Reversal of Bortezomib Resistance in Myelodysplastic Syndrome Cells by MAPK Inhibitors

Yingxing Yue[1º], Ying Wang[2º], Yang He[2º], Shuting Yang[1º], Zixing Chen[2], Yuanyuan Wang[2], Shanshan Xing[1], Congcong Shen[1], Hesham M. Amin[3], Depei Wu[2]*, Yao-Hua Song[1]*

1 Cyrus Tang Hematology Center, Jiangsu Institute of Hematology, First Affiliated Hospital, Soochow University, Suzhou, China, 2 Jiangsu Institute of Hematology, The First Affiliated Hospital of Soochow University, Key Laboratory of Thrombosis and Hemostasis of Ministry of Health, Suzhou, China, 3 Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, Texas, United States of America

Abstract

The myelodysplastic syndromes (MDS) comprise a heterogeneous group of malignant neoplasms with distinctive clinicopathological features. Currently, there is no specific approach for the treatment of MDS. Here, we report that bortezomib (BTZ), a proteasome inhibitor that has been used to treat plasma cell myeloma, induced G2/M phase cycle arrest in the MDS cell line SKM-1 through upregulation of Wee1, a negative regulator of G2/M phase transition. Treatment by BTZ led to reduced SKM-1 cell viability as well as increased apoptosis and autophagy. The BTZ-induced cell death was associated with reduced expression of p-ERK. To elucidate the implications of downregulation of p-ERK, we established the BTZ resistant cell line SKM-1R. Our data show that resistance to BTZ-induced apoptosis could be reversed by the MEK inhibitors U0126 or PD98059. Our results suggest that MAPK pathway may play an important role in mediating BTZ resistance.

Introduction

The myelodysplastic syndromes (MDS) are a group of clonal disorders characterized by ineffective hematopoietic cell production and variable risk of transformation to acute myeloid leukemia (AML). Treatment options are limited and targeted therapies are not available for MDS. Hematopoietic stem cell transplantation (HSCT) strategies may improve long-term survival in some young patients. However, MDS is primarily a disease of elderly people who are often intolerant to aggressive therapies such as HSCT and chemotherpeutics.

It has been shown that the proteasome inhibitor bortezomib (BTZ) is effective in the treatment of plasma cell myeloma [1] [2] [3]. More recently, BTZ demonstrated some promise in the treatment of MDS and AML [4–7]. In a phase I clinical trial, BTZ combined with weekly idarubicin successfully induced hematologic response in AML patients who have prior history of MDS [5]. Similarly, in a phase I/II trial, BTZ and low dose cytarabine arabinoside showed clinical response in 36% of high-risk MDS patients [7]. These studies also demonstrated that BTZ is more effective when combined with other chemotherapeutic agents for treating high-risk MDS patients [5] [7]. Nonetheless, chemotherapy is usually associated with severe side effects that might lead to patient's death. Most likely, targeted therapies that selectively exploit specific survival molecules are more effective and notably associated with fewer side effects. The development of targeted therapies for MDS has been particularly challenging due to the complexity of the oncogenic systems contributing to the survival of MDS cells.

The MEK/ERK pathway plays key roles in controlling cell survival and cell cycle progression, and its deregulation is often implicated in developing drug resistance and cancer progression. Upregulation of p-ERK has been observed in the majority of AML cases [8,9], and elevated expression of ERK in AMLs is associated with a poor prognosis [10]. Furthermore, introduction of a constitutively activated form of MEK into hematopoietic stem cells causes myeloid malignancies such as MDS and myeloproliferative neoplasms [11]. Persistant activation of MEK/ERK pathway mediates drug resistance in leukemia cells [12–15]. These studies suggest that MEK/ERK pathway may play a role in the development of MDS and in mediating drug resistance.

In this study, we investigated the effects of BTZ in a human MDS cell line SKM-1. Our results demonstrated that p-ERK1/2 is highly expressed in SKM-1 cells. The expression of p-ERK1/2 was markedly decreased after treatment with BTZ. In contrast, treatment with BTZ resulted in upregulation of ERK in the BTZ-resistant cell line SKM-1R. However, the resistance to BTZ in SKM-1R cells was reversed by the MEK inhibitors U0126 and PD98059. This study provides the first evidence that MEK/ERK pathway mediates BTZ resistance and suggests that MEK/ERK inhibitors could be successfully used in conjunction with BTZ to overcome drug resistance in MDS.
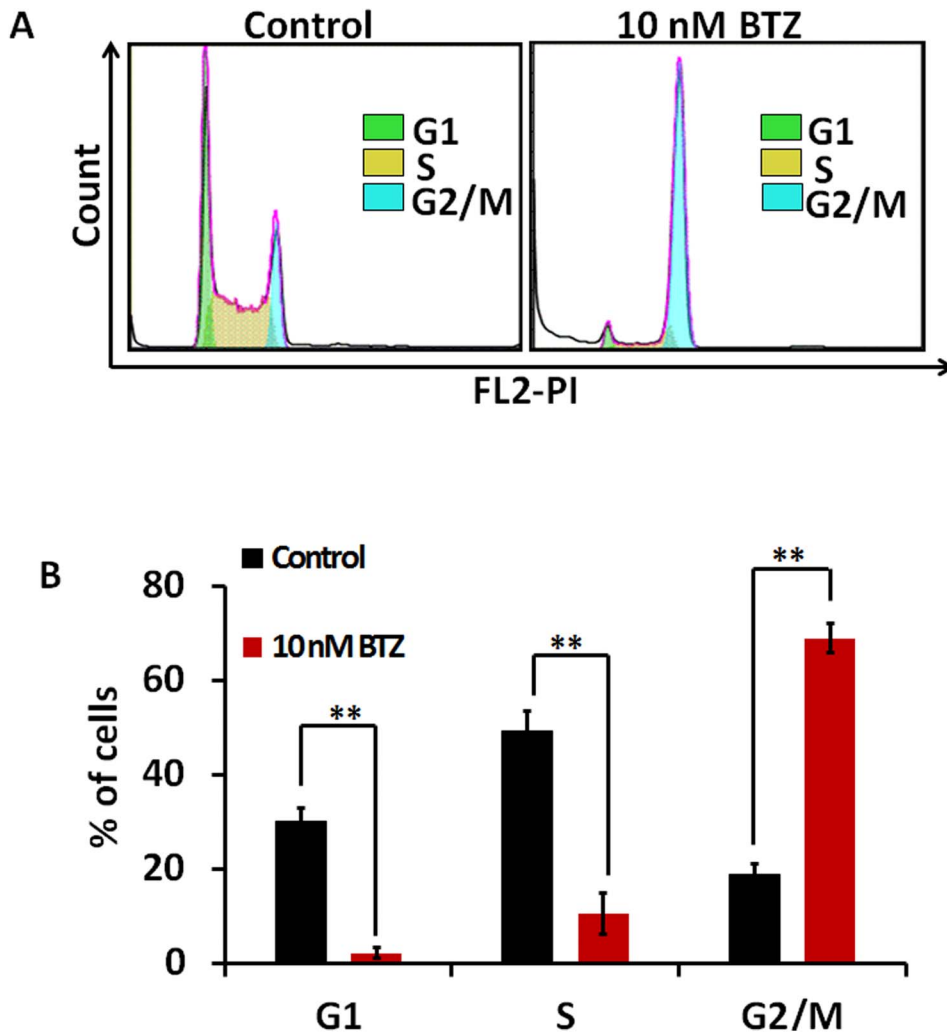
Figure 1. BTZ arrests SKM-1 cells at G2/M phase. A. SKM-1 cells were treated with vehicle (control) or 10 nM BTZ for 24 h and then stained with PI for FACS analysis. B. The distribution of cells in each cell cycle phase is shown. **$P<0.01$.
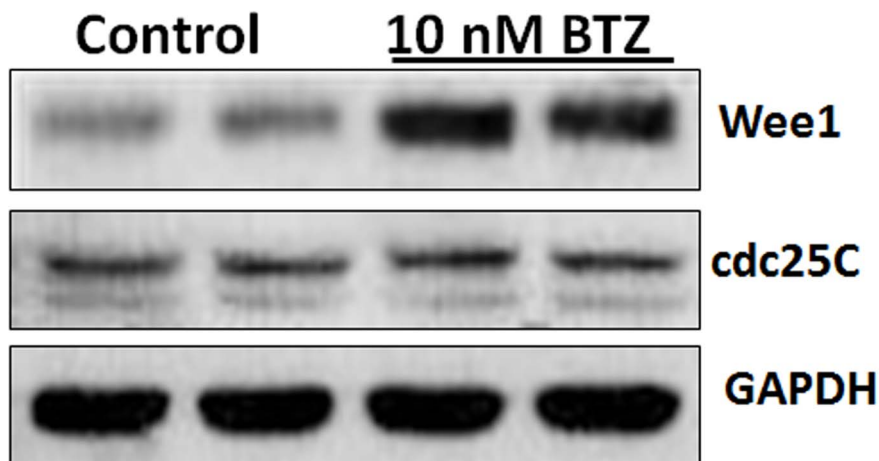doi:10.1371/journal.pone.0090992.g001

Figure 2. Effect of BTZ on the expression of cell cycle regulatory proteins Wee1 and cdc25C. SKM-1 cells were treated with 10 nM BTZ and the expression of Wee1 and cdc25C was analyzed by immunoblotting. GAPDH was used as loading control.
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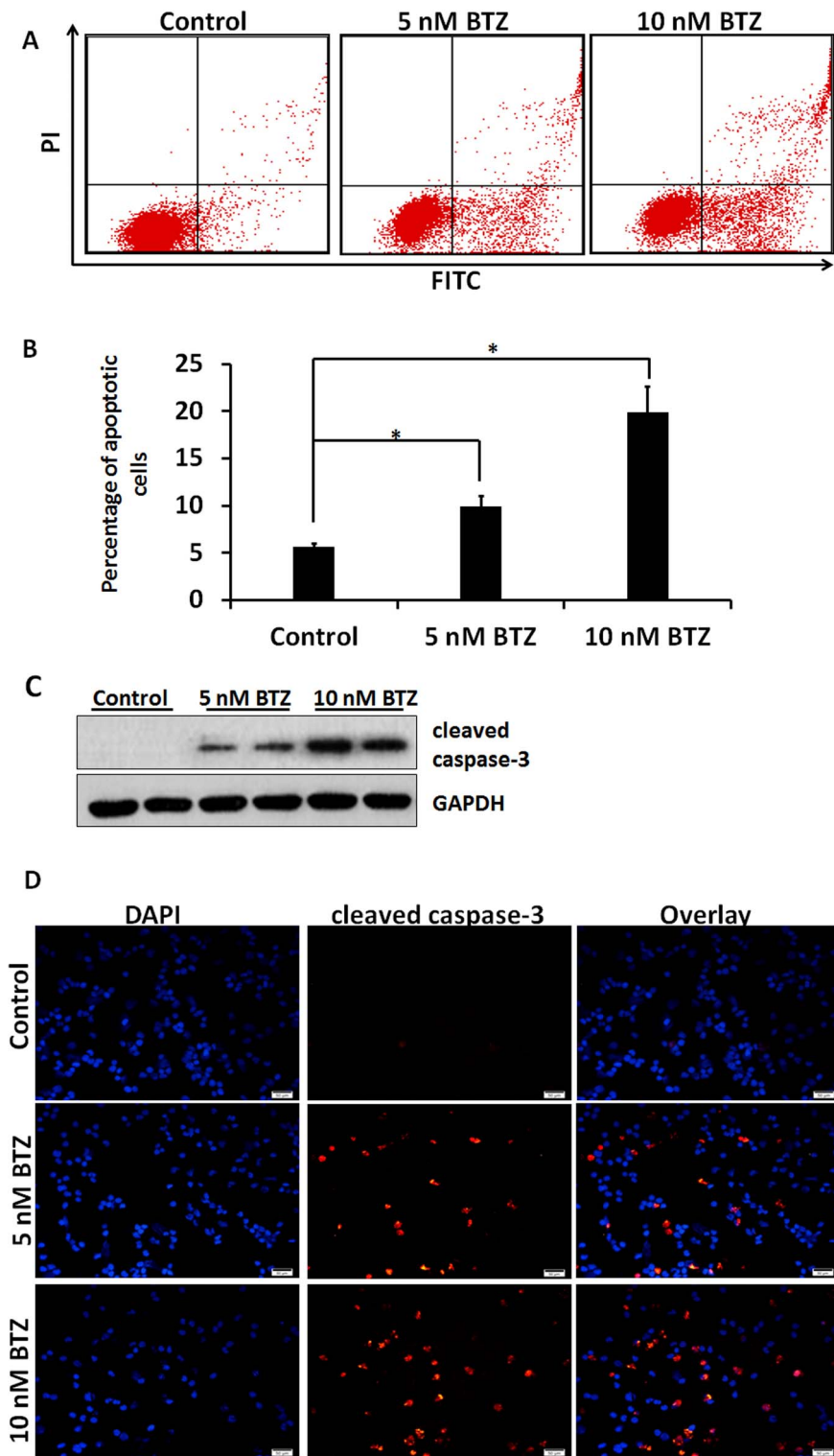**Figure 3. BTZ induces apoptosis.** A. Representative flow cytometry scatter plots showing cells undergoing apoptosis in response to BTZ. B. Bar graph shows percentage of SKM-1 cells undergoing apoptosis in response to 5 and 10 nM BTZ for 24 h. C. Immunoblot analysis of cleaved caspase-3. D. Immumofluorescence staining of cleaved caspase-3. Bar 50 μm. *$P < 0.05$.

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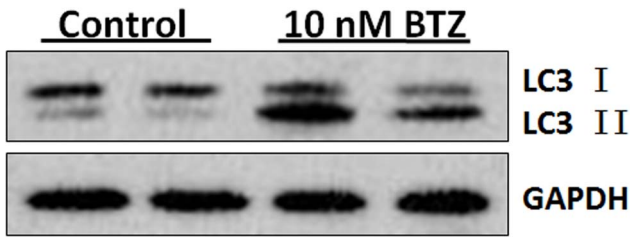
Figure 4. BTZ induces autophagy in SKM-1 cells. SKM-1 cells were treated with 10 nM BTZ for 24 h and immunoblotted for microtubule-associated light chain (LC3-I and LC3-II). GAPDH was used as loading control.
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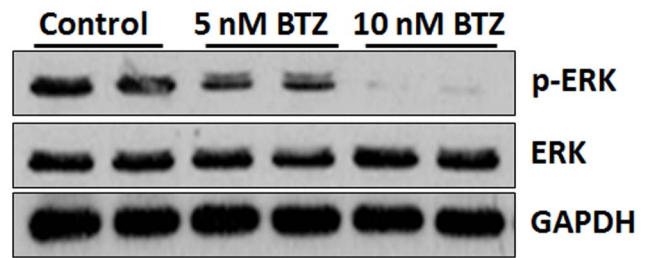
Figure 5. ERK1/2 expression in SKM-1 cells. Immunoblotting of SKM-1 cells shows that total and p-ERK1/2 are both expressed in untreated SKM-1 cells (control). However, BTZ inhibited the expression of p-ERK1/2 but not total ERK1/2. GAPDH was used as loading control.
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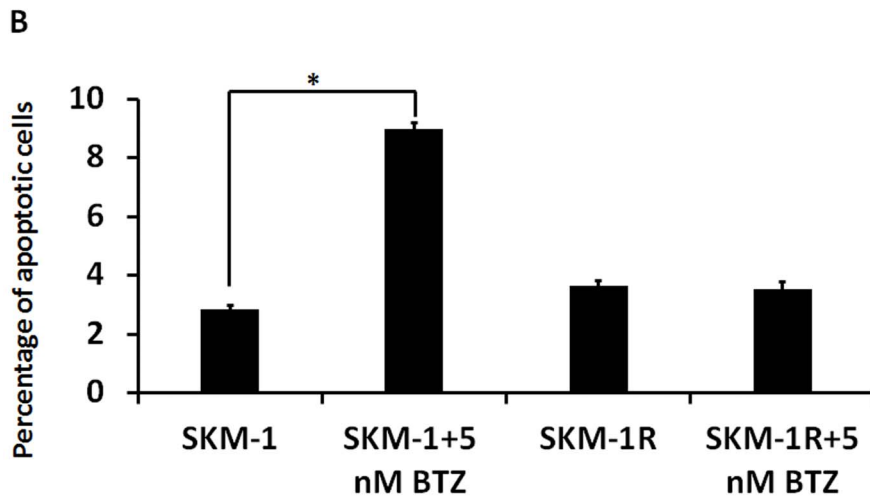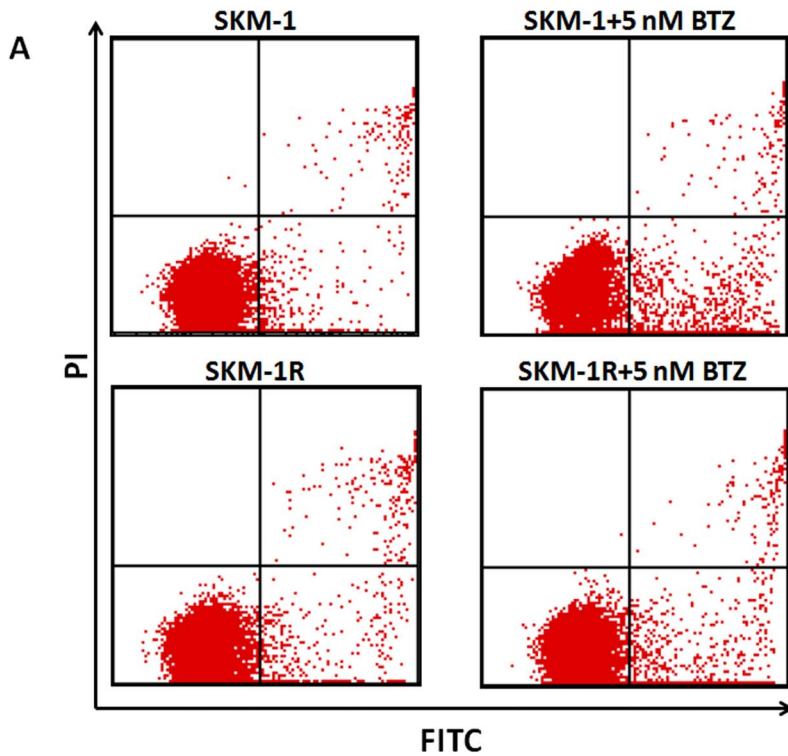
Figure 6. SKM-1R cells are resistant to BTZ induced apoptosis. A. Flow cytometry scatter plots of the apoptosis assay. B. Bar graph shows percentage of SKM-1 and SKM-1R cells undergoing apoptosis in response to a 24 h treatment with 5 nM BTZ. *$P<0.05$.
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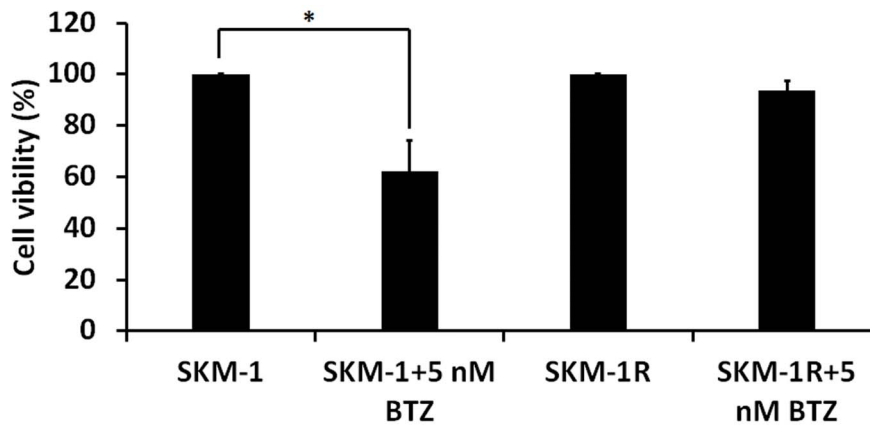
Figure 7. Cell viability in response to BTZ treatment. SKM-1 and SKM-1R cells were exposed to 5 nM BTZ for 24 h and the viable cell number was evaluated by MTT assay. The result showed a reduction in cell viability by treating wild type SKM-1 cells with 5 nM BTZ with no notable effect on SKM-1R cells. *$P<0.05$.
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Materials and Methods

Cell Culture and Reagents

The human MDS cell line SKM-1 has been described previously [16]. SKM-1 cells were maintained in RPMI −1640 with 20% fetal calf serum (HyClone), 100 U/ml penicillin and 100 μg/ml streptomycin in 5% CO_2 at 37°C. The BTZ-resistant SKM-1 cell line was established by repeated exposure of the cells to 5 nM of BTZ for 24 hours followed by 2 weeks recovery over a period of 3 months. MEK inhibitors PD98059 and U0126 were purchased from Cell Signaling Technology.

MTT Assay

Cell viability was assessed by the MTT assay. MTT reagent was purchased from Sigma. Human SKM-1 cells were treated with BTZ in 96 well plates at the density of 2×10^4/well in each experiment. After 24 h, MTT assay was performed. The absorbance was measured at 490 nm by a micro-plate reader (Spectra Max M5).

Measurement of Apoptosis and Cell Cycle

Apoptosis was assessed by flow cytometry (FACS Calibur Flow Cytometer, BD Biosciences) for Annexin V and propidium iodide (PI) staining (kit from Roche). Cells that are positive for Annexin V but negative for PI are considered undergoing apoptosis. Cell cycle analysis was performed by flow cytometry for PI staining (Sigma).

Figure 8. Expression of LC3 in BTZ resistant SKM-1R cells. Cell lysates were subjected to immunoblot analysis using specific antibody against LC3. Conversion of LC3-I to LC3-II was not observed in SKM-1 cells treated with BTZ.
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Immunoblotting

SKM-1 cells were washed with PBS and then lysed in a buffer containing 20 mM HEPES (pH 7.2), 10% glycerol, 150 mM NaCl, 1%TritonX-100, 50 mM NaF, 1 mM Na3VO4, 10 ug/ml leupeptin, 10 ug/ml aprotinin, and 1 mM PMSF. Proteins were separated by SDS–polyacrylamide gel electrophoresis and blotted onto a PVDF membrane (MILLIPORE). Primary antibodies (Cell Signaling Technology) were directed against Wee1 (1:1000), cdc25C (1:1000), p-ERK (1:1000), ERK (1:2000), LC3 (1:2000), cleaved caspase-3 (1:1000) and GAPDH (1:5000). Secondary antibodies were anti-rabbit IgG (1:5000, Cell Signaling) and anti-mouse IgG (1:5000, Sigma). Antigen–antibody complexes were visualized by enhanced chemi- luminescence (PerkinElmer).

Immunofluorescence

Cells were fixed with 4% paraformaldehyde and incubated with blocking buffer containing 1% BSA and 0.1% Triton X-100. After blocking, cells were incubated overnight in the primary antibody (cleaved caspase-3, 1:400). After washing, cells were incubated in goat anti-rabbit IgG-Alexa Fluor R 568 (Molecular Probes, diluted 1:500). Cells were then rinsed and counterstained with DAPI. Images were taken by fluorescence microscope (Leica DM2000).

Statistical Analysis

Data are presented as means ± SD. Student t test was used to determine the significance of the differences between variables. $P<0.05$ was considered statistically significant.

Result

BTZ Causes Cell Cycle Arrest in G2/M Phase via Increasing Wee1 Expression

The effect of BTZ on cell cycle progression in SKM-1 cells was evaluated using flow cytometric analysis. After treatment with 10 nM BTZ for 24 h, the percentage of cells in G1 and S phases decreased 28% and 29% respectively, whereas the percentage of cells in G2/M phase increased 51% (Fig. 1). Our results indicate that BTZ is able to arrest SKM-1 cells at G2/M.

Entry of eukaryotic cells into M-phase of cell cycle is controlled by cdc2 kinase which is activated by cdc25C and inactivated by Wee1. At the G2/M transition, Wee1 is downregulated while cdc25C is upregulated [17]. The finding of a G2/M arrest by BTZ led us to examine the expression levels of Wee1 and cdc25C. Fig. 2
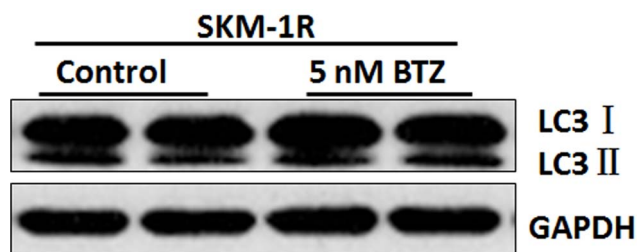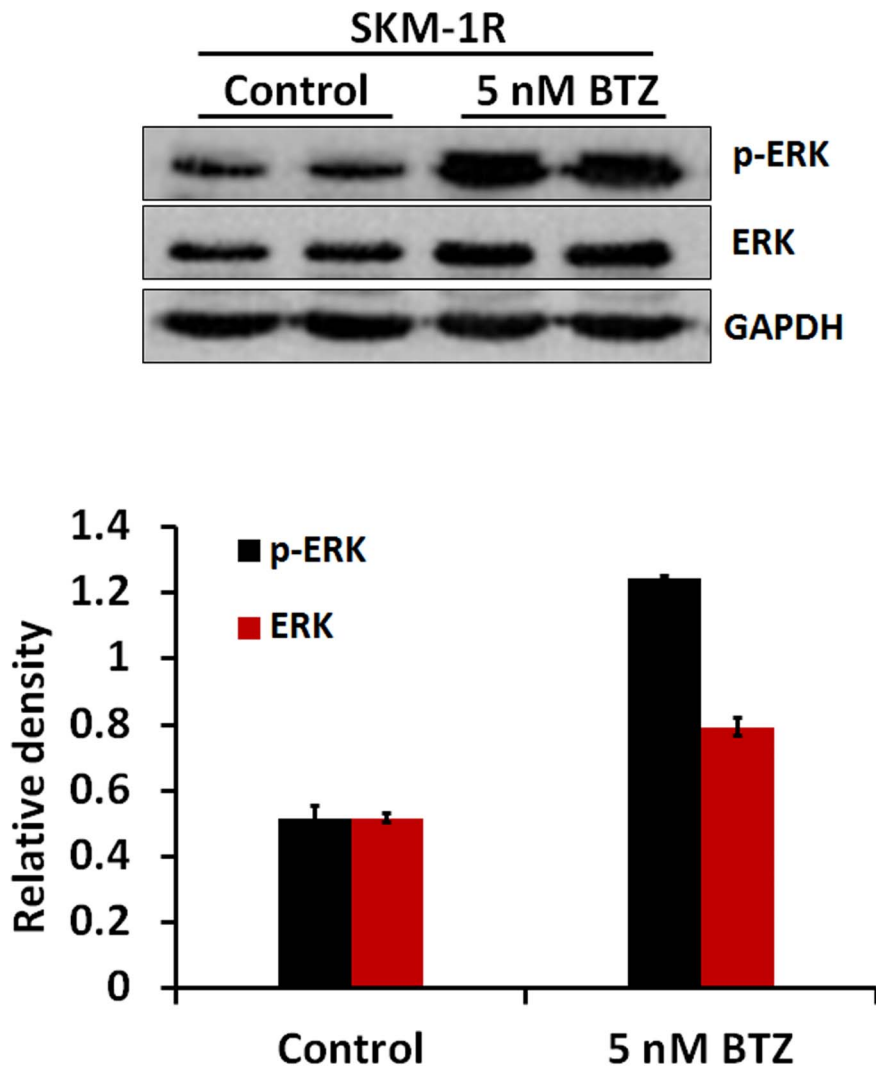
Figure 9. ERK1/2 expression in BTZ resistant SKM-1R cells. Immunoblotting of SKM-1R cells shows that both total and phospho-ERK1/2 are upregulated in response to BTZ treatment. Protein expression levels were quantified by ImageJ. Total and phospho-ERK1/2 levels were normalized to GAPDH.

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shows that treatment with 10 nM BTZ for 24 h resulted in increased Wee1 protein expression, while the protein level of cdc25C was not altered. These data suggest that increased Wee1 protein expression contributes to cdc2 inhibition and subsequent G2/M cell cycle arrest following BTZ treatment. Previous studies have shown that cell cycle arrest at G2/M phase often leads to induction of apoptosis and cell death [18].

BTZ Induced Cell Death in SKM-1 Cells is Mediated by Down-regulation of p-ERK

Recent studies showed that BTZ induced apoptosis in plasma cell myeloma cells [19,20]. The ability of BTZ to induce apoptosis in SKM-1 cells was evaluated by flow cytometry. As shown in Fig. 3A and 3B, at 24 h, BTZ caused an increase of the percentage of apoptotic cells in a concentration-dependent manner. Immunoblot analysis and immunofluorescence staining of cleaved caspase-3 further confirmed apoptosis (Fig. 3C,D).

It has been shown that BTZ has the capacity to induce autophagic cell death in MDS cells [21]. The induction of autophagy was evaluated in the SKM-1 cells. LC3-II has been used as a marker for autophagy. During induction of autophagy, LC3-I present in the cytoplasm is converted to LC3-II which is membrane bound and correlated with the extent of autophagosome formation [22]. As shown in Fig. 4, an increase of LC3-II levels is evident in BTZ treated cells.

MAPK Inhibitors Overcome BTZ Induced Drug Resistance

Given the established role of MAPKs in controlling cell survival, we evaluated if MAPK pathway could be involved in BTZ induced apoptosis. The expression of p-ERK1/2 and total ERK1/2 was analyzed by immunoblotting. SKM-1 cells show constitutively high expression of p-ERK/ERK (Fig. 5). Treatment with BTZ dramatically suppressed the expression of p-ERK1/2 but not total ERK1/2 at 24 h (Fig. 5).

Most patients who receive the first treatment with BTZ develop resistance to this drug [1]. In order to elucidate the mechanisms of drug resistance, we established a BTZ resistant cell line SKM-1R. Figure 6 demonstrated the induction of apoptotic cell death in SKM-1 cells treated with 5 nM BTZ by Annexin/PI staining. However, the percentage of cells undergoing apoptosis in BTZ
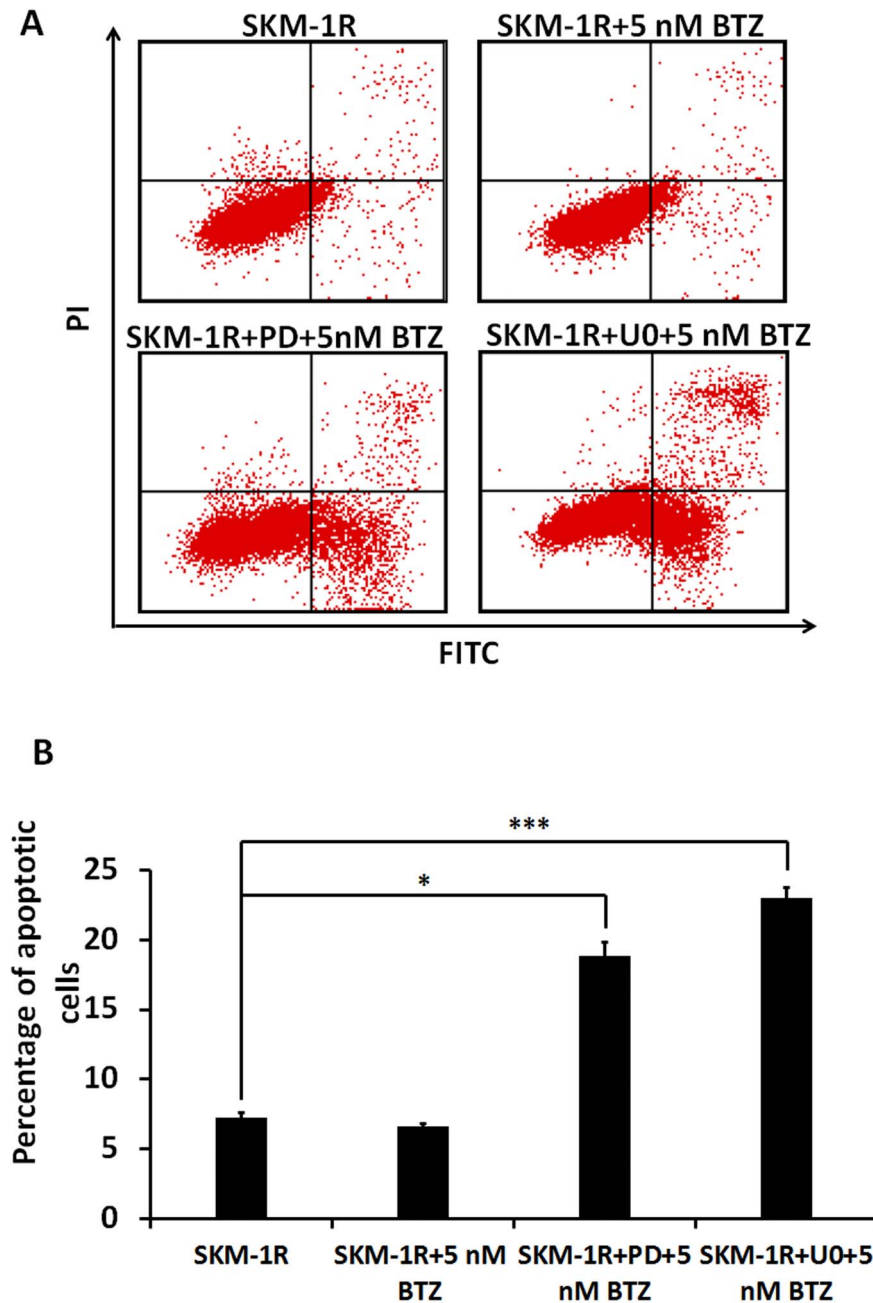
Figure 10. Reversal of BTZ resistance by MEK inhibitors in SKM-1R cells. A. Flow cytometry scatter plots showing Annexin V staining of SKM-1R cells. B. Bar graph shows percentage of SKM-1R cells undergoing apoptosis in response to a 24 h exposure to 5 nM BTZ. Both PD98059 (PD) and U0126 (U0) significantly increased the percentage of cells undergoing apoptosis. *$P<0.05$, ***$P<0.001$.
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treated SKM-1R cells was not different from the untreated SKM-1R cells. The observation that SKM-1R cells are resistant to BTZ induced apoptosis is consistent with our findings that cell viability did not decrease in BTZ treated SKM-1R cells (Fig. 7). Furthermore, activation of autophagy was not observed in SKM-1R cells treated with 5 nM BTZ (Fig. 8). We next evaluated the expression of ERK1/2 in SKM-1R cells. In contrast to wild type SKM-1 cells, the expression of both total and phospho-ERK1/2 was upregulated by BTZ in SKM-1R cells (Fig. 9).

To further explore the role of MAPK pathway in the development of BTZ resistance in SKM-1R cells, the effects of MAPK inhibitors U0126 and PD98059 on BTZ-induced apoptosis were examined. Flow cytometry analysis showed that resistance to BTZ induced apoptosis was reversed by PD98059 and U0126 (Fig. 10A,B).

Discussion

Despite the clinical success of BTZ in treating plasma cell myeloma, resistance to this drug remains a significant problem [1]. A phase 2 study showed that the rate of response to BTZ was only 35% and patients developed resistance to BTZ 12 months after the initial treatment [2]. A recent study showed that only 40% patients

with relapsed plasma cell myeloma responded to the retreatment [3].

Significant progress has been made in elucidating molecular mechanisms of BTZ resistance. Oerlemans et al [23] found an Ala49Thr mutation residing in a highly conserved BTZ-binding pocket in the proteasome β5-subunit (PSMB5) protein in BTZ resistant human myelomonocytic THP1 cells. The mutation resulted in overexpression of the PSMB5 protein [23]. Upregulated expression of the PSMB5 gene was confirmed in bone marrow cells of multiple myeloma patients who developed BTZ resistance [24]. Ria et al [25] suggested that HIF-1α may also play a role in developing BTZ resistance by inducing angiogenesis in tumors. Relative few studies can be found in the literature regarding BTZ and AML. Most of these articles documented the effectiveness of BTZ for a subset of AML patients. BTZ resistant AML cell lines have not been established so far. However, it has been shown that persisting NF-κB activity may be responsible for the survival of CD34(+) AML cells after BTZ treatment [26]. When the IKK inhibitor BMS-345541 was used in combination with BTZ, the survival of CD34(+) AML cells was reduced [26], which suggest that NF-kB may be involved in BTZ resistance in AML. Bcl-2 overexpression has been suggested as a potential mechanism of BTZ resistance in human lymphoid cells [27]. Overexpression of heat shock proteins and T-cell factor 4 has been reported in BTZ resistant B-lymphoma cells [28]. Nonetheless, the molecular mechanisms/signaling pathways that mediate BTZ resistance in cancer cells including MDS remain largely unknown.

In the present study, our data revealed that BTZ resistant SKM-1R cells are characterized by upregulation of ERK. These findings indicate that upregulation of ERK activity may be a potential mechanism for BTZ resistance. MEK/ERK pathway is activated in aggressive CKS1B-overexpressing plasma cell myeloma cells [29]. Higher expression levels of p-ERK in human prostate cancer samples were associated with tumor progression [30]. Hyperactivation of the MAPK pathway has also been implicated in the development of neurofibromatosis type 1-associated leukemia [31]. Our findings are consistent with these previous reports. We further showed that resistance to BTZ can be reversed by PD98059 and U0126, suggesting that MEK/ERK pathway may be a potential target for MDS patients who developed resistance to BTZ. Our findings are in line with previous report that ERK

inhibitors synergized with BTZ on anticancer effects in medulloblastoma cells [32].

Using the MUTZ-1 cell line, Huang et al found no notable changes in p-ERK1/2 after BTZ treatment [33]. The difference in p-ERK expression in response to BTZ treatment may be explained by the nature of the two different cell lines. MUTZ-1 was isolated from the malignant cells of a 5-year-old girl with MDS [34], whereas SKM-1 was established from leukemic cells of a 76-year-old patient [16]. These two cell lines exhibit different chromosome abnormalities.

Autophagy is a mechanism that degrades dysfunctional cellular components through lysosomes. The activation of autophagy may lead to either cell death or increased survival depending on cell types and conditions. For example, BTZ-induced autophagy led to cell death in MDS/AML cells [21]. Consistent with these previous findings, our present study showed that BTZ induced the conversion of LC3I to LC3II in wild type SKM-1 cells but not in BTZ resistance SKM-1R cells. Our results suggest that autophagic cell death may contribute, at least in part, to BTZ-induced cell cycle arrest and apoptosis.

In some cases, the LC3-I to LC3-II conversion is necessary but not sufficient to trigger cell autophagy. Further studies are needed to ascertain the role of autophagy in BTZ induced cell death by silencing autophagy related proteins such as ATG5, ATG7 and ATG8 in Skm-1 cells.

In conclusion, the present study demonstrates that BTZ induces cell cycle arrest, apoptosis and autophagy in SKM-1 cells. The cytotoxic effects of BTZ appeared to depend, at least in part, on the inhibition of the MEK/ERK pathway. The BTZ resistant SKM-1R cells are characterized by upregulation of ERK. Based on the present observations, a combinational therapy of BTZ and MAPK inhibitors may be an effective complement to current therapeutic approaches for MDS.

Author Contributions

Conceived and designed the experiments: YXY YW YH STY ZXC YYW SSX CCS HMA DPW YHS. Performed the experiments: YXY YW YH STY. Analyzed the data: YXY YW YH STY ZXC YYW SSX CCS HMA DPW YHS. Wrote the paper: YXY YW YH STY ZXC YYW SSX CCS HMA DPW YHS.

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