Addition of vasopressin synthetic analogue [V⁴Q⁵]dDAVP to standard chemotherapy enhances tumour growth inhibition and impairs metastatic spread in aggressive breast tumour models

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Abstract [V⁴Q⁵]dDAVP is a novel 2nd generation vasopressin analogue with robust antitumour activity against metastatic breast cancer. We recently reported that, by acting on vasopressin V2r membrane receptor present in tumour cells and microvascular endothelium, [V⁴Q⁵]dDAVP inhibits angiogenesis and metastatic progression of the disease without overt toxicity. Despite chemotherapy remaining as a primary therapeutic option for aggressive breast cancer, its use is limited by low selectivity and associated adverse effects. In this regard, we evaluated potential combinational benefits by adding [V⁴Q⁵]dDAVP to standard-of-care chemotherapy. In vitro, combination of [V⁴Q⁵]dDAVP with sub-IC₅₀ concentrations of paclitaxel or carmustine resulted in a cooperative inhibition of breast cancer cell growth in comparison to single-agent therapy. In vivo antitumour efficacy of [V⁴Q⁵]dDAVP addition to chemotherapy was first evaluated using the triple-negative MDA-MB-231 breast cancer xenograft model. Tumour-bearing mice were treated with i.v. injections of [V⁴Q⁵]dDAVP (0.3 µg/kg, thrice weekly) in combination with weekly cycles of paclitaxel (10 mg/kg i.p.). After 6 weeks of treatment, combination regimen resulted in greater tumour growth inhibition compared to monotherapy. [V⁴Q⁵]dDAVP addition was also associated with reduction of local aggressiveness, and impairment of tumour invasion and infiltration of the skin. Benefits of combined therapy were confirmed in the hormone-independent and metastatic F3II breast cancer model by combining

[V⁴Q⁵]dDAVP with carmustine (25 mg/kg i.p.). Interestingly, [V⁴Q⁵]dDAVP plus cytotoxic agents severely impaired colony forming ability of tumour cells and inhibited breast cancer metastasis to lung. The present study shows that [V⁴Q⁵]dDAVP may complement conventional chemotherapy by modulating metastatic progression and early stages of microtumour establishment, and thus supports further pre-clinical testing of the compound for the management of aggressive breast cancer.

Keywords Vasopressin analogue · Synthetic peptide derivative · [V⁴Q⁵]dDAVP · V2r agonist · Chemotherapy · Combinational therapy · Hormone-independent breast cancer · Antimetastatic

Abbreviations

[V ⁴ Q ⁵]dDAVP	1-deamino-4-valine-5-glutamine-8-D-arginine vasopressin
ANG	Angiostatin
ANOVA	Analysis of variance
AVP	Arginine vasopressin
DAB	3,3-diaminobenzidine
dDAVP	Desmopressin or 1-deamino-8-D-arginine vasopressin
D-MEM	Dulbecco's modified Eagle's medium
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
HER2/neu	Human epidermal growth factor receptor 2
HRP	Horseradish peroxidase
IC ₅₀	50 % inhibitory concentration
IHC	Immunohistochemistry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PKA	cAMP-dependent protein kinase

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PR	Progesterone receptor
TNBC	Triple negative breast cancer
V2r	Vasopressin type 2 receptor
vWF	Von Willebrand factor

Background

Breast cancer is the most frequent cancer among women worldwide, and the second cause of cancer-related death in more developed regions [1]. Despite major advances in detection, surgical techniques and adjuvant therapy, up to one third of patients with early-stage breast cancer will develop distant metastases after primary treatment [2]. With a median survival of 2–3 years [3], metastatic breast cancer is still considered an incurable disease and remains a major therapeutic challenge.

Vasopressin (AVP) and its receptors were proposed as attractive targets for breast cancer therapy almost two decades ago, when AVP gene-related products were identified as a feature of all breast cancer subtypes [4]. Desmopressin (1-deamino-8-D-arginine vasopressin, also designated as dDAVP), a 1st generation synthetic derivative of AVP, is a safe haemostatic compound with proven anticancer efficacy in multiple preclinical studies and veterinary clinical trials [5–7]. In contrast to AVP, which binds to the different vasopressin receptors, desmopressin is a selective agonist for the vasopressin type 2 membrane receptor (V2r) [8]. This receptor is expressed in endothelial cells and in many types of cancer, including lung, colorectal and breast cancer [9, 10]. By acting on small vessels, desmopressin induces a rapid increase in circulating von Willebrand factor (vWF) by stimulating their release from Weibel-Palade bodies mainly from microvascular endothelial cells [11]. Interestingly, recent studies have shown that vWF is a key regulator of metastasis, playing a protective role against tumour cell dissemination [12]. On the other hand, by acting directly on V2r-expressing cancer cells, desmopressin limits its growth and favours the production of antiangiogenic factor angiotensin (ANG), thus reducing tumour angiogenesis [6].

In search of novel peptide analogues with enhanced anticancer efficacy, lead compound dDAVP was rationally modified, and the novel analogue [V⁴Q⁵]dDAVP (1-deamino-4-valine-5-glutamine-8-D-arginine vasopressin) was developed [13]. 2nd generation sequence modifications, mainly aimed at the N-terminal loop of the molecule, were introduced. This region has a key role in ligand-receptor interaction and antitumour activity [14, 15]. Our group recently reported [V⁴Q⁵]dDAVP antitumour activity in preclinical models of breast cancer. Intravenous administration of the compound dramatically decreased distant

spread of aggressive mammary tumours, displaying greater antimetastatic efficacy than parental dDAVP. In athymic mice bearing breast cancer xenografts, [V⁴Q⁵]dDAVP treatment also reduced tumour growth and microvessel density. Cytostatic effects of [V⁴Q⁵]dDAVP against breast cancer cells were associated with V2r-activated signal transduction involving adenylate cyclase/cAMP/PKA axis activation, and partial cell cycle arrest [16]. Taken all together, [V⁴Q⁵]dDAVP seems to produce a dual anti-metastatic and antiangiogenic effect, breaking the cooperative interplay of tumour and endothelial cells during disease progression.

Treatment strategies for metastatic breast cancer have evolved in recent years. However chemotherapy remains a core component for the majority of patients. Unfortunately, there are important limitations with its use, including tumour drug resistance caused by prior exposure, and risk of cumulative toxicities due to lack of selectivity [17]. In this setting, potential combination of chemotherapy with selective cytostatic agents such as the novel compound [V⁴Q⁵]dDAVP is highly interesting.

In the present work, we investigated the ability of [V⁴Q⁵]dDAVP in combination with cytotoxic therapy to impair V2r-expressing breast cancer progression. With that purpose, [V⁴Q⁵]dDAVP was added to antimetabolic agent paclitaxel or alkylating drug carmustine for the treatment of MDA-MB-231 breast cancer xenografts in nude mice or highly metastatic F3II mouse mammary tumours in syngeneic immunocompetent host, respectively. During concurrent administration of cytotoxic drugs plus [V⁴Q⁵]dDAVP, mammary tumour growth and aggressiveness was severely impaired compared to control or single treatment groups. In the carmustine-[V⁴Q⁵]dDAVP treatment regimen, mice receiving combined therapy not only showed a marked reduction of final tumour burden and skin tumour infiltration, but also a complete inhibition of spontaneous metastatic progression to the lung. Cooperative anticancer activity was confirmed *in vitro*, where combination treatments caused a significant reduction in cancer cell proliferation, colony formation and multicellular tumour spheroid growth. The present work shows that novel peptide [V⁴Q⁵]dDAVP addition to standard-of-care chemotherapy could represent an interesting therapeutic approach for the treatment of aggressive breast cancer, suppressing primary tumour progression as well as spread and development of distant metastasis.

Methods

Cell lines

MDA-MB-231 human breast carcinoma cell line (ATCC HTB-26) was obtained from the American Type Culture

Collection. MDA-MB-231 is a triple-negative breast cancer (TNBC) cell line which lacks the oestrogen receptor (ER) and the progesterone receptor (PR), and doesn't overexpress the human epidermal growth factor receptor 2 (HER2/neu). These cells also express low levels of claudin-3, claudin-4 and Ki-67 [18]. MCF-7 (ATCC HTB-22) is a ER-positive/PR-positive hormone-responsive luminal breast carcinoma cell line [18]. The F3II murine breast carcinoma cell line is a metastatic variant derived from a clone of a spontaneous BALB/c mouse mammary tumour [19, 20]. It is a V2r-positive hormone-independent tumour cell line which expresses low levels of HER2/neu. F3II cells grow as poorly differentiated, spindle-cell carcinoma tumours with a prominent local invasiveness, a high angiogenic response, and a 90–100 % incidence of lung metastases when inoculated s.c. into syngeneic mice. All tumour cell lines were grown in Dulbecco's modified Eagle's medium (D-MEM, Gibco, Rockville, MD, USA) plus 10 % fetal bovine serum (FBS), 2 mM glutamine and 80 µg/ml gentamycin in monolayer culture, at 37 °C in a humidified atmosphere of 5 % CO₂. All cells were harvested using a trypsin/EDTA solution (Gibco, Rockville, MD, USA) diluted in phosphate buffered saline (PBS).

Peptide synthesis

[V⁴Q⁵]dDAVP synthesis was conducted in solid phase, using the tea-bag strategy and N α -Fmoc protection [13], and was purified by reversed-phase high-performance liquid chromatography. Quantification was performed using a commercial standard (BCN Peptides, Barcelona, Spain).

Cell proliferation

Antiproliferative effect against rapidly growing tumour cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St Louis, MO, USA). Briefly, cells were plated in 96-well flat bottom plates at a density of 2.5×10^3 per 200 µl in complete D-MEM, allowed to attach overnight, and then treated for 72 h with increasing concentrations of [V⁴Q⁵]dDAVP, antimitotic agent paclitaxel (Taxol, Bristol-Myers Squibb) or alkylating drug carmustine (BiCNU, Bristol-Myers Squibb). In vitro experiments were performed using nanomolar and low micromolar concentrations of the peptide, a range consistent with the in vivo dosage, as reported [13, 15, 16]. Following previously published protocols, breast cancer cells were treated using low nanomolar concentrations of paclitaxel (0.5–10 nM) or micromolar concentrations of carmustine (5–250 µM) [21, 22]. Combination studies using [V⁴Q⁵]dDAVP (1 µM) plus sub-50 % inhibitory concentrations (IC₅₀) of paclitaxel (1 nM) or carmustine (75 µM), depending on cell type,

were also conducted. MTT reagent was added to each well and the plate incubated for 4 h. After solubilisation using dimethyl sulfoxide the absorbance of each well was measured at 570 nm. The optical density of untreated control cells was taken as 100 % viability.

Animals

Outbred athymic female N:NIH(S)-nu mice and inbred immunocompetent female BALB/c AnN aged 8 weeks were purchased from UNLP (Universidad Nacional de La Plata, Buenos Aires, Argentina), and, after randomization, housed at 5 mice per cage in our animal house facility at the National University of Quilmes. Food and water was provided ad libitum and general health status of the animals was monitored daily.

Breast cancer xenografts in nude mice

In order to generate breast cancer xenografts, a 300 µl suspension containing 5×10^6 MDA-MB-231 tumour cells, D-MEM and MatrigelTM (BD Biosciences, San Jose, CA, USA) was injected subcutaneously in athymic mice. MatrigelTM was used to favour graft survival and decrease tumour latency, as reported [23, 24]. Tumours were measured periodically with a caliper and tumour volumes were calculated by the formula: $0.52 \times \text{width}^2 \times \text{length}$. Treatment started 14 days after cell inoculation, when tumours were detected by palpation, followed by histologic confirmation, and reached volumes of about 50 mm³. In vivo combination studies were performed by administering suboptimal 10 mg/kg i.p. doses of paclitaxel once weekly during 6 weeks, alone or with the addition of [V⁴Q⁵]dDAVP. Novel peptide was injected intravenously at 0.3 µg/kg, being a therapeutically relevant dose with well-recognised antitumour properties in mice [16]. Since it is known that peptides such as parental compound dDAVP can induce tachyphylaxis with daily applications [11], [V⁴Q⁵]dDAVP was administered on a thrice-weekly basis until the end of the protocol. When the paclitaxel group exhibited signs of ulceration and necrosis (day 80), animals were photographed at high resolution. Effect of combinational therapy on survival was also evaluated. Animals were euthanized by cervical dislocation when humane tumour burden limits (>1000 mm³) were reached [25].

Mammary tumour progression in syngeneic BALB/c mice

To generate tumours in immunocompetent hosts, 2×10^5 F3II cells were injected subcutaneously in syngeneic BALB/c mice. Treatment started 7 days later, when tumours were detected by palpation and reached volumes of about 50 mm³.

Carmustine was administered by weekly intraperitoneal injections of 20 mg/kg, alone or in combination with [V^4Q^5]dDAVP (administered as mentioned previously), until the end of the protocol. On day 50, F3II tumour-bearing animals were sacrificed and necropsied. To investigate the presence of spontaneous metastases, lungs were removed, fixed in Bouin's solution and macroscopic lung nodules were counted under a dissecting microscope. Lungs were further processed for paraffin embedding and metastatic lesions were confirmed after histopathological analysis.

Immunohistochemical staining for V2r

MDA-MB-231 breast tumour samples were fixed in 10 % formalin, embedded in paraffin, and tissue sections of 4 μ m were cut and placed on silane coated slides for immunohistochemistry (IHC). Sections were dewaxed and pretreated in 0.01 M sodium citrate buffer (pH 6.0) and microwaved for antigen retrieval. In order to block endogenous peroxidase, sections were then incubated with 3 % hydrogen peroxide for 10 min. Immunostaining was performed using polyclonal rabbit antibodies raised against the N-terminal extracellular domain of human V2r (cat. Number V5514; 1:100 dilution, Sigma-Aldrich) at 4 °C overnight and later incubation with the horseradish peroxidase/3, 3-diaminobenzidine (HRP/DAB) anti-mouse/rabbit Detection Kit (Abcam) for 30 min. Sections were counterstained with haematoxylin. MCF-7 breast cancer xenografts generated in nude mice [26] were used as a positive control for V2r expression [15, 16, 27].

Clonogenic assay

Colony forming ability was examined by the 2D clonogenic assay for human breast cancer cells. MDA-MB-231 cells were grown in complete medium with [V^4Q^5]dDAVP (1 μ M) in combination with paclitaxel (1 nM). Complete medium with tested compounds was renewed after 72 h. 7 days after cell seeding, clonogenic cultures were fixed and stained with crystal violet, and colonies of more than 50 tumour cells were counted.

Multicellular tumour spheroid formation

Cell culture dishes (24-well plates) were precoated with 100 μ l of a 1:3 dilution of MatrigelTM in D-MEM. A 200 μ l suspension containing 6×10^3 MDA-MB-231 cells in a MatrigelTM/D-MEM mixture (1:5 volume ratio) was added as a second layer. After the cell layer was set complete, FBS supplemented medium was added over the top with [V^4Q^5]dDAVP (1 μ M), alone or in combination with paclitaxel (1 nM). Saline vehicle was used in control group. Complete medium with or without drugs was changed every

3 days, without disturbing the matrix-embedded colonies. Photos were taken after 14 days of culture. Spheroid formation was analyzed using a Nikon TE-2000 microscope and the NIS-Elements software (Nikon, Tokyo, Japan).

Statistics

All statistical analyses were performed using the PRISM 6, version 6.01 (GraphPad Software Inc, La Jolla, CA, USA). Tukey's multiple comparisons test was used after ANOVA analysis. Dunn's multiple comparisons test was used after Kruskal–Wallis analysis. In tumour progression protocols, growth rates represent the slopes of the linear regressions of the tumour volumes over time. Differences between proportions in different experimental groups were evaluated by non-parametric one-tailed binomial test. Log-rank test and Cox regression analysis was applied to establish the association of treatment with survival. Differences were considered statistically significant at a level of $P < 0.05$. Data were presented as mean $\pm$ SD or SEM.

Results

In vitro cooperative anticancer effects of [V^4Q^5]dDAVP in combination with chemotherapy

Evaluation of potential combinational benefits of [V^4Q^5]dDAVP plus cytotoxic agents was conducted on exponentially growing MDA-MB-231 and F3II breast cancer cells. On both cell lines, [V^4Q^5]dDAVP incubation using a low micromolar concentration range resulted in mild cytostatic effects, inhibiting cell proliferation up to 30 % at higher concentrations (Fig. 1a). Treatment of different breast cancer cells with paclitaxel or carmustine significantly decreased cell viability in a dose dependent manner (Fig. 1b). The IC₅₀ values of paclitaxel or carmustine on MDA-MB-231 or F3II cells were found as 7.2 nM and 131.6 μ M, respectively, in accordance with the previously reported data [21, 22]. After a 72-hour exposure, incubation with [V^4Q^5]dDAVP combined to a sub-IC₅₀ concentration of paclitaxel (1 nM) or carmustine (75 μ M) resulted in a cooperative growth-inhibitory effect reducing 41 % of cancer cell proliferation over control group (Fig. 1c), while [V^4Q^5]dDAVP or chemotherapy single treatments showed significantly weaker cytostatic effects.

[V^4Q^5]dDAVP plus paclitaxel or carmustine combination regimens result in greater breast tumour growth inhibition

Along with doxorubicin and cyclophosphamide, antimitotic drug paclitaxel is one of the most commonly used

Fig. 1 Antiproliferative effects of [V⁴Q⁵]dDAVP in combination with chemotherapy on breast cancer cells. Proliferation was evaluated on log-phase growing breast cancer cells and measured by the MTT assay after 72 h exposure to drugs. **a** [V⁴Q⁵]dDAVP (0.1–1.5 μM) effect *on vitro* growth of MDA-MB-231 and F3II breast cancer cells. **b** Cytotoxic activity of paclitaxel (0.5–10 nM) and carmustine (5–250 μM) on MDA-MB-231 (left) and F3II (right) breast cancer cells. **c** Effect of [V⁴Q⁵]dDAVP (1 μM) addition to sub-IC₅₀ concentrations of paclitaxel (1 nM) or carmustine (75 μM) on MDA-MB-231 (left) or F3II (right) breast cancer cells. Data are presented as mean ± SEM and are representative of at least three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 *versus* control. ††*P* < 0.01 and †††*P* < 0.001 [V⁴Q⁵]dDAVP plus chemotherapy *versus* monotherapies. ANOVA followed by Tukey's test

chemotherapeutic agents in patients with TNBC [28–30]. In order to continue the exploration of possible combinational anticancer effects, MDA-MB-231 tumour-bearing nude mice were treated with novel analogue [V⁴Q⁵]dDAVP plus suboptimal doses of paclitaxel (Fig. 2a).

Combined therapy showed an enhanced antitumour efficacy *versus* single-drug regimens. Comparative analyses of tumour growth rates revealed that [V⁴Q⁵]dDAVP in addition to paclitaxel rendered cooperative therapeutic benefits during the period of concomitant use, slowing cancer progression by nearly 80 % compared to control group (4.90 ± 0.60; 3.62 ± 0.31; 1.51 ± 0.46 and 0.98 ± 0.34 for control, paclitaxel, [V⁴Q⁵]dDAVP or combination; tumour growth rates in mm³/day, mean ± SEM).

Results were confirmed after intravenous administration of [V⁴Q⁵]dDAVP in addition to weekly cycles of carmustine, on tumour progression in BALB/c mice bearing F3II tumors (Fig. 2b). The alkylating agent carmustine, a cytotoxic drug known to be active in the metastatic and hormone-independent F3II mammary carcinoma [22], is used for the treatment of different aggressive solid tumours including metastatic breast cancer [31, 32]. In this experimental setting, all treatments were capable of slowing down disease progression *versus* control. However, tumours from [V⁴Q⁵]dDAVP plus carmustine-treated mice grew at a significantly lower rate compared to tumors from animals administered with [V⁴Q⁵]dDAVP or carmustine in single-agent regimens (48.9 ± 3.4; 29.2 ± 1.7; 32.4 ± 3.0 and 18.5 ± 1.2 for control, carmustine, [V⁴Q⁵]dDAVP or combination; tumour growth rates in mm³/day, mean ± SEM).

Modulation of local tumour aggressiveness after [V⁴Q⁵]dDAVP addition was also observed in the MDA-MB-231 xenograft model. Saline vehicle- and, especially paclitaxel-treated mice, displayed tumours with visible skin infiltration and superficial necrosis (Fig. 3). Interestingly, adding [V⁴Q⁵]dDAVP to taxane-based chemotherapy inhibited tumour infiltration and necrosis, preserving superficial layers of skin.

Despite paclitaxel regimen ending after 6 weeks of treatment, [V⁴Q⁵]dDAVP therapy continued until a tumour burden of 1000 mm³ was reached and animals were euthanized. Sustained intravenous administration of [V⁴Q⁵]dDAVP, in combination or monotherapy regimen, resulted in a significant increase in survival in comparison to control or paclitaxel treatment alone (Fig. 4). Estimated median survival values for control, paclitaxel, [V⁴Q⁵]dDAVP and [V⁴Q⁵]dDAVP plus paclitaxel groups were 87, 85, 100 and 104 days, respectively.

V2r is expressed by human breast cancer xenografts

Expression of membrane receptor V2r by MDA-MB-231 cells growing in nude mice was assessed by IHC. Subcutaneous breast cancer xenografts displayed a diffuse membrane distribution of V2r throughout the tumour (Fig. 5a, b). These findings are consistent with prior studies where V2r expression by *in vitro* growing MDA-MB-231

Fig. 2 [V⁴Q⁵]dDAVP plus chemotherapy combinational treatment abrogates mammary tumour growth. **a** Xenogeneic MDA-MB-231 TNBC model. Subcutaneous xenografts were generated by injecting MDA-MB-231 cells in the flank of athymic nude mice. After 14 days animals were treated with [V⁴Q⁵]dDAVP (0.3 µg/kg i.v. thrice weekly) in combination or not with weekly cycles of antimitotic agent paclitaxel (10 mg/kg i.p). Curves represent tumour volume in mice receiving saline vehicle (control), paclitaxel, [V⁴Q⁵]dDAVP or [V⁴Q⁵]dDAVP plus paclitaxel over time. **b** Syngeneic F3II hormone-independent breast carcinoma model. Subcutaneous tumours were generated by injecting F3II cells in the flank of BALB/c mice.

After 7 days animals were treated with [V⁴Q⁵]dDAVP (0.3 µg/kg i.v. thrice weekly) in combination or not with weekly cycles of alkylating agent carmustine (20 mg/kg i.p) until the end of the protocol. Curves represent tumour volume in mice receiving saline vehicle (control), carmustine, [V⁴Q⁵]dDAVP or [V⁴Q⁵]dDAVP plus carmustine over time. Statistical analysis was conducted on tumour growth rates calculated from day 33 or 11 onwards in the MDA-MB-231 or F3II models, respectively. Data are presented as mean ± SEM. (5 or 6 per experimental group) ****P* < 0.001 versus control. ††*P* < 0.01 and †††*P* < 0.001 [V⁴Q⁵]dDAVP plus chemotherapy versus monotherapies. ANOVA followed by Tukey's test

Fig. 3 [V⁴Q⁵]dDAVP addition to chemotherapy reduces tumour progression and prevents infiltration of the skin in MDA-MB-231 xenografts. Representative photographs of nude mice bearing human

breast cancer xenografts were taken 80 days after tumour cell s.c. inoculation, and are representative of 5 or 6 animals per experimental group

cells was detected by immunofluorescence and RT-PCR [15, 16]. However, in vivo confirmation of therapeutic target V2r expression by MDA-MB-231 has never been

reported. As expected, MCF-7 xenografts displayed an intense membrane staining pattern for the V2r and were used as positive controls [15, 16, 27] (Fig. 5c, d).

Fig. 4 Effect of [V⁴Q⁵]dDAVP treatment, alone or in combination with paclitaxel, on survival of mice bearing human breast cancer xenografts. Kaplan-meier survival plot for control, paclitaxel, [V⁴Q⁵]dDAVP or [V⁴Q⁵]dDAVP plus paclitaxel-treated groups. After subcutaneous tumours reached a humane tumour burden of 1000 mm³, the mice were euthanized by cervical dislocation. n = 5–6 animals per experimental group. ***P* < 0.01, [V⁴Q⁵]dDAVP or [V⁴Q⁵]dDAVP plus paclitaxel *versus* control or paclitaxel alone. Log-rank test (Mantel Cox), $X^2 > 7.2$

[V⁴Q⁵]dDAVP plus paclitaxel treatment reduces clonogenic growth and three-dimensional tumour spheroid formation

Colony forming ability is a hallmark of metastatic outgrowth. Given that MDA-MB-231 cells are poorly metastatic in athymic nude mice, we further evaluated direct drug combination effect on *in vitro* establishment and growth of MDA-MB-231 colonies. In that regard, 2D and 3D low density cell culture systems were used. After 7 days of treatment, [V⁴Q⁵]dDAVP plus paclitaxel severely impaired colony forming ability in human breast cancer cells reducing clonogenic growth by 64.4 % *versus* control (Fig. 6a, c). In contrast with drug combination regimen, peptide therapy or taxane-based chemotherapy alone caused ~40 % reduction on colony formation. Additionally, anticancer efficacy was assessed on long-term three dimensional multicellular tumour spheroid cultures, where incubation with [V⁴Q⁵]dDAVP, alone or with chemotherapy during 14 days, significantly reduced establishment of MDA-MB-231 3D colonies (Fig. 6b, c).

[V⁴Q⁵]dDAVP in combination to chemotherapy reduces local aggressiveness and prevents metastatic spread of highly invasive mammary tumours

In line with previous reports [22], after ~30 days of subcutaneous F3II breast cancer cell inoculation in BALB/c mice, tumour skin infiltration occurred in 100 % of animals belonging to control group. Interestingly, [V⁴Q⁵]dDAVP plus carmustine was the only therapeutic regimen capable of significantly reducing down to 40 % the incidence of tumour invasion in the superficial dermis (Fig. 7a). At the

end of the protocol, mice were sacrificed and spontaneous lung metastatic lesions were counted. 100 % of vehicle-treated mice displayed macroscopic lung metastases, with a maximum of 13 visible lesions per animal. As expected, weekly cycles of carmustine caused a marked decrease of metastatic progression. Remarkably, [V⁴Q⁵]dDAVP, in combination with standard chemotherapy or administered as monotherapy, was able to completely abolish metastatic progression to lungs (Fig. 7a, b).

Discussion

To our knowledge, this is the first report showing the therapeutic benefits of combining standard chemotherapy with a novel AVP peptide analogue for the treatment of aggressive breast cancer. As previously mentioned, [V⁴Q⁵]dDAVP is a 2nd generation derivative of AVP, conserving several structural features of the well-known haemostatic and antitumoral drug desmopressin. This novel compound is a result of a structure-based drug design aiming to increase its biological activity [8, 13, 15, 16]. As well as desmopressin, [V⁴Q⁵]dDAVP is a selective agonist of V2r cell membrane receptor. This receptor subtype is expressed in microvascular endothelium and breast cancer cells [16, 27, 33]. In the present study we evaluated combinational benefits on two V2r⁺ highly aggressive breast cancer models, the human triple-negative MDA-MB-231 and the murine metastatic F3II cell lines.

In this work we show that combination-therapy using paclitaxel plus [V⁴Q⁵]dDAVP caused a greater reduction in MDA-MB-231 xenograft growth compared to taxane or [V⁴Q⁵]dDAVP monotherapies. Cooperative inhibition of cell growth was confirmed in log-phase breast cancer cell cultures incubated with [V⁴Q⁵]dDAVP and suboptimal concentrations of paclitaxel. Direct cytostatic action of [V⁴Q⁵]dDAVP on human breast cancer cells was previously associated with cell cycle arrest and V2r signalling, involving intracellular cAMP elevation and PKA activation [16]. It has been reported that cell cycle arrest and apoptosis induction by paclitaxel on MDA-MB-231 cells is caused by hyperphosphorylation and inactivation of anti-apoptotic protein Bcl2. This event is mediated by PKA activation and can be blocked by the PKA inhibitor Rp-cAMP [34]. Additionally, it was recently postulated that suppression of PKA enzymatic activity by type II- α regulatory subunit markedly increased cancer cell survival after paclitaxel treatment [35]. In this regard, several authors have described the cAMP/PKA signalling axis as a growth suppressor system in breast cancer [27, 36, 37]. Sharing the same antimitogenic signalling pathways could explain cooperative antitumour effects between [V⁴Q⁵]dDAVP and taxane treatment. However, the possibility that other

Fig. 5 Vasopressin receptor type 2 (V2r) is widely expressed in MDA-MB-231 breast cancer xenografts. V2r detection was performed by IHC on formalin-fixed paraffin-embedded tumour xenografts using primary antibodies against human V2r, a HRP/DAB detection method (brown stain) and haematoxylin counterstaining (dark blue stain). **a** and **b** representative microphotographs of

MDA-MB-231 xenografts growing in nude mice, 400 $\times$ and 1000 $\times$ magnification, respectively. **c** and **d** MCF-7 xenografts were generated in nude mice and used as positive control for V2r expression. 400 $\times$ and 1000 $\times$ magnification, respectively. Scale bars: (left) 50 and (right) 20 μ m

underlying mechanisms account for the combined anti-neoplastic action cannot be ruled out.

V2r agonists such as desmopressin or [V⁴Q⁵]dDAVP are able to reduce tumour angiogenesis by favouring the proteolytic conversion of plasminogen to ANG, a natural occurring antiangiogenic factor. V2r stimulation in breast cancer cells seems to induce ANG production by increased secretion of urokinase-type plasminogen activator (uPA) and matrix metalloproteinases [6]. Interestingly, it was also reported that in vitro exposure of mammary carcinoma cells to paclitaxel, not only modulates the polymeric status of the microtubular cytoskeleton, but also enhances cancer cell-derived uPA and MMP-9 activity [38]. Increment in protease production and secretion caused by [V⁴Q⁵]dDAVP and taxane treatment could ultimately result in augmented ANG production and angiogenesis blockade. Nonetheless, a paradoxical role of the uPA-MMP pathway in castration-resistant prostate cancer cells was recently reported. Treatment with desmopressin plus docetaxel inhibited growth and invasiveness of PC3 cells. Surprisingly, antitumour activity was mediated by down-regulation of both uPA and matrix metalloproteinases MMP-2 and

MMP-9 [39]. In toto, evidence suggest that up- or downregulation of protease activity after desmopressin or [V⁴Q⁵]dDAVP plus taxane therapy might depend on tumour type.

Additionally, we showed that [V⁴Q⁵]dDAVP plus chemotherapy treatment severely inhibits human breast cancer colony formation. These results suggest that, besides endothelium-mediated responses in host, direct V2r stimulation in breast cancer cells using [V⁴Q⁵]dDAVP, in addition to standard chemotherapy, might also affect early stages of microtumour establishment and residual disease.

In hormone-resistant breast cancer patients, metastatic relapse usually occurs in the lung and is strongly related to the severity of the disease and fatal outcome. It was recently reported by our group that sustained use of intravenous [V⁴Q⁵]dDAVP dramatically reduces distant spread of aggressive mammary tumours [16]. With the purpose of assessing combined therapy on hormone-independent metastatic breast cancer, [V⁴Q⁵]dDAVP was added to alkylating drug carmustine for the treatment of F3II tumours. Here we confirmed that administration of clinically relevant doses of [V⁴Q⁵]dDAVP completely inhibits the establishment of metastatic lung tumours and that

Fig. 6 [V⁴Q⁵]dDAVP in combination with chemotherapy impairs colony forming ability of human breast cancer cells. **a** Effect of [V⁴Q⁵]dDAVP (1 μ M) plus paclitaxel (1 nM) on monolayer clonogenic growth of MDA-MB-231 cells. After 7 days of incubation colonies were fixed, stained and quantified using optic microscopy. **b** Effect of [V⁴Q⁵]dDAVP plus paclitaxel on 3D breast cancer cell cultures. MDA-MB-231 cells were seeded at low density in a MatrigelTM mixture with complete medium and treated for 14 days. At the end of the protocol fully formed spheroids were counted.

Representative photomicrographs of MatrigelTM embedded spheroids belonging to different experimental groups. Scale bar: 200 μ m, 40 $\times$ magnification. **c** Quantitative analysis of colony formation as examined by the clonogenic assay (2D) or the multicellular tumour spheroid assay (3D). Data are presented as mean $\pm$ SEM. Results are representative of at least three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 versus control. †*P* < 0.05, [V⁴Q⁵]dDAVP plus paclitaxel versus paclitaxel. ANOVA followed by Tukey's test

combination with chemotherapy does not affect the pre-clinical antimetastatic activity of the peptide.

It is known that stimulation of V2r present in endothelium by i.v. injection of desmopressin induces the systemic release of vWF from microvascular cells [11]. vWF is considered a multifunctional protein involved in haemostasis [33], vascular normalization [40], tumour cell apoptosis and metastatic resistance [41, 42]. Using vWF-knockout mice, it was shown that absence of vWF leads to increased metastatic potential of i.v. injected tumour cells.

vWF seems to limit tumour cell adhesion in metastatic sites and favor apoptosis induction in malignant cells through binding to integrin α V β 3. Furthermore, it was reported that aggressive breast cancer cells with high levels of ADAM28, a disintegrin and metalloproteinase that binds and cleaves vWF, are able to counterbalance the proapoptotic function of vWF [41]. Preclinical studies in mice and veterinary clinical trials in dogs with advanced breast cancer, showed that administration of perioperative desmopressin inhibits locoregional and distant spread, and

Fig. 7 [V⁴Q⁵]dDAVP complements chemotherapy by reducing local aggressiveness and inhibiting spontaneous metastatic progression of F3II mammary tumours. **a** Incidence of local skin infiltration and metastatic spread to lung. Skin infiltration proportions in F3II tumours were analyzed at day 33. At the end of the tumour progression protocol animals were sacrificed and spontaneous lung metastases were counted. ****P* < 0.001, non-parametric one-tailed binomial

proportions test, setting the expected outcome for skin infiltration and metastatic progression to 100 %, according to prior studies [16, 22]. [†]*P* < 0.05, [V⁴Q⁵]dDAVP plus carmustine *versus* carmustine. **b** Pulmonary macroscopic metastases quantification. Data are expressed as scatter dot blot with median. ***P* < 0.01 *versus* control. Kruskal–Wallis followed by Dunn’s test

increases survival of treated animals, respectively [5, 43]. Due to its haemostatic and antimetastatic activity, desmopressin has just been evaluated as adjunctive therapy in a phase II dose-escalation trial in patients with breast cancer (NCT01606072). Administration of i.v. desmopressin, before and after surgical resection of primary tumour, was well tolerated and treatment was associated with reduction of intraoperative bleeding, higher circulating vWF levels and a drop in circulating tumour cells after surgery [44].

The present preclinical work has multiple strengths. It includes original and robust evidence of combinational therapeutic benefits by adding [V⁴Q⁵]dDAVP to standard chemotherapy used in the management of TNBC and metastatic hormone-independent breast cancer. In addition, compounds were tested in vitro and in vivo on well-characterized and widely used murine and human experimental models. However, our work has several limitations. By gene expression analysis, the majority of TNBCs display a basal-like profile, characterized by high expression of Ki-67, cytokeratin 5/6 and epidermal growth factor receptor (EGFR). Despite being a widely used TNBC model, MDA-MB-231 is a breast cancer cell line which belongs to the claudin-low molecular subtype [18]. These cells express several stem cell markers and are associated with tumour initiating properties [45]. In order to extrapolate our findings to all TNBC subtypes, future studies should include other tumour cell lines with basal-like molecular characteristics.

With the purpose of evaluating tumour response to combinational therapy, we transplanted tumour cells under the

skin of mice in order to develop tumours. Besides mimicking the whole process of tumour progression, experimental models should reproduce the tumour microenvironment as closer to the clinical setting as possible [46]. Given that [V⁴Q⁵]dDAVP targets both tumour and stroma by stimulating V2r present in cancer cells and microvascular endothelium, the influence of tumour microenvironment should not be neglected. In this regard, the use of orthotopic models where breast cancer cells are transplanted into the mammary fat pad, is more appropriate and should be taken into account for future studies.

We recently published that, at doses 300-fold higher than that required for effective anticancer activity, [V⁴Q⁵]dDAVP was well tolerated in acute toxicology studies [16]. In this work, animals belonging to [V⁴Q⁵]dDAVP or combinational experimental groups received up to 45 i.v. treatments over a 15-week period of time with no signs of toxicity, further confirming good tolerability and promising anticancer activity. It should be borne in mind that syndrome of inappropriate antidiuretic hormone secretion (SIADH) may occur in patients with breast cancer. This paraneoplastic syndrome is characterized by euvoletic hyponatremia as a consequence of tumour-secreted vasopressin [47, 48]. In this clinical setting, due to the potential effect on kidney V2r and water reabsorption, [V⁴Q⁵]dDAVP should be administered only under strict monitoring of patient serum sodium level and fluid intake and output in order to avoid hyponatremia-associated complications.

Despite the considerable progress made in the treatment of aggressive breast cancer in recent years, challenges still

remain. Chemotherapy remains a core therapeutic component for the majority of metastatic breast cancer patients. However, its effectiveness is limited by drug resistance, associated toxicity and the difficulty in finding optimal combination with biologic therapy [49]. Integration of novel and promising anticancer agents such as [V⁴Q⁵]dDAVP into current breast cancer management is highly challenging. We believe that the present work provides promising evidence that [V⁴Q⁵]dDAVP may complement conventional chemotherapy by reducing tumour aggressiveness and metastatic progression of the disease, and supports the continuation of preclinical testing of the compound.

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Authors contributions JG, GVR and DFA participated in the conception and design of this work. JG, MP, MBP and GVR developed the methodology. JG, MP, DEG, GVR and DFA were in charge of data acquisition and analysis. JG and DFA participated in the writing and revision of the manuscript. DFA supervised the study.

Compliance with ethical standards

Conflict of interest We acknowledge that DEG, GVR and DFA serve in a consultant/advisory role for Elea Laboratories S.A.C.I.F. y A. and Chemo-Romikin S.A. All other authors have no conflict of interests to declare.

Ethics approval All animal protocols have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the U.S. National Institutes of Health and were approved by our institutional Animal Care Committee.

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