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Original article

Proteasome inhibitor bortezomi-induced the apoptosis of laryngeal squamous cell carcinoma Hep-2 cell line via disrupting redox equilibrium

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ABSTRACT

Bortezomib, a proteasome inhibitor, has been therapeutic effects in some solid tumors. In the present work, the effects of bortezomib on laryngeal squamous cell carcinoma Hep-2 cell line was investigated and the role of redox equilibrium was explored. The results showed that bortezomib decreased the cell viability and increased the apoptosis in Hep-2 cells. In addition, bortezomib overproduced reactive oxygen species (ROS) and induced the loss of mitochondrial membrane potential ($\Delta\Psi_m$). And the activity of caspase-3 increased. N-acetyl-L-cysteine (L-NAC), a ROS scavenger, alleviated oxidative stress and inhibited the apoptosis induced by bortezomib. However, buthionine sulfoximine (BSO), an inhibitor of GSH synthetase, aggravated bortezomib-induced oxidative stress and apoptosis. So bortezomi-induced the apoptosis of Hep-2 cells via disrupting redox equilibrium.

1. Introduction

Laryngeal carcinoma is a common malignant tumor in the head and neck, and has a high incidence and mortality rates in northeast China. Pathologically, about 93–99% of laryngeal carcinomas are laryngeal squamous cell carcinoma (LSCC) and constitutes approximately 2–3% of all malignant tumors [1,2]. Since larynx has a crucial physiological function in respiration and phonation, LSCC has a severe impact on the quality of life of patients [3]. Although many treatments are used against LSCC, its overall survival rate has not increased (at approximately 35–70%). It is mainly due to uncontrolled recurrence and local lymph node metastasis [4]. Therefore, novel therapeutic methods need to be found for LSCC.

Bortezomib (Velcade, Millennium Pharmaceuticals, Inc., Cambridge, MA, and Johnson & Johnson Pharmaceutical Research & Development, L.L.C., Raritan, NJ) is the first proteasome inhibitor approved by Food and Drug Administration and European Medicines Agency for the treatment of newly diagnosed multiple myeloma and relapsed/refractory multiple myeloma and mantle-cell lymphoma clinically [5,6]. In a phase 2 study of relapsed and refractory myeloma, 27 percent of heavily pretreated patients had a complete or partial response with bortezomib [7]. In addition, it is reported that bortezomib has strong potential antitumor effects in experimental studies. Bortezomib has also inhibited several solid tumors including gastric, prostate, hepatocellular and pancreatic cancer [8–11]. In the present work, the effects of bortezomib on LSCC Hep-2 cell line were investigated. It was measured the cell viability, apoptosis, reactive oxygen species (ROS), mitochondrial membrane potential ($\Delta\Psi_m$) and caspase-3 activity.

2. Materials and methods

2.1. Materials

RPMI 1640 and fetal bovine serum were purchased from Gibco. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), 2′, 7′-dichlorfluorescein-diacetate (DCFH-DA), rhodamine 123, N-acetyl-L-cysteine (L-NAC) and buthionine sulfoximine (BSO) were purchased from Sigma. The proteasome inhibitor, bortezomib (PS-341, Velcade) was purchased from Millenium Pharmaceuticals, Inc. ApoAlert™ CPP32/caspase-3 assay kit was purchased from CLONTECH Laboratories Inc.

2.2. Cell culture and drug treatments

Hep-2 cells were cultured in RMPI 1640 with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air environment. The media were changed every other day. After Hep-2 cells were

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seeded and cultured for 24 h bortezomib was added with L-NAC or BSO coculturing for another 24 h.

2.3. MTT assay

The cell viability was determined using MTT assay. Hep-2 cells were added with MTT solution (5 mg/ml in PBS) and incubated for 4 h at 37 °C. The media were removed and DMSO was then added. The absorbance was measured at 540 nm by a microplate reader. The results were expressed by percent of control.

2.4. Measurement of apoptotic rates

Hep-2 cells were harvested and fixed with 70% ice-cold ethanol for 1 h. Then Hep-2 cells were dyed with PI at 37 °C in the dark for 45 min. The apoptotic cells were measured with a FACScan flow cytometer (Becton Dickinson).

2.5. Measurement of intracellular ROS level

The intracellular ROS level was detected by the dye DCFH2-DA. Hep-2 cells were washed and suspended in RPMI 1640 medium without serum. Then Hep-2 cells were incubated with 50 μmol/L DCFH2-DA for 30 min and washed with PBS. After cells were centrifuged the supernatants were removed, and then Hep-2 cells were mixed with PBS containing 1% Triton X-100. The DCF fluorescence intensity were detected with a fluorescence microplate reader (excitation wavelength: 485 nm and emission wavelength: 530 nm). The results were expressed as percentage of control.

2.6. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was assessed by the fluorescent dye rhodamine 123. Hep-2 cells were mixed with 5 mg/L rhodamine 123 for 30 min at 37 °C in the dark and then washed with PBS. The fluorescence intensity was measured (excitation wavelength: 488 nm and emission wavelength: 510 nm) using a fluorescence microplate reader. $\Delta\Psi_m$ was expressed as percentage of control.

2.7. Measurement of caspase-3 activity

Caspase-3 activity was measured according to the manual of the ApoAlert™ CPP32/Caspase-3 assay kit. Hep-2 cells were lysed and centrifuged to take the supernatant. After incubation for 1 h at 37 °C the absorbance of the chromophore p-nitroanilide was measured at 405 nm using a microplate reader. The standard curve were made by the absorbance of *p*-nitroanilide standard reagent in lysis buffer (up to 20 nM). One unit of the enzyme was defined as the activity producing 1 nmol of *p*-nitroanilide. The data were expressed by percentage of control.

2.8. Statistical analysis

Data are presented as the mean ± SEM and analyzed by ANOVA $p < 0.05$ was considered to be significant.

3. Results

3.1. Bortezomib decreased the cell viability in Hep-2 cells

The effect of bortezomib on cell viability in Hep-2 cells was investigated using MTT assay. Hep-2 cells were treated with different concentrations of bortezomib (10, 20, 30, 40 and 50 nM) for 24 h and the cell viability decreased in a concentration-dependent manner

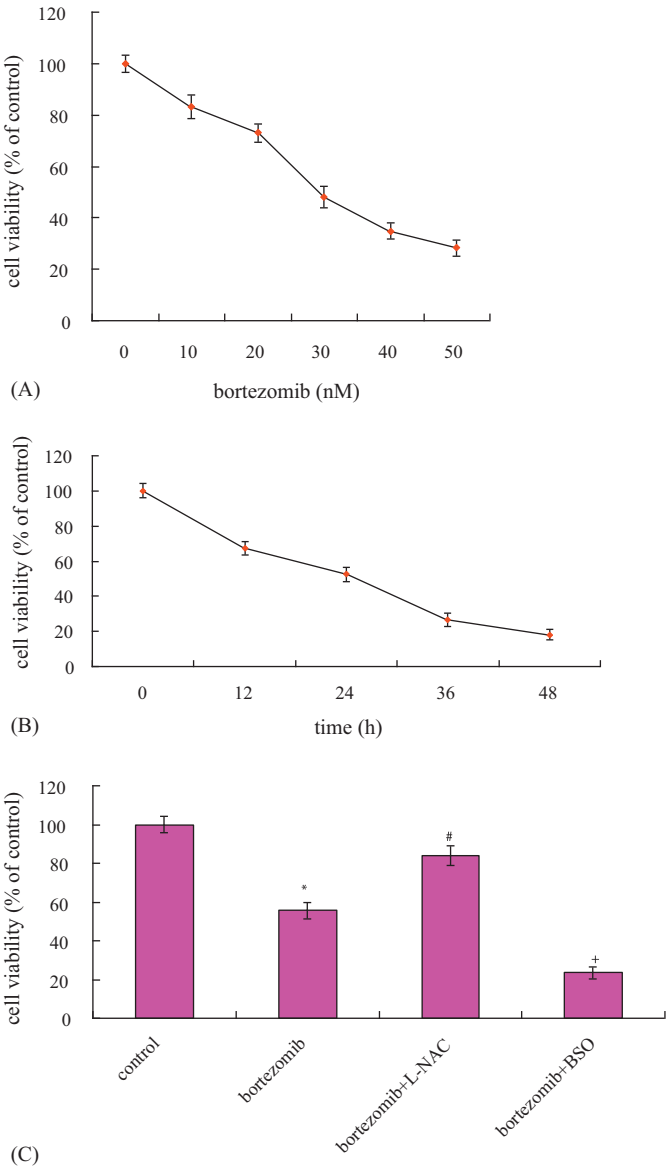

Fig. 1. The effects of bortezomib on cell viability in Hep-2 cells. A. Hep-2 cells were treated with different concentrations of bortezomib (10, 20, 30, 40 and 50 nM) for 24 h. B. Hep-2 cells were treated with 30 nM bortezomib for 12, 24, 36 and 48 h. C. Hep-2 cells were pretreated with L-NAC or BSO for 2 h and added 30 nM bortezomib co-culturing for 24 h. $^{*}P < 0.05$ compared with control. $^{\#}P < 0.05$ compared with bortezomib group. $^{+}P < 0.05$ compared with bortezomib group.

(Fig. 1A). Then Hep-2 cells were exposed to 30 nM bortezomib for 12, 24, 36 and 48 h and the cell viability decreased in a time-dependent manner (Fig. 1B). In Hep-2 cells L-NAC or BSO was pretreatmented for 2 h and added 30 nM bortezomib co-culturing for 24 h. The results showed that L-NAC alleviated the decrease of cell viability induced by bortezomib. However, BSO, an inhibitor of γ-glutamylcysteine synthetase, aggravated the cytotoxicity of bortezomib in Hep-2 cells (Fig. 1C).

3.2. Bortezomi-induced the apoptosis in Hep-2 cells

The apoptosis of Hep-2 cells were measured induced by bortezomib. Hep-2 cells were treated 30 nM bortezomib for 24 h and the apoptotic rate was increased significantly. L-NAC decreased the apoptotic rates of Hep-2 cells compared with bortezomib group. However, BSO increased the apoptotic rates compared with bortezomib group (Fig. 2).