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The effects of bortezomib alone or in combination with 5-fluorouracil on proliferation and apoptosis of choriocarcinoma cells

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Summary

Purpose: To investigate the effects of bortezomib alone and in combination with 5-fluorouracil (5-FU) on proliferation and apoptosis in the human choriocarcinoma cell line JEG-3. *Materials and Methods:* Cells were treated with bortezomib, 5-FU or with a combination. Proliferation and apoptosis were measured. NF-κB protein expression was examined using immunofluorescence. *Results:* Following treatment with ten nM bortezomib, rates of apoptosis were significantly higher than controls ($p < 0.05$) and NF-κB expression increased. 5-FU at 0.025 μg/ml or 0.25 μg/ml resulted in 60.1 ± 0.4% and 67.0 ± 0.2% growth inhibition, respectively, an increase compared to individual treatment ($p < 0.05$). Apoptosis in cells treated with bortezomib +5-FU was significantly higher than either treatment alone ($p < 0.05$). Inhibition of proliferation by the combination treatment was synergistic. *Conclusion:* Bortezomib alone or in combination with 5-FU inhibited JEG-3 cell proliferation and induced apoptosis by increasing NF-κB expression. Combination treatment exerted synergistic effects on growth inhibition.

Key words: Bortezomib; 5-fluorouracil; Apoptosis; Choriocarcinoma.

Introduction

Choriocarcinoma is the most malignant form of gestational trophoblastic neoplasm (GTN) [1]. Chemotherapy is the preferred treatment for choriocarcinoma, which can be cured using combination therapy [2]. 5-Fluorouracil (5-FU) and methotrexate are commonly used in clinical treatment of choriocarcinoma; however, drug resistance and relapse are reported in some patients [3]. An increased dose of conventional chemotherapy drugs can improve efficacy; however, serious physical and psychological side-effects are associated with this treatment [4]. It is, therefore, essential to identify novel chemotherapeutic agents for optimized treatment protocols that minimize side-effects and increase sensitivity to chemotherapy. Bortezomib is a novel anti-tumor, targeted therapeutic drug [5]. It has been shown that bortezomib has promising antitumor activity and increases sensitivity to chemotherapy in a number of preclinical cancer cell-line models *in vivo* and *in vitro*. Bortezomib has been successfully applied in treatment of refractory recurrent multiple myeloma [6]. The current study demonstrates that bortezomib, either alone or in combination with 5-FU, can inhibit the proliferation of choriocarcinoma JEG-3 cells and induce apoptosis. Furthermore, bortezomib and 5-FU exert synergistic effects on the growth inhibition of JEG-3 cell lines.

Materials and Methods

Cell culture

The human choriocarcinoma cell line JEG-3 was cultured in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotic mixture (100 U/ml 100 μg/ml penicillin and streptomycin) at 37ºC.

Cell proliferation and apoptosis assays.

JEG-3 cells were subjected to one of three treatments consisting of either bortezomib alone (one, ten, or 50 nM), 5-FU alone (0.025 μg/ml or 0.25 μg/ml), bortezomib + 5-FU (ten nM bortezomib + 0.025 μg/ml, or 0.25 μg/ml 5-FU) or left untreated. The CCK-8 assay was used to measure inhibition of cell proliferation. Briefly, cells were seeded in 96-well plates (3 x 10^3 cells/well), incubated for 24 hours followed by treatment with inhibitors at various concentrations. Cells were then incubated for 24, 48 or 72 hours at 37ºC. Ten microliters of the CCK-8 assay reagent were added to each well, followed by an incubation for four hours at 37ºC. Plates were shaken and the absorbance was measured at 450 nm in a plate reader. Inhibition was calculated as: 1-(A1-AC)/(A2-AC)×100%. (A1 is the OD value of the treatment group, A2 is the OD value of the control group)

Flow cytometry analysis

Annexin V/propidium iodide (PI) staining and flow cytometry (FCM) were utilized to analyze apoptosis rates. Cells were washed twice with cold BioLegend cell staining buffer and resuspended in Annexin V Binding Buffer at a concentration of 1×10^6 cells/ml. One hundred microliters of the cell suspension were transferred to a five-ml test tube and five μl of Annexin V-FITC and ten μl of PI

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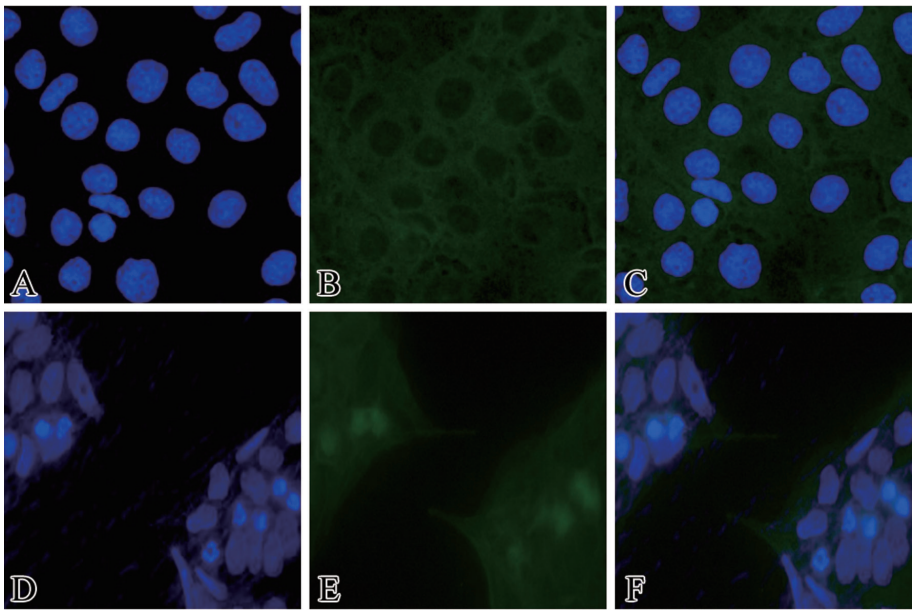

Figure 1. — Expression of NF-κB in bortezomib-treated JEG-3 cells. Nucleus staining (A), NF-κB staining (B), and merged image (C) of control cells at ×400 magnification. Nucleus staining (D), NF-κB staining (E) and merged image (F) of cells treated with ten nM bortezomib at ×400 magnification.

solution were added. Cells were gently vortexed and incubated for 15 minutes at 25°C in the dark. Four hundred microliters of Annexin V Binding Buffer were added to each tube and samples analyzed by FCM. Annexin V+ and PI- cells were identified as early apoptotic samples.

Immunofluorescence

Immunofluorescence was used to detect NFκ-B expression. Grow cells in defined media to a concentration of 1-5*10^7. Added were 0.6 ml of 4% paraformaldehyde solution directly to the cells and incubated with gentle shaking for ten minutes at the same temperature as growth. Slides were washed once with PBS. Supernatant was removed and 1° antibody (diluted in PBS) was added, and then incubate in a moist chamber for at least 24 hours. Excess solution was aspirated and washed three times with PBS. Twenty µl of 2° antibody were added (diluted in BSA/PBS) and place in dark/moist chamber for one hour. Excess was aspirated and washed three times in PBS. Hoechst 33258 was added to wells and allowed to incubate for a few minutes. Excess was aspirated and washed once with PBS, and allowed slides to air dry. Wells were covered with anti-fade reagent and cover slip- seal ed with clear nail polish.

Statistical analysis

Unless otherwise specified, all data are presented as mean ± SD (standard deviation). Comparison of means was accomplished using single factor analysis of variance and the least significant difference (LSD) test. Synergy was calculated using the formula: CDI = AB/A * B, where AB is the OD value of the combination treatment group, A is the OD of the single treatment and B is the OD of the control group. CDI < 0.85 indicates synergistic effects with the two drugs [5, 6] (CDI: combination drug index).

Results

Effects of bortezomib on proliferation and apoptosis of choriocarcinoma cells

JEG-3 cells were treated with ten nM bortezomib for 24, 48, or 72 hours. The resulting percentage of growth inhibition was 22.50 ± 0.46%, 41.45 ± 0.61%, and 58.43 ± 0.65% after 24, 48, and 72 hours, respectively. Differences compared to controls were statistically significant ($p < 0.01$). Treatment with one, ten or 50 nM bortezomib for 48 hours resulted in growth inhibition of 17.21 ± 0.51%, 41.45 ± 0.61%, and 58.40 ± 0.46%, respectively, compared with the control group. Differences were statistically significant ($p < 0.05$). Effects were time and concentration dependent. Apoptosis was measured following treatment with ten nM bortezomib for 24, 48, or 72 hours. The percentage of cells undergoing apoptosis was 4.71 ± 0.22%, 16.61 ± 0.81%, and 26.98 ± 0.31% for the 24-, 48-, and 72-hour groups, respectively. The number of apoptotic cells in treatment groups was significantly higher than the control group ($p < 0.05$). The expression of NF-κB in apoptotic cells treated with bortezomib was enhanced, as determined by immunofluorescence (Figure 1).

Effects of bortezomib in combination with 5-FU in JEG-3 cells

Inhibition of JEG-3 proliferation at 24, 48, and 72 hours following treatment with ten nM bortezomib + 0.25 µg/ml 5-FU was 34.76 ± 0.33%, 67.00 ± 0.29%, and 71.56 ± 0.25%, respectively. Inhibition with bortezomib + 0.025 µg/ml 5-FU was 27.85 ± 0.33%, 60.16 ± 0.40%, and 67.00 ± 0.10% at 24, 48, and 72 hours, respectively. Compared to bortezomib treatment alone which showed levels of inhibition of 21.89 ± 0.47%, 41.46 ± 0.14%, and 58.71 ± 0.48%

Table 1. — *CDI of bortezomib in combination with 5-FU.*

Bortezomib (nM)	5-FU (μg/ml)	A450 ($\bar{x} \pm SD$)	Inhibition (%)	CDI
-	0.025	0.914 ± 0.003	12.8	
-	0.25	0.862 ± 0.002	17.6	
10	-	0.613 ± 0.001	41.4	
10	0.025	0.417 ± 0.002	60.1	0.744
10	0.25	0.345 ± 0.002	67.0	0.652

at each time point, respectively, or the 5-FU group, inhibition was increased. The difference was statistically significant ($p < 0.05$). The CDI of cytotoxicity of bortezomib in combination with 5-FU at 0.025 μg/ml or 0.25 μg/ml were 0.744 and 0.652, respectively, lower than 0.85. Results are shown in Table 1.

Apoptosis in JEG-3 cells following treatment with bortezomib alone or in combination with 5-FU

The percentage of apoptotic cells in the ten nM bortezomib + 0.25 μg/ml 5-FU treatment group was 19.44 ± 0.38%, significantly higher than the 0.25 μg/ml 5-FU group (11.39 ± 0.59%) or the ten nM bortezomib group (17.30 ± 0.28%). The differences were statistically significant ($p < 0.05$). Results are shown in Figure 2.

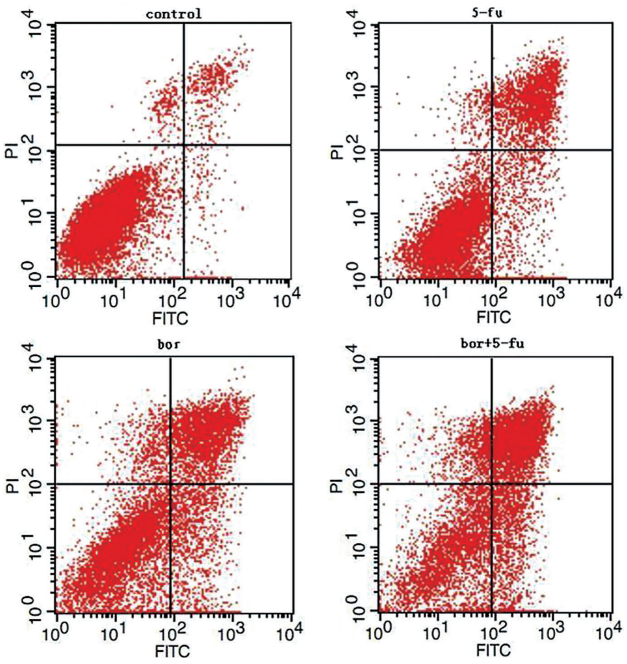

Figure 2. — Percentage of apoptotic JEG-3 cells after treatment with bortezomib alone or in combination with 5-FU as determined by FACS.

Discussion

Proteasomes are widely distributed in eukaryotic cells and participate in the degradation of most intracellular proteins, including those involved in cell cycle regulation and apoptosis [7, 8]. With an increased understanding of proteasomes in recent years, the antitumor effects of proteasomal inhibitors have become an important topic of research. Bortezomib is the first treatment in more than a decade to be approved by the Food and Drug Administration for treatment of patients with multiple myeloma. The effects of bortezomib result from the degradation of regulatory proteins, anti-proliferation effects, the promotion of apoptosis, and anti-angiogenesis effects, which inhibit tumor activity [4, 9]. The present study showed that bortezomib suppressed tumor cell proliferation and induced apoptosis, demonstrating promising prospects for clinical application [10-12].

5-FU is a common chemotherapeutic drug for the treatment of choriocarcinoma. Unfortunately, although it shows some promise in the clinic, its effective dose and toxic dose are close which often cause adverse reactions such as nausea, vomiting, diarrhea, bone marrow suppression, and in serious cases can cause heart toxicity, necrosis of liver cells, and nerve system toxicity [13]. In addition, drug resistance and relapse have been reported. Identification of new and effective drugs is, therefore, an important area of research. Recent studies have demonstrated that bortezomib treatment results in cell death in many different types of tumor cells, such as prostate cancer, breast cancer, small cell lung cancer, leukemia, and lymphoma [14, 15]. It can also be applied with chemotherapeutic drugs to increase the sensitivity of tumor cells to chemotherapy drugs [16-18]. To date, its anti-tumor effects in choriocarcinoma cells have not been examined.

The present study examined the effects of bortezomib and its combination with 5-FU on the proliferation and apoptosis of JEG-3 choriocarcinoma cells. The authors found that, compared with the control group, the inhibition of cell proliferation in bortezomib-treated cells was significantly higher ($p < 0.05$), and that the effects were time and dose-dependent. Al-Eisawi *et al.* showed that bortezomib increased the sensitivity of ovarian cancer cells to cisplatin chemotherapy drugs [16]. In the present study, the authors combined ten nM bortezomib with either 0.025 μg/ml or 0.25 μg/ml 5-FU. The level of inhibition of proliferation in cells treated with bortezomib + 5-FU was significantly higher than in cells treated with either compound alone ($p < 0.05$). The CDI of cytotoxicity of bortezomib in combination with 5-FU was lower than 0.85. These results are similar to those obtained by Weng *et al.* [19] and Jandial *et al.* [20]. The present authors demonstrated in the study of Weng *et al.* that combined treatment with cisplatin and proteasome inhibitors significantly enhances cytotoxicity against ovarian cancer cells, comparing with those agents used alone [19]. This study showed that low doses of 5-FU

combined with bortezomib could enhance the inhibitory effects on choriocarcinoma cell growth and promote apoptosis. The combination of 5-FU with bortezomib had synergistic effects in JEG-3 cells ($p < 0.05$). In addition, treatment with ten nM bortezomib alone or in combination with 0.25 μg/ml 5-FU resulted in 19.44 + 0.38% of cells undergoing apoptosis in the combination treatment group, which was greater than the number of apoptotic cells following bortezomib treatment alone (17.30 + 0.28%) or 5-FU alone (11.39 + 0.59%; $p < 0.05$). This study suggests that bortezomib combined with 5-FU exerts synergistic effects in promoting apoptosis of choriocarcinoma cells. The study by Hougardy *et al.* showed that proteasome inhibitors sensitize HPV-positive human cervical cancer cells to rhTRAIL-induced apoptosis [21].

NF-κB is a pleiotropic transcription factor, which is activated by a broad variety of stimuli such as growth factors, cytokines, ionizing radiation, ultraviolet light, and chemotherapeutic drugs. NF-κB regulates the expression of a large number of genes, which have important functions in inflammation, apoptosis, proliferation, and angiogenesis. NF-κB shows constitutive or increased activity in a wide variety of tumors and plays a crucial role in neoplastic transformation [22]. Under normal conditions, NF-κB and the inhibitory protein I kappa B predominate in the cytoplasm, with NF-κB in an inactive form. Proteasome inhibitors can inhibit protease activity, thereby inhibiting I kappa B degradation and preventing NF-κB release. This results in inhibition of the NF-κB gene transcription and promotes apoptosis. In many different types of tumor cells, proteasome inhibition causes cell death by blocking NF-κB activity [23, 24]. The present study demonstrates bortezomib-induced apoptosis through enhancement of NF-κB expression. Dolcet *et al.* also reported that in endometrial cancer cells, bortezomib increased rather than inhibited NF-κB activity [25]. These studies suggest that the mechanism of bortezomib in promoting apoptosis varies in different types of cells.

Conclusion

The present authors evaluated the effects of bortezomib and 5-FU on choriocarcinoma JEG-3 cells. The experimental results showed that bortezomib combined with 5-FU can enhance inhibitory effects and promote apoptosis in choriocarcinoma cells, and does so in a synergistic manner. Bortezomib-promoted apoptosis may be related to the activation of NF-κB; however, the detailed mechanism needs further study. In view of relapse, drug resistance and serious chemotherapy side effects in choriocarcinoma treatment, the present study provides a potential new avenue for treatment of choriocarcinoma, where proteasome inhibitors are expected to be applied to treatment.

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