SPANDIDOS PUBLICATIONS

ONCOLOGY LETTERS 12: 1605-1609, 2016

Potential suppressive effects of gentian violet on human breast cancer MDA-MB-231 cells *in vitro*: Comparison with gemcitabine

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Received October 22, 2015; Accepted June 7, 2016

DOI: 10.3892/ol.2016.4773

Abstract. Gentian violet (GV), a cationic triphenylmethane dye, is used as an antifungal and antibacterial agent. Recently, attention has been focused on GV as a potential chemotherapeutic and antiangiogenic agent. The present study was undertaken to determine the suppressive effects of GV on human breast cancer MDA-MB-231 cells *in vitro*. The proliferation of MDA-MB-231 cells was suppressed by culture with GV (1-200 nM). The suppressive effects of GV on cell proliferation were not potentiated in the presence of various inhibitors that induce cell cycle arrest *in vitro*. This finding suggested that GV inhibits G1 and G2/M phase cell cycle arrest in MDA-MB-231 cells. The suppressive effects of GV on proliferation are mediated through the inhibition of various signaling pathways or nuclear transcription *in vitro*. Moreover, the suppressive effects of GV on cell proliferation were compared with that of gemcitabine, a strong antitumor agent that induces nuclear DNA damage. Notably, the culture with gemcitabine >50 nM suppressed cell proliferation, while the effects of GV were observed at >1 nM. The suppressive effects of gemcitabine on cell proliferation were not potentiated by GV. Overall, the present study demonstrated that GV exhibits a potential suppressive effect on the proliferation of human breast cancer MDA-MB-231 cells *in vitro*.

Introduction

Various types of cancer induce bone metastasis, which leads to serious bone loss and fractures. Bone metastasis occurs in 70-80% of patients with advanced stage breast cancer (1-4), and induces severe pathological bone fractures, pain, hypercalcemia, and spinal cord and nerve-compression syndromes (3,5). This bone disorder is frequently causes morbidity and mortality. Tumor invasion of the bone tissues is associated with the recruitment of osteoclasts and osteoblasts, resulting in growth factor liberation from the bone matrix. Furthermore, these growth factors can enhance tumor growth, resulting in a cycle of bone metastasis (4,5).

Breast cancer cells promote osteoclast formation via the secretion of osteoporotic cytokines, including parathyroid hormone-related peptide, tumor necrosis factor-α (TNF-α), prostaglandin E_2, leukemia inhibitory factor, and interleukin-1, -6, -8, -11, -15 and -17 (4,6). Constitutively-activated nuclear factor-κB (NF-κB) in breast cancer cells has been shown to play a crucial role in osteolysis, which stimulates osteoclastogenesis. Moreover, breast cancer cells stimulate the production of granulocyte macrophage colony-stimulating factor, which enhances development from monocytes to osteoclasts (7). In addition, progesterone receptor-positive mammary epithelial cancer cells express receptor activator of NF-κB ligand (RANKL), which mediates the proliferation of epithelial cells and carcinogenesis (8). In addition, breast cancer cells suppress the function of osteoblasts. This is demonstrated by an increase in apoptosis and a decrease in proteins required for new bone formation (6). Bone loss induced by breast cancer bone metastasis is based on activated osteoclastic bone resorption and suppressed osteoblastic bone formation.

Bisphosphonate or anti-RANKL antibody (denosumab) is used as the current standard care for patients with bone metastasis (9). Bisphosphonate inhibits osteoclastic bone resorption, but does not possess osteogenic effects. Denosumab suppresses osteoclast maturation by inhibiting the binding of RANKL to RANK, which is the receptor of RANKL in preosteoclasts and mature osteoclasts. These drugs target bone resorption mediated through osteoclasts. However, agents that stimulate osteogenic bone formation to repair bone destruction have been poorly developed.

Gentian violet (GV), a triaminophenylmethane dye, has been used extensively in medicine for a century, and it has a potent anti-microbial action (10). Furthermore, recent studies have suggested the angiogenic and anticancer properties of GV, and this chemical is currently experiencing renewed interest in medical applications (11,12). Our recent study demonstrated that GV inhibits nuclear factor-κB (NF-κB) activity, and that this agent can potently enhance osteoblast differentiation

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Key words: gentian violet, human breast cancer, MDA-MB-231 cells, cell proliferation, cell cycle, cell death

and mineralization, but suppress the differentiation to osteoclasts (13). Thus, GV may regulate the differentiation of bone cells *in vitro*. Further development of GV as an anti-osteoporotic and/or anti-inflammatory agent may be expected.

Moreover, GV may possess preventive effects on bone loss induced by cancer cell bone metastasis. However, the anticancer effects of GV on human breast cancer bone metastatic cells have been poorly investigated. The present study was undertaken to determine whether GV exhibits a suppressive effect on the proliferation of human breast cancer MDA-MB-231 cells *in vitro*. The results showed that GV potently suppresses the proliferation of human breast cancer MDA-MB-231 cells.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose, L-glutamine and sodium pyruvate, and antibiotics [penicillin and streptomycin (P/S); 5,000 U/ml and 5,000 μg/ml, respectively] were purchased from Gibco Laboratories (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Gentian violet, sodium butyrate, roscovitine, sulforaphane, PD98059, staurosporine, wortmannin, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Gemcitabine was obtained from Hospira, Inc. (Lake Forest, IL, USA). Gemcitabine was diluted in phosphate-buffered saline (PBS) and other reagents were dissolved in 100% ethanol to use in the experiments.

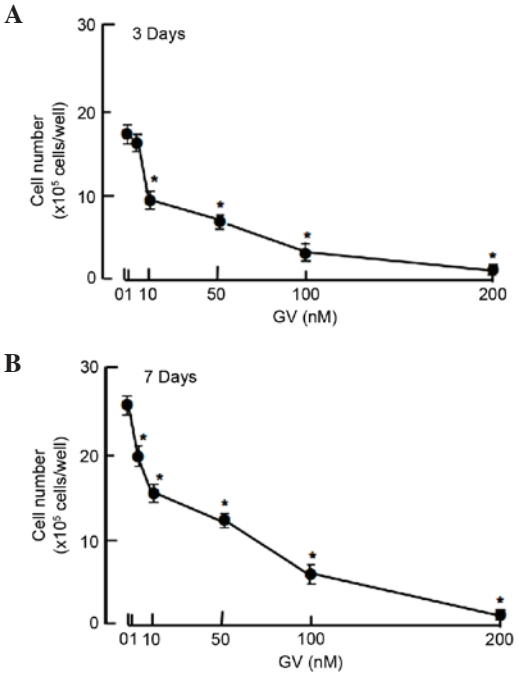

Figure 1. Gentian violet (GV) potently suppresses MDA-MB-231 human breast cancer cell proliferation *in vitro*. Cells were cultured in Dulbecco's modified Eagle's medium in the presence or absence of GV (1-200 nM) for (A) 3 or (B) 7 days. After culture, the number of attached cells on each dish was counted. Data are presented as the mean ± standard deviation of 2 replicate wells per dataset using different dishes and cell preparations. *P<0.001 vs. control (one-way analysis of variance, Tukey-Kramer post-hoc test).

Cancer cells. MDA-MB-231 human breast cancer cells lack the receptors for progesterone, estrogen and human epithelial growth factor receptor 2, and are therefore considered as triple negative (14). However, MDA-MB-231 cells do express epithelial growth factor receptor (EGFR) at high levels, and activation of this receptor and its downstream signaling events enhance the migration, proliferation, invasion and progression of the malignant phenotype of breast cancer cells (14). The present study used estrogen-independent bone-seeking triple negative human breast cancer MDA-MB-231 cells ($1x10^6$ cells/ml of DMEM containing 10% FBS and 0.1% P/S), which were stored at -80˚C. The cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

Proliferation in cancer cells. The breast cancer MDA-MB-231 cells ($1x10^5$/ml per well) were cultured in a 24-well plate using DMEM containing 10% FBS and 1% P/S in the presence or absence of GV (1, 10, 50, 100 or 200 nM) for 1, 3, 7 or 14 days in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37˚C (15-17). In separate experiments, the MDA-MB-231 cells ($1x10^5$/ml per well) were cultured in DMEM containing 10% FBS and 1% P/S in the presence of either ethanol (0.1% final concentration; control), sodium butyrate (10 and 100 μM), roscovitine (10 and 100 nM), sulforaphane (1 and 10 nM), PD98059 (1 μM), staurosporin (0.1 μM), wortmannin (1 μM), DRB (1 μM) or gemcitabine (100 nM) for 3-7 days. Subsequent to the culture process, the cells were detached from each culture dish and counted (16,17). In addition, to determine the effects of GV on MDA-MB-231 cells that reached confluence, the cells ($1x10^5$ cells/ml per well) were cultured using a 24-well plate in DMEM containing 10% FBS and 1% P/S in the absence of GV for 7 days until they reached confluence, and then the cells were cultured in the presence of GV (1, 10, 50, 100 or 200 nM) for 3 days (18). Following this, the cells were detached from each culture dish and counted.

Cell counting. Following trypsinization of each culture dish using 0.2% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free PBS for 2 min at 37˚C, detached cells from the dishes were collected after centrifugation at 150 x g for 5 min (16-18). The cells were resuspended in PBS solution and stained with eosin. Cell numbers were counted under a microscope (Olympus MTV-3; Olympus, Tokyo, Japan) using a hemocytometer plate. For each dish, the average of two counts was used. Cell number is shown as the number per well of each plate.

Statistical analysis. Statistical significance was determined using GraphPad InStat version 3 for Windows XP (GraphPad Software Inc., La Jolla, CA, USA). Multiple comparisons were performed by one-way analysis of variance with Tukey-Kramer multiple comparisons post-hoc test for parametric data as indicated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

To determine the effects of GV on the proliferation of human breast cancer MDA-MB-231 cells *in vitro*, the cancer cells were

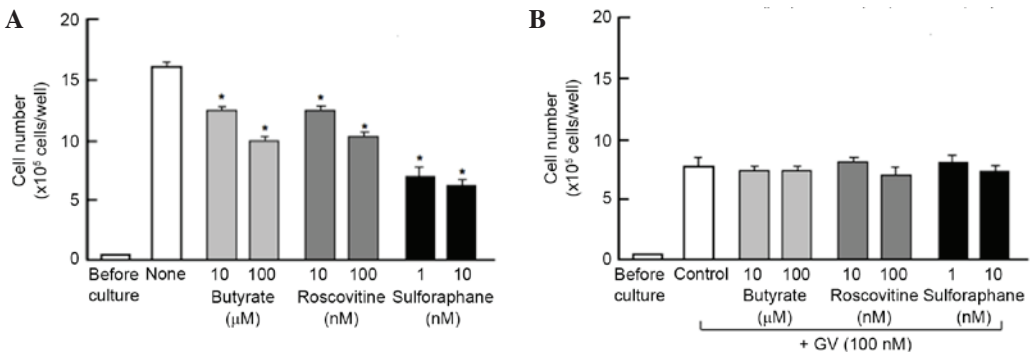

Figure 2. Gentian violet (GV) induces cell cycle arrest in human breast cancer MDA-MB-231 cells *in vitro*. Cells were cultured for 3 days in the (A) absence or (B) presence of GV (100 nM) with or without butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM). After culture, the number of attached cells on each dish was counted. Data are presented as the mean ± standard deviation of 2 replicate wells per dataset using different dishes and cell preparations. *P<0.001 vs. control (white bar) (one-way analysis of variance, Tukey-Kramer post-hoc test).

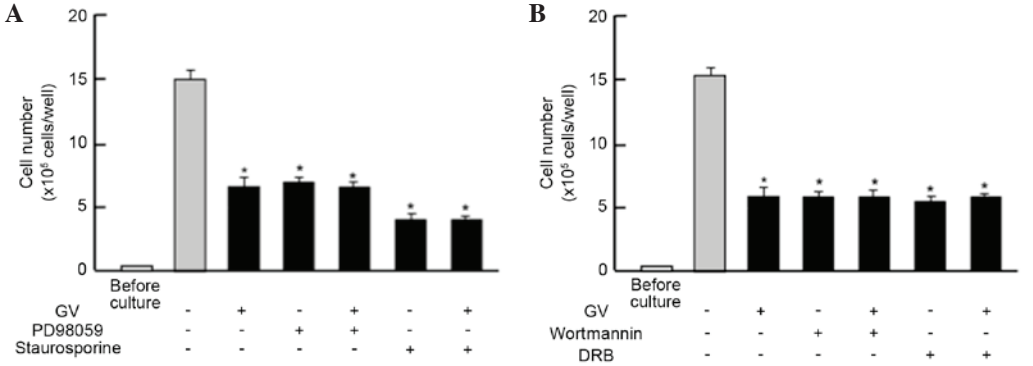

Figure 3. Suppressive effects of gentian violet (GV) on cell proliferation are not altered in the presence of inhibitors associated with intracellular signaling and transcriptional activity in MDA-MB-231 human breast cancer cells *in vitro*. (A) Cells were culture for 3 days in the presence of GV (100 nM) with or without PD98059 (1 μM) or staurosporin (0.1 μM). (B) Cells were cultured for 3 days in the presence of GV (100 nM) with or without wortmannin (1 μM) or DRB (1 μM). After culture, the number of attached cells on each dish was counted. Data are presented as the mean ± standrad deviation of 2 replicate wells per dataset using different dishes and cell preparations. *P<0.001 vs. control (grey bar). DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

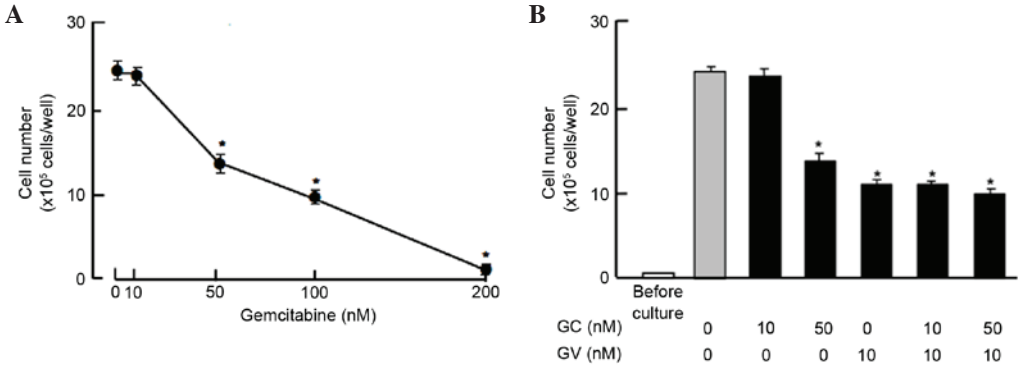

Figure 4. Suppressive effects of gentian violet (GV) on human breast cancer MDA-MB-231 cells show potential as compared with gemcitabine (GC) *in vitro*. (A) Cells were cultured for 7 days in the presence of gemcitabine (10, 50, 100 or 200 nM). (B) Cells were cultured for 7 days in the presence of GV (10 nM) with or without gemcitabine (10 or 50 nM). After culture, the number of attached cells on each dish was counted. Data are presented as the mean ± standard deviation of 2 replicate wells per dataset using different dishes and cell preparations. *P<0.001 vs. control (grey bar) (one-way analysis of variance, Tukey-Kramer post-hoc test).

cultured in the presence of GV for 3 or 7 days. Cell numbers were increased with increasing culture periods. This increase was suppressed after culture with GV (1-200 nM) for 3 (Fig. 1A) and 7 (Fig. 1B) days. Thus, GV was found to exhibit suppressive effects on the proliferation of the MDA-MB-231 cells *in vitro*. In addition, the MDA-MB-231 cells that reached confluence after culture for 7 days were cultured for an additional 3 days in the presence of GV (1-200 nM). Cell number was significantly (P=0.001) decreased after culture with GV (10-200 nM) (data not shown), suggesting that GV partly stimulates cell death.

To determine a mechanistic characterization, the present study determined whether the suppressive effects of GV on the proliferation of MDA-MB-231 cells are altered using various inhibitors that induce cell cycle arrest *in vitro*

(Fig. 2). Cells were cultured for 3 days with or without butyrate (10 and 100 μM), roscovitine (10 and 100 nM) or sulforaphane (1 and 10 nM) (17,19,20). The proliferation of the MDA-MB-231 cells, which were cultured in the absence of GV, was suppressed in the presence of these inhibitors (Fig. 2A). The suppressive effects of these inhibitors on cell proliferation was not altered in the presence of GV (100 nM) (Fig. 2B). This finding suggested that GV induces G1 and G2/M phase cell cycle arrest in MDA-MB-231 cells.

Next, the study determined whether the suppressive effects of GV on the proliferation in MDA-MB-231 cells are changed by various signaling factors that suppress proliferation. The suppressive effects of GV (100 nM) on the proliferation of the MDA-MB-231 cells were not altered in the presence of PD98059 (1 μM), an extracellular signal-regulated kinase (ERK) inhibitor (21), or staurosporin (0.1 μM), an inhibitor of protein kinase C (22) (Fig. 3A). In addition, the suppressive effects of GV on cell proliferation were not enhanced in the presence of wortmannin (1 μM), an inhibitor of phosphatidylinositol 3-kinase (PI3K) (23), or DRB (1 μM), an inhibitor of transcriptional activity with RNA polymerase II inhibition (24) (Fig. 3B).

Moreover, the suppressive effects of GV on the proliferation of the MDA-MB-231 cells were compared with those of gemcitabine, a strong antitumor agent that induces nuclear DNA damage (25). Culture with gemcitabine (50, 100 and 300 nM) for 7 days suppressed cell proliferation, while such effects were not observed at a concentration of 10 nM gemcitabine (Fig. 4A). The suppressive effects of gemcitabine (10 and 50 nM) on proliferation were not potentiated in the presence of GV (10 nM), which exhibited suppressive effects on the proliferation of the MDA-MB-231 cells (Fig. 4B).

Discussion

The present study demonstrated that GV exhibits a potent suppressive effect on the proliferation of human breast cancer MDA-MB-231 cells *in vitro*. The suppressive effects of GV on cell proliferation were characterized using various factors that inhibit cell cycle-related signaling processes. The suppressive effects of GV on the proliferation of the MDA-MB-231 cells were not changed by the presence of butyrate, roscovitine or sulforaphan, which induce cell cycle arrest. Roscovitine is a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2m and cdk5 (19), sulforaphane induces G2/M phase cell cycle arrest (20) and butyrate induces the inhibition of G1 progression (17). In the present study, GV was suggested to induce G1 and G2/M phase cell cycle arrest in the MDA-MB-231 cells.

The suppressive effects of GV on the proliferation of the MDA-MB-231 cells were not altered in the presence of various inhibitors that regulate intracellular signaling pathways *in vitro*. The suppressive effects of GV on cell proliferation were not potentiated in the presence of PD98059, an inhibitor of the ERK/mitogen-activated protein kinase (MAPK) signaling pathway (21), staurosporin, an inhibitor of the calcium-dependent protein kinase C signaling pathway (22), or wortmannin, an inhibitor of the PI3K/Akt signaling pathway (23). GV appeared to suppress cell proliferation, which is mediated through the inhibition of various signaling pathways associated with ERK/MAPK, calcium and PI3/Akt in breast cancer MDA-MB-231 cells.

Moreover, the suppressive effects of GV on cell proliferation were not altered in the presence of DRB, an inhibitor of transcriptional activity with RNA polymerase II inhibition (24). GV may also suppress transcriptional activity in the nuclei of MDA-MB-231 cells. Gemcitabine is an antitumor agent that induces nuclear DNA damage (25). This agent suppresses cell proliferation and stimulates apoptotic cell death in various types of cancer cells. In the present study, the effects of GV on proliferation and cell death were not enhanced in the presence of gemcitabine in the MDA-MB-231 cells, suggesting that GV partly acts in a process involved in the action of gemcitabine. Notably, GV exhibited suppressive effects on cell proliferation at lower concentrations compared with gemcitabine, indicating that GV exhibits a potential effect in breast cancer cells. GV may provide a useful tool as a novel antitumor agent.

GV has been shown to potently prevent TNF-α-induced suppression of osteoblastic mineralization and RANKL-induced stimulation of osteoclastogenesis by antagonizing the activation of NF-κB signaling in preosteoblastic cells and RAW267.4 preosteoclastic cells *in vitro* (13). Moreover, GV has been demonstrated to potently prevent suppressed osteoblastic mineralization and enhanced osteoclastogenesis induced by MDA-MB-231 cells in bone marrow culture *in vitro* (26). From these findings, it has been suggested that GV exhibits a potent suppressive effect on the activation of NF-κB signaling in MDA-MB-231 cells.

In conclusion, the present study demonstrated that GV potently suppresses the proliferation of human breast cancer MDA-MB-231 cells *in vitro*, and that this effect of GV has potential compared with that of gemcitabine, which is clinically used as an anticancer drug (25). GV may be a novel useful tool in the prevention and therapy of breast cancer *in vivo*.

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