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Original Article

α2β1 Integrin specific inhibitor BTT-3033 promotes paclitaxel-induced apoptosis in human ovarian cancer cells

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Abstract

Background and purpose: The new plan of using molecular targeted agents in combination with cytotoxic drugs may represent a promising strategy to increase the efficacy of chemotherapy. Hence, we examined whether α2β1 integrin-specific inhibitor, BTT-3033, could modulate the susceptibility of OVCAR3 and SKOV3 ovarian cancer cells to paclitaxel (PTX).
Experimental approach: Ovarian cancer cell lines were treated with BTT-3033 and different concentrations of PTX. To determine the mechanisms involved in the PTX/BTT-3033 combination-induced cell death, cell viability, apoptosis, reactive oxygen species (ROS) production, mitochondrial membrane potential (MMP), and caspase-3 activity were evaluated.
Findings/Results: Both BTT-3033 (≥ 1 μM) and PTX (≥ 0.01 μM) suppressed the proliferation of OVCAR3 and SKOV3 cells in a concentration-related manner. Pretreatment with BTT-3033 (1 μM), followed by PTX-induced synergistic antiproliferative effects, decreased the IC_{50} values of PTX from 0.45 to 0.03 μM in OVCAR3 and 0.35 to 0.02 μM in SKOV3 cells. All of the coefficients of drug interaction for various PTX and BTT-3033 combinations were found to be less than 1. Moreover, PTX/BTT-3033 combination induced more apoptotic cells (from 4.2% to 87.0% in OVCAR3 and 2.4% to 88.5% in SKOV3) than PTX alone. Combination therapy also decreased MMP and increased the caspase-3 activity. Additionally, we found that the PTX/BTT-3033 combination enhanced ROS production in OVCAR3 and SKOV3 cells.
Conclusion and implications: BTT-3033 has demonstrated the ability to enhance the susceptibility of ovarian cancer cells to PTX by inducing MMP loss, ROS production, and mitochondrial apoptosis, therefore this combination therapy might represent a promising strategy for ovarian cancer treatment.

Keywords*:* Apoptosis; BTT-3033; Drug interaction; Ovarian cancer; Paclitaxel.

INTRODUCTION

Integrins are a family of α/β heterodimeric transmembrane receptors that mediate cell adhesion to the surrounding extracellular matrix. Integrins transduce biochemical signals to promote cell survival, proliferation, differentiation, adhesion, and migration (1,2). These receptors are also involved in regulating diverse cellular functions essential to tumor development and progression, including cell growth, invasion, and migration, and their differential expression in tumors has been used to generate targeted inhibitors (1,2). In addition, recent studies have shown that integrins contribute to the chemoresistance of cancer cells (1,2).

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The α2β1 integrin, which binds collagen, protects various solid tumors, including breast cancer (3), head and neck cancer (4), pancreatic cancer (5), small cell lung cancer (6,7), and ovarian cancer (8) from chemotherapy- and radiation-induced apoptosis.

Integrin α2β1 can protect tumor cells against chemotherapy-induced apoptosis through numerous intracellular mechanisms (3-9). The α2β1 integrin/phosphoinositide 3-kinase (PI3K)/AKT pathway has been reported as a crucial mediator of resistance to paclitaxel (PTX) and vincristine in breast cancer (3). Integrin α2β1/p38 mitogen-activated protein kinase (MAPK)/reactive oxygen species (ROS) pathway has been proposed as a mechanism for reducing A431 carcinoma cell line sensitivity to PTX (9). Moreover, α2β1 integrin inhibited apoptosis of pancreatic cancer cells induced by 5-fluorouracil by upregulating the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family member, Mcl-1 (myeloid cell leukemia-1) protein (5). Integrin α2β1 was also shown to decrease PTX effects on ovarian cancer cells through PI3K/AKT signaling, supporting a role for this receptor in the chemotherapy resistance of ovarian cancer cells (8).

PTX has been widely used as a common treatment in cancer therapy. This drug acts as a microtubule stabilizing agent, interfering with the mitosis of proliferating tumor cells, thus inducing apoptosis (10,11). It has been reported that less than 33% of patients with ovarian cancer respond to current chemotherapy due to the development of chemoresistance (11). It is suggested that utilizing integrin inhibitors as an adjuvant for chemotherapy might help overcome drug resistance. Several methods for modulation of integrin signaling have been discovered including small-molecule inhibitor BTT-3033 (12). BTT-3033 is a sulfonamide derivative developed to inhibit α2β1 integrin through binding to the α2I domain (12). BTT-3033 has been reported to be effective in suppressing the cell growth of prostate cancer cells (13). We have previously demonstrated that BTT-3033 induced the loss of mitochondrial integrity and the overproduction of ROS to increase prostate cancer cell apoptosis (13). However, the mechanism by which BTT-3033 modulates chemoresistance remains unclear. In the current study, we examined whether α2β1 integrin specific inhibitor, BTT-3033, could modulate the susceptibility of ovarian cancer cells to PTX and investigated the cellular mechanisms by which BTT-3033 could enhance PTX-induced apoptosis.

MATERIALS AND METHODS

Chemical and assay kits

BTT-3033 (Cat No. 4724), a selective inhibitor of integrin α2β1, was procured from Tocris Co. (Tocris Bioscience, Ellisville, MO, USA). 5,5',6,6'-etrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Cat No. M2128), and propidium iodide (PI) were purchased from Sigma-Aldrich company (Germany). Marker Gene™ live cell fluorescent ROS detection kit was obtained from Marker Gene Technologies (Marker Gene Technologies, Inc. Eugene, USA). Caspase-3 assay kit (Cat No. K533) was purchased from Biovision (Mountain View, CA, USA). The annexin V-fluorescein isothiocyanate (FITC) apoptosis staining/detection kit was bought from Abcam (Cambridge, UK). Stock solutions of PTX and BTT-3033 were prepared in dimethyl sulfoxide (DMSO). For each experiment, two drugs were freshly diluted in RPMI-1640 medium (Gibco Life Technologies, Grand Island, NY, USA) with the desired concentration. The final concentration of DMSO did not exceed 1%.

Cell culture

The National Cell Bank of Iran (NCBI, Pasteur Institute of Iran) provided OVCAR3 and SKOV3 ovarian cancer cell lines. Cells were propagated in a humidified incubator in Roswell Park Memorial Institute (RPMI-1640) medium containing 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA) and 1% penicillin/streptomycin (Gibco, USA) at 37 °C with 5% CO_2.

Cellular viability test

Cell viability was determined colorimetrically using the MTT dye reduction assay, as described previously (14). After OVCAR3 and SKOV3 cells (7000 cells/well)

were seeded in a 96-well plate overnight, three conditions of drug treatment were set: (1) cells treated with PTX (0, 0.001, 0.01, 0.1, or 1 μM) for 48 h; (2) cells treated with BTT-3033 (0, 0.1, 1, 10, or 50 μM) for 48 h; and (3) cells treated with BTT-3033 (1 μM) for 2 h, following incubated with PTX (0-1 μM) for 48 h. Then, 20 μL MTT reagent (final concentration of 0.5 mg/mL) was added directly into each well, and incubated for 4 h. Subsequently, media was removed and all wells were treated with 150 μL DMSO. Cell viability was quantified by measuring the optical density (OD) values of the absorbance at 570 nm by using a microplate reader (BioTek Instruments, Winooski, VT, USA). The growth inhibitory effect was expressed as the half maximal inhibitory concentration (IC_{50}) estimated from the dose-response curve (GraphPad Prism software). The effects of drug interaction were assessed by the coefficient of drug interaction (CDI), which was determined as follows:

$$\mathrm{CDI} = \frac{AB}{\mathrm{A} \times \mathrm{B}}$$

Where AB is the ratio of the cell viability treated with PTX/BTT-3033 combination to that of the control group and A or B is the ratio of the viability of cells treated with only BTT-3033 or PTX to that of the control group. Therefore, CDI value < 1, = 1, or > 1 shows that the drugs' interactions are synergistic, additive, or antagonistic, respectively (15).

Cell apoptosis assay by flow cytometry

Cell apoptosis was analyzed by staining the cells with annexin V-FITC/PI solution, followed by flow cytometry. Briefly, OVCAR3 and SKOV3 cells were incubated with different concentrations of PTX (0, 0.001, 0.01, 0.1, or 1 μM) alone or in combination with BTT-3033 (1 μM) for 48 h. Both attached and floating cells were collected, washed twice with phosphate-buffered saline (PBS), and resuspended in 150 μL of annexin-binding buffer. Next, 5 μL of annexin V-FITC and 1 μL of PI solution (50 ng/mL) were added and incubated for 15 min at room temperature in a dark place. The stained cells were evaluated by FACS Calibur flow cytometer (BD Bioscience, Franklin Lakes, NJ).

Measurement of ROS generation

Intracellular ROS generation in OVCAR3 and SKOV3 cells was measured with a fluorescent probe Dichloro-dihydro-fluorescein diacetate (DCFH-DA). After exposure to PTX at 0, 0.001, 0.01, 0.1, or 1 μM, alone or in combination with BTT-3033 (1 μM) for 48 h, cells were incubated with DCFH-DA (20 μM) in HEPES buffer (40 mmol/L, pH 7.4) for 30 min in a dark place at 37 °C. The fluorescence intensity was detected using a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

Mitochondrial membrane potential analysis

The mitochondrial membrane potential (MMP) of treated cells was measured with JC-1 fluorescent dye which can enter the mitochondrial matrix according to the MMP level (14). OVCAR3 and SKOV3 cells were seeded in black clear-bottom 384-well plates and treated with different concentrations of PTX (0, 0.001, 0.01, 0.1, or 1 μM) alone or in combination with BTT-3033 (1 μM) for 48 h. Then, cells were incubated with JC-1 in 40 mM HEPES buffer (pH 7.4) containing JC-1 (2.5 mM), glucose (4.5 g/L), and NaCl (0.65%) for 30 min. After that, fluorescence was evaluated at two excitation/emission wavelength pairs using a microplate reader. The ratio between the measured red (590 nm) and green (540 nm) fluorescence intensities indicated an alteration in MMP.

Caspase-3 activity assay

Caspase-3 activity in OVCAR3 and SKOV3 cells was assessed using a caspase colorimetric assay kit (14). Briefly, 3×10^5 cells were incubated overnight and treated with increasing concentrations of PTX (0-1 μM) alone or in combination with BTT-3033 (1 μM) for 48 h. Following the treatment, cells were lysed to collect their intracellular contents. Then, the supernatant was collected and evaluated for protease activity by adding a caspase-specific peptide conjugated with p-nitroanaline. After 1 h incubation at 37 °C, the amount of released p-nitroaniline was measured at 405 nm by a microplate reader.

Statistical analysis

All experiments were carried out in triplicate and the results were expressed as mean ± SD. The results were considered significant at $P < 0.05$. Student's t-test was applied to determine differences between two groups and one-way ANOVA followed by Dunnett's post hoc test was used to identify significant differences among multiple groups. The data were analyzed by GraphPad Prism 8.0.1 software (GraphPad Software, La Jolla, CA, USA).

RESULTS

The effect of BTT-3033 on the viability rate of OVCAR3 and SKOV3 cells

To demonstrate the inhibitory effect of BTT-3033 on the viability of ovarian cancer cells, cells were incubated in the presence of BTT-3033 (0, 0.1, 1, 10, or 50 µM) for 48 h, and cell viability was determined using the MTT assay. As shown in Fig. 1A and B, BTT-3033 (≥ 1 µM) significantly and concentration-dependently decreased the viability of OVCAR3 and SKOV3 cells compared to the control cells. Cytotoxic effects of BTT-3033 were observed at concentrations between 1 and 50 µM (80.3% to 38.2% for OVCAR3 and 83.5% to 44.2% for SKOV3 cells) in comparison to the control (Fig. 1A and B). The IC_{50} values of BTT-3033 in OVCAR3 and SKOV3 cells were 29.6 µM and 44 µM, respectively.

The effect of PTX on the viability rate of OVCAR3 and SKOV3 cells

PTX (≥ 0.01 µM) showed inhibitory effects on the growth rate of cells in a concentration-dependent manner, and the IC_{50} values of PTX in OVCAR3 and SKOV3 cells were 0.45 µM and 0.33 µM, respectively (Fig. 1C and D). In the presence of 0.001, 0.01, 0.1, and 1 µM PTX, the cell viability rate of OVCAR3 cells was 89.5%, 73.9%, 66.5%, and 41.5%, respectively (Fig. 1C). The viability rate of cells induced by PTX (0.001, 0.01, 0.1, and 1 µM) in SKOV3 cells was 91.7%, 77.0%, 65.5%, and 37.6%, respectively (Fig. 1D).

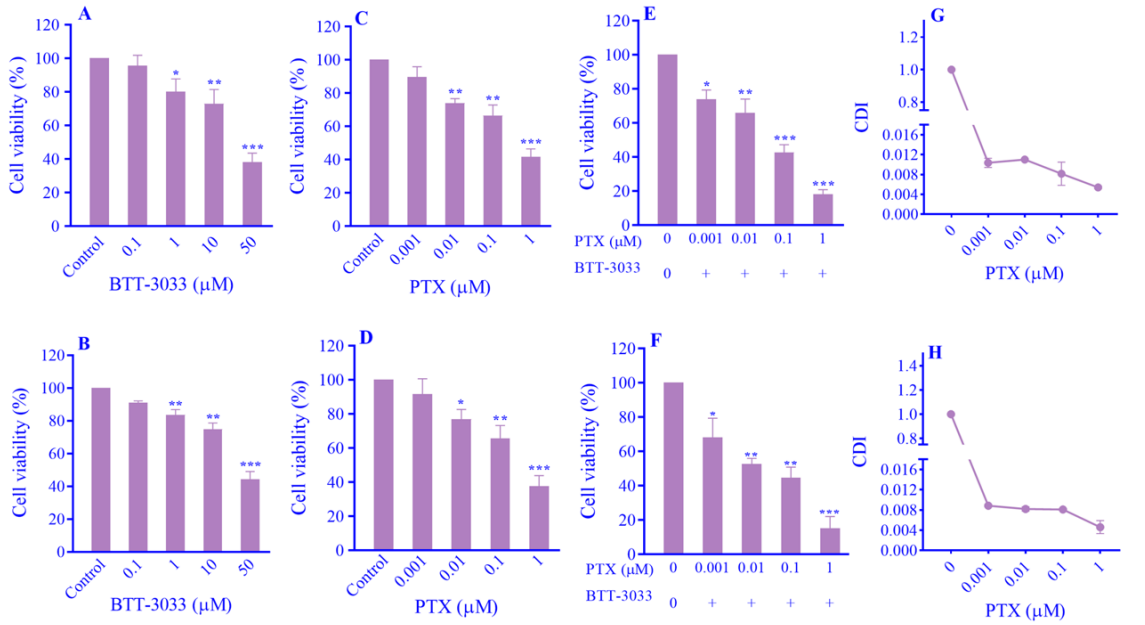

Fig. 1. Effect of BTT-3033 on PTX-induced cytotoxicity in ovarian cancer cells. BTT-3033 (≥ 1 µM) significantly decreased the viability of (A) OVCAR3 and (B) SKOV3 cells. PTX reduced the viability of (C) OVCAR3 and (D) SKOV3 cells. Results showed that the combination of BTT-3033 (1 µM) with PTX (0, 0.001, 0.01, 0.1 or 1 µM) induced a higher growth inhibition rate in (E) OVCAR3 and (F) SKOV3 cells than PTX alone. (G and H) The synergistic antiproliferative effect of PTX combined with BTT-3033 on the growth of OVCAR3 and SKOV3 cells. Data are presented as the means ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences compared to the control group. PTX, Paclitaxel; CDI, coefficient of drug interaction.

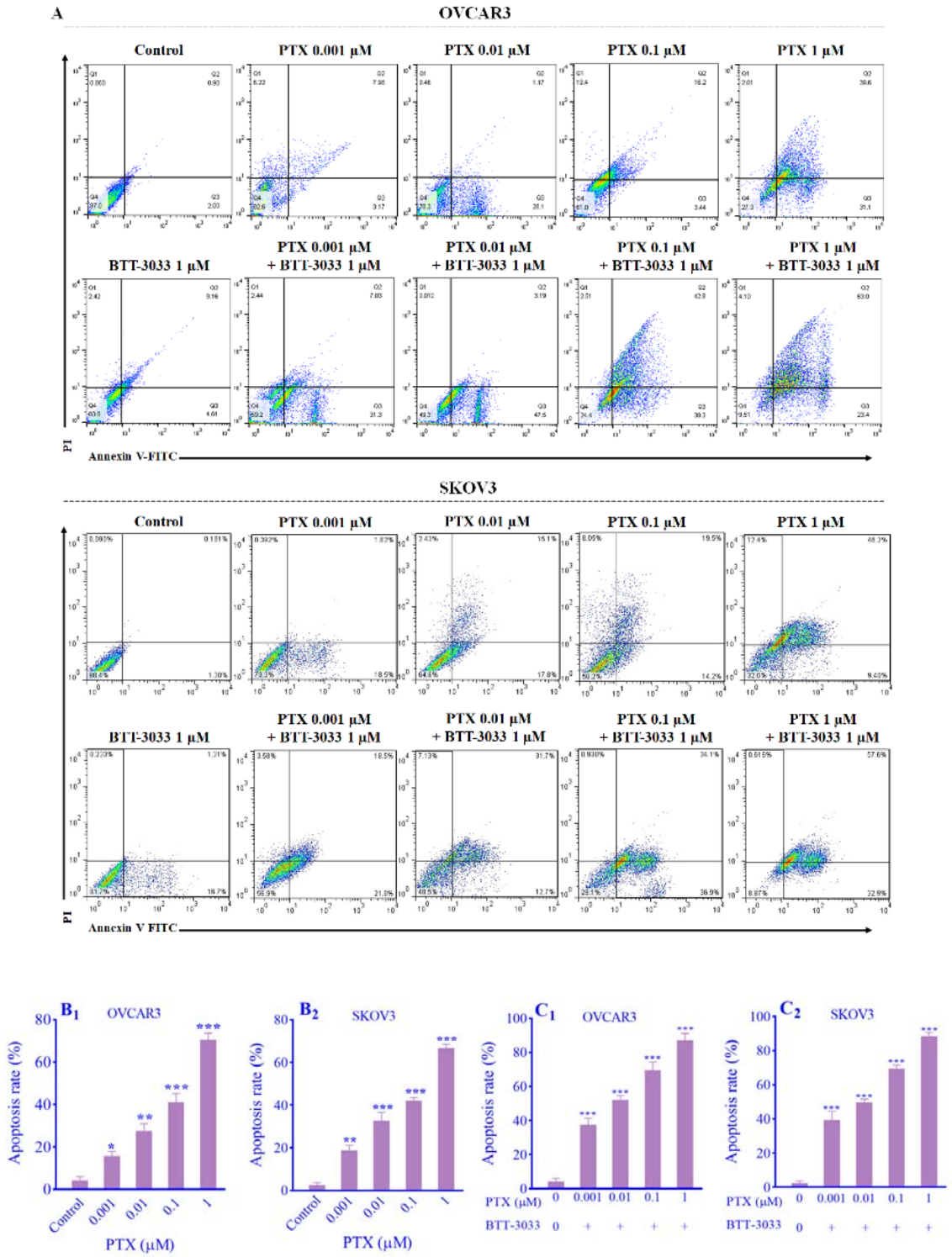

Fig. 2. Apoptotic effect of PTX/BTT-3033 combination on OVCAR3 and SKOV3 cells. (A) Flow cytometric analysis of ovarian cancer cells after treatment with different concentrations of PTX (0.001, 0.01, 0.1, or 1 μM) or BTT-3033 (1 μM) followed by PTX (0.001, 0.01, 0.1, or 1 μM) for 48 h. Compared to (B_1 and B_2) PTX alone, (C_1 and C_2) pretreatment with BTT-3033 followed by PTX induced more apoptotic cells. Data are presented as the means ± SD from three independent experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ indicate significant differences compared to the control group. PTX, Paclitaxel.

The effect of BTT-3033 on PTX-induced cytotoxicity in OVCAR3 and SKOV3 cells

We extended our investigations to the effect of a low-concentration BTT-3033 (1 µM) combined with different concentrations of PTX (0-1 µM) in OVCAR3 and SKOV3 cells. Pretreatment of cells with 1 µM BTT-3033 followed by 0.001, 0.01, 0.1, or 1 µM of PTX, resulted in significant inhibition of cell growth greater than that obtained with PTX alone (the IC_{50} value of the combination was 0.03 µM in OVCAR3 and 0.02 µM in SKOV3 (Fig. 1E and F). As expected, according to the CDI values shown in Fig. 1G and H, PTX, and BTT-3033 yielded synergistic interactions across a wide concentration range (CDI < 1). These results showed that the growth inhibition rate of OVCAR3 and SKOV3 cells treated with the PTX/BTT-3033 combination was significantly higher than those that occurred with PTX alone.

The effect of PTX/BTT-3033 combination on apoptosis of OVCAR3 and SKOV3 cells

To further determine whether BTT-3033 sensitizes for PTX treatment by inducing apoptosis, we assessed the apoptotic status by staining the cells with annexin V-FITC/PI solution, followed by flow cytometry. Cells were treated with BTT-3033 (1 µM), PTX (0.001, 0.01, 0.1, or 1 µM), or a combination of the two drugs for 48 h. The apoptosis in OVCAR3 and SKOV3 cells increased from 4.2% and %2.4 in control groups to 70.3% and 66.6% in the groups treated with PTX at 1 µM, respectively (Fig. 2A and B). Compared to the groups treated with PTX alone, PTX/BTT-3033 combination therapy induced more apoptosis (Fig. 2C). Apoptotic rates for the combination of BTT-3033 (1 µM) and PTX (0.001, 0.01, 0.1, or 1 µM) were 37.6%, 52.3%, 69.5%, and 87.0% in OVCAR3 cells, respectively. In SKOV3 cells, the percentage of apoptotic rates induced by BTT-3033 (1 µM) treatment followed by PTX at 0.001, 0.01, 0.1, or 1 µM was 39.3%, 49.8%, 69.5%, and 88.5%, respectively. These data support the idea that BTT-3033 might sensitize OVCAR3 and SKOV3 cells to PTX by inducing apoptosis.

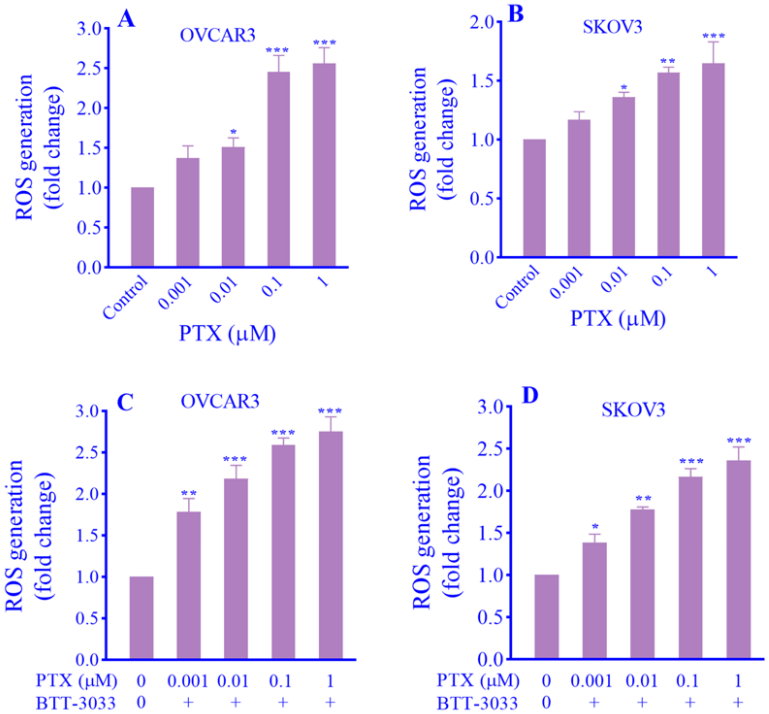

Fig. 3. Effect of BTT-3033 on PTX-induced ROS production in OVCAR3 and SKOV3 cells. Compared to (A and B) 48-h PTX treatment alone, (C and D) 48-h pretreatment with BTT-3033 followed by PTX caused a further increase in ROS production in OVCAR3 and SKOV3 cells. Data are presented as the means ± SD from three independent experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences compared to the control group. PTX, Paclitaxel; ROS, reactive oxygen species.

The effect of BTT-3033 on PTX-induced ROS production in OVCAR3 and SKOV3 cells

The effect of the PTX/BTT-3033 combination on intracellular ROS production was assessed. DCFH-DA is a nonfluorescent dye and is deacetylated by cellular esterases to 2,7-dichlorodihydrofluorescein, which is then oxidized to fluorescent 2,7-dichlorofluorescein (DCF) *via* ROS. The fluorescence intensity of DCF is used to quantify the level of ROS. PTX (≥ 0.01 μM) exhibited a marked increase in ROS levels in a concentration-dependent manner (Fig. 3A and B). However, the relative fluorescence intensity increased after pretreatment with BTT-3033 (1 μM), followed by PTX (≥ 0.001 μM) compared to treatment with PTX alone (Fig. 3C and D). These results indicated that BTT-3033 strengthened the susceptibility of OVCAR3 and SKOV3 cells to PTX action which was likely mediated by ROS production.

PTX/BTT-3033 combination induces MMP loss in OVCAR3 and SKOV3 cells

Since high levels of ROS can damage mitochondrial membranes (16), we next estimated the loss of MMP in treated cells using fluorescent cationic JC-1 dye. In healthy cells with a normal MMP, the cationic JC-1 is accumulated within the mitochondrial matrix. In contrast, in the apoptotic cell, where the MMP is compromised, the cationic JC-1 does not accumulate in the mitochondria and this cell shows a lower fluorescence intensity. As shown in Fig. 4A and B, PTX alone (≥ 0.01 μM) induced MMP loss in OVCAR3 and SKOV3 cells. Additionally, treatment with BTT-3033 (1 μM), followed by PTX (≥ 0.001) caused a further increase in MMP loss (Fig. 4C and D).

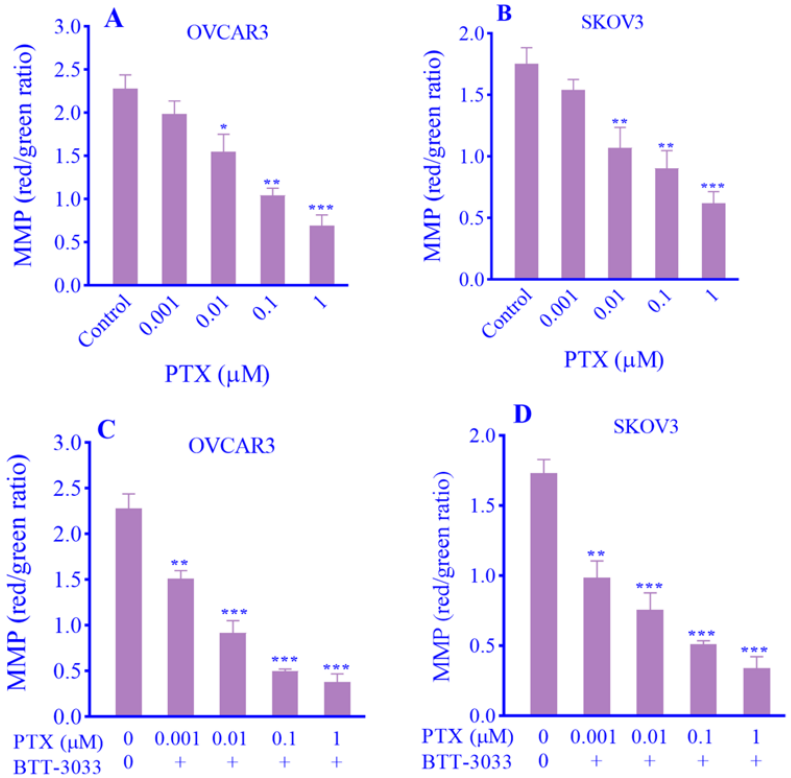

Fig. 4. Effect of PTX/BTT-3033 combination on MMP loss in OVCAR3 and SKOV3 cells. Compared to (A and B) PTX alone, (C and D) pretreatment with BTT-3033 followed by PTX resulted in a significantly higher MMP loss in OVCAR3 and SKOV3 cells. Data are presented as the means ± SD from three independent experiments. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001 indicate significant differences compared to the control group. PTX, Paclitaxel; MMP, mitochondrial membrane potential.

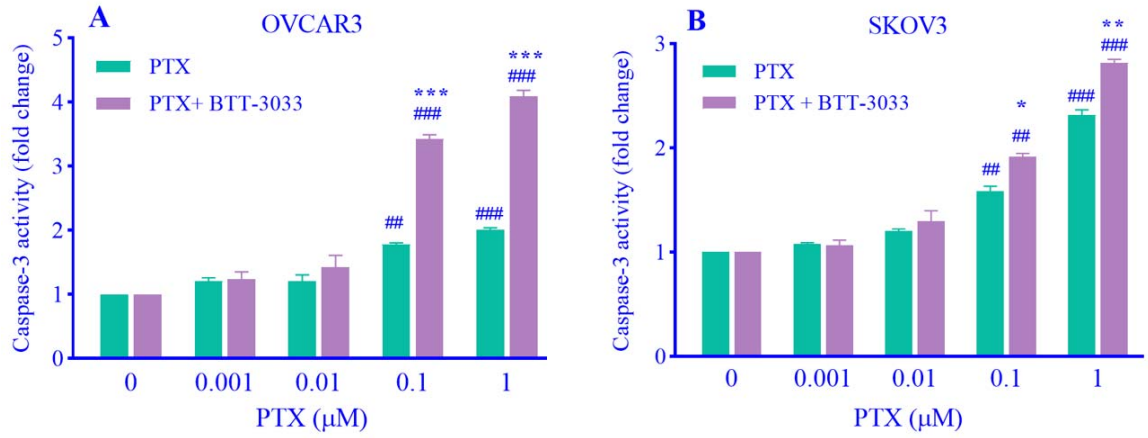

Fig. 5. Effect of PTX/BTT-3033 combination on caspase-3 activity in OVCAR3 and SKOV3 cells. Compared to (A and B) PTX alone, pretreatment with 1 μM BTT-3033 followed by 0.1 or 1 μM PTX induced more caspase-3 activity. Data are presented as the means ± SD from three independent experiments. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ indicate significant differences compared to the respective control group (PTX zero concentration); $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus respective PTX alone. PTX, Paclitaxel.

The effect of PTX/BTT-3033 combination on caspase-3 activity in OVCAR3 and SKOV3 cells

To evaluate the contribution of apoptotic mediators in the BTT-3033 enhancement of sensitivity to PTX, we assessed the caspase-3 activity. In OVCAR3 and SKOV3 cells after 48 h of incubation, PTX (≥ 0.1 μM) significantly increased caspase 3 activity in comparison to the control cells (Fig. 5A and B). It is worth mentioning that the combination of BTT-3033/PTX (0.1 and 1 μM) treatment significantly enhanced caspase-3 activity compared to PTX alone. OVCAR3 cells were more sensitive to BTT-3033/PTX treatment than SKOV3 cells, suggesting the involvement of caspase signaling in apoptosis induced by combination therapy.

DISCUSSION

Recently, integrins have been shown to play an important role in ovarian cancer chemoresistance (17-20). Clinical and basic studies revealed that α5 and β1 integrin subunits are upregulated in ovarian tumors and overexpression of α5 integrin is associated with ovarian cancer chemoresistance (17,18). Researchers also reported that α4β1 integrin inhibition makes peritoneal ovarian tumors more susceptible to carboplatin (19). Another study demonstrated that inhibition of β1 integrin increased the inhibitory effects of bevacizumab on migration, apoptosis, and adhesion of ovarian cancer cells (20). Meanwhile, the aberrant expression of integrin α2β1 in ovarian cancer was confirmed to be significantly correlated with poor clinical outcomes and chemoresistance (8,21,22). Furthermore, by using ovarian cancer cell lines and a series of ovarian tumor biopsies from microtubule-directed chemotherapeutic drugs resistant patients, it was found that α2β1 integrin contributes to PTX chemotherapy resistance and targeting α2β1 integrin *via* E7820 can reverse PTX resistance (8). We therefore hypothesized that the use of α2β1 integrin inhibitor, BTT-3033, in combination with PTX can improve the anti-tumor effects of PTX on ovarian cancer cells.

BTT-3033 is a highly selective inhibitor of integrin α2β1, and our recent study has demonstrated that BTT-3033 effectively inhibited the proliferation and epithelial-mesenchymal transition of prostate cancer cells and promoted apoptosis in these cells by suppressing α2β1 integrin/mitogen-activated protein kinase 7 signaling (12,13). Although the anticancer activities of BTT-3033 are now well documented, little is known about its role in suppressing the growth of ovarian cancer cells both as a single agent and in combination with

chemotherapeutic drugs such as PTX. The present study provides significant evidence of the synergistic effect of BTT-3033 and PTX drugs. The results of the MTT assay showed that the PTX/BTT-3033 combination had a greater inhibiting capacity on cell viability (lowest IC_{50}) compared to PTX alone. Moreover, the CDIs for various PTX/BTT-3033 combinations were less than 1, indicating a significant synergistic interaction between PTX and BTT-3033. It has been revealed that α2β1 integrin signaling is a key survival pathway in drug-induced apoptosis of tumor cells and that activation of this signaling may contribute to the induction of drug resistance (2,3,5,23). The role of integrin α2β1 in drug resistance could be explained by the role of this receptor in regulating multiple pro-survival pathways, including the PI3K/AKT, MAPK/extracellular-signal-regulated kinase (ERK), and Bcl-2 proteins (3,5,9,23). Studies have reported that changes in the function of this signaling affect the sensitivity of tumor cells to chemotherapy (3,5,9). Research on breast cancer demonstrated that β1 integrin mediates cisplatin resistance by promoting focal adhesion kinase/PI3K/AKT signaling in MCF7 breast cancer cells, and MAPK pathway in triple-negative breast cancer cells (24). Moreover, cancer-associated fibroblasts promote tamoxifen resistance by β1 integrin/EGFR/ERK signaling (25). In line with the mentioned findings, the results of the present study suggest that blocking the α2β1 integrin with the specific inhibitor BTT-3033 could enhance the cytotoxic effect of PTX on ovarian cancer cells.

A major cytotoxic effect of chemotherapy agents is the induction of apoptosis (26,27). Chemotherapy-induced apoptosis involves the activation of the mitochondrial death pathway, which is controlled by the balance between pro- and anti-apoptotic Bcl-2 family proteins (26,27). The over-activation of pro-apoptotic proteins leads to increased mitochondrial membrane permeability and subsequent cytochrome c release, which consequently activates the caspases cascade (16,28-30). Stabilization of microtubules by PTX impairs mitosis and exerts an anti-tumor effect (10). In addition to its effects on microtubules, PTX was found to induce apoptosis through BH3-only protein Bim (Bcl-2 interacting mediator of cell death) in breast cancer (31). Resistance to apoptosis has been reported to be a key factor contributing to tumor chemoresistance (32). Meanwhile, activation of integrins has been known to trigger resistance to apoptosis, possibly by modulating the expression of Bcl-2-associated death protein, Bcl-2, Bcl-xL, survivin, Mcl-1, p27Kip, and p21 (2). In the present study, ROS generation, MMP loss, and caspase 3 activity were evaluated as possible mechanisms of action for the PTX/BTT-3033 combination. The results showed that PTX/BTT-3033 combination induced more apoptotic OVCAR3 and SKOV3 cells compared to PTX alone. ROS are critical regulators of cell apoptosis. PTX has been reported to induce ROS production by increasing the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (33). ROS has also been reported as one of the mechanisms of BTT-3033-induced apoptosis (13). Consistent with these studies, our results revealed that BTT-3033 increased PTX-induced ROS production in OVCAR3 and SKOV3 cells, indicating the potential for PTX/BTT-3033 combination to increase ROS generation in ovarian cancer cells. Moreover, we found that treatment with BTT-3033 followed by PTX caused a further increase in MMP loss compared with PTX alone. Furthermore, the combination of BTT-3033 and PTX treatment successfully increased caspase-3 activity, especially in OVCAR3 cells. Engagement of α2β1 integrin with its ligand collagen I has been shown to suppress doxorubicin-induced mitochondrial depolarization, cytochrome c release, and activation of caspase-9 and -3 (34). Besides, Aoudjit *et al*. reported that β1 integrin in breast cancer cells can inhibit apoptosis induced by PTX and vincristine by preventing the release of cytochrome c from the mitochondria (3). These findings suggest that integrin α2β1 provides tumor cells with a survival advantage against drug-induced apoptosis. These results corroborate our recent finding that BTT-3033, as α2β1 integrin specific inhibitor, increased PTX-induced cell death in ovarian cancer cells.

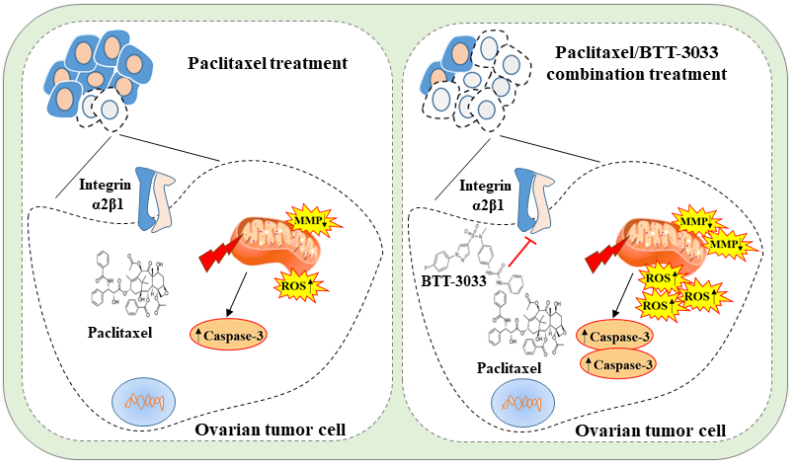

Fig. 6. Schematic representation of BTT-3033-induced sensitivity to paclitaxel in ovarian cancer cells. BTT-3033 as α2β1 integrin-specific inhibitor sensitizes ovarian cancer cells to paclitaxel by inducing MMP loss, ROS production, and mitochondrial death. MMP, mitochondrial membrane potential; ROS, reactive oxygen species.

CONCLUSION

The results of the present study demonstrate the synergistic effect of PTX and BTT-3033 on OVCAR3 and SKOV3 tumor cells, and this effect might be attributed to an increase in the level of apoptosis. Besides, our results suggest that BTT-3033 might enhance PTX cytotoxicity by causing MMP loss, ROS production, and caspase 3 activation (Fig. 6). Therefore, the PTX/BTT-3033 combination may represent a viable and promising alternative therapeutic strategy for ovarian cancer treatment. Further studies are required to clarify the detailed mechanisms involved.

Acknowledgments

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Conflict of interest statement

All authors declared no conflicts of interest in this study.

Authors' contributions

M. Aghaei and Z. Babaei conceived and designed the study, and prepared the manuscript with input from all authors. M. Amani, S.S. Ghorbanhosseini, and M. Aghaei performed the experiments. Z. Babaei analyzed the data. M. Aghaei and M. Minaiyan critically revised the manuscript. Z. Babaei and M. Amani contributed equally to this work. The finalized article was read and approved by all authors.

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