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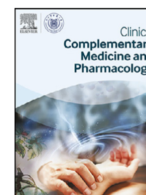
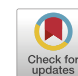


Chemosensitizer Effect of Violacein on Cisplatin-treated Bladder Cancer Cells



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ABSTRACT

Background: Bladder cancer is the tenth most common cancer worldwide. Considering its high prevalence (vulnerability to multiple recurrences and progression despite local therapy), which leads to a substantial health service burden, it becomes necessary to develop new strategies to increase the effectiveness of bladder tumor therapy. Natural compounds with antiproliferative effect on cancer cells could be a good choice for co-adjuvant chemotherapy. Microorganisms are one of the main sources for natural compounds. Pigments extracted from the cold-adapted microorganisms can contribute to the development of a broader range of applications in biotechnology. Violacein is a purple pigment commonly produced by many bacterial strains. We have previously shown that very low concentrations of violacein extracted from *Janthinobacterium* sp. produced an antiproliferative effect on HeLa cells.

Objective: With the aim to determine if violacein has an antiproliferative activity on bladder cancer cells, as well as to test if it has synergistic effects on cisplatin treated cells *in vitro*, T24 and 253J cell lines (derived bladder cancer cells from carcinoma *in situ* and retroperitoneal metastasis, respectively) were exposed to different concentrations of violacein in the presence or absence of cisplatin.

Methods: i) Resazurin assay and flow cytometry were performed in two bladder cancer-derived cell lines, namely T24 and 253J, to see if violacein affects cell viability and induce cell death. ii) To find out whether violacein sensitizes bladder cancer cells to cisplatin, the drug interaction among different doses of cisplatin and violacein was analyzed, as well their combination index was determined. iii) The effect of violacein to induce primary genetic damage was determined through the analysis of induced micronuclei frequency and γH2AX foci, as well as performing the comet assay.

Results: The half-maximal inhibitory concentration of violacein at 24 h for both cell lines were around 500 nM, and decreased below 400 nM in combination with 10 µM of cisplatin, indicating antiproliferative and sensitizing effects of violacein to cisplatin in both cell lines tested. A clear cell cycle delay, as well as an increase in the percentage of cell death was observed by flow cytometry at 300 nM of violacein, either alone or in combination with cisplatin. On the other hand, the analysis of the micronucleus frequency did not evidence an increase in genetic damage. Moreover, in combined treatments with cisplatin there was a slight decrease on micronucleus induction. Besides, the induction of genetic damage was not observed through comet assay when cells were treated with violacein alone, however, when cells were treated with violacein in the presence of cisplatin (10 µM). The production of genetic damage was diminished in T24 or 253J cells. By the same token, increase in the frequency of γH2AX foci by violacein was not observed at any tested dose in both cell lines.

Conclusion: It was shown that violacein has an *in vitro* antiproliferative effect in bladder cancer cell lines, sensitizing them to cisplatin. Interestingly, at doses tested, violacein did not induce genotoxicity and reduce the genotoxic effect produced by cisplatin.

1. Introduction

Bladder cancer is the tenth most common malignancy worldwide, with 549,000 new cases and 200,000 deaths per year (Sung et al., 2021). This cancer has a high mortality rate (between 30% and 70% according to the disease stage) and high recurrence, showing up to 70% reappearance 5 years after the first diagnosis (Loras et al., 2018). Moreover, bladder cancer is one of the most-costly cancers among the elderly people. Cisplatin (cis-diamminedichloridoplatinum (II) or CDDP) is a well-

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known chemotherapeutic drug. It was approved in 1978 by the Food & Drug Administration (FDA, USA) as an agent for the chemotherapeutic treatment of bladder and testicular cancer (Zhu et al., 2016). CDDP has a pleiotropic effect, both in the cytoplasm and in the nucleus, but its main effect is due to its ability as an intercalating agent between the DNA purine bases. This agent mainly produces inter-strand crosslinks, and also DNA single-strand breaks, as well as interferes with the DNA repair mechanisms (Dasari and Tchounwou, 2014). Therefore, CDDP can kill tumor cells causing DNA damage and subsequently inducing apoptosis (Dasari and Tchounwou, 2014; Galluzzi et al., 2012). It also produces several side effects, and in most cases, tumor cells develop mechanisms of resistance, thus limiting its use as a chemotherapeutic agent (Castedo et al., 2018; Cheung-Ong et al., 2013). To overtake these side effects, a combination of two or more chemotherapeutic agents has been assayed. For example, it has been tested with non-conventional agents such as phytochemicals, including the flavonoid quercetin (Sun et al., 2019), or the suberoylanilide hydroxamic acid, SAHA (Ryu et al., 2019).

On the other hand, natural compounds with antiproliferative effect on cancer cells could be a good choice for co-adjuvant chemotherapy. Microorganisms are one of the main sources for natural compounds. Bacteria coming from the Antarctic region become of the utmost interest during last years. Bacterial pigments constitute one of the emerging fields of research since they offer promising opportunities for different applications. Besides their use as safe coloring agents in the cosmetic and food industry, bacterial pigments also possess biological properties such as antimicrobial, antiviral, antioxidant and anticancer activities (Azman et al., 2018). More recently, it has been investigated the expression of different pigments from several strains of Antarctic bacteria (Marizcurrena et al., 2019). One of them, a purple violet pigment (PVP) known as violacein, is commonly produced by many bacterial strains including *Chromobacterium, Janthinobacterium, Alteromonas, Duganella, Massilia, Pseudoalteromonas,* and *Collimonas*, among others (Alem et al., 2020; Choi et al., 2015; Durán et al., 2012), and has emerged as one promissory antiproliferative compound in HeLa cells as we have previously shown (Alem et al., 2020). Although violacein has shown anti-tumor activity in different tumor cell lines (Alem et al., 2020; Alshatwi et al., 2016), there are no previous reports about the effect of violacein in bladder cancer cells. Interestingly, violacein sensitized colorectal cancer-derived cells to 5-fluorouracil (Kodach et al., 2006) and HeLa cell to CDDP (Alem et al., 2020).

Considering the unique qualities of these pigments obtained from cold-adapted microorganisms will contribute to the development of a broader range of applications in biotechnology (Sajjad et al., 2020), in the current work it is aimed to find new cancer-fighting molecules by studying the possible antiproliferative and genotoxic activities of violacein in two bladder cancer cell lines (T24 and 253J), as well as its ability to sensitize cells to CDDP. For this purpose we used a highly pure violacein, a purple violet pigment produced by an Antarctic microorganism identified as *Janthinobacterium* sp. (Alem et al., 2020).

2. Materials and Methods

2.1. Cell lines and culture conditions

Bladder cancer cell lines were obtained from ABAC (Asociación Banco Argentino de Células; *http://www.abac.org.ar/*). T24 and 253J cells, derived from transitional cell carcinoma (Bubeník et al., 1973) and a retroperitoneal metastasis (Elliott et al., 1974), respectively; were grown in Mac Coy's 5A Medium (McCoy's) from Gibco (Gaithersburg, Maryland, USA), supplemented with 1 mM L-glutamine, 1% w/v penicillin–streptomycin and 10% v/v fetal bovine serum (FBS, Capricorn Scientific, Ebsdorfergrund, Germany). Cell cultures were maintained in a 5% CO_2 atmosphere at 37°C.

2.2. Viability testing

Violacein was extracted from the antartic *Janthinobacterium* sp, collected near the Artigas Antarctic Scientific Base [Base Científica Antártica Artigas (BCAA); 62° 11′ 4″ S; 58° 51′ 7″ W], at the Nebles Point, Collins Glacier (King George Island, Fildes Peninsula, Antarctica), authenticated by Dr. Castro-Sowinsky (Adjunct Professor from Biochemistry and Molecular Biology Department, Faculty of Sciences, UdelaR) and purified by Alem et al. (Alem et al., 2020). Briefly, a bacteria culture (1.5 L) growth during 96 h in a bioreactor, and then mixed with 0.5 M NaCl and 200 mL of butanol (incubated at 65°C for 15 min). The purple butanolic phase was dried in a rotary evaporator equipment. Violacein was obtained with a modified technique from Rettori and Durán (Rettori and Durán, 1998), using a chromatographic procedure with silica gel (Sigma 381276) as stationary phase and ethyl acetate as mobile phase; the eluted fraction was vacuum-dried. Then, the powder was washed twice with 100 mL hexane and 100 mL dichloromethane, and the pellet was dried again. Then hot methanol (99%, 45°C) was added until the pigment dissolved; finally, two volumes of cold water were added and the mix was incubated at 4°C overnight, for pigment precipitation. The pigment was identified by Nuclear Magnetic Resonance (NMR) spectroscopy as violacein and its purtity was tested by HPLC-UV-Vis (Alem et al., 2020).

Cell viability was tested by the Resazurin (RZ) assay (Sittampalam et al., 2004). Briefly, 5×10^3 cells (100 μL) were plated in 96-well plates for 24 h. Then, culture medium was removed and purified violacein (dissolved in dimethyl sulfoxide, DMSO) was added at different final concentrations, ranging from 0.075 to 1.4 μM. Besides, combined treatments with 10 μM CDDP (Sigma-Aldrich, USA), with and without violacein (0.075–1.4 μM) were also assayed. Control cells were only exposed to DMSO (Sigma-Aldrich, USA), since violacein is resuspended on it. After 20, culture medium was replaced by 100 μL of 0.025 mg•mL^{-1} RZ (Sigma-Aldrich, USA) suspended in sterile PBS, and incubated for 4 h at 37°C. RZ fluorescence (530 nm Ex/590 nm Em) was measured using a microplate spectrophotometer (Varioskan Flash Microplate spectrophotometer; Thermo Fisher, Finland). The IC_{50} correspond to the agent concentration that reduces fluorescence of samples cells to 50% relatively to control ones, (just treated with DMSO), determined by linear regression analysis employing the following equation:

$$F = \left(F_{Max}\right)/\left(1 + 10^{\left(\mathrm{LogIC}_{50} - \mathrm{LogC}\right)b}\right)$$

where F is the fluorescence value at each violacein concentration, F_{Max} is the fluorescence value of the control sample, C is the violacein concentration and b is the curve slope.

The combination index (CI) between violacein and CDDP was carried out using the Compusyn software (ComboSyn, Inc., USA), a computational tool based in isobologram analysis of drug interaction (Huang et al., 2019), using the following equation:

$$CI = (D)_1/\left(D_x\right)_1 + (D)_2/\left(D_x\right)_2$$

where $(D_x)_1$ and $(D_x)_2$ are the doses of drug 1 and 2 that inhibits x% of every of them. $(D)_1$ is the portion of drug 1 which in combination with $(D)_1$ + $(D)_2$ inhibits x%. In the same way, $(D)_2$ is the portion of drug 2 which in combination with $(D)_1$ + $(D)_2$ inhibits x% (Chou, 2014). A CI higher than 1 indicate an antagonistic effect, a CI equal to 1 indicate an additive effect, and a CI less than 1 indicate synergistic effects, respectively.

2.3. Cell-cycle analyses

For cell-cycle evaluation (Nair and Manohar, 2021), 5×10^5 cells were plated into 100 mm diameter culture plates. Twenty-four hours later, cells were treated with 0.3 and 0.5 μM violacein, in the presence or absence of CDDP (10 μM) for 24 h. Then, cells were collected and fixed with ethanol 70% at -20°C for 30 min, centrifuged, suspended in PBS,

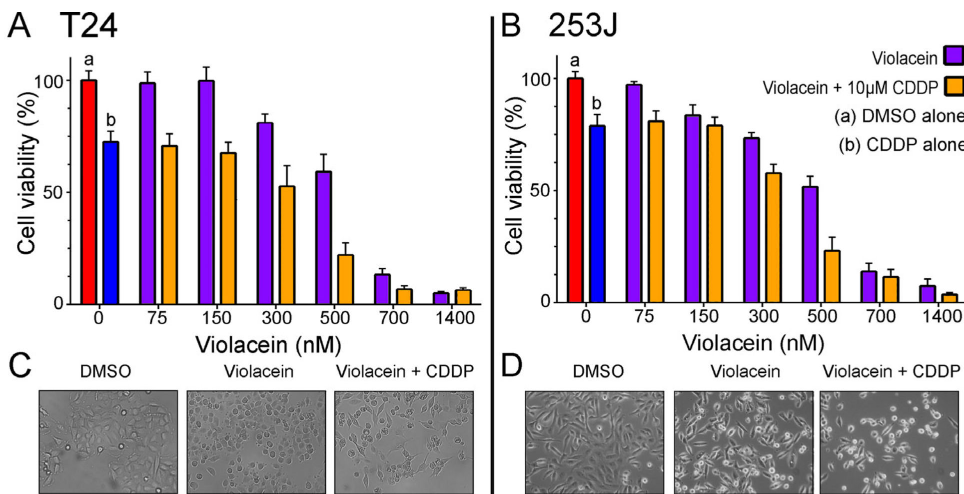

Fig. 1. Cell viability of bladder cancer cell lines treated with violacein and with violacein and CDDP. Violacein viability testing (75 nM – 1400 nM) (gray columns) or in combination with 10 μM CDDP (pattern columns) was evaluated using the Resazurin method at 24 h, in T24 (A) and 253J (B) cells. Abscises represent mean ± SD of cell viability (%). Three independent experiments were carried out. (a) DMSO treatment; (b) 10 μM CDDP treatment. (C) and (D) are pictures taken at 100× magnification under phase contrast transmitted light inverted microscope of T24 and 253J cells, respectively, under different treatment conditions, such as, DMSO, violacein alone and violacein plus cisplatin (CDDP).

and treated with 50 μg•mL^{-1} RNAse (at 37°C for 30 min). Finally, cells were stained with 50 μg•mL^{-1} propidium iodide (Sigma-Aldrich, USA) and immediately analyzed in a MoFlo Astrios EQ cell sorter (Beckman Coulter, USA) using a blue laser (488 nm) and a 100 μm nozzle (operating at 25 psi). The calibration of the cytometer and its daily quality control was carried out with 3.0 μm Ultra Rainbow Fluorescent Particles (Spherotech, USA). A minimal of ten thousand events were assured. The forward scatter signal (FSC) was detected in FSC1 by placing the P1 mask in front of the detector. FSC1 photomultiplier data with P1 mask and side scatter data (Side Scatter, SSC) were obtained on a linear scale. For cell-cycle analysis, PI emitted fluorescence was detected using a 620/29 band-pass filter with linear amplification. Doublets were excluded using 620/29-Area versus 620/29-Width graphs using Kaluza software (Beckman Coulter, USA). Markers were placed in the DNA content histograms (620/29-A), identifying the relative percentages of cells in G1, S and G2/M as well as Sub-G1.

2.4. Cytokinesis-block micronucleus test

The micronucleus test was performed as described previously (Alem et al., 2020). Briefly, 1×10^5 cells were plated into 35 mm plates. Twenty-four hours later, cells were treated with 0.15 and 0.3 μM violacein in the presence or absence of CDDP (10 μM), and placed at 37°C and 5% CO_2. A negative control with DMSO was included. Six hours later Cytochalasin B (Sigma-Aldrich, USA) (3 μg•mL^{-1}) was added and cells were incubated for additional 18 h at 37°C and 5% CO_2. A similar experimental design was done with the addition of 4 mM Caffeine B (Sigma-Aldrich, USA) during 24 h, with the aim to block G2 checkpoint and allow cells to reach division. Then, cells were trypsinized and centrifuged at 1000 rpm for 5 min at room temperature. The supernatant was discarded, and the cell pellet was treated with 1 volume of cold KCl hypotonic solution (75 mM) for five minutes and 3 volumes of methanol: acetic acid (3:1) fixative was added and centrifuged as above mentioned. Finally, cells were fixed in pure methanol plus 1% of acetic acid and spread onto a cold glass slide. Cell nucleus were stained with PI (20 μg•mL^{-1}) and analyzed under a fluorescence microscope (Zeiss, Axioplan II, USA). To evaluate the cytokinesis-block proliferation index or CBPI, (CBPI = (MC +2BC+3TC+4FC))/N; MC = mononucleated cell, BC binucleated cell, TC three nucleated cell, FC four nucleated cell, N= total cell), one thousand cells per experimental point were scored. For micronucleus counting, one thousand binucleated cells were scored per experimental point (Fenech, 2007).

2.5. Comet assay

The genotoxic damage induced in 24 h of treatment was evaluated by alkaline single-cell gel electrophoresis (comet assay) (Tice et al., 2000). Briefly, 1 × 10^5 cells were plated into 35 mm plates and incubated for 24 h. Afterward, cells were treated with 0.3 and 0.5 μM violacein, in the presence or absence of CDDP (10 μM) for 24 h. A negative control (DMSO) and a 100 μM hydrogen peroxide as positive control was included. The cells were further incubated for 3 h at 37°C and 5% CO_2. Then, cells were trypsinized and resuspended in PBS. The slides were prepared by mixing cell suspension with 0.5% low melting point agarose. Cells were lysed by immersion in cold lysis buffer (10 mM Tris-HCl pH 10, 2.5 M NaCl, 100 mM EDTA and 1% Triton X-100) for 1 h at 4°C. The electrophoresis was performed in a horizontal electrophoresis chamber filled with an alkaline electrophoresis buffer (300 mM NaOH pH 13 and 1mM EDTA) at 0.7 V•cm^{-1} (300 mA) for 15 min at 4°C. Samples were washed with neutralization buffer (0.4 mM Tris-Cl pH 7.5) three times for 5 min at room temperature and finally stained with 50 μl PI (10 μg•mL^{-1}). Comet Imager (MetaSystems, Altlußheim, Germany) software was used for the semi-quantitative analysis. Two slides were scored and 50 nuclei per slide were measured. Olive Tail Moment was used to estimate genetic damage.

2.6. γH2AX immunocytochemistry assay

The DNA damage induced by 24 h of violacein treatment in the presence or absence of CDDP was evaluated by quantification of the mean fluorescence intensity (MFI) of γH2AX (Pérez et al., 2019). Briefly, cells were plated into a 24-well plate at densities of 6×10^4 per well and allowed to attach for 24 h in a humidified incubator with a 5% CO_2 atmosphere at 37°C. Afterward, cells were treated with 0.3 and 0.5 μM violacein, in the presence or absence of CDDP (10 μM) for 24 h. The positive control was performed employing hydrogen peroxide at 100 μM for 3 h. After 24 h of treatment, cells were fixed in 4% paraformaldehyde for 15 min and permeabilized with blocking buffer (0.05% Triton X-100, 1% BSA in PBS) for 40 min at RT. Permeabilized cells were incubated with the anti-γH2AX antibody (1/500, ab26350; Abcam, Cambridge, Massachusetts, USA) overnight at 4°C. Secondary antibody (1:1000, goat anti-mouse conjugated with Alexa 488; Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was incubated for 45 min at room temperature. DAPI (300 nM; Invitrogen) was employed as DNA counterstaining. Finally, slides were mounted using Prolong Glass Antifade (Invitrogen, Thermo Scientific, USA). Immunolabeled cells were visualized using a Zeiss LSM800 confocal microscope (Zeiss, Oberkochen,

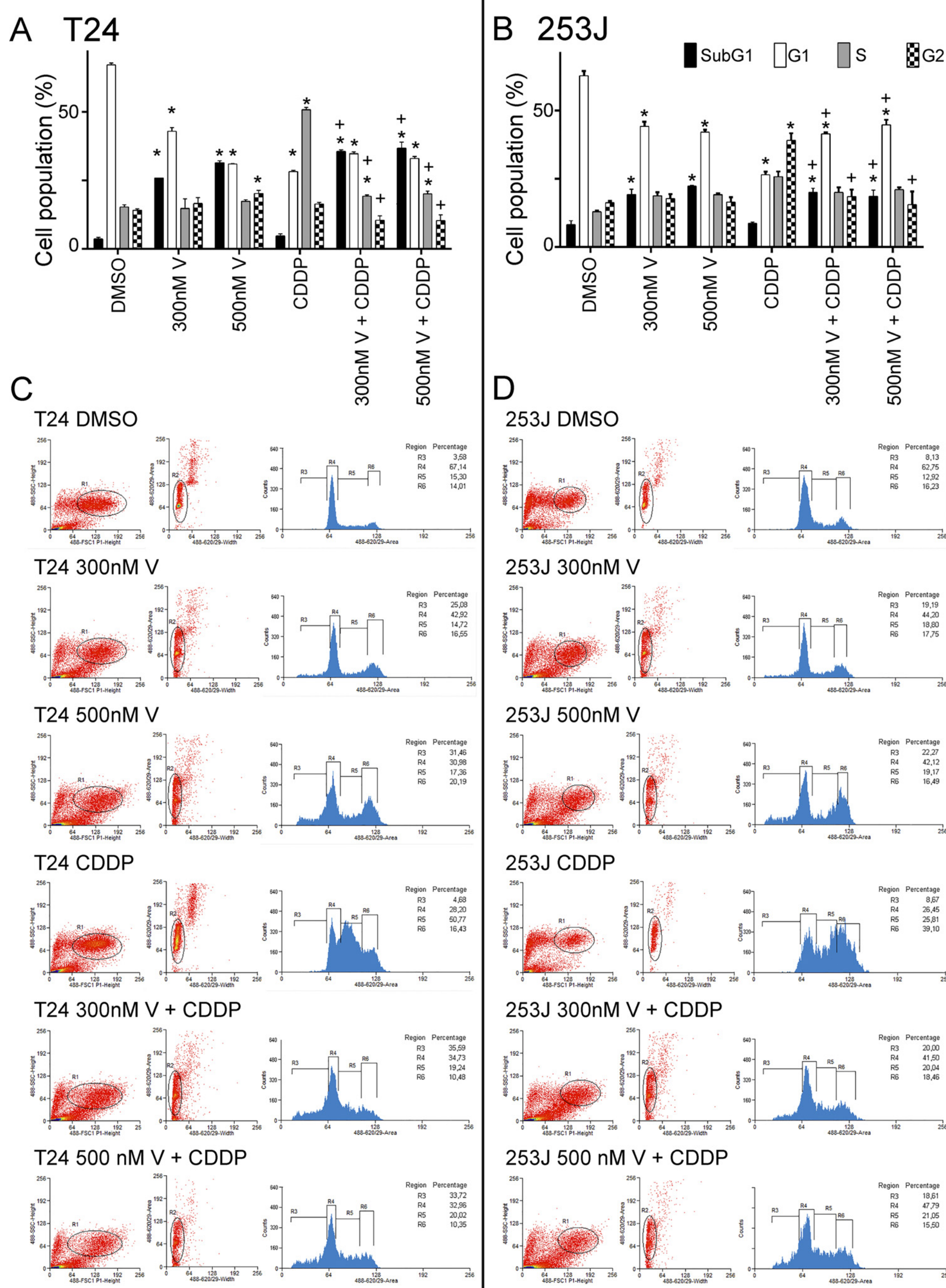

Fig. 2. Analysis of the cell cycle by flow cytometry. In **(A)** Cell cycle profiles of T24, and in **(B)** 253J cells treated with violacein (V) or with violacein plus CDDP (10 μM) are shown. Abscises shows the cell percentage (%) in different phases of the cell cycle. SubG1 (black), G1 (white), S (gray) and G2 (pattern) cell cycle phases were recorded. We have collected more than 10000 events per experimental point. Three independent experiments were carried out. In **(C)** for T24 cells and in **(D)** for 253 cells are shown examples of scattered dot plots (FSC vs SSC) and the fluorescence of propidium iodide (620 nm-area vs 620 nm-width) for every experimental point, indicating the population of selected cells (R1 for the scattered plot and R2 for SubG1, G1, S, G2 cells excluding doublets). Besides, on the right side of every line of plots, DNA histograms including cells belonging to R1 plus R2 populations are also shown, estimating the percentage of cells in the SubG1, G1, S and G2 phases as R3, R4, R5 and R6 regions, respectively. DMSO: dimethyl sulfoxide; V: violacein; CDDP: cisplatin. Statistical analysis of every sample with respect to DMSO control sample was carried out using Tukey's Test, with a significance of * $P \leq 0.0001$. Statistical analysis of every CDDP + violacein sample with respect to CDDP alone was carried out using Tukey's Test with a significance of + $P \leq 0.0001$).

Germany), equipped with a Plan Apo N 63X oil NA 1.4 lens. The mean fluorescence intensity of γH2AX (Pérez et al., 2019) was measured in at least 200 nuclei per experimental condition using the Fiji Image J program (Schindelin et al., 2012).

2.7. Statistical analysis

All experiments were done in triplicate and at least two biological replicas were performed for every experimental condition. Data analyses and statistical calculations were performed using One-way ANOVA followed by Tukey's multiple comparisons test or Two-way ANOVA followed by Tukey's multiple comparisons test to grouped analysis.

3. Results

3.1. Viability testing

The half-maximal inhibitory concentration (IC_{50}) of violacein, estimated by linear regression analysis, either for T24 or 253J cells is shown in Fig. 1. The IC_{50} of violacein for T24 cells was 524.2 nM, and when used in combination with 10 μM of CDDP this value decreased up to 385.4 nM. Similarly, 253J cells showed an IC_{50} value of 490.4 nM at 24 h, while combined with 10 μM of CDDP this value decreased up to 375.8 nM.

We also determined the combination index (CI) for the quantitative determination of synergism, additive, or antagonism effects when cells were treated with violacein and/or CDDP (Table 1). Violacein and CDDP

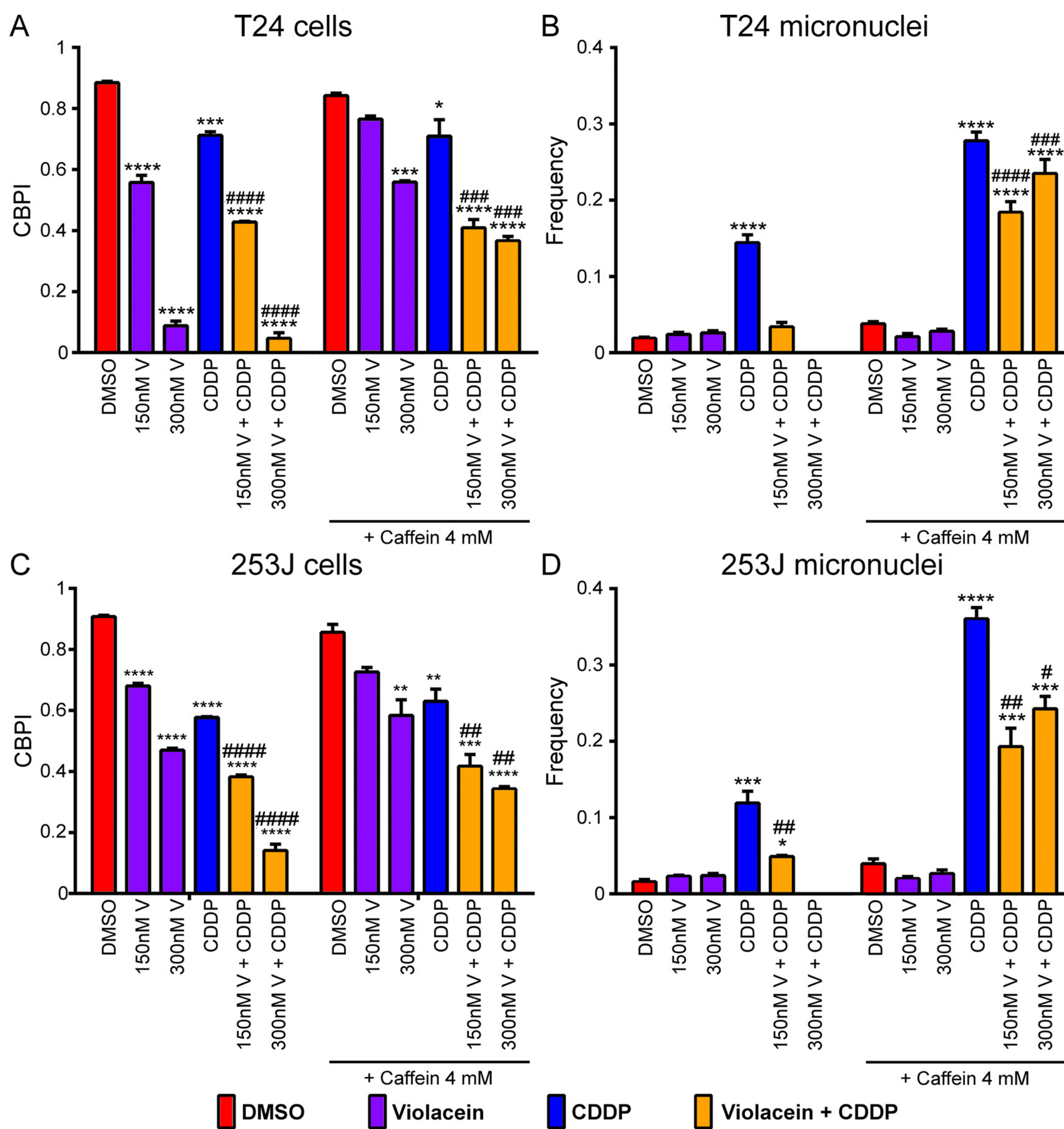

Fig. 3. Micronuclei test. CBPI (**A** and **C**) and micronuclei frequency (**B** and **D**) in T24 cells (**A** and **B**), and 253J cells (**C** and **D**) after their treatment with violacein (V), 10 μM CDDP, as well as with the mix of violacein and CDDP. Results from experiments performed with and without the addition of caffeine are also shown. The frequency means ± SD of three independent experiments is presented. Values with statistical significance are indicated according to the Tukey Test with respect to the control (DMSO): * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, and **** $P \leq 0.0001$; or regarding CDDP 10 μM: ## $P \leq 0.01$, ### $P \leq 0.001$ and #### $P \leq 0.0001$.

Table 1
Combination index (CI) values for violacein treatment of both bladder cancer cell lines in the presence of 10 μM of CDDP.

	Combination Index	
CDDP (10 μM)+Violacein (nM)	T24 cells	253J cells
75	1.77	1.10
150	1.03	1.04
300	0.58	0.97
500	0.50	0.67
700	0.66	0.62

showed an additive effect in T24 and 253J at the 150 nM dose (CI = 1.03 and 1.04, respectively). At higher doses (300, 500, and 700 nM) the effect was synergistic in T24 cell, while for 253J cells, an additive effect was observed for 300 nM violacein (CI = 0.97), while for higher doses (500 and 700 nM), the interaction of violacein and CDDP showed a synergistic effect (CI = 0.67 and 0.62, respectively).

3.2. *Cell cycle analysis*

The effect of violacein on the cell cycle, either in the presence or absence of CDDP, was analyzed by flow cytometry (Fig. 2). An increase in the percentage of cells at the sub-G1 phase was observed, indicating that violacein increases the cell death either alone or in combination with CDDP (Tukey's Test * $P \leq 0.0001$). As expected, it was observed that CDDP produces a stacking of cells at the S/G2-phases of the cell cycle (Tukey's Test + $P \leq 0.0001$). Interestingly, combined treatment showed a decrease in G2 blockage, as it could be expected with CDDP

alone, instead, an increase in cell death and a cell proliferation blockage were clearly observed.

3.3. Evaluation of genetic damage

To properly study the potential genotoxic effect of violacein, we assayed the sub-cytotoxic doses (at 150 and 300 nM, both below the IC_{50}) performing micronuclei tests in both bladder cancer cell lines (T24 and 253J). In Fig. 3, the cytokinesis-block proliferation index (CBPI) as well as the micronucleus frequency on T24 or 253J cells treated with violacein in the presence or absence of 10 μM CDDP is shown. As it can be observed in the CBPI graphic, violacein substantially reduced cell proliferation, especially in T24 cells, which turn difficult to obtain enough binucleated cells for micronucleus frequency analysis, therefore, 4 mM caffeine was added. The analysis of the frequency of micronucleus did not show an increase in genetic damage. On the contrary, there is a slight decrease in combined treatments, more pronounced in 253J cells.

In both cell lines, neither 150 nM nor 300 nM violacein induced micronuclei formation, while CDDP produces an increase in the frequency of micronuclei in binucleated cells as expected (FMN = 0.28). However, when the mix of violacein and CDDP was used, we detected a reduction in the CBPI (maybe due to a cell cycle-delay), and a significant decrease in the formation of micronuclei (compared with results obtained using 10 μM CDDP alone) when combined with 150 nM of violacein (FMN = 0.19), and 0.24 with 300 nM of violacein. Similarly, the genotoxic effect of violacein and CDDP in the 253J cell line showed a similar picture to the one described for T24. The FMN induced by 10 μM CDDP was 0.36, but in combination with either 150 or 300 nM of violacein decreased up to 0.19 and 0.24, respectively. For both cell lines, the higher dose of violacein (300 nM), alone or in combination with CDDP (10 μM), produced a clear cell cycle delay. With 150 nM of violacein, there was a moderate cell cycle delay allowing cells to reach mitosis, clearly enhanced when caffeine was employed (Fig. 3A and C). On the other hand, when higher dose of violacein was combined with 10 μM CDDP, a decrease in the frequency of micronuclei was observed (Fig. 3B and D).

In order to assure that no genetic damage was induced by violacein concentrations employed in the present work, two molecular approaches were employed, such as comet assay and γH2AX. As expected, CDDP and hydrogen peroxide (H_2O_2) did induce DNA damage in both cell lines as observed by comet assay, however, we did not observe genetic damage when cells were treated with violacein alone (150 and 300 nM) (Fig. 4). More interesting, a decrease in the production of the genetic damage in T24 and 253J cells was observed when cells were treated with violacein plus CDDP (10 μM) as compared with cells treated with CDDP alone (Fig. 4A and Fig. 4B), suggesting that violacein reduces the genotoxic effect of cisplatin, most probably due to cell cycle blocking in G1/S transition by violacein, diminishing CDDP effectiveness at S/G2 phases of the cell cycle.

On the other hand, the γH2AX assay showed that violacein did not induce genetic damage at any tested dose (150, 300 and 500 nM) in both cell lines, as it can be seen in Fig. 5A and B for T24 and in Fig. 5C and D for 253J. Positive controls performed with CDDP (10 μM) or with hydrogen peroxide showed an increase in the fluorescence intensity as expected. Cells treated with the mix of violacein and CDDP (10 μM) did not induce an increase in the fluorescence intensity as compared with cells treated with CDDP alone, but the fluorescence intensity was higher than the one observed in the control cells or violacein treated cells, in both cell lines. We did not perform the analysis of γH2AX assay in cells treated with violacein over 500 nM due to an increase in cell death produced by violacein as it was shown previously.

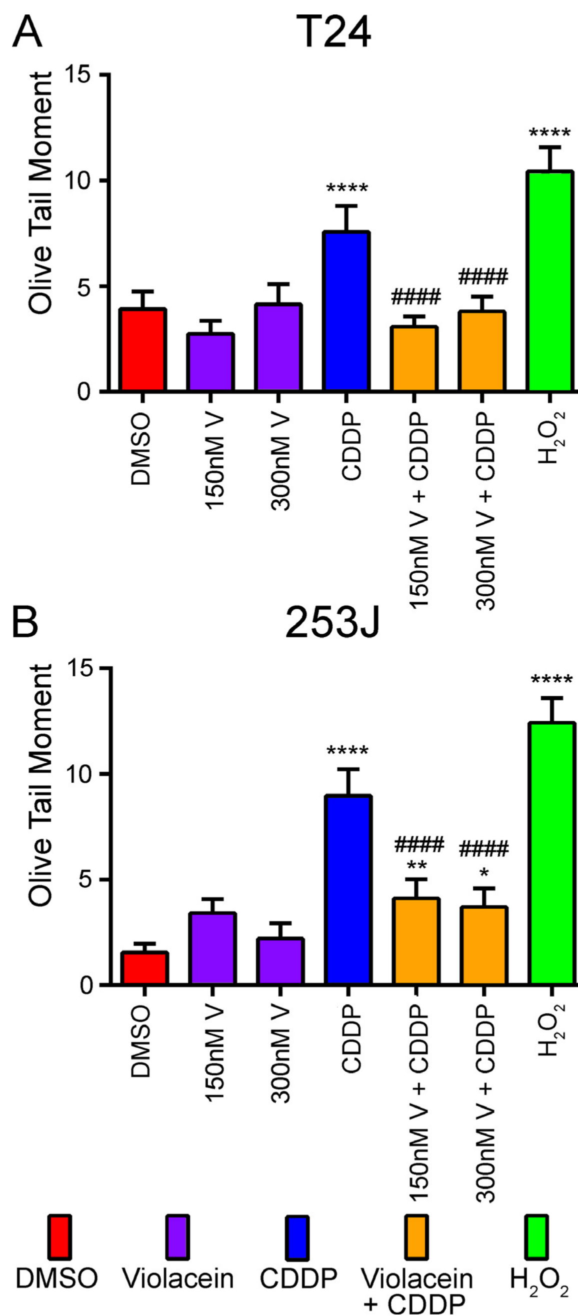

Fig. 4. Comet assay of bladder cancer cells. Analysis of the DNA damage induced by violacein (V) or by the mix of violacein and CDDP (10 μM) in T24 (**A**) and 253J (**B**) cells. Hydrogen peroxide was used as the positive control. Olive Tail Moment mean ± SD are shown. Values with statistical significance are shown according to the Tukey Test as compared with the control treatments as follows: with DMSO - * $P \leq 0.05$, ** $P \leq 0.01$, **** $P \leq 0.0001$; or with 10 μM CDDP - #### $P \leq 0.0001$.

4. Discussion

It has been previously reported that the purple violet pigment (PVP) extracted from *Janthinobacterium* sp. which absorbs UVA, UVB, and UVC radiation, possessed antimicrobial and anticancer activities (Durán et al., 2012; Koo et al., 2016). We have described here cytotoxicity and genotoxicity properties of the violacein pigment extracted from *Janthinobacterium* sp. brought from the Antarctic Region as described previously (Alem et al., 2020).

As it is shown in Fig. 1 viability of both cell lines was diminished in the presence of violacein. Cytotoxicity induced by violacein has been observed previously in V79 Chinese hamster lung fibroblasts (Melo et al., 2000) as well as in leukaemia cell lines (Ferreira et al., 2004; Melo et al., 2003). The way how violacein reduce cell viability was investigated in

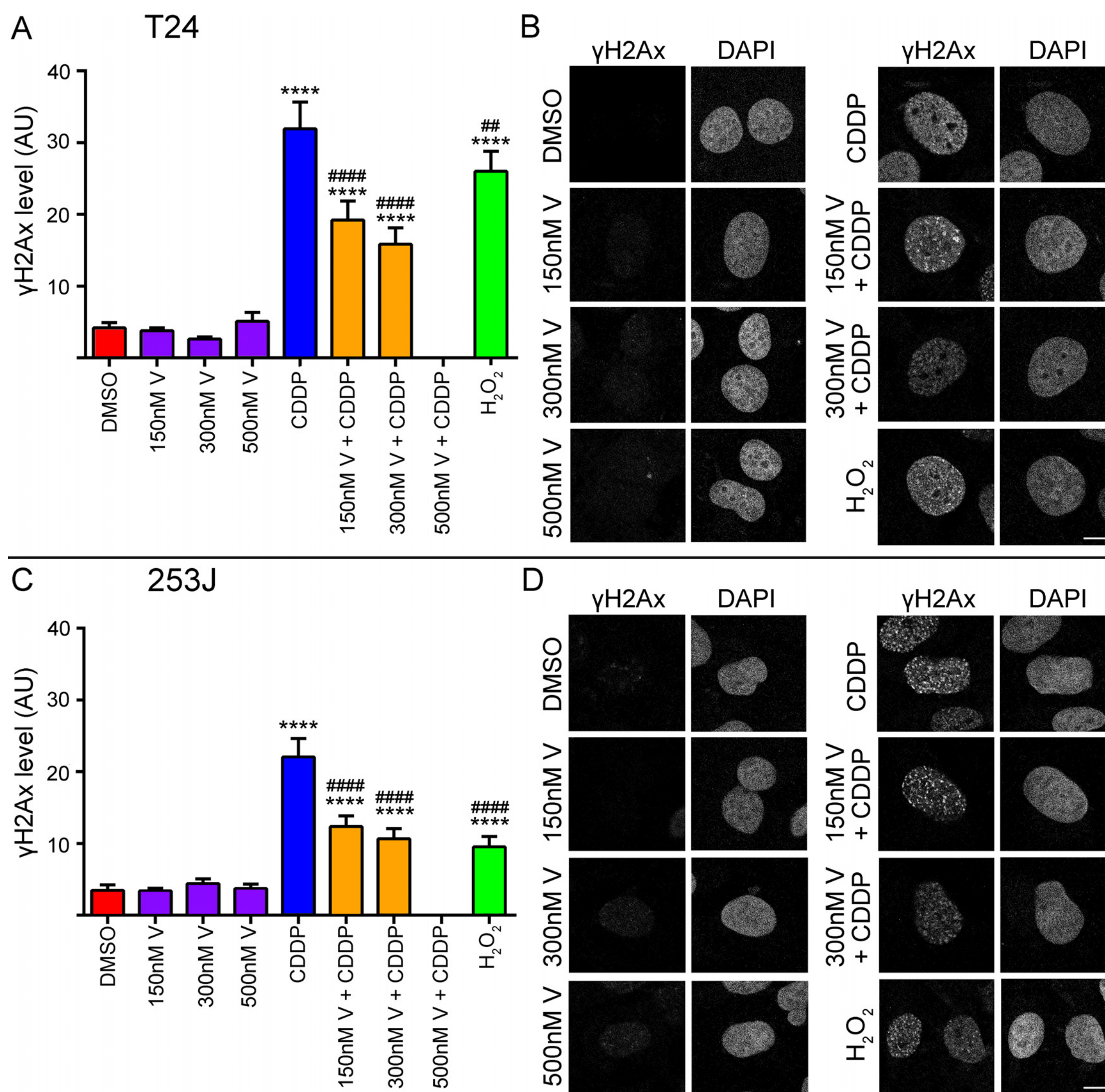

Fig. 5. ***γ*H2AX immunocytochemistry assay.** Analysis of the *γ*H2AX nuclear signal in bladder cancer cells treated with violacein (V), CDDP (10 μM), or with the mix (violacein and CDDP). Hydrogen peroxide was used as the positive control. A graphical representation of *γ*H2AX quantification is presented for T24 (**A**) and 253J (**C**) cell lines. Values with statistical significance are indicated according to the Tukey Test, as compared to the controls: DMSO - * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, or 10 μM CDDP - ## $P \leq 0.01$, ### $P \leq 0.001$ and #### $P \leq 0.0001$. Representative images of nuclei (DAPI staining) and *γ*H2AX signal (Alexa-488) for each condition with T24 (**B**) or 253J (**D**) cells are shown. Bar = 5 μm.

relation to the effect on the cell cycle of both cell lines employed. According to flow cytometry analysis there was observed an increase in the sub-G1 peak with violacein at cytotoxic doses tested in both bladder cancer cell lines. By the same token, increased cell death produced by violacein, mainly due to apoptosis induction, was described in colon cancer cells (de Carvalho et al., 2006; Kodach et al., 2006) as well as in breast cancer cells, where changes in gene expression were detected (Alshatwi et al., 2016). An interesting issue has been shown by Mojib et al. (Mojib et al., 2011) employing the PVP to treat a murine fibrosarcoma cell line which response in a dose and time response manner with respect to a mouse embryo cells that do not show cytotoxicity at same doses and time of treatments. Interestingly, it has been described that variation in violacein cytotoxicity are observed depending on the cell type, which could indicate that different cell-type-specific mechanisms could be activated. Similarly, several studies have been carried out previously to study apoptosis induction by violacein in different cancer cell lines with no agreement on which cell death mechanism could be activated. Finally, authors claimed that the programmed cell death activated by violacein should be specific for the cancer cell lines (Venegas et al., 2019). In this respect, it was evidenced through the CBPI analysis a clear reduction in the production of binucleated cells, indicating that violacein diminish cell proliferation and increase cell death. Interestingly, Leal et al have found that violacein toxicity could be associated with mitochondria damage, since an increase of membrane potential (hyperpolarization), even at the lowest violacein concentration tested, was detected in HeLa and MRC-5 cells (Leal et al., 2015).

Besides, it was observed that over 300 nM of violacein, a sensitizing effect was produced in both cell lines when they were exposed to CDDP, although a strong effect was seen in T24 cells, which could indicate that 253J cells would be more resistant to classical chemotherapy treatment (Table 1). The fact that violacein produce a decrease in G1 cells in both cell lines (more pronounced in T24 cells) increasing either the sub-G1 peak (death cells) or the number of cells accumulated in S phase which could favor the CDDP action, could be a possible mechanism to explain the synergistic effect of violacein to CDDP in both cell lines, especially at higher doses and in non-metastatic derived bladder cancer cells, like T24 cells. Similarly, it has been demonstrated a synergistic effect of violacein in colorectal cancer cells treated with 5-fluorouracil

(Kodach et al., 2006). Interestingly, the sensitizing effect of violacein to CDDP was obtained at very low concentration of violacein, suggesting that it could be a very promising molecule to be used in combination with classical cancer therapy. By the same token, it has been found that several compounds, such as curcumin and cryptotanshinone sensitize cells derived from ovarian cancer to CDDP at 10 μM (Yallapu et al., 2010; Yuan et al., 2017). However, underlying molecular mechanisms that produce the sensitizing effect to CDDP by violacein are still not known.

In this respect, it was investigated if the increase in cell death as well as the synergistic effect of CDDP on both cell lines occurred due to a genotoxicity effect of violacein. The analysis of the micronucleus frequency did not show an increase in genetic damage induced by violacein. On the contrary, it showed a decrease in genetic damage when combined with CDDP, although caffeine was added during cell culturing in order to overtake cell cycle blockage induced by violacein (Fig. 3). Besides, we evaluated the potential induction of primary DNA damage by using comet assay and studying the presence of the histone variant gamma H2AX. Comet assay allows the recognition of the primary DNA lesions that are converted into single-strand breaks by DNA repair processes of oxidative damage or any base damage induced by CDDP or violacein. On the other hand, γH2AX constitutes a sensor protein appearing nearby the DNA lesion as soon as the DNA damage response begins after genetic insult. In this respect, we have observed that violacein does not induce DNA damage, even at 500 nM. Interestingly, it was observed that violacein reduced the primary DNA damage produced by CDDP. In this respect, a positive genotoxic effect of violacein was previously described by Andrighetti-Fröhner et al but with a dose of 1.5 μM (Andrighetti-Fröhner et al., 2006).

Nearly three-fourths of patients with high-risk bladder cancer will relapse within the following few years of their diagnosis. Even though most patients do not die of primary bladder cancer, the vast majority endures the morbidity of recurrence and progression of their cancer (Wong et al., 2018). Besides, Astolfi et al have shown that the dosage of cisplatin constitutes the main factor influencing the severity of its adverse effects (Astolfi et al., 2013). Therefore, increasing efforts should be made to offer patients intravesical therapy for minimizing the incidence of recurrences (Chamie et al., 2013). Thus, the development of improved intravesical chemotherapies, for treating high-grade, non-muscle-invasive bladder cancer becomes of the utmost importance (Kim et al., 2015). For this reason, we choose cells derived from an *in situ* carcinoma as well as from metastatic bladder tumor cells to test sensitivity to violacein alone or in combination with a classical chemotherapy agent (CDDP).

Currently, many investigators are studying if different natural molecules (from plants or microbes) or combinations of molecules could potentiate the anti-tumor activity of classical chemotherapy agents (Aumeeruddy and Mahomoodally, 2019; Wei et al., 2020; Xiao et al., 2019). For example, experimental evidence suggests that herbal extracts such as *Solanum nigrum* and *Claviceps purpurea* and isolated herbal compounds (e.g., curcumin and resveratrol) combined with antitumoral drugs have the potential to attenuate resistance against cancer therapy (Lin et al., 2020). Actually, several natural products, belonging to diverse chemical families, have shown to be potent chemosensitizers for tumor therapy, enhancing the cytotoxicity of conventional drugs. In this respect, our results suggest that violacein is an interesting agent to be incorporated in future cancer therapy since it allows the block of cancer cells growth, without increasing genomic instability. However, a better understanding of the cell death mechanisms induced by this compound will be useful to develop new therapeutic approaches.

5. Conclusion

It was shown that violacein has an *in vitro* antiproliferative and non-genotoxic effect in bladder cancer cell lines. Besides, violacein sensitized CDDP in bladder cancer cell lines, increasing cell death but reducing its genotoxic effect. Besides, it is worth to mention, that the synergistic effect observed when combining violacein and CDDP in killing bladder cancer cells (with no increase in the genetic damage), may diminish the side effects of classical chemotherapy, whose main target is the genetic material. Therefore, violacein could be considered as an antiproliferative drug to control the growth of bladder cancer cells. Nevertheless, to confirm this assumption, *in vivo* assays should be performed (using animal models) as well as a thorough analysis of its possible mechanism of action.

Ethical Approval

Not Applicable.

Date Availability

Supporting data is available upon request.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Diego Alem: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Lucía Canclini:** Investigation, Formal analysis, Writing – original draft. **Susana Castro-Sowinski:** Conceptualization, Supervision. **Wilner Martínez-López:** Conceptualization, Supervision, Writing – review & editing.

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Supplementary Materials

Nil.

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