

Original Article

Bryostatin 1 inhibits reduction in viability and induction of apoptosis by hydrogen peroxide in Saos-2, osteosarcoma cells through mitochondrial pathway

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Abstract: The present study was aimed to investigate the effect of bryostatin 1 on hydrogen peroxide (H₂O₂) induced inhibition in proliferation and induction of apoptosis in Saos-2, osteosarcoma cells. Cell proliferation was analyzed by MTT assay and induction of apoptosis using Annexin V-propidium iodide double staining by flow cytometry. The results revealed that pretreatment of Saos-2 cells with bryostatin 1 prevented H₂O₂ induced inhibition in cell proliferation and induction of apoptosis in concentration dependent manner. H₂O₂ reduced cell viability to 39.43% however, in bryostatin I (20 μM) pretreated cells the effect of H₂O₂ was prevented completely. Treatment of Saos-2 cells with 100 μM concentration of H₂O₂ induced apoptosis in 49.34 ± 3.47% and bryostatin I pretreatment for 12 h inhibited the apoptosis induction. The results from Western blot analysis showed increase in the activation of caspase-3 and -9 and reduction in the expression of Bcl-2 in H₂O₂ treated cells. However, pre-treatment for 12 h with bryostatin I before H₂O₂ treatment prevented increase in caspase-3 and -9 activation and decrease in Bcl-2 expression significantly (P<0.05). Thus bryostatin I inhibits H₂O₂ induced reduction in cell viability and induction of apoptosis through mitochondrial pathway. Therefore, bryostatin I can be of vital importance for treatment of osteoporosis.

Keywords: Osteoporosis, apoptosis, viability, expression, proliferation

Introduction

Bryostatin 1 has been isolated during the chemical investigation of a marine invertebrate *Bugula neritina* belonging to the Ectoprocta phylum [1]. Bryostatin 1, exhibits various biological activities which include strengthening of immune system, promotion of platelet accumulation, induction of differentiation and stimulation of haemopoiesis. However in a number of systems bryostatin 1 behaves differently from the phorbol esters. Studies have demonstrated that bryostatin 1 pre-treatment prevents phorbol ester induced effects in various types of cells. Activation of proteins requires significantly low doses of bryostatin 1 compared to that of phorbol ester [2, 3]. Bryostatin 1 treatment inhibits the induction and progress by tumor by phorbol esters [4]. Furthermore, bryostatin 1 inhibits ovarian, leukemia, reticulum, melanoma, renal and lung carcinoma cell proliferation

[1, 5-7]. Analysis of *in vivo* effect of the bryostatin 1 revealed a direct correlation with *in vitro* antitumor effect [7].

Apoptosis, programmed cell death plays an important role during the process of embryogenesis, treatment of cancer, induction of carcinoma, and providing immunity [8, 9]. Various factors including surface receptors, hormones, chemotherapeutic agents and oxidative stress are responsible for the induction and regulation of apoptosis. Most of the apoptosis inducing factors exhibit their effect through alteration of protein phosphorylation [10]. Production of reactive oxygen species (ROS) including, hydrogen peroxide (H₂O₂) and hydroxyl radical (OH) leads to induction of cell damage and ultimately death [11-13]. Although, antioxidants like glutathione peroxidase are present in the cells for neutralization of ROS species however, higher production of ROS induces apoptosis [14]. In

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the present study effect of bryostatin I on Saos-2 cell proliferation and induction of apoptosis by H₂O₂ treatment was investigated. Bryostatin I pretreatment prevented H₂O₂ induced inhibition in proliferation and induction of apoptosis.

Materials and methods

Reagents and antibodies

Bryostatin 1 used in this study was extracted from *Bugula neritina* and its stock solution was prepared in dimethyl sulfoxide (DMSO) which was stored at -20°C. H₂O₂ and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxym ethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) were purchased from Sigma (St. Louis, MO, USA).

Cell culture and treatment

Saos-2, osteosarcoma cell line was purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS, USA) and antibiotics. Incubation of the cells was performed in humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Cytotoxicity assay

The effect of H₂O₂ and bryostatin I on cell proliferation was analyzed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay. Saos-2 cells were distributed into 6-well plates (Nunc A/S, Roskilde, Denmark) at a density of 1 × 10⁵ cells per well in 2 ml RPMI-1640 and were incubated for 24 h. The cells were then treated with RPMI-1640 containing 2% FBS and various concentrations of bryostatin I (5, 10, 15, 20 and 25 µM/ml) for 12 h. Following incubation with bryostatin I, medium was replaced by RPMI-1640 containing H₂O₂ (100 µM) and incubated for 48 h. The control cultures were treated with DMSO or H₂O₂ alone. Following incubation, 50 µl of MTT solution (5 µg/ml) was put into each of the well and incubation was continued for 1 h more. 100 µl of DMSO was added to each well of the plate and then optical density (OD) was recorded at 570 nm using an EL800 Universal Microplate Reader (BioTek Instruments Inc., Winooski, VT, USA).

Annexin assay

The effect of H₂O₂ and bryostatin I on induction of apoptosis in Saos-2 osteosarcoma cells was analyzed using fluorescein isothiocyanate (FITC)-Annexin V Apoptosis Detection kit I (BD Biosciences, San Jose, CA, USA). Apoptosis induction was analyzed according to the manufacturer's instructions. The cells were seeded at a density of 1 × 10⁶ cells per well onto the 100 mm culture dishes and treated with various concentrations of bryostatin for 12 h followed by H₂O₂ (100 µM) for 48. The control cultures were incubated with H₂O₂ or DMSO alone. Following incubation, the cells were harvested and centrifuged for 10 min at 500 × g. The cell pellets obtained were put in Annexin V binding buffer containing 140 mM CaCl₂, 10 mM HEPES/NaOH and 2.5 mM MgCl₂. To each of the well was then added 5 µl each of FITC-conjugated Annexin V and propidium iodide (PI) and incubation was continued under dark conditions for 20 min. Fluorescence-Activated Cell Sorting (FACScan) instrument (Becton-Dickinson, Franklin Lakes, CA, USA) was used for the analysis of the apoptotic cells.

Western blot analysis

Saos-2 cells treated with bryostatin I and H₂O₂ or with H₂O₂ alone were put in RIPA buffer (Rockland, Gilbertsville, PA, USA) supplemented with 5 µM AEBSF, 1.5 µM aprotinin, 10 µM E-64, 0.01 µM leupeptin and phosphatase inhibitors [1 mM sodium orthovanadate (Na₂VO₄), 1 mM sodium molybdate (Na₂MoO₄), 4 mM sodium tartrate dehydrate and 2 mM imidazole]. The cells were placed on ice for 45 min and then centrifuged at 10,000 × g for 30 min at 4°C. Bicinchoninic protein assay kit (Pierce, Rockford, IL, USA) was used for the determination of concentration of proteins. The cell lysates were isolated on 10% SDS-PAGE gel and then transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) using electroblotting. The membranes were incubated with primary antibodies overnight at 4°C. The polyclonal antibodies against caspase-3 and -9 (Chemicon, Temecula, CA, USA) and rabbit anti-Bcl-2 (Cell Signaling Technologies, Beverly, MA, USA) were used to probe the proteins. Anti-β-actin (Santa Cruz, CA, USA) was used as the internal control. Following incubation the membranes were washed twice with

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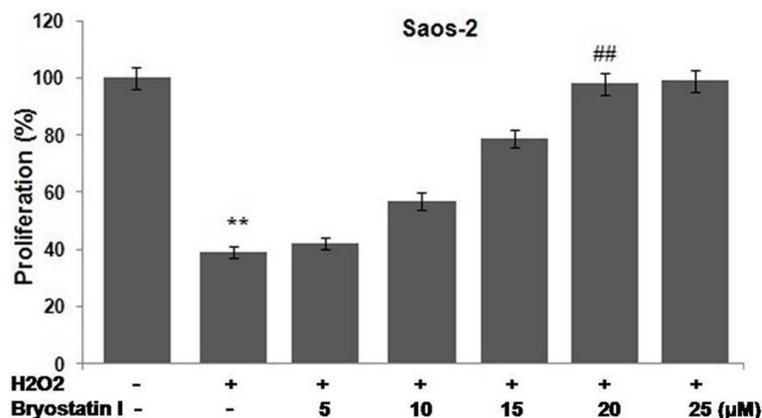


Figure 1. Effect of H₂O₂ and bryostatin I on the proliferation rate of Saos-2 osteosarcoma cells. The cells were pretreated with various concentrations of bryostatin I before H₂O₂ treatment or with H₂O₂ alone and then analyzed using MTT assay. **P<0.01 compared with control group; #P<0.05 compared with control group.

PBS and Tween-20 for 20 min and subsequently incubated with horseradish peroxidase-conjugated polyclonal horse anti-rabbit (1:2,000; Cell Signaling Technology, Inc.) for 1 h. The enhanced chemiluminescence kit (Intron Biotechnology Inc., Seongnam, Korea) was used for developing the blot.

Statistical analysis

All the presented data are expressed as the mean ± standard deviation (SD). Comparison of the data between different groups of cells was performed using Student's t-test and data analysis using Microsoft Excel 2010 software (Microsoft Corporation, Redmond, WA, USA). One-way analysis of variance (ANOVA) was also used. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of bryostatin I and H₂O₂ on proliferation of Saos-2 cells

Incubation of Saos-2 cells with H₂O₂ at a concentration of 100 μM for 24 h reduced the proliferation rate to 39.43% compared to 100% in the control. However, Saos-2 cells pretreated with various concentrations of bryostatin I for 12 h followed by H₂O₂ treatment showed inhibition of reduction in cell proliferation in a concentration dependent manner. The various concentrations of bryostatin I used to prevent the H₂O₂ induced inhibition in the cell proliferation

were 5, 10, 15, 20 and 25 μM. The H₂O₂ induced inhibition in cell proliferation was prevented completely by pretreatment with 20 μM concentration of bryostatin I (**Figure 1**).

Effect of H₂O₂ and bryostatin I on morphological alterations in Saos-2 cells

Treatment of Saos-2 cells with 100 μM concentration of H₂O₂ for 48 h caused alterations in the cell morphology. The H₂O₂ treated cells showed condensation of chromatin material, degeneration of cells and the cells became

rounded in shape. However, pretreatment with bryostatin I for 12 h prevented the H₂O₂ induced alterations in the Saos-2 cell morphology (**Figure 2**).

Effect of bryostatin I on H₂O₂ induced apoptosis in Saos-2 cells

Treatment of Saos-2 cells with 100 μM concentration of H₂O₂ led to induction of apoptosis in 49.34 ± 3.47% cells compared to 2.23 ± 0.11% cells in the control (P<0.05) after 48 h. However, when Saos-2 cells were pretreated with bryostatin I for 12 h followed by incubation with H₂O₂ the induction of apoptosis was inhibited in concentration dependent manner. In H₂O₂ treated cells, pretreatment with 5, 10, 15, 20 and 25 μM concentration reduced rate of apoptosis to 47.46 ± 3.13, 24.25 ± 2.75, 8.65 ± 2.32, 2.79 ± 1.14, 2.23 ± 0.46%. Thus bryostatin I pretreatment for 12 h caused a significant decrease in the H₂O₂ induced apoptosis in Saos-2 cells compared to H₂O₂ treated cells (P<0.01) (**Figure 3**).

Effect of bryostatin I and H₂O₂ on expression of Bcl-2 and activation of caspase-3 and -9

Western blot analysis was used to analyze the effect of H₂O₂ and bryostatin I on the expression of Bcl-2, activation of caspase-3 and -9. Incubation of Saos-2 cells with H₂O₂ reduced the expression of Bcl-2 significantly compared to the untreated control cells. The activation of

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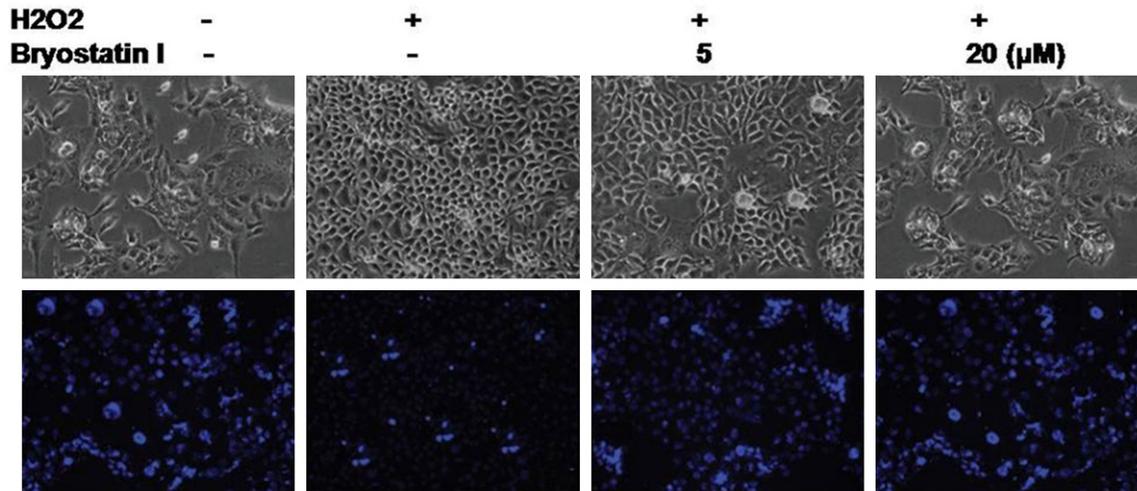


Figure 2. Effect of bryostatin I on the H₂O₂ induced alterations in morphology of Saos-2 osteosarcoma cells. The cells were pretreated with various concentrations of bryostatin I before H₂O₂ treatment or with H₂O₂ alone and then analyzed using MTT assay. (magnification, × 200).

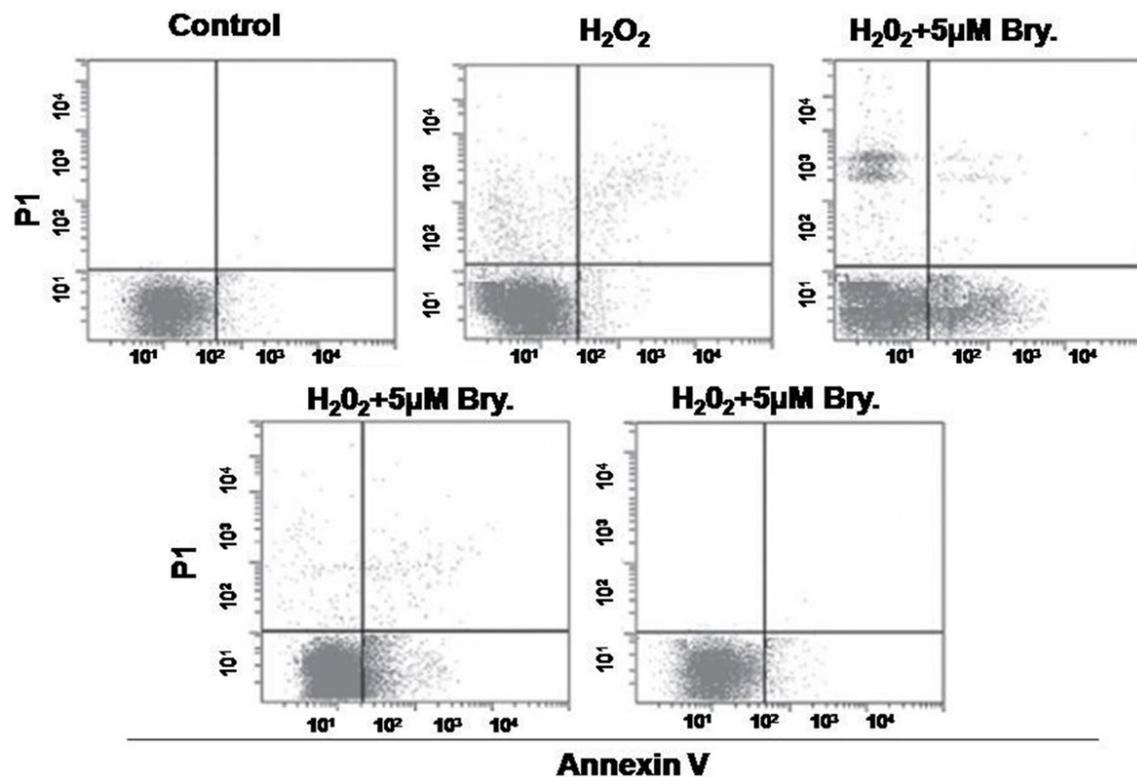


Figure 3. Bryostatin I pretreatment prevents apoptosis in Saos-2 cells induced by H₂O₂. Following pretreatment with bryostatin I before H₂O₂ or with H₂O₂ alone the cells were analyzed using Annexin V and propidium iodine double staining by flow cytometry.

caspase-3 and -9 was increased significantly by H₂O₂ treatment. On the other hand, in Saos-2 cells pretreated with bryostatin I followed by H₂O₂ treatment, the enhanced expression of

Bcl-2 was inhibited. The activation of caspase-3 and -9 enhanced by H₂O₂ was reduced by pretreatment with bryostatin I for 12 h (P>0.001) (**Figure 4**).

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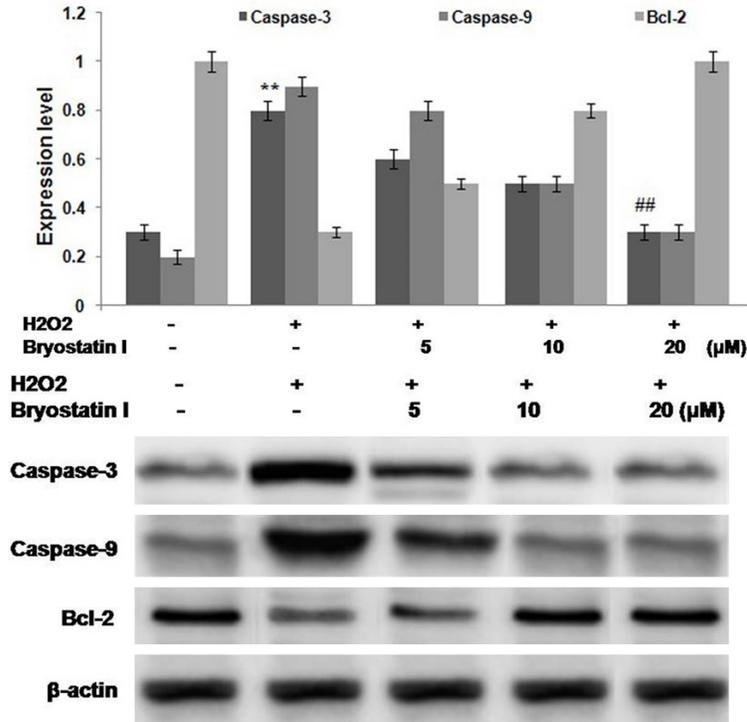


Figure 4. Bryostatin I pretreatment inhibits H_2O_2 induced increase in expression of caspase-3 and -9 and reduction in Bcl-2. The cells were treated with bryostatin I before H_2O_2 or with H_2O_2 alone and then subjected to western blot analysis. ** $P < 0.01$ compared with H_2O_2 treatment alone, * $P < 0.01$ compared with control.

Discussion

The present study demonstrated the effect of bryostatin I on the H_2O_2 induced inhibition in osteosarcoma cell proliferation and induction of apoptosis. Generation of higher level of reactive oxygen species (ROS) including, nitric oxide, hydrogen peroxide (H_2O_2) and hydroxyl free radical (OH) causes cell damage and ultimately apoptosis [11-13]. The proliferation rate of the cells decreases with the increase in production of ROS [15]. In the present study, addition of H_2O_2 to Saos-2 cell cultures significantly inhibited the rate of proliferation. Pretreatment of Saos-2 cells with bryostatin I for 12 h before incubation with H_2O_2 prevented the inhibition of cell proliferation.

Oxidative stress and several other factors responsible for induction of apoptosis in various types of cells [8, 9]. In the present study incubation of Saos-2 cells with H_2O_2 induced morphological alteration like cell rounding and disruption. However, pretreatment of Saos-2 cells with bryostatin I before H_2O_2 addition pre-

vented from alterations in the morphology. In the cell cultures incubated with H_2O_2 the proportion of apoptotic cells was found to be significantly higher compared to the control cell cultures. But in cell cultures, pretreated with bryostatin I before H_2O_2 addition, the induction of apoptosis was prevented. To investigate the mechanism of prevention of H_2O_2 induced apoptosis in Saos-2 cells, the expression of caspases and Bcl-2 was analyzed.

Caspases after activation either by mitochondrial [16], death receptor [17] or endoplasmic reticulum pathways [18] play a vital role in the regulation of cell apoptosis [19]. In the present study, incubation of Saos-2 cells with H_2O_2 increased the expression of cleaved caspase-3 and 9. Bryostatin I pretreatment for 12 h prevented the H_2O_2 induced increase in the expression level of cleaved caspase-3 and -9. Another factor involved in the regulation of cell death through mitochondrial pathway is the Bcl-2 [20]. In the present study reduction in expression of Bcl-2 by H_2O_2 was also prevented by pretreatment with bryostatin I.

In summary, bryostatin I prevented from H_2O_2 induced reduction in cell proliferation and induction of apoptosis in Saos-2 cells through reduction in the activation of caspase-3 and -9 and increase in the expression of Bcl-2 expression. Therefore, bryostatin I can be of vital importance for treatment of osteoporosis.

Disclosure of conflict of interest

None.

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