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Graphical abstract

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ISG20L2 Suppresses Bortezomib Anti-Myeloma Activity by Attenuating Bortezomib Binding to PSMB5

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Conflict of Interest Statement

The authors have declared that no conflict of interest exists.
Abstract

The proteasome inhibitors (PIs) bortezomib and carfilzomib, which target proteasome 20S subunit beta 5 (PSMB5) in cells, are widely used in multiple myeloma (MM) treatment. In this study, we demonstrated the role of interferon-stimulated 20 kD exonuclease-like 2 (ISG20L2) in MM PI resistance. Gain- and loss-of-function studies showed that ISG20L2 suppressed MM cell sensitivity to PIs in vitro and in vivo. Patients with ISG20L2^{low} MM had a better response to PIs and a longer overall survival than patients with ISG20L2^{high} MM. Biotinylated-bortezomib pull-down assays showed that ISG20L2 competed with PSMB5 in binding to bortezomib. The surface plasmon resonance (SPR) assay further confirmed the direct binding of bortezomib to ISG20L2. In ISG20L2^{high} MM cells, ISG20L2 attenuated the binding of bortezomib to PSMB5, resulting in lower inhibition of proteasome activity and therefore less bortezomib-induced cell death. Overall, we identified a novel mechanism by which ISG20L2 conferred bortezomib resistance on MM. The expression of ISG20L2 correlated with MM PI responses and patient treatment outcomes.
Introduction

Multiple myeloma (MM) is a refractory hematological cancer characterized by monoclonal plasma cell accumulation in bone marrow(1). Over the past decades, the use of proteasome inhibitors (PIs) has significantly improved the prognosis of MM patients. Bortezomib (BTZ), also known as Velcade or PS-341, was the first PI approved by the United States Food and Drug Administration for treating MM. Due to its effectiveness and good tolerability, BTZ has become central to various MM treatment regimens(2). However, BTZ resistance is common in patients with relapsed and refractory MM, and the underlying mechanisms are still not fully understood.

MM with chromosome 1q amplification (+1q) is considered high risk and is likely to respond poorly to BTZ(3-5). We identified interferon-stimulated 20 kD exonuclease-like 2 (ISG20L2) in our study of prognostic genes in 1q. ISG20L2 is in the 1q23 region of chromosome 1. Upregulation of ISG20L2 was previously found in 1q-amplified MM patients(6). Another report showed that ISG20L2 was among the top 34 genes that could predict a high risk of progression from smoldering MM to symptomatic MM(7). Nevertheless, the roles of ISG20L2 in MM progression remain obscure. ISG20L2 is known as an exoribonuclease(8). Microarray-based analysis showed that ISG20L2 expression correlated with overall survival (OS) in MM. In this study, we show that ISG20L2 confers BTZ resistance on MM cells via a novel mechanism.
Results

Identification of Chromosome 1q Prognostic Gene Expression in Multiple Myeloma

MM with +1q resulted in the amplification of multiple 1q genes (9). Therefore, we included all protein-encoding genes in 1q (1q12-1q44) in our analysis. According to the Ensembl Genomes website (https://ensemblgenomes.org/), a total of 1984 protein-coding genes were located on 1q (1q12-1q44). Using the GSE2658 dataset, we selected genes overexpressed in +1q MM in a copy number-dependent manner and further identified prognostic genes. We identified 5 candidate genes, *ILF2*, *VPS72*, *ISG20L2*, *CDC42SE1* and *TADA1*. In this study, we focused on *ISG20L2* function in MM (Supplementary Figure 1A). *ISG20L2* is located in the 1q23 region. In clinical practice, 1q21 probes are often used by fluorescence in situ hybridization (FISH) assays to identify the amplification of +1q (6). We performed dual-color FISH assays using 1q21 and 1q23 probes, showing that the amplifications of 1q21 and 1q23 were identical in the tested primary MM cells and human MM cell lines (Supplementary Figure 1B, C). MM cell lines or patients with a high copy number of 1q showed high ISG20L2 expression (Figure 1A, B). We observed higher ISG20L2 expression at both the mRNA and protein levels in +1q MM patients than in patients without +1q (Figure 1C, D). Finally, we verified the prognostic value of *ISG20L2* expression in different MM datasets. *ISG20L2*\textsuperscript{high} MM had inferior OS (Figure 1E; Figure 1F, second panel). Interestingly, for MM patients without +1q, *ISG20L2* expression also correlated with MM OS (Figure 1F, third panel). For +1q MM, the *ISG20L2*\textsuperscript{high} patients had a worse
prognosis, but without statistical significance (Figure 1F, fourth panel). This may be attributed to the limited number of +1q patients (n=93) in the dataset (GSE13591) or the already high expression of ISG20L2 in +1q MM. These findings offer proof of the prognostic value of ISG20L2 in MM. Importantly, previous studies have identified multiple functional 1q genes in myeloma pathogenesis and chemoresistance(6, 7, 10). The correlation of ISG20L2 expression and other prognostic factors in the +1q cohort has not yet been determined. Our understanding of the roles of ISG20L2 in MM is still very limited.

ISG20L2 Regulates Multiple Myeloma Drug Sensitivity to Proteasome Inhibitors

To examine ISG20L2 function, we generated human MM cell lines with consistent ISG20L2 knockdown (ISG-KD) and overexpression (ISG-OE) (Supplementary Figure 2A). ISG20L2 knockdown did not affect cell growth of ARD in vitro (Supplementary Figure 2B) but enhanced its sensitivity to BTZ or carfilzomib (CFZ) (Figure 2A). ISG20L2 knockdown did not significantly change MM sensitivity to dexamethasone (DEX, 40 µM), doxorubicin (DOX, 2 µM) or melphalan (MEL, 15 µM) at the concentration we used. Further studies may be required to test the functions of ISG20L2 in MM multidrug resistance. ISG-KD ARD cells were sensitive to a wide dose range (3.125 to 50 nM) of BTZ. When more than 80% of both CTR-KD and ISG-KD cells were killed at 12.5 nM BTZ, BTZ still induced more late apoptotic (Annexin-V+/PI+) cell population in ISG-KD cells (Supplementary Figure 2C). ISG-OE AMO1 cells showed decreased sensitivity to PIs compared with CTR cells (Figure 2B).
Apoptotic caspase-3 fragmentation indicated differences in BTZ-induced cell death in control cells and ISG-KD or ISG-OE cells (Figure 2C). Similar results were also observed in the ISG-KD human MM cell line KMS-11 (Supplementary Figure 2D-G). To test the above results in vivo, we generated a human MM xenograft mouse model using CTR-KD and ISG-KD ARD cells. Tumor-bearing mice were treated with a low dose of BTZ (0.75 mg/kg, twice weekly for four weeks) using PBS (phosphate-buffered saline) as a control. The tumor burden was examined by in vivo luminescence assay (Figure 2D) and circulating monoclonal protein (Figure 2E). Although CTR-KD and ISG-KD MM exhibited similar growth in vivo without drug treatment, ISG-KD MM was more sensitive to BTZ treatment than CTR-KD MM in vivo. ISG-KD MM-bearing mice survived longer than CTR-KD MM-bearing mice after BTZ treatment (Figure 2F).

Gene expression profiling datasets from MM patients also suggested that ISG20L2 expression correlated with patients’ response to PIs. In the GSE9782 dataset(11), patients in the trial were classified as achieving complete response, partial response, minimal response, no change, or progressive disease. We defined the complete response, partial response and minimal response groups as “Response to BTZ”, while the no change and progressive disease groups were defined as “No response to BTZ”. The patients who had higher ISG20L2 expression at diagnosis did not respond to BTZ than those who responded to BTZ (Figure 3A). Using the MMRF CoMMpass dataset(12), we analyzed data from patients who had been treated with PIs (BTZ or CFZ). After PI treatment, patients who had PD (progressive disease) had higher ISG20L2 expression
than those with CR (complete response), VGPR (very good partial response) or PR (partial response). Patients with SD (stable disease) had higher median and mean values of \textit{ISG20L2} expression than the CR, VGPR and PR groups, but the difference was only statistically significant when compared with the PR group (Figure 3B). Considering the different 1q amplification statuses of patients, for +1q patients, only one patient ended in the PD group, which was not sufficient for statistical analyses. The expression of \textit{ISG20L2} was slightly higher in the SD group than in the CR, VGPR and PR groups, but the difference was not statistically significant. We found that in patients without +1q, \textit{ISG20L2} expression was higher in the PD group than in the CR, VGPR and PR groups. No +1q patients with SD had higher \textit{ISG20L2} expression than the CR, VGPR and PR groups, but the difference was not statistically significant when compared with the CR group (Supplementary figure 3A). For patients treated with the PI-based regimen, those with no progressive disease had lower \textit{ISG20L2} expression than those with at least one progressive disease (Figure 3C). Patients with at least one progressive disease had higher \textit{ISG20L2} expression, regardless of their 1q amplification status (Supplementary figure 3B). Most patients who received PI-based therapy and died from disease progression had \textit{ISG20L2} upregulation during the treatment course (Figure 3D). Of note, the above statistically significant comparison results based on MM patient data were quantitatively modest.

To summarize, we found that ISG20L2 regulated MM PI sensitivity in vitro and in vivo. MM patients with higher \textit{ISG20L2} expression were less sensitive to PIs and had inferior treatment outcomes.
ISG20L2 Competes with PSMB5 in Bortezomib Binding

We examined proteasome activity in CTR-KD versus ISG-KD ARD cells. PI exposure resulted in greater proteasome inhibition in ISG-KD cells than in CTR-KD cells (Figure 4A); conversely, less proteasome inhibition was observed in ISG-OE cells (Figure 4B). Unfolded protein response (UPR) signaling is known to mediate PI-induced MM cell death(13, 14). Western blot results showed that BTZ treatment induced upregulation of ATF-4, ATF-6, CHOP, P-PERK (T982), XBP-1s and P-eIF2α (S51) in ISG-KD ARD cells (Figure 4C, left; Figure 4D). Consistent results were observed in ISG-OE AMO1 cells (Figure 4C, right).

PSMB5, the beta 5 subunit of the 20S proteasome complex, was identified as a molecular target of BTZ(15). We synthesized biotinylated bortezomib (BTZ-b) (Figure 5A). The compound BTZ-b induced the same pattern of cell death in CTR-KD versus ISG-KD ARD cells as that observed with BTZ treatment (Figure 5B). A competition BTZ-b pull-down assay showed that the addition of BTZ to ARD cell lysate decreased the binding of PSMB5 to BTZ-b (Figure 5C). This result suggested that the BTZ-b analog mimicked BTZ in PSMB5 binding. The BTZ-b pull-down assay using ARD cell lysate showed that both PSMB5 and ISG20L2 could be pulled down by BTZ-b (Figure 5D). More PSMB5 was pulled down in ISG-KD MM than in CTR-KD MM (Figure 5D), while ISG20L2 knockdown had a mild effect on the expression of the 20S proteasome beta subunits (Supplementary Figure 4A). Consistent results were observed in BTZ-b pull-down assays using ISG-OE MM cell lysate (Figure 5E) or
PSMB5 knockdown MM cell lysate (Supplementary Figure 4B). Next, we used recombinant ISG20L2 (rISG) and PSMB5 (rB5) proteins to repeat the pull-down assay (Figure 5F). The addition of rISG decreased the pull-down of PSMB5 by BTZ-b in a dose-dependent manner. To confirm the specific binding of BTZ to ISG20L2, we performed surface plasmon resonance (SPR) assays, confirming that the binding affinity of BTZ to ISG20L2 ($K_D=26.71 \mu M$) is comparable to that of BTZ binding to PSMB5 ($K_D=25.05 \mu M$) (Figure 5G and Supplementary Figure 5A). Additionally, through SPR assays, we also confirmed the specific binding of CFZ to ISG20L2, and the affinity between ISG20L2 and CFZ ($K_D=8.712 \mu M$) was similar to that between PSMB5 and CFZ ($K_D=15.31 \mu M$) (Supplementary Figure 5B and C). Based on our findings, we proposed the mechanism of ISG20L2-induced MM resistance to PIs (Figure 5H). In $ISG20L2^{low}$ MM cells, BTZ mainly bound to the beta5 subunit of the proteasome complex and inhibited proteasome activity. This inhibition resulted in an unfolded protein response in MM cells and eventual cell death. In contrast, in $ISG20L2^{high}$ MM, significant amounts of BTZ bound to ISG20L2 instead of the proteasome complex. Therefore, $ISG20L2^{high}$ MM was less sensitive to BTZ. To our knowledge, the ISG20L2 protein structure has yet to be revealed. Crystallographic data showing binding of BTZ with ISG20L2 might provide confirmative evidence of the PI resistance mechanism that we proposed here.

Discussion

Resistance to PIs, such as BTZ and CFZ, remains a major limitation for the full
application of drugs in MM treatment. The proteasome beta 5 subunit, encoded by the PSMB5 gene, is the primary molecular target of BTZ. PSMB5 gene mutations that affected the structure of the BTZ-binding pocket of the PSMB5 protein resulted in impaired BTZ binding and therefore decreased drug sensitivity(16, 17). Since second generation PI, CFZ, targeted proteasome 20S beta5 subunit as well, many MM with BTZ-resistance mutations in PSMB5 also responded poorly to CFZ or ixazomib(18, 19). In addition to mutations, the abundance of the PSMB5 protein also correlated with BTZ resistance. MM with high PSMB5 expression had decreased BTZ sensitivity(20, 21). These findings might indicate that the compound-to-target ratio was also critical to achieve effective proteasome inhibition in BTZ treatment. In our study, we showed that a high level of ISG20L2 prevented BTZ from binding to its anti-MM target PSMB5. Therefore, reduced proteasome inhibition, weaker downstream UPR signaling and less BTZ-induced cell death were observed.

The function of ISG20L2 in cells is still largely unknown. Previously, Coute Y et al. reported that ISG20L2 was an exoribonuclease involved in ribosomal subunit biosynthesis(8). Recently, several independent omics analyses identified ISG20L2 as a prognostic marker in different human cancers, including hepatocellular carcinoma(22-24), lung adenocarcinoma(25), and breast cancer(26). Using MM gene expression profiles, we also found that high ISG20L2 expression was associated with inferior MM OS. Furthermore, we addressed the negative impact of ISG20L2 on MM prognosis by showing that ISG20L2 might function as an interference target of BTZ in MM cells. Mounting evidence suggests that PI resistance is mediated by multiple mechanisms.
Our findings demonstrated a role for ISG20L2 in MM PI resistance. However, how much ISG20L2 contributes to MM patient resistance to PI-based therapy was not determined in our study.

To our knowledge, PIs are not used in the treatment of hepatocellular cancer, lung cancer or breast cancer. Therefore, the oncogenic role of ISG20L2 across cancers remains uncertain and requires further investigation.

Methods and Materials

Patient samples

Bone marrow aspirations from 76 multiple myeloma (MM) patients were used in this study. Patient samples were obtained from the tissue bank of the Department of Hematology, West China Hospital, Sichuan University. This study was approved by the Ethical Committee of West China Hospital, Sichuan University (Protocol No. 114). Written informed consent was obtained from the patients or their legal guardians for sample collection and usage.

Bone marrow mononuclear cells were isolated by Ficoll density gradient centrifugation. CD138+ cells were sorted by immune magnetic beads (Miltenyi Biotec, Cat No. 130-051-301, MA, USA) according to the manufacturer’s protocol.

Myeloma gene expression profile datasets

The MM gene expression profile (GEP) datasets GSE13591, GSE755, GSE2658 and GSE9782 were downloaded from the NCBI Gene Expression Omnibus database.
The corresponding clinical information was obtained from the Oncomine database (https://www.oncomine.org). The GEP and clinical information from the Multiple Myeloma Research Foundation (MMRF) CoMMpass study were downloaded from the MMRF web portal (https://research.themmrf.org).

For the GSE755 dataset, CD138⁺ plasma cells were isolated from newly diagnosed MM patients (n=173) BM. The gene expression profiles were assessed using the Affymetrix U95Av2 microarray platform. For the GSE2658 dataset, CD138⁺ plasma cells were isolated from newly diagnosed MM patients (n=559) BM. The patients were subsequently treated with high-dose therapy and stem cell transplantation, referred to as total therapy 2 (TT2) and total therapy 3 (TT3). The gene expression profiles were assessed using the Affymetrix U133Plus2.0 microarray platform. For the GSE9782 dataset, plasma cells were negatively selected from 264 relapsed MM patients. Those patients were enrolled in phase II/III clinical trials for bortezomib (PS-341). The gene expression profiles were assessed using the Affymetrix U133A/B microarray platform.

Reagents and antibodies

Bortezomib (Cat No. S1013), carfilzomib (Cat No. S2853), melphalan (Cat No. S8266), dexamethasone (Cat No. S1322) and doxorubicin (Cat No. S1208) were purchased from Selleck Chemicals (TX, USA). Recombinant human PSMB5 protein was custom-made by MedChemExpress (Shanghai, China). Recombinant human ISG20L2 protein was custom-made by MerryBio Co., Ltd. (Nanjing, China).
Western blot antibodies against PSMB5 (Cat No. sc-393931), PSMD8 (Cat No. sc-514053), PSMD3 (Cat No. sc-393588), PSMC5 (Cat No. sc-390631), and eIF2α (Cat No. sc-133132) were purchased from Santa Cruz Biotechnology (CA, USA). Western blot antibodies against ISG20L2 (Cat No. 24639-1-AP) and GAPDH (Cat No. 60004-1-lg) were purchased from Proteintech Group (IL, USA). ATF-4 (Cat No. 11815), ATF-6 (Cat No. 65880), cleaved caspase 3 (Cat No. 9661), P-p38MAPK (T180/Y182) (Cat No. 9211), p38MAPK (Cat No. 9212), P-eIF2α (S51) (Cat No. 3398), and XBP-1s (Cat No. 27901) were obtained from Cell Signaling Technology (MA, USA). Western blot antibodies against P-PERK (T982) (Cat No. WL05295), PERK (Cat No. WL03378), and CHOP (Cat No. WL00880) were obtained from Wanleibio (Shenyang, China).

Cell culture

The human MM cell lines ARD, KMS-11, AMO1, and IM-9 were kindly provided by Dr. Qing Yi, Methodists Hospital, Houston, Texas, US. The human MM cell line OCI-My5 was kindly provided by Dr. Jumei Shi, Tongji University School of Medicine, Shanghai, China. All of the above cell lines were cultured in RPMI-1640 medium (HyClone, Cat No. SH30809.01, UT, USA) supplemented with 10% fetal bovine serum (GeminiBio, Cat No. 900-108, CA, USA) at 37°C and 5% CO₂. All cell lines were authenticated by short tandem repeat (STR) profiling and tested for mycoplasma contamination before use.

Lentivirus packaging and infection
The pLKO.1 control vector (Sigma–Aldrich, Cat No. SHC002, MO, USA) and pLKO.1 plasmids containing human-ISG20L2-shRNAs (sh1: TRCN0000233064 and sh2: TRCN0000233062) were used to produce CTR-KD and ISG-KD viruses, respectively. ISG20L2 cDNA was cloned into the pLEX-MCS plasmid (Thermo Fisher Scientific, Cat No. OHS4735, MA, USA) to construct the ISG-OE expression vector, while the empty pLEX-MCS plasmid was used as a control (CTR). Lentiviral particles were generated by transfection of HEK293T cells by calcium phosphate precipitation as described earlier(27).

Quantitative RT–PCR

Total RNA was extracted from cells with TRI reagent (MRC, Cat No. TR118, OH, USA). cDNA was synthesized by HiScript II Q RT SuperMix (Vazyme, Cat No. R223-01, Nanjing, China) following the manufacturer’s protocol. The expression of target genes was analyzed by RT–PCR using SYBR green real-time PCR Master Mix (Bimake, Cat No. B21202, Shanghai, China). The following primers were used for RT–qPCR:

GAPDH:
Forward: ACAACTTTTGTTATCGTGGAAGG
Reverse: GCCATCACGCCACAGTTTC

ISG20L2:
Forward: GAGACTCCTACGGTCGATGG
Reverse: GGTTGGGTGCTATTGATCTTTG
Cell proliferation assay

Cell Counting Kit-8 (CCK8) (Beijing 4A Biotech, Cat No. AS-20739, Beijing, China) was used to detect cellular proliferation. Cells (2x10^3 cells per well) were seeded in 96-well plates in triplicate and examined at 0, 24, 48 and 72 hours after seeding. Cells were incubated with CCK8 solutions for 2 hours, and absorbance was measured at 450 nm by a microplate luminometer (Molecular Devices, SpectraMax 190, CA, USA).

Apoptosis assay

MM cells were treated with different agents and stained with Annexin V-fluor647 and propidium iodide (Beijing 4A Biotech, Cat No. FXP023). Stained cells were examined by flow cytometry (Beckman Coulter, Navios EX, IN, USA).

Fluorescence in situ hybridization (FISH)

FISH assays were performed as described earlier(28). The 1q23 probe, which covered the ISG20L2 gene, was labeled with green fluorescence dye (Anbiping Group, Guangdong, China). The 1q21 probe was labeled with red fluorescence dye (Anbiping Group, Cat. No. F.01124-01). The probe information is summarized in Supplementary Table 1. The results were visualized by a BX51 fluorescence microscope (Olympus, Tokyo, Japan), and the images were captured by a FISH imaging system (CytoVision, Leica Biosystems, IL, USA).
Animal study

The animal study was approved by the West China Hospital Animal Ethics Committee. A human MM xenograft mouse model was established as previously described(29). CTR-KD or ISG-KD (sh1 sequence was used) ARD cells were intravenously injected (one million cells per mouse) into 7-week-old female severe immunodeficient NOD-Prkdc<sup>scid</sup> IL2rg<sup>iml</sup>/Bcgen mice (Biocytogen, Beijing, China), ten mice per group. On day 14 after tumor cell inoculation, the mice injected with CTR-KD or ISG-KD cells were randomly divided into two groups (vehicle control and BTZ treatment group, 5 mice per group). The treatment groups were intraperitoneally injected with a low dose of BTZ (0.75 mg/kg, twice a week) for four weeks, while the control groups were injected with PBS. The mice were subjected to weekly bioluminescence imaging (The IVIS<sup>®</sup> Spectrum, PerkinElmer, MA, USA) to monitor the tumor burden. Mouse serum was collected for ARD monoclonal protein examination using ELISA for human kappa light chain (Abcam, Cat. No. ab157709, MA, USA). The mice were sacrificed when they reached preestablished endpoints: paraplegia, lethargy or body weight loss of more than 20%. The overall survival of the mice was recorded and compared.

Proteasome activity assay

Proteasome-Glo<sup>™</sup> Chymotrypsin-Like Cell-Based Assay (Promega, Cat. No. G8662, WI, USA) was used to determine the intracellular proteasome activity.
**Biotin pull-down assay**

Biotinylated bortezomib (BTZ-b) was synthesized and purified by Z.Y. with the protocol undisclosed. Streptavidin-coated magnetic beads were obtained from Thermo Fisher Scientific (Dynabeads™ MyOne™ Streptavidin T1, Cat. No. 65602). The BTZ-b pull-down assay was performed using a standard protocol. In brief, Dynabeads™ magnetic beads (100 µl per sample pulldown) were washed twice with PBS and incubated with BTZ-b (1 mM, 2 µl) in PBS at 4°C for 2 h with gentle rotation. Then, the beads were washed twice with ice-cold PBS, resuspended in cell lysate (1 mg/ml, 500 µl PBS) or recombinant protein (1 µg/ml, 500 µl PBS), and incubated at 4°C overnight with gentle rotation. Subsequently, the magnetic beads were washed five times with ice-cold PBS, and bound protein was released by adding SDS-PAGE loading buffer. Biotin pull-down served as a negative control.

**Surface plasmon resonance (SPR) assay**

The binding behavior of BTZ or CFZ with ISG20L2 or PSMB5 was measured using SPR on a Biacore X100 system (GE Healthcare). Recombinant ISG20L2 or PSMB5 was immobilized on a CM5 chip through its amine groups. Successful immobilization of ISG20L2 or PSMB5 was confirmed by an ~13000 resonance unit (RU) or an ~10000 resonance unit (RU) increase in the sensor chip, respectively. After immobilization, BTZ or CFZ was diluted in running buffer (1xPBS, pH 7.4, 0.05% surfactant P20) at the indicated concentrations and injected at 30 µL/min for 2 min. Following analyte injection, running buffer flowed through the sensor surface for a 3
min period for dissociation. The response was determined as a function of time. $K_D$ values were calculated with Biacore X100 Evaluation software, Version 2.0.2.

**Statistical analysis**

Statistical analyses were performed in GraphPad Prism 8.0.2 software. Significance between 2 groups was determined by two-tailed Student’s t test. One-way ANOVA was performed to estimate differences among three or more groups. Two-way ANOVA was performed to compare the differences in the peripheral blood light chain of mice in different treatment groups over time. Patient survival and tumor-bearing mouse survival were analyzed by the log-rank (Mantel-Cox) test. The results are presented as the mean ± standard deviation. $P \leq 0.05$ was considered statistically significant.

**Study approval**

The use of human BM samples was approved by the Ethical Committee of West China Hospital, Sichuan University (Protocol No. 114). Written informed consent was obtained from the patients or their legal guardians for sample collection and usage. The animal study was approved by the West China Hospital Animal Ethics Committee.

**Author Contributions**

Y.Y., Y.G. and J.H. performed most of the experiments; Y.Z. initiated the project and designed the studies; Y.Z. and J.H. wrote the manuscript; Z.Y. synthesized biotinylated-
bortezomib; H.L., F.W., J.X., Y.C., H.D., Z.L., Y.Q., L.Z., T.N. and T.L. performed the experiments and provided critical suggestions.

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References


**Figures**

**A)** Western blot showing the expression of ISG20L2 in five human MM cell lines (OCI-My5, IM-9, AMO1, ARD, KMS-11).

**B)** In the myeloma patient gene expression dataset GSE2658, the expression of ISG20L2 in primary MM cells increased with the copy number of 1q21 (n=248; patients with 2 copies=134, patients with 3 copies=70, patients...
with 4 or more copies=44). One-way ANOVA with a post hoc LSD-t test was performed. 

$P$ values are shown as follows: **$P \leq 0.01$; **** $P \leq 0.0001$. 

C) RT–qPCR results showed $ISG20L2$ expression in primary MM cells of 76 patients diagnosed at West China Hospital (WCH). The 1q amplification status of patients was determined by FISH assay. Student’s t test was performed. $P$ values is shown as **** $P \leq 0.0001$. 

D) Dot blot assay of ISG20L2 in the 61 MM patients mentioned in Figure 1C (left blots). The dot intensities of ISG20L2 relative to GAPDH were calculated (right column). Student’s t test was performed. $P$ values is shown as **** $P \leq 0.0001$. 

E) In myeloma patient gene expression datasets GSE9782 (left, $n=264$) and GSE2658 (right, $n=559$), Kaplan–Meier curves showed the correlation between $ISG20L2$ expression and overall survival (OS) in MM patients. $P$ values were calculated by Mantel–Cox test. 

F) In the GSE13591 dataset ($n=186$), Kaplan–Meier curves showed the survival of different MM patient groups. $P$ values were calculated by Mantel–Cox test.
Figure 2. ISG20L2 regulates multiple myeloma cell sensitivity to proteasome inhibitors

A) CTR-KD and ISG-KD (sh1 used) ARD cells were treated with different drugs for 24 h. Bortezomib (BTZ, 5 nM), carfilzomib (CFZ, 3.5 nM), doxorubicin (DOX, 2 µM), melphalan (MEL, 15 µM) and dexamethasone (DEX, 40 µM) were used. Cell apoptosis was analyzed by flow cytometry with Annexin V and PI double staining (left), and the
percentage of apoptotic cells was quantified (right, n=3). Student’s t test was performed. 

*P* values are shown as follows: ns=*P*>0.05; ** *P*≤0.01; *** *P*≤0.001.  

**B**) AMO1 cells infected with control virus (CTR) or ISG20L2-overexpressing virus (ISG-OE) were treated with BTZ (5 nM) and CFZ (3.5 nM) for 24 h. Cell apoptosis was analyzed (left), and the results were quantified (right, n=3). Student’s t test was performed. *P* values are shown as ** *P*≤0.01.  

**C**) CTR-KD vs ISG-KD ARD cells and CTR vs ISG-OE AMO1 cells were treated with BTZ (5 nM for 12 hours). Western blot results of cleaved-caspase 3 and GAPDH are shown.  

**D**) CTR-KD or ISG-KD (sh1 used) ARD cells expressing luciferase were intravenously injected into B-NDG mice to establish a human MM xenograft mouse model. Bioluminescence imaging indicated a correlation between ISG20L2 expression and MM sensitivity to BTZ in vivo (n=5 for each group).  

**E**) Monoclonal protein (human kappa light chain secreted by ARD cells) levels in mouse peripheral blood were examined by ELISA. Two-way ANOVA with Tukey’s post hoc test was performed. (**** *P*<0.0001 for BTZ-treated CTR-KD vs ISG-KD mice.)  

**F**) Survival curves of B-NDG mice with different treatments. The Mantel–Cox test was performed. (** *P*<0.01 for CTR-KD vs ISG-KD mice treated with BTZ.)
Figure 3. ISG20L2 expression correlates with the response of multiple myeloma patients to proteasome inhibitors

A) In dataset GSE9782, 169 patients with MM received BTZ treatment. We analyzed ISG20L2 expression in MM patients who responded to BTZ treatment (R, n=85, ISG20L2 expression: 8.009 to 351.6, median value=118.6) and who did not respond to BTZ treatment (NR, n=84, ISG20L2 expression: 13.89 to 443.7, median value=134.5). Student’s t test was performed. P values is shown as *P≤0.05.

B) In the MMRF CoMMpass dataset, analyses of the correlation between ISG20L2 expression and patients’ response status to PIs (BTZ or CFZ) treatment. Patients were stratified based on response degrees after treatment as CR (complete response, n=176, ISG20L2 expression: 7.612 to 64.42, median value=24.83), VGPR (very good partial response,
n=344, ISG20L2 expression: 4.044 to 98.31, median value=21.94), PR (partial response, 
n=100, ISG20L2 expression: 5.433 to 56.73, median value=21.07), SD (stable disease, 
n=33, ISG20L2 expression: 12.84 to 56.51, median value=27.83) and PD (progressive 
disease, n=6, ISG20L2 expression: 16.14 to 68.98, median value=33.86). One-way 
ANOVA with post hoc LSD-t test was performed. \(P\) values are shown as follows: 
ns=\(P>0.05\); \(*P\leq0.05\); \(**P\leq0.01\). C) In the MMRF CoMMpass dataset, for the patients 
who received PI treatment, ISG20L2 expression was analyzed in patients without PD 
(n=376, ISG20L2 expression: 4.044 to 108.9, median value=20.88) and in patients with 
at least one PD (n=287, ISG20L2 expression: 6.284 to 98.31, median value=25.33). 
Student’s t test was performed. \(P\) values is shown as **** \(P\leq0.0001\). D) In the MMRF 
CoMMpass dataset, for the patients who received PI-based therapy and died from 
disease progression, 20 patients had sequential BM GEP data. ISG20L2 expression 
increased during the treatment. Student’s t test was performed. \(P\) values is shown as 
*\(P\leq0.05\).
Figure 4. ISG20L2 regulates ubiquitin–proteasome-related cell signaling

A) CTR-KD and ISG-KD ARD cells that were treated with BTZ (5 nM, 1.5 h, left) and CFZ (3.5 nM, 1.5 h, right). The solvent DMSO served as a negative control. Proteasome activity was determined by measuring the fluorescence (amc) intensity released by the cleavage of the fluorogenic substrate suc-LLVY-amc (n=6). Student’s t test was performed. P values are shown as **** P≤0.0001. B) Proteasome activity of CTR and ISG-OE AMO1 cells pretreated with BTZ (5 nM, 1.5 h) or CFZ (3.5 nM, 1.5 h) (n=6). Student’s t test was performed. P values are shown as **** P≤0.0001. C) CTR-KD vs. ISG-KD ARD cells, as well as CTR vs. ISG-OE AMO1 MM cells, were treated with BTZ (5 nM, 4 h). The cell lysates were used for western blotting to examine UPR pathway activation. The UPR pathway components ATF-6, PERK, P-PERK(T982),
eIF2α, P-eIF2α(S51), ATF-4, CHOP and XBP-1s were analyzed. p38MAPK and P-p38MAPK (T180/Y182) were also examined. D) Western blot results showed the signaling molecules in UPR and ER-stress pathways in CTR-KD vs. ISG-KD ARD cells, which were treated with BTZ (5 nM) for 1 h or 6 h.
Figure 5. ISG20L2 competes with PSMB5 in bortezomib binding

A) Molecular structure of biotinylated BTZ (BTZ-b). B) Flow cytometry-based apoptosis assay to examine the cytotoxicity of BTZ-b in CTR-KD vs. ISG-KD (sh1) ARD cells. The cells were treated with BTZ-b (2 μM) for 24 h. Dimethyl sulfoxide (DMSO)- or biotin-treated cells served as controls (n=3). Student’s t test was performed. P value is shown as *P≤0.05. C) Biotinylated-BTZ pull-down assay using ARD cell
lysat in the presence of BTZ to examine BTZ competition with BTZ-b in PSMB5 binding. BTZ was added to the cell lysate to a final concentration of 200 μM and incubated with BTZ-b-coated beads for competition. **D)** Biotinylated BTZ pull-down assay using CTR-KD and ISG-KD ARD cell lysates. Biotin served as a negative control for pull-down. The pull-down precipitate and the whole-cell lysates (input) were subjected to western blotting. **E)** Biotinylated BTZ pull-down assay using CTR and ISG-OE AMO1 cell lysates. **F)** Biotinylated BTZ pull-down assay using recombinant ISG20L2 (rISG) and PSMB5 (rB5) proteins. The recombinant protein was dissolved in PBS. The final concentration of rB5 was 0.5 μg/ml (+), while the final concentration of rISG was 0.25 μg/ml (+) or 1.5 μg/ml (++). Using the gray intensity of PSMB5 pull-down by BTZ-b alone as a control, ImageJ software was used to determine the levels of PSMB5 pull-down by BTZ-b with rISG competition. Numbers indicated on the lane. **G)** BTZ binds to ISG20L2 as shown by SPR measurements. Graphs of equilibrium response unit (RU) responses versus compound concentrations were plotted. The estimated K_D is 26.71 μM. **H)** Schematic mechanism of ISG20L2-induced MM BTZ resistance.