

Violacein anticancer activity is enhanced under hypoxia

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Received November 28, 2014; Accepted December 29, 2014

DOI: 10.3892/or.2015.3781

Abstract. Current cancer treatments of solid tumours such as chemotherapy and radiotherapy, have yet to produce effective therapeutic results due to non-specific targeting. This has led to many complications, such as toxicities in cancer patients. The ability of natural compounds in inducing programmed cell death (apoptosis), a process dysregulated in cancer cells, has been extensively studied in recent studies. This study assessed the anti-proliferative activity of violacein in a number of human cancer cell lines under normal and hypoxic conditions. Furthermore, we investigated its effects in a tumour-bearing subcutaneous mouse model. We also examined the ability of a tumour-targeting *Salmonella* strain to produce violacein for local delivery within the tumour microenvironment. The results showed that hypoxia significantly increased the cytotoxic effects of violacein. The most significant reduction in the IC₅₀ was in the HT29 (12.6-fold) and HCT116 (4.8-fold) colon cancer cell lines, HN5 head and neck squamous carcinoma cell line (6.5-fold), and MCF-7 breast ductal carcinoma cell line (4-fold). Among the cell lines tested for active caspase-3/7 activity, violacein only increased caspase-3/7 activity in the A549 non-small lung cancer cell line. *In vivo* efficacy of violacein showed that HN5 tumour-bearing mice had regressed tumours during the treatment period and survival increased. The results also showed that transfer of the violacein biosynthetic cluster into the oncolytic strain VNP20009 of *Salmonella* resulted in the production of active violacein, suggesting targeted delivery of violacein by VNP20009. Taken together, our study has shown that hypoxia synergises the effects of violacein and the results from the *in vivo* administration of violacein require further investigation of violacein as an anticancer chemotherapeutic.

Introduction

Cancer is one of the leading causes of mortality in humans. However, this problem is exasperated by the complex molecular nature of cancer and therefore requires a complex therapeutic strategy. Efforts have focused on the ability of natural compounds to induce programmed cell death and activate the relevant apoptotic cascades (1-4).

Violacein is a violet pigment that is produced from tryptophan by action of a cluster of five genes, designated as *vioABCDE*, naturally by the bacterium *Chromobacterium violaceum* (5,6). It has significant biological and pharmacological activities, including antimicrobial, antiparasitary, anti-diarrhoeal, and antifungal activities (7-12). Violacein has been shown to possess anticancer properties (13-21) and has been overexpressed in *E. coli* K12 JM109 by cloning of the violacein gene cluster (22). Ahmetagic *et al* found that expression of the violacein gene cluster in the pPSX vector (JM109 *E. coli* K12 strain) and mutations in the promoter region produced hyper-producing strains of violacein (22).

Anticancer studies of violacein have shown efficacy in a number of cell lines of both neoplastic and haematological malignant origins. The most effective activity of violacein was against MOLT-4 leukaemia, NCI-H460 non-small-cell lung cancer and KM12 colon-cancer cell lines (GI₅₀ ~30-60 nM) (23). Furthermore, Saraiva *et al* found that uveal melanoma cell lines, 92.1 and OCM-1, were found to be sensitive to violacein (GI₅₀ ~1.69-2.21 μ M) (21). Additionally, violacein was found to inhibit the growth and proliferation of colorectal cancer cell lines (16). The mechanism of action in HCT116 cells was identified as induction of apoptosis. Violacein was found to potentiate the effects of 5-fluorouracil in HCT116 cells. The *in vivo* efficacy of violacein has been assessed in intraperitoneal models of Ehrlich ascites tumour, which showed increased lifespan in tumour-bearing mice (13).

In terms of the mechanism of action of violacein, its role in the induction of apoptosis has been investigated and evidence suggests cell type-dependent activity (23). Findings of previous studies have indicated caspase-dependent apoptosis and production of reactive oxygen species as the mechanism of action (14,16). The most comprehensive mechanistic study was undertaken by Ferreira *et al* and Kodach *et al* on leukaemia cells and colorectal cancer cells, respectively (15,16). Violacein was found to activate TNF receptor 1-mediated apoptosis in HL60

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Abbreviations: IC₅₀, 50% inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Key words: violacein, chemotherapy, hypoxia, oncolytic *Salmonella*

leukaemia cells (15). Furthermore, in HCT116 cells violacein was found to block cell cycle at G1, upregulate the expression of p53, p27 and p21 levels and decrease cyclin D1 expression. Most significantly, violacein was found to inhibit the phosphorylation of Akt, a serine-threonine-specific protein kinase involved in the proliferation and survival of many cancer types (16).

This study aimed to compare the cytotoxic effects of violacein in a number of cancer cells under normoxic and hypoxic conditions. Furthermore, we investigated the *in vivo* effect of violacein in subcutaneous tumour models. The results showed that hypoxia significantly potentiated the cytotoxic effects of violacein. Violacein was also found to cause tumour regression and have an increased lifespan in tumour-bearing mice. The results suggest additional investigations into the potential antitumour capacity of violacein.

Materials and methods

Mammalian cell culture conditions. Human cancer cell lines, A549 (lung), PC3 (prostate), HCT116 (colon), HT29 (colon), MCF-7 (breast), A431 (melanoma), HN5 (head and neck), and HeLa (cervix), were obtained from the American Type Culture Collection. The cell lines were cultured in Dulbecco's modified essential medium (Invitrogen-Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 110 mg/l sodium pyruvate, 25 mM HEPES, 100 U/ml of penicillin and 100 µg/ml of streptomycin solution, in an atmosphere of 5% CO₂ and 95% air at 37°C. Hypoxic conditions were achieved by placing culture plates in a GasPak EZ pouch (BD) and incubating at 37°C.

Expression and extraction of violacein. Violacein was expressed in the recombinant *E. coli* strain JM109 containing the violacein expression vector pBSX-vio-opv. This strain was kindly provided by Professor John Pemberton, The University of Queensland, Australia. The recombinant violacein expression vector pBSX-vio-opv was also transformed into the tumour-targeting *Salmonella typhimurium* strain, VNP20009 (24). Rosenberg *et al.*, showed that VNP20009 is able to colonise the tumour microenvironment 10,000-fold higher than that in liver in mice (24). The violacein-producing strains were cultured on PYE agar for 48 h until dark purple colonies were visible, indicating the production of violacein (22). The colonies were subsequently scraped from the culture plates and resuspended in water and centrifuged at 14,000 x g. After centrifugation, the cells were resuspended in ethanol to extract the violacein. After centrifugation at 14,000 x g the supernatant, containing the violacein, was collected and stored at 4°C until further use. The concentration of the extracted violacein was determined according to the methods of Durán *et al.* (25).

In vitro efficacy of violacein. Cytotoxicity of violacein in all the cell lines was studied by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (Invitrogen). Cells were seeded at a density of 1x10⁴ cells per well in a 96-well plate. After seeding, the cells were exposed to violacein treatment for another 24 h, and then MTT reagent was added following the manufacturer's instructions. The absorbance was read at 570 nm and IC₅₀ values were calculated by Prism 5 (GraphPad Software).

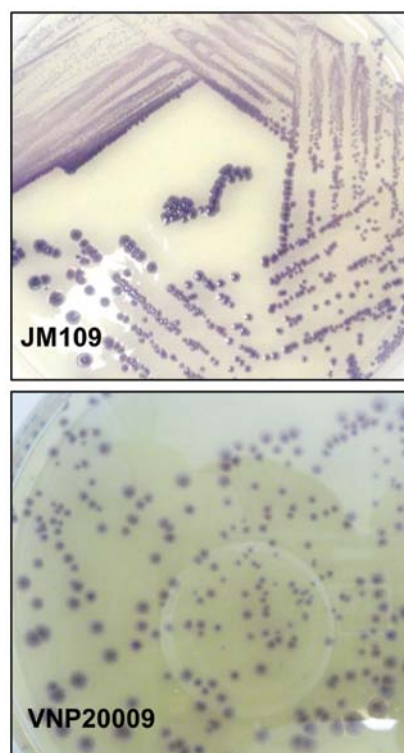


Figure 1. Production of violacein in *E. coli* JM109 and *Salmonella typhimurium* (*S. typhimurium*) VNP90001. The violacein biosynthetic cluster in the pBSX-vio-opv vector was transferred into *E. coli* JM109 and *S. typhimurium* VNP90001. Violacein production was induced by culturing the bacteria on PYE media. Purple pigmentation is indicative of violacein production.

Apoptosis assays. Cancer cell lines were seeded at a density of 1x10⁴ cells per well in white µClear 96-well plates (Greiner) and exposed to violacein for 24 h. Caspase-3 activity was evaluated using Caspase-Glo 3/7 assay systems (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase intensity was measured by POLARstar Omega at 24 h post-violacein addition.

Mouse xenograft models and violacein antitumour evaluation. Experiments involving animals were approved by the Griffith University animal ethics committee (AEC No. MSC/01/08). To establish mouse models, 5x10⁶ HN5 cells in PBS (200 µl) were subcutaneously injected into the right flank of female BALB/c nude mice. When tumours reached a size of 320 mm³, the mice (aged 8 weeks and weighing 16-18 g) were randomly divided into two groups (6 mice per group): the treatment group, which was administered 0.7 mg/kg body weight violacein, while an equal amount of injection solution without violacein was administered to the control group. Treatments were delivered by intraperitoneal injection. Tumour volume was measured using a calliper and calculated using the formula: 0.5(l x w²). When the tumour volume reached 1 cm³, mice were sacrificed by cervical dislocation.

Statistical analysis. Experiments were repeated at least three times to obtain statistically significant data. The results are presented as means with standard error of mean. The Student's t-test was used to analyse the data and p<0.05 was considered statistically significant.

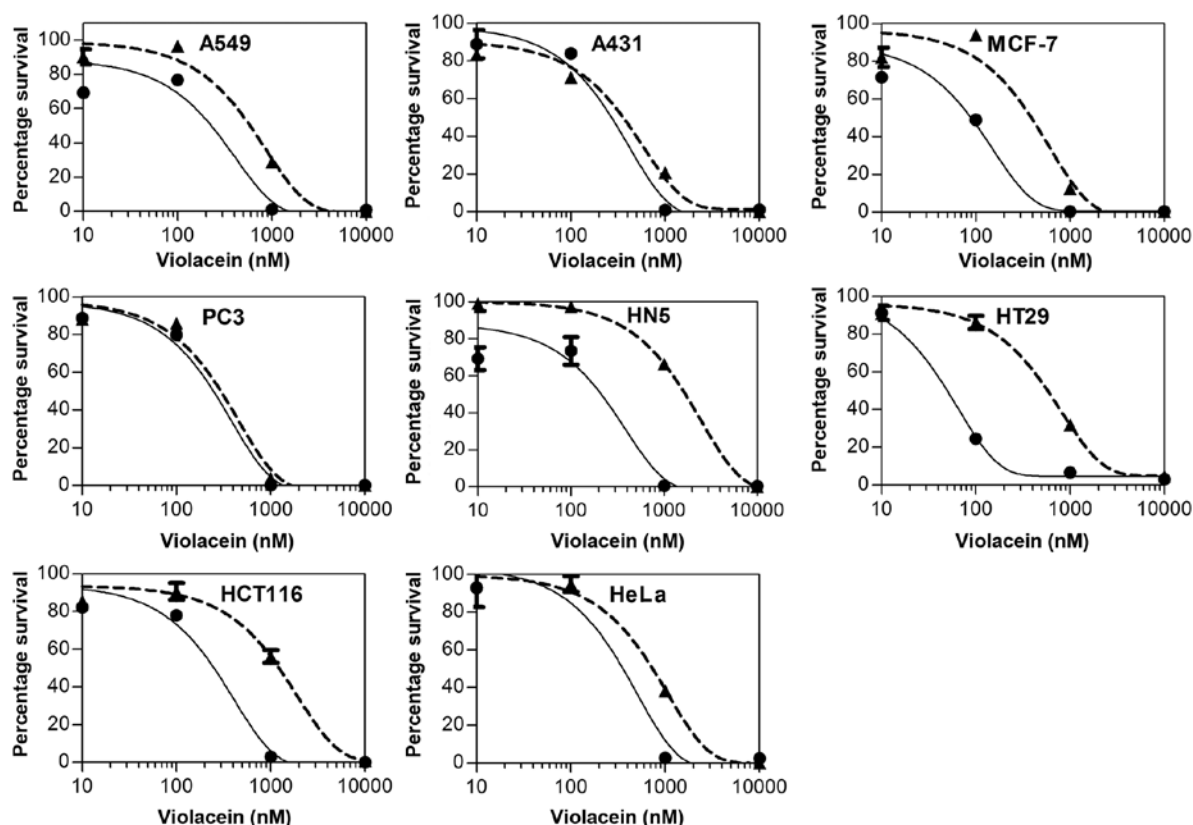


Figure 2. Violacein anti-proliferative effect on cancer cells. MTT assays were performed on several cancer cell lines to ascertain the anticancer effects of violacein. The solid line is the dose response in hypoxic conditions, while the dotted line is under normal cell culture conditions. Violacein was shown to be more effective under hypoxia in most cell lines.

Results

Production of violacein. Recombinant *E. coli* and *Salmonella typhimurium* strain VPN20009 containing the violacein biosynthetic clusters were cultured on PYE agar. After 48 h intense purple-coloured colonies were visible in the two strains (Fig. 1). The intense purple colourisation in the VPN20009 plates showed that it is capable of producing violacein from the pBSX-vio-opv expression vector. Following extraction and purification, the violacein content was measured by spectrophotometry at 570 nm. From a batch of 20 plates, 5 ml of 3 mM violacein was extracted in ethanol. This extract was subsequently used in the cytotoxic assays and in the xenograft tumour models.

Violacein is a potent inhibitor of cancer cell proliferation. To examine the effects of violacein on cells MTT proliferation assays were used. MTT was converted to formazan by living cells and was detected by spectrophotometric quantification. Violacein was tested at a concentration range of 0-10 μ M to yield a dose response for subsequent IC_{50} calculation. The results showed that violacein was highly potent against A549 non-small cell lung cancer, A431 Melanoma, MCF-7 breast cancer, PC3 prostate cancer, HT29 colon cancer, and HeLa cervical cancer (Fig. 2). Furthermore, violacein was effective against HCT116 colon and HN5 head and neck cancer, although not as potent as against the other cell lines. The dose response curve was used to calculate IC_{50} concentrations for the various cell lines (Table I). Based on the IC_{50} values

Table I. Anticancer activity of violacein.

Cell line	Tissue origin	Violacein IC_{50} (nM)		Fold drop IC_{50}
		Hypoxia	Normoxia	
A549	Lung	285.5	601.8	2.1
A431	Skin	288.2	412.6	1.4
MCF-7	Breast	105.6	421	4
PC3	Prostate	268.5	318.2	1.2
HCT116	Colon	283.5	1348	4.8
HT29	Colon	45.2	569.4	12.6
HN5	Head and neck	268.4	1738	6.5
HeLa	Cervix	351.9	744	2.1

Bold, cell lines with significantly lower IC_{50} under hypoxic conditions.

violacein was most effective against A431, MCF-7 and PC3 cancer cells, while HN5 and HCT116 were the least affected. A microscopic analysis of the treated cells revealed fewer and dead cells compared to the non-treated group (Fig. 3). Furthermore, the morphology of violacein-treated cells was round compared to a flat appearance of the control cells.

Hypoxia sensitises cancer cells to violacein. To determine the effects of violacein on cancer cells in hypoxic conditions,

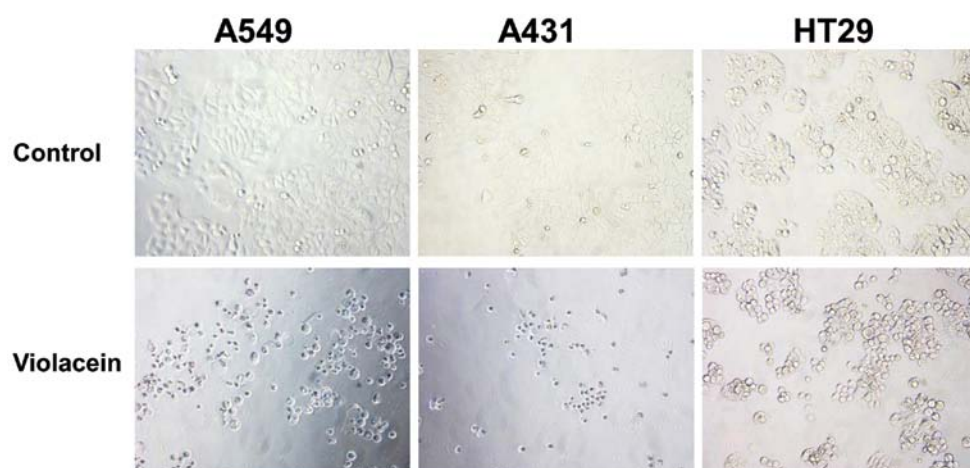


Figure 3. Microscopic analysis of cancer cells treated with violacein. Treated cells show a round morphology compared to non-treated cells. Violacein-treated cells are fewer in number and are detached from the culture plate surface.

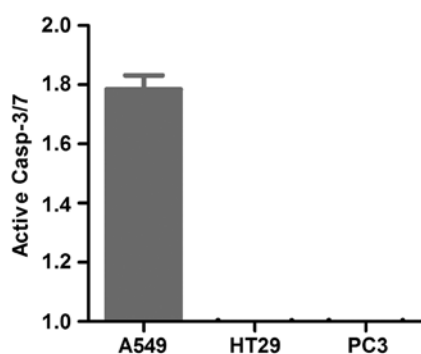


Figure 4. Caspase 3/7 activity in cancer cells induced by violacein. Active caspase-3/7 was only induced in A549 cells, while the remaining cells showed no change.

the cells were cultured in a GasPak EZ pouch (BD) at 37°C. MTT proliferation assays showed that hypoxia sensitised all the cancer cells to violacein. The most significant effect was on MCF-7 breast ductal carcinoma, HCT116 and HT29 colon cancer, and HN5 head and neck squamous carcinoma cells (Fig. 2). Furthermore, a dose response curve was used to calculate the IC_{50} values for violacein under hypoxia and normoxia. It was found that HT26 was 12.6-fold more sensitive to violacein under hypoxia when compared to normoxic conditions (Table I). The IC_{50} values for PC3 prostate cancer and A431 melanoma cells were not significantly affected by hypoxia.

Violacein activates caspase-dependent apoptosis in A549 cells only. To determine whether violacein activates caspase dependent apoptosis, the cells were seeded in 96-well white walled plates, exposed to violacein and the caspase-3/7 activity was measured. Caspase-3/7 activity was induced in A549 non-small cell lung cancer cells only when treated with violacein (Fig. 4). The remaining cell lines tested had similar caspase-3/7 activity in the treated and non-treated cells.

In vivo activity of violacein. Having established the efficacy of violacein in the *in vitro* culture system we elucidated its activity in xenograft tumour models. Head and neck squamous

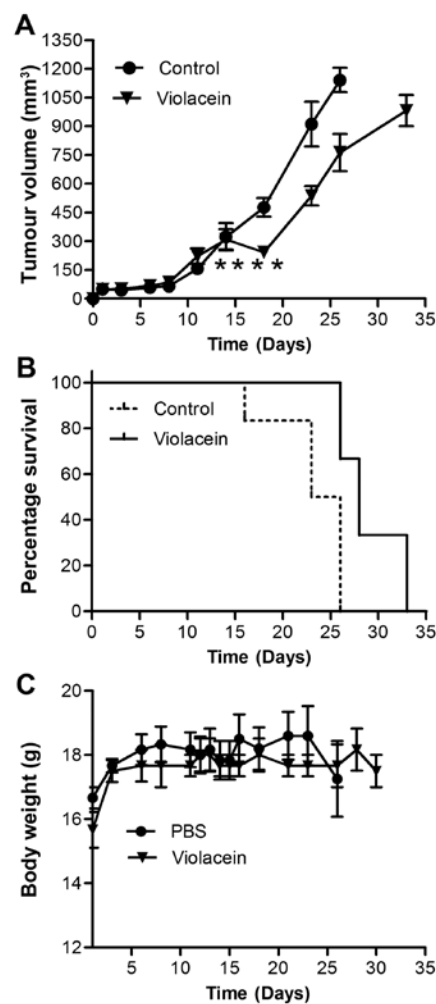


Figure 5. *In vivo* efficacy of violacein against mice tumour models. (A) The effect of violacein on tumour volume compared to control groups. (B) Survival of tumour-bearing mice in response to violacein or vehicle (Control). (C) Body weight of mice during the treatment period.

carcinoma subcutaneous xenograft models were established in BALB/c nude mice and treated with daily doses of 0.7 mg/kg of body weight for 4 days. It was found that tumours treated with violacein regressed during the treatment period while

the non-treated control tumours continued to grow (Fig. 5). Furthermore, violacein-treated tumours had a significantly slower growth rate compared to the control groups. It was also found that violacein extended the life span of animals during the treatment period, however, the mortality rate reached similar levels as that of the controls when treatment was terminated. Violacein administration did not affect the weight of the animals and treated animals were not any different to controls in terms of behaviour or phenotypic features.

Discussion

Cancer remains a devastating disease inflicting significant mortality rates to humans worldwide despite significant breakthroughs in its diagnosis and treatment. Natural compounds have been extensively studied for their biological activities and in particular their ability to induce programmed cell death or apoptosis in cancer cells (1-4). Understanding the mechanisms by which these drugs kill cancer cells provides the possibility of engineering novel drugs with improved anticancer activity.

Violacein produced by the bacterium *Chromobacterium violaceum*, a broad spectrum bioactive compound, has been shown to have anticancer activity (23). Our study focused on understanding the mechanisms of action of violacein in cancer cells.

Cell proliferation assays were used to determine the *in vitro* effects of violacein on different cancer cell lines. Our results indicated a broad range and cell-specific violacein-mediated cytotoxicity. Furthermore, active caspase-3/7 measurements showed that apoptosis was induced only in A549 cells, indicating a differential effect of violacein on inducing apoptosis. A number of other investigators have shown that violacein induces apoptosis in colorectal cancer cells (16), Ehrlich ascites tumour cells (13), and HL60 leukemia cells (15). Notably, Ferreira *et al* found that violacein only induced apoptosis in HL60 leukemia cells and was ineffective in other types of leukemia cells indicating a differential apoptosis-inducing activity (15). Furthermore, we found that the anti-proliferative activity of violacein increased when the cells were exposed to hypoxic conditions. In addition, the most significant effect was observed in HT29 (colon cancer) and HN5 (head and neck cancer) cells showing a 12.6- and 6.5-fold decrease, respectively, in IC₅₀ from normoxic conditions. Of note, chemotherapeutic drugs that target proliferating cells are less effective in hypoxic conditions when cancer cells are quiescent (26). Furthermore, it has been well established that the quiescent cells, especially cancer stem cells, are involved in tumour progression and metastasis (27). This has limited the use of many chemotherapeutic, making them mostly palliative. The apparent increased *in vitro* activity of violacein in hypoxic conditions suggests a capacity in killing non-proliferative cells, a property with potential promise in combating chemoresistant cells.

Since violacein can be produced in *E. coli* carrying the recombinant plasmid pBSX-vio-opv, we determined the ability of the tumour-colonising *Salmonella typhimurium* strain VPN20009 to produce violacein from pBSX-vio-opv. Our results showed that this strain was capable of producing violacein when transformed with the pBSX-vio-opv plasmid. Furthermore, cytotoxicity assays showed that the violacein

produced was effective in killing cancer cells. Combining the tumour-colonising property of *Salmonella typhimurium* strain VPN20009 with violacein expression offers a potential target delivery system for violacein to the tumour core (28). Future studies are needed to ascertain the efficacy of this genetically modified strain.

The efficacy of violacein was also assessed *in vivo* on xenograft tumour models of HN5 (head and neck cancer) cells in mice. Our results showed that violacein regressed tumour volume and increased survival. However, tumour volumes reached the same levels as those of the controls when treatment was stopped. Additional studies with a prolonged dose regime are likely to yield an improved response to violacein. The *in vivo* results are encouraging for future studies to employ the tumour-targeting ability of VNV20009 (24,29) for the local production of violacein in the tumour microenvironment.

In conclusion, our findings confirm the anti-proliferative properties of violacein in a number of cancer cell lines and that this activity is increased in hypoxia-induced cells. Furthermore, violacein can be expressed in the antitumour *Salmonella typhimurium* strain VPN20009, a potentially promising strategy to deliver violacein locally to tumours. Our study further showed that violacein apoptotic activity is differential and *in vivo* violacein showed anti-proliferative activity against subcutaneous head and neck tumours. The findings of this study require further studies to ascertain the anticancer properties of violacein and its local delivery by genetically modified tumour-colonising bacteria harbouring the violacein biosynthetic cluster.

Acknowledgements

This study was supported by the Dr Jian Zhou Smart State fellowship from the Queensland government, and grants from the National Health and Medical Research Council and Cancer Council, Queensland to M.Q.W. The National Natural Science Foundation of China (grant no. 81160297) supported Tiefeng Xu. We would like to thank other members of the Wei Laboratory for their support and helpful comments. Special thanks is extended to Professor John Pemberton for providing the violacein producing *E. coli* strain (pBSX-vio-opv).

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