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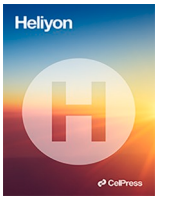

GRP78 inhibitor HA15 increases the effect of Bortezomib on eradicating multiple myeloma cells through triggering endoplasmic reticulum stress

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A R T I C L E I N F O

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A B S T R A C T

Bortezomib (BTZ), a selective proteasome inhibitor, exhibits a significant efficacy in the therapy of multiple myeloma (MM) partly through triggering endoplasmic reticulum (ER) stress-dependent apoptosis. However, sensitivity to BTZ varies greatly among patients. ER stress functions as a double-edged sword in regulating cell survival depending on cell context and ER stress extent. The major aim of this study was to investigate whether GRP78 inhibitor, HA15, increased the therapeutic effect of BTZ on MM to through further increasing ER stress and shifting the balance towards cell apoptosis. The biological role of BTZ and HA15 was assessed using Cell counting kit- (CCK-) 8, colony formation, and Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) assay. We found that BTZ combined with HA15 remarkably decreased MM cell viability more effective than BTZ monotherapy, though low dose of HA15 did not exhibit a significant cytotoxicity to MM cells. BTZ combined with HA15 also repressed colony formation ability of MM cell and accelerated MM cell apoptosis compared with BTZ monotherapy. Mechanistically, HA15 synergized with BTZ to trigger ER stress, as evidence by significantly increased expression of ER stress markers (GRP78, ATF4, CHOP, and XBP1). Importantly, unfolded protein response (UPR) inhibitor significantly damaged the effect of BTZ combined with HA15 on accelerating MM cell death. *In vivo*, combination treatment with BTZ and HA15 inhibited tumor growth more effective than BTZ alone, whereas these effects were blocked by UPR inhibitor. Taken together, these results demonstrate that ER stress is a critical pathway in regulating MM cell survival, and that combination treatment with BTZ and HA15 may be an effective strategy to treat MM patients that fail to respond to BTZ monotherapy.

1. Introduction

Multiple myeloma (MM) is the second most frequent hematological malignancy derived from B-lymphocyte, with an estimated 170,000 new cases and estimated 110,000 deaths in 2020 [1,2]. Despite great progression in the treatment of MM like proteasome inhibitors, monoclonal antibodies, and immunomodulatory agents, this disease has a high rate of recurrence and remains incurable [3,4]. As a proteasome inhibitor, bortezomib (BTZ) is applied in general treating refractory or relapsed MM [5], but resistance to BTZ-based regimens hampers its clinical application [6,7].

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The endoplasmic reticulum (ER) is a vital organelle responsible for protein synthesis, folding, assembly, and degradation [8]. Enhanced protein biosynthesis is required in cancer cells because of their rapid proliferation [9]. Protein misfolding inevitably occurs in the process, ultimately resulting in an excessive amassing of misfolded proteins in ER, which is called ER stress and meanwhile unfolded protein response (UPR) and ER-associated degradation (ERAD) is initiated to maintain protein homeostasis [9–11]. Several key proteins have been identified in ER stress such as chaperone protein glucose-regulated protein 78 (Grp78, also called binding immunoglobulin protein BiP), protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 4 (ATF4), transcription factor C/EBP homologous protein (CHOP), and X-box binding protein (XBP1) [12]. Abnormal expression of these proteins is significantly associated with drug resistance and disease prognosis [13,14]. For instance, aspirin acts as an anti-tumor role in MM by inducing ATF4/CHOP pathway activation [15]. Low expression of XBP1 is associated with BTZ resistance in MM [16].

The proteasome is an effective target in the treatment of different types of tumors such as liver cancer [17], lung cancer [18], and MM [19]. BTZ, a proteasome inhibitor, has been widely applied in treating hematological malignancy and clinical trials in solid tumors [17]. However, prolonged therapy with BTZ inevitably results in chemoresistance and the mechanism underlying BTZ resistance remains unclear. Mounting studies have demonstrated that ER stress is a crucial factor for tumor initiation and progression, and BTZ resistance of cancer cells [13,20,21]. ER stress activates UPR, which contributes to cell survival through accelerating misfolded proteins degradation and thus sustain intracellular homeostasis [22,23]. Although BTZ can efficiently kill MM cells through activating ER stress-dependent cell apoptosis, the degree of ER stress may not sufficient to clear tumor cells and thus MM cells acquire BTZ resistance. Further increase of ER stress by combination treatment with BTZ and other drugs may be an effective strategy for clearing MM cells.

HA15 is a potent inhibitor of ER chaperone GRP78/BiP and exhibits a significant effect on inhibiting cancer cell survival and overcoming drug resistance [24,25]. GRP78 inhibition by HA15 accelerates lung cancer cell apoptosis through activating ER stress and autophagy [26]. HA15 accelerates MM cell death and overcomes BRAF inhibitor resistance through inducing ER stress [24], indicating its potential role in MM treatment. In the study we demonstrated that combination treatment with HA15 and BTZ decreased MM cell viability more prominently than BTZ monotherapy, suggesting that HA15 is an effective chemosensitizer in MM treatment.

2. Materials and methods

2.1. Cell culture

Two human MM cell lines (NCI–H929 and U266), obtained from ATCC (Manassas, VA, USA), were cultivated in RPMI 1640 (Invitrogen, CA, USA) containing 10% FBS (TIANHANG, Zhejiang, China). Cells were maintained in a humidified 5% CO2 incubator at 37 °C.

2.2. Cell viability

NCI–H929 or U266 cells were seeded in 96-well plate at 2×10^3 cells/well. MM cell viability was assessed using CCK-8 kit (Beyotime, Shanghai, China) after treatment with BTZ (0, 1, 2, 4, 8, and 16 nM) in the presence or absence of HA15 (0, 1, 2, 4, 8, 16, and 32 μM). After treatment for 48 h, cells were treated with CCK-8 reagent (10 μL) for 55 min at 37 °C. The absorbance was obtained at 450 nm with a microplate reader (FK-SY96S, Shandong, China) to calculate relative cell viability.

2.3. Colony formation assay

NCI–H929 cells (approximately 500 cells/well) or U266 cells (approximately 400 cells/well) were seeded in 12-well plate and treated with BTZ (4 nM) in the presence or absence of HA15 (1 μM) for 48 h. After washing thrice with PBS, cells were cultured in methylcellulose medium (StemCell Technologies, Vancouver, Canada) for 14 days in a humidified 5% CO2 incubator at 37 °C. Colonies were fixed with 4% PFA and stained with 0.1% crystal violet for 15 min, and colonies defined as a cluster of more than 50 cells per colony were calculated with an inverted microscope (OCEANHOOD, Shanghai, China).

2.4. TUNEL

MM cell apoptosis was assessed using TUNEL staining. NCI–H929 cells or U266 cells were seeded in 12-well plate and treated with BTZ (4 nM) in the presence or absence of HA15 (1 μM) for 48 h. Then cells were fixed with 4% PFA for 15 min, stained with TUNEL reagent (Beyotime) for 50 min at 37 °C, and observed with a fluorescence microscopy (DMI4000B, Leica, Wetzlar, Germany).

2.5. Western blot

NCI–H929 cells or U266 cells (1×10^6) were seeded in 6-well plate, treated with BTZ (4 nM) in the presence or absence of HA15 (1 μM) for 48 h, and then were lysed with RIPA buffer (Beyotime) supplemented with protease inhibitor mix (Thermo Fisher Scientific, MA, USA). Total protein concentration was measured with a BCA protein assay kit (Beyotime). Equal amount of protein (approximately 30 μg) was separated using 10% SDS-PAGE, and then transferred to PVDF membranes (Beyotime). After treatment with blocking buffer (Beyotime), membranes were incubated with antibodies against Bax (1:3500, ab32503, Abcam, CA, USA), Bcl2 (1:1500, ab182858, Abcam), GRP78 (1:1000, ab21685, Abcam), ATF4 (1:1000, ab216839, Abcam), CHOP (1:1000, ab11419,

Abcam), XBP1 (1:1500, ab37152, Abcam), and β-actin (1:300, ab8226, Abcam) for 1 h at room temperature. After washing thrice with TBST, membranes were incubated with HRP-labelled secondary anti-rabbit/mice for 1 h at room temperature. Lastly, immunoblot was visualized with an ECL kit (Pierce, Rockford, USA).

2.6. In vivo xenograft tumor experiments

Animal experiments were approved by the Animal Ethics Committee of Shanghai municipal Hospital of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine (NO.20222190) following the ARRIVE guidelines to reduce mice suffering [27]. Female BALB/c nude mice aged 6–8 weeks (Shanghai Model Organisms Center, Shanghai, China) were housed in pathogen-free conditions with a 12 h light-dark cycle and fed *ad-libitum* with water and food. MM mouse xenograft model was generated through subcutaneously injecting NCI–H929 cells (1×10^7 cells in 1:1 ratio of RPMI 1640 and phenol red-free Matrigel (BD Biosciences, CA, USA)) into dorsal flank of nude mice (n = 3). When tumor volume reached approximately 100 mm^3, tumor-bearing mice were treated with the following schedule and dose: i) intraperitoneal (i.p.) injection of BTZ (1 mg/kg twice weekly), ii) i.p. injection of BTZ (1 mg/kg twice weekly) and HA15 (0.5 mg/kg twice weekly), iii) i.p. injection of BTZ (1 mg/kg twice weekly) and HA15 (0.5 mg/kg twice weekly), and oral gavage of GSK2606414 (50 mg/kg once per day), iv) vehicle (control). Tumor volume was monitored at the indicated time-points.

2.7. Statistical analysis

Data are showed as the mean ± standard error of mean from three separate experiments. Statistical analysis was conducted with GraphPad Prism 7 (Version X, CA, USA). $p < 0.05$, calculated by the Student's *t*-test or the ANOVA followed by Scheffé test, was seemed as a significant difference.

3. Results

3.1. BTZ combined with HA15 remarkably decreased MM cell viability

To investigate the effect of BTZ combined with HA15 on accelerating MM cell death, the role of BTZ and HA15 in regulating cell

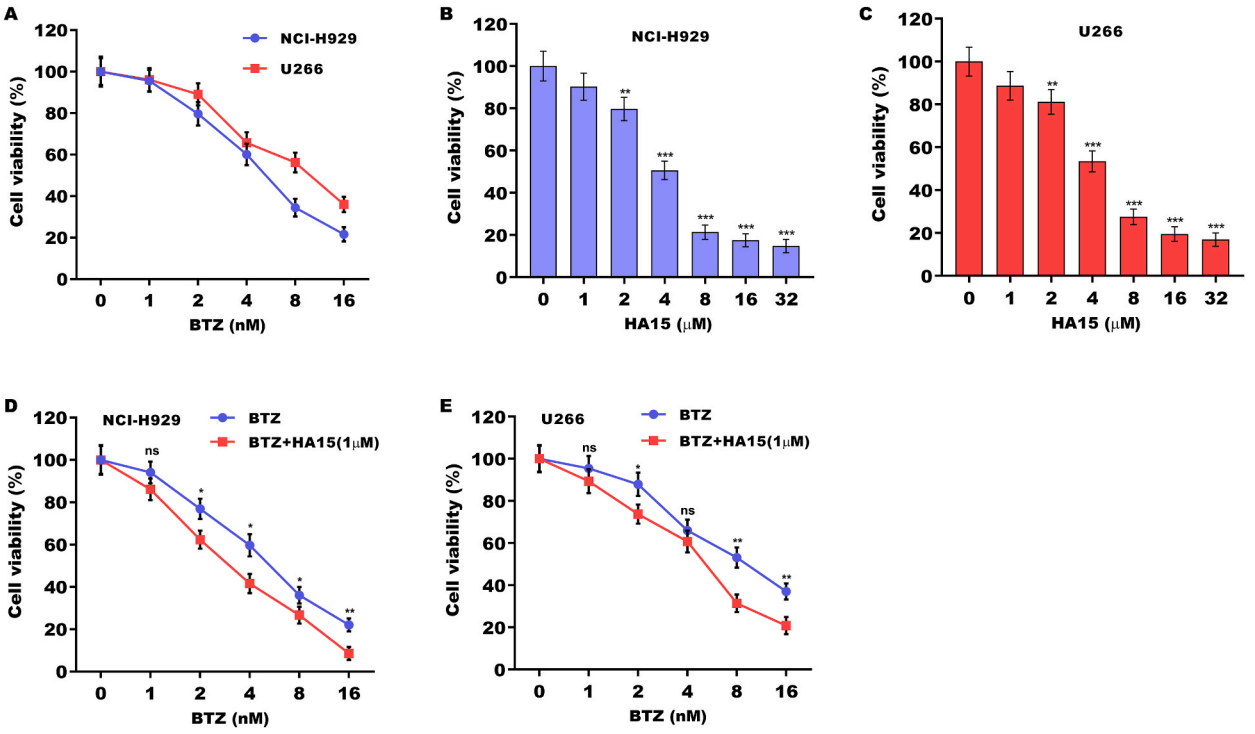

Fig. 1. BTZ combined with HA15 remarkably decreased MM cell viability. (A) NCI–H929 and U266 cells were treated with different doses of BTZ for 48 h and then cell viability was assessed using CCK-8. NCI–H929 (B) and U266 (C) cells were treated with different doses of HA15 for 48 h and then cell viability was assessed using CCK-8. NCI–H929 (D) and U266 (E) cells were treated with different doses of BTZ in the presence or absence of HA15 (1 μM) and then cell viability was assessed using CCK-8. The Student's *t*-test was used to analyze the difference between two groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, no significant.

viability was first assessed in two MM cell lines (NCI–H929 and U266), respectively. As shown in Fig. 1A, BTZ treatment effectively decreased MM cells viability in a dose-dependent manner. Fig. 1B and C showed that 2–32 μM of HA15 showed a significant cytotoxicity to MM cells, but low dose of HA15 (1 μM) did not possess the potential to repress cell viability. Interestingly, 1 μM of HA15 synergistically increased the cytotoxicity of BTZ to the two MM cell lines (Fig. 1D and E).

3.2. HA15 synergized with BTZ to repress MM cell colony formation ability

To further assess whether HA15 increased the sensitivity of MM cells to BTZ, NCI–H929 and U266 cells were treated with 4 nM of BTZ in the presence or absence of HA15 (1 μM), and then colony formation assay was carried out to determine the effect of BTZ, HA15, and BTZ combined with HA15 on regulating MM cell growth. Fig. 2A and B showed that 1 μM of HA15 did not affect NCI–H929 cell growth compared with control, but BTZ significantly repressed NCI–H929 cell growth compared with control. Importantly, BTZ combined with HA15 remarkably decreased NCI–H929 cell growth compared with BTZ monotherapy (Fig. 2A and B). Similarly, BTZ combined with HA15 remarkably decreased U266 cell growth compared with BTZ monotherapy (Fig. 2C and D).

3.3. HA15 synergized with BTZ to accelerate MM cell apoptosis

The effect of BTZ, HA15, and BTZ combined with HA15 on regulating MM cell apoptosis was next assessed using TUNEL assay and Western blot analysis. Fig. 3A and B showed that 1 μM of HA15 did not affect NCI–H929 cell apoptosis compared with control, but BTZ significantly accelerated NCI–H929 cell growth compared with control. Importantly, BTZ combined with HA15 further accelerated NCI–H929 cell apoptosis compared with BTZ monotherapy (Fig. 3A and B). Similarly, BTZ combined with HA15 remarkably accelerated U266 cell apoptosis compared with BTZ monotherapy (Fig. 3C and D). The protein expression of Bax and Bcl-2 was assessed using Western blot analysis in NCI–H929 and U266 cells after treatment with BTZ, HA15, and BTZ combined with HA15. Fig. 3E–H showed that BTZ combined with HA15 further enhanced Bax protein expression and reduced Bcl-2 protein expression in NCI–H929 (Fig. 3E and F) and U266 (Fig. 3G and H) cells compared with BTZ monotherapy. These results demonstrate that HA15 synergizes with

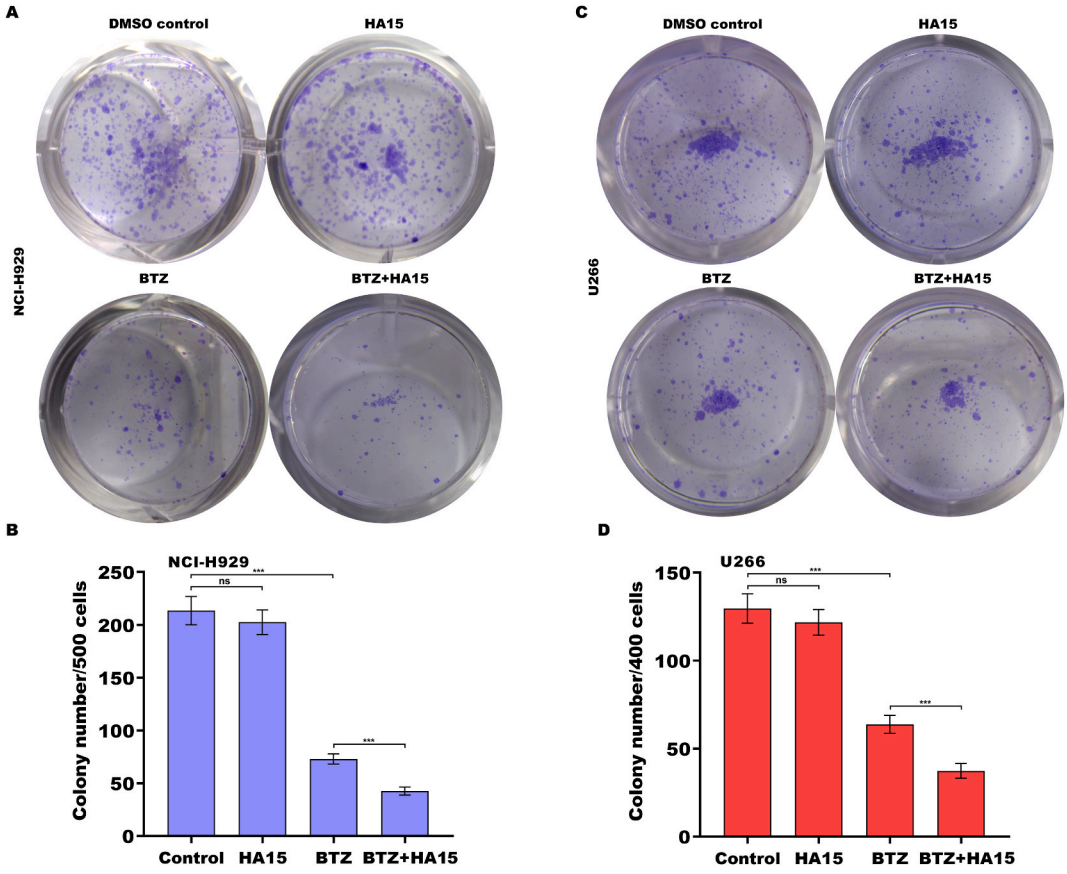

Fig. 2. HA15 synergized with BTZ to repress MM cellgrowth. NCI–H929 cell colony formation ability (A) was assessed after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM) and quantitative analysis (B) was carried out. U266 cell colony formation ability (C) was assessed after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM) and quantitative analysis (D) was carried out. The ANOVA (Scheffé test) was used to analyze the difference among groups. ***$p < 0.001$, ns, no significant.

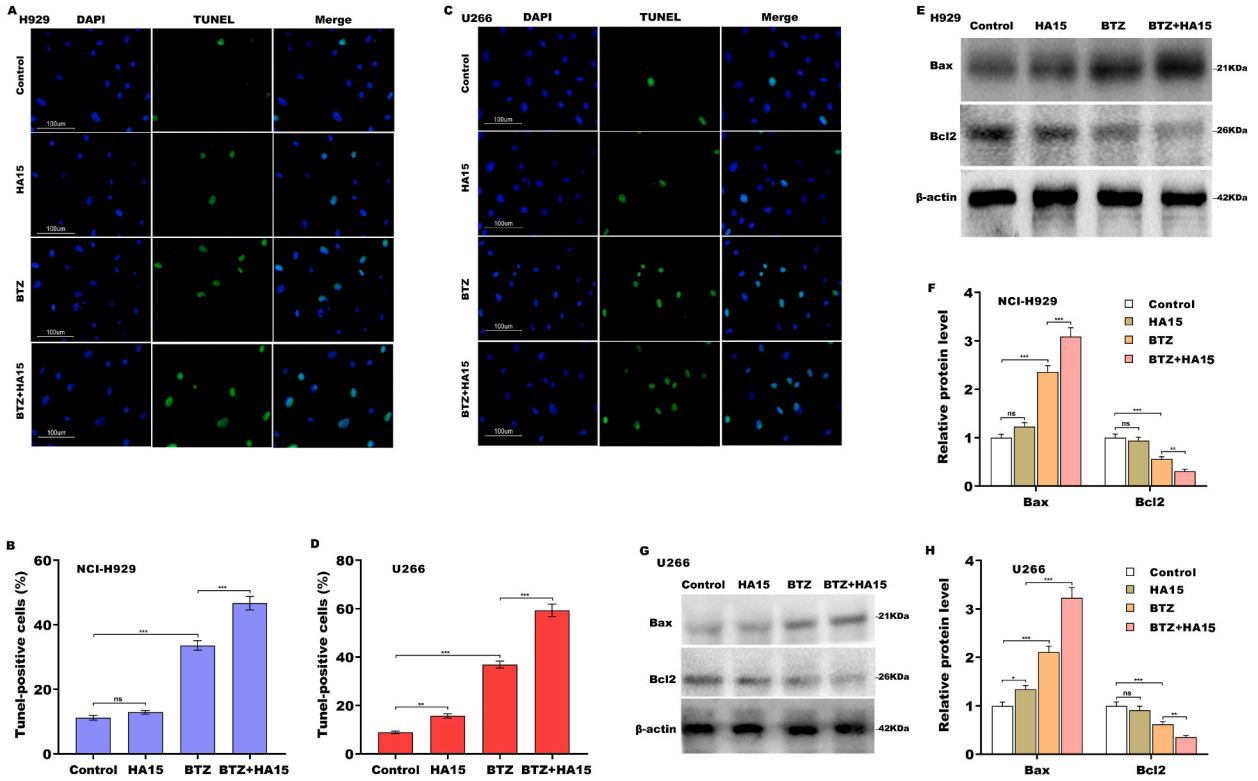

Fig. 3. HA15 synergized with BTZ to accelerate MM cellapoptosis. NCI–H929 cell apoptosis (A) was assessed after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM) and quantitative analysis (B) was carried out. U266 cell apoptosis (C) was assessed after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM) and quantitative analysis (D) was carried out. Western blot (E) and quantitative analysis (F) of Bax and Bcl2 protein expression in NCI–H929 cells after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM). Western blot (G) and quantitative analysis (H) of Bax and Bcl2 protein expression in U266 cells after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM). The ANOVA (Scheffé test) was used to analyze the difference among groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, no significant.

BTZ to accelerate MM cellapoptosis.

3.4. HA15 further increased BTZ-induced ER stress in MM cells

Given that HA15 is a potent inhibitor of GRP78 [24], which exerts a critical role in regulating ER stress and UPR [28,29], we next explored whether HA15 further increases BTZ-triggering ER stress. To this end, MM cells were treated with 4 nM of BTZ in the presence or absence of HA15 (1 μM) and then ER stress was assessed by analyzing the expression of ER stress markers. Low dose of HA15 did not trigger ER stress in NCI–H929 cells, whereas BTZ induced a significant ER stress compared with control (Fig. 4A and B). As expected, BTZ combined with HA15 further triggered ER stress in NCI–H929 cells compared with BTZ monotherapy (Fig. 4A and B). Similarly, BTZ combined with HA15 also promoted ER stress in U266 cells compared with BTZ monotherapy (Fig. 4C and D).

3.5. UPR inhibitor damaged the effect of BTZ combined with HA15 on inducing MM cell death.

Finally, the biological role of UPR activation in BTZ combined with HA15-triggered MM cell death was explored. To this end, MM cells were treated with BTZ combined with HA15 in the presence or absence of GSK2606414, a selective PERK inhibitor, and cell viability was assessed using CCK8 assay. Fig. 5A and B showed that GSK2606414 markedly decreased the effect of BTZ combined with HA15 on accelerating NCI–H929 and U266 cell death. Furthermore, combination treatment with BTZ and HA15 inhibited tumor growth more effective than BTZ alone, while these effects were blocked by GSK2606414 (Fig. 5C and D). There was no significant difference in body weight among the groups (Fig. 5E). These results demonstrate that HA15 increases the effect of BTZ on eradicating MM cells through triggering ER stress.

4. Discussion

As a specific inhibitor of GRP78/BiP, HA15 can eradicate MM cells and overcome resistance of MM cells to BRAF inhibitor [24]. HA15 also sensitizes adrenocortical carcinoma cells to mitotane by activating ER stress [25]. Given the role of BTZ in eradicating MM cells and activating ER stress, here we investigated whether combination treatment with HA15 and BTZ accelerates MM cell death more efficient than BTZ monotherapy. The current results demonstrate that, i) Combined treatment with BTZ and HA15 decreases MM

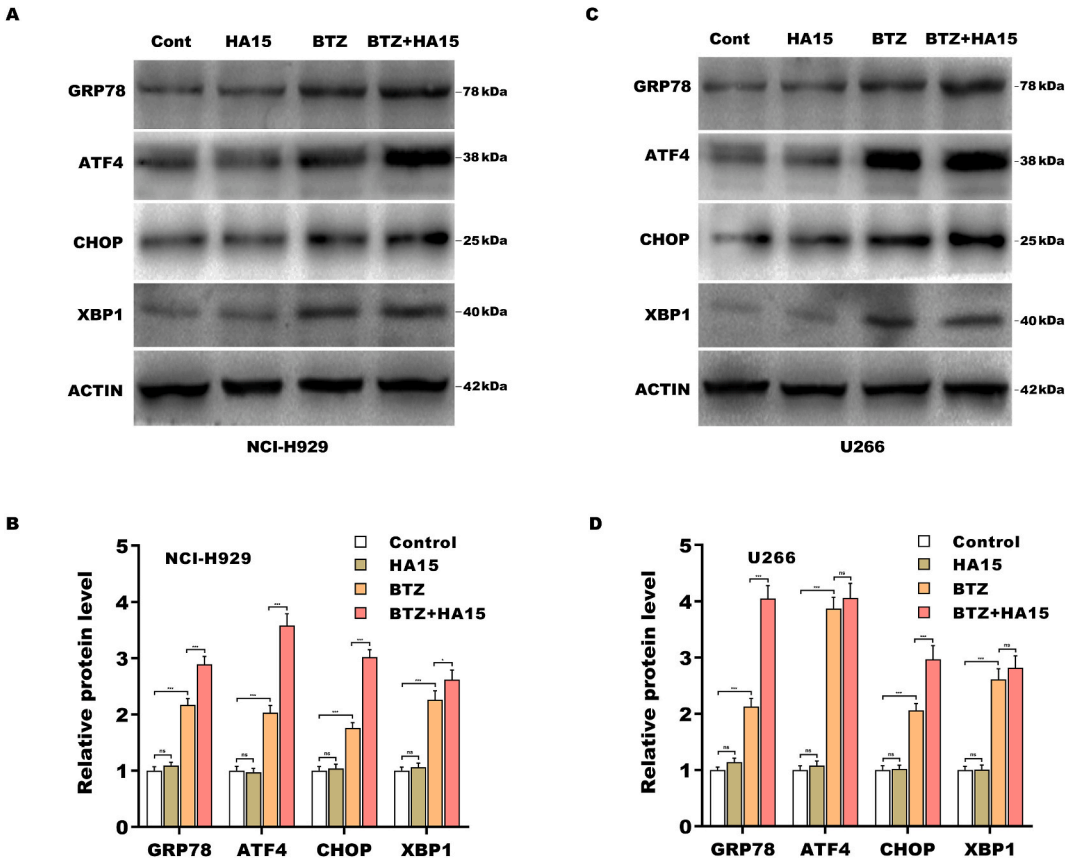

Fig. 4. HA15 further increased BTZ-induced ER stress in MM cells. Western blot (A) and quantitative analysis (B) of GRP78, ATF4, CHOP, and XBP protein expression in NCI–H929 cells after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM). Western blot (C) and quantitative analysis (D) of GRP78, ATF4, CHOP, and XBP protein expression in U266 cells after treatment with BTZ (4 nM) in the presence or absence of HA15 (1 μM). The ANOVA (Scheffé test) was used to analyze the difference among groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, no significant.

cell viability and colony formation ability compared with BTZ alone, ii) HA15 synergizes with BTZ to accelerate MM cellapoptosis, iii) HA15 further increases BTZ-induced ER stress in MM cells, iv) UPR inhibitor damages the effect of BTZ combined with HA15 on accelerating MM cell death. These results reveal that HA15 increases the therapeutic effect of BTZ on patients with MM by inducing ER stress.

In accordance with cell context and ER stress extent, ER stress exerts a double-edged sword in maintaining cell survival [30]. Basal ER stress is required for cancer cell survival because rapid proliferation inevitably results in an excessive amassing of misfolded proteins in ER. After ER stress activation, UPR and ERAD is initiated to maintain cellular homeostasis through degrading misfolded proteins [31–33]. ER stress is also closed associated with chemotherapy resistance in different types of cancer. Several critical proteins in UPR pathway, including GRP78, ATF4, and XPB1, regulate drug resistance. Pharmacological or genetic inhibition of ATF4 facilitates resistance of liver cancer cells to BTZ, whereas ATF4 over-activation sensitizes cancer cells to BTZ [17]. Low XBP1 level leads to BTZ resistance in MM and thus measurement of XBP1 level can predict BTZ resistance [16]. Given the positive correlation of low XBP1 and ATF4 level with ER stress, we conclude that MM cells acquire BTZ resistance partly through alleviating ER stress activation. Therefore, it is vital to further increase ER stress extent by pharmacological intervention in BTZ-resistant MM cells.

GRP78 (also known as BiP or HSPA5), located at ER membrane, acts as a critical function on protein folding, assembly, and degradation [8]. GRP78 is necessary to regulate UPR through evading retention mechanisms and translocating to cell surface [34]. Cell surface GRP78 (csGRP78) expression is upregulated with disease progression and strongly increased in refractory MM [35]. Mounting evidence has demonstrated that GRP78 contributes to cancer cell survival and chemoresistance through correcting misfolded proteins and facilitating recovery from ER stress [34,36], and that GRP78 inhibition is an effective strategy to kill cancer cells. Pharmacological intervention of GRP78 by HA15 accelerates lung cancer cell apoptosis through activating ER stress and autophagy [26]. Combined treatment with HA15 and mitotane accelerates adrenocortical carcinoma cell death through activating ER stress pathways [25]. HA15 also overcomes intrinsic resistance of glioblastoma cells to Ubiquitin-activating enzyme 1 inhibitor TAK-243 [21]. However, little is known whether HA15 can sensitize MM cells to BTZ by further increasing of ER stress. In the study we demonstrated that combination treatment with HA15 and BTZ remarkably decreased MM cell viability and clone formation ability, and accelerated MM cell apoptosis compared with BTZ monotherapy, though low dose of HA15 did not exhibit a significant cytotoxicity to MM cells. Mechanistically,

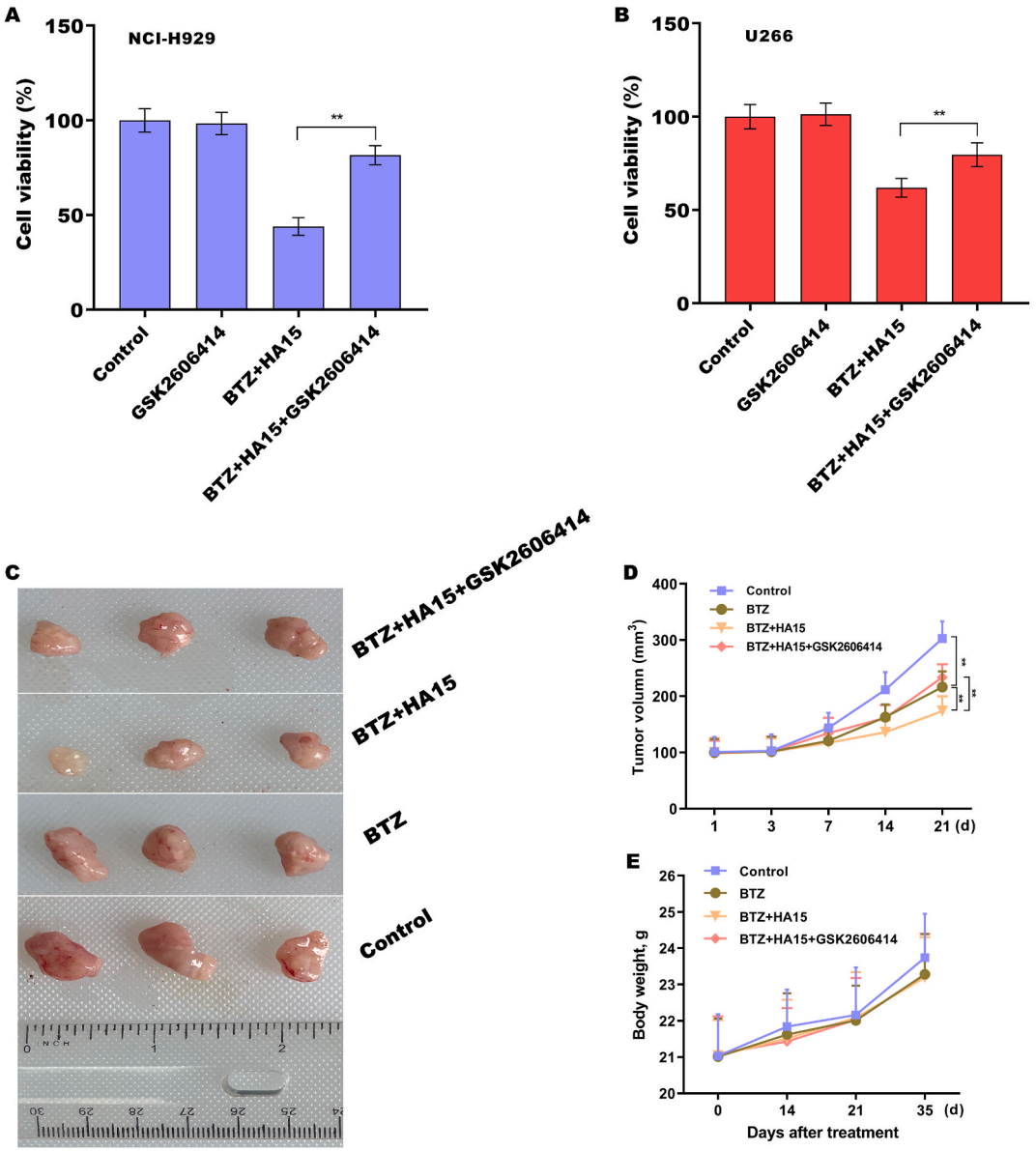

Fig. 5. GSK2606414 damaged the effect of BTZ combined with HA15 on inducing MM cell death.NCI–H929 (A) and U266 (B) cells were treated with BTZ (4 nM) and HA15 (1 μM) in the presence or absence of GSK2606414 (1 μM) for 48 h, and then cell viability was assessed using CCK-8. (C and D) MM mouse xenograft model was generated through subcutaneously injecting NCI–H929 cells into nude mice. When tumor volume reached approximately 100 mm^3, tumor-bearing mice were treated with i.p. injection of BTZ (1 mg/kg twice weekly), i.p. injection of BTZ (1 mg/kg twice weekly) and HA15 (0.5 mg/kg twice weekly), i.p. injection of BTZ (1 mg/kg twice weekly) and HA15 (0.5 mg/kg twice weekly), and oral gavage of GSK2606414 (50 mg/kg once per day), and tumor volume was calculated at the indicated time. (E) Body weight was surveyed at the different time points. The ANOVA (Scheffé test) was used to analyze the difference among groups. **$p < 0.01$.

HA15 synergized with BTZ to further increase ER stress. Therefore, UPR inhibitor significantly damaged the effect of combination treatment on accelerating MM cell death. Conclusion: These data suggest that combination treatment with BTZ and HA15 is an effective strategy to treat MM patients that fail to respond to BTZ monotherapy.

Author contribution statement

Yirong Chen: Performed the experiments; Wrote the paper.
Yuchen Tao: Performed the experiments; Analyzed and interpreted the data.
Kexin Hu: Analyzed and interpreted the data.
Jiahui Lu: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supp. material/referenced in article.

Ethics approval and consent to participate

Not applicable.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e19806.

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