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Research paper

Homoharringtonine enhances bortezomib antimyeloma activity in myeloma cells adhesion to bone marrow stromal cells and in SCID mouse xenografts

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ABSTRACT

Despite the great progress in the treatment, multiple myeloma (MM) still remains incurable. Bortezomib (BTZ), a reversible inhibitor of the 26S proteasome, is very effective against MM but unable to eradicate the MM cells in bone marrow niche eventually causing the disease relapse. Homoharringtonine (HHT) is a known anti-leukemia drug that inhibits MM both in vitro and in vivo. This study aimed to investigate whether HHT could potentiate the anti-tumor activity of BTZ in MM cells cocultured with bone marrow stromal cells and in vivo xenograft models. We found that coculture of myeloma cells with a human stroma cell line significantly decreased the sensitivity of myeloma cells to BTZ treatment. HHT inhibited the proliferation of MM cells and potentiated the anti-myeloma effects of BTZ by inhibition of both canonical and noncanonical NF-κB pathways. HHT also enhanced the anti-myeloma effect of BTZ in vivo xenograft models. Taken together, our data suggest that HHT can enhance the anti-myeloma activity of BTZ both in vitro and in vivo, which may represent a new clinical treatment in MM.

1. Introduction

Multiple myeloma (MM) is a malignant plasma cell disease characterized by the abnormal proliferation of plasma cells in the bone marrow (BM), leading to the generation of monoclonal immunoglobulin or light chain (M protein) [1]. Despite the great progress in the treatment of MM, this disease is still incurable. Bortezomib (BTZ), a reversible inhibitor of the 26S proteasome, exhibits a significant overall response rate against MM patients and has been approved by the US Food and Drug Administration (FDA) for MM treatment in 2003 [2,3]. However, relapse of MM is a major problem even following a combination chemotherapy with BTZ [4,5]. A large body of evidence has demonstrated that the BM stroma can protect MM cells from BTZ and other traditional chemotherapeutic drugs, partially responsible for MM relapse [6–8]. How to eradicate MM cells in BM niche remains a major clinical challenge. Therefore, there is an urgent need to find a novel agent in combination with BTZ to exhibit high performance and low toxicity in anti-multiple myeloma treatment.

Homoharringtonine (HHT) is an anti-leukemia drug that is extracted from the herb *Cephalotaxus mannii* found in southern China, and has been applied in the treatment of a variety of hematologic malignancies since 1970s [9–11]. It has been shown that the VHD program (HHT, vincristine, dexamethasone) that included HHT for the treatment of two cases of refractory MM obtained good results [12]. The studies further confirmed that HHT also exerted a significant inhibitory effect on MM cells both in vitro and in vivo xenograft model [13–15]. Recently, HHT and BTZ were shown to synergistically inhibit the growth of high risk MDS cell line SKM-1 and induce apoptosis via inhibition of Akt and nuclear factor κB (NF-κB) signaling pathway [16]. However, whether and how HHT can potentiate the anti-myeloma effects of BTZ against MM cells in the BM stroma and the related mechanisms are still elusive

NF-κB plays an important role in the occurrence and development in MM that is known to highly express NF-κB [17,18]. In resting cells, NF-κB and inhibitor of NF-κB (IκB) kinase can form a complex in the cytoplasm as an inactive form. When cells are stimulated by an extracellular signal, IκB can be phosphorylated and degraded by IκB kinase (IKK) to expose the nuclear localization site of NF-κB. Then, the free NF-κB can rapidly migrate to the nucleus and bind to the specific κB sequence, thereby inducing the transcription of relevant genes [19]. As a proteasome inhibitor, BTZ can inhibit proteasomes and therefore stabilize IκB, thus preventing the release of NF-κB and having an anti-tumor effect [20]. After contact with the BM stroma, the expression of

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NF-κB in MM cells is further enhanced [21–23]. Therefore, we hypothesize that BM stroma could protect MM cells through activation of NF-κB pathway and HHT in combination with BTZ has a synergistic anti-myeloma effect by regulating the NF-κB pathway. In this study, the HS-5 cells which are the BM fibroblast cells transformed with HPV-16 E6/E7 were used as an in vitro model of human bone marrow stroma. We first employed a co-culturing system composed of U266 cells and HS-5 cells and detected their sensitivity to BTZ in MM cell cultured alone, or adhered to HS-5 cells followed by examining an activation of NF-κB pathway. Furthermore we evaluated the anti-myeloma effect of HHT in combination with BTZ in primary MM cells and in vivo xenografts.

2. Materials and methods

2.1. Cell lines, primary MM cells and cell culture

The human multiple myeloma cell line U266 was purchased from JENNIO Biological Technology (Guangzhou, China), whose authenticity has been confirmed by short tandem repeat (STR) analysis matching perfectly the reference cell line U266 in the ATCC STR database. The human stromal cell line HS-5 was purchased from the Biomedicine and Health of the Chinese Academy of Sciences (Guangzhou, China). Primary MM cells were isolated from the patients in the Fujian Medical University Union Hospital with informed consent and institutional review board approval. The bone marrow aspirates were processed by Ficoll density gradient centrifugation to isolate mononuclear cells followed by $CD138^+$ selection according to the manufacturer's instructions (Miltenyi Biotec, Shanghai, China). The MM patients' data is presented in Table 1. The cell lines and primary cells were cultured in a humidified incubator at 37 °C and 5% CO_2 in RPMI 1640 medium (Gibco, CA) containing 10% fetal bovine serum (FBS, Gibco, CA) and conventional concentrations of penicillin and streptomycin (Sigma-Aldrich, MO).

2.2. Drugs

Homoharringtonine (Harbin, China) was dissolved in sterile PBS to prepare a 1 mg/ml stock solution and was stored at −80 °C. Bortezomib was purchased from Xian Janssen (Xian, China).

2.3. Co-culture system and magnetic bead sorting

1.0×10^5 HS-5 cells were seeded into 12-well plate first and 2.0×10^5 U266 cells were then seeded into the plate when the HS-5 cells reached 70–80% confluence. After coculture for 48 h, the adherent MM cells were collected with nylon mesh (400 mesh, Becton and Dickinson Company, Franklin Lakes, NJ) to create a single cell suspension. $CD138^+$ magnetic beads (Miltenyi Biotec Technology & Trading, Shanghai, China) were used to sort the adhered MM cells. The purity of the MM cells was > 90% as determined by flow cytometry.

Table 1
MM patients' clinical characteristics.

Sample No	Gender/Age	Subtype	Stage (D-S)	FISH
1	M/42	IgA,λ	II	nuc ish(GLP 1q21) × 3[112/200], nuc ish(IGH × 2) (5′IGH sep 3′IGH × 1) [72/200]
2	F/76	λ	III	nuc ish(GLP RB1) × 1[134/200], nuc ish(GLP D13S319) × 1[122/200], nuc ish(IGH × 2)(5′IGH sep 3′IGH × 1) [90/200]

2.4. Measurement of cell proliferation and inhibitory effect

Cell proliferation was measured by absolute viable cell counts or CCK-8 assay. The cell counts were determined by trypan blue dye exclusion over a 5-day period. The inhibitory effect was measured according to the manufacturer's instruction of the CCK-8 Kit (Dojindo, Japan). Briefly, 10 μl of CCK8 was added into each well and mixed in the dark. After incubation at 37 °C for 2 h, the absorbance value (OD) at the dual wavelengths of 450 nm/630 nm was detected using a microplate reader. The rate of inhibition of cell proliferation was calculated as follows: inhibition rate% = (1 – the average OD value of the drug-treated cells/the average OD value of the control group) × 100%. Each experiment was repeated three times.

2.5. Western blot analysis

Western blot experiments were carried out as previously described [24]. Briefly, total and nuclear protein were extracted from U266 cells or primary MM cells according to the manufacturer's instruction (Thermo, Shanghai, China). Isolated proteins (40 μg) were resolved using 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes, which were blocked with 5% BSA in TBST [10 mM Tri-HCl pH 7.6, 150 mM NaCl, and 0.1% Tween 20]. The membranes were incubated overnight at 4 °C with the appropriate primary antibodies. Antibodies anti-p65 (1:400, 7970), anti-p50 (1:200, 7971), anti-p52 (1:1000, 7972), anti-IκBα (1:5000, 32041) were purchased from Abcam (Cambridge, MA). The anti-histone 3 polyclonal antibody (1:1000, 9715), anti-GADPH polyclonal antibody (1:1000, 2118) and horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000, 7074) were purchased from Cell Signal Technology (Danver, MA). Primary antibodies were diluted in TBS-T/5% milk or BSA according to the manufacture instructions. After 24 h, blots were washed with TBS-T and incubated for 1 h at room temperature with the appropriate secondary antibody tagged with horseradish peroxidase (Cell Signaling Technologies). Finally, the antibody-labeled proteins were detected using an ECL system. GADPH and Histone 3 were used as the internal control for cytoplasmic proteins and nuclear proteins respectively.

2.6. Apoptosis assay

Apoptosis was detected by flow cytometry with fluorescein isothiocyanate (FITC)-labeled Annexin-V/propidium iodide (PI) double staining (Roche, Shanghai). Cells were collected and resuspended in 500 μl binding buffer. Then 5 μl Annexin V-FITC and 5 μl of PI were added. Upon incubation in the dark at room temperature for 5 min, the samples were analyzed using flow cytometer (Miltenyi Biotec, Germany).

2.7. SCID mouse xenograft model

5×10^6 U266 cells were subcutaneously injected into the subscapularis in female SCID mice (Shanghai, China) with the age of 6 weeks. The tumor volume was measured every two days and calculated with the following formula: V = [tumor length in mm × (tumor width^2 in mm)]/2. CD38 and CD138 expression in SCID mouse xenografts was detected by immunohistochemical analysis (IHC) and antibodies of anti-CD38, anti-CD138 were purchased from Maxin (Fuzhou, China). When the tumor volume reached 100 mm^3, the mice were randomly assigned into 4 groups (n = 10/group). The use of animals was approved by the Committee of Research Animals of Fujian Medical University. The control group was intraperitoneally treated with the same volume of normal saline. The HHT group was administered with HHT at 3 mg/kg from day 1–5 and day 11–15. The BTZ group received BTZ at 0.1 mg/kg on day 1, 5, 11 and day 15. The combination group was treated with both HHT and BTZ. Tumor size and animal body weights were measured every two days. The mice were sacrificed 24 h