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Induction of endoplasmic reticulum stress by bortezomib sensitizes myeloma cells to DR5-mediated cell death

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TNF-related apoptosis-including ligand/Apo2 (TRAIL)-mediated immunotherapy is an attractive anti-tumor modality with high tumor specificity. In order to improve its therapeutic efficacy, we further need to implement a novel maneuver for sensitization of malignant cells to TRAIL. Bortezomib (BTZ), a novel anti-myeloma (MM) agent, potently induces endoplasmic reticulum (ER) stress to cause apoptosis. Here, we explored the roles of BTZ in the cytotoxicity of anti-TRAIL receptor agonistic antibodies against MM cells with special reference to ER stress. BTZ enhanced the expression of death receptor 5 (DR5) but not DR4 in MM cells at surface protein as well as mRNA levels. However, the DR5 expression was not affected by BTZ without ER stress induction in MM cells with a point mutation in a BTZ-binding proteasome β_5 subunit. Tunicamycin, an ER stress inducer, was able to enhance the DR5 expression even in the BTZ-resistant MM cells, suggesting the role of ER stress in up-regulation of DR5 expression. Interestingly, BTZ facilitated extrinsic caspase-mediated apoptosis by anti-DR5 agonistic antibody in MM cells along with reducing c-FLICE-like interleukin protein, a caspase 8 inhibitor. These results suggest that BTZ enhances DR5 expression and its downstream apoptotic signaling through ER stress to sensitize MM cells to TRAIL-mediated immunotherapy.

Key words: multiple myeloma, bortezomib, TRAIL, DR5, ER stress

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Introduction

Multiple myeloma (MM) still remains incurable because of its resistance to chemotherapeutic drugs and an escape from tumor immune surveillance, although new agents as well as high dose chemotherapy followed by stem cell transplantation have been introduced into a clinical practice with better quality of therapeutic response and outcome. Therefore, alternative approaches are needed to overcome drug resistance to improve the therapeutic outcome in patients with MM.

Immunotherapies have been getting generally accepted as attractive treatment options for yet incurable malignancies by conventional chemotherapeutic agents. One such approach is a TNF-related apoptosis-including ligand/Apo2 (TRAIL)-mediated immunotherapy[1–3]. TRAIL binds to two different proapoptotic receptors, death receptor 4 (DR4) and DR5. Unlike Fas ligand and TNF-α, TRAIL is able to induce cell death in malignant cells with marginally affecting normal tissues; TRAIL-mediated immunotherapy is, therefore, regarded as an attractive tumor-specific strategy against various cancers, including MM[4–6].

However, weak expression of the TRAIL receptors as well as the suppression of their downstream pro-apoptotic signaling often cause malignant cell resistance to TRAIL; and sensitization of malignant cells to TRAIL has become a major issue in the TRAIL-mediated immunotherapy. To restore the sensitivity to TRAIL, we need to develop novel therapeutic maneuvers to up-regulate surface TRAIL receptors along with stimulation of DR-mediated pro-apoptotic signaling.

The proteasome inhibitor bortezomib (BTZ) is widely used in treatment of MM with improved response rates in patients with both relapsed/refractory and newly diagnosed MM [7]. BTZ induces misfolded protein accumulation in MM cells followed by endoplasmic reticulum (ER) stress-associated apoptosis [8,9]. However, the effects of ER stress induced by BTZ on TRAIL-mediated MM cell death are largely unknown. In the present study, we therefore aimed to clarify the role of BTZ on TRAIL receptor editing and TRAIL-mediated cell death in MM cells with special reference to ER stress. We demonstrated here that BTZ enhanced the surface expression of DR5 but not DR4 in MM cells and its downstream apoptotic signaling through the induction of ER stress to sensitize MM cells to an anti-DR5 agonistic antibody.

Materials and Methods

Reagents

Bortezomib was purchased from Millenium Pharmaceuticals, Inc. (Cambrigde, MA, USA). Rabbit monoclonal antibodies against caspase 9, caspase 3, cleaved caspase 3, poly (ADP-ribose) polymerase (PARP), and mouse monoclonal antibodies against activating transcription factor 4 (ATF4) and C/EBP-homologous protein (CHOP) were purchased from Cell Signaling Technology Japan (Tokyo, Japan). Mouse monoclonal antibodies against caspase 8, c-FLICE-like interleukin protein (c-FLIP) and β-actin were obtained from Medical and Biotechnological Laboratories (Nagoya, Japan), Santa Cruz Biotechnology (Santa Cruz, CA), Abcam (Cambridge, UK) and Sigma (Saint Louis, MO), respectively. FITC-conjugated mouse monoclonal antibodies against human DR4 and DR5 were from Biolegend (San Diego, CA). Horseradish peroxidase-conjugated goat anti-mouse antibody was from Invitrogen Life Technologies (Carlsbad, CA). The human monoclonal anti-DR5 agonistic antibody R2-E11 was a kind gift from from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan).

Cells and cultures

The use of human samples was approved by the Institutional Review Board at University of Tokushima (Tokushima, Japan), and informed consent was obtained according to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh peripheral blood from healthy donors [10]. Primary MM cells were purified from bone marrow mononuclear cells (BMMCs) using CD138 microbeads and a magnetic cell sorting system (Miltenyi Biotec, Auburn, CA). Human MM cell lines, RPMI 8226, KMS-11 and U266, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human MM cell line OPC was established in our laboratory [11]. The human MM cell line INA-6 was kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany). The human MM cell lines OPM-2 was purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). BTZ-resistant MM cell lines with a point mutation in the β_5 subunit of a 26S proteasome, KMS-11/BTZ and OPM-2/BTZ, were kindly provided from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan) [12]. Cells were cultured in RPMI1640 (Sigma) supplemented with 5% FCS (Life Technologies, Grand island, NY), penicillin (100 units/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO_2.

Flow cytometry

Cell preparation and staining for flow cytometry were performed as described previously [13]. Briefly, cells were incubated in 100 μl PBS with 2% human γ-globulin with saturating concentrations of different FITC-conjugated monoclonal antibodies on ice for 40 minutes. They were then washed and analyzed by flow cytometry using EPICS-Profile (Coulter Elecronics, Hialeah, FL).

Cell viability and apoptosis assay

MM cells were incubated with various concentrations of BTZ with or without TRAIL agonistic antibody at 37°C for 48 hours. Viable cell numbers were measured by a cell proliferation assay using 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (WST-8; Kishida Chemical, Osaka, Japan). Apoptosis in MM cells was evaluated by staining the cells with an annexinV-FITC and propidium iodide labeling kit (MEBCYTO Apoptosis Kit; MBL, Nagano, Japan) according to the manufacturer's instruction.

Western blot analysis

Cells were collected and lysed in a lysis buffer (Cell Signaling, Beverly, MA) supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Sigma). The cell lysates were subjected to SDS-PAGE on a 10% polyacrylamide gel, and then transferred to polyvinylidene difluoride membranes (Millpore, Billerica, MA). The membranes were blocked with 5% non-fat dry milk in TBS with 0.01% Tween 20 for 1 hour at room temperature and incubated for 16 hours at 4°C with the primary antibodies. After washing, a secondary horseradish peroxidase-conjugated antibody was added

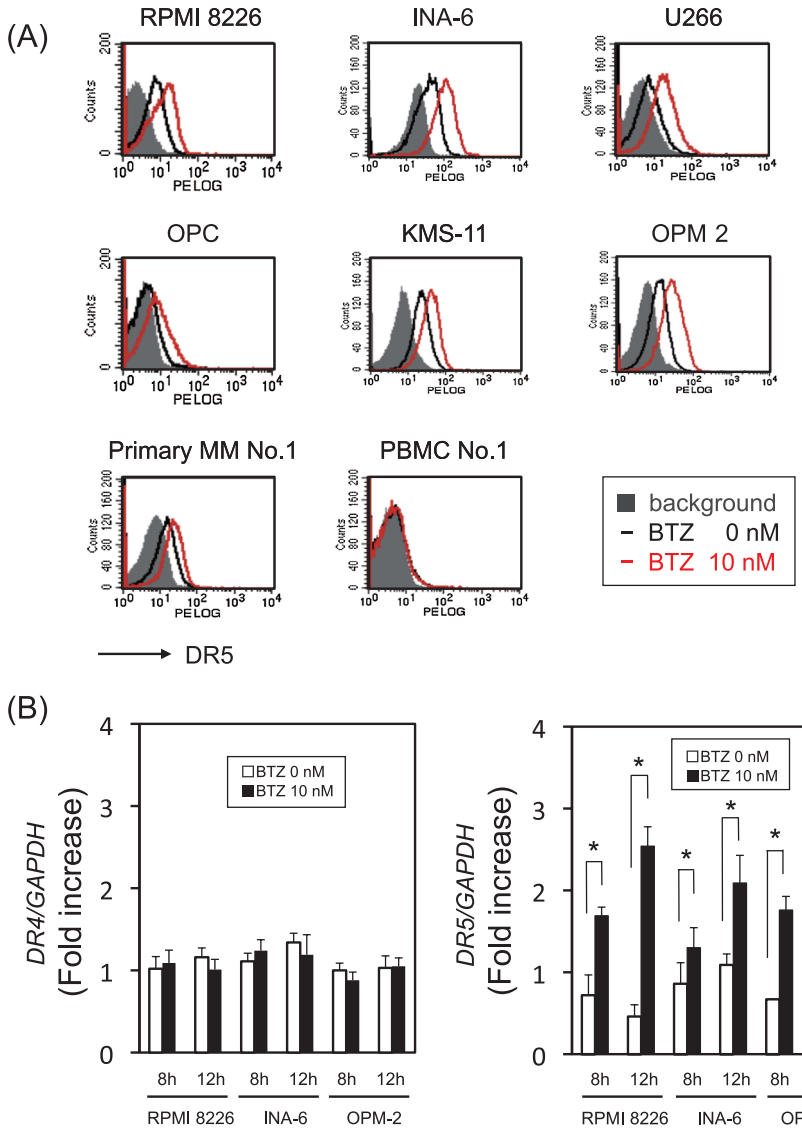

Figure 1. Up-regulation of DR5 expression in MM cells by BTZ. (A) MM cell lines, primary MM cells, and peripheral blood mononuclear cells (PBMCs) were incubated with BTZ at 10 nM for 24 hours, and the surface expression of DR5 was analyzed by flow cytometry. (B) RPMI 8226, INA-6 and OPM-2 cells were incubated with BTZ at 10 nM for different periods as indicated. *DR4* and *DR5* mRNA expression was determined by real time RT-PCR. *GAPDH* was used as an internal control. *$P < 0.05$.

and the membranes were developed using the enhanced chemiluminescence plus Western blotting detection system (American Biosciences, Piscataway, NJ).

Quantitative real-time PCR

Cells were harvested and total RNA was extracted from cells using TRIZOL reagent (Invitrogen). Equal amounts of total RNA were subjected to reverse transcription using Superscript II (Invitrogen). Real-time PCR was performed using Platinium SYBR Green qPCR SuperMix UDG with Rox (Invitrogen) with the following amplification program: one cycle of 50°C for 2 minutes and 95°C for 2 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. The reaction was followed by a melting curve protocol according to the specifications of the ABI 7300 (Applied Biosystems, Foster City, CA, USA). Primers used were as follows: *DR4* sense 5′-AAGTTTGTCGTC GTCGGGGTCCT-3′ and antisense 5′-GGTGGACACACTCTCCCA AAGGGC-3′, *DR5* sense 5′-TCTCCTGAGATGTGCCGGAAGTGCC-3′ and antisense 5′-GCTGGGACTTCCCCACTGTGCTTT-3′, *GAPDH* sense 5′-AATCCCATCACCATCTTCCA-3′ and antisense 5′-TGGAC TCCACGACGTACTCA-3′. Products were run on 2% agarose gels containing ethidium bromide.

Statistical analysis

Comparisons between experimental data were performed by one-way analysis of variance (ANOVA) or one-sided, paired t-test. *P* below .05 was considered statistically significant.

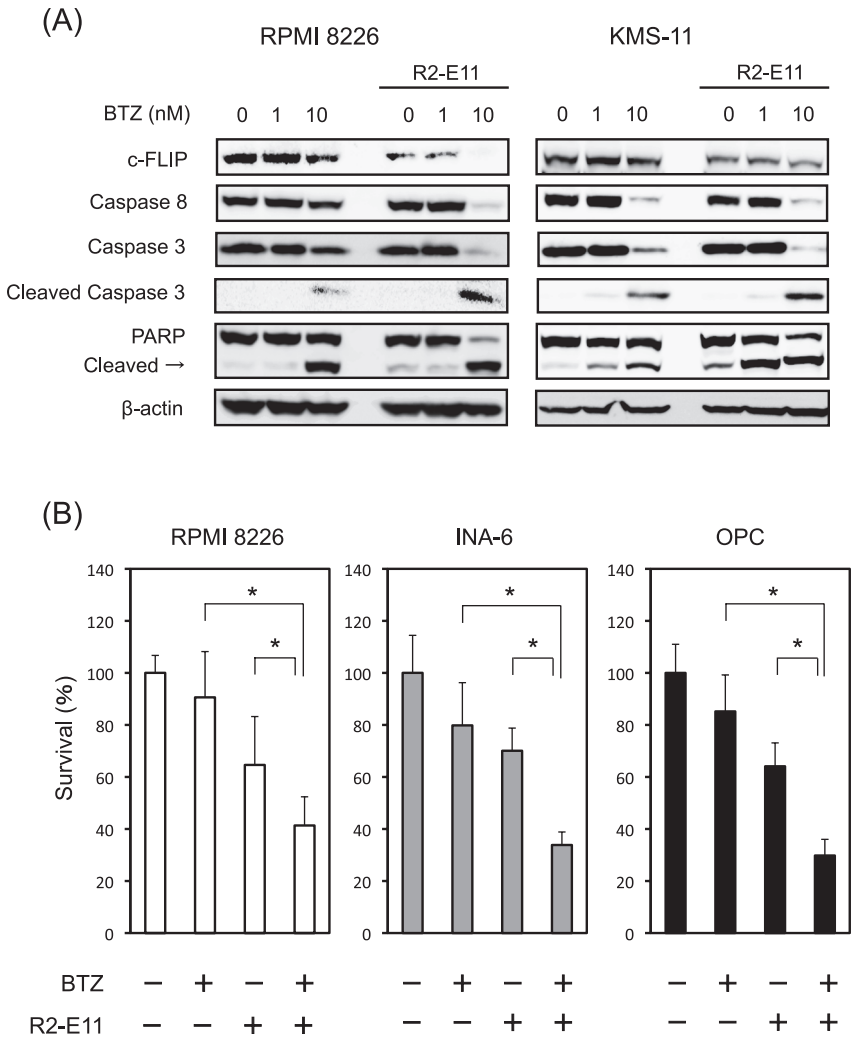

Figure 2. Induction of caspase activation and cell death in MM cells. (A) RPMI 8226 and KMS-11 cells were treated with BTZ at the indicated concentrations or the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL alone or both in combination for 24 hours. The protein levels of c-FLIP, caspase 8, caspase 3, and PARP were analyzed by Western blotting. β-actin was used as a protein loading control. (B) RPMI 8226, INA6 and OPC cells were treated with BTZ or the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL alone or both in combination for 48 hours. Cell viability was analyzed by WST-8 assay. *$P < 0.05$.

Results and Discussion

BTZ up-regulates DR5 expression in MM cells

We first examined whether BTZ affects the surface expression of TRAIL receptors, DR4 and DR5, on MM cells. BTZ at 10 nM up-regulated the surface level of DR5 on primary MM cells as well as all MM cell lines tested (Fig. 1A). However, BTZ did not up-regulate the surface level of DR4 on MM cells (data not shown). Real time RT-PCR demonstrated BTZ increased the *DR5* mRNA expression by BTZ in RPMI 8226, INA-6 and OPM-2 MM cells (Fig. 1B), suggesting the up-regulation of DR5 at transcriptional levels.

BTZ enhances anti-DR5 agonistic antibody-mediated activation of the extrinsic apoptotic pathway and death in MM cells

Because BTZ up-regulates DR5 expression in MM cells, we next looked at the effects of BTZ on anti-DR5 agonistic antibody-mediated activation of the extrinsic apoptotic pathway and death in MM cells. BTZ at 10 nM induced the activation of caspase 8 and caspase 3, and the cleavage of caspase 3 and PARP along with decreasing c-FLIP protein levels in RPMI 8226 and KMS-11 cells (Fig. 2A). Treatment with the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL in combination with BTZ at 10 nM markedly reduced c-FLIP protein levels and enhanced the activation of caspase 8 and the cleavage of caspase 3 and PARP, although the anti-DR5

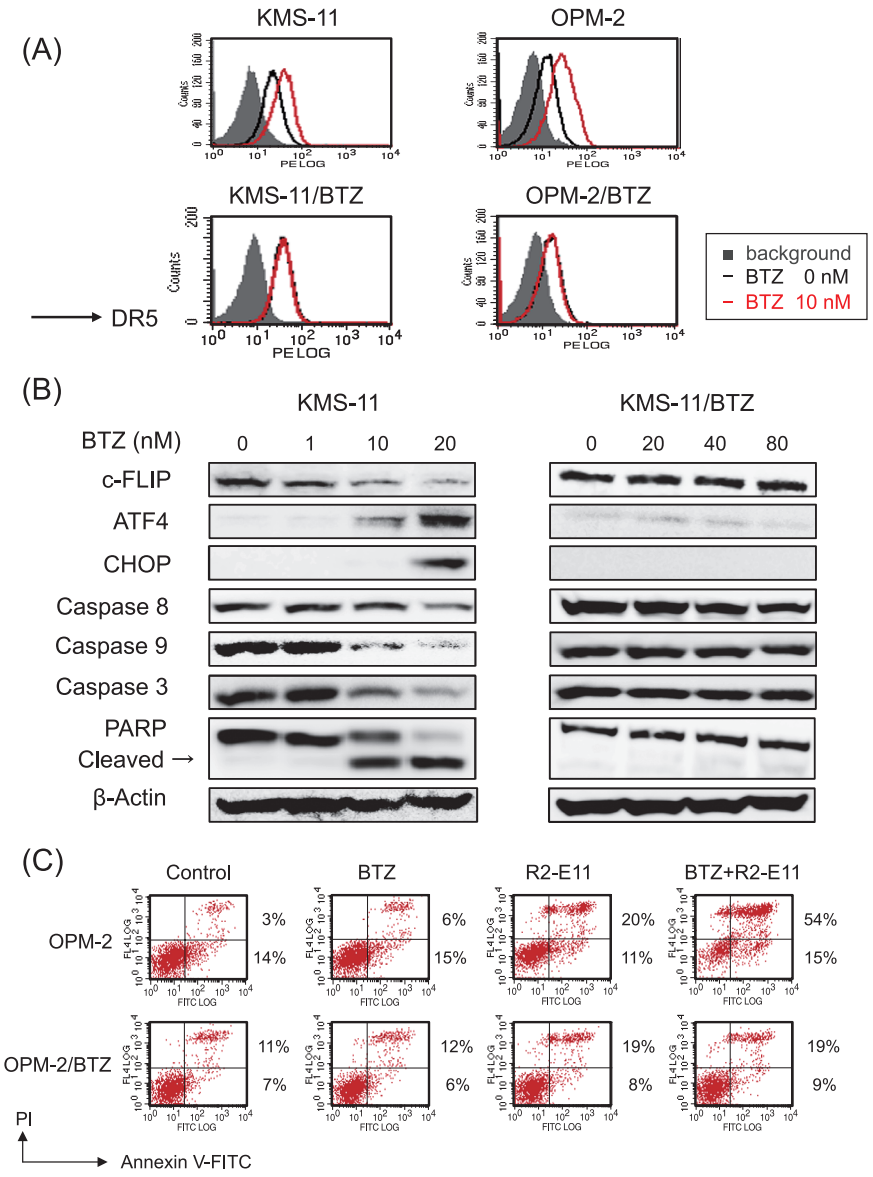

Figure 3. Up-regulation of DR5 as well as ER stress by BTZ was absent in BTZ-resistant MM cells. (A) KMS-11 and OPM-2 cells, and their derived BTZ-resistant cell lines with a point mutation in a β_5 subunit (KMS-11/BTZ and OPM-2/BTZ cells, respectively) were treated with BTZ at 10 nM for 24 hours. The surface expression of DR5 was analyzed by flow cytometry. (B) KMS-11 and KMS-11/BTZ cells were treated with BTZ at the indicated concentrations for 24 hours. The protein levels of c-FLIP, ATF4, CHOP, caspase 8, caspase 9, caspase 3, and PARP were analyzed by Western blotting. β-actin was used as a protein loading control. (C) OPM-2 and OPM-2/BTZ cells were treated with BTZ or the anti-DR5 agonistic antibody R2-E11 at 500 ng/mL alone or both in combination for 48 hours, and stained with annexin V-FITC and propidium iodide (PI). Cells were then analyzed by flow cytometry to determine the percentage distribution of cells displaying annexin V staining (early apoptosis) or both annexin V and PI staining (late apoptosis).

agonistic antibody R2-E11 alone showed only marginal effects on these caspase and PARP cleavage. Consistently, BTZ and R2-E11 in combination cooperatively enhanced cell death in RPMI 8226, INA6 and OPC cells, whereas BTZ or R2-E11 alone at this experimental condition only partially induced MM cell death (Fig. 2B). These results suggest that BTZ potentiates DR5-mediated activation of the extrinsic apoptotic pathway and cell death in MM cells.

BTZ does not up-regulate DR5 expression in MM cells with a β_5 subunit mutation

To further clarify the role of ER stress induced by BTZ on DR5 expression and DR5-mediated cytotoxicity, we examined the effects of BTZ, using KMS-11 and OPM-2 cells with a point mutation in the β_5 subunit of a 26S proteasome, KMS-11/BTZ and OPM-2/BTZ, respectively, which are resistant to BTZ-induced cell death. Although BTZ up-regulated DR5 expression on parental KMS-11 and OPM-2 cells, the DR5 up-regulation by BTZ was completely absent in the BTZ-

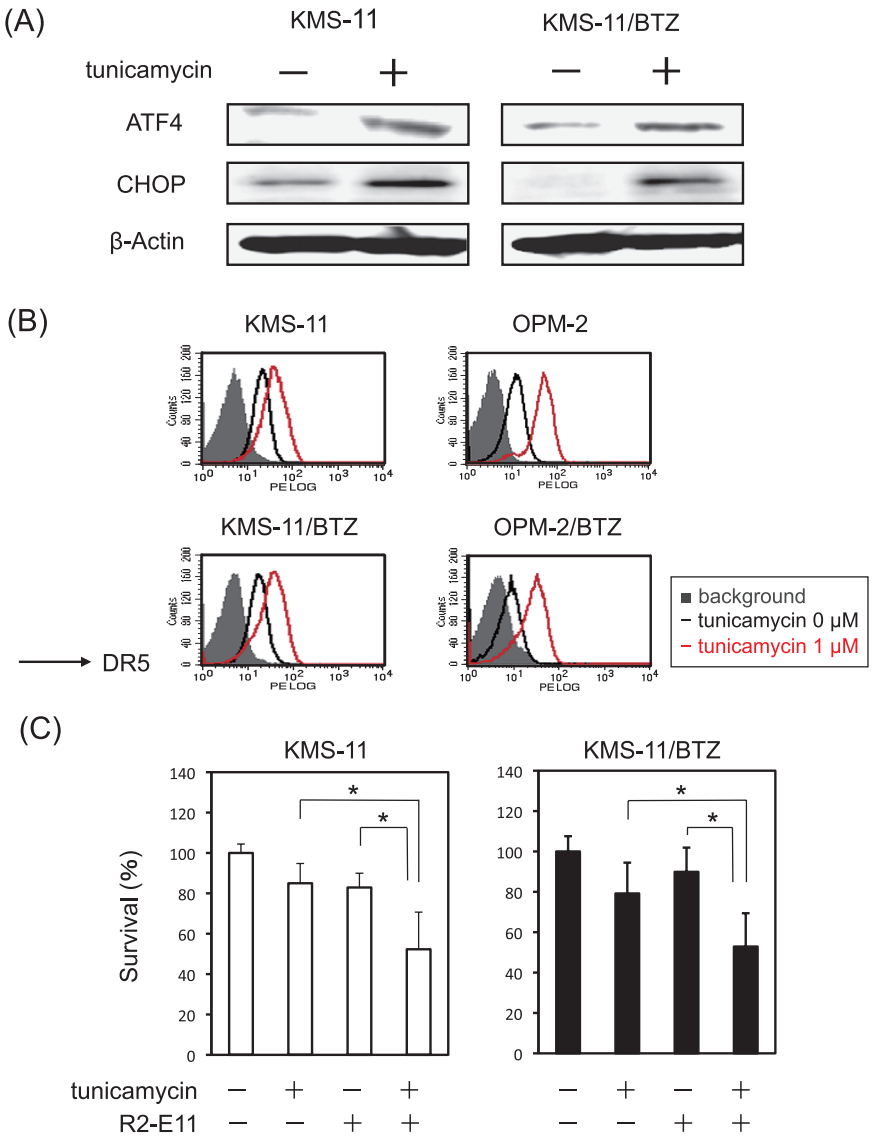

Figure 4. Induction of ER stress and DR5 in BTZ-resistant MM cells by tunicamycin. (A) KMS-11 and KMS-11/BTZ cells were incubated with tunicamycin at 1 μM for 24 hours. Cell lysates were then extracted, and the protein levels of ATF4 and CHOP were analyzed by Western blotting. (B) KMS-11 and OPM-2 cells, and their derived BTZ-resistant cell lines (KMS-11/BTZ and OPM-2/BTZ cells, respectively) were treated with tunicamycin at 1 μM for 24 hours. The surface expression of DR5 was analyzed by flow cytometry. (C) KMS-11 and KMS-11/BTZ cells were treated with tunicamycin at 1 μM or the anti-DR5 agonistic antibody R2-E11 at 100 ng/mL alone or both in combination for 48 hours. Cell viability was analyzed by WST-8 assay.*$P < 0.05$.

resistant KMS-11/BTZ and OPM-2/BTZ cells (Fig. 3A). Treatment with BTZ at 10 nM or more increased ATF4 and CHOP protein levels along with the activation of caspase 8, caspase 9 and caspase 3, and the cleavage of PARP in KMS-11 cells (Fig. 3B). However, these effects of BTZ were not observed in the BTZ-resistant KMS-11/BTZ cells. Consistently, BTZ did not induce apoptosis in the OPM-2/BTZ cells (Fig. 3C). The cytotoxic effects of the anti-DR5 agonistic antibody R2-E11 were equally observed in the parental and mutated OPM-2 cells. However, the enhancement of cell death by R2-E11 in combination with BTZ was only observed in the parental OPM-2 cells but not in the mutated ones. Therefore, the induction of ER stress and thereby DR5 up-regulation appears to be responsible for the enhancement of anti-MM effects of the combinatory treatment with an anti-DR5 agonistic antibody and BTZ.

DR5 expression is up-regulated in the BTZ-resistant MM cells under ER stress by tunicamycin

In order to further clarify the relationship between ER stress and the up-regulation of DR5, we looked at the effects of ER stress induced by the ER stress inducer tunucamycin on DR5 expression and death in the parental and β_5 subunit-mutated MM cells. Tunicamycin at 1 μM was able to increase ATF4 and

CHOP protein levels in BTZ-resistant β_5 subunit-mutated KMS-11/BTZ cells as well as their parental cells (Fig. 4A), and up-regulate the DR5 expression on the surface of both the parental and mutated KMS-11 and OPM-2 cells (Fig. 4B). Consistent with the up-regulation of the surface DR5 expression, tunicamycin at 1 μM was able to induce significant cytotoxic effects equally on both KMS-11 cells and bortezomib-resistant KMS-11/BTZ cells in combination with R2-E11 at 100 ng/mL, although tunicamycin or R2-E11 alone only minimally affected the viability of these cells in this experimental condition (Fig. 4C). These results further corroborated the critical role of ER stress in the up-regulation of DR5 in MM cells and anti-DR5 agonistic antibody-mediated MM cell death.

Because *DR5* has been demonstrated to be one of the target genes of CHOP [14,15] and ATF3 [16] induced downstream of ATF4, the up-regulation of DR5 in MM cells by BTZ is suggested at least in part due to the induction of ATF4 through ER stress. Collectively, BTZ enhances DR5 expression and its downstream apoptotic signaling through ER stress to sensitize MM cells to TRAIL-mediated immunotherapy. Furthermore, BTZ also induces death receptor-independent apoptosis as a result of excessive ER stress, which may cooperatively enhance MM cell death in combination with anti-DR5 agonistic antibody.

We have previously reported that MM cells post-translationally down-modulate the cell surface expression of DR4 but not DR5 through ectodomain shedding by endogenous TNF-α converting enzyme (TACE), and that TACE inhibition is able to restore cell surface DR4 levels and the susceptibility of MM cells to TRAIL or an agonistic antibody against DR4 [17]. TACE-mediated shedding appears to be an important mechanism for the reduction of surface DR4 levels on MM cells, which may blunt TRAIL-mediated apoptosis by surrounding immune cells expressing TRAIL to protect MM cells. Thus, DR4 and DR5 editing and expression on the surface of MM cells appear to be differentially regulated. BTZ and TACE inhibitors seem good options to revitalize TRAIL-mediated immunotherapy whose therapeutic efficacy has been limited as a single treatment modality. The combination of TRAIL-mediated immunotherapy with BTZ and/or TACE inhibitors is warranted for further study in patients with MM.

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Conflicts of Interest Disclosures

The authors declare no competing financial interests related to this work.

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