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Original Article

cAMP induces cell apoptosis in multiple myeloma and overcomes bortezomib resistance

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Abstract: The acquired resistance to bortezomib represents a major obstacle for multiple myeloma (MM) treatment. Studies revealed that the treatment with cyclic adenosine monophosphate (cAMP) may be a promising strategy for MM therapy. Therefore, the present study aimed to explore the mechanism of action of cAMP in MM cells. Our results showed that 8-CPT-cAMP and bortezomib synergistically induced growth inhibition and apoptosis in MM bortezomib-resistant cell lines and primary MM cells, in which protein kinase A (PKA) activation was involved. Furthermore, 8-CPT-cAMP induced the degradation of cyclinD1 and downregulation of myeloid cell leukemia-1 (Mcl-1). Moreover, 8-CPT-cAMP enhanced endoplasmic reticulum stress caused by bortezomib. A synergy between bortezomib and cAMP was also revealed in a murine MOPC315 xenograft model, which was evidenced by the significantly inhibited tumor growth and the improved multiple cancer-related parameters by the combination of the cAMP-elevating compound forskolin and bortezomib. Taken together, this study suggests that the treatment with cAMP may be a promising strategy for enhancing the therapeutic efficacy of bortezomib in MM treatment.

Keywords: Multiple myeloma, cAMP, bortezomib, apoptosis

Introduction

Multiple myeloma (MM) is one of the common hematologic malignancies, which is characterized by clonal B-cell-derived plasma cells accumulation in the bone marrow and accounts for approximately 1% of all neoplastic disorders [1, 2]. Even though bortezomib therapy advanced the treatment for MM [3, 4], off-target toxicities and drug-resistance development have been reported in MM treatment [5-7]. Therefore, further investigations are required to identify the drug resistance mechanisms and explore novel therapeutic strategies to overcome bortezomib resistance.

Recently, several studies have been focused on the development of a combination of bortezomib and inhibitors of signal transduction pathways to enhance antitumor efficacy and restore the sensitivity of MM cells to bortezomib [8, 9]. Their results strongly supported the notion that combination therapy was an effective approach to overcome the pitfalls of the treatment with bortezomib only. Cyclic adenosine monophosphate (cAMP), one of the common second messengers, was found to be involved in hematopoietic cell proliferation, apoptosis, and differentiation [10]. Previous studies reported that cAMP signaling stimulation induced apoptosis in both steroid-sensitive and -resistant MM cells [11, 12]. In addition, downregulation of myeloid cell leukemia-1 (Mcl-1) was involved in cAMP-induced apoptosis in MM [13]. Furthermore, it was suggested that intracellular cAMP elevation kills MM cells *in vitro* and inhibits MM development *in vivo* [14], and multiple mechanisms were involved in the process [15]. A recent examination revealed that the natural compound forskolin, a cAMP-elevating agent,

synergized with dexamethasone to induce cell death in MM cells [16].

Therefore, we hypothesized that cAMP may sensitize MM cells to bortezomib, especially the bortezomib-resistant cells. In the present study, we found that cAMP induced cell apoptosis and overcame bortezomib resistance in MM both *in vitro* and *in vivo*.

Materials and methods

Cell culture and reagents

Human MM cell lines NCI-H929 (referred to as H929), U266, and the murine MM cell line MOPC-315, purchased from the American Type Culture Collection (ATCC, USA), were cultured in RPMI-1640, supplemented with 10% (v/v) fetal calf serum (FCS; Gibco, USA) and 1/100 penicillin-streptomycin (Gibco, USA). HEK-293T cell line was obtained from ATCC. Then, bone marrow stromal cells (BMSC) and HEK-293T were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone, Logan, UT, USA) supplemented with 10% (v/v) FCS and 1/100 penicillin-streptomycin. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

H929 bortezomib resistance (referred to as H929-R) cells and U266 bortezomib resistance (referred to as U266-R) cells were established by consecutive stimulation with a gradually increasing bortezomib dosage (See Supplementary material). In brief, H929 and U266 cells were gradually exposed to the increased dose of bortezomib (from an initial dose of 4 nM to a final dose of 50 nM within 12 months with a gradient of 4 nM/month). Further, the cells were maintained in 50 nM bortezomib for three months and cultured in a bortezomib-free medium for two weeks prior to further analysis.

Bortezomib (Sigma Aldrich, USA) and 8-CPT-cAMP (Sigma Aldrich, USA) were dissolved in PBS as a stock solution at concentrations of 10 mM and 10 mM, respectively. Forskolin was dissolved in dimethyl sulfoxide (DMSO) to obtain a stock solution at a concentration of 10 mM. H-89 (N-(2-[p-bromocinnamylamino] ethyl)-5-isoquinolinesulfonamide) was obtained from Calbiochem (San Diego, CA, USA) and dissolved in 50% ethanol with a stock solution at 10 mM.

Primary MM cells isolation

Mononuclear cells (MNCs) were isolated from the bone marrow of MM patients by Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA) density sedimentation. $CD138^+$ cells were isolated from MNCs using EasyStep $CD138^+$ magnetic nanoparticles (Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's instructions. Written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki [17], and the study protocol was approved by the Clinical Investigational Reviewing Board of the Shanghai Jiao Tong University School of Medicine, China.

Cell apoptosis

The U266, H929, U266-R, and H929-R cells were exposed to bortezomib, 8-CPT-cAMP, or their combination. Cell apoptosis was measured by an Annexin-V apoptosis detection kit (BD Pharmingen, USA) following the manufacturer's instructions. Data were collected and analyzed by FlowJo software (BD Biosciences, San Diego, CA, USA).

Synergism calculation

The synergism between bortezomib and 8-CPT-cAMP was determined by the combination index (CI) method using the CompuSyn software (ComboSyn Inc., Paramus, NJ, USA). Briefly, cell viability was determined by PI exclusion and expressed as the fractional inhibition by the individual drug or the drug combination. CI values greater than 1 indicated antagonism, a CI value of 1 indicated an additive effect, and CI values of less than 1 indicated synergy.

Western blot analysis

After treatment, cells were collected and lysed in RIPA lysis buffer (Beyotime, China). Protein concentration was determined by the Bradford assay. Equal amounts (10 μg/lane) of protein were separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE, 10%-12%). Proteins were transferred to a PVDF membrane (Millipore, USA) and then blocked with 5% (w/v) non-fat milk at room temperature for 2 h. Afterwards, primary antibodies against PARP-1, caspase-3, PKA-cα, cyclinD1, Mcl-1, Bcl-2, Bax, CHOP, and ATF-4 with a dilution of 1:1,000

Table 1. Primers for RT-PCR

	Forward (5'-3')	Reverse (5'-3')
CyclinD1	GCGTACCCTGACACCAATCTC	CTCCTCTTCGCACTTCTGCTC
Mcl-1	GGGCAGGATTGTGACTCTCATT	GATGCAGCTTTCTTGGTTTATGG
CHOP	AGGTCCTGTCCTCAGATGAAAT	CAGGGTCAAGAGTAGTGAAGGTTT
ATF-4	CCTTCGACCAGTCGGGTTTG	CTGTCCCGGAAAAGGCATCC
GAPDH	CATCAAGAAGGTGGTGAAGC	ACCACCCTGTTGCTGTAG

were incubated overnight at 4°C. Subsequently, horseradish peroxidase (HRP)-conjugated secondary antibodies were incubated at room temperature for 1 h. Protein bands were detected by electrochemiluminescence (ECL) (Amersham, USA) according to the manufacturer's instructions. All antibodies (PARP-1, caspase-3, PKA-cα, cyclinD1, Mcl-1, Bcl-2, Bax, CHOP, and ATF-4) used in the present study were purchased from Santa Cruz Biotechnology, USA. Densitometry of proteins was performed by Quantity One software (Bio-Rad, Hercules, CA, USA).

Real-time PCR (RT-PCR)

Total RNA was extracted using the TRIzol (Invitrogen) method. Complementary DNA (cDNA) was transcribed using a reverse transcription kit (Promega, Japan) according to manufacturer's instructions. The expression of CyclinD1, Mcl-1, CHOP, ATF-4, and GAPDH was detected by real time (RT-PCR) using the ABI PRISM 7900 system (Applied Biosystems, USA). The following RT-PCR cycling conditions were used: 95°C for 5 min; 40 cycles at 95°C for 5 s and at 60°C for 30 s; and then elongation was performed at 60°C for 10 min. The primers utilized are listed in **Table 1**. The relative mRNA expression of the target genes was analyzed by the $2^{-\Delta\Delta Ct}$ method, and GAPDH served as an internal control.

RNA interference and transfection

Complementary oligonucleotides pairs targeting PKA were designed (Top: 5'-GACAAACAGAAGGTGGTGATTCAAGAGATCACCACCTTCTGTTTGTCTTTTTTACGCGT-3', Down: 5'-ACGCGTAAAAAAGACAAACAGAAGGTGGTGATCTCTTGAATCACCACCTTCTGTTTGTC-3'). Non-target control (NC) shRNA was synthesized by Transgene (Jiangsu, China) and was then annealed and ligated to the PSIREN-RetroQ Vector (Clontech, USA). shRNA-carrying retroviruses were produced in 293T cells and were further used to infect H929 cells. Stably transfected cells were selected by treatment with puromycin (2.0 μg/mL, Calbiochem, USA) for 96 h.

Co-culture

Bone marrow stromal cells (BMSCs) were isolated from bone marrow aspirates of MM patients as previously described [18]. For the cell co-culture experiment, 5×10^5 BMSCs cells were seeded in a 10-cm Petri dish. Next, 24 h after BMSCs seeding, 5×10^5 $CD138^+$ cells were added into the dish, followed by 24-h incubation and collection of the cells for further use.

Cell morphology

After treatment, approximately 1×10^4 cells were cast on slides by using Thermo Shandon Cytospin (Thermo Fisher Scientific, USA). Cell morphology was analyzed by May-Grunwald-Giemsa staining and observed under a light microscope (Nikon, Japan).

Xenograft mouse model

Female BALB/c nu/nu mice aged 4-6 weeks, obtained from Silaike (Shanghai, China), were kept under specific-pathogen-free (SPF) conditions and used according to the Shanghai Medical Experimental Animal Care guidelines.

A number of 1×10^6 MOPC-315 cells were injected subcutaneously with matrix gel into the right hips. When tumor was palpable, the mice were divided into a control, bortezomib, forskolin, and bortezomib + forskolin groups (n = 4/group). Forskolin (5 mg/kg/d, intraperitoneal injection, i.p.), bortezomib (0.8 mg/kg, twice weekly by intravenous injection, i.v.), bortezomib plus forskolin (forskolin was injected four hours after bortezomib), or vehicle (8% DMSO and 92% saline) were administered. Body weight was monitored daily. Tumor sizes were measured with calipers, and the tumor volumes were calculated by the formula: tumor volume = $0.5 \times a \times b^2$, where "a" is the length and "b" is the width. Animal study protocols were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine.

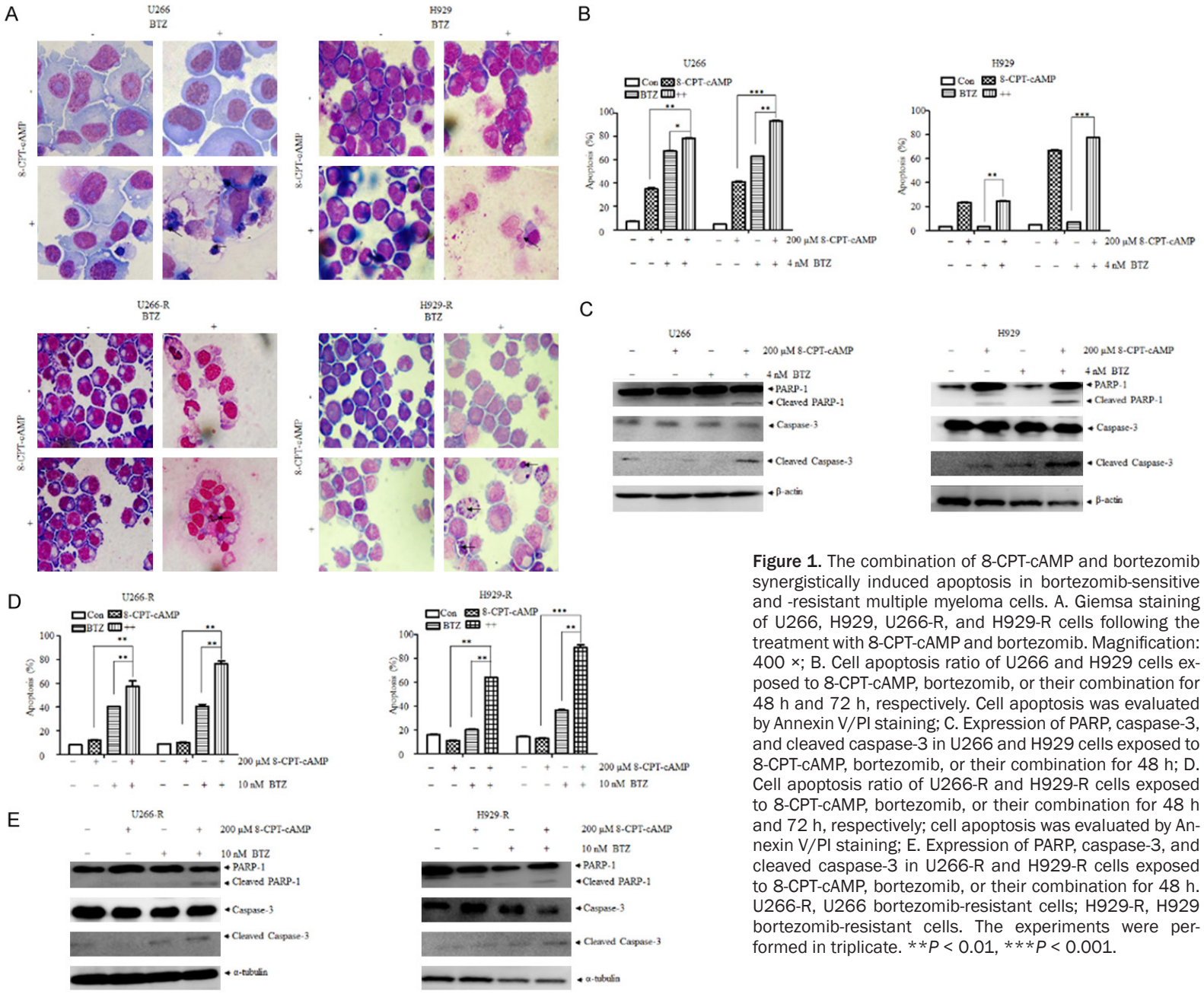


Figure 1. The combination of 8-CPT-cAMP and bortezomib synergistically induced apoptosis in bortezomib-sensitive and -resistant multiple myeloma cells. A. Giemsa staining of U266, H929, U266-R, and H929-R cells following the treatment with 8-CPT-cAMP and bortezomib. Magnification: 400 ×; B. Cell apoptosis ratio of U266 and H929 cells exposed to 8-CPT-cAMP, bortezomib, or their combination for 48 h and 72 h, respectively. Cell apoptosis was evaluated by Annexin V/PI staining; C. Expression of PARP, caspase-3, and cleaved caspase-3 in U266 and H929 cells exposed to 8-CPT-cAMP, bortezomib, or their combination for 48 h; D. Cell apoptosis ratio of U266-R and H929-R cells exposed to 8-CPT-cAMP, bortezomib, or their combination for 48 h and 72 h, respectively; cell apoptosis was evaluated by Annexin V/PI staining; E. Expression of PARP, caspase-3, and cleaved caspase-3 in U266-R and H929-R cells exposed to 8-CPT-cAMP, bortezomib, or their combination for 48 h. U266-R, U266 bortezomib-resistant cells; H929-R, H929 bortezomib-resistant cells. The experiments were performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$.

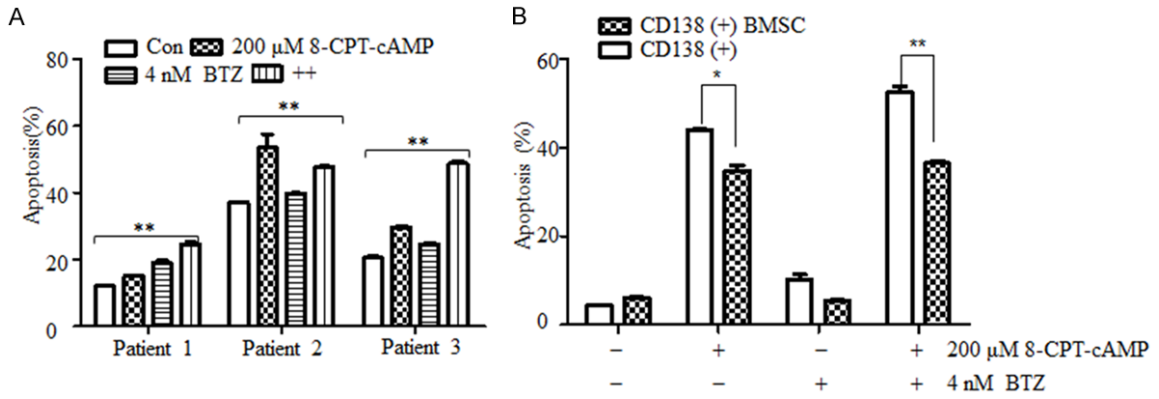


Figure 2. The combination of 8-CPT-cAMP and bortezomib synergistically induced apoptosis in primary CD138$^+$ MM cells. A. Primary CD138$^+$ cells isolated from MM patients were exposed to 8-CPT-cAMP, bortezomib, or their combinations for 24 h; cell apoptosis was evaluated by Annexin V/PI staining; B. CD138$^+$ MM cell co-cultured with bone marrow stromal cells (BMSCs) for 24 h and treated with 8-CPT-cAMP, bortezomib, or their combination for 24 h; cell apoptosis was evaluated by Annexin V/PI staining. *P < 0.05, **P < 0.01. The experiments were performed in triplicate.

Histology

The expression of PCNA, cyclinD1, and Mcl-1 in MOPC-315 nude mice xenografts were analyzed using immunohistochemistry staining. Sections were deparaffinized, heat-treated with citrate buffer (pH 6.0) for 15 min. Next, endogenous peroxidase was blocked with 1% hydrogen peroxide for 30 min, and the non-specific-binding sites were blocked with 2% (w/v) goat serum for 30 min. The sections were then incubated with the primary antibodies against PCNA, TUNEL, cyclinD1, and Mcl-1 1:100 overnight at 4°C. Further, the biotinylated secondary antibody was incubated for 1 h. Avidin-biotin-peroxidase complex (Beyotime, China) was then added, and the color was developed by 3-3'-diaminobenzidine. Counterstaining was performed with hematoxylin and eosin (HE). All steps were performed at room temperature, and the sections were observed under a confocal microscope (Leica, Japan). Five random fields were selected.

Statistical analysis

The paired-samples *t*-test was applied to check the significance in the cell-line experiments using the PASW Statistic 18 software (IBM, Armonk, New York, USA) for Windows. The data were expressed as mean ± SEM. The Wilcoxon signed rank test was used in the *in vivo* experiments, to determine the significance of the between-group differences. A two-way *P*-value less than 0.05 was considered statistically significant.

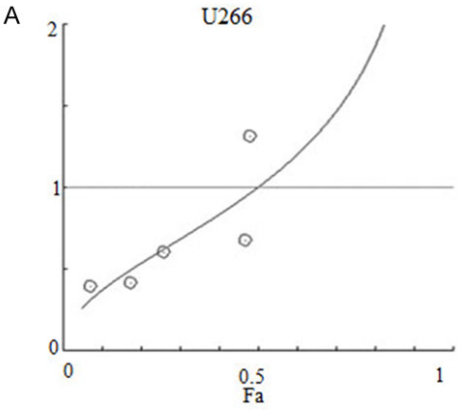
Results

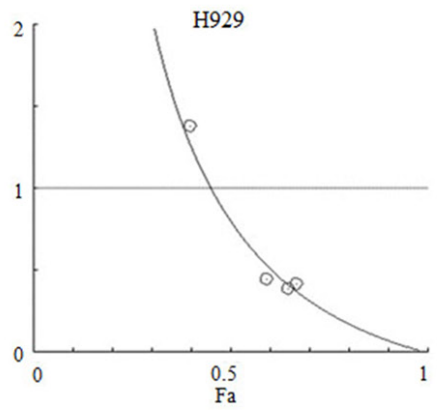
8-CPT-cAMP synergizes with bortezomib to induce MM cell apoptosis

A previous study indicated that 8-CPT-cAMP, a cell membrane-permeable cAMP analog, induced cell apoptosis and elevation of intracellular cAMP in MM cells, exhibiting attractive anti-cancer efficacy in MM animal models [14]. U266 and H929 cells treated with a combination of bortezomib and 8-CPT-cAMP showed an increasing proportion of condensed cytoplasms, ruptured nucleuses, and marginal chromatins as well as apoptotic bodies, which indicated the rising of cell death ratio. However, no prominent changes were observed in the presence of each agent alone (**Figure 1A**). This phenomenon was confirmed by Annexin V/PI staining, which suggests that 8-CPT-cAMP enhances the antitumor efficacy of bortezomib (**Figure 1B**). Moreover, the elevation of cleaved PARP and cleaved caspase-3 further reaffirmed these results (**Figure 1C**).

Next, we aimed to evaluate the effects of 8-CPT-cAMP in bortezomib-resistant MM cells. As expected, 8-CPT-cAMP synergized with bortezomib to induce marked morphological changes in U266-R and H929-R cells (**Figure 1A**). 8-CPT-cAMP alone did not significantly induce

A

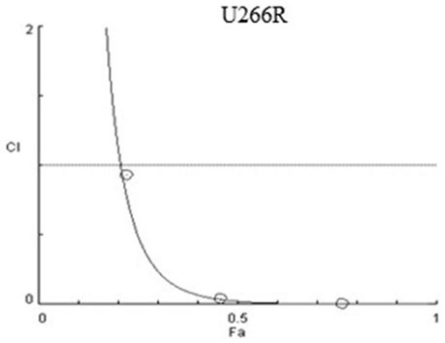


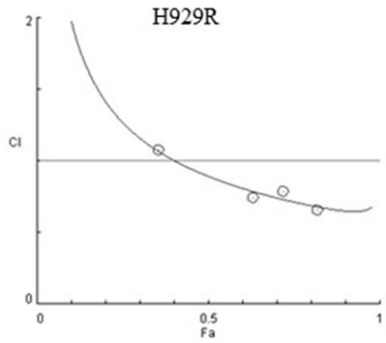


8-CPT-cAMP(μM)	BTZ (nM)	Fa	CI Value
50	1	0.173	0.41974
100	2	0.259	0.60719
200	4	0.467	0.68005
400	8	0.48	1.31607

8-CPT-cAMP(μM)	BTZ (nM)	Fa	CI Value
100	2	0.39883	1.38296
200	4	0.59199	0.44537
300	6	0.64664	0.38865
400	8	0.66729	0.41841

B





8-CPT-cAMP(μM)	BTZ (nM)	Fa	CI Value
50	10	0.1498	2.58
100	20	0.2215	0.93
200	40	0.4597	0.037
400	80	0.76489	0.001

8-CPT-cAMP(μM)	BTZ (nM)	Fa	CI Value
100	20	0.356	1.077
200	40	0.630	0.745
300	60	0.721	0.78
400	80	0.821	0.659

Figure 3. 8-CPT-cAMP and bortezomib have synergetic effects. CI analysis of the combination of 8-CPT-cAMP and bortezomib in (A) U266 and H929 cells and in (B) U266-R and H929-R cells. U266-R, U266 bortezomib-resistant cells; H929-R, H929 bortezomib-resistant cells. The experiments were performed in triplicate.

cell apoptosis in U266-R and H929-R cells. However, it dramatically enhanced the cell apoptotic effects of bortezomib (**Figure 1D**) and this was supported by the upregulation of cleaved PARP and cleaved caspase-3 (**Figure 1E**). To further verify that 8-CPT-cAMP was synergic with bortezomib in inducing MM cell apoptosis, bone marrow stromal cells (BMSCs) were isolated from three bortezomib-resistant MM patients. As depicted in **Figure 2A**, **2B**, it is

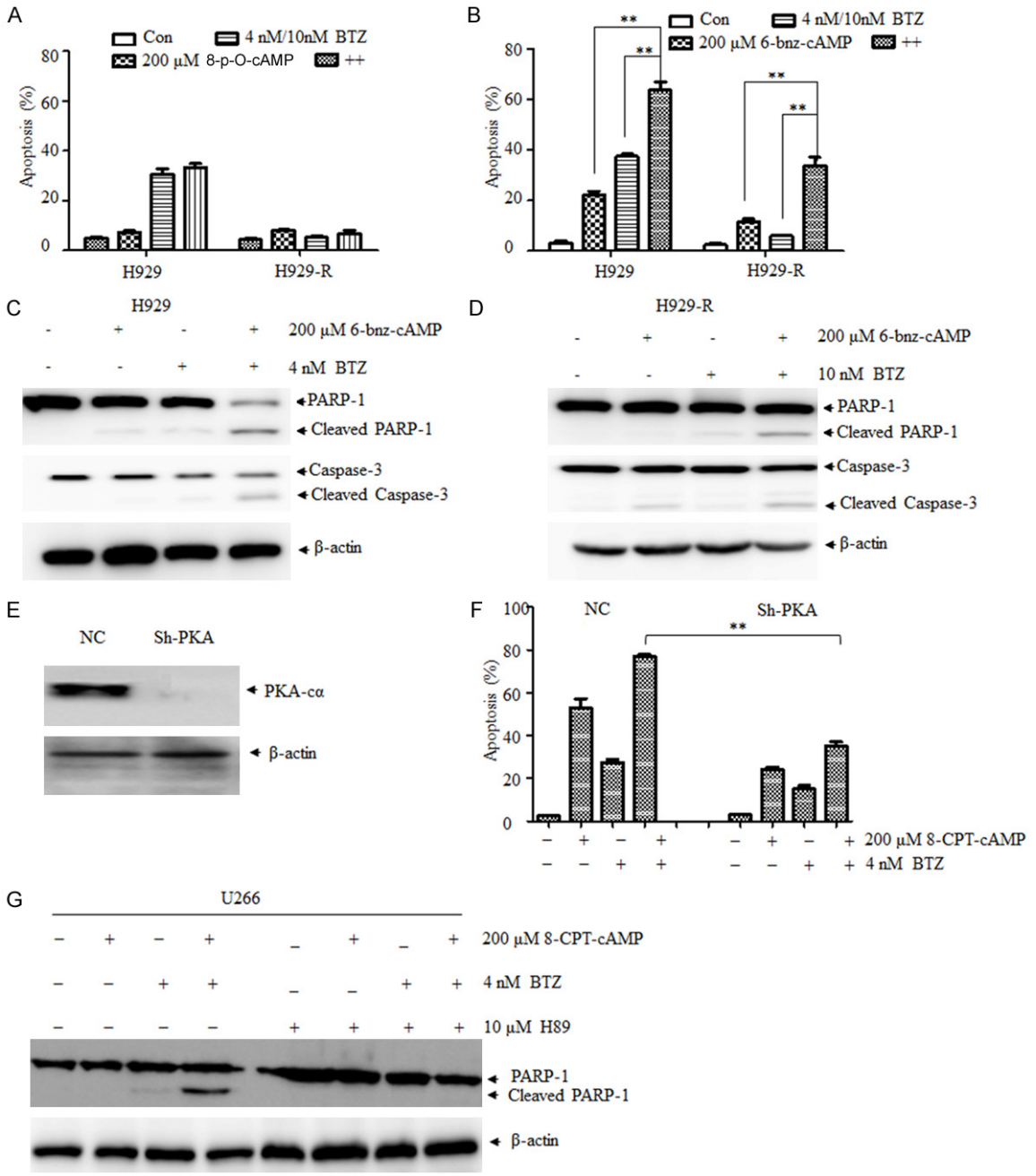


Figure 4. PKA is involved in the cell growth inhibition and apoptosis induced by bortezomib and cAMP. A. Cell apoptosis ratio of H929 and H929-R cells exposed to 8-p-O-cAMP (Epac activator), bortezomib, or their combination for 48 h, respectively. Cell apoptosis was evaluated by Annexin V/PI staining; B. Cell apoptosis ratio of H929 and H929-R cells exposed to 6-bnz-cAMP (PKA activator), bortezomib, or their combination for 48 h, respectively. Apoptosis was evaluated by Annexin V/PI staining; C. H929 cells were exposed to bortezomib, 6-bnz-cAMP, or their combination for 48 h, and the expression of PARP and caspase-3 was measured by Western blot; D. H929-R cells were exposed to bortezomib, 6-bnz-cAMP, or their combination for 48 h, and the expression of PARP and caspase-3 was measured by Western blot; E. Knockdown of PKA in H929 cells by stably transfected with non-specific shRNA pairs (normal control, NC) or shRNA pairs against PKA-Cα; F. PKA-silenced H929 cells were exposed to 8-CPT-cAMP, bortezomib, or their combination for 48 h. Apoptosis was evaluated by Annexin V/PI staining; G. The expression of PARP and caspase-3 was measured by Western blot after U266 cells were treated with 8-CPT-cAMP, bortezomib, H89 (PKA inhibitor), or their combination for 48 h. H929-R, H929 bortezomib-resistant cells. Epac, exchange protein directly activated by cAMP; PKA, protein kinase A. $^{**}P < 0.01$. Each experiment was performed in triplicate.

noteworthy that the combined treatment with 8-CPT-cAMP and bortezomib significantly promoted the apoptosis levels in BMSCs, which was confirmed by similar results, observed in $CD138^+$ co-cultured BMSCs. The additive or synergistic cytotoxicity effect of the combination of bortezomib and 8-CPT-cAMP was further analyzed using the Chou-Talalay method. In the four tested MM cell lines (U266, H929, U266-R, and H929-R), the CI values between 8-CPT-cAMP and bortezomib treatments were less than 1 (**Figure 3A**, **3B**), which suggested that the combination of these two drugs had synergistic effects in inducing MM cell apoptosis. Therefore, these results indicate that 8-CPT-cAMP synergizes with bortezomib in inducing MM cell apoptosis.

PKA activation was involved in bortezomib and cAMP-induced cell growth inhibition and apoptosis

As known, protein kinase A (PKA) is the main downstream effector protein in triggering biological responses. Therefore, the activators of PKA (6-Bnz-cAMP) and exchange protein directly activated by cAMP (Epac, 8-pCPT-2'-O-Me-cAMP) were further used to examine the potential role of PKA in the cell growth inhibition and apoptosis induced by cAMP and bortezomib. As can be seen in **Figure 4B**, 6-Bnz-cAMP inhibited the proliferation of both H929 and H929-R cells and enhanced bortezomib-induced cell apoptosis as indicated by Annexin V/PI staining, the upregulation of cleaved caspase-3, and cleaved PARP (**Figure 4C**, **4D**), as well as by the morphological changes observed (data not shown). Conversely, 8-pCPT-2'-O-Me-cAMP, a specific Epac activator, did not synergize with bortezomib to induce cell apoptosis (**Figure 4A**). These observations suggest that the activation of PKA was involved in the synergistic effect of the combination of cAMP and bortezomib in inducing apoptosis of MM cells. To confirm the validity of this hypothesis, the expression of PKA in H929 cells was silenced (**Figure 4E**). As expected, PKA knockdown significantly decreased the 8-CPT-cAMP- and bortezomib-induced cell apoptosis (**Figure 4F**). Furthermore, the addition of H89 (a specific PKA inhibitor) blocked the apoptosis-inducing effect triggered by bortezomib and 8-CPT-cAMP (**Figure 4G**). Taken together, these results suggested that PKA was involved in 8-CPT-cAMP- and bortezomib-induced apoptosis.

Downregulation of cyclinD1 and Mcl-1 was involved in 8-CPT-cAMP- and bortezomib-induced cell death

Our previous results showed that 8-CPT-cAMP and bortezomib inhibit MM cell proliferation and induce cell apoptosis [15]. Nevertheless, the underlying mechanism is not completely understood. As illustrated in **Figure 5A**, **5B**, the expression of cyclinD1 and Mcl-1 was significantly downregulated in both cell lines treated with the combination of 8-CPT-cAMP and bortezomib. In contrast, the expression of Bax and Bcl-2 was not significantly altered post-treatment. To clarify the mechanisms of the downregulation of cyclinD1 and Mcl-1, H929-R cells were treated with 8-CPT-cAMP in the presence or absence of the protein synthesis inhibitor cycloheximide (CHX). The half-life of cyclinD1 was reduced by the addition of CHX, whereas the half-life of Mcl-1 was not significantly changed (**Figure 5C**, **5D**). Moreover, the expression of these two genes at the mRNA level was measured by RT-PCR, which showed that the expression of cyclinD1 at the transcriptional level was not disturbed; however, downregulation of Mcl-1 might have been caused by a decrease in mRNA levels (**Figure 5E**, **5F**). These findings suggested that 8-CPT-cAMP-mediated cyclinD1 and Mcl-1 downregulation occurred at the protein and transcriptional level, respectively.

8-CPT-cAMP enhanced bortezomib-induced endoplasmic reticulum stress

It has been reported that bortezomib selectively inhibits the 26S proteasome and leads to the accumulation of misfolded proteins in MM cells, resulting in endoplasmic reticulum (ER) stress, followed by a coordinated cellular unfolded protein response (UPR) [19, 20]. As illustrated in **Figure 6A**, the expression of the ER stress markers, transcription activating factor-4 (ATF4) and CCAAT/enhancer-binding protein homologous protein (CHOP), at the mRNA level was significantly increased in H929 and H929-R cells treated with bortezomib alone. Moreover, this upregulation was further enhanced by the addition of 8-CPT-cAMP. In line with RT-PCR results, the expression of ATF4 and CHOP were dramatically increased at the

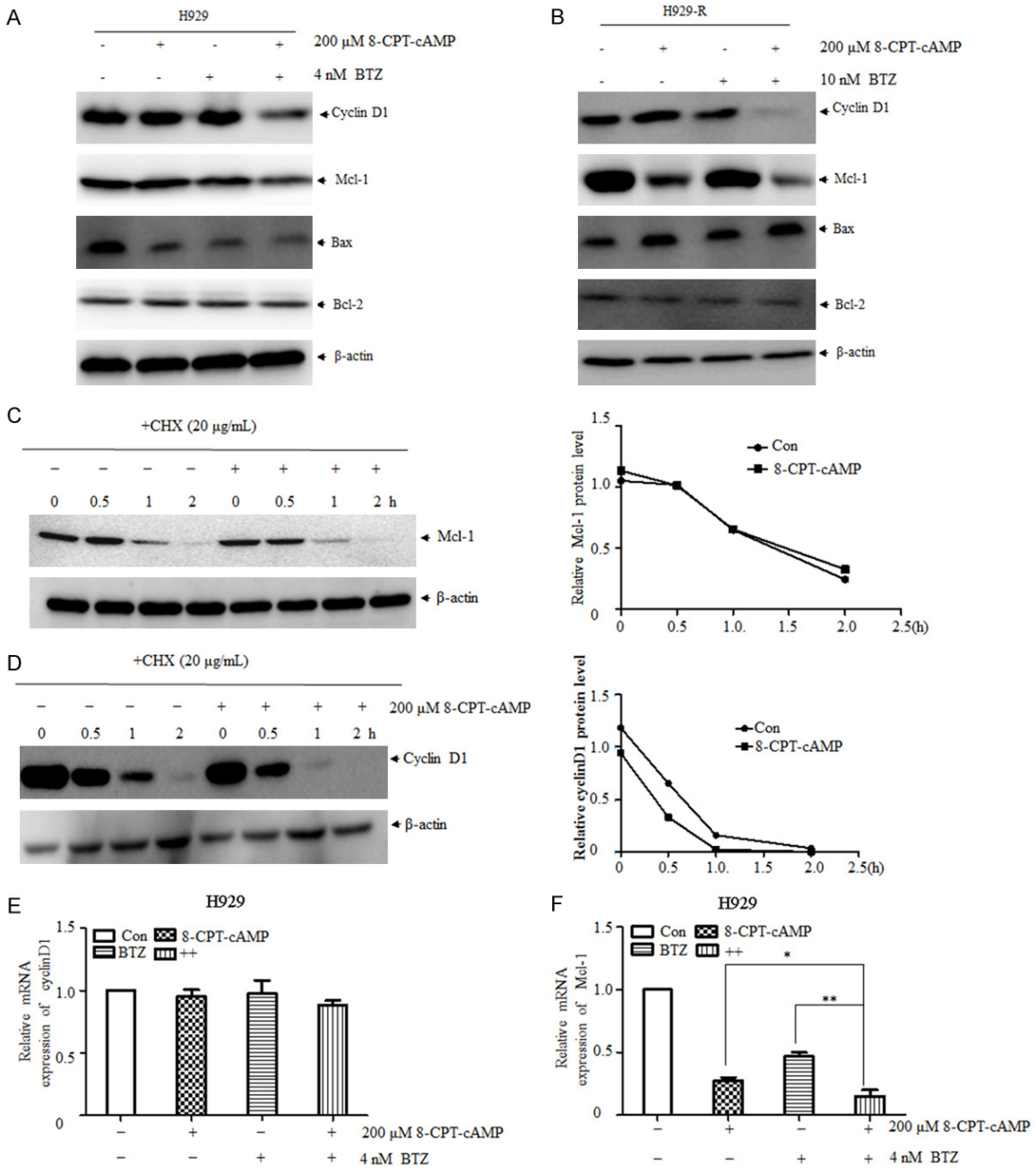


Figure 5. CyclinD1 and Mcl-1 downregulation was involved in 8-CPT-cAMP- and bortezomib-induced cell death. H929 (A) and H929-R (B) cells were exposed to 8-CPT-cAMP, bortezomib, or their combination for 48 h. The expression levels of cyclinD1, Mcl-1, Bax, and Bcl-2 were measured by immunoblot analysis. Cycloheximide (CHX) was used to detect the half-life of Mcl-1 (C) and cyclinD1 (D) affected by 8-CPT-cAMP in H929-R cells. Densitometry of proteins was performed; blot density was normalized to β-actin expression levels. After the treatment with 8-CPT-cAMP, bortezomib, or their combination, the expression of cyclinD1 (E) and Mcl-1 (F) at the mRNA level was assessed by RT-PCR. H929-R, H929 bortezomib-resistant cells. $^{**}P < 0.01$, $^{***}P < 0.001$. Each experiment was performed in triplicate.

protein level post-bortezomib treatment, whereas 8-CPT-cAMP enhanced the upregulation of these proteins induced by bortezomib (**Figure 6B**). These results indicated that ER stress was induced by bortezomib, and 8-CPT-cAMP promoted this effect to a certain extent.

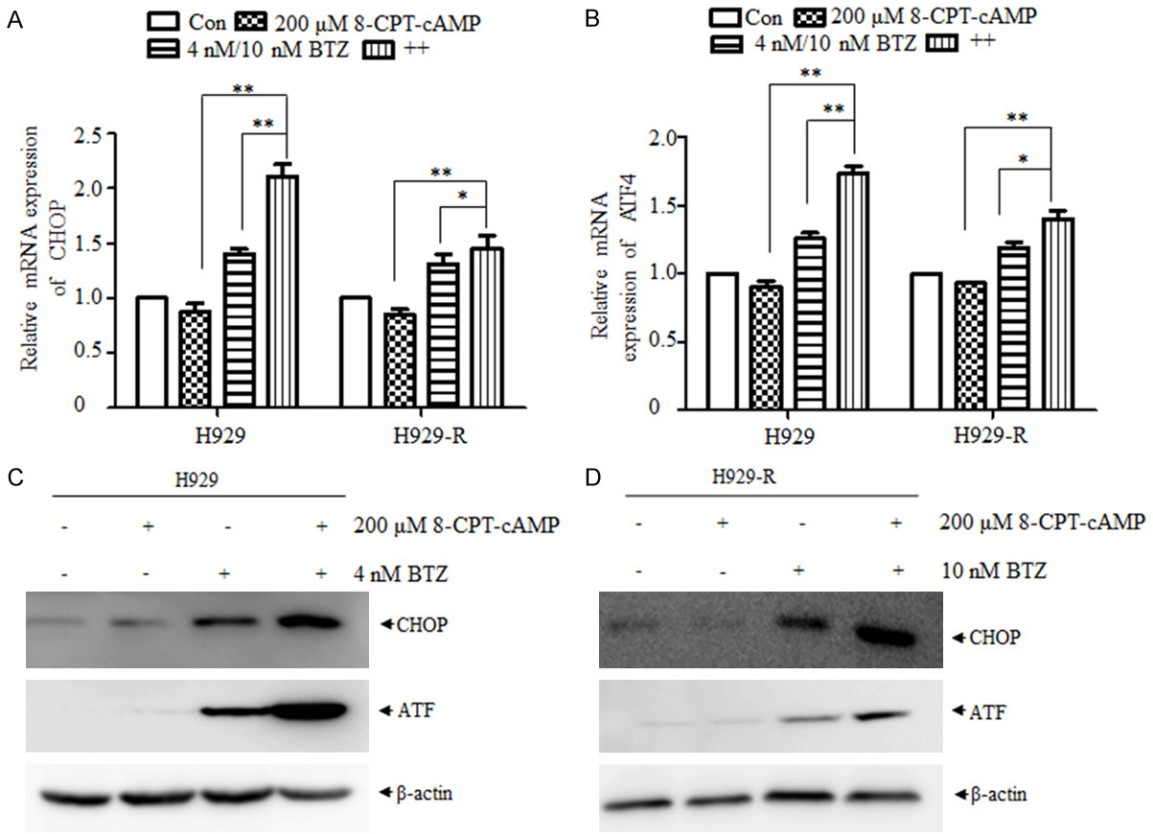


Figure 6. 8-CPT-cAMP enhanced the ER stress induced by bortezomib. The expression of CHOP and ATF4 (ER stress markers) was measured at the mRNA (A, B) and protein (C, D) levels after the treatment of H929 and H929-R cells with 8-CPT-cAMP, bortezomib, or their combination for 24 h in. H929-R, H929 bortezomib-resistant cells; ER, endoplasmic reticulum. *$P < 0.05$, **$P < 0.01$. The experiments were performed in triplicate.

Forskolin and bortezomib synergistically inhibits MM cell death in vivo

Finally, we investigated the *in vivo* efficacy of forskolin (a cAMP-elevating compound) and bortezomib in the MOPC-315 xenograft model. The combination of forskolin and bortezomib inhibited more the significantly tumor growth than the treatment with forskolin or bortezomib alone (**Figure 7A-C**). Furthermore, no significant body weight loss was observed in the groups, indicating adequate tolerance to the combination of forskolin and bortezomib (**Figure 7D**). Importantly, the forskolin and bortezomib combination significantly inhibited the proliferation and enhanced the cell death of tumor cells, as indicated by PCNA and TUNEL staining (**Figure 7E**). Furthermore, the expression of cyclinD1 and Mcl-1 in tumor tissues was significantly attenuated by the combination of forskolin and bortezomib (**Figure 7E**), which was in accordance with results obtained from cells experiments. These data further supported the notion that cAMP synergized with bortezomib to induce cell death of MM cells both *in vitro* and *in vivo*.

Discussion

The cAMP is a multifunctional second messenger that has been used in the treatment of hematological malignancies and solid tumors. In the present study, we demonstrated that cAMP synergizes with bortezomib to induce MM cell death and overcomes bortezomib resistance both *in vitro* and *in vivo*. Therefore, targeting cAMP may enhance the anti-proliferative and pro-apoptotic activities of bortezomib.

Currently, the combination chemotherapy based on proteasome inhibitors (PI) or immunomodulatory drugs (IMiDs) is the backbone of MM therapy. Bortezomib-containing regimens, including CyBorD (cyclophosphamide, bortezomib, and dexamethasone) and RVD (lenalido-

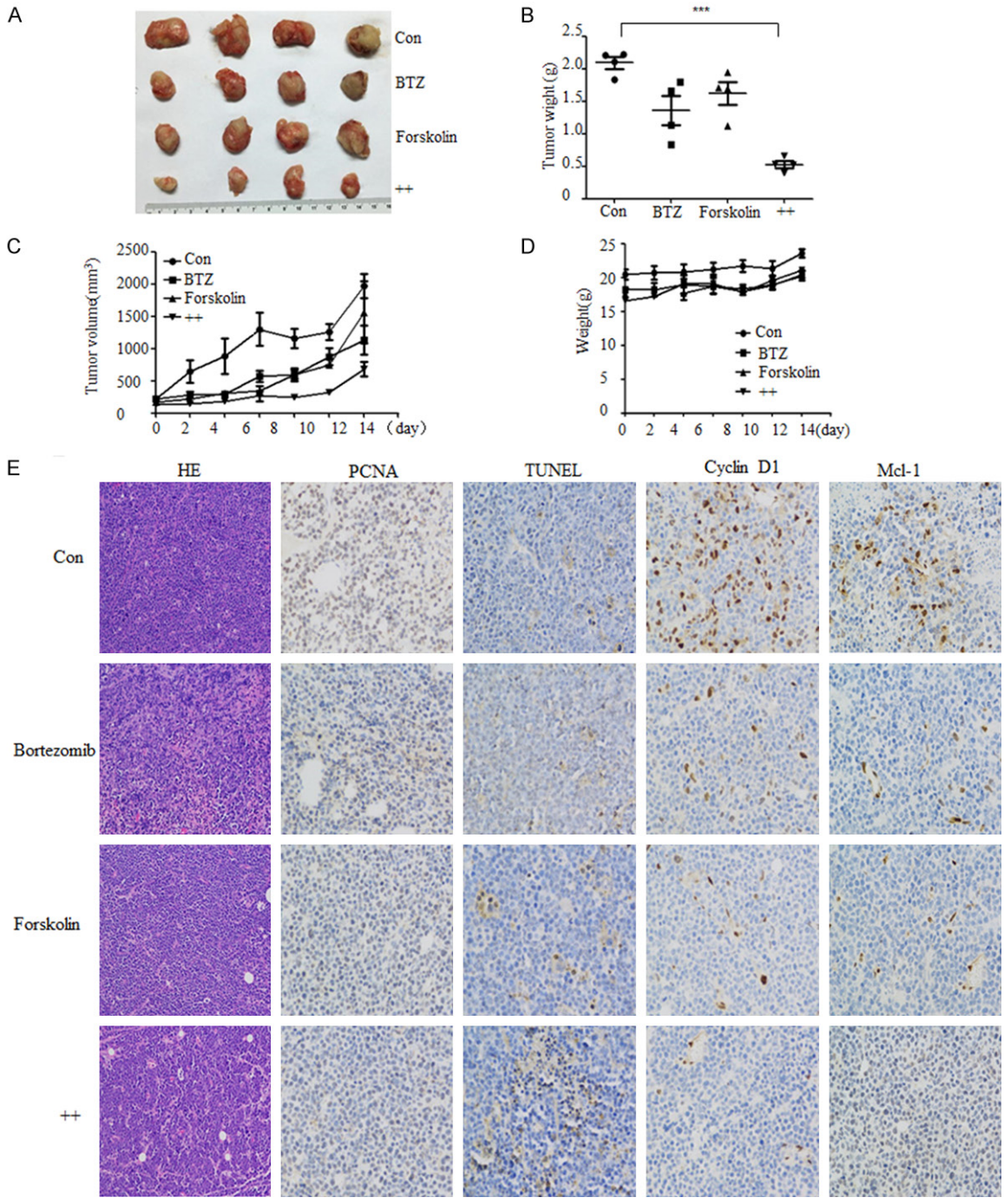


Figure 7. The combination of forskolin and bortezomib inhibits myeloma tumor growth *in vivo*. A xenograft MOPC315 MM mouse model was established, in which mice were administered with forskolin (5 mg/kg/d, i.p.), bortezomib (0.8 mg/kg, twice a week, i.v.), or their combination for two weeks. A. Tumor volume; B. Tumor weight; C. Consecutive measurement of tumor volume; D. Body weight of mice; E. Hematoxylin-eosin staining for histology, the expression of PCNA, TUNEL, cyclinD1, and Mcl-1 were analyzed by immunohistochemistry (IHC). ***$P < 0.001$. Scale bar = 50 μm.

mide, bortezomib, and dexamethasone), have been highlighted as frontline regimens for newly diagnosed or relapsed/refractory MM [21, 22]. However, the majority of MM patients relapse due to drug resistance [2, 23]. The combinations of bortezomib and novel agents,

including monoclonal antibodies, inhibitors of deacetylases, and phosphatidylinositol 3-kinase (PI3K) inhibitors, have yielded promising results [24, 25]. The cAMP signaling pathway has been identified as a key regulator of hematopoietic cell proliferation, differentiation, and apoptosis [10, 26-28]. In our previous study, we reported that a cAMP-modulating agent induced cell cycle arrest and apoptosis in MM cells [15]. In the present investigation, we found that cAMP synergized with bortezomib to trigger apoptosis in MM cells. Strikingly, cAMP substantially enhanced the apoptosis induced by low-dose bortezomib treatment not only in bortezomib-sensitive U266 and H929 parental cells, but also in U266-R and H929-R cells. Moreover, this ability of cAMP was also observed in isolated primary CD138-positive cells from recurrent MM patients as well as in *in vivo* studies. These results were in accordance with previously published data showing synergistic inhibition of bortezomib and forskolin on MM cells apoptosis [16].

Previous reports have indicated that cAMP functions *via* Epac and PKA, which are the main downstream effectors of cAMP in lymphoid cells [29-31]. The present study demonstrated that the activation of cAMP/Epac pathways did not affect cell growth and apoptosis of MM cells, whereas the activation of cAMP/PKA pathway did. Besides, the knockdown of PKA or the administration of an antagonist of PKA significantly abrogated the inhibition of cell growth and apoptosis, induced by the combination of cAMP and bortezomib, which confirmed that PKA was involved in cAMP- and bortezomib-induced cell death. In contrast, the role of the cAMP/PKA pathway in MM tumor cell pathophysiology and bortezomib resistance remains to be further studied.

It has long been recognized that interventions resulting in growth inhibition or apoptosis of cells influence the central regulators of cell cycle or apoptosis [18, 32]. This study showed that the exposure of MM cells to 8-CPT-cAMP led to dramatic downregulation of the expression of cyclinD1, which is in agreement with the findings of previous studies [33, 34]. The accumulation of Mcl-1 (one of the antiapoptotic Bcl-2 family proteins) has been shown to induce MM cell resistance to bortezomib-induced lethality [35], which was associated with the selective activation of the ATF4 signaling branch of the UPR [36, 37]. Our examination revealed that the activation of the cAMP signaling pathway led to rapid downregulation of Mcl-1 at the transcriptional and protein levels. This observation is in line with the results obtained by Virginie *et al.* that cAMP induced the downregulation of Mcl-1 in MM cells [13]. Interestingly, no significant expression modulation of cell cycle or apoptosis regulatory proteins was observed in the presence of a low dose of bortezomib alone, and 8-CPT-cAMP the applied alone exhibited lower apoptotic activity than drug combinations, which suggests that additional signals are involved in the synergistic induction of apoptosis.

In the case of MM, extensive immunoglobulin production sensitizes MM cells for ER stress-induced apoptosis. Thus, one approach for MM cell apoptosis is to trigger ER stress signaling pathways [38, 39]. In this context, two strategies may be taken into account: (1) interference with the ER function and (2) target inhibition of ER-associated degradation pathway to dispose misfolded proteins [40-42]. In this study, we found that bortezomib exposure triggered ER stress signaling, which was confirmed by the upregulation of the ER stress markers ATF4 and CHOP [37, 43]. Moreover, we observed that 8-CPT-cAMP synergized with bortezomib to enhance ER stress, which might be associated with cAMP signaling-mediated suppression of the homeostasis of the ER.

In conclusion, the present study demonstrated that synergism between bortezomib and cAMP triggers cell apoptosis in bortezomib-sensitive and -resistant MM cells, which might, to some extent, provide rational support for the clinical evaluation of the combination of bortezomib and cAMP signaling modulator in relapsed MM patients who are resistant to bortezomib.

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Disclosure of conflict of interest

None.

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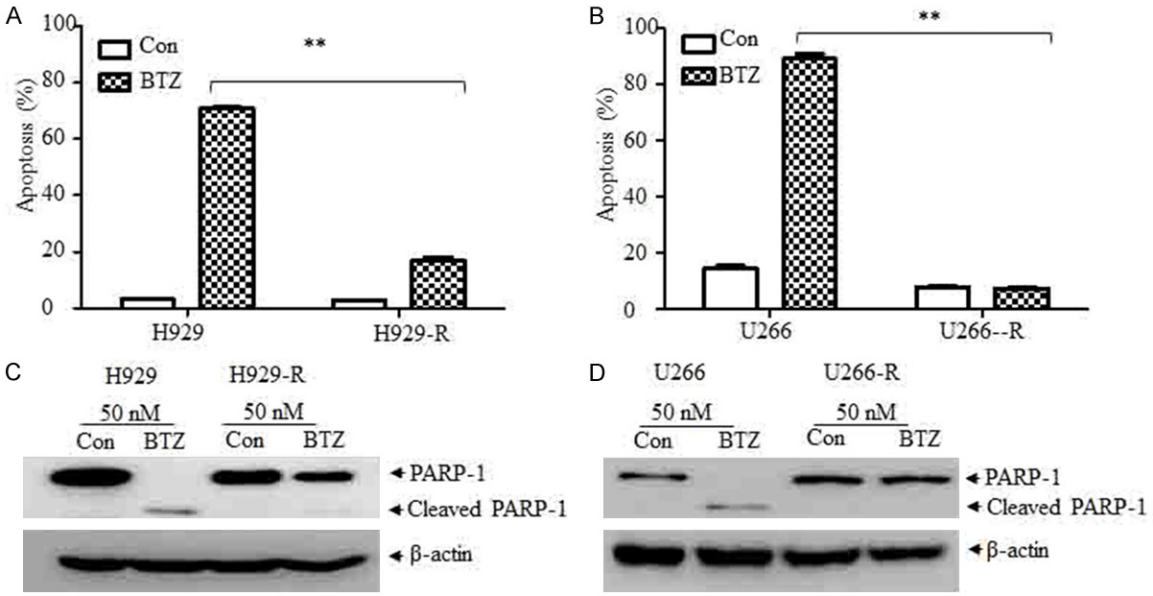
Supplementary material

To examine the effects of 8-CPT-cAMP in bortezomib-resistant cells, we initially established two bortezomib-resistant MM cell lines, named U266-R and H929-R. As can be seen in Supplementary Figure 1, U266-R and H929-R cells were resistant, in contrast to the control cells. Moreover, 50 nM of bortezomib significantly increased cleaved PARP (an indicator of apoptosis) expression in the control MM cells, whereas no cleaved PARP was observed in U266-R and H929-R cells (Supplementary Figure 1). These results indicated that bortezomib-resistant MM cells were successfully established and could be further used.



Supplementary Figure 1. Bortezomib-resistant MM cells were successfully established. Cell apoptosis ratio of H929 and H929-R (A), and U266 and U266-R (B) cells exposed to bortezomib (50 nM) for 24 h, respectively. Cell apoptosis was evaluated by Annexin V/PI staining. The expression of PARP and its cleaved form was assessed by Western blot post-bortezomib (50 nM) exposure for 24 h in H929 and H929-R (C) and U266 and U266-R (D) cells. $**P < 0.001$. The experiments were performed in triplicate.