Evaluation of the anti-proliferative activity of violacein, a natural pigment of bacterial origin, in urinary bladder cancer cell lines

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Abstract. Violacein is a natural pigment, a pyrrolidone, and a bisindole derived from the condensation of two tryptophan molecules, which gives a blue violet color to several gram-negative violacein-producing bacteria. Violacein production provides a competitive advantage against antagonistic species or predators. In addition, the compound has antibacterial, antifungal, antiviral, and antioxidant activities. Several studies on colon, breast, and head and neck cancer lines have already demonstrated the anti-proliferative potential of violacein. Bladder cancer is one of the most common types of cancer in urology. The therapeutic approach is mainly based on surgery, chemotherapy, and immunotherapy. The aim of the present study was to evaluate the anti-proliferative activity of violacein against the human bladder cancer cell lines, HTB4 (T24) and HTB9 (5637), using low-grade and high-grade transitional cell carcinoma models, respectively, which has never been assayed before. For this purpose, the potential violacein anti-proliferative effect on T24 and 5637 cells was evaluated by studying the cell viability, proliferation, cell cycle, and caspase-3 activation. The results showed that violacein had anti-proliferative activity in the two cell lines, which was greater for the second-stage bladder cancer cell line (5637), and a different mode of action against the two cell lines.

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Introduction

In recent decades, many natural molecules of bacterial origin have been tested to evaluate their anti-proliferative activity. Pigments produced by microbes, such as melanin, flavin, phenazine, and violacein, are medically interesting and are well known to show cytotoxic, antioxidant, antimicrobial, and antimalarial activities (1). Violacein is a member of the class of water-soluble hydroxyindoles, it is formed by the condensation of two modified tryptophan molecules, with a molecular weight of 343.33 ($C_{20}H_{13}N_3O_3$) (Fig. 1) (2). It is a natural pigment that confers a blue violet color to bacteria that produce it, such as *Chromobacterium violaceum (C. violaceum)*, *Janthinobacterium lividum (J. lividum)*, *Alteromonas luteoviolacea*, *Pseudoalteromonas luteoviolacea*, and *Duganella* sp. (3). These bacteria are all gram-negative, facultative aerobes, and saprophytes belonging to Proteobacteria phylum. They are mainly isolated from soil and water in tropical and subtropical regions and are able to survive in hostile environmental conditions (3). In *J. lividum* and *C. violaceum*, the production of violacein and biofilm appears to be regulated by a common metabolic pathway (4). Violacein production is a response to environmental stress and a key factor associated with survival in hostile conditions (5).

Several studies have shown that this substance possesses different activities: antibacterial (6), anti-fungal (7), trypanocidal (8), immunomodulating, analgesic, antipyretic (9), and anti-proliferative (9,10). Violacein is able to induce apoptosis in HL60 leukemic cells through the activation of caspases (11), which play an important role in the induction of programmed cell death. The same study also showed the anti-cancer activity of violacein versus leukemia cells of the chronic myeloid line (K562 cell line). Furthermore, violacein appears to be non-toxic in peripheral blood cells at a concentration of 700 nM (11). Programmed cell death is one of the biological events underlying the regulation of tissue homeostasis, but it also plays a role in the elimination of damaged, stressed, or infected cells. The modalities of cell death can be traced back to two models: death suffered, whose morphological aspect is necrosis, and cell death or suicide, whose typical form (also physiological) is apoptosis (12,13). Necrosis

causes the involvement of the structures surrounding inflammation. Apoptosis is a form of programmed cell death that does not induce inflammation and helps to keep the number of cells stable (14). Apoptosis is characterized by a series of dramatic disturbances in the cellular architecture that not only contribute to cell death, but also prepare cells for phagocyte removal and prevent unwanted immune responses. Much of what occurs during the breakdown phase of apoptosis is orchestrated by members of the cysteine protease caspase family (12). Alterations of these mechanisms are responsible for the onset of severe diseases (12). Corroborating data on the anti-cancer activity of violacein can be found in studies carried out on colon (15), breast (16), and head and neck (17) cancer cell lines. Kodach *et al* demonstrated that, in the HCT116 cell line, violacein enhances the cytotoxic effect of 5-fluorouracil, a chemotherapeutic agent used in the treatment of colorectal cancer (CRC) (18). The literature data show that in Caco-2 cells, violacein causes tumor cell apoptosis, stimulating the production of ROS, the release of cytochrome c and calcium into the cytosol, and the activation of caspase-3 (15,19).

Bladder cancer is the second most frequent tumor of the urinary tract, it is more common in men than in women (at a ratio of 4:1) (20), representing the seventh and seventeenth most diagnosed cancer types in the male and female population worldwide, respectively (21). It mainly affects individuals aged 50-70 years (20) and the incidence and mortality of this cancer are influenced by different risk factors, such as tobacco smoking and prolonged exposure to aromatic polyamines (22). At diagnosis, bladder cancer is superficial in 75% of cases with a disease limited to the mucosa (stage Ta, CIS) or submucosa (stage T1). The main therapeutic strategies for urinary bladder tumor is surgery followed by adjuvant and neoadjuvant therapy, such as single immediate, post-operative intravesical instillation of chemotherapy or repeated instillations, based on prognosis (21).

Novel drugs represent an innovative pharmaceutical tool for implementation of the current therapies for this type of cancer (23). Owing to its antibacterial and antitumoral properties, through bladder instillations, violacein may represent a favorable therapeutic option as an anticancer agent in both pre- and post-surgery. To this purpose, the aim of the present study was to investigate, for the first time, the anti-proliferative activity of violacein against human bladder carcinoma cell lines T24 and 5637, respectively, transitional cell carcinoma (low grade) and grade II carcinoma (high grade), and to explore the mode of action of this substance on these cell lines. The present preliminary *in vitro* study on bladder carcinoma cell lines may represent the first step in the design of violacein as a potential antineoplastics drug.

Materials and methods

General. To evaluate the anti-proliferative activity of violacein against urinary bladder cancer, the proliferation and cell viability of two bladder cell lines, T24 and 5637, were initially assessed following treatment with violacein. Cell proliferation and viability were measured using the MTT assay, and the viable cell count was conducted using the trypan blue assay. Furthermore, the cytotoxic effect induced by violacein in the two cell lines was examined through observation of the cell

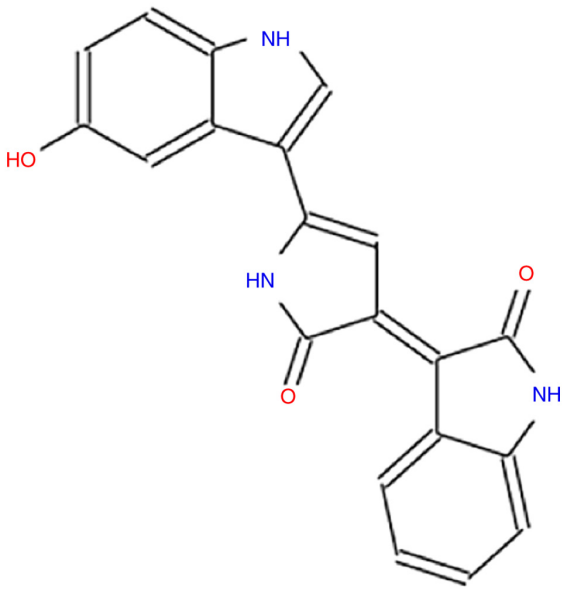

Figure 1. The 2D representation of the molecular structure of violacein (drawing made by the authors).

nuclei, which were stained with DAPI fluorophore, using fluorescence microscopy. Subsequently, using colorimetric assays and flow cytometry, the cell cycle of T24 and 5637 following violacein treatment, which were labelled with propidium iodide (PI) was examined. In addition, the activation of the caspase-3 protease was assessed by detecting the antibody directed against caspase-3 cleaved.

Materials. Violacein, dimethylsulfoxide (DMSO), methanol, and acetic acid were purchased from Sigma-Aldrich. The PI was obtained from Biotium Corporate Headquarters. The MTT assay and DAPI were purchased from SERVA Electrophoresis GmbH. The T24 and 5637 cell lines were purchased from the American Type Culture Collection (ATCC). Media, RPMI-1640, Trypsin + EDTA, phosphate-buffered saline (PBS), penicillin-streptomycin, and sera for the cell culture were obtained from Corning and were endotoxin-free. For the immunofluorescence experiments, anti-cleaved caspase-3 primary antibody (1:20 dilution; ab2302) and goat anti-rabbit IgG-H&L polyclonal secondary antibody (1:5 dilution; ab150077; Alexa Fluor® 488) were purchased from Abcam plc. RNase A and Lab-Tek™ II Chamber Slide™ were purchased from Thermo Fisher Scientifics Baltics UAB. Tween-20 solution and paraformaldehyde solution 4% in PBS were obtained from Bio-Rad Laboratories Srl.

Cell cultures. The human urinary bladder carcinoma T24 and 5637 cells were cultured in RPMI-1640, without phenol red, supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The T24 and 5637 cells were obtained from ATCC and were grown in RPMI-1640, supplemented with 10% FBS, according to international guidelines. Mycoplasma testing was performed for the cell lines. The cells were incubated at 37˚C in a humidified atmosphere of 5% CO_2 for two weeks prior to experimental use. To subculture the T24 and 5637 cells, they were washed with PBS and incubated with 0.25% trypsin

and 1 mM EDTA at 37˚C for 3-5 min (T24) and 5-7 min (5637). Then, the detached cells were resuspended in a fresh serum-containing medium to inactivate the trypsin and transferred to new flasks.

Cell proliferation assay (MTT). The T24 and 5637 cells were seeded in parallel in 96-well plates at a density of 5.000 cells/well. The next day, the cells were exposed to different concentrations of violacein (0.1, 0.2, 0.3, 0.5, and 1 μM) for 24 h. Two cellular controls were set up in parallel, one containing only the cell culture medium (RPMI-1640 supplemented with 10% FBS) and the other containing the cell culture medium with an added percentage of DMSO used to resuspend violacein (0.1% v/v). After the treatment, the cells were treated with 20 μl of 5 mg/ml MTT in PBS, and the plates were incubated for 4 h in the dark at 37˚C and 5% CO_2. Then, the medium was removed, and the formed formazan salt was dissolved by adding 100 μl of DSMO to each well. The optical density was measured at 570 nm, with the reference set at 620 nm, using an automatic microplate reader, Tecan Sunrise (24). The assay was carried out in triplicate. To understand whether the cytotoxic effect was permanent or reversible, the same test was performed 48 h after the end of the violacein treatment. Briefly, after the treatment with different concentrations of violacein, the medium (RPMI-1640 supplemented with 10% FBS) was discarded, and fresh RPMI-1640 medium with 10% of FBS was added to the cells, which were then incubated at 37˚C and 5% CO_2 for 48 h. Then, the MTT test was carried out as described above. The assay was performed in triplicate. No absorbance differences were found between the two cell controls, indicating that the percentage of DMSO used was nontoxic for the cells (Fig. S1). For this reason, only fresh medium with added DMSO was used as a negative control in the following tests.

Vital staining with trypan blue. The cell viability was assessed using the dye, trypan blue. T24 and 5637 cells seeded in 6-well plates at a density of $1x10^6$ cell/well were exposed to different concentrations of violacein (0.1, 0.2, 0.3, 0.5, and 1 μM) and to the cell medium alone as a control. At the end of the treatment, the medium was collected and centrifuged at 1,200 x g for 5 min at 4˚C to recover the dead cells present in the suspension. The cells still adhering to the wells were detached by trypsinization and centrifuged at 1,200 x g for 5 min at 4˚C, and the pellet was recovered and resuspended in 1 ml of a complete medium (RPMI-1640 supplemented with 10% FBS) also containing the dead cells that were previously recovered. An aliquot was then taken and added to an equal volume of trypan blue (0.4%). The cells were then counted using an optical microscope via a Burker's chamber (25). The assay was carried out in triplicate.

DAPI images. To evaluate the presence of morphological changes characteristic of the cytotoxic effect, the T24 and 5637 cells were seeded at a density of $2x10^5$ cells/well in 8-well Lab-Tek chamber slides and exposed to 1 μM of violacein or a fresh medium (as a control) for 24 h at 37˚C. Briefly, after the violacein treatment, the medium was removed, and the remaining adhering cells were washed with PBS and fixed with fixative (3:1 methanol/acetic acid) for 10 min at room temperature (rt). Subsequently, the cells' nuclei were stained with a solution of 1 μg/ml DAPI in PBS at 37˚C for 15 min. Excess solution dye was removed, the polystyrene chambers were detached, and the remaining slides were directly examined under a fluorescence microscope (330-380 nm), Leica DM IRBE.

Cell cycle analysis. Flow cytometric analysis of the cells was performed using a MACSQuant Analyzer 10-flow cytometer (Miltenyi Biotec Inc.). To analyze the cell cycle distribution, the T24 and 5637 cells were treated with 1 μM of violacein and a fresh medium (as control) for 24 h. After the treatment, the medium was removed and centrifuged at 1,200 x g for 5 min at 4˚C to recover the cellular pellet, while the adhering cells were collected by trypsinization. After fixation with 75% cold ethanol, the cells were washed in PBS and resuspended in 1 ml of PBS containing 0.5 mg/ml of RNase A and 0.01 mg/ml of PI and incubated at room temperature in the dark for 30 min. The percentage of cells in the sub-G1, G0/G1, S, and G2/M phases of the cell cycle was analyzed using the FlowJo software (Becton, Dickinson and Company).

Caspase-3 activation. An indirect staining assay by flow cytometry was used to test the caspase-3 activation. Briefly, the two cell lines were seeded at a concentration of $5x10^5$ cells/well in 12-well plates. The following day, the cells were exposed to 1 μM of violacein or a fresh medium only (as a control) for 1, 3, 6, or 24 h. After the treatment, the cells were resuspended in a 4% paraformaldehyde solution in PBS and kept on ice for 30 min. The cells were then centrifuged at 300 x g for 5 min at rt, and the pellet was resuspended in 0.5 ml of 0.2% Tween solution in PBS. After three washes, the cell pellets were resuspended in a staining solution containing the primary antibody (ab2302; anti-cleaved caspase-3 antibody) and incubated for 10 min at rt. At the end of the incubation, the cells were washed with PBS and then incubated for 30 min at room temperature in a staining solution containing the secondary antibody (ab150077). Subsequently, the obtained solutions were transferred into appropriate tubes for flow cytometric analysis.

Statistical analysis. Each assay was replicated at least three times, and statistical significance was determined using GraphPad Prism 9 statistical software package (GraphPad Software, Inc.). Data are expressed as means ± standard deviation (SD). Multiple comparisons among group mean differences were analysed with one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. The Student's t-test was used to compare paired and un-paired samples. The means of the same group under two separate scenarios, were compared with paired t-test (e.g., Fig. 2). The means of two independent or unrelated groups (e.g., Fig. 3), were compared with the unpaired t-test. Differences were considered significant when $P<0.05$.

Results

Cell proliferation activity and viability. After 24 h of exposure to different violacein concentrations (0.1, 0.2, 0.3, 0.5, and 1 μM), the cytotoxic effect of violacein on T24 and 5637 cells was evaluated through the measurement of cell viability by

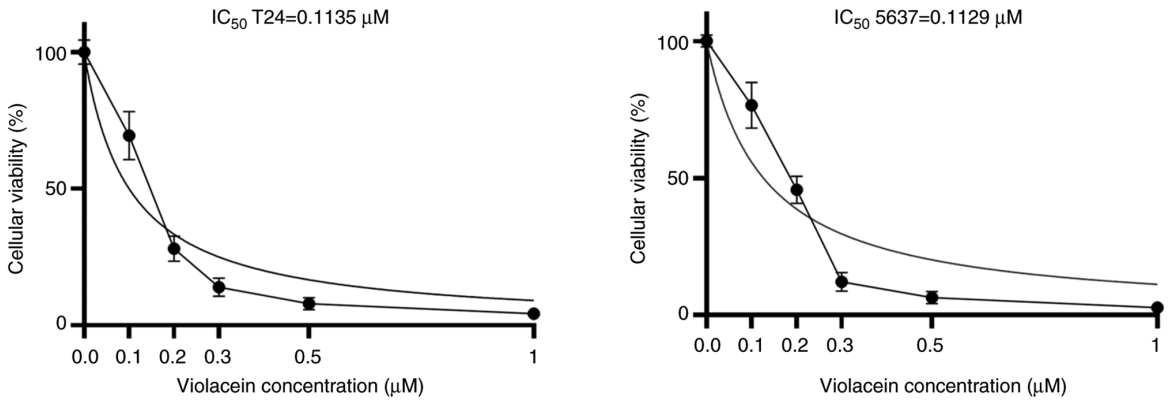

Figure 2. Dose-response curve obtained with the MTT assay after 24 h of contact with increasing concentrations of violacein. The data are presented as the mean ± SD.

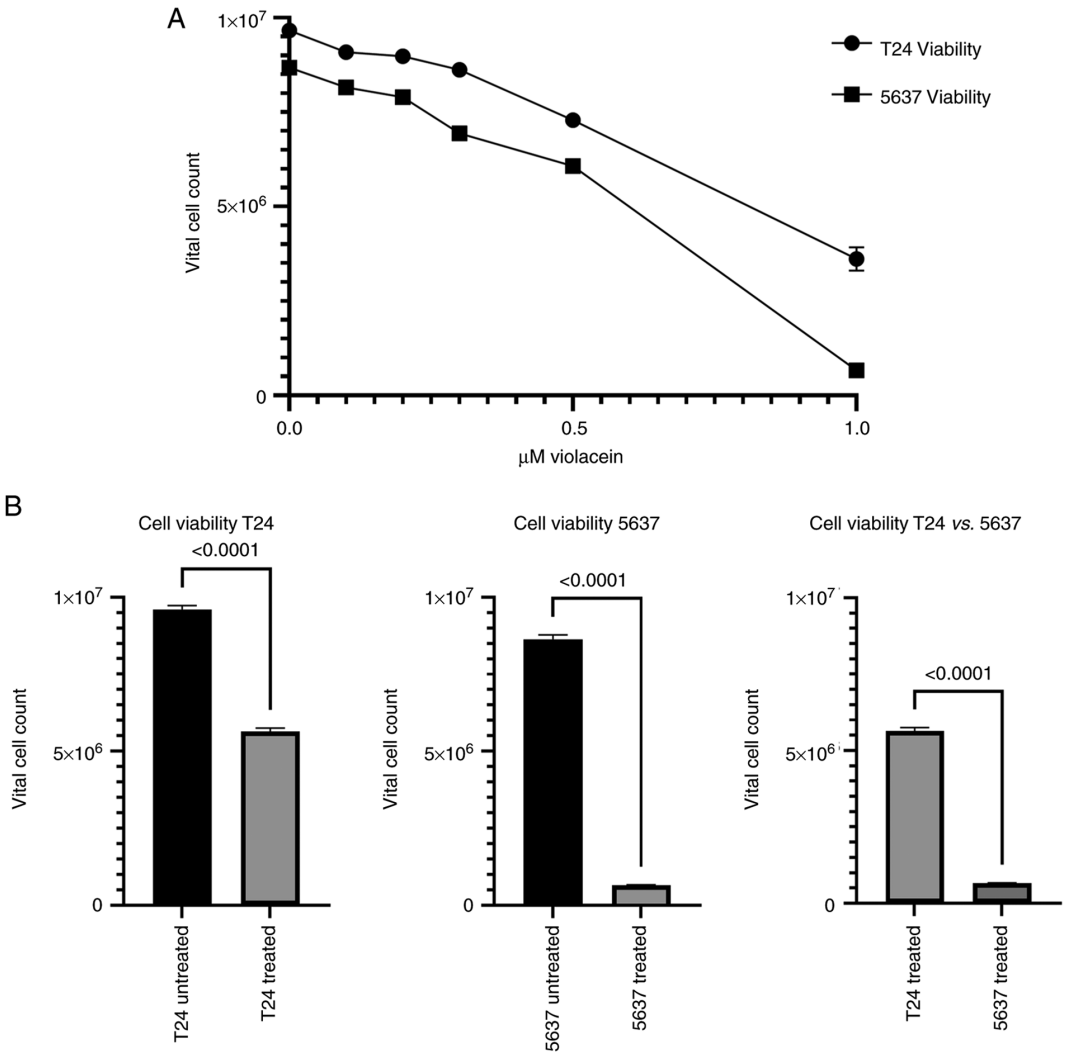

Figure 3. Trypan blue assay. (A) Cell viability of T24 and 5637 cell lines, represented as logarithmic scale (y-axis), treated with increasing concentrations of violacein (x-axis), for 24 h. The data are presented as the means ± SD. (B) Histogram plot for each cell line, relative to the number of live cells after the treatment with 1 μM of violacein for 24 h, compared with their respective untreated cells (resuspended in fresh medium with additional DMSO). A histogram plot showing a comparison of the two treated cell lines is shown on the right of panel 3B. The data are presented as the mean ± SD, P<0.001. The assays were carried out in triplicate.

MTT assay, and a vital count was assessed with trypan blue dye. The assay was based on the principle that proliferating cells are capable of transforming tetrazolium salts (MTT) into formazan. No significant changes were observed in the absorbance values after the restoration of the fresh medium for 48 h, compared to the values obtained by the MTT assay, which

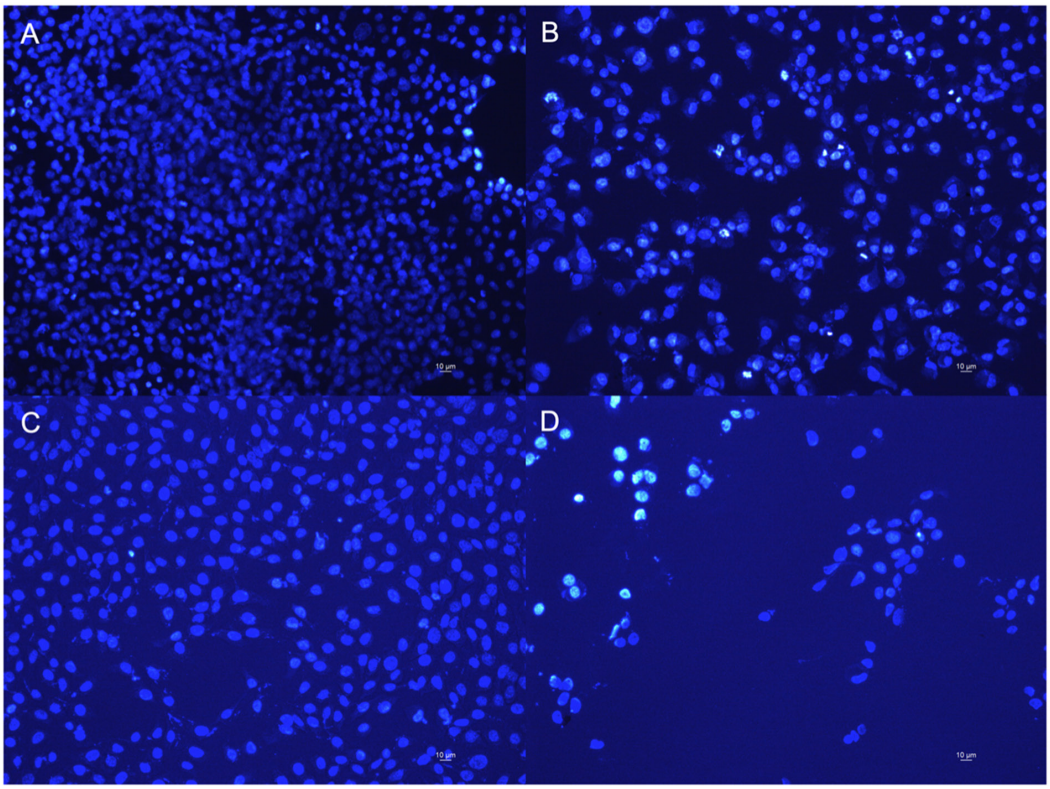

Figure 4. Violacein exposure alters the morphology of T24 and 5637 cell nuclei. DNA staining with DAPI. (A) Untreated cells of T24 (resuspended in fresh medium with additional DMSO), (B) T24 cells treated with 1 μM of violacein for 24 h, (C) untreated cells of 5637 (resuspended in fresh medium with additional DMSO), and (D) 5637 cells treated with 1 μM of violacein for 24 h. The images shown are representative of the DAPI staining of three independent experiments. Magnification, x100. Scale bars, 10 μm.

was performed immediately after the treatment (Fig. S2), indicating that violacein induces a permanent cytotoxic effect. As shown in Fig. 2, cell viability was evident in both cell lines (T24 and 5637), but inhibited by the violacein treatment in a dose-dependent manner, with an IC_{50} value of 0.1135±0.05 μM for the T24 cell line and an IC_{50} value of 0.1129±0.1 μM for the 5637 cells. To determine whether a reduction in cell proliferation also corresponded to an increase in cell mortality, the vital count with trypan blue dye was added to the MTT assay. Although inhibition of the cell proliferation had already started at a violacein concentration of 0.1 μM (Fig. 2), only at the 1 μM dose was there a significant parallel increase in cell mortality. In fact, compared to untreated cells, treatment with 1 μM of violacein caused a significant decrease in the cellular viability in both cell lines ($P<0.0001$) (Fig. 3A and B). In order to confirm the difference between the cell lines used with respect to violacein sensitivity, the viability level in T24 and 5637 cells treated for 24 h with 1 μM of violacein was examined (Fig. 3B). A 2% viability reduction for T24 and 10% for 5637 ($P<0.0001$), when compared with their respective untreated cells, was observed (Fig. 3B). The 5637 cell line, a model of second-grade bladder cancer, showed a greater sensitivity to violacein, compared to the T24 cell line.

Morphological evaluation of cell nuclei with DAPI. In order to evaluate the cytotoxic effect induced by violacein, the nuclei of the two cell lines were stained with DAPI, a fluorescent DNA stain, and visualized under a widefield optical fluorescence microscopy (Leica DM 5000B). From the images detected (Fig. 4), a decrease of the number of nuclei per field is evident in both cell lines after the treatment, compared to the untreated cells. On the other hand, the nuclei show a normal phenotype and homogeneous light emission signal in the untreated cells, while the nuclei of the violacein-treated cells show a greater fluorescence intensity, indicative of the damage. These results corroborate our cytotoxicity data.

Cell cycle. To investigate the mechanism underlying the action of violacein in the two cell lines, analysis of the progression of the cell cycle after the violacein treatment was performed. The cell cycle distribution analysis of both T24 and 5637 cell lines was performed after 24 h of 1 μM violacein exposure (Fig. 5). As shown in Fig. 5 and reported in Table I, the T24 G0/G1 population treated with violacein decreased by 20%, compared to the untreated cells, while the treatment caused an increase of 18% in the progression of S-phase blocking cells in G2/M phase. The cell diminution in the G0/G1 phase caused an increase in sub-G1 particles, with 2.5% more apoptotic cells after the treatment, compared to the untreated cells. The cell cycle analysis of 5637 cells revealed a possible different mode of action of violacein, with respect to what was observed in the T24 cells, with an increase in the G0/G1 population, compared to the untreated cells (25%), indicating a block in this phase, with a decrease of the cell population in the S phase (11.5%). A significant 1.2% increase in the sub-G1 cell fraction, probably representing apoptotic cells, compared to the untreated cells, was observed. This fraction would seem to be greater in the T24 cell line, compared to 5637.

Table I. Value for the cell percentage in each phase of the cell cycle.

Cell cycle phase	T24 cells		5637 cells	
	Ctrl	Treated with 1 μM of Violacein	Ctrl	Treated with 1 μM of Violacein
Sub-G1	6.9±0.5	9.4±0.5[b]	7.9±0.4	8.7±0.5[a]
G0/G1	65.6±3	46.6±2[b]	24.8±1	48.6±2[b]
S	13.5±1	31.4±2[b]	39.2±2	27.7±1[b]
G2/M	14.0±2	12.6±0.5[a]	28.1±3	15.0±2[b]

Values for the cell percentage in each phase of the cell cycle, treated with 1 μM of violacein for 24 h or untreated and resuspended in fresh medium with additional DMSO (Ctrl) are reported. The data present the mean percentage of cells ± SD and are calculated from three independent experiments. [a]P<0.05; [b]P<0.01.

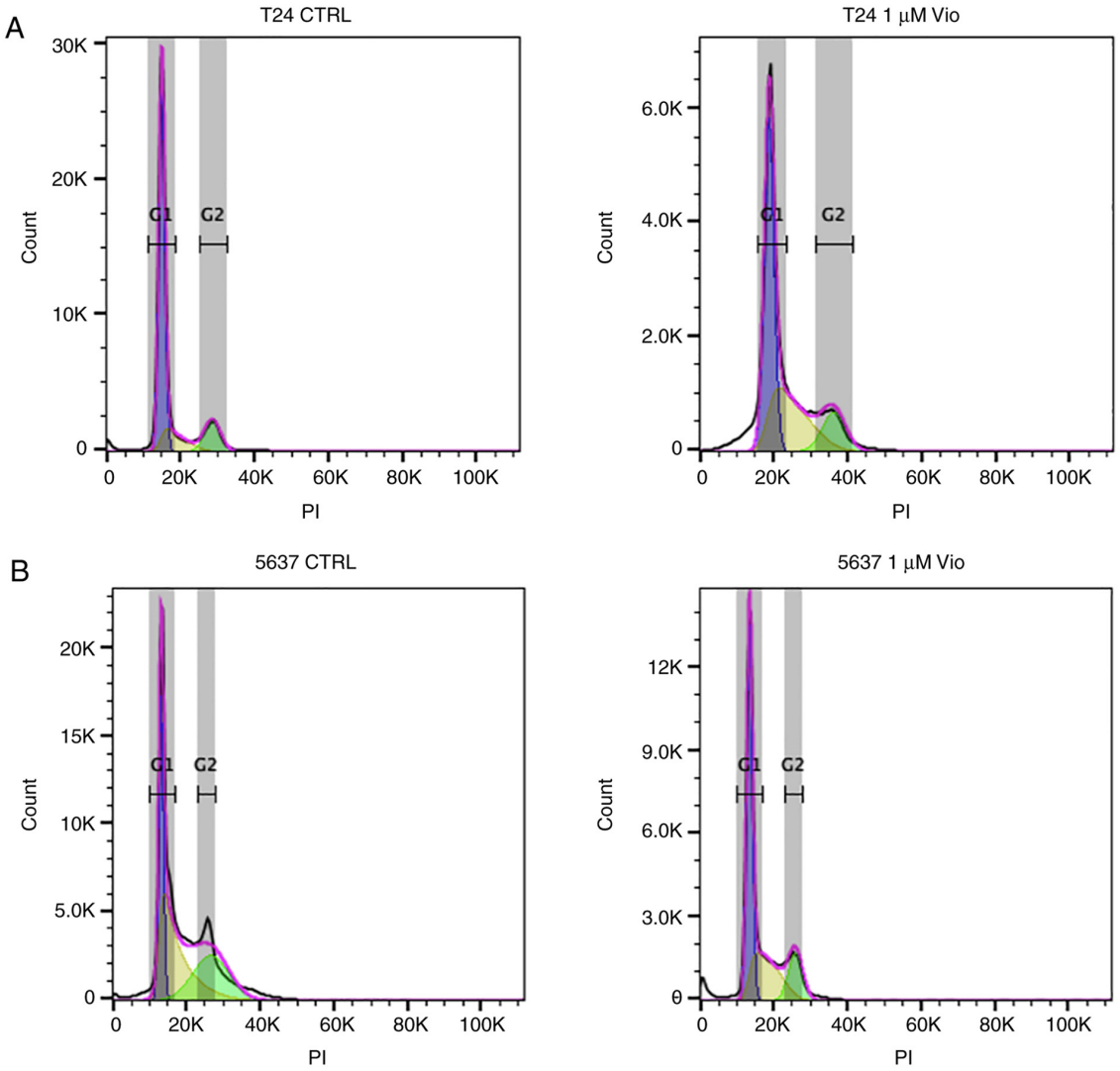

Figure 5. Distribution of T24 and 5637 cells in the different cell cycle phases. (A) Cell cycle progression in T24 violacein-treated cell lines and untreated cell lines (CTRL) resuspended in fresh medium with additional DMSO. (B) Cell cycle progression in 5637 violacein-treated cell lines and untreated cell lines (CTRL) resuspended in fresh medium with additional DMSO. The distribution of T24 and 5637 cells in the different phases of the cell cycle was analyzed by flow cytometry using PI staining, after 24 h of cell exposure to a concentration of 1 μM of violacein. The panels show representative flow cytometry profiles obtained from three independent experiments. Count, cell counts; K, x1,000; PI, propidium iodide.

Active caspase-3 production. A distinctive feature of the early stages of apoptosis is the activation of caspase enzymes, which participate in the cleavage of protein substrates and the subsequent disassembly of the cell. Caspase-3, a fundamental mediator of apoptosis, also termed the death protease, is an effector caspase that cuts precise protein substrates, thus

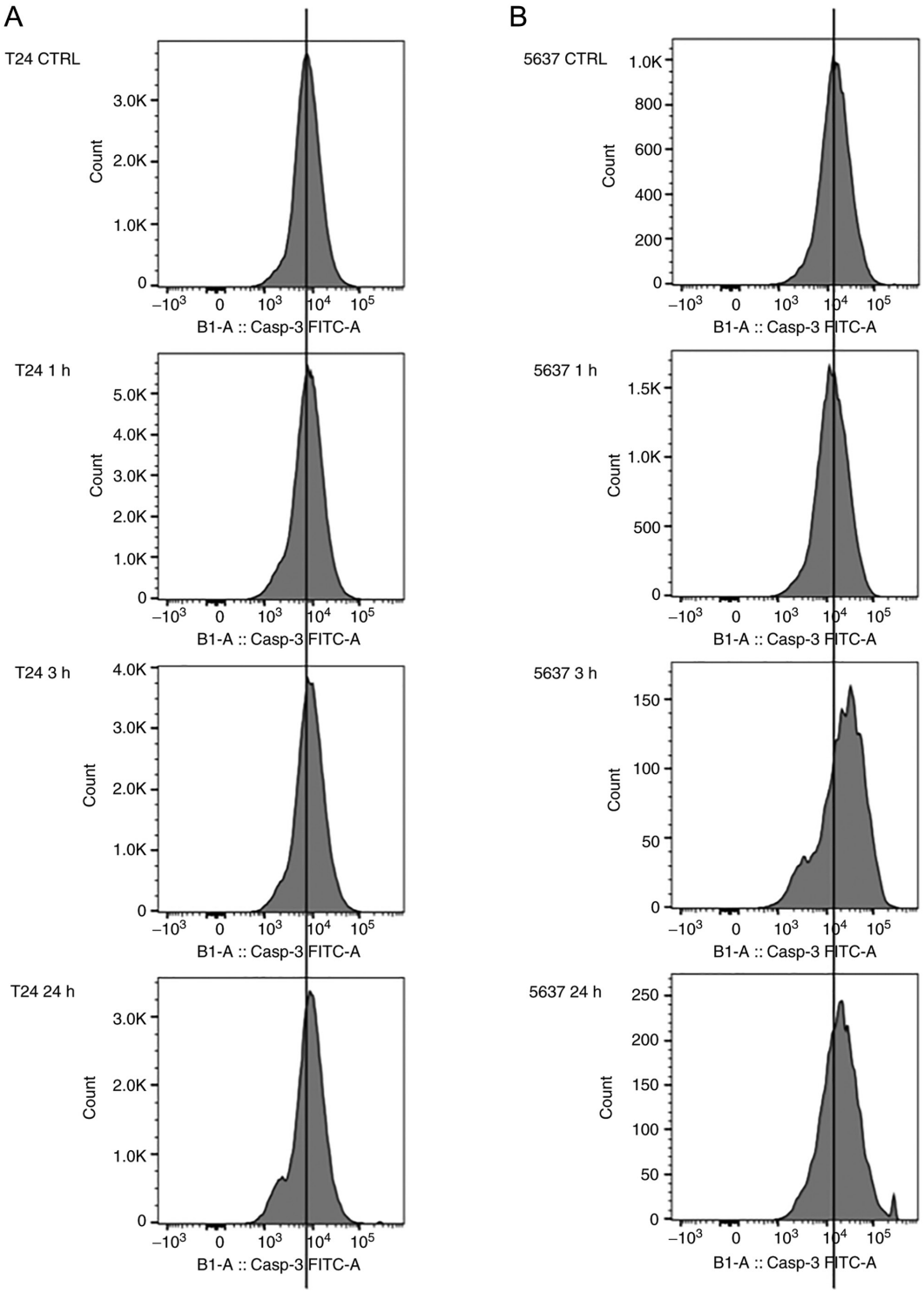

Figure 6. Caspase-3 activation. Histograms of the median cell fluorescence intensity. The median cell fluorescence intensity is directly related to the active caspase-3 content. The straight line indicates the median fluorescence of the untreated cells (CTRL) resuspended in fresh medium with additional DMSO in the two cell lines. (A) T24 cells after the treatment with 1 μM of violacein for 1, 3, and 24 h; (B) 5637 cells after the treatment with 1 μM of violacein for 1, 3, and 24 h. The panels are representative of the flow cytometry profiles obtained from three independent experiments.

initiating the apoptotic process (26). To highlight the onset of the apoptotic process, after the violacein treatment, caspase-3 activation was evaluated by caspase-3 fluorometric assay.

The two cell lines were subjected to a treatment of 1 μM violacein for 1, 3, or 24 h. The activation index of caspase-3 induced by the violacein treatment was determined as the ratio between the fluorescence values of both the treated and the untreated cells. In the two cell lines, the maximum expression of active caspase-3 was obtained after 3 h of the treatment (Fig. 6). After 24 h of violacein exposure, the expression level of caspase-3 returned to the untreated cell values (Fig. S3). The expression of the active caspase-3 was higher in the 5637 cell line, with double the value for Median Fluorescence Intensity, compared to the untreated cells (Figs. 6 and 7).

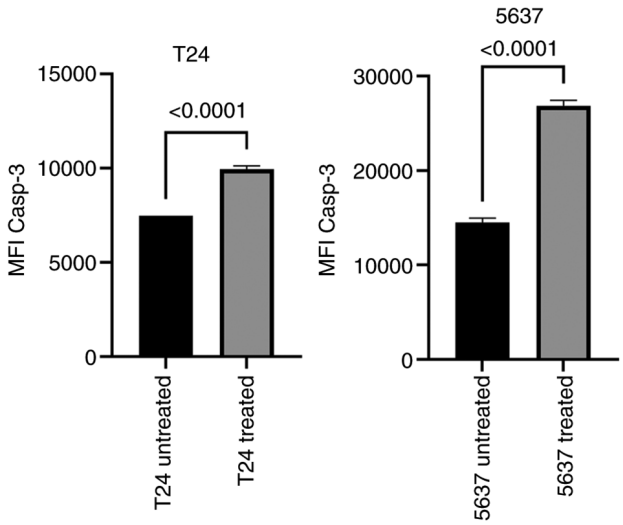

Figure 7. Histogram plot for each cell line, relative to the median of the fluorescence intensity values of the cells based on the caspase-3 activation (MFI Casp-3), after the treatment with 1 μM of violacein for 3 h, compared with their respective untreated cells resuspended in fresh medium with additional DMSO. The data are presented as the mean ± SD; P<0.001. The assays were carried out in triplicate.

Discussion

The therapeutic approach for bladder cancer involves interventions frequently used in combinations: surgery, chemotherapy, or immunotherapy (21). Several natural compounds with the ability to induce programmed cell death (apoptosis), causing minimal damage to the surrounding cells and tissues, are currently under evaluation. Among these compounds, the pigment, violacein, has been highlighted for its anti-proliferative activity against specific cell lines (17). The violacein anti-proliferative activity appears to be due to the induction of the apoptotic process, as already shown in several cancer models (15-17,27). In the present study, the anti-proliferative activity of this substance was evaluated against two cell lines, T24 and 5637, which are models of human transitional cell carcinoma, low-grade, and high-grade, respectively. The effect of violacein on these cells of the same derivation tissue, but at different tumor stages, has, to the best of our knowledge, never been previously tested.

The DAPI staining assay confirmed the cytotoxicity data of the present study showing, after 24 h of treatment with violacein, a decrease in nuclei per field and an increased fluorescence intensity for the nuclei of violacein-treated cells, indicative of damage, thereby corroborating the cytotoxicity data obtained in the present study. In order to better understand the type of death activated by violacein further studies are necessary including semi-quantitative analysis with DAPI, and assay with annexin V-FITC and PI, as in the current study. Exploring violacein activity, a proliferation inhibitory effect and an activation of the caspase-3 protease were identified in the two cell lines, with a greater antiproliferative activity against the second-grade tumor model cell line, i.e., 5637. Moreover, from the cell cycle analysis, a different violacein mode of action on these cell lines emerged. In particular, violacein appears to block T24 cells in the S phase of the cell cycle, thus preventing progression in the G2/M division phase, while the 5637 cell line appeared to be blocked in phase G1. Furthermore, a significant increase in sub-G1 particles (probably representing apoptotic cells) after the violacein treatment in both cell lines was observed. The distribution of T24 and 5637 cells in the different cell cycle phases showed a higher apoptotic cell percentage in the T24 cell line, compared to 5637 (2.5 versus 1.2%, respectively). The violacein apoptotic induction was evaluated by the expression of active caspase-3 in the lines employed, after treatment with violacein. Caspase-3 is a protease synthesized as an inactive zymogen in the cytosol of mammalian cells. It is rapidly activated through a specific cascade of events in the executive phase of apoptosis (28). A significant increase in active caspase-3 expression was observed in the two cell lines after 3 h of treatment with violacein. The 5637 cell line showed both a basal expression of caspase-3 and a higher cellular proliferation activity, than the T24 cells. The results of the present study support the hypothesis that violacein may have different modes of action on these cell lines possibly due to molecular differences among cells. However, further investigations are required to gain a better understanding of the violacein-induced cell death mechanism in bladder cancer cells, with particular attention to the differences observed between the two cell lines. Further studies are also needed to determine how violacein affects caspase-3, directly or indirectly. Moreover, for the 5637 cell line, the apparent discrepancy in the percentage of death and apoptotic cells obtained through the vital count with trypan blue (Fig. 3B) and the cell cycle analysis (Fig. 4; Table I), respectively, could be supported by the co-existence of different mechanisms of cell death. In addition, the present preliminary *in vitro* study has shown, through classical assays based on biochemical parameters and morphological changes, the cytotoxic power of violacein against bladder cancer cells assuming activation of the apoptotic pathway. In order to identify molecular changes characteristic of apoptosis and to attest the anti-proliferation activity of violacein, further experiments, such as western blot analysis of caspase-3, Bcl-2, Bax, and proliferating cell nuclear antigen (PCNA), are needed. Furthermore, to investigate the death activated by violacein, to allow the distinction between necrotic and apoptotic cells, a valid approach was represented by flow cytometry analysis of annexin V/PI assay. Future studies should examine the effect of violacein in more advanced bladder cancer cell lines. In fact, in the case of advanced and metastatic disease the tumor extends through the bladder wall until it invades the pelvic and abdominal wall with the ability to invade distant organs. In this case, the total removal of the tumor was technically impossible and treatment with chemotherapy to eradicate the tumor cells appears to be the main therapeutic option. In this context, violacein may represent a potential valid therapeutic strategy or an alternative/adjuvant to existing ones focused on killing cancer cells, especially against those at a more advanced stage. This preliminary *in vitro* study on bladder carcinoma cell lines may be the first step in the design of violacein as a potential antineoplastics drug.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BN conceptualized and designed the study, performed the experimental part of the study, analyzed all the results, wrote the manuscript and acquired the funding necessary for the study. MAZ analyzed and interpreted the data regarding the cell cycle analysis. GR analyzed results obtained from the MTT assay and revised the manuscript. MRC analyzed the data regarding the cell cycle analysis, and visualized and critically revised the manuscript. LM supervised the experiments, performed the MTT assay and wrote and critically revised the manuscript. FP conceptualized the study, supervised experiments, analyzed the results and wrote and revised the manuscript. SS conceptualized and designed the study, supervised the experiments, analyzed results, wrote the manuscript and critically revised it. SS and FP confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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