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Violacein synergistically increases 5-fluorouracil cytotoxicity, induces apoptosis and inhibits Akt-mediated signal transduction in human colorectal cancer cells

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Despite recent additions to the armory of chemotherapeutic agents for colorectal cancer (CRC) treatment, the results of chemotherapy remain unsatisfactory. 5-Fluorouracil (5-FU) still represents the cornerstone of treatment and resistance to its actions is a major obstacle to successful chemotherapy. Therefore, new active agents in CRC and agents that increase the chemosensitivity of cancer cells to 5-FU are still urgently required. Violacein, a pigment isolated from *Chromobacterium violaceum* in the Amazon river, has a diverse spectrum of biological activities, and represents a novel cytotoxic drug with known antileukemic properties. To assess the suitability of violacein as a chemotherapeutic agent in CRC its cytotoxic effects were evaluated both as a single agent and in combination with 5-FU. Its underlying mechanisms of action were further investigated by studying its effects on the cell cycle, apoptosis and cell survival pathways [phosphatidylinositol-3-kinase/Akt, p44/42 mitogen activated protein kinase and nuclear factor κB (NF-κB)] in colon cancer cell lines. Violacein inhibits the growth of all four colon cancer cell lines tested. It induces apoptosis, and potentiates the cytotoxic effect of 5-FU in a poorly differentiated microsatellite unstable cell line (HCT116). Violacein causes cell cycle block at G_1, upregulates p53, p27 and p21 levels and decreases the expression of cyclin D1. Violacein leads to dephosphorylation of retinoblastoma protein and activation of caspases and a pancaspase inhibitor abrogates its biological activity. Our data provide evidence that violacein acts through the inhibition of Akt phosphorylation with subsequent activation of the apoptotic pathway and downregulation of NF-κB signaling. This leads to the increase in chemosensitivity to 5-FU in HCT116 colon cancer cells. Taken together, our findings suggest that violacein will be active in the treatment of colorectal tumors and offers new prospects for overcoming 5-FU resistance.

Abbreviations: CI, combination index; CRC, colorectal cancer; DMSO, dimethyl sulfoxide; 5-FU, 5-fluorouracil; GSK-3α/β, glycogen synthase kinase 3α/β; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; PI3 kinase, phosphatidylinositol-3-kinase; Rb, retinoblastoma protein.

Introduction

Violacein, a purple-colored pigment produced by one of the strains of *Chromobacterium violaceum* found in the Amazon river, Brazil, is an indole derivative characterized as 3-(1,2-dihydro-5-(5-hydroxy-1H-indol-3-yl)-2-oxo-3H-pyrrol-3-ilydene)-1,3-dihydro-2H-indol-2-one (1) (Figure 1A). The biosynthesis and biological properties of violacein (2,3) have attracted attention due to its antitumoral (4,5), antiulcerogenic (6), antileishmanial (7), antibacterial and antiviral (1) activities. Animal experiments during the evaluation of the ability of violacein to prevent gastric ulceration in mice show that the oral administration of violacein in doses up to 10 mg/kg does not result in any toxic effect in the animals (6). The cytotoxicity of violacein to V79 fibroblasts was determined in previous experiments ($IC_{50} = 7$ μM) when cancer cells [leukemia cells, lung cancer and colorectal cancer (CRC) cell lines] were found to be very sensitive to violacein treatment (8). A very recent study has shown a potent antileukemic effect of violacein in HL60 cells, while cytotoxic effects in relevant concentrations in untransformed cells have not been found (9).

CRC is the second leading cause of cancer death in the USA and Europe (10). Conventional chemotherapy of CRC with 5-fluorouracil (5-FU) in combination with other agents improves overall and disease-free survival of patients after surgery (11). When given alone in advanced disease, 5-FU produces response rates of between 11 and 17% and a median survival time of ~1 year (12). Combination with newer medicines such as irinotecan or oxaliplatin improves the response rates for advanced CRCs to 40–50% and increases the median survival time to 15–20 months (13,14). Despite advances in therapy, the prognosis of advanced CRCs remains poor due to resistance of cancer cells to conventional chemotherapeutic drugs, so the search for new alternatives is needed.

Self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis are the hallmarks of cancer (15). Defects in apoptosis are an important aspect not only for tumorigenesis but for the development of resistance to anticancer drugs (16). Reactivation of the apoptotic cascade in apoptosis-reluctant cancer cells via selective inhibition of survival pathways [Raf, mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3 kinase), Akt and nuclear factor κB (NF-κB)] and activation of extrinsic and intrinsic apoptotic pathways can be approached through synergistic combination of two agents (17).

CRCs also vary in their initial response to 5-FU. For example, microsatellite unstable CRC cell lines show growth

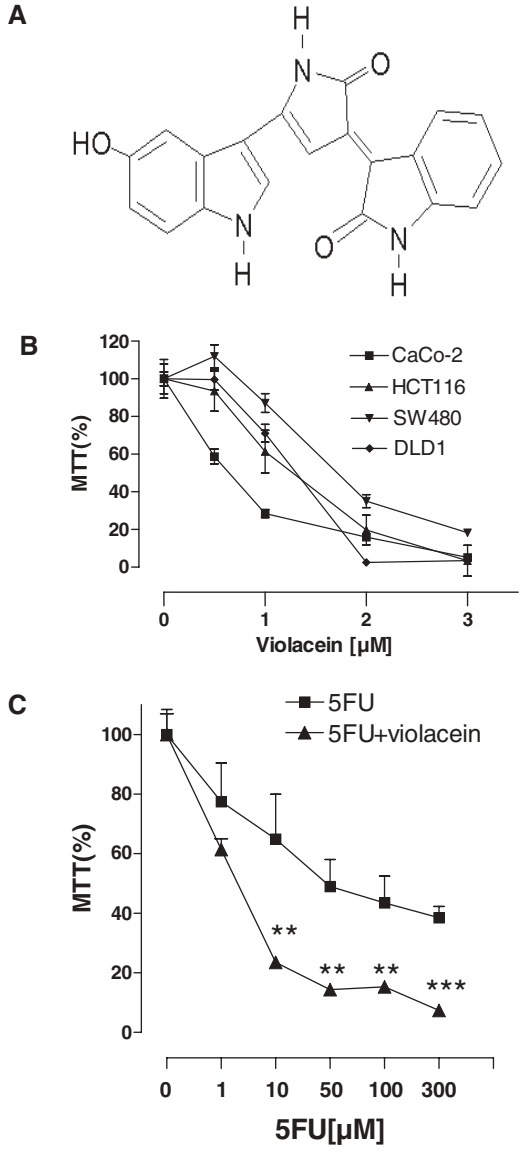

Fig. 1. Cytotoxicity of violacein in CRC cell lines and synergistic cytotoxic interaction of 5-FU and violacein. (**A**) Chemical structure of violacein. (**B**) The MTT assay was performed in four colon cancer cell lines treated for 48 h with various concentrations of violacein. Values obtained in the absence of compound have been set at 100%. Results represent the mean ± standard deviation ($n = 10$) of three independent experiments. (**C**) HCT116 cells were seeded treated with 5-FU (1–300 μM) or 5-FU in combination with violacein (1 μM), and the MTT assay was performed. Control cells were treated with DMSO (5-FU experiment) or with violacein (1 μM) (combined treatment experiment). Values are expressed as percentage of living cells relative to the control with control values set at 100%. Results represent the mean ± standard deviation ($n = 10$) of three independent experiments.

advantage and tolerance to 5-FU treatment in comparison with mismatch repair (MMR) proficient cell lines, and these results suggest that a deficient MMR system reduces 5-FU cytotoxicity (18). A recent clinical study on hereditary non-polyposis CRC patients showed no differences between patients treated with and without adjuvant 5-FU (19). The importance of microsatellite instability (MSI) status of patients with CRC for outcome of chemotherapy with 5-FU has been suggested by other clinical trials showing improved survival in patients with microsatellite stable tumors compared with untreated patients and no survival benefits in patients with microsatellite unstable tumors (20).

The above mentioned considerations prompted us to investigate the effect of violacein on colon cancer cell lines. The development of violacein as a prospective new therapeutic agent depends on its ability to target colon cancer cells, its ability to work in combination with established compounds perhaps sensitizing drug-resistant malignant cells, and the establishment of its molecular mechanisms of action. In this study we first examined the cytotoxic effect of violacein as a single agent on four colon cancer cell lines. Subsequently, we studied the combination of violacein and 5-FU on a poorly differentiated microsatellite unstable human colon cancer cell line (HCT116). Finally, we investigated the mechanism of action of violacein by assessing its effects on the cell cycle, apoptosis and survival pathways. Our results suggest that violacein has potential as a novel and active chemotherapeutic agent in CRC. The ability of violacein to restore apoptosis of cancer cells, block cell cycle progression, inhibit survival pathways and increase the sensitivity of cells to 5-FU could provide the basis for the incorporation of this compound into the combined therapy of CRC.

Materials and methods

Reagents and antibodies

Antibodies against p21 (rabbit polyclonal), p27 (mouse polyclonal), p53 (mouse monoclonal), NF-κB p50 (goat polyclonal), p65 (rabbit polyclonal), IkBα (rabbit polyclonal) and β-actin (goat polyclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin D1 antibodies (mouse monoclonal) were purchased from NeoMarkers (Fremont, CA). Antibodies against cleaved caspase 3 (rabbit polyclonal), pGSK3α/β (Ser21/9) (rabbit polyclonal), phospho-retinoblastoma protein (pRb) (rabbit polyclonal), Bad (rabbit polyclonal), phospho-Bad (Ser136) (rabbit polyclonal), FADD (Fas associated death domain protein; goat polyclonal), phospho-Akt (Thr308) (rabbit polyclonal), phospho-p44/42 MAPK (Thr202/Tyr204) (E10) (mouse monoclonal), phospho-IkBα (Ser32/36) (mouse monoclonal), phospho-PTEN (Ser380) (rabbit polyclonal) were all purchased from Cell Signaling Technology (Beverly, MA). Anti-TCF4 antibodies (mouse monoclonal) were purchased from Upstate (Lake Placid, NY). Horseradish peroxidase (HRP)-linked secondary goat anti-rabbit, rabbit anti-goat, goat anti-mouse antibodies were bought from Dako (Glostrup, Denmark). 5-FU was from Sigma (Schnelldorf, Germany). Stock solution of 5-FU was prepared in dimethyl sulfoxide (DMSO) and stored at −20°C. Z-VAD(OMe)-FMK was from Instruchemie (Delfzijl, The Netherlands), Z-LE(OMe)HD(OMe)-FMK and Z-IE(OMe)TD(OMe)-FMK were from Kordia Life Sciences (Leiden, The Netherlands). Stock solutions of 20 mM of all caspase inhibitors were prepared in DMSO and stored at −20°C. Violacein was extracted from *C.violaceum* (CCT 3496), purified according to the method of Rettori and Durán (2) and dissolved in DMSO.

Cell culture

CACO-2, DLD-1, SW480 and HCT116 colon cancer cell lines were obtained from the ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland) with 4.5 g/l glucose and L-glutamine. This was supplemented with penicillin (50 U/ml) and streptomycin (50 μg/ml) and, when serum was used, with 10% fetal calf serum (FCS) (Gibco). Cells were grown in monolayer in a humidified atmosphere containing 5% CO_2. In all experiments the concentration of DMSO in control and treated samples was equal (0.1%).

Immunoblotting

Cells at 60–80% confluence from 6-well plates were washed in ice-cold phosphate-buffered saline (PBS) and scraped into 200 μl of 2× sample buffer [125 mM Tris–HCl (pH 6.8), 4% SDS, 2% β-mercaptoethanol, 20% glycerol and 1 mg bromophenol blue]. Protein concentration was measured using the RC DC protein assay kit (Bio-Rad, CA) according to the manufacturer's instructions. The lysates were sonicated and then heated at 95°C for 5 min. From each sample, 50 μg of protein was loaded onto SDS–PAGE and blotted onto PVDF membrane (Millipore, Bedford, MA). The blots were blocked with 2% low-fat milk powder in Tris-buffered saline (TBS) with 1% Triton (TBST)

for 1 h at room temperature and washed 3× for 10 min in TBST before overnight incubation at 4°C with primary antibody in primary antibody buffer (TBST with 0.2% low-fat milk powder). Blots were then washed 3 × 10 min in TBST and incubated for 1 h at room temperature in 1/2000 HRP-conjugated secondary antibody in block buffer. After a final 3 × 10 min wash in TBST, blots were incubated for 5 min in Lumilite plus (Boehringer-Mannheim, Mannheim, Germany) and then chemiluminescence detected using a Lumi-Imager (Boehringer-Mannheim).

NF-κB translocation

Cells at 60–80% confluence from 6-well plates were washed in ice-cold PBS and scraped into 200 μl ice-cold cell extract buffer [10 mM HEPES–KOH (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)]. The cells were kept on ice for 10 min, vortexed for 10 s, and centrifuged at 4°C for 30 s at 14 000 r.p.m. The supernatant was discarded and the pellet was resuspended in 30 μl of nuclear extraction buffer [20 mM HEPES–KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF], placed on ice for 20 min, and centrifuged at 4°C for 2 min at 14 000 r.p.m. The supernatant was saved as the nuclear extract and used in western blotting assay.

PI3 kinase assay

HCT116 cells at 60–80% confluence from 6-well plates were washed in ice-cold PBS and lysed in 500 μl ice-cold lysis buffer [137 mM NaCl, 20 mM Tris—HCl (pH 7.4), 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1% NP-40 and 1 mM PMSF]. PI3 kinase was immunoprecipitated with 5 μl of rabbit antibody against full-length PI3 kinase (which coprecipitates the p110 catalytic subunit of PI3 kinase) and 60 μl of Protein A–Sepharose beads Amersham Pharmacia Biotechnology (Roosendaal, The Netherlands). PI3 kinase activity in the immunoprecipitates was analyzed with PI3 kinase enzyme-linked immunosorbent assay (ELISA) (from Echelon Biosciences, Salt Lake City, UT) according to the manufacturer's instructions. Briefly, immunoprecipitated enzyme and $PI(4,5)P_2$ substrate were incubated for 1 h at room temperature in the reaction buffer. Kinase reaction was stopped by pelleting the beads by centrifugation and transferring the reaction mixture to the incubation plate and incubated overnight at 4°C with a $PI(3,4,5)P_3$ detector protein, then added to the $PI(3,4,5)P_3$-coated microplate for 1 h for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric detection (absorbance was measured at 450 nm) is used to detect $PI(3,4,5)P_3$ detector protein binding to the plate. The colorimetric signal is inversely proportional to the amount $PI(3,4,5)P_3$ produced by PI3 kinase. The expression levels of the PI3 kinase components p85 and p110 for each time point were detected by western blot analysis of pelleted beads.

MTT assay and cytotoxicity analysis

Cells were trypsinized and taken up in DMEM with 0.5 % FCS and 5 × 10^3 cells were seeded in flat-bottomed tissue culture treated 96-well plates BD Falcon™ (Alphen aan den Rijn, The Netherlands); and allowed to adhere for 12 h. Cells were then stimulated with different concentrations of violacein or 5-FU for 24 and 48 h. After treatment, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution was added (final concentration 0.5 mg/ml and stock solution 5 mg/ml MTT in PBS), for 3 h. Medium was discarded and cells were lysed in acidified 2-propanol. Absorbance was measured at 550–560 nm. The combined cytotoxic effect of 5-FU with violacein was determined by combination index (CI)-isibologram using CalcuSyn software (Biosoft, Cambridge, UK).

Annexin V and 7-amino-actinomycin D assays

Control and violacein-treated cells were collected and resuspended in 1× binding buffer [0.01 M HEPES–NaOH (pH 7.4), 0.14 mM NaCl and 2.5mM $CaCl_2$] at a concentration of 2 × 10^7 cells/ml. Subsequently, 100 μl of cell suspension was transferred to a 5 ml tube and 5 μl each of Annexin V-APC and 7-amino-actinomycin D (7-AAD) was added. The cells were incubated at room temperature for 15 min, after which 400 μl of 1× binding buffer was added, and apoptosis analyzed by flow cytometry (Becton Dickinson FACSCalibur, Rockville, MD) and data were analyzed using the software Cell Quest Pro BD Biosciences Pharmingen (Erembodegem, Belgium).

Flow cytometry analysis

Cells were plated at a density of 5 × 10^5 cells/plate in 60 mm tissue culture dishes in culture media (described above) 1 day before the agent treatment. At the indicated time, control and treated cells were washed once with PBS, harvested by trypsinization, collected, and then resuspended in 300 μl of PBS and fixed with 5 ml of ice-cold 75% ethanol. After fixation for at least 3 h at 4°C, the cells were sedimented by centrifugation and resuspended in PBS containing 1 mg/ml each of glucose and RNase A up to 1 × 10^6 cells/ml and incubated at room temperature for 30 min. Subsequently propidium iodide solution (1 mg/ml in distilled water) was added to each sample and incubated in the dark for an additional 30 min. The samples were analyzed with flow cytometry (Becton Dickinson FACScan). Analysis was performed after 10 000 counting events. Histograms were analyzed using Modifit software (Veriety Software House, Topsham, ME).

Plasmids and transfection procedure

HCT116 cells were transiently transfected either with pSG-Gag-Akt (kindly provided by Professor P.J.Coffer, University Hospital Utrecht, The Netherlands) a plasmid vector encoding a version of Akt that is constitutively activated, or pmaxGFP control vector (from amaxa GmbH, Cologne, Germany) using Lipofectamine Plus (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. Efficiency of transfection was determined by the measurement of GFP-positive cells and was at least 90%.

Statistical analysis

Statistical analysis was performed using two-tailed Student's *t*-test, and $P < 0.05$ was considered significant. Data are expressed as the mean and standard deviation.

Results

Effect of violacein on the growth of CRC cell lines and combined growth-inhibitory synergy of 5-FU and violacein in HCT116 cells

In order to establish the cytotoxicity of violacein on CRC cells four cell lines were tested and cell viability was assessed using the MTT assay. Violacein exerts a growth-inhibitory effect in all colon cancer cell lines tested. Figure 1B illustrates that violacein inhibits the growth of CACO-2, HCT116, SW480 and DLD-1 cells after 48 h of incubation and displayed an IC_{50} value ∼1–2 μM.

To test the combined effect of 5-FU and violacein on cell growth, HCT116 cells were cultured with 5-FU (1–300 μM) with or without violacein (1 μM) for 48 h before MTT analysis. Figure 1C demonstrates that the growth inhibitory effect of 5-FU on HCT116 cell line was significantly enhanced by violacein. To determine if the combined effect of violacein and 5-FU is synergistic, the effect of these two drugs on HCT116 cells was subjected to CI-isobologram analysis (21). The results indicate a synergistic cytotoxic effect between violacein and 5-FU at all concentrations of 5-FU > 1 μM (Table I).

Activation of apoptosis and enhancement of 5-FU induced apoptosis by violacein

To further investigate the underlying mechanism for observed reduction in cell viability using the MTT assay, we determined the sensitivity of HCT116 cells to violacein-induced apoptosis and evaluated whether violacein could increase the apoptosis induced by 5-FU. Cells were incubated either with violacein (0.25–3 μM), or with 5-FU (1–10 μM), or with 5-FU (1–10 μM) + violacein (1 μM). Apoptosis was then quantified by flow cytometry. Violacein causes a dose-dependent increase in apoptosis in HCT116 cells (Figure 2A) and enhances 5-FU-induced apoptosis (Figure 2B). Importantly, while a concentration of 1 μM violacein does not cause apoptosis after 24 h of incubation and 5-FU 10 μM leads to 14% apoptosis induction, combined treatment results in 33% apoptotic HCT116 cells (Figure 2B).

Effects of violacein and combined violacein/5-FU treatment on the cell cycle

To determine the effect of violacein on the cell cycle progression of HCT116 cells, flow cytometry analysis was performed on cells treated with various concentrations of violacein, 5-FU, or a combination of both. Administration of violacein results in a concentration-dependent increase of ∼10% in the proportion of the cells in G_1 phase with a corresponding decrease in the

Table I. Combination index (CI) of 5-FU + violacein for HCT116 cell line

5-FU (μM)	Violacein (μM)	CI
1	1	1.302
10	1	0.861
50	1	0.776
100	1	0.794
300	1	0.682

The combined cytotoxic effect of 5-FU with violacein was determined by CI-isibologram using CalcuSyn software. CI: CI >1, $=1$ and <1 indicate antagonistic, additive and synergistic effects, respectively.

proportion of the cells in S and G_2/M phases in comparison with control cells (Figure 3A) while cells treated with 5-FU alone do not show any changes in the proportions of the cells in G_1 phase (Figure 3B). Combined administration of violacein with 5-FU increases the proportion of cells in G_1 phase to ~20% (Figure 3B and C) and decreases the amount of the cells in S and G_2/M phases compared with violacein and 5-FU given alone. In combined treatment experiments the sub-G_1 fraction, representing apoptotic cells, increases to 18% compared with 4% in controls (sub-G_1 fraction increases to 10% with violacein treatment and no changes are observed with 5-FU alone). The above data show that violacein and 5-FU have combined cytostatic and cytotoxic effects in HCT116 cells.

To elucidate the specific cell cycle regulatory proteins responsible for the G_1 block mediated by violacein in HCT116 cells, we performed western blot analysis on protein extracts from HCT116 cells treated with 1 μM violacein at different time points. In agreement with the observed cell cycle arrest induced by violacein, we found a time-dependent upregulation of p21, p27 and p53 protein expression (Figure 3D). Furthermore, a significant reduction of cyclin D1 expression and downregulation of pRb were detected (Figure 3D). Our results imply that violacein-dependent cell cycle arrest in G_1 is induced via upregulation of Kip/Cip cyclin-dependent kinase inhibitors with consistent downregulation of cyclin D1 and dephosphorylation of pRb. The level of cyclin D1 as well as p53, p21 and p27 could be regulated by the PI3 kinase/Akt pathway, suggesting that violacein may inhibit the Akt survival pathway with subsequent cell cycle block.

Effects of violacein on the activation of apoptotic pathways

To obtain further insight into the mechanism of violacein-induced apoptosis, we first assessed the involvement of caspase 3. We performed western blotting on HCT116 cells treated with violacein at different time points and used anti-cleaved caspase 3 antibodies. The activation of caspase 3 can already be observed after 45 min (Figure 4A).

In order to confirm that activation of caspases culminates in the apoptosis of HCT116 cells, we treated these cells with violacein or violacein in combination with a pancaspase inhibitor. Subsequently cell viability was determined using the MTT assay. A pancaspase inhibitor significantly reduces the effect of violacein on cell viability, confirming that violacein-induced cell death is a caspase-dependent process (Figure 4B).

In order to gain more information about the apoptotic mechanism induced by violacein, we assessed the involvement of caspases 8 and 9 in the death process. For this purpose specific caspases 8 and 9 inhibitors were used. Each

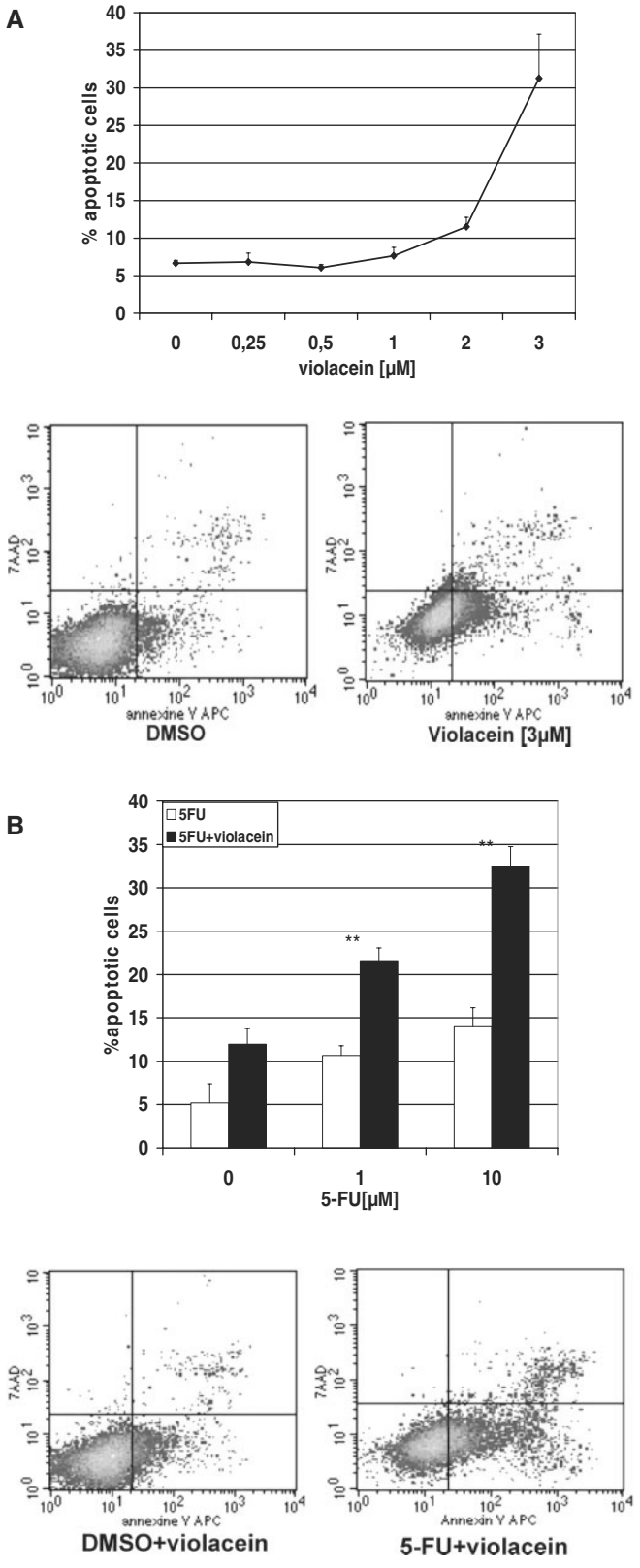

Fig. 2. Effect of violacein on HCT116 cells apoptosis induction. HCT116 cells were treated for 24 h either with violacein (0.25–3 μM), or with 5-FU (1–10 μM), or with 5-FU (1–10 μM) + violacein (1 μM) and harvested for quantification of apoptosis by flow cytometry measured as the percentage of Annexin V-APC positive cells. Control cells were treated with DMSO (5-FU experiment and violacein experiments) or with violacein (1 μM) (combined treatment experiment). (**A**) violacein treatment results in dose-dependent augmentation of apoptosis. (**B**) violacein enhances 5-FU induced apoptosis ($^{**}P < 0.01$).

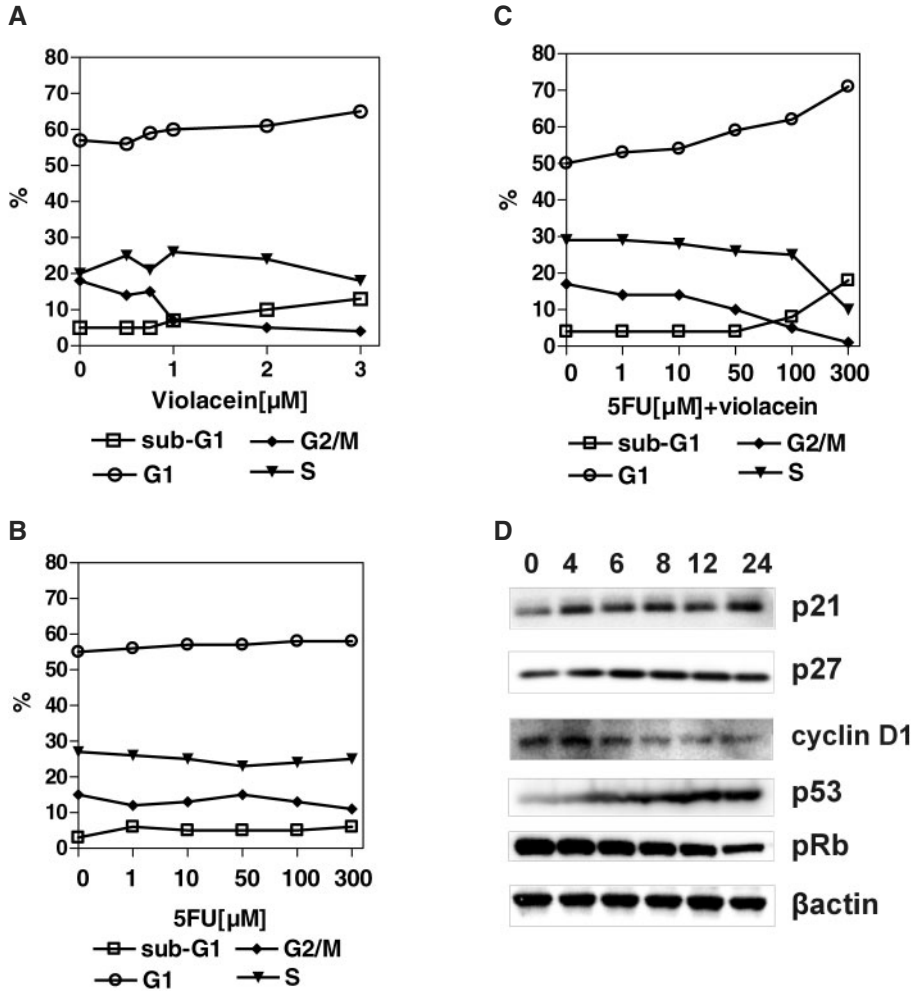

Fig. 3. Analysis of the effects of 5-FU and violacein on the cell cycle. HCT116 cells were grown to 60% confluence, treated for 24 h either with violacein (0.25–3 μM), or with 5-FU (1–10 μM), or with 5-FU (1–10 μM) + violacein (1 μM), harvested, fixed with ethanol, and stained with propidium iodide. The stained cells of each sample were analyzed by flow cytometry. Control cells were treated with DMSO (5-FU experiment and violacein experiments) or with violacein (1 μM) (combined treatment experiment). (**A**) Violacein induces a concentration-dependent increase in the proportion of the cells in G_1 phase and decrease in the S and G_2/M phases. (**B** and **C**) combined administration of violacein with 5-FU synergistically induces cell cycle arrest in G_1 phase and decreases the amount of the cells in S and G_2/M phases compared with violacein and 5-FU given alone. (**D**) Regulation of G_1-related proteins by violacein. Western blot analysis was performed on HCT116 cells treated with violacein (1 μM) for various times (shown in hours). 50 μg of protein from cell lysates was loaded per line, and expression of p21, p27, p53, cyclin D1 and Rb were analyzed by blotting with the corresponding specific antibody. Results representative of three independent experiments are presented.

inhibitor was added to HCT116 cells 30 min before violacein treatment and cell viability was determined using the MTT assay. Both caspases 8 and 9 inhibitors reduce the effect of violacein on cell growth suggesting that these caspases are both involved in violacein-induced cell death (Figure 4C).

To elucidate the upstream pathway leading to caspase 3 activation, western blot analysis was performed. Violacein treatment results in increased levels of pro-apoptotic Bad protein and a decrease in phosphorylated Bad^{Ser136}, thereby promoting apoptosis. These results indicate that Bcl-2 family proteins, members of the intrinsic apoptotic pathway, are involved in violacein-induced apoptosis. As phosphorylation of the pro-apoptotic protein Bad at Ser136 occurs via the serine-threonine kinase Akt, our findings suggest the involvement of Akt in the mechanism of violacein action. The level of FADD was reduced by violacein treatment implying that the extrinsic apoptotic pathway is not primarily involved in cell death caused by violacein (Figure 4D).

Effects of violacein on survival pathways

The PI3 kinase/Akt pathway is an important regulatory pathway governing the apoptotic response. We therefore investigated the effect of violacein on this pathway. As the activity of Akt is regulated by phosphorylation, we examined the level of phosphorylated Akt^{Thr308} in HCT116 cells treated with violacein at different time points and found time-dependent downregulation of phosphorylated Akt^{Thr308} (Figure 5A). As confirmation we also show a decrease in the phosphorylation of GSK-3α/$\beta^{Ser21/9}$ a known downstream target of Akt (Figure 5A). In order to examine the influence of violacein on PI3 kinase, we performed immunoprecipitation of PI3 kinase from HCT 116 cells treated with violacein and measured the PI3 kinase activity by competitive ELISA. Compared with PI3 kinase activity from control cells, violacein does not affect the PI3 kinase activity or the expression of the PI3 kinase components (p85α and p110α) (Figure 5B). We evaluated the level of phosphorylated $PTEN^{Ser380}$ (phosphatase and tensin homologue deleted on chromosome 10) as PTEN is a

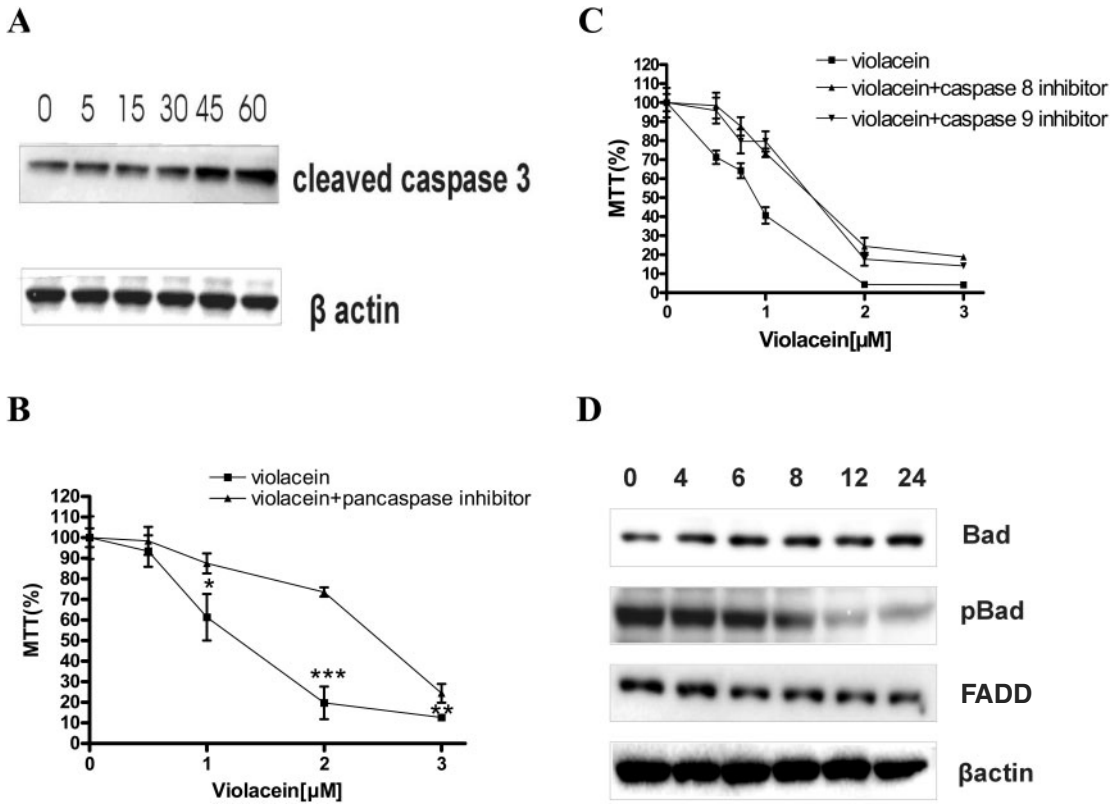

Fig. 4. Effect of violacein on the activation of apoptotic pathways. (**A**) Immunoblot of HCT116 cells treated with violacein (1 μM) for various times (shown in minutes). 50 μg of total protein from cell lysates was loaded per lane and blotted with anti-cleaved caspase 3 antibodies. Equal loading was confirmed by showing equal β-actin levels. (**B**) HCT116 cells were treated with different concentrations of violacein (0.5–3 μM) for 24 h with and without caspases inhibitor Z-VAD(OMe)-FMK. Cell viability was assessed by the MTT assay ($^{*}P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.001$). (**C**) HCT116 cells were treated with different concentrations of violacein (0.5–3 μM) for 24 h with and without caspase 8 inhibitor Z-IE(OMe)TD(OMe)-FMK or caspase 9 inhibitor Z-LE(OMe)HD(OMe)-FMK. Inhibitors were added to HCT116 culture plates at 20 μM, 30 min before violacein treatment. Cell viability was assessed using the MTT assay. (**D**) Immunoblot of HCT116 cells treated with violacein (1 μM) for various times (shown in hours). 50 μg of total protein from cell lysates was loaded per lane and blotted with antibodies against Bad, phospho-Bad and FADD. Equal loading was confirmed by showing equal β-actin levels.

major negative regulator of the PI3 kinase/Akt pathway and found no changes after violacein treatment (Figure 5A). These results suggest that violacein inhibits Akt activity in a PI3 kinase- and PTEN-independent manner.

We assessed the activity of the p44/42 MAPK pathway, another major pathway involved in cellular proliferation and apoptosis. The level of phosphorylated P44/42 $MAPK^{Thr202/Tyr204}$ is not affected by violacein treatment suggesting that the MAPK pathway is not affected by violacein (Figure 5A).

To further test the hypothesis that violacein-induced apoptosis is mediated through the inhibition of the Akt pathway, we transiently transfected HCT116 cells with a plasmid expressing a constitutively active form of Akt (pSG-Gag-Akt). Control cells were transfected with pmaxGFP plasmids and 24 h after transfection, cells were treated with DMSO or different concentrations of violacein (0.5–3 μM) for 48 h and then the MTT assay and Annexin V measurements were performed. HCT116 cells transfected with pSG-Gag-Akt show an increase in cell survival, relative to control cells (Figure 5C). While violacein strongly induces apoptosis in HCT116 cells transfected with pmaxGFP (9% apoptosis with 0.5 μM violacein up to 84% apoptosis with 3 μM violacein) cells transfected with pSG-Gag-Akt were far more resistant to violacein-induced apoptosis (5% apoptosis with 0.5 μM violacein up to 16% apoptosis with 3 μM violacein) as shown in Figure 5D. In all tested concentrations the percentage of apoptotic cells was lower in pSG-Gag-Akt transfected cells compared with pmaxGFP transfected cells, and with concentrations of 2 and 3 μM of violacein the difference was significant ($P < 0.001$), suggesting that the induction of apoptosis by violacein in HCT116 cells results, at least in part, from an inhibition of the Akt pathway.

Finally, we investigated the influence of violacein on the activity of the NF-κB pathway as activation of NF-κB blocks apoptosis and promotes cell proliferation in colon cancer. We show a time-dependent decrease of expression of p50 and p65 subunits of NF-κB with violacein treatment (Figure 5E). Inactivation of NF-κB is confirmed by direct observation of the decrease in nuclear translocation of NF-κB as determined by western blot analysis of nuclear extracts of HCT116 cells (Figure 5F). NF-κB is present in the cytosol in an inactive state complexed with the inhibitory IkB proteins. Activation occurs via phosphorylation of IkBα at Ser32/Ser36, resulting in the release and nuclear translocation of NF-κB. To further confirm violacein-induced downregulation of the activity of NF-κB pathway, we performed western blot for phosphorylated $IkB\alpha^{Ser32/36}$. Violacein treatment leads to a decrease in IkBα phosphorylation (Figure 5E) suggesting that violacein blocks IkBα phosphorylation preventing its

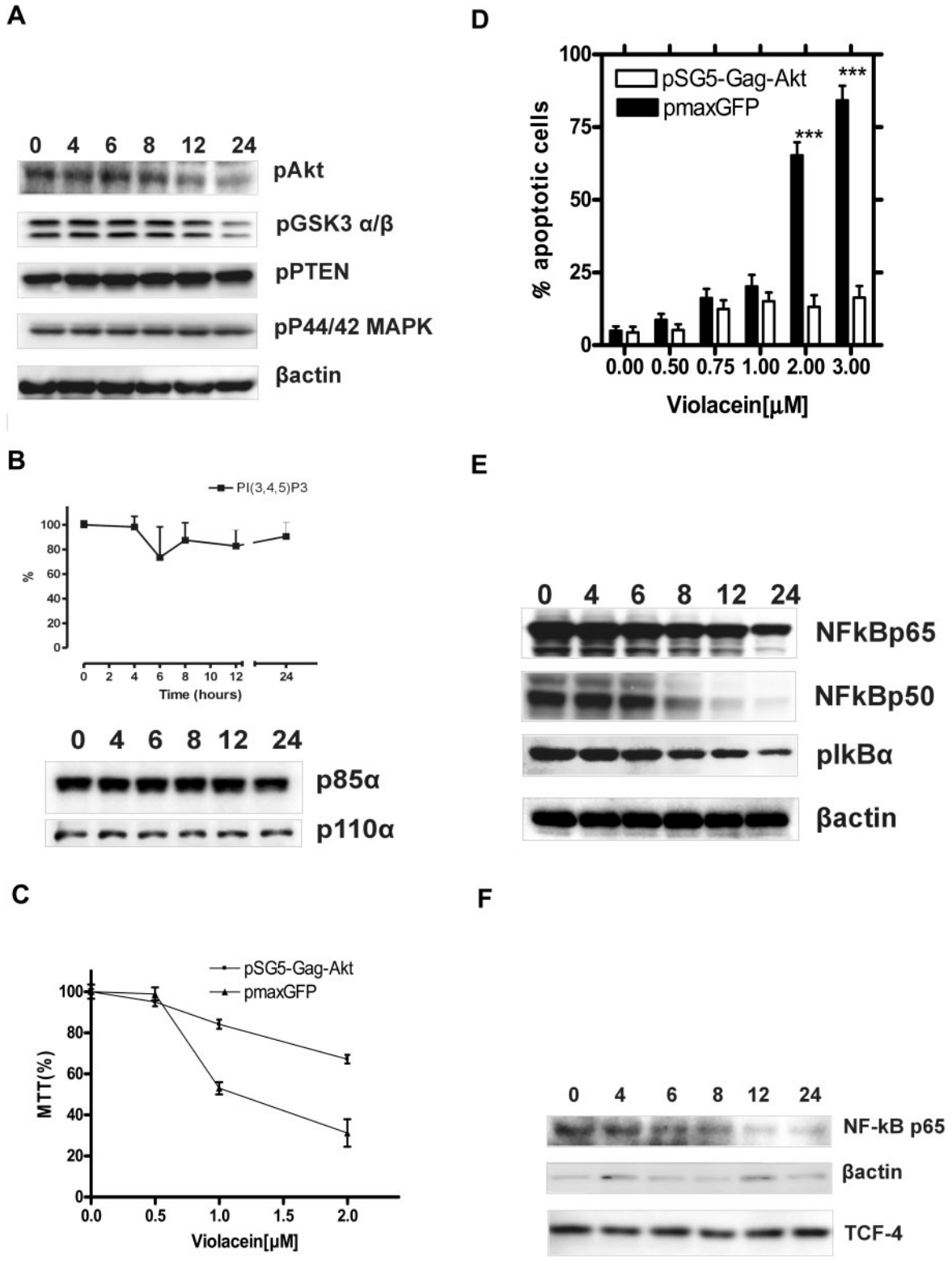

Fig. 5. Violacein inhibits the activity of Akt and NF-κB signaling pathways. HCT116 cells were treated with violacein (1 μM) for various times (shown in hours). (**A**) 50 μg of total protein from cell lysates was loaded per line and western blot analysis was performed. Violacein treatment caused time-dependent downregulation of phosphorylated Akt, decreased the phosphorylation of its downstream target GSK-3α/β, but had no effect on phosphorylation of PTEN and phosphorylation of P44/42 MAPK. (**B**) PI3 kinase was immunoprecipitated and its activity was analyzed as described in Materials and methods. The data were quantified and expressed as a percentage of control. The expression levels of the PI3 kinase components p85α and p110α for each time point were detected by western blot analysis. (**C**) HCT116 cells were transfected either with pmaxGFP or pSG5-Gag-Akt, and after 24 h, were treated with DMSO or different concentrations of violacein for 48 h. (**D**) HCT116 cells were transfected either with pmaxGFP or pSG5-Gag-Akt, and after 24 h, were treated with DMSO or different concentrations of violacein for 48 h. Apoptosis was measured by flow cytometry as the percentage of Annexin V-APC positive cells ($^{***}P < 0.001$). (**E**) 50 μg of total protein from cell lysates was loaded per lane and western blot was performed. A time-dependent decrease of the expression of p50 and p65 subunits of NF-κB and decreased phosphorylation of IkBα were observed. (**F**) Nuclear extracts of HCT116 cells were subjected to immunoblot analysis with NF-κB p65 antibody. Lack of β-actin shows effective nuclear fractionation, and TCF4 levels were used to prove equal loading.

ubiquitination and further degradation thus preventing nuclear translocation of NF-κB.

Discussion

To combat the problem of chemoresistance new alternative effective agents are urgently needed in the treatment of CRC. Natural products are an important source of potential chemotherapeutic agents (22,23). Violacein has attracted attention as a possible candidate for cancer treatment due to its high cytotoxicity in transformed cells and low toxic effect in relevant concentrations in untransformed cells (6,8,9). Earlier studies screening a large panel of cancer cell lines have already shown that violacein is promising with respect to leukemia, lung cancer and CRC (8). Our work has specifically concentrated on CRC, a major malignancy with insufficient treatment options and one of the leading causes of cancer death in western countries. We find that violacein is a highly effective cytotoxic compound for CRC cells. Combined treatment with violacein and 5-FU synergistically potentiates the cytotoxic effect of 5-FU in a microsatellite unstable cell line, HCT116. We further investigated the mechanisms involved in this effect by studying the effects of violacein on apoptosis, the cell cycle and major cellular survival pathways. We find that violacein reactivates the apoptotic cascade in colon cancer cells, which we have confirmed using multiple methodologies. We tried to elucidate whether this occurs primarily through the intrinsic mitochondrial pathway or through the extrinsic apoptotic pathway using specific inhibitors of caspases 8 and 9 and by western blot analysis of components of both pathways. While both the caspases 8 and 9 inhibitors seem to block the effects of violacein to a similar extent this can perhaps be explained by the extensive crosstalk between these two apoptotic pathways. Our findings of reduced levels of FADD and increases in the levels and activity of Bad lead us to suggest that the mitochondrial pathway is primarily affected, although our data are not conclusive.

Violacein treatment alters the expression of proteins involved in the cell cycle such as p21 and p27, downregulates the level of cyclin D1 and blocks pRb phosphorylation, thus leading to the arrest of cancer cells in G_1 that we observe using flow cytometry.

Analysis of components of the MAPK, NF-κB and Akt and survival pathways show that violacein has no effect on the MAPK pathway but reduces the activity of both the NF-κB and Akt pathways. In the MAPK pathway levels of phosphorylated p44/42 $MAPK^{Thr202/Tyr204}$ remain unchanged with violacein treatment. Violacein decreases the expression of p50 and p65 subunits of NF-κB, reduces $IkB\alpha^{Ser32/36}$ phosphorylation and inhibits nuclear translocation of NF-κB consistent with downregulation of the NF-κB pathway and violacein also reduces levels of phosphorylated Akt^{Thr308} suggesting that violacein blocks Akt signaling. This last action of violacein is particularly interesting when trying to explain the data as a whole as Akt inhibition could explain most of the various effects of violacein observed.

The apoptotic response to conventional chemotherapy may be augmented by the inhibition of the Akt pathway. Akt directly phosphorylates several components of the cell death machinery and in this way protects cells from death and inhibits apoptosis (24). Phosphorylation of the pro-apoptotic protein Bad by Akt, inhibiting Bad function, has been described previously (25). The observed effects of violacein on Bad and phosphorylated Bad are therefore consistent with inhibition of Akt.

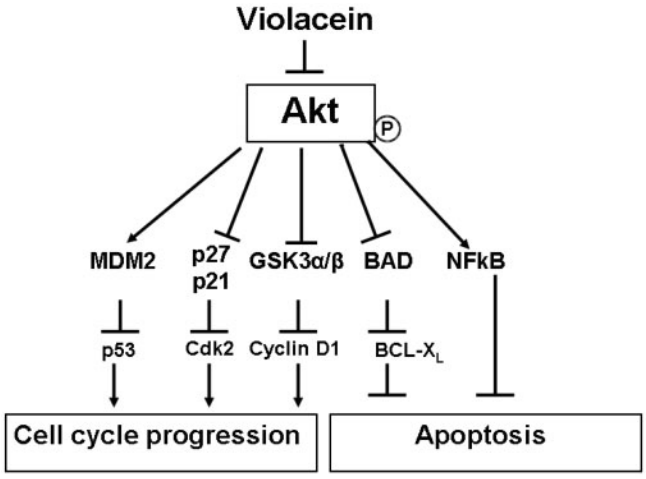

Fig. 6. Schematic representation of the proposed molecular mechanism of violacein action.

Akt is also known to modulate the NF-κB pathway. Akt leads to the activation of the NF-κB pathway via phosphorylation and activation of IkB kinase, which induces degradation of the NF-κB inhibitor, IkB ultimately leading to increase nuclear translocation of NF-κB (26). A variety of human cancers have demonstrated constitutively high levels of NF-κB activation (27). Exposure of cancer cells to anticancer drugs can induce the activation of the NF-κB pathway leading to the expression of anti-apoptotic genes (28) and resistance to chemotherapy (29). CRC cell lines resistant to thymidylate synthase inhibitors, such as 5-FU, demonstrate NF-κB mRNA and protein overexpression and high NF-κB nuclear accumulation. Inhibition of NF-κB activity can enhance the cytotoxic effect of irinotecan and tumour necrosis factor α (TNFα) (29,30). Our observation of inhibition of the NF-κB pathway, possibly through Akt downregulation, could also be responsible for the proapoptotic effect of violacein and for sensitizing of HCT116 cells to 5-FU.

Akt is also known to have effects on various components of the cell cycle. Akt affects levels of cyclin D through its actions on glycogen synthase kinase 3α/β (GSK-3α/β) (31) and effects on both p21 and p27 are also described (32,33). Thus the effects of violacein on the cell cycle are also consistent with Akt inhibition.

To provide further evidence for Akt being a crucial target of violacein we further investigated the effects of violacein on another Akt target, p53. Akt is known to promote cell survival through activation of the p53-binding protein MDM2, resulting in enhanced p53 degradation (34). We find that the level of p53 is increased due to violacein treatment. We show that transfection with constitutively active Akt blocks the effects of violacein and finally we show that two upstream modulators of Akt activity, PI3 kinase and PTEN are not affected by violacein.

Our data provide evidence that violacein is a promising chemotherapeutic agent that acts by blocking Akt activation and inducing apoptosis thus increasing the chemosensitivity of colon cancer cells to 5-FU treatment (Figure 6).

Conflict of Interest Statement: None declared.

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