Arsenic trioxide potentiates the effect of bortezomib on the proliferation and apoptosis of multiple myeloma cell line KM₃

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Abstract  Objective: The aim of our study was to investigate the effect of bortezomib in combination with arsenic trioxide (As₂O₃) on the proliferation and apoptosis in the human multiple myeloma cell line KM₃.  Methods: KM₃ cells were cultured with different concentrations of bortezomib, As₂O₃ alone or in combination for different times. Cell proliferation was analyzed by MTT assay, and the IC₅₀ was calculated. Cell morphology was observed under the light microscope (using Wright-Giemsa stain) and electric microscope. Agarose gel electrophoresis was used to evaluate DNA content. Flow cytometry was used to examine Annexin V-FITC/PI stain and mitochondrial transmembrane electric potential (ΔΨm).  Results: Bortezomib and As₂O₃ alone both inhibited KM₃ cell proliferation in a time and dose dependent manner, with the IC₅₀ were 0.27, 3.10 μmol/L, respectively; the inhibiting rate on KM₃ cells of bortezomib plus As₂O₃ was significantly higher than bortezomib alone (18.22 ± 1.04)% vs. (13.18 ± 1.29)%, P < 0.05; A series of typical morphological features of apoptosis and a typical DNA ladder were observed in KM₃ cell treated with 0.25 μmol/L bortezomib for 48 h, which showed increased Annexin V positivity and decreased ΔΨm. The apoptosis rate induced by bortezomib plus As₂O₃ was also significantly than that of induced by bortezomib alone.  Conclusion: Bortezomib could inhibit the proliferation while induce apoptosis of KM₃ cells which may be through decreased ΔΨm. Bortezomib had enhanced inhibitory effect with As₂O₃ on the growth of KM₃ cell (P < 0.05). As₂O₃ enhances the apoptosis effects of bortezomib on KM₃ cell.

Key words  bortezomib; arsenic trioxide; apoptosis; multiple myeloma

Mutiple myeloma (MM) is a B-cell derived neoplasm. Although traditional combination chemotherapy and high dose chemotherapy with autologous stem cell support have certain efficiency, few patient remain in long term remission. Proteasome inhibitor bortezomib is a new targeted anti-tumor drug and has been successfully applied to treat MM, especially when combined with liposomal Doxorubicin and dexamethasone [1]. Arsenic trioxide (As₂O₃) has some affections on relapsed or refractory MM. This study was aimed to investigate the effects of bortezomib alone or in combination with As₂O₃ on the proliferation and apoptosis of MM cell line KM₃.

Materials and methods

Cell line

Mutiple myeloma cell line KM₃ was kindly provided by Professor Jian Hou (Affiliated Changzheng Hospital of the 2nd Military Medical College, Shanghai). 2 × 10⁶ cells/mL was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/mL) and streptomycin (100 μg/mL). Cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂. Then the cells were separated to several parts every 2–3 days and the cells in exponential growth phase were used in the experiments. Trypan blue staining was used to test cells' vitality. The stain resist rate was above 98%.

Reagents

Bortezomib was provided by Xian-Janssen Pharmaceutical Ltd. (China). As₂O₃ (5055 mol/L) was purchase

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from Harbin Yida Pharmaceutical Ltd. Company (China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) was purchased from American Chemical Company (USA). Apoptosis DNA ladder was obtained from R&D systems (USA). Annexin V-FITC related cell apoptosis kits were purchased from BD (USA). Kits for examining Δψm were purchased from Stratagene (USA). RPMI1640, FBS was purchased from Gibco Company (USA).

Drug concentration

According to the data of our preliminary experiments and literatures [2], the concentrations used in this experiment was 1.0 to 15 μmol/L for As2O3 and 0.1 to 5.0 μmol/L for bortezomib. Prior to MTT assay, the drug was added into the culture media for 24, 48 or 72 h.

MTT assay

The cells in exponential growth phase were cultured in RPMI-1640 supplemented with 10% FBS after washed by fresh culture solution. 1 × 10^3–3 × 10^6 cells/mL with a volume of 100 μL were seeded in a 96-well plate. The drug was added to each well respectively. Each concentration repeated three times. Wells in blank control group only was added to each well. After precipitation dissolved, absorbance (A570) at 570 nm was removed and 100 µL DMSO was added to each well. The plate was incubated at 37℃ MTT reagent (5 g/L, 10 µL) was added into each well and 100 µL. The plate was incubated for 24, 48 or 72 h. Then included cell culture fluid RPMI 1640 with a volume of 200 µL were added and mixed gently. After a reaction for 15 min away from light at room temperature, 300 μL binding buffer was added and the samples were examined within 1 h. Early apoptotic cells were defined as Annexin V–PI– positive cells. Both the cell populations were merged for calculation of cell apoptotic percentage. KM3 cells were incubated with bortezomib (0.25 μmol/L) + As2O3 for 24 h, then collected and centrifuged in cold PBS (4℃) (300 × g, 5 min). The cell pellets were washed twice and resuspended in 200 µL binding buffer. Annexin V-FITC (10 μL) and PI (5 μL) were added and mixed gently. After a reaction for 15 min away from light at room temperature, 300 μL binding buffer was added and the samples were examined within 1 h. Early apoptotic cells were defined as Annexin V–PI– positive cells. Both the cell populations were merged for calculation of cell apoptotic percentage. KM3 cells were incubated with bortezomib (0.25 μmol/L) for 0 h, 24 h, 48 h and 72 h. Then Annexin V was examined according to the methods described above.

Calculation of IC50

The half cell growth inhibitory concentration (IC50) was the concentration when growth of 50% KM3 was inhibited. IC50 was calculated by a method of Probit regression analysis.

Observation of cell morphology

Light microscope

The KM3 cells were fixed and dried in air, stained with Wright-Gimsa staining and then observed, and photographed using light microscope (10 × 100).

Electron microscope

The ultrastructure of apoptotic cells were observed by transmission electron microscope. KM3 cells were incubated with bortezomib (0.125, 0.25, 0.5 μmol/L) for 0 h or 24 h. Approximately 1 × 10^6 cells were collected and washed by cold PBS (4℃) and centrifuged (300 × g, 5 min). The cells were washed twice and fixed in 2.5% glutaraldehyde. Slides were observed and photographed under the electron microscope (performed in the Department of Electron Microscope of Zhejiang University School of Medicine, China).

DNA assay

Cells (1 × 10^6) were collected and washed twice with PBS. DNA was extracted from cells lysed according to protocols along with the Annexin V kit for cell apoptosis assay. Concentration of As2O3 and bortezomib used in this experiment was 2.0 μmol/L and 0.125, 0.25, 0.5 μmol/L respectively. KM3 cell suspension were treated with (1) Control; (2) Bortezomib (0.125, 0.25, 0.5 μmol/L, incubated for 0, 24, 48, 72 h; (3) Bortezomib (0.25 μmol/L) + As2O3 for 24 h, then collected and centrifuged in cold PBS (4℃) (300 × g, 5 min). The cell pellets were washed twice and resuspended in 200 μL binding buffer. Annexin V-FITC (10 μL) and PI (5 μL) were added and mixed gently. After a reaction for 15 min away from light at room temperature, 300 μL binding buffer was added and the samples were examined within 1 h. Early apoptotic cells were defined as Annexin V–PI– and later apoptotic cells were defined as Annexin V–PI+ positive cells. Both the cell populations were merged for calculation of cell apoptotic percentage. KM3 cells were incubated with bortezomib (0.25 μmol/L) for 0, 24, 48 h and 72 h. Then Annexin V was examined according to the methods described above.

Δψm assay

Determination of Δψm were conducted by flow cytometry according to the protocol provided along with the Annexin V kit for cell apoptosis assay. Concentration of As2O3 and bortezomib used in this experiment was 2.0 μmol/L and 0.125, 0.25, 0.5 μmol/L respectively. KM3 cell suspension were treated with (1) Control; (2) Bortezomib (0.125, 0.25, 0.5 μmol/L, incubated for 0, 24, 48, 72 h; (3) Bortezomib (0.25 μmol/L) + As2O3 for 24 h, then collected and centrifuged in cold PBS (4℃) (300 × g, 5 min). The cell pellets were washed twice and resuspended in 200 μL binding buffer. Annexin V-FITC (10 μL) and PI (5 μL) were added and mixed gently. After a reaction for 15 min away from light at room temperature, 300 μL binding buffer was added and the samples were examined within 1 h. Early apoptotic cells were defined as Annexin V–PI– and later apoptotic cells were defined as Annexin V–PI+ positive cells. Both the cell populations were merged for calculation of cell apoptotic percentage. KM3 cells were incubated with bortezomib (0.25 μmol/L) for 0 h, 24 h, 48 h and 72 h. Then Annexin V was examined according to the methods described above.

Definition of drug interaction

The effect of a drug combination is determined in ref-