Arsenic trioxide and bortezomib interact synergistically to induce apoptosis in chronic myelogenous leukemia cells resistant to imatinib mesylate through Bcr/Abl-dependent mechanisms

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Abstract. Arsenic trioxide (As_2O_3) and the proteasome inhibitor bortezomib (BTZ) have been used successfully to treat acute promyelocytic leukemia and multiple myeloma. Their synergistic effects with other anticancer drugs have been widely studied. In this study, interactions between As_2O_3 and BTZ were examined in imatinib-resistant Bcr/Abl$^+$ (K562r) cells. Co-treatment of K562r cells with subtoxic concentrations of As_2O_3 (2 μM) and BTZ (24 nM) resulted in a synergistic enhancement in growth inhibition and apoptosis, as demonstrated by increased annexin V staining. These events were associated with activation of protein kinase Cδ, poly ADP-ribose polymerase cleavage and caspase-3 activation, along with downregulation of Bcr/Abl mRNA and Bcr/Abl protein expression levels during apoptosis. In addition, reactive oxygen species were downregulated during combined treatment in K562r cells. Collectively, these findings suggest that BTZ and As_2O_3 act synergistically to induce apoptosis in K562r cells. Therefore, further studies are required to assess the potential of BTZ and As_2O_3 combinatory treatment of chronic myeloid leukemia, particularly using imatinib-resistant Bcr/Abl$^+$ clones.

Introduction

Chronic myelogenous leukemia (CML) is a myeloproliferative disorder of the bone marrow stem cells characterized by genetic translocation between chromosomes 9 and 22. This translocation results in the fusion of Bcr on chromosome 22 with Abl on chromosome 9, leading to the expression of the Bcr/Abl fusion oncoprotein. This fusion protein exhibits constitutively active kinase activity (1), transducing signals to a variety of downstream survival pathways, including the mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade, Akt, and the signal transducers and activators of transcription (STATs) and nuclear factor κB (NF-κB) pathways (2-4). A number of anti-apoptotic proteins, such as B-cell lymphoma extra large, are upregulated as a result of the activation of these pathways. Collectively, these events provide Bcr/Abl$^+$ cells with a survival advantage over normal cells, due to their reduced capacity to undergo apoptosis. Furthermore, Bcr/Abl$^+$ cells have exhibited varying degrees of resistance towards conventional cytotoxic drugs (5-7), until the recent clinical application of imatinib mesylate (Gleevec®, STI-571). Imatinib has been widely demonstrated to be effective in the treatment of CML through inhibition of the tyrosine kinase activity of Bcr/Abl. However, acquired resistance to imatinib can occur through various mechanisms, thereby leading to continued disease progression. Thus, the development of alternative approaches to the treatment of CML is required.

Bortezomib (BTZ), also known as Velcade® or PS-341, has been shown to act as an inducer of apoptosis in multiple myeloma cells. BTZ exerts its effects through a number of signaling cascades, predominantly the NF-κB pathway, ultimately inducing apoptosis and thereby reversing drug resistance and improving prognosis (8,9). The administration of BTZ as a combination treatment with other agents has been widely investigated; the cyclin-dependent kinase inhibitor flavopiridol and the histone deacetylase inhibitor suberoylanilide hydroxamic acid have both been shown to act synergistically with BTZ to induce apoptosis in Bcr/Abl$^+$ cells (10,11).

Arsenic trioxide (As_2O_3) is a chemotherapeutic agent that acts by inducing apoptosis and differentiation and can uniquely induce complete remission in the majority of patients with acute promyelocytic leukemia (12-14). Furthermore, studies have shown that As_2O_3 alone, and in combination with other compounds, induces apoptosis and/or growth arrest in Bcr/Abl$^+$ cells (15-17). However, high concentrations of As_2O_3

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were used in these studies, and, due to the toxic nature of arsenic, its use has been limited in clinical practice. In 2007, Yan *et al* (18) showed that clinically achievable concentrations of As_2O_3 could act synergistically with BTZ to successfully induce apoptosis in imatinib-sensitive Bcr/Abl+ (K562) cells, in which protein kinase Cδ (PKCδ) activation played a critical role.

This study aimed to investigate the effects of the combination of As_2O_3 and BTZ on apoptosis in imatinib-resistant Bcr/Abl+ cells and to determine whether this combinatory approach warranted further investigation as a potential therapeutic strategy for the treatment of CML, particularly for cases exhibiting imatinib resistance.

Materials and methods

Reagents. Imatinib (STI571) was purchased from Selleck Chemicals (Houston, TX, USA), prepared as a 1 mM stock solution in dimethyl sulfoxide and stored at -20˚C. BTZ (Millennium Pharmaceuticals Inc., Cambridge, MA, USA) was dissolved in phosphate-buffered saline (PBS) and stored at -20˚C until use. As_2O_3 (Sigma, St. Louis, MO, USA) was dissolved in 1.0 N NaOH and then diluted to 1 mM with PBS.

Cell culture and viability assay. K562 and K562r cells (provided by Professor J.V. Melo, Department of Haematology, Imperial College of London, London, UK) were cultured in RPMI-1640 media supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA) in a humidified atmosphere with 5% CO_2 at 37˚C. K562r cells were cultured in 1 μM imatinib to maintain drug-resistance. Cell viability and inhibition of cell growth were estimated using a Cell Counting kit 8 (Dojindo Laboratories, Kumamoto, Japan).

Apoptosis assessment. Apoptosis was measured using a fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection kit (BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's instructions. Following treatment with As_2O_3 and/or BTZ, $1x10^5$ K562r cells were collected, washed with cold PBS and resuspended with binding buffer. A total of 5 μl annexin V-FITC was added and cells were incubated for 5 min in the dark. Following incubation, 10 μl propidium iodide was added and the samples were incubated for an additional 3 min. Binding buffer (400 μl) was then added and cells were analyzed by flow cytometry.

Western blot analysis. Equal amounts of protein were loaded on 8-14% SDS-polyacrylamide gels, subjected to SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, UK). The blots were stained with 0.2% Ponceau S red to ensure equal protein loading. Following blocking with 5% nonfat milk in PBS, the membranes were probed with primary antibodies targeting c-Abl, PKCδ, poly (ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and cleaved caspase-3 (Cell Signaling Technology, Inc., Beverly, MA, USA). Membranes were subsequently incubated with horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling Technology, Inc.) and the signal was detected using a chemiluminescence Phototope®-HRP kit (Cell Signaling Technology, Inc.). Blots were stripped and reprobed with a mouse anti-β-actin monoclonal antibody (Oncogene, San Diego, CA, USA) as a loading control.

Quantitative polymerase chain reaction (qPCR) analysis of Bcr/Abl mRNA. Total RNA was isolated by TRIzol™ reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and RNA was treated with DNase (Promega Corp., Madison, WI, USA). Complementary DNA was synthesized according to the manufacturer's instructions. The analysis of Bcr/Abl and β-actin was performed by qPCR using SYBR Green PCR Master Mix reagents (Applied Biosystems, Foster City, CA, USA) using an ABI PRISM 7900 system (Applied Biosystems). The specific primers used for detecting p210 Bcr/Abl were as follows: Forward primer (5'-CTGGCCCAACGATGGCGA-3') and reverse primer (5'-CACTCAGACCCTGAGGCTCAA-3'). Primers were synthesized by Sangon Biotechnology (Shanghai, China).

Measurement of reactive oxygen species (ROS). Cells were incubated with either As_2O_3, BTZ or the two in combination for the indicated times. Following incubation, cells were washed twice with PBS and treated with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes®/Invitrogen Life Technologies) for 30 min at 37˚C, in the dark. Cells were then washed with PBS once. Red fluorescence was detected by fluorescence-activated cell sorting at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Statistical analysis. All experiments were repeated in triplicate. The SPSS 11.0 (SPSS Inc., Chicago, IL, USA) software package was used for statistical analysis. $P<0.05$ was considered to indicate a statistically significant difference. Results are presented as the mean ± standard deviation.

Results

K562r cells are more resistant than K562 cells to imatinib treatment. In order to determine the resistance characteristics of K562r cells, K562 (imatinib-sensitive) or K562r (imatinib-resistant) cells were incubated with varying concentrations of imatinib. Of note, 0.5 μM imatinib induced 50% inhibition of K562 cell growth, whereas up to 10 μM imatinib was required to inhibit 50% cell growth in K562r cells (Fig. 1A and B). Western blot analysis showed that 1 μM imatinib caused the downregulation of Bcr/Abl protein expression in K562 cells, whereas 25 μM imatinib was required to observe measurable downregulation in K562r cells (Fig. 1C and D).

BTZ synergistically interacts with As_2O_3 to induce apoptosis in K562r cells. Treatment with either BTZ or As_2O_3 alone inhibited cell growth in K562r cells in a dose- and time-dependent manner (Fig. 2A and B). To evaluate the potential synergistic effects of these two agents, BTZ and As_2O_3 were used at concentrations of 24 nM and 2 μM, respectively, the individual half maximal concentration (IC_{50}) of these agents in the inhibition of K562r cell growth. Neither of these concentrations elicited significant apoptosis-inducing effects. After 24-48 h of combined incubation with BTZ and As_2O_3, growth inhibition and apoptosis induction were significantly enhanced in K562r

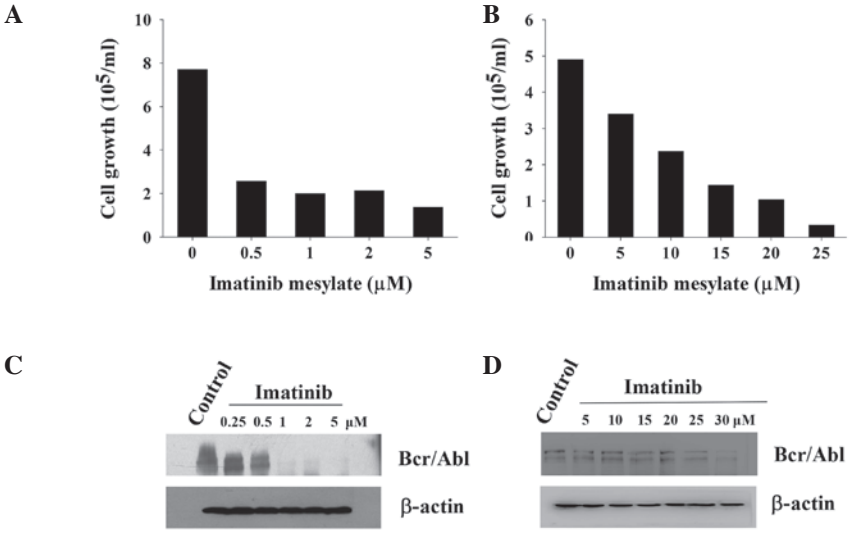

Figure 1. K562r cells are more resistant to imatinib treatment than K562 cells. Cells were exposed to the indicated concentrations of imatinib mesylate for 48 h and (A and B) cell growth and (C and D) Bcr/Abl protein expression in (A and C) K562 and (B and D) K562r cells were measured.

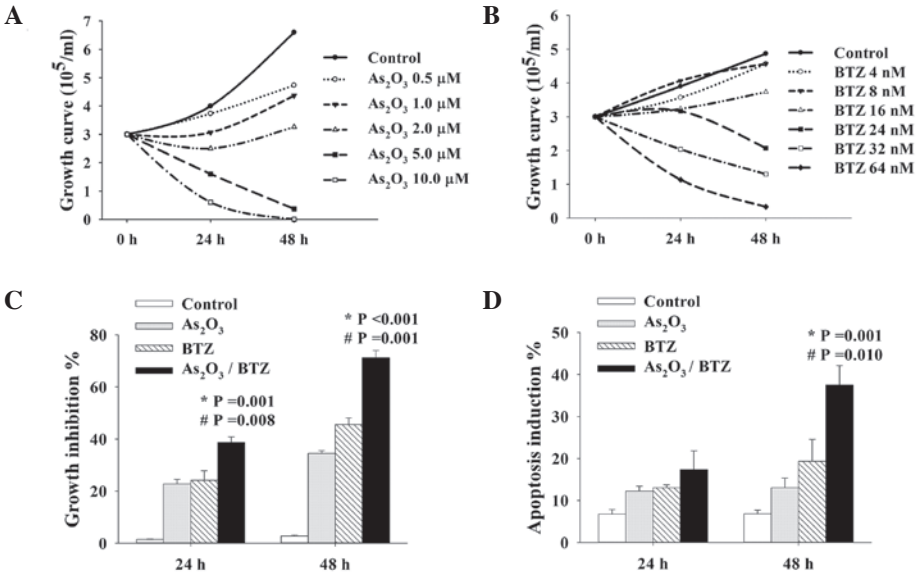

Figure 2. BTZ and As_2O_3 synergistically induce growth arrest and apoptosis of K562r cells. K562r cells were exposed to the indicated concentrations of (A) As_2O_3 and (B) BTZ for 48 h and the growth curves were measured. A total of 2 μM As_2O_3 and/or 24 nM BTZ were selected for combined treatment, and the (C) growth inhibition and (D) annexin V^+ apoptotic cells were measured. Error bars represent standard deviation, from three independent experiments. *P-value versus As_2O_3 treatment; #P-value versus BTZ treatment. As_2O_3, arsenic trioxide; BTZ, bortezomib.

cells as compared with those administered a single treatment (P<0.05) (Fig. 2C and D).

Combined treatment with BTZ and As_2O_3 results in enhanced caspase-3 activation and PARP and PKCδ cleavage. Western blot analysis was performed to assess apoptosis-associated events during combined treatment with BTZ and As_2O_3 in K562r cells. Treatment with 24 nM BTZ or 2 μM As_2O_3 alone resulted in only minimal effects, whereas combined treatment with BTZ and As_2O_3 for up to 48 h resulted in a significant increase in caspases-3 activation, as well as enhanced PARP and PKCδ cleavage (Fig. 3).

Combined treatment with BTZ and As_2O_3 downregulates Bcr/Abl mRNA and protein expression. The Bcr/Abl kinase can inhibit apoptosis through multiple mechanisms, leading Bcr/Abl^+ cells to develop resistance to apoptosis induced by conventional agents (5-7). In the present study, the effects of 24 nM BTZ and/or 2 μM As_2O_3 on Bcr/Abl mRNA and protein expression were examined. The results showed that single treatment with either BTZ or As_2O_3 alone could decrease Bcr/Abl mRNA and protein expression to a moderate extent; however, combined treatment with the two agents resulted in a significant decrease in Bcr/Abl expression in K562r cells (P<0.01), most markedly after 48 h of incubation (Fig. 4A and B). This effect was further confirmed by the Bcr/Abl protein downregulation analyzed by western blotting.

BTZ enhances As_2O_3-induced downregulation of ROS in K562r cells. As_2O_3 has been reported to exert

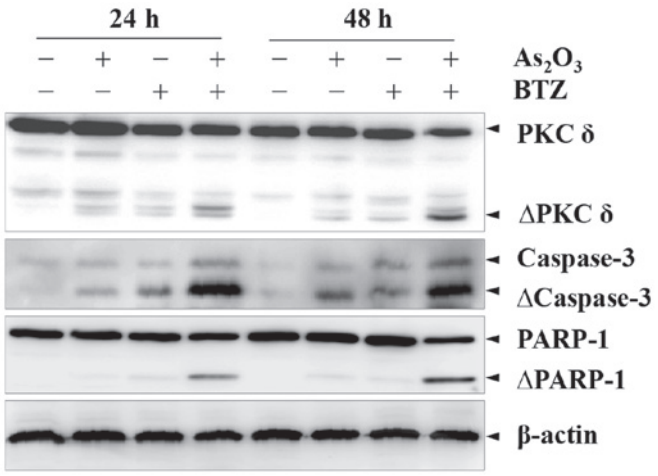

Figure 3. BTZ and As_2O_3 synergistically enhance caspase-3 activation as well as PKCδ and PARP cleavage in K562r cells. K562r cells were exposed to 24 nM BTZ and/or 2 μM As_2O_3 for 48 h. The indicated proteins were detected by western blotting with β-actin as loading control. PARP, poly ADP-ribose polymerase; PKCδ, protein kinase Cδ; As_2O_3, arsenic trioxide; BTZ, bortezomib.

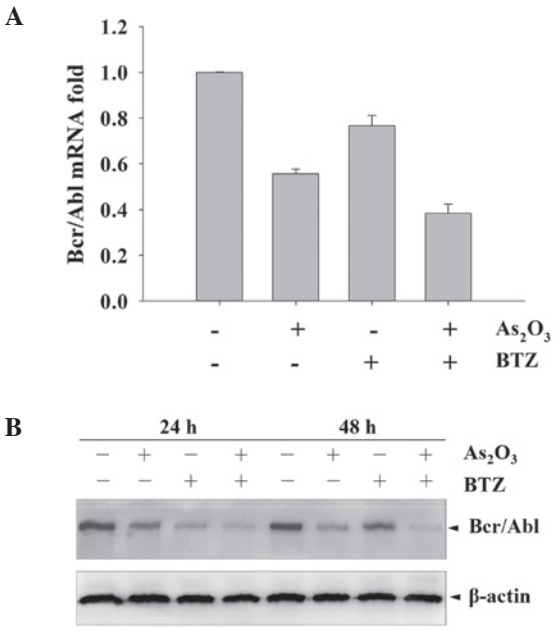

Figure 4. BTZ and As_2O_3 synergistically downregulate the expression of Bcr/Abl mRNA as well as Bcr/Abl protein levels. K562r cells were exposed to 24 nM BTZ and/or 2 μM As_2O_3 for 24-48 h. (A) Quantitative polymerase chain reaction was used to detect the Bcr/Abl mRNA level, expressed as the fold-change compared with the control level. (B) Western blotting was used to detect Bcr/Abl protein expression. β-actin was used as a loading control. Error bars represent standard deviation, from three independent experiments. As_2O_3, arsenic trioxide; BTZ, bortezomib.

pro- or anti-apoptotic effects via manipulating cellular oxidative stress, whilst BTZ is considered to be an inducer of ROS (19-21). The effects of As_2O_3 and/or BTZ on ROS production in K562r cells were next investigated. The results showed that treatment with As_2O_3 alone exhibited a marginal decrease in ROS production, whilst that with BTZ elicited an increase in ROS levels. Of note, although As_2O_3 and BTZ exerted opposing effects, combined treatment with both

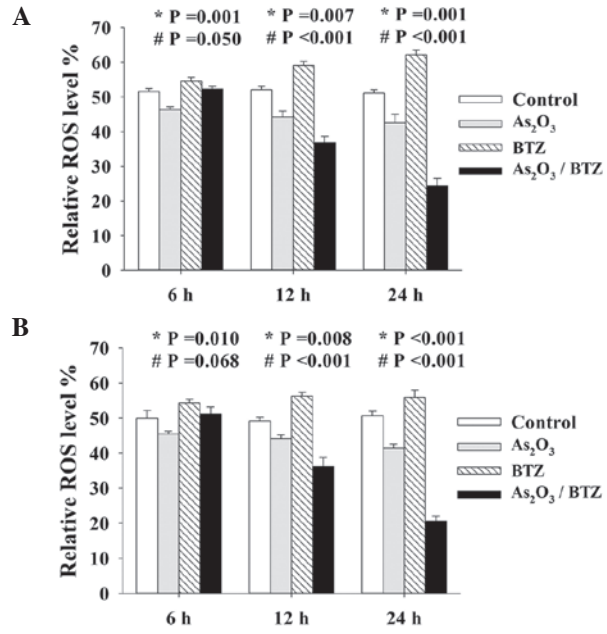

Figure 5. ROS production is significantly downregulated in BTZ/As_2O_3-treated Bcr/Abl+ cells. (A) K562r cells were exposed to 24 nM BTZ and/or 1 μM As_2O_3 for 6, 12 and 24 h and the relative ROS level was measured using direct fluorescent antibody staining. (B) A parallel experiment was performed in K562 cells. *P-value versus As_2O_3 treatment; #P-value versus BTZ treatment. Error bars represent standard deviation, from three independent experiments. As_2O_3, arsenic trioxide; BTZ, bortezomib; ROS, reactive oxygen species.

agents significantly downregulated ROS production. Parallel experiments were performed in K562 cells and comparable results were produced (Fig. 5A and B).

Discussion

Despite the success of imatinib, the emergence of drug resistance and disease progression in CML remains a continuing problem (22). In the present *in vitro* study, it was found that the IC_{50} of imatinib in K562r cells was up to 20-fold greater than that in K562 cells and clinically unachievable concentrations of up to 20 μM were required to downregulate Bcr/Abl protein. Thus, the identification of alternative drugs or targets is of great importance. It has been suggested that, instead of inhibiting the tyrosine kinase activity, targeting the signaling pathways downstream of Bcr/Abl or downregulating Bcr/Abl expression could be an effective strategy to overcome imatinib resistance (23).

Following the success of BTZ in the treatment of myeloma, proteasome inhibitors have gained interest as novel treatment strategies for cancer. As summarized by a number of studies (10,11,18,24), proteasome inhibitors, including BTZ, may exhibit anti-leukemic activities when used alone and may increase the sensitivity of cancer cells to various anti-cancer agents, including flavopiridol, histone deacetylase inhibitors and As_2O_3. The combination treatment of As_2O_3 and BTZ has been widely reported to induce apoptosis in numerous leukemia cell lines (18,25-27). Among these studies, a report by Yan *et al* (18) showed that As_2O_3 and BTZ interacted synergistically to enhance apoptosis in imatinib-sensitive K562 cells, predominantly through PKCδ activation and decreased NF-κB activity. In the present study, the data showed that clinically

achievable concentrations of BTZ and As_2O_3 could synergistically inhibit cell growth and induce apoptosis in K562r cells that were resistant to imatinib treatment. Thus, these data support the potential use of this combinatory therapy in the treatment of imatinib-resistant leukemia.

The caspase cascade is a protease system that directly facilitates apoptosis. Activation of caspase-3, the endpoint of this cascade, is critical in cleaving numerous substrates, including the repair enzyme PARP, leading to DNA strand breaks and eventually apoptosis. Caspase-3-mediated cleavage of PARP has been shown to induce apoptosis in various malignant cell lines, including Caco-2 colon cancer cells (28). Consistent with previous reports, the present study also identified that the serial activity of caspases was involved in inducing apoptosis in combined treatment with BTZ and As_2O_3 in CML cells, further supporting the use of this treatment in chemotherapies for CML (18,26-27).

The Bcr/Abl kinase plays a key role in the pathogenesis of Bcr/Abl$^+$ malignancies by blocking apoptosis through multiple pathways, including NF-κB, Akt and STAT signaling cascades (29). As reported both *in vitro* and *in vivo*, the overexpression of Bcr/Abl kinase represents a major mechanism of resistance to imatinib (22,30). Wei *et al* (31) reported that alantolactone could significantly induce apoptosis in K562r cells and that this mechanism involved the knockdown of Bcr/Abl protein expression, resulting in increased apoptosis. Thus, 24 nM BTZ and 2 μM As_2O_3 were tested in combination in K562r cells and the expression levels of Bcr/Abl were analyzed. Of note, while each agent alone minimally affected the expression of Bcr/Abl, combined treatment led to a marked downregulation of Bcr/Abl mRNA and protein, thus providing important insights into the mechanisms through which these drugs exert their effects.

ROS act as important regulators of apoptosis through numerous signaling pathways (19). A previous study reported that As_2O_3 and BTZ alone or in combination with other agents could induce ROS production and subsequent apoptosis in several cell lines, including NB4 cells (20). However, certain studies have shown contradictory results, such as a study by Stepnik *et al* (21), which showed that 5 μM As_2O_3 induced >20-fold increase in the generation of ROS in HL-60 cells after 6 h of exposure, whereas a 35% decrease was noted in K562 cells under the same conditions. This indicated that the ROS response in cells was strongly dependent on the cell origin, concentration of drugs, time of exposure and other parameters (21). Additionally, the Bcr/Abl kinase is known to enhance ROS production in certain Bcr/Abl-expressing hematopoietic cells and imatinib could effectively reduce ROS production during apoptosis (32-34). In the present study, the effects of the BTZ/As_2O_3 combination on ROS production at 6, 12 and 24 h of incubation were investigated. Of note, 2 μM As_2O_3 moderately decreased ROS levels, while 20 nM BTZ had only a minor promoting effect on ROS levels; however, the combined treatment markedly downregulated ROS production in a time-dependent manner. These data showed that BTZ/As_2O_3 treatment could directly reduce ROS production in K562r cells and that Bcr/Abl downregulation further enhanced this effect.

Collectively, this study showed that BTZ and As_2O_3 synergistically induced apoptosis in Bcr/Abl$^+$ K562r cells via caspase-3 activation and downregulation of Bcr/Abl kinase; reduced ROS production appeared to be involved in the underlying mechanism. These findings indicate that combined treatment of BTZ and As_2O_3 may be a potential therapeutic regimen for the treatment of imatinib-resistant CML.

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