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ClC5 Decreases the Sensitivity of Multiple Myeloma Cells to Bortezomib via Promoting Prosurvival Autophagy

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Resistance to bortezomib (BZ) is the major problem that largely limits its clinical application in multiple myeloma treatment. In the current study, we investigated whether ClC5, a member of the chloride channel family, is involved in this process. The MTT assay showed that BZ treatment decreased cell viability in three multiple myeloma cell lines (ARH77, U266, and SKO-007), with IC_{50} values of 2.83, 4.37, and 1.91 nM, respectively. Moreover, BZ increased the conversion of LC3B-I to LC3B-II and expressions of beclin-1 and ATG5, concomitantly with a decreased p62 expression. Pharmacological inhibition of autophagy with 3-MA facilitated cell death in response to BZ treatment. Additionally, BZ increased ClC5 protein expression in ARH77, U266, and SKO-007 cells. Knockdown of ClC5 with small interfering RNA sensitized cells to BZ treatment, and upregulation of ClC5 induced chemoresistance to BZ. Furthermore, ClC5 downregulation promoted BZ-induced LC3B-I to LC3B-II conversion and beclin-1 expression, whereas overexpression of ClC5 showed the opposite results in ARH77 cells. Finally, BZ induced dephosphorylation of AKT and mTOR, which was significantly attenuated by ClC5 inhibition. However, ClC5 upregulation further enhanced AKT and mTOR dephosphorylation induced by BZ. Our study demonstrates that ClC5 induces chemoresistance of multiple myeloma cells to BZ via increasing prosurvival autophagy by inhibiting the AKT–mTOR pathway. These data suggest that ClC5 may play a critical role in future multiple myeloma treatment strategies.

Key words: Bortezomib (BZ); Multiple myeloma; Chemoresistance; Autophagy; ClC5; AKT–mTOR pathway

INTRODUCTION

Multiple myeloma is a frequent malignancy of plasma cells that is characterized by bone destruction, anemia, hypercalcemia, renal disease, and immunodeficiency[1–3]. Its incidence varies globally from 1/100,000 people in China[4]. Patients with multiple myeloma often succumb to recurrence or increased susceptibility to viral, bacterial, and fungal infection, which are the major underlying cause of death[5]. For the last decades, extensive research efforts have sought to characterize the molecular mechanisms or targets that contribute to the development of multiple myeloma[6]. Although the survival rates could now exceed 10 years due to the use of various novel treatment options including bortezomib (BZ), thalidomide, lenalidomide, and hematopoietic stem cell transplantation, multiple myeloma is still an incurable hematological malignancy[7,8]. The major problem in multiple myeloma treatment is drug resistance that is produced by high-dose chemotherapy[4,5]. Therefore, there is an unmet clinical need for developing more effective treatments to facilitate the sensitivity of multiple myeloma cells to chemotherapy.

Increasing lines of evidence indicate that the proteasome is necessary for tumor cell angiogenesis, growth, and apoptosis[9–11]. Proteasome inhibition has been implicated as an important therapeutic strategy for the treatment of multiple myeloma[12]. BZ is the first proteasome inhibitor to be introduced for clinical treatment and is predominantly used in combination with prednisolone or thalidomide in multiple myeloma patients[13,14]. BZ inhibits nuclear factor κ light chain enhancer of activated B cell (NF-κB) activation and antiapoptotic B-cell lymphoma 2 (Bcl-2) expression and disrupts the balance between protein biosynthesis and protein degradation, leading to cell death by apoptosis[11,14]. However, emerging studies have suggested that increasing administration of BZ can eventually develop drug resistance[12,15,16]. While there are several possible explanations involved in the resistance to BZ in multiple myeloma cells, such as insulin growth

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factor-1, proteasome subunit β5 mutation, and the differentiation status of myeloma cells[17–19], the exact mechanisms have not yet been fully understood.

Autophagy is a highly conserved catabolic process that could digest and recycle cellular proteins and organelles to maintain cellular homeostasis[20]. Multiple research studies have shown that autophagy serves as a potential mechanism for the development of chemoresistance in cancer cells[11,12,21]. Moreover, it should be noted that autophagy exerts different roles in regulating chemoresistance toward various antineoplastic therapies[20,21]. Recent findings support the theory that the protective role of autophagy may promote the survival of tumor cells to overcome the stress caused by chemotherapy[22,23]. On the contrary, autophagy-activated cell death is also the predominant mechanism that enhances chemotherapy sensitivity[24,25].

Growing evidence has shown that chloride channels are critical for growth control in a number of cell types, including rat aortic smooth muscle cells, glioma cells, and mouse liver cells[26–28]. Chloride channel 5 (ClC5), a member of the chloride channel family, is primarily found to be expressed in the kidney[29]. It has been demonstrated that ClC5 is expressed in glioma cells, native leukocytes, and leukemic cells[30–32]. Several microRNAs (miRNAs) hosted in the ClC5 locus could increase the resistance of chronic lymphocytic leukemia cells to chemotherapy[33]. Moreover, the expression of ClC5 was cell cycle dependent in myeloid cells[29]. In the present study, we set out to examine the role of autophagy in BZ resistance and the crosstalk between autophagy and ClC5 in multiple myeloma cells under BZ exposure. Our findings suggest, through different multiple myeloma cell lines, that ClC5 may contribute to the evasion of multiple myeloma cell death in response to BZ through enhancing prosurvival autophagy.

MATERIALS AND METHODS

Materials and Reagents

Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), streptomycin, penicillin, and glutamine were purchased from Gibco (Grand Island, NY, USA). BZ was obtained from Selleckchem (Houston, TX, USA) and dissolved in 10 mM ethanol. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-methyladenine (3-MA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The 3-MA was dissolved in dimethyl sulfoxide (DMSO) to make stock solutions of 1 mM. Antibodies targeting microtubule-associated protein 1A/1B light chain 3-I/II (LC3B-I/II), autophagy protein 5 (ATG5), phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa (p62), phosphorylated AKT (p-AKT), AKT, phosphorylated mechanistic target of rapamycin (p-mTOR), and mTOR were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies targeting beclin-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibody of ClC5 was purchased from Abcam (Cambridge, MA, USA). All secondary horseradish peroxidase (HRP)-linked antibodies were from Thermo (Wilmette, IL, USA).

Cell Culture

The human multiple myeloma cell lines ARH77, U266, and SKO-007 were acquired from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 medium supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 4 mM glutamine in a humidified atmosphere with 5% CO_2 at 37°C.

Cell Viability Assay

The cell viability was detected using the MTT assay. ARH77, U266, or SKO-007 cells were seeded in 96-well plates at a density of 10^4 cells per well. After the indicated treatments, the cells were incubated with MTT at a final concentration of 10 μg/ml for 4 h (5% CO_2, 37°C). The medium was removed, and the cells were dissolved in DMSO. The absorbance was measured at a wavelength of 490 nm using a microplate reader (Bio-Tek, Winooski, VT, USA). The value of IC_{50} was calculated with the SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA).

Western Blotting Analysis

The cells were lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Jiangsu, P.R. China) containing protease and phosphatase inhibitors (Roche Applied Science, Indianapolis, IN, USA) at 4°C. The lysates were centrifuged at 12,000×*g* for 15 min at 4°C, and the supernatants were harvested. The protein content was quantified by the Enhanced BCA Protein Assay Kit (Beyotime). Equal amounts of protein were supplemented with concentrated sodium dodecyl sulfate (SDS) sample buffer and denatured at 100°C for 5 min. Forty micrograms of protein was separated on a 10%–12% polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked with 5% nonfat milk at room temperature for 1 h and then probed with primary antibodies including anti-LC3B-I/II (1:1,000), anti-beclin-1 (1:500), anti-ATG5 (1:2,000), anti-p62 (1:500), anti-ClC5 (1:500), anti-p-AKT (1:1,000), anti-AKT (1:1,000), anti-p-mTOR (1:1,000), anti-mTOR (1:1,000), and anti-GAPDH (1:4,000). The immunoreactive proteins were detected with HRP-linked secondary antibodies (1:1,000) and an enhanced chemiluminescence reagent (Beyotime).

Densitometry of bands was quantified using a Molecular Imager, ChemiDoc XRS System (Bio-Rad).

Knockdown of ClC5 by siRNA

The stealth small interfering RNA (siRNA) targeting ClC5 (5′-GCACTTCCATCATTCATTT-3′) was synthesized and purchased from Invitrogen (Carlsbad, CA, USA). ARH77, U266, or SKO-007 cells were electroporated with either ClC5 siRNA (si-ClC5; 40 nM) or negative siRNA (NS; 40 nM) using the Nucleofector Kit (Lonza, Basel, Switzerland). The cells were then replaced with RPMI-1640 medium containing 10% FBS and incubated for 48 h at 37°C.

ClC5 Transfection

Full-length cDNA for human ClC5 was purchased from OpenBioSystems (Huntsville, AL, USA) and then was amplified and cloned into pCMV-Tag2 (Invitrogen). ClC5 plasmid DNA (2 μg) was transfected using Lipofetamine Plus reagent diluted in Opti-Minimum Essential Medium (Opti-MEM; Invitrogen) for 48 h according to the supplier's instructions.

Statistical Analysis

All data were given as mean±SEM. Statistical analysis of the data between the control and treatment groups was performed using one-way analysis of variance (ANOVA) or the unpaired two-tailed Student's *t*-test. Statistical analysis was performed by the SPSS 17.0 statistical software. A value of $p<0.05$ indicated statistical significance.

RESULTS

BZ Induced Cell Growth Inhibition and Autophagy in Multiple Myeloma Cell Lines

The MTT assay showed that BZ inhibited the cell viability of ARH77, U266, and SKO-007 cells in a dose-dependent manner. After 48 h of BZ treatment, the IC_{50} value of BZ was 2.83±0.15, 4.37±0.21, and 1.91±0.11 nM in ARH77, U266, and SKO-007 cells, respectively (Fig. 1A). A 3, 4.5, and 2 nM dose of BZ was therefore used for ARH77, U266, and SKO-007 cells, respectively. Autophagy has been demonstrated to play an important role in the development of chemoresistance in multiple myeloma[34]. LC3B-I conversion to LC3B-II is a hallmark of autophagic activity, and Western blotting showed that BZ treatment significantly promoted the conversion of LC3B-I to LC3B-II in ARH77, U266, and SKO-007 cells. Furthermore, BZ treatment also increased the expression of beclin-1 and ATG5, along with decreased expression of p62, suggesting BZ induces autophagy in multiple myeloma cells (Fig. 1B). To further explore whether autophagy serves as a survival or cell death mechanism that facilitates or attenuates chemoresistance of multiple myeloma cells to BZ treatment, all three multiple myeloma cell lines were treated with BZ in the presence or absence of 3-MA (1 μM) for 48 h, and cell viability was measured. The 3-MA treatment alone had little influence on the viability of ARH77, U266, and SKO-007 cells at a dose of 1 μM. However, combined treatment with BZ and 3-MA further decreased cell viability, compared with cells treated with BZ alone (Fig. 1C). These data suggest that autophagy is likely to be a prosurvival mechanism that attenuates the chemosensitivity of multiple myeloma cells to BZ treatment.

BZ Increased ClC5 Expression in Multiple Myeloma Cells

ClC5 has been found to be expressed in several cancer cell types, and its expression has been demonstrated to be associated with proliferation of myeloid cells[27,29,33]. To investigate whether ClC5 is involved in BZ resistance of multiple myeloma cells, the effect of BZ on ClC5 expression was examined. Western blotting analysis showed that BZ treatment significantly increased ClC5 expression in ARH77 cells (Fig. 2A). In addition, we confirmed these phenomena in two other multiple myeloma cell lines, U266 (Fig. 2B) and SKO-007 (Fig. 2C). These results indicate that the increased ClC5 expression may be associated with BZ resistance of multiple myeloma cells.

Knockdown of ClC5 Facilitated Multiple Myeloma Cell Sensitivity to BZ

To clarify the function of ClC5 in BZ resistance, ClC5 siRNA was transfected into ARH77, U266, and SKO-007 cells, respectively. The data revealed that ClC5 protein expression was markedly decreased by more than 65% in ClC5 siRNA-transfected cells (Fig. 3A). Furthermore, all three multiple myeloma cell lines were transfected with ClC5 siRNA, followed by incubation with BZ. Inhibition of ClC5 had no effect on the viability of multiple myeloma cells under the basal level (data not shown). Nevertheless, ClC5 knockdown further decreased the cell viability of multiple myeloma cells with BZ treatment (Fig. 3B).

Upregulation of ClC5 Attenuated Chemosensitivity of Multiple Myeloma Cells to BZ

To further evaluate the role of ClC5 in regulating BZ resistance in multiple myeloma cells, we overexpressed ClC5 in ARH77, U266, and SKO-007 cells. Expression of the ClC5 plasmid showed increased ClC5 expression, whereas no increase in the empty vector group was observed (Fig. 4A). Moreover, overexpression of ClC5 had little influence on the viability of multiple myeloma cells under the basal level (data not shown). However, cell survival was restored in cells that were treated with ClC5 plasmid and BZ (Fig. 4B), suggesting that ClC5 overexpression induces BZ resistance.

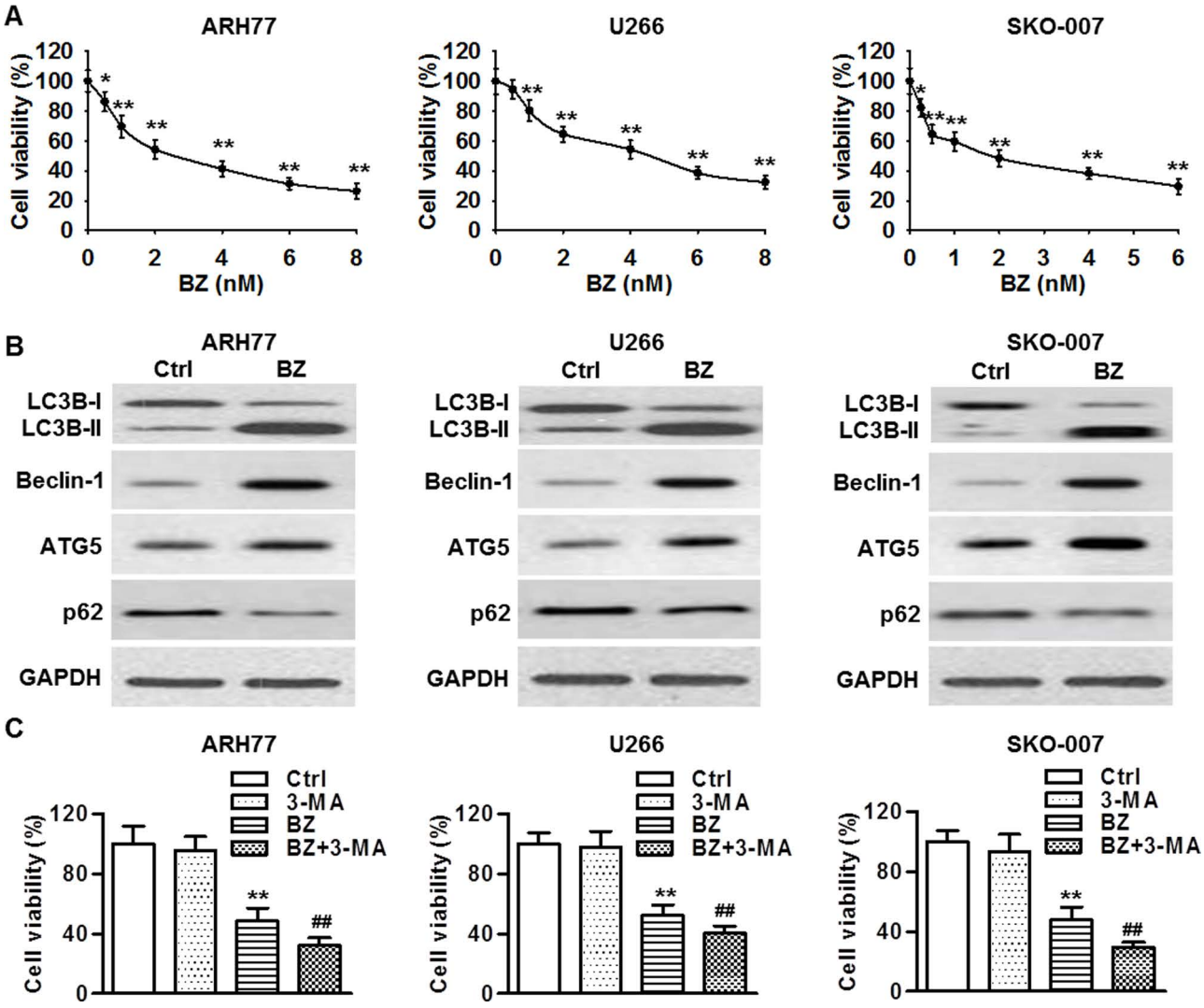

Figure 1. Bortezomib (BZ) treatment induced cell growth inhibition and autophagy in multiple myeloma cell lines. (A) ARH77, U266, and SKO-007 cells were treated with various concentrations of BZ for 48 h. The viability of multiple myeloma cells was decreased in a dose-dependent manner. *$p<0.05$, **$p<0.01$ versus control (0 nM), $n=8$. (B) The protein expressions of microtubule-associated protein 1A/1B light chain 3-I/II (LC3B-I/II), beclin-1, autophagy protein 5 (ATG5), and phosphotyrosine-independent ligand for the Lck SH2 domain of 62 kDa (p62) were determined by Western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the loading control. (C) All three multiple myeloma cell lines were treated with BZ in the presence or absence of 3-methyladenine (3-MA; 1 μM) for 48 h, and cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **$p<0.01$ versus control; ##$p<0.01$ versus BZ, $n=6$.

ClC5 Enhanced BZ-Induced Autophagy via Inhibition of the AKT–mTOR Pathway

Our above results showed that ClC5 knockdown or inhibition of autophagy further augmented the BZ-induced decrease in multiple myeloma cell viability. To explore the association between ClC5 and autophagy in regulating multiple myeloma cell sensitivity to BZ, we first analyzed the effect of ClC5 on autophagic markers in ARH-77 cells. Although the conversion of LC3B-I to LC3B-II and beclin-1 expression remained unchanged in cells transfected with ClC5 siRNA, blockade of ClC5 markedly inhibited the BZ-induced conversion and beclin-1 expression (Fig. 5A). On the contrary, overexpression of ClC5 significantly enhanced the conversion of LC3B-I to LC3B-II and beclin-1 expression induced by BZ (Fig. 5B). The data indicate that ClC5 downregulation facilitates multiple myeloma cell sensitivity to BZ via suppression of autophagy. AKT–mTOR is an important signaling pathway in the regulation of autophagic activity[22]. We next investigated whether AKT–mTOR signaling was also involved in the mechanism by which ClC5

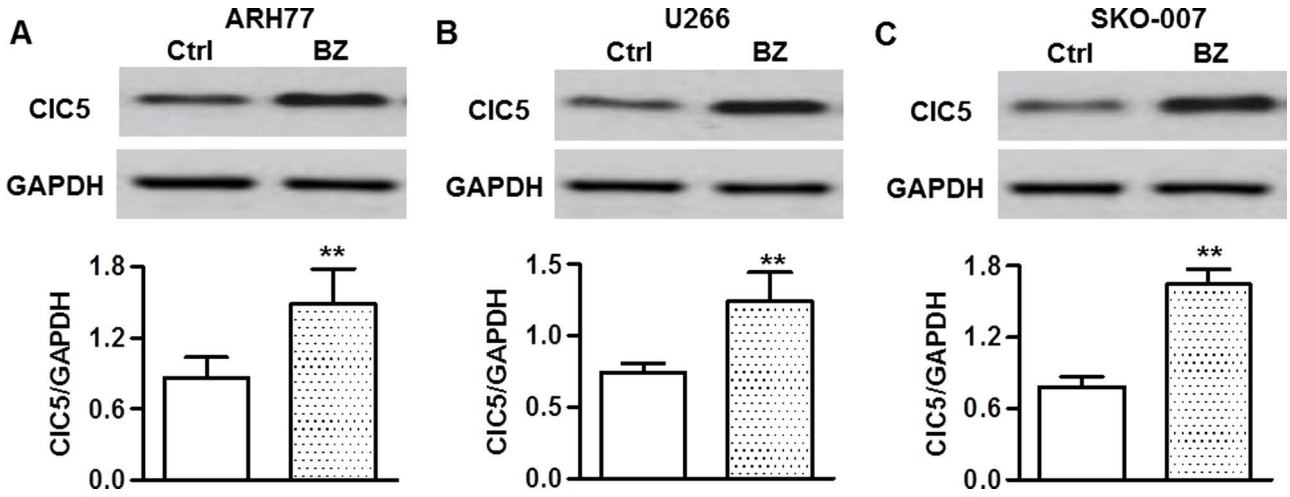

Figure 2. BZ increased chloride channel 5 (ClC5) expression in multiple myeloma cells. ARH77 (A), U266 (B), and SKO-007 (C) cells were treated with BZ for 48 h. The protein expression of ClC5 was analyzed by Western blotting. GAPDH was used as a loading control. $^{**}p<0.01$ versus control, $n=6$.

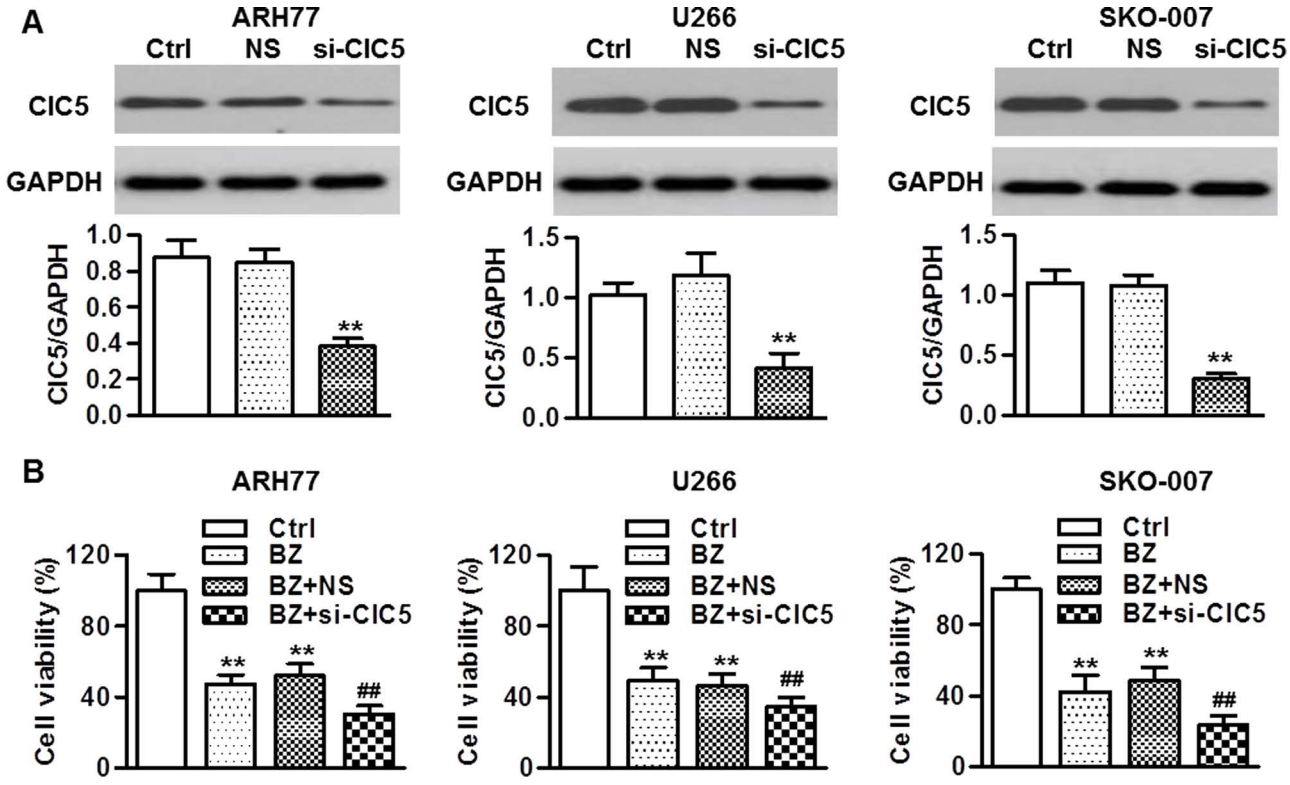

Figure 3. Knockdown of ClC5 enhanced BZ-induced cell growth inhibition. (A) Transfection efficiencies of ClC5 small interfering RNA (siRNA) were determined. Multiple myeloma cells were transfected with 40 nM ClC5 siRNA (si-ClC5) or negative siRNA (NS) for 48 h. ClC5 expression was examined by Western blotting. GAPDH was used as the loading control. $^{**}p<0.01$ versus control, $n=6$. (B) After 48 h of siRNA transfection, the cells were then incubated with BZ for another 48 h. MTT assay was used to test cell viability. $^{**}p<0.01$ versus control; $^{\#\#}p<0.01$ versus BZ, $n=8$.

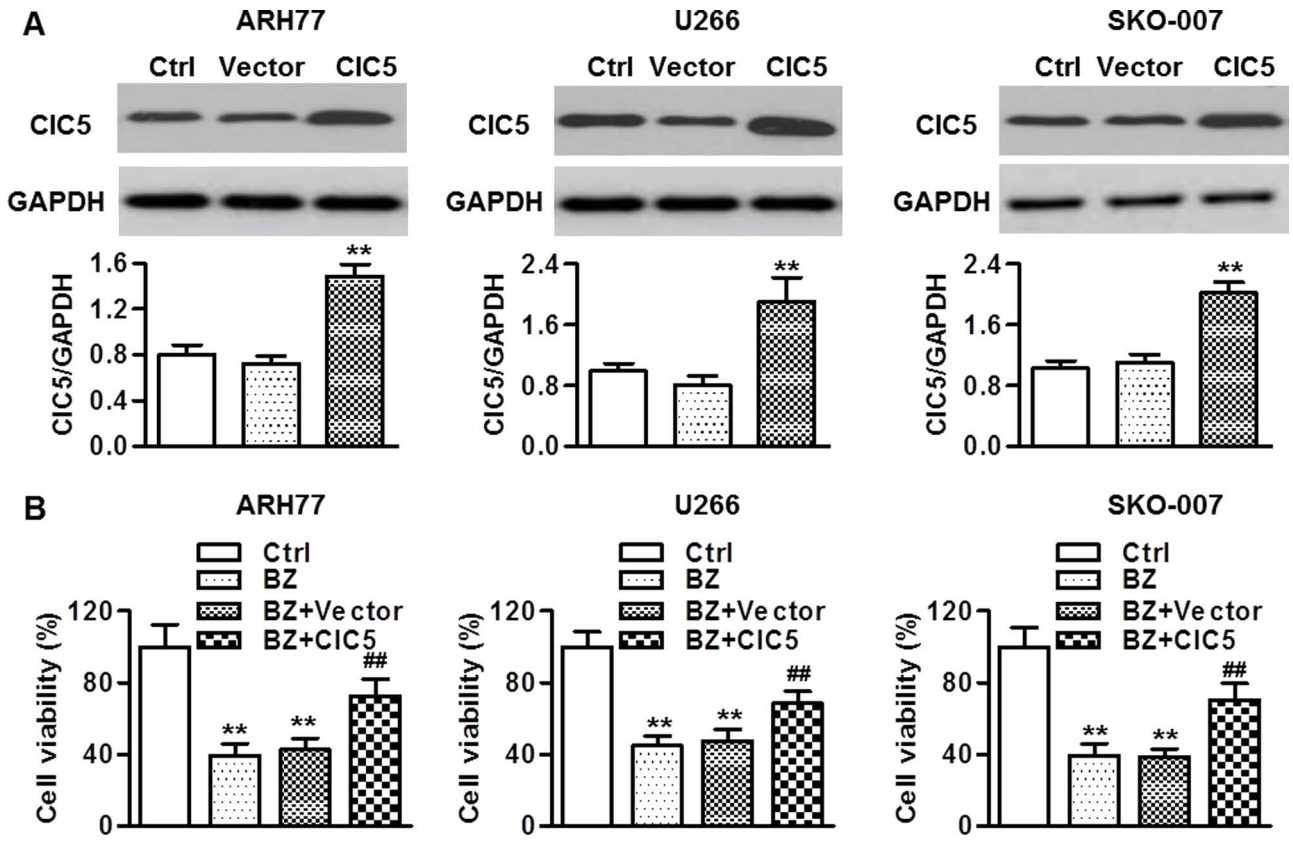

Figure 4. ClC5 upregulation decreased the sensitivity of multiple myeloma cells to BZ treatment. (A) The cells were transfected with ClC5 plasmid for 48 h. Overexpressing efficiencies were detected by Western blotting. GAPDH was used as a loading control. $^{**}p<0.01$ versus control, $n=6$. (B) After transfection with the ClC5 plasmid for 48 h, the cells were then treated with BZ for 48 h. The viability of multiple myeloma cells was examined by MTT assay. $^{**}p<0.01$ versus control; $^{\#\#}p<0.01$ versus BZ, $n=6$.

inhibition suppresses autophagy in multiple myeloma cells. Western blotting showed that the phosphorylation of AKT and mTOR in ARH77 cells was markedly inhibited after BZ treatment. However, ClC5 downregulation significantly attenuated the dephosphorylation of AKT and mTOR (Fig. 5C). On the other hand, upregulation of ClC5 further enhanced the inhibitory effect of BZ on AKT–mTOR signaling (Fig. 5D). Collectively, the results demonstrate the involvement of AKT–mTOR signaling in ClC5-mediated autophagy.

DISCUSSION

In the present study, we explored the relationship between ClC5 and BZ resistance in multiple myeloma cells. Our results are the first to demonstrate that ClC5 is involved in multiple myeloma cell chemoresistance to BZ. The findings can be summarized as follows: 1) BZ treatment induced autophagy, and inhibition of autophagy augmented the cytotoxic activity of BZ against multiple myeloma cells. 2) ClC5 expression was increased after treatment with BZ. Knockdown of ClC5 enhanced BZ chemosensitivity in multiple myeloma cells, whereas ClC5 upregulation inhibited the BZ-induced decrease in cell viability. 3) The inhibitory effect of ClC5 on BZ sensitivity may be associated with increased autophagy by inactivating the AKT–mTOR signaling pathway.

Multiple studies have shown that BZ is able to induce autophagy in different cancer cell types. For example, BZ could induce autophagy via endoplasmic reticulum stress in breast cancer cells[16,35]. Moreover, BZ has the ability to induce autophagy through a mitochondria–caspase-dependent pathway in chondrosarcoma cells[36]. Our results showed that BZ was capable of increasing autophagy in multiple myeloma cells, which was supported by the increased expression of LC3B-II, beclin-1, and ATG5. In fact, this was in line with an earlier study[12]. Furthermore, the function of autophagy is controversial in cancer; likely this could be a survival or cell death mechanism. On the one hand, autophagy appears to function as a tumor suppressor[24,25]. On the other hand, it has been proposed as a prosurvival mechanism that resists cell death[22,23].

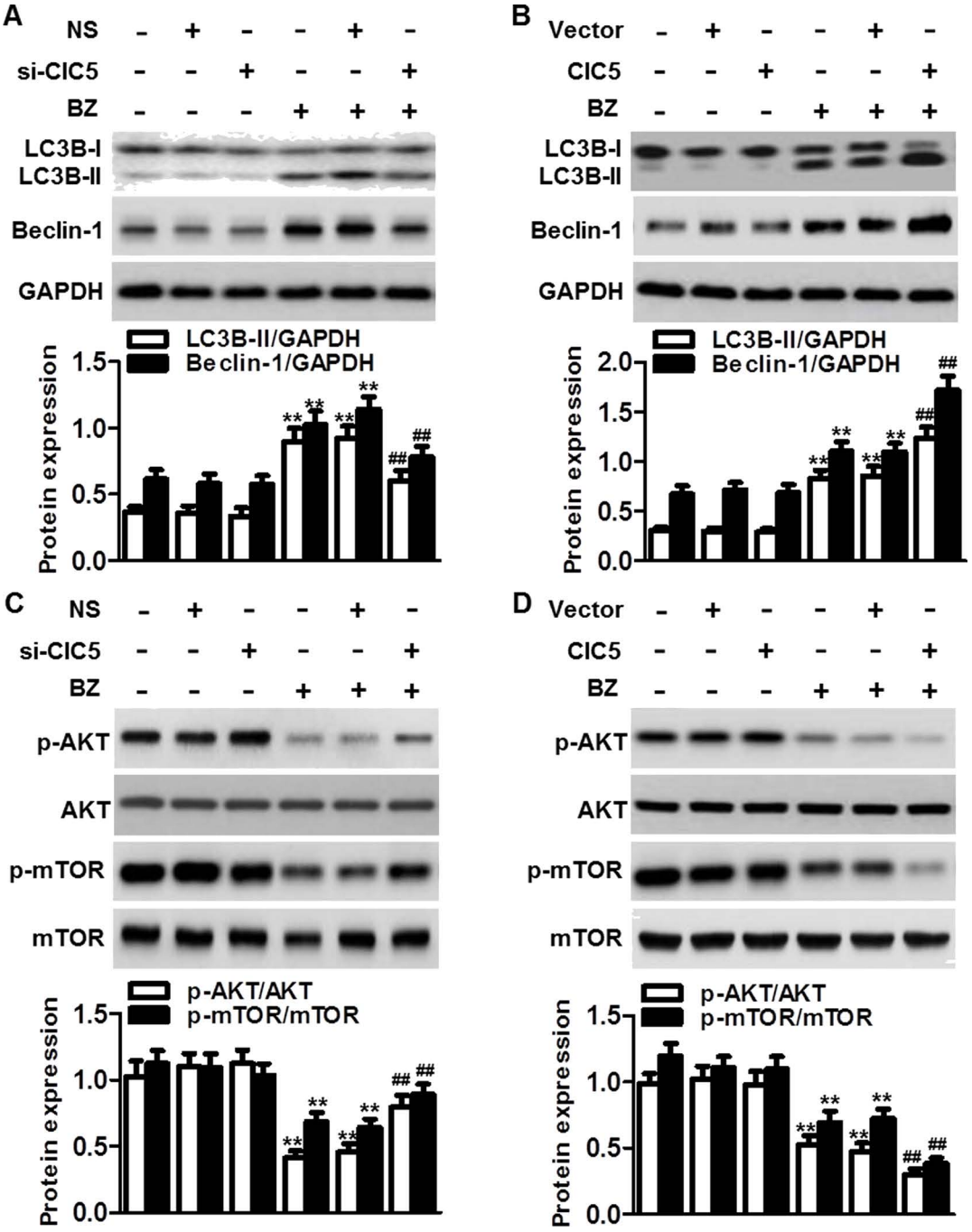

Figure 5. ClC5 facilitated BZ-induced autophagy by inactivating the AKT–mTOR signaling pathway. ARH77 cells were transfected with ClC5 siRNA (A) or plasmid (B) for 48 h and then treated with or without BZ for another 48 h. The protein expressions of LC3B-I/II and beclin-1 were analyzed by Western blotting. GAPDH was the loading control. $^{**}p<0.01$ versus control; $^{\#\#}p<0.01$ versus BZ, $n=6$. (C) Knockdown of ClC5 downregulation attenuated BZ-induced dephosphorylation of AKT and mechanistic target of rapamycin (mTOR). Ratios of phosphorylated to unphosphorylated AKT and mTOR, respectively, are depicted. (D) ClC5 upregulation further enhanced the inactivation of AKT–mTOR signaling induced by BZ. $^{**}p<0.01$ versus control; $^{\#\#}p<0.01$ versus BZ, $n=4$.

Despite the confusing role of autophagy, there is increasing evidence showing that autophagy is associated with the resistance of cancer cells to BZ therapy. For instance, the development of BZ-resistant breast cancer cells was accompanied with increased autophagy, and increased autophagy could attenuate apoptotic death[35,37]. Moreover, inhibition of autophagy sensitized cervical cancer cells to BZ therapy[10]. Our data in this study revealed that inhibition of autophagy with 3-MA further augmented the effect of BZ on cell death in multiple myeloma cells. This was consistent with a previous study showing that blockade of autophagy with another autophagy inhibitor, bafilomycin A1, enhanced BZ-induced apoptosis in multiple myeloma cells[34].

On the basis of the above observations, targeting protective autophagy may be an alternative approach to enhance the efficacy of BZ therapy. Our findings showed that BZ exposure markedly increased ClC5 expression in different multiple myeloma cell lines. The expression of ClC5 was positively correlated with the increased level of autophagy, indicating that ClC5 may have a role in autophagy-mediated chemoresistance under BZ treatment. To the best of our knowledge, there have been only a few reports showing expression of ClC5 in several cancer cells[30,32,33]. The function of ClC5 in cancer cell growth or chemosensitivity is still unknown. In the current study, we investigated the non-ion channel function of ClC5 in mediating BZ sensitivity in multiple myeloma cells. Our results showed that the BZ-induced decrease in cell viability was suppressed by ClC5 overexpression, whereas ClC5 knockdown exhibited opposite effects. Moreover, ClC5 further promoted autophagy that in part attenuated drug-induced cell death. These data clearly support the notion that ClC5 plays a critical role in the chemosensitivity of BZ by mediating autophagy.

It is well known that the AKT pathway has an important role in regulating human cancer cell survival[38]. Dysregulation of the AKT pathway can be observed in cancers, which may be involved in the resistance mechanisms of tumor cells[15,38]. Phosphorylation of AKT can activate the serine/threonine kinase mTOR that acts as an important upstream regulator of autophagy[24]. Related studies have demonstrated that inhibition of the AKT–mTOR pathway results in autophagic activation[22,39]. In our study, the results showed that BZ dephosphorylated AKT as well as mTOR in multiple myeloma cells, which was further enhanced by ClC5 overexpression. The results strongly indicate that the AKT–mTOR signaling pathway may contribute to BZ-induced autophagy, and ClC5 is required for the process involved.

In conclusion, our study demonstrates that BZ induces protective autophagy, at least in part, through inhibiting the AKT–mTOR signaling pathway. ClC5 attenuates BZ sensitivity in multiple myeloma cells through enhancing autophagy. These findings suggest that inhibition of ClC5 may be a potential strategy to overcome the chemoresistance of BZ for the treatment of multiple myeloma.

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