions 2 and 3 are hydrophobic or aromatic residues, and positions 4 and 5 are polar or charged residues; these shape the peptide cycle. Positions variability in the different VT-like peptides is observed at positions 2–5 and at position 8 [43,44]. All VT/AVP/OT peptides of vertebrates have Tyr in position 2, Pro in position 7 and all forms of VT/AVP have a basic residue at 8 (Arg or Lys), indicating that these might be preserved positions. Thus, there are only five or six positions at which a change might be tolerated.

Neurohypophyseal arginine VT is the ancestral neuropeptide homologous to OT/AVP in vertebrates [45,46] and appears in Agnatha. Several VT/AVP/OT like peptides are found in others vertebrates: isotocin–mesotocin-OT line concerned with reproduction in addition to arginine VT (*Chondrichthyes*, *Actinopterygii*, *Amphibia*, *Reptilia* and *Aves*) and AVP (*Mammalia*) [47].

In invertebrates, VT/AVP/OT signaling system has been isolated from several species. In annelid and nematoda OT-related peptides were also identified [48–50]. In molluscs, cephalotocin, sepiatocin and conopressin are present [51,52]. Different types of inotocin (INT) were found in insects [53]. Gruber *et al.* discovered the INT sequences and their possible receptors in the genome of several species of ants [43], with amino-acid variations in positions 2 and 4. The receptor sequence in ants also shows a high similarity with other insects, such as the beetle [53,54], suggesting that they may also have a similar function. This neuropeptide system, within the basal orders of Holometabola (complete metamorphosis), is limited to Coleoptera and others insects basal groups [55]. The INT system appears to be restricted to basal holometabolus insects, whereas the other higher orders have lost it. In the beetle, *Tribolium castaneum*, the INT receptor is mainly expressed in the head and in the Malpighian tubules or hindgut (low expression here), thus that INT does not stimulate water reabsorption in insects as does mammalian AVP [56]. The INT might play a key role in the development of the animal due to the INT receptor has high expression in the early larval stages, in eggs and pupae [53,57]. Aikins *et al.* propose that INT stimulates

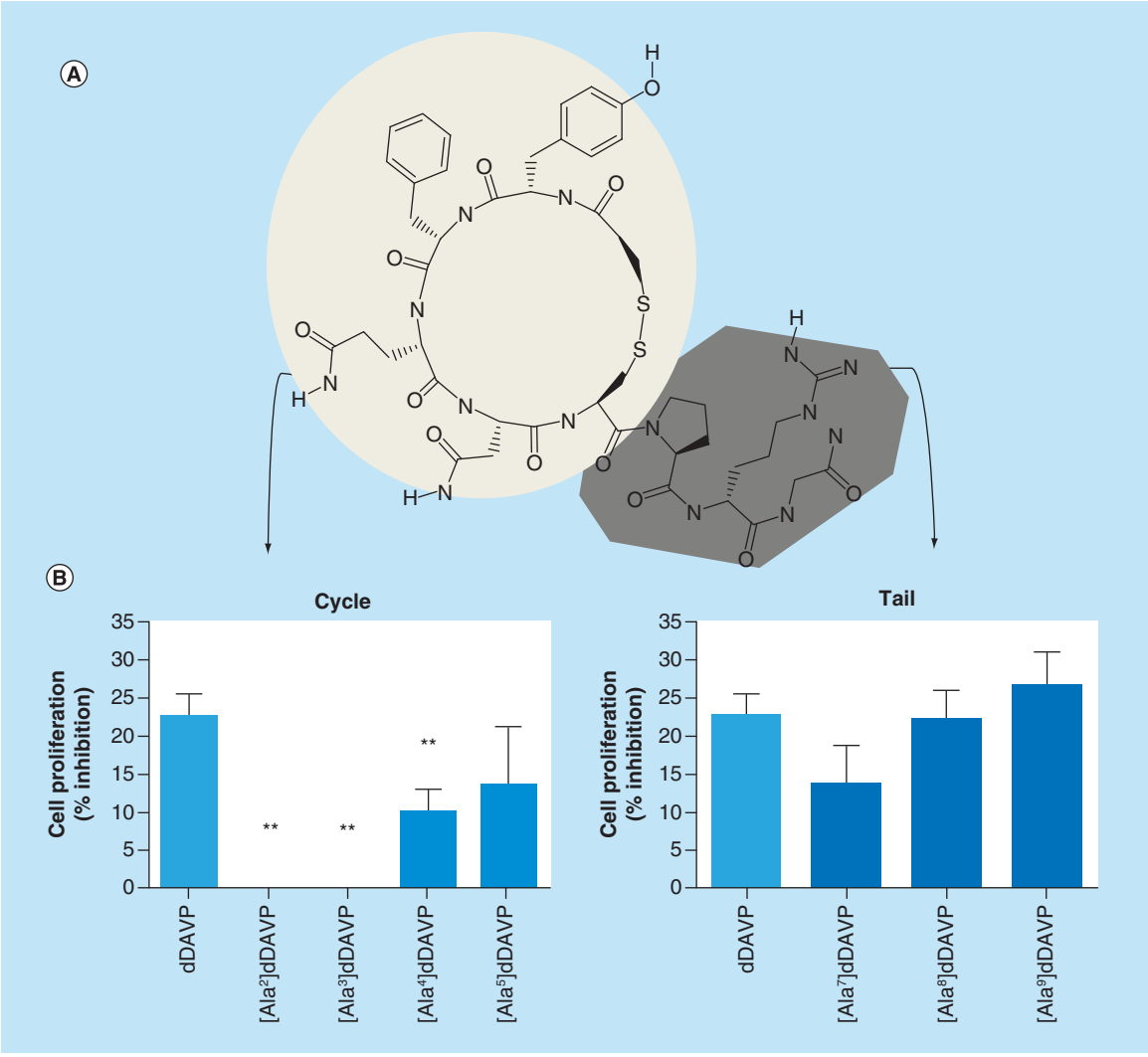

Figure 3. Alanine-scanning technique of desmopressin on aggressive lung cancer cells. (A) Nonapeptide sequence and structure of the parental compound desmopressin. **(B)** Proliferation inhibition after a 72 h exposure on log-phase growing cells at a peptide concentration of 1000 nM. Data represent mean ± standard error of the mean, ANOVA followed by Dunnett's test.
**$p < 0.01$ analog versus desmopressin.

(directly or indirectly) the secretion of diffusible diuretic factors, which act on the Malpighian tubules for dieresis in *T. castaneum* (Table 1) [58].

Based on the above data, a new analog was synthetized: 1-deamino-2-leucine-3-isoleucine-4-threonine-8-D-AVP (dDInotocin; dDINT; MpaLITNCPrG*) (Figure 4). A similar approach has been used and reported recently where Di Giglio and coworkers have designed an evolutionary-related analog replacing Arg8 of INT by D-Arg8 leading to V1AR-antagonist ([D-Arg8]-inotocin) with a low potency allosteric agonism on V2R [64]. However, unlike others, our work focuses on: AVP analog development for potential application as anticancer therapy; AVP analogs with strict agonist activity on tumor cell V2R; and preservation of key structural modifications present in dDAVP for enhancement of biological activity. This strategy led us to design an analog of the insect INT: dDINT. This peptide has been chosen for its natural physiological activity related with growth modulation and cell differentiation that occurs during metamorphosis, two processes that are also relevant in the development and progression of cancer. As we already mentioned, dDINT presents deamination of Cys1 in order to improve the half-life of the molecule and an L-Arg by D-Arg substitution at position 8 to markedly impair the V1R-dependent vasopressor activity. Biological activity of dDINT was evaluated in V2R-expressing SCLC NCI-H82 cells.

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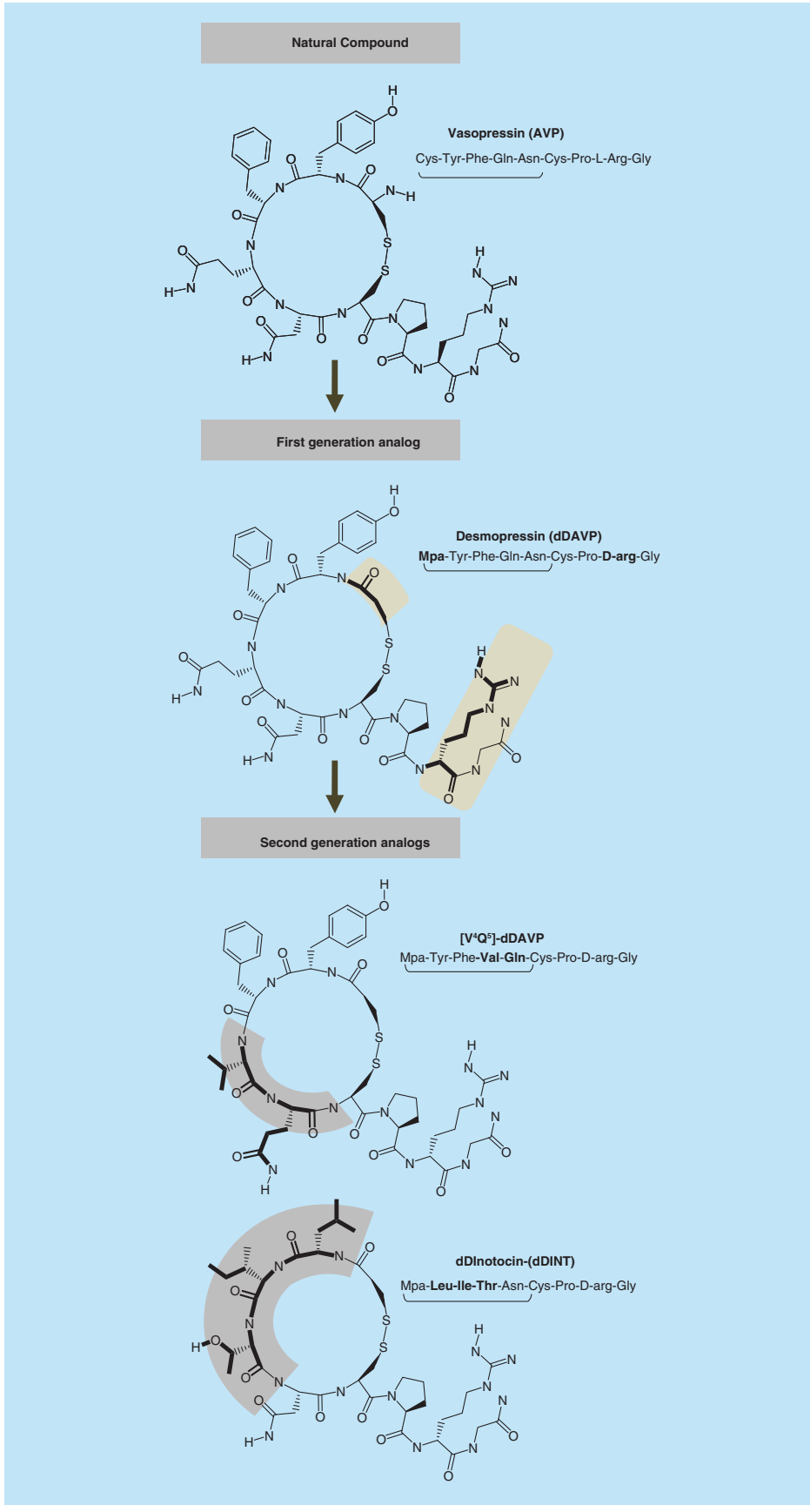

Figure 4. Nonapeptide sequence and structure of natural, first- and second-generation compounds. Natural compound: arginine vasopressin, first-generation compound: desmopressin, and second-generation compounds: [V^4Q^5] desmopressin and dDINT. Chemical structures drawing were made with BIOVIA draw 65].

Table 1. Molecular variants of vasotocin/arginine vasopressin/oxytocin-like neuropeptide in Metazoa.

Neuropeptide (like AVP/OT)	Sequence	Phylum/class/specie	Function	Study (year)
Metazoa AVT	CFVRNCPPG†	Precursor	Precursor	Douzery *et al.* (2004) [42]; Gruber (2014) [59]
Annetocin	CFVRNCPTG†	Annelida/oligochaeta (subclass)/*Eisenia foetida*	Related OT: reproduction and gut motility	Oumi *et al.* (1994) [49]
Lys-Conopressin G	CFIRNCPKG†	Annelida/Hirudinea (subclass)/*Whitmania pigra, Erpobdella octoculata*	Reproduction and osmoregulation	Salzt *et al.* (1993) [48]
Nematocin	CFLNSCPYRRY†	Nematode/Secernentea/*Caenorhabditis elegans*	Reproduction and learning	Beets *et al.* (2013) [50]
Cephalotocin	CYFRNCPIG†	Mollusca/Cephalopoda/*Octopus vulgaris*	Contractile activity (penis, oviduct, vena cava muscles); reproduction and blood circulation	Henry *et al.* (2013) [52]
Sepiatocin	CFWTTCPIG†	Mollusca/Cephalopoda/*Sepia officinalis*		
Conopressin G	CFIRNCPKG†	Mollusca/Gastropoda/*Conus geographus*	Lys or Arg in position 8; defense	Nielsen *et al.* (1994) [51]
Conopressin S	CIIRNCPKG†	Mollusca/Gastropoda/*Conus striatus*		
Inotocin	CLIVNCPRG†	Arthropoda/Insects/*Camponotus floridanus*	Stimulation of carbohydrate and lipid mobilization; ecdysis	Gruber and Muttenthaler (2012) [43]
	CLITNCPRG†	Arthropoda/Insects/*Bettles (Tribolium castaneum); Locust (Locusta migratoria) and Ants species (Atta cephalotes, Harpegnathos saltator, Nasonia vitripennis*)	Development and cell differentiation; ecdysis; indirect diuresis	Proux *et al.* (1987) [60]; Dulcis *et al.* (2005) [61]; Kim *et al.* (2006) [62]; Zitnan *et al.* (2007) [56]; Stafflinger *et al.* (2008) [53]; Li *et al.* (2008) [54]; Aikins *et al.* (2008) [57]; Hauser *et al.* (2008) [63]; Gruber and Muttenthaler (2012) [43]
Vertebrata AVT	CYIQNCPRG†	Chordata/Vertebrata (subphylum)/Agnatha	Ancestral neuropeptide homologous to OT and AVP in vertebrates	Koehbach *et al.* (2013) [46]; Mayasich and Clarke (2016) [45]
		Chordata/Vertebrata (subphylum)/Chondrichthyes	Duplication gene AVT (AVT + OT) electrolyte balance	Kiss and Mikkelsen (2005) [47]
		Chordata/Vertebrata (subphylum)/Actinopterygii	Electrolyte homeostasis becomes apparent	Kiss and Mikkelsen (2005) [47]
		Chordata/Vertebrata (subphylum)/Retilian/Aves		Kiss and Mikkelsen (2005) [47]
Isotocin	CYISNCPIG†	Chordata/Vertebrata (subphylum)/Actinopterygii	Reproduction	Kiss and Mikkelsen (2005) [47]
OT	CYIQNCPLG†	Chordata/Vertebrata (subphylum)/Mammalia/Eutheria	Reproduction (Duplication gene AVT, Arg8 to Leu8)	Kiss and Mikkelsen (2005) [47]
MT	CYIQNCPIG†	Chordata/Vertebrata (subphylum)/Reptilia and Aves	Reproduction	Kiss and Mikkelsen (2005) [47]
AVP	CYFQNCPRG†	Chordata/Vertebrata (subphylum)/Mammalia/Prototheria and Eutheria	Electrolyte balance and homeostasis	Kiss and Mikkelsen (2005) [47]

† Amidation C-terminal.
AVP: Arginine vasopressin; AVT: Arginine vasotocin; MT: Mesotocin; OT: Oxytocin; VT: Vasotocin.

Drug design based on a rational approach using the Ala scanning technique

The rational design of drugs based on SAR studies constitutes an almost indispensable tool in the development of new medicines, contributing to an increase in the chances of success and a substantial decrease in costs. SAR studies relate features of a chemical structure to a property, effect or biological activity. In the pharmaceutical and chemical industries, SAR studies have long been used to design chemicals with desirable properties [66].

One of the methodological approaches most used for the rational design based on SAR is the Ala-Scan; in this work we conduct an Ala-Scan study of dDAVP in order to identify the amino acids that are crucial for its antiproliferative activity on lung cancer cells. Ala-substituted peptides were studied on aggressive NCI-H82 cells. Antiproliferative action was decreased up to 100% when amino acids 2 and 3 were replaced (Figure 3B left) and up to 50% when amino acid in position 4 was substituted. No change in cytostatic capacity was observed when arginine and glycine were substituted (positions 8 and 9, respectively) (Figure 3B right), and partially reduced when positions 5 and 7 were replaced (Figure 3B). This analysis highlighted the critical role of the amino acids located

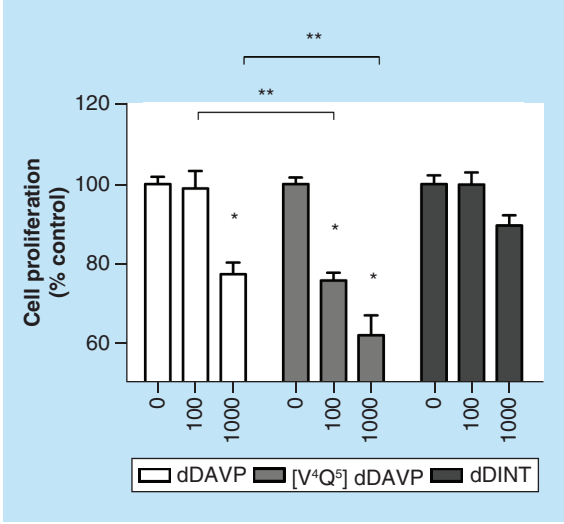

Figure 5. Effect on cellular proliferation of analogs designed by evolutionary perspective: dDINT, and by rational approach: [V^{4}Q^5] desmopressin, on aggressive lung cancer cells. Effect of analogs, [V^{4}Q^5] desmopressin and dDINT, (100 and 1000 nM) on log-phase growing cells was measured using the MTS assays. Results are expressed as mean ± 95% CI. Two-way ANOVA followed by mean 95% CI comparison post hoc.
*$p < 0.001$ (corresponding to each analog vs control).
**$p < 0.001$ (same concentration: analog vs desmopressin).

at the loop of the peptide in accordance with our previous results on MDA-MB-231 breast cancer cells [31] and also with the postulated by Czaplewski *et al.* using a docking approach. This group reported that the N-terminal hydrophobic positions, Cys-1-Tyr-2-Phe-3, could sit on the floor of the V2R cavity, while Arg-8 could be proximal to one of the extracellular loops of the V2R [67].

It was previously reported that all vertebrates have tyrosine in position 2, one of the positions that completely abolished the agonist effect of V2R when Ala replaced; and proline in position 7, one position that causes a partial loss of the antiproliferative effect when Ala substituted [46,47].

Based on the above-mentioned results and previously reported information, a new analog was synthetized replacing glutamine for valine in position 4 and asparagine for glutamine in position 5. Amino acid positions 4 and 5 belong to the peptide conformational loop which has a key role in ligand–receptor interaction and antiproliferative activity. Hydrophobicity enhancement at position 4 could result in an increase in affinity for the V2R which has a deep cavity on the extracellular side containing hydrophobic moieties [22,68]. Manning *et al.* determined three main structural modifications of AVP which determine an important enhancement in the antidiuretic/vasopressor activity [69]. Two of these modifications are present in dDAVP and were mentioned before: the deamination of position 1 and substitution of l- for d-arginine at position 8. The third modification, enhancement of lipophilicity at position 4 is present in the dV4DAVP intermediate analog described by Manning *et al.*, a more potent and selective V2R agonist and antagonist for V1R than the widely used dDAVP which was evaluated in human receptor binding assays [70]. The conservative substitution at position 5 was introduced in order to improve the stability of the analog, based on its distinctive susceptibility to the deamidation process [71,72]. Our previous work showed that a conservative substitution at position 5 (Asn for Gln) improves the antiproliferative activity compared with the analog [V4]dDAVP in the human breast cancer cell line MCF-7 (data not shown). Thus, the resulting peptide 1-deamino-4-valine-5-glutamine-8-D-arginine vasopressin ([V^{4}Q^5] dDAVP; MpaYFVQCPrG*) was evaluated as an antiproliferative agent in SCLC NCI-H82 (Figure 4).

Evaluation of antiproliferative effect based on V2R stimulation in NCI-H82 cells by new analogs, dDINT and [V^{4}Q^5] dDAVP

We assayed the antiproliferative action of the novel analogs, [V^{4}Q^5] dDAVP and dDINT, in comparison to first-generation derivative dDAVP on log-phase growing SCLC NCI-H82. [V^{4}Q^5] dDAVP analog showed a major antiproliferative effect than parental peptide dDAVP at high and low concentrations, reducing cell proliferation by up to 40% at 1000 nM concentration (Figure 5B). As demonstrated by Manning *et al.* [22], increasing hydrophobicity at position 4 enhances the interaction of AVP analogs with V2R (tenfold higher affinity for the human V2R than dDAVP). [V^{4}Q^5] dDAVP reported to be a potent agonist for the V2R in MCF-7 and MDA-MB-231 breast cancer cells [28]. We also tested these analogs on V2R expressing normal endothelial cells. [V^{4}Q^5] dDAVP but no dDAVP modulates the angiogenesis *in vitro* reducing capillary-like tube formation by human lung microvascular endothelial cells (HMVEC-L) [28]. However, the new analog dDINT reduced tumor cell growth by only 10% at

the maximum concentration assayed displaying a markedly reduced efficacy as a cytostatic agent compared with dDAVP (Figure 5A).

The results of Ala-Scan technique, as well as the previous reports by Manning and others [68], could explain why the analog dDINT did not display significant antiproliferative activity. Despite similarity of function between AVP and INT, the amino acids present in the cycle are very different from those found in AVP. These amino acids are responsible for the specificity of the interaction with V2R binding pocket. When amino acids of hydrophobic nature at positions 2 and 3 are substituted by a neutral aliphatic amino acid such as Ala, the antiproliferative effect is completely lost. In dDINT these aromatic amino acids were replaced with two amino acids that are also neutral aliphatic like Ala. This change could impair the effect on proliferation. Based on the findings of Di Giglio [58], an allosteric modulation in V2R is not discarded. As we have been able to determine here, this modulation could be responsible for the mild but not significant antiproliferative effect observed (10% inhibition of cell proliferation) with the dDINT analog in the tumor cells studied. However, to establish any interaction with vasopressin receptors it is necessary to perform pharmacology assays and study in a more profound way if dDINT could act as antagonist on the V1aR and as allosteric modulator of the V2R.

Loss of [V^4Q^5] dDAVP antiproliferative effect by V2R chemical blockade or gene silencing

Cytostatic effect of [V^4Q^5] dDAVP was completely abolished by the selective V2R antagonist chemical tolvaptan, indicating that observed antiproliferative activity mainly results from V2R activation (Figure 6A). Additionally, to confirm that [V^4Q^5] dDAVP acts by means of V2R, we interfered with the expression of the targeted receptor. V2R was reduced using siRNA on NCI-H82 cells. As shown in Figure 5C, V2R expression decreased by up to 50%. In accordance with this, the inhibitory effects of [V^4Q^5] dDAVP on cell proliferation were significantly attenuated when compared with control siRNA (Figure 6B). We thus showed that [V^4Q^5] dDAVP acts via V2R. These results are in consistence with the findings by Keegan *et al.* (2006), where the use of satavaptan (another nonpeptidic V2R antagonist) blocked the mild cytostatic effects of dDAVP on cancer cells [15]. This result complements the already reported findings by our group, where the gene silencing of V2R expression significantly attenuated the inhibitory effects of [V^4Q^5] dDAVP on cell proliferation [25].

Conclusion

The development of safe, adequate and effective medicines for the treatment of diseases is a task that requires the interaction of different disciplines. Medicinal chemistry is advocated to the design and synthesis of new bioactive compounds from the biological information previously generated. This includes evolutionary, physiological and computational information generated by other fields using a vast plethora of methodological approaches.

Our laboratory has been working for years in the Rresearch and development of new peptide compounds for the treatment of different types of cancer, focusing on tumors with few therapeutic alternatives.

In this work, we demonstrated a close relationship between the loop of dDAVP and its antiproliferative activity assayed on aggressive SCLC cells. Therefore, the new V2R-selective analog [V^4Q^5] dDAVP was developed using SAR studies on lead compound dDAVP in SCLC. In comparison to lead compound, the novel compound [V^4Q^5] dDAVP showed higher cytostatic effect on SCLC cells, constituting an interesting and potential therapeutic application for this type of tumor. Although the design through an evolutionary analysis emphasizing the physiological function was not effective in this case, it is a valid and useful tool for future drug design and development efforts aimed at disorders related to V1R or V2R.

Future perspective

SCLC is a rare and aggressive tumor with NE features, accounting for approximately 15% of bronchogenic carcinomas. Even though initially is responsive to chemotherapy and radiotherapy, eventually develops resistance to treatment and patients with advanced disease have a median survival of <12 months [68]. Regardless of stage, the current prognosis for patients with SCLC is unsatisfactory despite improvements in diagnosis and therapy made over the past 25 years, because SCLC has a greater tendency to be widely disseminated and symptoms are often nonspecific [73]. Given the aggressiveness of the disease and the urgent need of novel therapeutic approaches, our studies were conducted on human SCLC model: NCI-H82 cells, a highly aggressive cell line that expresses high levels of V2R, as it was demonstrated in this work. This line represents a valuable disease model of high recurrence and drug resistance for the preclinical development of new antitumor compounds as [V^4Q^5]dDAVP. Owing to the similarity with the lead peptide aspects such as syndrome of inappropriate antidiuretic hormone secretion,

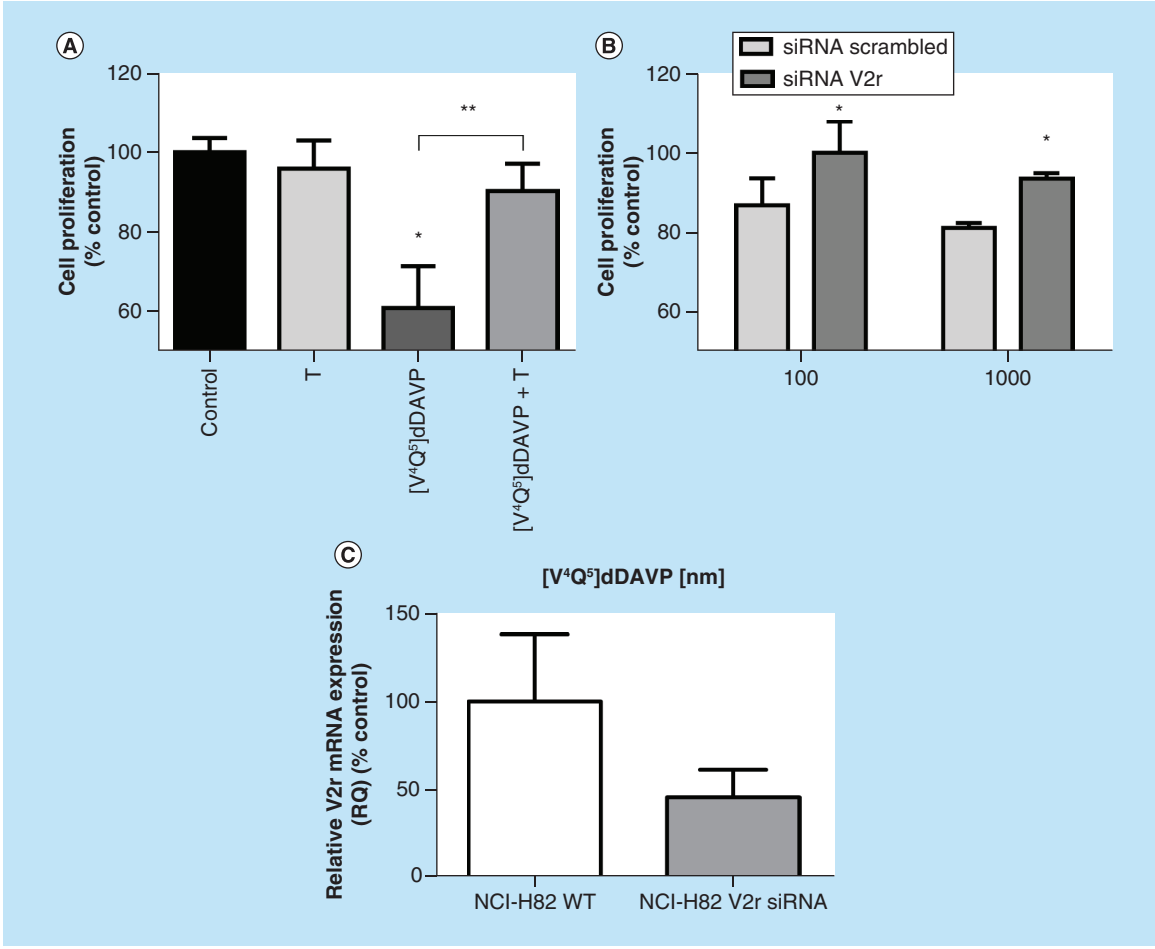

Figure 6. V2R chemical blockage and silencing on NCI-H82 cells. (A) Blockade of cytostatic effect of [V^4Q^5] dDAVP (at 1000 nM) by the selective and competitive V2R antagonist tolvaptan (at 1000 nM). **(B)** NCI-H82 cells were transfected with V2R siRNA or control siRNA and plated in 96-well plates and then treated with different concentrations (100 and 1000 nM) of [V^4Q^5] dDAVP for 72 h. **(C)** Expression of V2R in the silenced NCI-H82 with V2R siRNA by quantitative RT-PCR (Figure 1A). Cell growth was measured by colorimetric MTS assay. Results are expressed as mean ± 95% CI. Two-way ANOVA followed by mean 95% CI comparison post hoc.
*$p < 0.001$ (analog vs control).
**$p < 0.05$ ([V^4Q^5] dDAVP vs [V^4Q^5] dDAVP + T).
T: Tolvaptan; [V^4Q^5] dDAVP + T: [V^4Q^5] dDAVP plus tolvaptan

half-life and behavioral effects of the new analog [V^4Q^5]dDAVP should be taken into account in future studies. Preliminary pharmacological/toxicological studies were already carried out in rats and no substantial differences were found with dDAVP in doses up to 300-fold greater than those producing an antitumor effect *in vivo* [28]. Nevertheless, [V^4Q^5]dDAVP would be administered only under strict monitoring of patient serum sodium level and fluid intake/output in order to avoid hyponatremia-associated complications. Also, there should be paid special attention to the risk of tissue accumulation. The risk of hyponatremia should be taken into account, particularly in elderly patients receiving hypotonic solutions or after frequent, repeated doses of the analog. Caution is also recommended in small children [74,75]. All the previous exclusion criteria from our Phase II clinical trial of dDAVP in breast cancer [41] will be taken into account to any future consideration of clinical trial for [V^4Q^5]dDAVP. Exclusion criteria included pregnancy or breast-feeding, hormonal treatment, known hypersensitivity to dDAVP or AVP, severe von Willebrand's disease or hemophilia, syndrome of inappropriate antidiuretic hormone secretion, renal impairment or hyponatremia, diabetes type I or II, among others.

The synthetic parental analog dDAVP differs from the AVP by deamination of cysteine in position 1 (arrowhead), which prolongs its half-life. This modification is also present in the analog [V^4Q^5]dDAVP and why a similar half-life

is expected. Intravenous injection of dDAVP induces a rapid release of multimeric forms of von Willebrand factor (VWF) from microvascular endothelial cells, reaching peak levels at about 60 min and having a plasma half-life of 8–10 h [76]. Moreover, it is known that the hemostatic dosage is higher than the dose used for antidiuresis. Maximal antidiuretic effect is already achieved with low doses, while duration of hemostatic effect tends to prolong with increasing doses [75].

The search for V2R agonist analogs avoids the effects that the interaction with V1AR could cause on behavior. Even when the passage of these peptides across the blood–brain barrier is limited [77], and the analog is a selective agonist for V2R, its possible effects remain to be analyzed.

We believe that the search and functional characterization of AVP analogs from natural sources in combination with rational design based on SAR and computational studies may be a suitable strategy for the development of selective drugs for the treatment of human diseases such as cancer, especially tumors with few therapeutic alternatives as aggressive SCLC. This work constitutes a starting point for future studies. The remaining challenge is to optimize and combine the information obtained by each methodology achieving an overcoming product. Further sequence alignment studies and computational biology analysis, among others, are necessary and fundamental to continue with the study of new enhanced activity analogs. Taken all these data into account we believe that the search and functional characterization of AVP neuropeptides analogs from natural sources in combination with rational design based on SAR and computational studies may be a suitable strategy for the development of selective drugs for the treatment of human diseases such as cancer.

Summary points

- We developed arginine vasopressin analogs with strict agonist activity on tumor cell V2R preserving key structural modifications present in desmopressin (dDAVP) for enhancement of biological activity.
- The new V2R selective analog [V^4Q^5] dDAVP was developed using structure–activity relationship studies on lead compound dDAVP in small-cell lung cancer (SCLC).
- As an alternative approach, the novel peptide dDINT was designed from an arginine vasopressin-related peptide present in insects and its biological activity was also evaluated.
- In comparison to lead compound the novel compound [V^4Q^5] dDAVP showed higher cytostatic effect on SCLC cells.
- Despite the fact that additional studies are mandatory to further characterize its biological activity, results show that [V^4Q^5] dDAVP has potential therapeutic application in SCLC.

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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