Caspase-8 regulates the anti-myeloma activity of bortezomib and lenalidomide

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List of non-standard abbreviations: IMiDs, Immunomodulatory Drugs; Thal, thalidomide; Len, lenalidomide; Pom, pomalidomide; Btz, bortezomib; CRBN, cereblon; CASP-8, caspase-8; shRNA, short hairpin RNA
Abstract

Proteasome inhibitors and immunomodulatory drugs (IMiDs) are two major types of drugs for treatment of multiple myeloma. Although different combination therapies for myeloma have been developed and achieved high responsive rate, these strategies frequently result in drug resistance. Therefore, it is necessary to explore new molecular mechanisms and therapeutic approaches to fulfill this unmet medical need. Here, we find that proteasome inhibitor bortezomib (Btz) causes cereblon (CRBN) cleavage and caspase-8 (CASP-8) is responsible for this cleavage. Either inhibition or genetic depletion of CASP-8 decreased the CRBN cleavage upon Btz treatment, which could potentiate the anti-myeloma activity of IMiD lenalidomide (Len). This work suggests that administration of CASP-8 inhibitors might enhance the overall effectiveness of Btz/Len-based therapeutic treatment for myeloma patients.

SIGNIFICANCE STATEMENT: Caspase-8 activation upon bortezomib treatment results in the cleavage of cereblon, a substrate receptor of the cullin 4-RING E3 ligase, which is responsible for the degradation of two transcription factors IKZF1 and IKZF3 in the presence of immunomodulatory drugs including lenalidomide. The administration of caspase-8 inhibitor may enhance the anti-myeloma activity of the combination therapy with bortezomib and lenalidomide to multiple myeloma.
Introduction

Multiple myeloma is the second most common hematological malignancy and is characterized by abnormal proliferation of malignant plasma cells (Mao et al., 2011; Kazandjian, 2016; van Nieuwenhuijzen et al., 2018). Proteasome inhibitors such as bortezomib (Btz) and carfilzomib, and immunomodulatory drugs (IMiDs) including thalidomide (Thal) and its structural analogs lenalidomide (Len) and pomalidomide (Pom), have been approved by FDA for the treatment of myeloma (Goldschmidt et al., 2019). Although this disease is treatable, it is currently incurable due to the frequent occurrence of drug resistance, leading to relapsed and/or refractory myeloma (Sonneveld et al., 2016). Therefore, it is important to explore new molecular mechanisms and develop novel strategies for overcoming drug resistance, promoting the death or inhibiting the growth of myeloma cells.

Several mechanisms of action of Btz have been proposed in the treatment of myeloma. Under physiological conditions Btz preferentially targets the proteasome subunit β5 and impairs the proteasome function through inhibiting its chymotrypsin-like activity (Berkers et al., 2005). The crystal structure of yeast 20S proteasome and Btz complex revealed that Btz interacts with multiple amino acid residues in several proteasome subunits and exhibits different degree of inhibition to the chymotrypsin-like and caspase-like activities in vitro. Btz could not only inhibit the degradation of IκB and then prevent the NF-κB signaling pathway (Karin et al., 2004), but also increase the expression of NOXA through upregulating c-Myc, thus exhibiting the anticancer activity against myeloma cells (Chen et al., 2011). In addition, at high concentrations, Btz could enhance the trypsin-like activity of the proteasome (Groll et al., 2006). It has been discovered that Btz exhibited pleiotropic effects on the treatment of myeloma through targeting DNA repair, cell cycle regulation, unfolded protein response, and apoptotic and classical stress response pathways including caspase-9 (CASP-9)-mediated intrinsic and CASP-8-mediated extrinsic cell death cascades (Chauhan et al., 2005). However, myeloma patients frequently develop resistance to Btz due to mechanisms such as dysregulation of gene and microRNA expression (Yang and Lin, 2015). Therefore, new proteasome inhibitors such as carfilzomib, ixazomib, marizomib, oprozomib, and delanzomib have been developed for the treatment of myeloma (Chauhan et al., 2005; Allegra et al., 2014).

The primary target of Thal is cereblon (CRBN) (Ito et al., 2010). CRBN is a substrate receptor of cullin-4 RING E3 ligase (CRL4^{CRBN}), including damage-specific DNA-binding protein 1 (DDB1), cullin-4A/B, and RING-box protein ROC1, which could enhance the
ubiquitination and degradation of proteins (Angers et al., 2006; Jackson and Xiong, 2009; Ito et al., 2010; Xu et al., 2013). Len and its structural analogs bind to CRBN and recruit the neo-substrates such as transcription factors IKZF1 (Ikaros) and IKZF3 (Aiolos). Subsequently, IKZF1 and IKZF3 are ubiquitinated by CRL4\textsuperscript{CRBN} E3 ligase, leading to their degradation through the ubiquitin-proteasome system, which would eventually inhibit the proliferation of myeloma cells (Krönke et al., 2014; Lu et al., 2014). Interestingly, long-term Len treatment of myeloma cells results in the reduction of CRBN at the mRNA and protein levels (Lopez-Girona et al., 2012; Gandhi et al., 2014), which may be the molecular mechanisms by which Len exhibits resistance in the relapsed myeloma.

To overcome the resistance to single drug treatment, combination therapies of IMiDs and proteasome inhibitors are current clinical treatments for the newly identified and relapsed/refractory myeloma (Richardson et al., 2009; Richardson et al., 2010). The primary mechanisms of action are distinct for proteasome inhibitors and IMiDs because proteasome inhibitors block the degradation of ubiquitinated proteins while IMiDs promote the ubiquitin-mediated degradation of a specific subset of proteins including IKZF1 and IKZF3. It is interesting to examine whether mechanisms discovered in one type of therapy could be used for the improvement of the other type of therapy (Zhang et al., 2013).

In this work, we first examined the CRBN stability in several myeloma cell lines upon Btz treatment. Surprisingly, we discovered that Btz induced CRBN cleavage. Then, we used pharmacological inhibition and Western blotting to explore the molecular mechanism by which CRBN was cleaved and found that Btz-induced CRBN cleavage was mediated by CASP-8 both in cell lines and in primary cells. Using both pharmacological inhibition and genetic knockdown, we further investigated the effect of CASP-8 inactivation on the viability of myeloma cells upon Btz and Len treatment. Taken together, our work revealed that CRBN was cleaved by CASP-8 activation in myeloma cells upon Btz treatment and reduction of CASP-8 could enhance the anti-myeloma activity of Btz and Len. This study might suggest a potential new combination therapy for multiple myeloma patients.

**Materials and Methods**

**Materials**

SUMOylation inhibitor anacardic acid (S7582), Btz (S1013), brefeldin A (s7046), CASP-3 inhibitor z-DEVD-fmk (S7312), CASP-8 inhibitor z-IETD-fmk (S7314), cisplatin (S1166), Len (CC-5013), and Pom (S1567) were purchased from Selleck; calpain inhibitor calpeptin (sc-202516) was from Santa Cruz biotechnology; MG132 (CC2775) was from ChemCatch;
tunicamycin (T7765) was from Sigma; TRAIL (abs04233) was from Absin; pan-caspase inhibitor z-VAD-fmk (C1202) was from Beyotime Biotechnology.

Anti-CASP-8 antibody (BA2143) was purchased from Boster Biological Technology; anti-CASP-3 (9662S), anti-cleaved CASP-3 (9661S), anti-PARP1 (9532S), and rabbit anti-CRBN (71810S) antibodies were from Cell Signaling Technology; anti-GAPDH (60004-1-Ig) antibody was from ProteinTech Group; anti-BAX (CPA1091) and anti-BID (CPA4351) antibodies were from Cohesion Biosciences; anti-ubiquitin (Ub, sc-8017) antibody was from Santa Cruz Biotechnology; anti- \( \beta \)-actin antibody was from BD Pharmingen. Mouse anti-CRBN antibody was a kind gift from Dr. Xiu-Bao Chang (Mayo Clinic College of Medicine, USA) (Xu et al., 2016). Control IgG was from Beyotime Biotechnology and secondary antibodies (sheep anti-mouse IgG-HRP and anti-rabbit IgG-HRP) were from Jackson ImmunoResearch Laboratories (USA).

**Cell culture**

HEK293T, K562, LP1, MM1.S, NCI-H1688, and RPMI8226 cells were obtained from American Type Culture Collection (ATCC). CAG cells were a kind gift from Dr. Joshua Epstein (University of Arkansas for Medical Sciences, Little Rock, AK) (Borset et al., 2000). Multiple myeloma cell lines (MM1.S, LP1, RPMI8226, CAG), leukemia cell line (K562), and small cell lung cancer cell line (NCI-H1688) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplied with 10% fetal bovine serum (FBS, Lonsera), 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin (HyClone). HEK293T cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, HyClone) supplied with 10% FBS, 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin.

The multiple myeloma patient was recruited and deidentified at the Weill Cornell Medicine. The bone marrow specimens were obtained under informed consent as part of an Institutional Review Board approved study. The human CD138\(^{+}\) primary myeloma cells were isolated from bone marrow according to the previously described method (Huang et al., 2012). For Western blotting analysis, cells from patients were first treated with DMSO or CASP-8 inhibitor z-IETD-fmk (20 \( \mu \)M) for 1 h and then with DMSO or Btz (20 nM) for 15 h in U-bottom plate. Cells were lysed and cell lysates were analyzed by immunoblotting.

**Construction of stable shCASP-8 expressing myeloma cells**

The shCASP-8 plasmid was constructed according to the published procedure (Moffat et al., 2006; Liu et al., 2021). To obtain the control and the stable shCASP-8 expressing myeloma
cell lines, MM1.S cells were infected with the lentiviral particles expressing shLacZ and shCASP-8 and then selected with puromycin (1 μg/mL) for two weeks.

**Western blotting analysis**

Proteins in cell lysates were separated by SDS-PAGE and then transferred to PVDF membrane. The PVDF membranes were incubated with primary and second antibodies for immunoblotting analyses (Tao et al., 2018; Guo et al., 2019).

**Cell viability measurement**

Myeloma cells were seeded in 96-well plates and treated with Btz and Len at the indicated concentrations. Cells were then analyzed with cell counting kit-8 assay (CCK-8, Beyotime Biotechnology) according to the manufacturer’s protocol (Ni et al., 2021). Experiments were performed for three biological replicates (three independent experiments) and Student’s t-test was used to calculate the P-value.

**Propidium iodide (PI) staining**

The stable shCASP-8 expressing MM1.S cells were treated with 10 nM Btz for 24 h and then cultured in fresh medium containing 10 μM Len for 4 days. The cells were further incubated with PI and Hoechst for 5 min, and then washed one time with phosphate-buffered saline (PBS). Images were captured using a inverted Olympus IX71 fluorescence microscope.

**Results**

**Bortezomib (Btz) induces the cleavage of CRBN in myeloma cells**

The proteasome inhibitor Btz is the first FDA-approved drug targeting the ubiquitin-proteasome system for the treatment of multiple myeloma (Hideshima et al., 2001; Jung et al., 2004). However, the molecular mechanism by which Btz exhibits anti-myeloma activity is not completely elucidated. IMiDs such as Len and Pom are used for the treatment of myeloma in combination with Btz and dexamethasone (Paludo et al., 2017). Recently, it has been discovered that IMiDs promote the ubiquitination and degradation of IKZF1 and IKZF3, resulting in reduced proliferation of myeloma cells (Krönke et al., 2014; Lu et al., 2014). However, it is unknown whether and how Btz regulates CRBN and its associated function. To explore this possibility, we treated the myeloma cell lines MM1.S, RPMI8226, and LP1 with different concentrations of Btz for 24 h. We found that Btz could induce the apoptosis of myeloma cells, as evidenced by the decrease of the apoptotic marker, the full
length poly(ADP-ribose) polymerase 1 (PARP1), and the increase of its cleaved form (Fig. 1A-C), which is consistent with previous results (Chauhan et al., 2005). Interestingly, we discovered a band slightly below the full length CRBN in the Western blotting analysis of CRBN after Btz treatment. This result suggests that CRBN is cleaved and the cleaved fragment is stable in MM1.S cells after Btz treatment (Fig. 1A-C). However, the Btz could not induce the cleavage of CRBN and apoptosis in HEK293T cells (Supplemental Fig. 1). These results indicate that the Btz-mediated CRBN cleavage is cell-type specific.

**Bortezomib activates CASP-8 pathway**

Our previous study revealed that CASP-8 activation could induce the cleavage of CRBN (Zhou et al., 2020). Here, we discovered that Btz treatment resulted in the cleavage of CRBN (Fig. 1). We then sought to investigate whether Btz could activate CASP-8 pathway. To test this, we treated the myeloma cell line MM1.S with Btz and used anti-ubiquitin immunoblotting to confirm the effectiveness of Btz. We further discovered that Btz induced the decrease of full-length CASP-8, suggesting the possible activation of CASP-8 (Fig. 2A). To further validate the activation of CASP-8, we immunoblotted its substrates BID and CASP-3 (Li et al., 1998; Luo et al., 1998). The immunoblotting images showed that the full-length BID was decreased and the cleaved CASP-3 was increased, indicating the activation of CASP-8 upon Btz treatment (Fig. 2A). Moreover, Btz could also induce the activation of CASP-8 and the cleavage of CRBN in myeloma cell lines RPMI8226 and LP1 and in leukemia cell line K562 (Fig. 2B-D), although CRBN was not completely cleaved in K562 cells compared with three myeloma cell lines. These results clearly demonstrated that Btz induced CRBN cleavage in myeloma and leukemia cells, which is unexpected since Btz was thought to function as a proteasome inhibitor to block protein degradation.

Next, we asked whether CRBN cleavage is a general phenomenon upon the induction of apoptosis in myeloma cells. To test this, we used several drugs to induce apoptosis in MM1.S cells. Although Btz, MG132, tunicamycin, brefeldin A, and cisplatin led to different degree of apoptosis based on the observation of PARP1 cleavage, only Btz and MG132 resulted in CRBN cleavage in MM1.S cells (Fig. 3A), indicating that CRBN cleavage is dependent of a specific signaling pathway. Treating MM1.S cells with Btz revealed that CRBN cleavage was time-dependent and the cleavage at 24 h was more pronounced than that at 12 h (Fig. 3B). In addition, CRBN cleavage exhibited a Btz concentration dependent manner (Fig. 3B). A similar result was also observed in K562 cells although less CRBN cleavage was detected even at a higher concentration of Btz (Fig. 3D).
CRBN cleavage is inhibited by a pan-caspase inhibitor

We next sought to ask whether caspase pathway activation is responsible for the Btz-induced CRBN cleavage. It has been reported that Btz can activate calcium-activated protease calpain (Li et al., 2010) and caspases (Uddin et al., 2008). Therefore, we used a calpain inhibitor calpeptin and a pan-caspase inhibitor z-VAD-fmk to pretreat MM1.S cells before Btz treatment. Immunoblotting analysis showed that pretreatment with z-VAD-fmk but not calpeptin prevented Btz-mediated CRBN cleavage (Fig. 4A-B). The cleavage of BAX, a Bcl-2 family member, could be partially blocked by calpeptin and z-VAD-fmk, which is consistent with previous studies that BAX can be cleaved either by calpain (Toyota et al., 2003) or by caspases (Choi et al., 2001). BID, another Bcl-2 family member, which can be cleaved by caspases (Li et al., 1998), was also reduced in myeloma cells after Btz treatment (Fig. 2-4) and its reduction was partially blocked by caspase inhibition but not by calpain inhibition (Fig. 4A-B). Taken together, these data indicate that Btz can activate caspases and lead to CRBN cleavage in myeloma cells.

IMiDs such as Thal, Len, and Pom affect CRBN stability (Liu et al., 2015). However, Pom does not alter the Btz-induced CRBN cleavage in myeloma cells (Fig. 4C). Covalent attachment of the small ubiquitin-related modifier (SUMO) to protein substrates (SUMOylation) can also regulate protein stability through different molecular mechanisms including proteasomal degradation (Bies et al., 2002; Ghioni et al., 2005; Fei et al., 2006). To investigate whether SUMOylation affects the Btz-induced CRBN cleavage in myeloma cells, we used anacardic acid to block the formation of E1-SUMO conjugates (Fukuda et al., 2009). This treatment did not affect Btz-induced CRBN cleavage in MM1.S (Fig. 4D) and RPMI8226 cells (Supplemental Fig. 2). Taken together, these experiments demonstrated that caspases can cleave CRBN in myeloma cells upon Btz treatment and this cleavage is not affected by calpeptin, IMiDs, and anacardic acid.

CASP-8 is responsible for the Btz-induced CRBN cleavage

CASP-9 is the initiator caspase of intrinsic pathway (Jin and El-Deiry, 2005) whereas CASP-8 is the initiator caspase for the extrinsic pathway such as extracellular apoptosis-inducing ligand (i.e., TRAIL) induced apoptosis (Crowder and El-Deiry, 2012). Although different caspase cascades are activated in extracellular and intracellular stimulus-induced apoptosis, CASP-3 is the critical executioner caspase and is responsible for proteolysis of the majority of substrates in the terminal phase of apoptosis (Walsh et al., 2008; Han et al., 2014). It has been
reported that Btz can activate all these three caspases (Chauhan et al., 2005). We, therefore, investigated which caspase was responsible for Btz-induced CRBN cleavage. The result showed that CASP-8 specific inhibitor z-IETD-fmk but not CASP-3 specific inhibitor z-DEVD-fmk blocked Btz-induced CRBN cleavage (Fig. 5A-B), indicating that CASP-8 cleaves CRBN in myeloma cells upon Btz treatment.

Experiments with primary myeloma cells isolated from bone marrow of a myeloma patient further confirmed that Btz induced the cleavage of CRBN and the CASP-8 inhibitor prevents this cleavage (Fig. 5C). Furthermore, CRBN did not regulate the Btz-induced apoptosis (Supplemental Fig. 3), suggesting that CRBN was not involved in CASP-8-dependent apoptosis, which is consistent with our previous study (Zhou et al., 2020).

**CASP-8 regulates the anti-myeloma activity of Btz and Len**

The activation of CASP-8 resulted in the cleavage of CRBN, leading to the reduction in the CRBN stability and the increase in the anti-myeloma activity of Len (Zhou et al., 2020). To further examine the effect of CASP-8 on the anti-myeloma activity of Btz in combination with Len, we established MM1.S cell lines stably expressing shCASP-8 or control shLacZ. The cell counting kit-8 (CCK-8) analysis showed that the cell viability was significantly reduced upon CASP-8 knockdown after the Btz and Len treatment although CASP-8 knockdown alone had no effect on cell viability. The propidium iodide (PI) staining showed that CASP-8 knockdown did not affect the Btz-induced apoptosis in combination with Len (Supplemental Fig. 4). Taken together, these results indicate that CASP-8 knockdown could significantly increase the anti-myeloma activity of Btz and Len (Fig. 5D).

**Discussion**

IMiDs bind to CRBN and enhance the ubiquitination and degradation of neo-substrates (Krönke et al., 2014; Krönke et al., 2015; An et al., 2017; Donovan et al., 2018; Matyskiela et al., 2018). It has also been observed that short term (1-3 day) and long term (2-6 month) IMiD treatment changes CRBN protein levels albeit through different molecular mechanisms (Zhu et al., 2011; Liu et al., 2015). In this study, we found that CRBN can be cleaved in myeloma cells upon Btz treatment (Fig. 1-3) and further demonstrated this cleavage is mediated by CASP-8 (Fig. 4-5). Because CRBN cleavage is concomitant with the apoptotic cell death induced by Btz (Fig. 1-4), we envisaged two possible outcomes of Btz-induced CRBN cleavage. The first one is that the cleaved CRBN product may play a specific role in the regulation of Btz-induced cell death. The second one is that CRBN protein level may be
controlled by Btz, thus affecting the therapeutic effectiveness of IMiDs on multiple myeloma. Our experiments demonstrated that CRBN knockdown did not alter the Btz-induced apoptotic cell death in NCI-H1688 cells (Supplemental Fig. 3), which rules out the first possibility. Experiments with pharmacological inhibition and genetic depletion in myeloma cell lines (Fig. 1-5) and primary myeloma cells (Fig. 5) supported the second hypothesis.

Btz-mediated CRBN cleavage was also observed in primary myeloma cells and this cleavage was abolished by CASP-8 inhibition. Our experiments revealed a novel concept that CASP-8 inactivation enhanced the anti-myeloma activity of Btz in combination with IMiDs. This suggests that combination therapy with Btz, IMiDs, and CASP-8 inhibitor might improve the patient outcome.

Currently, combination therapies with Btz, Len, and dexamethasone (Paludo et al., 2017) are the most effective treatment for myeloma. However, it is still not clear why two drugs, Btz and Len, which function in two different ways, inhibiting protein degradation and promoting the degradation of two transcription factors, together work effectively in the treatment of myeloma patients (Wang et al., 2021). Here, we discovered that CASP-8 inhibition potentiates the anti-myeloma activity of Btz in combination with Len. Therefore, their combination therapy enhances the overall effectiveness of myeloma patients. As Btz induces CRBN cleavage while CRBN is required for the anti-proliferation effect of Len in myeloma cells, further administration of a CASP-8 inhibitor to recover the full length CRBN might enhance the overall effectiveness of IMiD-based combination therapy. Together with previous discovery, our results suggest that administration of Btz and Len at different time might also enhance anti-myeloma activity. This work might provide a potential combination therapy for myeloma patients.

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Footnotes

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Figure legends

Fig. 1. Bortezomib (Btz) induces cereblon (CRBN) cleavage in multiple myeloma cell lines. MM1.S (A), RPMI8226 (B), and LP1 (C) cells were treated with DMSO, 2 nM, 5 nM, 10 nM, 50 nM, or 500 nM Btz for 24 h and the resulting cell lysates were immunoblotted with the indicated antibodies. The asterisk (*) at the anti-CRBN blot indicated the cleaved CRBN. Clvd: cleaved.

Fig. 2. Btz results in the activation of CASP-8 in multiple myeloma and leukemia cell lines. MM1.S (A), RPMI8226 (B), LP1 (C), and K562 (D) cells were treated with DMSO or Btz (0.5 µM) for 24 h and the resulting cell lysates were immunoblotted with the indicated antibodies. The asterisk (*) at the anti-CRBN blot indicated the cleaved CRBN. Clvd: cleaved. The anti-ubiquitin (Ub) blotting was used to validate the effectiveness of Btz treatment.

Fig. 3. Btz induces CRBN cleavage in a time- and dose-dependent manner. (A) Btz and MG132, but not tunicamycin, brefeldin A or cisplatin, caused CRBN cleavage in myeloma cells. MM1.S cells were treated with the indicated compounds (DMSO, 0.5 µM Btz, 10 µM MG132, 1 µg/mL tunicamycin, 1 µg/mL brefeldin A, or 5 µg/mL cisplatin) for 24 h. (B) MM1.S cells were treated with Btz (0.5 µM) for 0, 12, and 24 h. (C) MM1.S cells were treated with Btz (0, 0.1, or 0.5 µM) for 24 h. (D) K562 cells were treated with Btz (0, 0.5, or 1.0 µM) for 24 h. The resulting cell lysates were subjected to immunoblotting analysis.

Fig. 4. Btz-induced CRBN cleavage is blocked by a pan-caspase inhibitor. MM1.S cells were pretreated with DMSO, 10 µM calpain inhibitor calpeptin (A), 10 µM pan-caspase inhibitor z-VAD-fmk (B), 2 µM pomalidomide (Pom) (C), or 10 µM SUMOylation inhibitor anacardic acid (D) for 30 min, and then treated with DMSO or 0.5 µM Btz for 24 h. Cell
lysates were immunoblotted with the indicated antibodies.

**Fig. 5. CASP-8 cleaves CRBN and regulates the anti-myeloma activity of Btz and Len.**

(A-B) MM1.S cells were pretreated with DMSO, 40 µM CASP-3 inhibitor z-DEVD-fmk (A), or 40 µM CASP-8 inhibitor z-IETD-fmk (B) for 30 min, and then treated with 0.5 µM Btz for 24 h. (C) CD138⁺ primary myeloma cells isolated from the bone marrow of a myeloma patient were pretreated with 20 µM z-IETD-fmk for 1 h and further treated with DMSO or 20 nM Btz for about 15 h. Cell lysates were immunoblotted with the indicated antibodies. (D) The stable shCASP-8 expressing MM1.S cells were treated with 10 nM Btz for 24 h and then cultured in fresh medium containing 10 µM Len for 4 days. The relative cell viability was measured with CCK-8 assay. Quantitative data (mean ± SD) from three independent experiments. Statistics: one-way ANOVA with Tukey’s multiple comparison test. **: $P < 0.01$, ***: $P < 0.001$. 

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Fig. 1 (Zhou et al)
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