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Original Research Article

Resveratrol increases the sensitivity of multiple myeloma cells against bortezomib via Hedgehog signaling pathway

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Abstract

Purpose: *To investigate the effect of resveratrol (RSV) on bortezomib (BTZ)-resistant multiple myeloma (MM) cells, and to elucidate the underlying mechanism of action.*
Methods: *H929 cell lines were exposed to BTZ for 8 months to establish BTZ-resistant MM cell model. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Apoptosis was measured using annexin V/propidium iodide (PI) staining while cell cycle analysis was evaluated by flow cytometry. The expression of Hedgehog (Hh) signaling proteins (sonic hedgehog (SHH), smoothened (SMO), and glioma-associated oncogene homolog (GLI)) was analyzed by western blot.*
Results: *H929R was confirmed as a MM cell line that is resistant to BTZ. RSV enhanced the sensitivity of H929R cells against BTZ via inhibition of cell viability and colony formation, induction of cell apoptosis and regulation of expression of apoptosis-related proteins. Furthermore, RSV inhibited the expression of Hh signaling proteins ($p < 0.05$.*
Conclusion: *RSV enhances the sensitivity of MM cells to BTZ, partly via Hh signaling pathway. Thus, Hh pathway is a probable target for MM treatment, and RSV has potentials for use in the clinical management of MM.*

Keywords: *Resveratrol, Bortezomib, Hedgehog signaling pathway, Multiple myeloma*

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INTRODUCTION

Multiple myeloma (MM), is characterized by clonal proliferation of malignant plasma cells, ranking the second most common hematological malignan [1]. In the past decades, supportive care strategies and new therapeutic agents (such as thalidomide, bortezomib (BTZ), and lenalidomide) have made significant breakthroughs in the treatment for MM[2]. However, MM is still incurable due to the resistance to drugs, particularly, BTZ). The mechanisms underlying the resistance against BTZ are complex, such as chromosomal translocations, oncogene mutations and disordered signaling pathways. Among them, Alonso *et al* reported that crosstalk between Hedgehog (Hh) and retinoid signaling alters the

MM microenvironment and generates BTZ resistance[3]. Other molecules are also involved the resistance against BTZ, including de-ubiquitinylating enzyme, ubiquitin receptor, serine synthesis, and Notch [4].

The Hh signaling pathway regulates the development and differentiation of tissues and organs during embryonic life. In mammals, this pathway can be activated when one of three ligands [Desert hedgehog, Indian hedgehog, or Sonic hedgehog (SHH)]bind to their receptors (Patched)[5]. Hedgehog receptor PTCH then relieve smoothened (SMO) and result in the translocation of the glioma-associated oncogene homolog (GLI) and the transcription of Hh target genes[6].Studies have reported that the dysregulation of the Hh pathway may lead to tumorigenesis, including hepatic carcinoma, lung cancer, pancreatic cancer, and MM [7]. Beside, activation of Hh signaling is associated with drug resistance of MM stem cells, and the drug resistance is inhibited by Hh blocking agents[8].

Resveratrol (RSV), abundant in grapes and grape products, was widely used as an antioxidant and anti-mutagenic agent [9] . It exerts many beneficial effects, including anti-inflammatory, antioxidant, cardiac protection, and anti-tumor and chemo-protective effects [10]. Previous studies reported that RSV inhibits the proliferation and invasion of MM cells, as well as cell cycle arrest and apoptosis [11]. Besides, in MM cells, resveratrol sensitizes apoptosis induced by carfilzomib via regulating oxidative stress [12]. Researchers reported the role of RSV in chemosensitization of cancers was associated with the modulation of drug efflux transporters, cell cycle, and autophagy through NF-κB and STAT pathways [13]. However, it is still unclear whether RSV has an effect on the resistance of MM against BTZ. Thus, the aim of present study was to characterize the effects of RSV on the chemoresistance against BTZ, and to identify the detail mechanism for this process.

EXPERIMENTAL

Cell culture

The H929 human MM cell line (ATCC® CRL-9068) was purchased from the American Type Culture Collection (Manassas, VA, USA). Bortezomib-resistant MM line was obtained by H929 exposure to BTZ for 8 months, with final concentration from 10 to 200 nM. The RPMI-1640 medium (10% fetal bovine serum) was changed twice each week by 50%. In this manner, the concentration was gradually increased to 200 nM, and H929R cells resistant to bortezomib were obtained after 8 months of culture.

Clonogenic assay

Clonogenic assays were performed as reported [14]. Cells were cultured with 10% FBS for 24 h and then with basal medium (containing 0.5% FBS) for3 weeks, and then stained with 0.5% Crystal Violet solution, followed by phosphate-buffered saline to remove excess dye. Each treatment was performed in triplicate.

MTT assay

The principle of the MTT assay is that dehydrogenase enzyme in metabolically active cells converts the MTT dye (Sigma, St. Louis, MO) to formazan crystals which are soluble in dimethyl sulfoxide (DMSO), which is quantified using a spectrophotometer. Cells were seeded in a 96-well plate to adhere overnight and treated with or without RSV for 48 h. Then, 20 µL of MTT solution was added and incubated for another 4 h. DMSO (150 µL) was used to dissolve the formazan, and then measured at 570 nm.

Cell cycle analysis

Cells were fixed in cold 70% alcohol overnight, permeabilized for 5 min with Triton X-100, and stained in a propidium iodide (PI) solution with a final concentration of 50 µg/mL (Sigma-Aldrich, St. Louis, MO, USA). The analysis was performed using a FACScan flow cytometer (BD Biosciences, Bedford, MA, USA).

Apoptosis analysis

Cells were seeded at a density of 5×10^5 cells, and 24 h later, the medium was replaced and added with RSV or BTZ combined with RSV (the final concentration for RSV was 10 and 20 µM for BTZ). The cells were collected after 72 h, resuspended, then stained with annexin V and PI solution (BD Biosciences), and finally analyzed using flow cytometry.

Western blot analysis

Cells were seeded, treated with different methods, and collected. After being lysed in RIPA lysis buffer, protein was quantified using the BCA method and resolved using SDS-PAGE and transferred onto a PVDF membrane. After blocking, the membranes were incubated with primary antibodies[cleaved caspase 3 (Cell Signaling Technology, Inc. (CST), Danvers, MA, #9661), cleaved caspase 9 (CST #9505), BAX (CST #5023), BCL-2 (CST #15071), GLI-1 (CST

#2643), SHH (CST #2207), and SMO (CST #4930)using a dilution of 1:1,000; Cell Signaling Technology, Danvers, MA, USA] followed by incubation with secondary antibody (1:10,000), and detection of proteins using an ECL Western Blotting Analysis System (AmershamGEHealthcare, Little Chalfont, UK).The blots were standardized to glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

One-way analysis of variance was used for statistical analysis. All data are expressed as mean ± standard deviation (SD). Half maximal inhibitory concentration (IC_{50}) values were calculated using linear regression analysis.

RESULTS

Confirmation of MM cell line (H929R) that resists to BTZ

H929 and H929R cells were cultured in media containing different concentrations of BTZ. The half-maximal inhibitory concentration (IC_{50}) was obtained using an MTT assay. Figure 1 A shows that BTZ effectively inhibited the proliferation of H929 cells (IC_{50} =13.45 nM), but H929R cells were more resistant to BTZ (IC_{50} = 143.7 nM). The colony formation results (Figure 1 B) showed that the number of colonies formed by H929 cells was lower than that of H929R cells ($p < 0.001$), which was consistent with the MTT assay results. Thus, H929R cells were used in the following study.

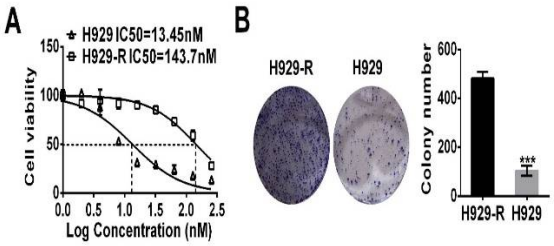

Figure 1: Viability and colony formation of bortezomib (BTZ)-treated H929R and H929 cells. (A) The viability of H929R and H929 cells treated with bortezomib (BTZ) measured using the MTT assay. (B) The colony formation of bortezomib (BTZ)-treated H929R and H929 cells analyzed using a clonogenic assay; $^{***}p<0.001$ vs. H929-R cells

RSV enhanced the sensitivity of H929R cells to BTZ

To investigate the effects of RSV on the sensitivity of H929R cells against BTZ, H929R cells were treated with different concentration (5, 10, 50 and 100μM) of RSV or various exposure times (12, 24, 36, 48 and 72 h). The viability of H929R cells decreased as RSV concentration increased (Figure 2 A). Viability was also decreased with longer incubation times (Figure 2 B). The IC_{50} of BTZ on H929R cells was decreased from 143.7 to 104.2 nM (Figure 2 C). These results indicated that RSV enhanced the sensitivity of H929R cells against BTZ.

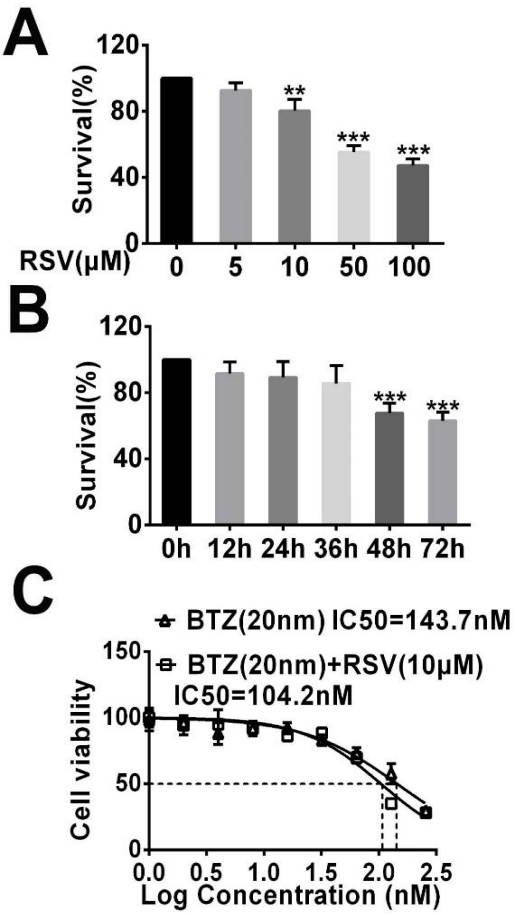

Figure 2: Effect of RSV on the viability of H929R cells. (A) The viability of H929R cells treated with different concentrations of RSV, followed by the MTT assay. (B) The viability of H929R cells with RSV treatment at various exposure periods, followed by the MTT assay. (C) The IC_{50} of BTZ on H929R cells. $^{*}p<0.05$ vs. bortezomib (BTZ) alone; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. BTZ alone

RSV enhanced sensitivity of H929R cells to BTZ via inhibition of cell viability, colony formation and induction of cell apoptosis

For further analysis, proliferation, viability, colony formation, and cell cycle analysis of H929R cells were performed. Figure 3A showed that BTZ did not inhibit the viability of H929R cells as compared with normal control group, confirming the resistant of H929R cells to BTZ. In contrast, RSV combined with BTZ treatment inhibited the proliferation of H929R when compared with BTZ

alone (p < 0.01), which indicated that RSV enhanced the sensitivity of H929R cells against BTZ through inhibiting cell viability. The colony formation results were consistent with the results of cell viability (Figure 3 B). To explore the mechanism of RSV-inhibited cell proliferation, the cell cycle was analyzed using flow cytometry. The amount of G1, S, and G2phases are shown in Figure 3 C. The fraction in the S phase was lower than that of RSV+BTZ group compared to the BTZ group.

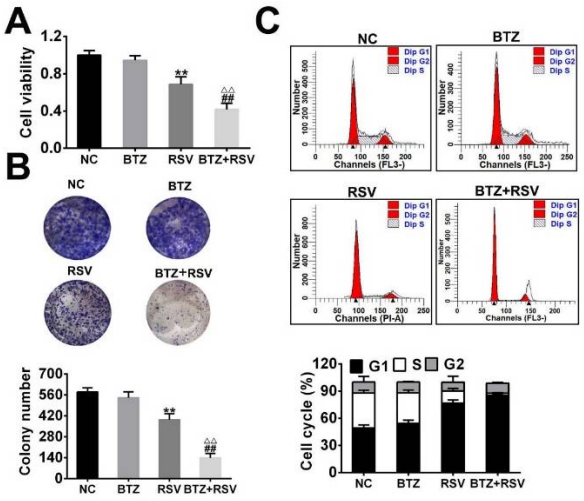

Figure 3: RSV enhanced the sensitivity of H929R cells against BTZ through inhibiting cell viabiliy and colony formation and inducing cell apoptosis. (A) The viability of H929R cells measuredusing the MTT assay. (B) The colony formation of H929R cells analyzed by the clonogenic assay. (C) The cell cycle was analyzed using flow cytometry; $^{**}p$ < 0.01 vs. NC; $^{\#\#}p$ < 0.01 vs. bortezomib (BTZ); $^{\triangle\triangle}p$ < 0.01 vs. RSV

RSV enhanced BTZ-induced apoptosis of H929R cells

As Resveratrol (RSV) enhanced the sensitivity of H929R against BTZ, the effects of RSV on the apoptosis of H929Rwere determined using annexin V/PI staining. Figure 4A showed that the proportion of apoptotic cells in the BTZ group was increased as compared to the normal control (p< 0.01). RSV combined with BTZ significantly increased the number of apoptotic cells (p< 0.01 vs. BTZ). To further verify the effects of RSV on BTZ-induced apoptosis of H929R, apoptosis-related proteins were measured using western blot analysis. Figure 4B showed that BTZ elevated the expression levels of activated caspases 3 and 9 (cleaved caspase 3 and 9) and a pro-apoptotic protein (BAX). Addition of RSV aggravated these results as compared with BTZ alone.

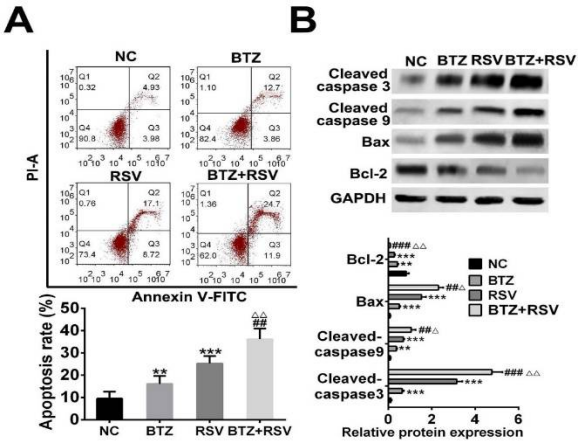

Figure 4: Resveratrol (RSV) enhanced the apoptosis of H929R cells.(A) Cell apoptosis by annexin V/PI staining. Q1: annexin-/PI+ necrotic cells, Q2: annexin+/PI+ late apoptotic cells, Q3: annexin-/PI- viable cells, Q4: annexin +/PI- early apoptotic cells. (B) The level of apoptosis-related proteins was determined using western blotting; $^{**}p$ < 0.01 and $^{***}p$ < 0.001 vs. normal control; $^{\#\#}p$ < 0.01 and $^{\#\#\#}p$ < 0.001 vs. bortezomib (BTZ); $^{\triangle\triangle}p$ < 0.01 and $^{\triangle\triangle\triangle}p$ < 0.001 vs. RSV

RSV inhibited the activation of the Hh signaling pathway of H929R cells

To reveal the underlying mechanism, Hh signaling proteins (SHH, SMO, and GLI-1) were analyzed using western blotting. Figure 5 showed that BTZ alone did not affect the expression of these three proteins, while RSV inhibited the expression of SHH (p < 0.001), SMO (p < 0.001), and GLI-1 (p < 0.01). RSV, when combined with BTZ, enhanced the effects of RSV on H929R cells, which indicated that RSV enhanced the sensitivity of H929R cells to BTZ partly through the Hh signaling pathway.

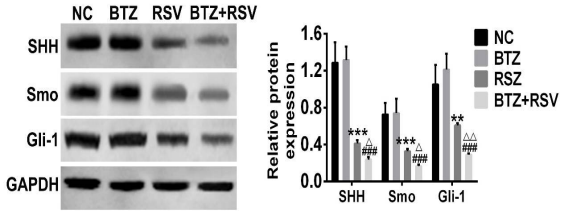

Figure 5: Effect of RSV on expression of Hedgehog signaling proteins (SHH, SMO, and GLI-1) analyzed by western blot; $^{**}p$ < 0.01 and $^{***}p$ < 0.001 vs. normal control; $^{\#\#\#}p$ < 0.001 vs. bortezomib (BTZ); $^{\triangle}p$ < 0.05 and $^{\triangle\triangle}p$ < 0.01 vs. RSV

DISCUSSION

Multiple myeloma (MM) is the second most common type of hematological cancer, with median survival of 3–4 years from the initial

diagnosis. Although the development of newly marketed proteasome inhibitors and immune modulators has improved the effectiveness of treatment, MM remains incurable for patients. Besides, chemotherapy resistance is still one of the important causes for failure of treatment. Studies have indicated that the use of Chinese Material Medica in clinical practice has achieved good effects in the treatment of MM. Resveratrol (RSV), a major active compound of stilbene phytoalexins, has been proven to be beneficial for health. The biological effects of RSV are diverse, including antioxidant activity, modulation of lipid and lipoprotein metabolism, antiplatelet aggregation, anticancer and estrogenic activity [15].

Studies have reported that RSV could be a novel agent for the treatment of MM. Researchers [12] found that RSV sensitized proteasome inhibitor-induced apoptosis via regulating oxidative stress in MM cells. Similarly, Jazirehi *et al* [16] reported that RSV sensitized paclitaxel-induced apoptosis by modifying the expression of apoptotic proteins and regulating the cell cycle. Other researchers investigated the mechanism of RSV on MM cell proliferation, apoptosis, and chemoresistance. Their results indicated that RSV inhibited MM cell proliferation, promoted apoptosis, and decreased chemoresistance via downregulation of STAT3 and nuclear factor-B-regulated anti-apoptotic gene expression [17].

Consistently, the present study confirmed the previous results that RSV sensitizes MM cells to BTZ (a proteasome inhibitor).Firstly, a BTZ-resistant MM cell line (H929R) was established to further verify the effects of RSV on the resistance of MM cells against BTZ. The results showed that RSV enhanced the sensitivity of H929R cells against BTZ through inhibiting cell viabiliy and colony formation, inducing cell apoptosis and regulating the expression of apoptosis-related proteins.

The mechanism of MM generation and development is complex, and it is very important to elucidate the pathogenesis of MM or the effect of drugs on the clinical treatment. Previous studies found that Wnt, STAT3, NF-κB , MAPK, AKT, and the Hh signaling pathway were involved in the proliferation, apoptosis, cell invasion, and chemoresistance of MM [18]. Particularly, Hh signaling pathway, a highly conserved system which regulates the development and differentiation of tissues, was recently confirmed to be associated with the progression of MM [19].

A phase I clinical study of the Hh pathway antagonist (BMS-833923) in patients with relapsed or refractory MM showed that inhibition of the Hh pathway effectively improved the treatment effect of MM patients[20]. Research also demonstrated that the Hh signaling pathway plays a key role in MM. Hedgehog activation was heterogeneous across the spectrum of MM tumor stem cells, to maintain a tumor stem cell compartment [21].The inhibition of Hh signaling inhibited clonogenic growth and self-renewal in MM cells [22].These results showed that RSV improved the sensitivity of MM cells to BTZ via inhibiting the activation of the Hh signaling pathway, although few studies had reported this mechanism of RSV on BTZ-resistant in cells.

CONCLUSION

RSV enhanced the sensitivity of H929R cells to BTZ via inhibition of cell viabiliy and colony formation, induction of cell apoptosis and regulation of the expression of apoptosis-related proteins. The underlying mechanism of action is associated with Hh signaling pathway. Hh pathway is, therefore, a target for MM treatment, and thus, RSV may be developed as a candidate for the therapeutic management of MM.

DECLARATIONS

Acknowledgement

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Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. RZ and YZ designed all the experiments and revised the paper. RM and ZJ carried out the experiments. LT and YZ wrote the manuscript.

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