Original Article
Sequential treatment with betulinic acid followed by 5-fluorouracil shows synergistic cytotoxic activity in ovarian cancer cells

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Abstract: Betulinic acid selectively inhibits the growth of ovarian carcinoma cell lines without affecting the normal cells. In the present study, the effect of 5-fluorouracil (5-FU) and betulinic acid (BA) combination on ovarian carcinoma cells was studied. The results demonstrated that ovarian carcinoma cells on concurrent or 5-FU followed by BA treatment show increased Sub-G1 cell population, increased rate of cell apoptosis and morphological changes in mitochondrial membrane. In OVCAR 432 cells treatment with sequential combination of 5-FU and BA increased the Sub-G1 cell population to 51.3% and growth inhibition rate of > 72%. However, exposure to BA before 5-FU treatment caused a decrease in rate of inhibition to < 35%. Treatment with combination of 5 µM of 5-FU and 1 µM of BA for 48 h, led to an induction of apoptosis in 79.7% and induced morphological changes in OVCAR 432 cells. The Western blot results showed high concentration of cytochrome c in the cell cytosol after 24 h of 5-FU and BA combination treatment. Treatment of BA-responsive RMS-13 cells with 5-FU and BA combination resulted in inhibition of GLI1, GLI2, PTCH1, and IGF2 genes. In addition, we found a significant reduction in hedgehog activity of RMS-13 cells after 5-FU and BA combination treatment by means of a hedgehog-responsive reporter assay. Therefore, 5-FU and BA combination can be a promising regimen for the treatment of ovarian carcinoma.

Keywords: Ovarian carcinoma, inhibition, morphological changes, hedgehog, apoptosis

Introduction

Ovarian carcinoma because of late stage detection is the most lethal gynecological malignancy. It is usually diagnosed after tumor cells are widely metastasized within the peritoneal cavity. In spite of advancement in modern cancer treatments like surgery and combination therapy with paclitaxel and carboplatin the death rate of patients is still high [1, 2]. All the treatments for ovarian carcinoma at present are inefficient. Radiation therapy has led to an average survival rate of 5-years in 10% [3-6] while as chemotherapy has led to a response rate from 3 to 30% [7-11]. The patients with less-than-25% and greater-than-75% cytoreduction have an average difference in survival of 11 months [12]. Therefore, the need for new treatment regimens with roles in ovarian carcinoma treatment is highly desired.

The 5-fluorouracil (5-FU) and oral fluoropyrimidines have been shown to be effective in many cancers including gastrointestinal tract cancers [13]. New generations of oral fluoropyrimidine such as S-1, which inhibit the degradation of 5-FU and capecitabine have been developed and put into use in the treatment of several solid tumors [14]. 5-FU and 4-HPR showed positive responses in combination with platinum based drugs or as a single agent in phase II trials respectively in ovary carcinoma [15-17].

Betulinic acid (Figure 1), has been reported to selectively inhibit the growth of lung, colon, prostate, and ovary cancer cells [18] without affecting the normal cell lines [19]. It possesses anti-tumor activity by inducing apoptosis in a variety of human cancers [18]. BA treatment in childhood tumors like Ewing sarcoma [20], neuroblastoma [21], and leukaemia [22] has been
Betulinic acid followed by 5-fluorouracil and ovarian cancer cells

significantly effective. BA treatment induces distinct morphological changes in sensitive cells [18] and has been found to be effective on both drug-resistant and drug-sensitive tumour cell lines [23].

Materials and methods

Chemicals

Betulinic acid and 5-fluorouracil were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-glutamine from Invitrogen (Carlsbad, CA, USA), 1% penicillin and streptomycin from Gibco, (Grand Island, NY, USA). Enhanced chemiluminescence (ECL), nitrocellulose membrane and ECL prime western blotting detection reagents were obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). Hoechst 33342, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1-methyl-4-phenylpyridinium (MPP+) dimethyl sulfoxide (DMSO) and b-actin were purchased from Sigma (St Louis, MO, USA).

Cell culture

The human ovarian cancer lines OVCAR 432, RMS-13 cell lines and NIH-3T3 fibroblasts were obtained from American Type of Collection centre (Manassas, VA, USA). The cells were maintained in minimum essential medium (MEM), containing 10% fetal bovine serum and antibiotics. The cell lines were maintained in a humidified incubator at 37°C and 5% CO₂.

Cytotoxicity assay

In 96-well culture plates 5 × 10⁴ cells were plated per well in MEM. To each well 200 µl containing different concentrations of BA, 5-FU and combination of 5-FU + BA in DMSO were added and the plates were incubated for 24 h at 37°C. After 24 h the medium was changed to drug free medium and incubation was continued for 5 days more. Alamar blue vital dye indicator assay [24] was used to measure the cell viability. Inhibition in cell growth is denoted as cytotoxicity and represented as concentration-response curve (surviving percentage versus drug concentration). The IC₅₀ is determined from a least squares regression fit to the linear portion of the curve.

Flow cytometry

The cells distributed at a density of 8 × 10⁵ onto 100 mm dishes were incubated at 37°C for 24 h. After 24 h, the cells were treated with 5-FU, BA or 5-FU + BA combination at IC₅₀ concentration. Nuclear staining for DNA content was performed after harvesting the cells at 12, 24 and 48 h from treatment. The untreated cells were also harvested at the same time points and used as control. The cells were treated with two solutions, a salt solution containing SDS and RNase A, second solution containing sucrose and citric acid. Additionally both solutions contained ethidium bromide. Becton Dickinson FACS can (cell Quest software) was used to acquire cell cycle data and Cell Cycle Muticycle (Phoenix Flow Systems, San Diego, CA) was used for analysis.

Assessment of apoptosis

The cells treated with BA, 5-FU and 5-FU + BA combination after harvesting were washed with PBS and fixed in formaldehyde at room temperature for 20 min. Then fixative was removed and cells were suspended in 50 µl of 8 µg/ml bisbenzimide trichloride dye (Hoechst-33258). On to the glass slides, 30 µl cell aliquots were fixed. Five hundred cells were counted and scored for the incidence of apoptosis using fluorescence microscope. All the measurements were performed in triplicates.

Western blot assay

In six well plates 1 × 10⁶ cells were plated per plate for 12 h and then treated with 5-FU (20 µM), BA (10 µM) or 5-FU (5 µM) + BA (1 µM) combination in the presence or absence of 50 µM zVAD. fmk. Vehicle treated cells were used.
Betulinic acid followed by 5-fluorouracil and ovarian cancer cells

Figure 2. Cytotoxic activity of BA and combination of 5-FU + BA against OVCAR 432 cells. A. Combination of 5-FU + BA increased the population of OVCAR 432 cells in Sub G1 phase compared to that of BA. B. Synergistic effect of BA on 5-FU depends on the schedule of exposure. Exposure of OVCAR 432 cells to 5-FU followed by BA or concurrently leads to synergistic effect. When BA is added before 5-FU treatment there is decrease in rate of inhibition.

Figure 3. Rate of apoptosis in OVCAR 432 cells using quantitative fluorescence microscopy. The combination of 5-FU + BA enhanced the rate of apoptosis in OVCAR 432 cells compared to that of BA used alone. All the results are mean of triplicate measurements.

5 × 10⁵ RMS-13 cells or NIH-3T3 fibroblasts were transfected with 900 ng of the reporter plasmid p11xGli or the empty control pGL3-TK (25) and 100 ng of the reference plasmid pRL-TK using FuGene 6 transfection reagent (Roche Diagnostics). 1 μg of an expression plasmid containing murine sonic hedgehog gene (Karolinska Institute, Stockholm, Sweden) was transfected into fibroblasts. After 24 h transfection, cells were treated with 5 μg/ml BA, 10 μg/ml 5-FU and BA + 5-FU combination, 10 μM cyclopamine (Toronto Research Chemicals, Toronto, Canada), or vehicle and cultured for 24 h. A Dual-Glo Luciferase Reporter Assay System (Promega, Madison, Wisconsin, USA) was used for determination of reporter gene activity. Firefly luciferase activity was normalised to Renilla luciferase activity. After 24 h of transfection total RNA was isolated using Trizol (Invitrogen) from fibroblasts.
Statistical analyses

The vehicle treated control cells were arbitrarily assigned 100% and other data were expressed in comparison to control. Data were analyzed by Dunnett’s multiple comparison test (Sigma Stat, Jandel, San Rafael, CA, USA). For all comparisons, differences were considered statistically significant at $P < 0.05$.

Results

Cytotoxicity of BA, 5-FU and their combination against ovarian carcinoma cells

The synergistic effect of sequential combination of 5-FU followed by BA on cell apoptosis in OVCAR 432 cells was determined by cytometric analysis of Sub-G1 cell population (Figure 2A). Treatment of OVCAR 432 cells with 5-FU and BA increased the cell population in sub-G1 to 23.4 and 27.9% respectively whereas in control only 16.2% cell population was in Sub-G1 phase. However, treatment of OVCAR 432 cells with sequential combination of 5-FU and BA increased the Sub-G1 cell population to 51.3%, which exceeded the additive effect due to BA and 5-FU when used separately. The concentration of BA that is effective in enhancing 5-FU cytotoxicity is significantly lower than $IC_{50}$ for this reagent alone. These results suggest that BA and 5-FU exhibit synergistic effect on induction of apoptosis in OVCAR 432 cells.

We observed that enhancement of 5-FU cytotoxicity by BA depends on exposure schedule (Figure 2B). Exposure to an $IC_{50}$ concentration of 5-FU for 24 h followed by BA at $IC_{50}$ for 24 h or both agents concurrently for 48 h resulted in growth inhibition rate of $> 72\%$ in OVCAR 432 cells. When BA is added before 5-FU treatment there is decrease in rate of inhibition to $< 35\%$ and when each agent is used separately, they produce $< 24\%$ inhibition of cell growth. Therefore, the mechanism of action of BA appears to be dependent on 5-FU.
Betulinic acid followed by 5-fluorouracil and ovarian cancer cells

Figure 5. 5-FU + BA combination blocks hedgehog signalling A. GLI1 expression of rhabdomyosarcoma cell lines. The RMS cell lines were lysed and the expression of GLI1 and β-actin was detected by western blot analysis. B. Hedgehog signalling dependency. The RMS cells were treated for 48 h with BA and combination of 5-FU + BA, GLI1, GLI2, PTCH1, and IGF2 mRNA expression from untreated and treated RMS cells was measured by quantitative real-time PCR in relation to the house-keeping gene TBP as a calibrator; *P < 0.05 (unpaired Student’s t-test). C. Hedgehog pathway activity. The RMS-13 cells were transiently transfected with 900 ng hedgehog-responsive reporter plasmid (p11xGli) or control plasmid (pGL3-TK) and treated for 24 h with 5 μM 5-FU + (1 Mm) BA and 10 μM cyclopamine or vehicle. Reporter assay experiments were repeated three times and transfections performed in duplicate; *P < 0.05 (unpaired Student’s t-test).

BA treatment increases apoptosis in 5-FU treated cell lines

We used quantitative fluorescence microscopy to observe apoptotic chromatin condensation. The results of the analysis showed that the combination of 5-FU + BA induces a higher rate of apoptosis than either agent used alone (Figure 3). Treatment of OVCAR 432 cells with 10 μM concentration of 5-FU for 24 h induced apoptosis in 5% and with 5 μM concentration of BA for 24 h induced apoptosis in 47.5% of the cells. However when OVCAR 432 cells were treated with a combination of 5 μM of 5-FU and 1 μM of BA for 48 h, there was an induction of apoptosis in 79.7% cells. Higher overall percentages of apoptosis are seen at latter points of time.

Effect of 5-FU and BA combination mitochondria in OVCAR 432 cells

We also analysed the effect of 5-FU + BA combination on morphological changes in OVCAR 432 cells. Compared to BA, the combination of 5-FU + BA induced morphological changes in OVCAR 432 cells at a concentration lower than IC50 of BA when used alone (Figure 4A). The results from flow cytometry showed that combination of 5-FU + BA induced significant rate of apoptosis after 48 h. When OVCAR 432 cells were treated with combination of 5-FU + BA along with caspase inhibitor, zVAD.fmk there was inhibition in rate of apoptosis (Figure 4B, 4C). This indicated in involvement of caspasas in 5-FU + BA-induced apoptosis. However treatment of OVCAR 432 cells with zVAD.fmk inhibited this effect. Therefore, combination of 5-FU and BA induces apoptosis through caspase dependent pathway.

The results from Western blot analysis showed high concentration of cytochrome c in the cell cytosol after 48 h of 5-FU + BA combination treatment. On the other hand cytochrome c concentration in the mitochondrial fraction inversely decreased (Figure 4D). The effect was most promising using 5-FU + BA combination compared to that BA alone. Together, these results clearly indicate that 5-FU + BA-induced apoptosis in OVCAR 432 cells is mediated by the intrinsic apoptotic pathway.

5-FU + BA combination specifically targets hedgehog signalling in RMS cells

Treatment of RMS-13 cells with BA and 5-FU + BA combination resulted in inhibition of GLI1, GLI2, PTCH1, and IGF2 genes. However inhibition caused by 5-FU + BA combination was much greater compared to BA alone (Figure 5B-E). We did not observe any significant effect in two GLI1-negative RMS cell lines RH-30 and RD (Figure 5A). Moreover, we found a significant reduction in hedgehog activity of RMS-13 cells after 5-FU + BA combination treatment by means of a hedgehog-responsive reporter assay. To examine whether this inhibition is dependent on hedgehog signalling components upstream of GLI1, RMS-13 cells were treated with 5-FU + BA combination in the presence or absence of 10 μM cyclopamine, a specific
Betulinic acid followed by 5-fluorouracil and ovarian cancer cells

However, the inhibitory effect of 5-FU + BA combination on GLI1 activity was found to be independent of cyclopamine treatment (Figure 3C), indicating that BA is able to selectively target hedgehog signalling on the level of GLI1.

In order to activate hedgehog pathway in untransformed NIH-3T3 cells, we expressed the ligand sonic hedgehog by transient transfection. This resulted in strong expression of the hedgehog target genes Gli1, Gli2, Ptc1, and Igf2 (Figure 6A, 6B). But exposure to hedgehog inhibitor, cyclopamine resulted in inhibition of expression of these hedgehog target genes. The use of 5-FU + BA combination led to inhibition of Gli1 and Gli2 expression without affecting Ptc1 and Igf2. These results

Figure 6. Hedgehog activation assay. The NIH-3T3 cells were transiently transfected with 1 μg sonic hedgehog (Shh) expression plasmid. For the inhibition of hedgehog signalling, cells were additionally exposed to 5-FU + BA combination and 10 μM cyclopamine (Cycl.) after 24 h transfection. Expression of the murine genes Shh, Gli1, Gli2, Ptc1, and Igf2 was determined after an incubation period of 48 h using quantitative real-time PCR in relation to the housekeeping gene TBP as a calibrator; *P < 0.05 (unpaired Student’s t-test).
led us to conclude that 5-FU + BA combination inhibits hedgehog signalling through GLI family transcription factors selectively.

Discussion

This study was carried out in order to investigate the potential of 5-FU in combination with BA in the treatment of ovary carcinoma. A synergistic effect was observed only when ovary carcinoma cells were treated with 5-FU followed by BA or concurrently in a sequence-dependent manner. Angiogenesis has been reported as a prerequisite for tumour growth beyond certain size as well as for metastatic spread and, therefore, is an attractive target for clinical tumor biologists [27]. There are reports that anti-angiogenic treatments synergise with traditional chemotherapeutic and radiotherapeutic regimens. BA is not only a cytotoxic effect against endothelial cells, but also inhibits tube-like structure formation of aortic endothelial cells [28, 29]. In the present study treatment of OVCAR 432 cells with sequential combination of 5-FU and BA increased the Sub-G1 cell population to 51.3%, which exceeded the additive effect due to BA and 5-FU when used separately. The concentration of BA that is effective in enhancing 5-FU cytotoxicity is significantly lower than IC$_{50}$ for this reagent alone. Obvious synergistic growth inhibition was observed only when OVCAR 432 cells were treated with 5-FU followed by BA. Based on the flow cytometric analysis, this synergism was attributable to the enhancement of apoptotic cell death. Treatment of OVCAR 432 cells with a combination of 10 µM of 5-FU and 5 µM of BA for 24 h, induced apoptosis in 49.7% cells.

The purpose of the present study was to provide experimental evidence in order to help develop chemotherapeutic regimens containing 5-FU and BA for ovary carcinoma. Compared to BA, the combination of 5-FU and BA induced morphological changes in OVCAR 432 cells at a concentration lower than IC$_{50}$ of BA when used alone. The results from flow cytometry showed that compared to BA-induced apoptosis which was significant after 48 h, the induction of apoptosis by combination of 5-FU and BA was significant after 24 h. When the OVCAR 432 cells were treated with combination of 5-FU and BA along with caspase inhibitor, zVAD.fmk there was inhibition in rate of apoptosis. This led to the conclusion that caspases were involved in 5-FU and BA-induced apoptosis.

The Western blot results showed high concentration of cytochrome c in the cell cytosol after 24 h of 5-FU and BA combination treatment. On the other hand cytochrome c concentration in the mitochondrial fraction inversely decreased. The effect was enhanced by 5-FU and BA combination compared to that BA alone.

Treatment of RMS-13 cells with BA and 5-FU + BA combination resulted in inhibition of GLI1, GLI2, PTCH1, and IGF2 genes. However inhibition caused by BA + 5-FU combination was much greater compared to BA alone. Moreover, we found a significant reduction in hedgehog activity of RMS-13 cells after BA + 5-FU combination treatment by means of a hedgehog-responsive reporter assay. To examine whether this inhibition is dependent on hedgehog signalling components upstream of GLI1, RMS-13 cells were treated with BA + 5-FU combination in the presence or absence of 7.5 µM cycloamine, a specific hedgehog signalling inhibitor [26]. However, the inhibitory effect of BA + 5-FU combination on GLI1 activity was found to be independent of cycloamine treatment, indicating that BA + 5-FU combination is able to selectively target hedgehog signalling on the level of GLI1.

In conclusion, BA + 5-FU combination has an important role in ovarian carcinoma treatment by inducing apoptosis through mitochondrial pathway and specifically targeting the hedgehog signalling pathway.

Disclosure of conflict of interest

None.

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Betulinic acid followed by 5-fluorouracil and ovarian cancer cells


