Bortezomib induces G_2-M arrest in human colon cancer cells through ROS-inducible phosphorylation of ATM-CHK1

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Received February 7, 2012; Accepted April 12, 2012

DOI: 10.3892/ijo.2012.1448

Abstract. Colorectal cancer (CRC) is one of the most common cancers; however, the development of drugs to treat the condition has reached a plateau. Bortezomib (PS-341, Velcade®) is a proteasome inhibitor approved for the treatment of hematological malignancies, including multiple myeloma. A few trials of bortezomib, alone or in combination chemotherapy, for CRC patients have been reported; however, the results were largely inconclusive. This may be related to a lack of understanding of the drug's mechanism of action. Although bortezomib is reported to induce apoptosis and cell cycle arrest in various human cancer cells, the inhibitory mechanism involved is not clear. In this study, the effect of bortezomib as a treatment for human CRC was examined *in vitro* using three CRC cell lines. Bortezomib induced G_2-M arrest in CRC cells. Investigation of G_2-M phase-related cell cycle proteins involved in the response to bortezomib revealed that the ataxia telangiectasia mutated (ATM)-cell cycle checkpoint kinase 1 (CHK1) pathway, but not ATM and Rad3-related (ATR), was activated, resulting in the inactivation of cdc2. Bortezomib caused an increase in intracellular reactive oxygen species (ROS) and treatment with the ROS scavenger NAC inhibited phosphorylation of ATM leading to a decrease in the number of cells in G_2-M phase. Thus, increased ROS levels after exposure to bortezomib resulted in ATM phosphorylation. In addition, knockdown of endogenous ATM by RNA interference resulted in decreased sensitivity to bortezomib. These results suggest that bortezomib induces G_2-M arrest through ROS-inducible ATM phosphorylation and demonstrate that bortezomib is a potential candidate for further investigations in the treatment for CRC patients.

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Key words: bortezomib, reactive oxygen species, ataxia telangiectasia mutated, G_2-M arrest, colon cancer

Introduction

Colorectal cancer (CRC) is the third most common cancer in Korea and at the time of diagnosis 50% of patients have advanced or metastatic disease and are potential candidates for systemic chemotherapy (1). The standard cytotoxic chemotherapy regimen for patients with metastatic colorectal cancer (mCRC) is still 5-fluorouracil (5-FU) plus either oxaliplatin (FOLFOX) or irinotecan (FOLFIRI), which have similar efficacy outcomes in the first-line setting (2-4). The recent introduction of targeted agents such as bevacizumab and cetuximab has significantly improved survival rates (5-9). However, cetuximab does not bring additional benefits to patients with mCRC containing mutant *KRAS*. Other genomic mutations, such as *PI3KCA* and *BRAF*, which are members of the downstream *EGFR* pathway, are also associated with cetuximab efficacy (10-12).

Mutations in the *KRAS* gene are detected in 30-40% of colorectal cancers, and mCRC patients with mutant *KRAS* cannot benefit from cetuximab-based chemotherapy, which prolongs survival in those with wild-type *KRAS*. The presence of wild-type *KRAS*, however, does not guarantee a response to cetuximab. For these reasons, much effort has been made to identify new drugs to treat mCRC and to decipher the underlying disease mechanisms in these patients; however, progress has not been significant.

Since its initial clinical trials as the first proteasome inhibitor and its proven efficacy for treating patients with multiple myeloma, lymphoma and other hematologic malignancies (13-15), bortezomib (PS-341, Velcade®) has recently been studied as a potential treatment for solid tumours. The drug is a proteasome inhibitor belonging to the family of peptide boronic acid analogues and induces apoptosis in human cancer

cell lines (13). However, results from the small number of trials of bortezomib with or without irinotecan for heavily-treated mCRC patients were disappointing (16,17). Only a single trial of bortezomib plus FOLFOX as a first-line therapy for untreated mCRC patients showed comparable antitumour activity (18). Dissimilar to the other cytotoxic agents, the antitumour activity of bortezomib is mainly dependent on its cytostatic effects and induction of apoptosis, rather than on direct killing. Thus, bortezomib alone may not be effective at promoting solid tumour shrinkage. However, synergistic effects may occur if bortezomib is used in combination chemotherapy; although the mechanism of action of bortezomib needs to be fully understood before combined agents can be appropriately selected.

Although there are several studies showing that bortezomib has inhibitory effects on various cancer cell lines including colon, the underlying mechanisms remain unclear. Therefore, the aim of this study was to investigate the inhibitory effects of bortezomib on human colon cancer cell lines and to characterize the main pathways involved.

Materials and methods

Cell lines and cultures. Three human colon cancer cell lines Colo320HSR, HT29 and DLD1 were cultured in a humidified, 5% CO_2 atmosphere at 37˚C in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) or Roswell Park Memorial Institute medium (RPMI-1640; Gibco-BRL) supplemented with 10% foetal bovine serum. Bortezomib was purchased from Millenium Pharmaceuticals, Inc. and prepared as a 2.6 mmol/l stock solution in sterile saline.

Evaluation of cell viability and cell cycle distribution. Cells were seeded at 10^5 cells/60-mm dish and allowed to attach for 24 h. The cells were then treated with bortezomib (5, 10 and 20 nmol/l) for 3, 6, 9, 12 and 24 h, after which cell viability was determined by trypan blue exclusion assay using at least 300-500 cells. For the analysis of cell cycle distribution, cells were seeded at 10^6 cells/60-mm dish, cultured and viability tested as above cells were then fixed with 70% ethanol and treated with staining solution (50 μg/ml propidium iodide, 50 μg/ml RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8). Fluorescence-stained cells were analysed by fluorescence-activated cell sorting (FACS) using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Immunoblotting. Cells were seeded into 60-mm dishes 24 h before treatment and then exposed to bortezomib for additional 24 h. After treatment, cells were scraped into radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl pH 7.4, 2 mM EGTA, 2 mM EDTA, 25 mM β-glycerophosphate, 1% Triton X-100 and 500 μl of 10% glycerol and protease inhibitors). Protein concentrations were determined using the Bradford assay, and 20 μg of total cellular protein/sample was resolved by SDS-PAGE and transferred to an Immobilon-PVDF membrane (Millipore Corporation, Billerica, MA, USA). Membranes were blocked using 5% nonfat milk powder in TBS-T buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.1% Tween-20) and probed with antibodies against phosphorylated-cdc2, ATM, phosphorylated-ATM (Ser1981), phosphorylated-ATR (Ser428) and phosphorylated-CHK1 (Ser317) (Cell Signaling Technology, Inc., Beverly, MA, USA). Primary antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-goat secondary antibodies as appropriate and visualized using enhanced chemiluminescence reagents (Amersham, Buckinghamshire, UK).

Detection of intracellular reactive oxygen species (ROS) production. ROS production was determined by staining with 20 μM 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma, St. Louis, MO, USA). Cells were incubated with DCF-DA for 30 min at 37˚C, washed, resuspended in PBS and analysed by FACS. Ten thousand cells were acquired using a flow cytometer (BD Biosciences). Hydrogen peroxide (H_2O_2) was used as a positive control and N-acetylcysteine (NAC) was used for detoxification of ROS.

RNA interference. Cells were transiently transfected with scrambled (control) siRNA or siRNA against ATM using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA). The sequences of scrambled- and ATM-specific siRNAs were 5'-AATTCTCCGAACGTGTCACGT-3' and 5'-AAGCGCCTGATTCGAGATCCT-3', respectively.

Statistical analysis. Comparisons were made using the paired Student's t-test to calculate the level of statistical significance in cell viability, cell cycle distribution and ROS production between groups.

Results

Bortezomib induces growth inhibition in human colon cancer cells. Induction of apoptosis and cell cycle arrest by bortezomib has been reported in human hepatic, mantle cell, prostate, ovarian and breast cancer cells (19-23). The present study investigated the inhibitory effects of bortezomib in human colon cancer cells.

Human colon cancer cell lines Colo320HSR and HT29, which harbour wild-type *KRAS*, and DLD1, which harbours mutated *KRAS*, were treated with various doses of bortezomib for 24 h and cell viability was measured using the trypan blue exclusion assay (Fig. 1). The number of cells in G_2-M phase increased significantly after exposure to bortezomib (Colo320HSR, $p=0.001$; HT29, $p<0.001$; DLD1, $p=0.012$), and each cell line showed differing levels of sensitivity to bortezomib (Fig. 2). Interestingly, the mutational status of *KRAS* did not appear to influence the inhibitory effect of bortezomib on cell proliferation.

Bortezomib induces G_2-M arrest by activating the ATM-CHK1 pathway. As shown in Fig. 2, the number of cells in G_2-M phase significantly increased after treatment with bortezomib. Therefore, changes in G_2-M phase-related cell cycle proteins in response to bortezomib were investigated. ATM is a serine/threonine kinase activated in response to DNA damage and regulates the signaling cascades that lead to cell cycle arrest, DNA repair or apoptosis. Phosphorylation of ataxia telangiectasia mutated (ATM) in Colo320HSR, HT29 and DLD1 cells

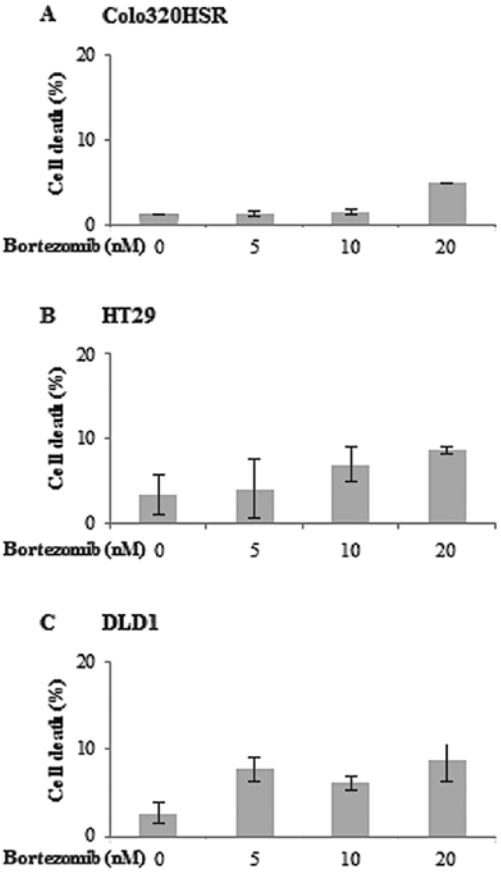

Figure 1. Cell viability after bortezomib treatment. Colon cancer cell lines were treated with bortezomib at the indicated doses for 24 h. Cell viability was measured using the trypan blue exclusion assay. Values are presented as the mean ± SE of 3 independent experiments performed in duplicate. Data from a representative experiment are presented. (A) Colo320HSR, (B) HT29 and (C) DLD1 cell lines.

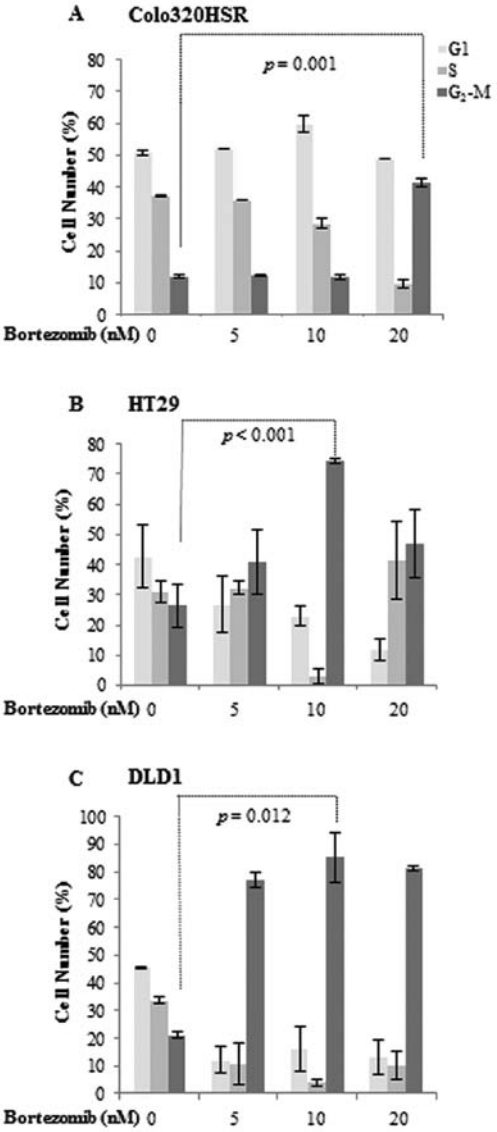

Figure 2. Bortezomib induced G_2-M arrest in various human colon cancer cells. Colon cancer cells were treated with bortezomib at the indicated doses for 24 h and then washed with PBS, trypsinized, fixed in 70% ethanol and collected in 1 ml of PI staining solution for FACS analysis. Values are presented as the mean ± SE of 3 independent experiments performed in duplicate. Data from a representative experiment are shown. (A) Colo320HSR, (B) HT29 and (C) DLD1 cell lines.

clearly increased after exposure to bortezomib, whereas phosphorylation of ATM-related ATR (ATM and Rad3-related) was not induced (Fig. 3). ATM protein, but not ATR, was also phosphorylated in three other colon cancer cells upon exposure to bortezomib (data not shown). Cell cycle checkpoint kinase 1 (CHK1) is a downstream target of ATM and amplifies the DNA damage signal whereas cell division cycle 2 (cdc2) promotes cell cycle progression. Consistently, phosphorylation of ATM causes both activation of CHK1 and inactivation of cdc2 (Fig. 3). Thus, bortezomib induces G_2-M arrest through activation of the ATM-CHK1 pathway in human colon cancer cells.

To further examine the relationship between bortezomib and the ATM-CHK1 pathway, the dependence of ATM on bortezomib-induced G_2-M arrest was analysed by ATM silencing using small interfering RNA (siRNA). The proportion of ATM siRNA-transfected Colo320HSR, HT29 and DLD1 cells in G_2-M phase significantly decreased compared with that in cells transfected with scrambled siRNA after exposure to bortezomib ($p<0.001$, $p=0.015$ and $p=0.003$, respectively) (Fig. 4). Consistent with this, ATM knockdown by siRNA led to a reduction in phosphorylated CHK1, which mirrored the reduction in non-phosphorylated ATM (Fig. 4), whereas transfection with ATR-siRNA did not affect bortezomib-induced G_2-M arrest (data not shown). Thus, bortezomib-induced G_2-M arrest is dependent on activation of the ATM-CHK1 pathway.

Bortezomib induces G_2-M arrest through ROS-inducible ATM activation. It was recently reported that proteasome inhibition by bortezomib increased the levels of intracellular ROS levels (19,24,25). Therefore, we next investigated whether bortezomib also increased the levels of intracellular ROS in human colon cancer cells. H_2O_2 was used as a positive control. Human colon cancer cell lines were treated with a dose of bortezomib that induced G_2-M arrest (see Fig. 2); 20 nM for Colo320HSR and HT29 and 10 nM for DLD1. Cells were then stained with DCF-DA and analysed by FACS to identify cells bearing ROS. Surprisingly, the levels of intracellular ROS increased significantly in Colo320HSR, HT29 and DLD1 cells after exposure to bortezomib (Fig. 5A-C). To confirm that exposure to bortezomib led to the generation of intracellular ROS, HT29 cells were treated with the ROS scavenger NAC. NAC treatment significantly blocked generation of ROS

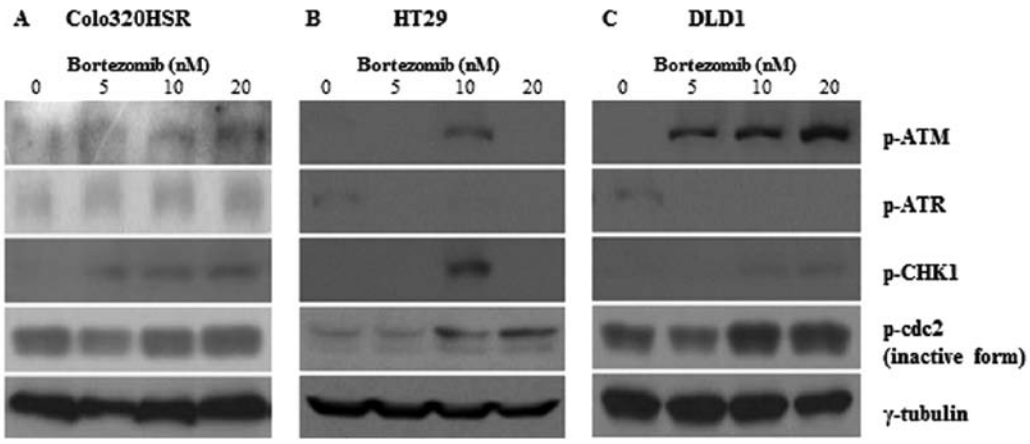

Figure 3. Bortezomib induces activation of ATM/CHK1 pathway. (A) Colo320HSR, (B) HT29 and (C) DLD1 cells were treated with bortezomib at the indicated doses for 24 h. Cell lysates were prepared and analysed by immunoblotting using antibodies against p-ATM, p-ATR, p-CHK1 and p-cdc2 (inactive form). γ-tubulin was used as a loading control.

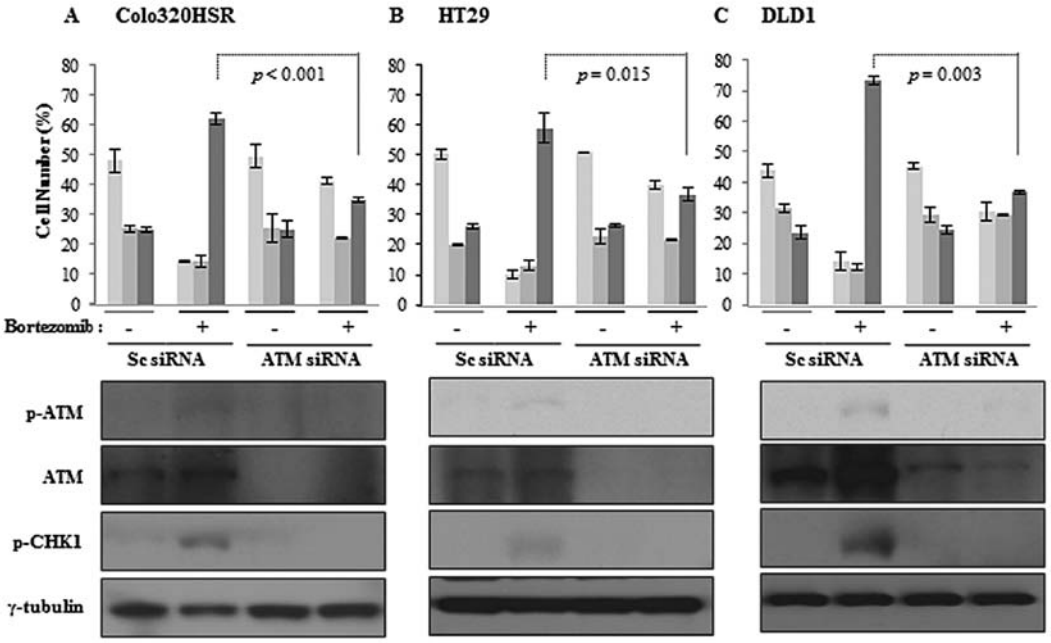

Figure 4. Activation of ATM is indispensable for bortezomib-induced G_2-M arrest. (A) Colo320HSR, (B) HT29 and (C) DLD1 cells were transfected with scrambled or ATM siRNA for 48 h and then treated with bortezomib 20 nM (Colo320HSR and HT29) or 10 nM (DLD1) for another 24 h. Cells were prepared for PI staining and FACS analysis. Cell lysates were immunoblotted using antibodies against p-ATM, ATM and p-CHK1. γ-tubulin was used as a loading control.

in response to bortezomib (p=0.01) (Fig. 5D). These results implied that increased ROS levels are associated with G_2-M arrest through activation of the ATM-CHK1 pathway in response to bortezomib.

To test this hypothesis, we investigated the effect of combined treatment with NAC and bortezomib on bortezomib-induced G_2-M arrest and phosphorylation of ATM. The proportion of Colo320HSR, HT29 and DLD1 cells treated with bortezomib plus NAC in G2-phase was significantly lower than that in cells treated with bortezomib alone (p<0.001, p=0.001, and p<0.001, respectively) (Fig. 6A-C). Notably, phosphorylation of ATM was completely inhibited after treatment with NAC, indicating that ATM phosphorylation is the result of ROS production following bortezomib treatment (Fig. 6D-F). In addition, the level of phosphorylated CHK1 paralleled the level of phosphorylated ATM. Taken together, these results suggest that bortezomib induces G_2-M arrest via ROS-inducible activation of the ATM-CHK1 pathway.

Discussion

This preclinical study showed that bortezomib induced G_2-M arrest in human colon cancer cell lines and that this G_2-M arrest resulted from increased production of intracellular ROS, which can induce DNA damage and subsequent activation of the ATM-CHK1 pathway. In the presence of DNA damage, cells activate multiple signalling pathways, known as the DNA damage response (DDR), to sense the extent of DNA damage and coordinate pathways that are associated with either cell cycle arrest to allow DNA repair, or regulation of senescence

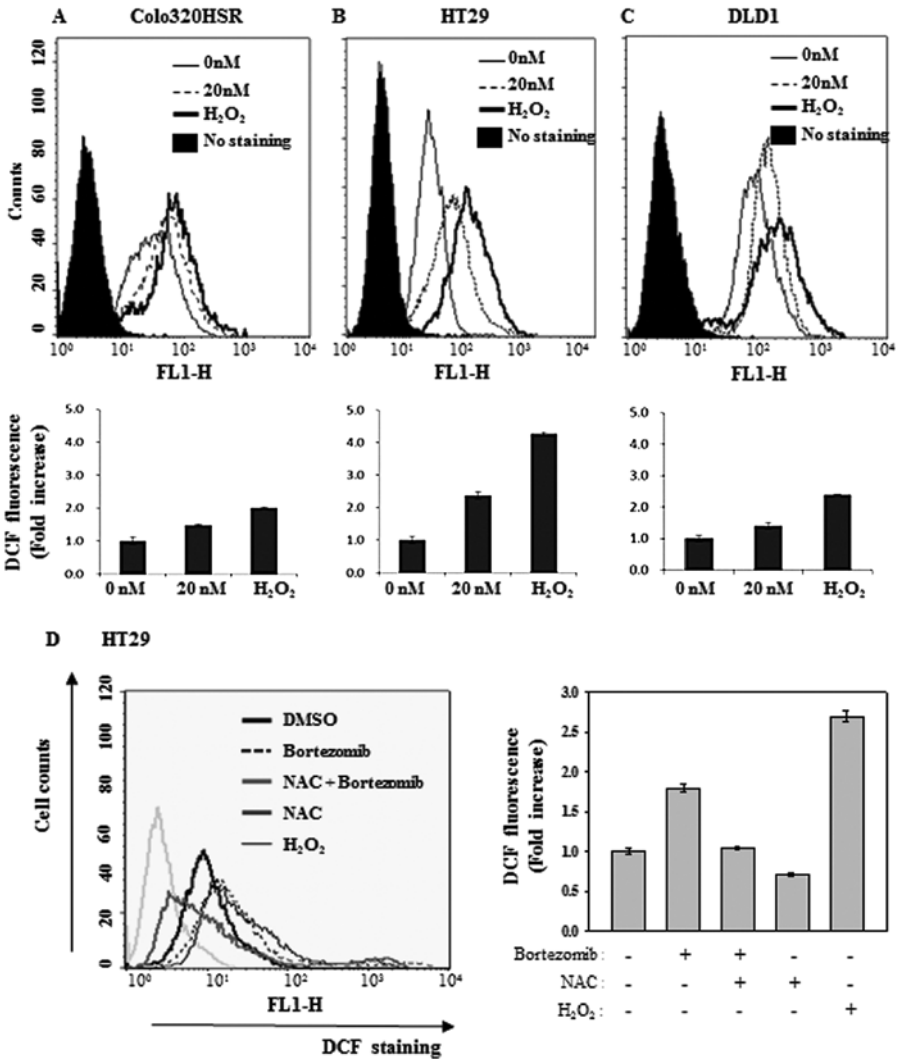

Figure 5. Bortezomib induces an increase of intracellular ROS level. (A) Colo320HSR, (B) HT29 and (C) DLD1 cells were treated with H_2O_2 or bortezomib at the indicated doses for 24 h, washed with PBS, incubated with 25 μM of DCF-DA for 30 min, and then washed with PBS, trypsinized and collected in 1 ml of PBS for FACS analysis. Representative results from 3 experiments are shown as mean fluorescence histograms (upper panels) and as -fold increases in DFC ± SE (lower panels). (D) HT29 cells were treated with bortezomib 10 nM for 24 h, washed and incubated with 25 μM of H2DCF-DA for 30 min, and then washed with PBS and analysed for ROS levels by FACS.

or apoptosis to prevent proliferation of genetically unstable cells (26). Inactivation of this response is regarded as important for oncogenic transformation or tumour progression, and recent studies show that several markers consisting of DDR checkpoints are expressed in human precancerous lesions (27,28). Furthermore, inactivation of crucial elements in the DDR checkpoints is essential for progression from a precancerous lesion to an invasive cancer (29), and patients bearing mutations in genes specific for DDR checkpoints are vulnerable to developing malignancies or suffer from severe genetic disorders exemplified by ataxia telangiectasia (AT) (30,31). ATM is a member of a PI3K-related protein kinase family and plays a central role in coordinating DDR, including cell cycle checkpoint control and DNA repair and apoptosis, together with ATR (32). The current study shows that phosphorylation of ATM was increased in human colon cancer cells treated with bortezomib and verified that the induction mechanism of G_2-M arrest after bortezomib treatment was associated with the ATM signalling pathway. However, activation of ATR was not observed in response to bortezomib and the role of ATR in the bortezomib-inducible DNA damage response remains to be elucidated.

In addition, bortezomib induced production of intracellular ROS and subsequent activation of the ATM-CHK1 pathway. ROS, mainly consisting of superoxide anion radical (O_2^-), singlet oxygen, H_2O_2 and the highly reactive hydroxyl radical, are harmful to cells or tissues. However, recent research indicates that ROS act as second messengers and are essential for cell signalling and various normal cellular processes (33,34). Non-lethal levels of intracellular ROS, particularly for long periods, play a role in many human diseases, especially cancers (35). ROS are oncogenic because they act in multiple signalling cascades related to malignant cell behaviour; however, ROS can also be used to kill cancer cells. ROS production is a mechanism shared by non-surgical cancer therapies including chemotherapy and radiotherapy (36,37). Hence, strategies to increase or decrease ROS levels must be considered carefully in cancer treatments (38). Recent studies have shown that bortezomib induces apoptosis in medulloblastoma, lymphoma and leukaemia cell lines through the generation of ROS (19,39,40).

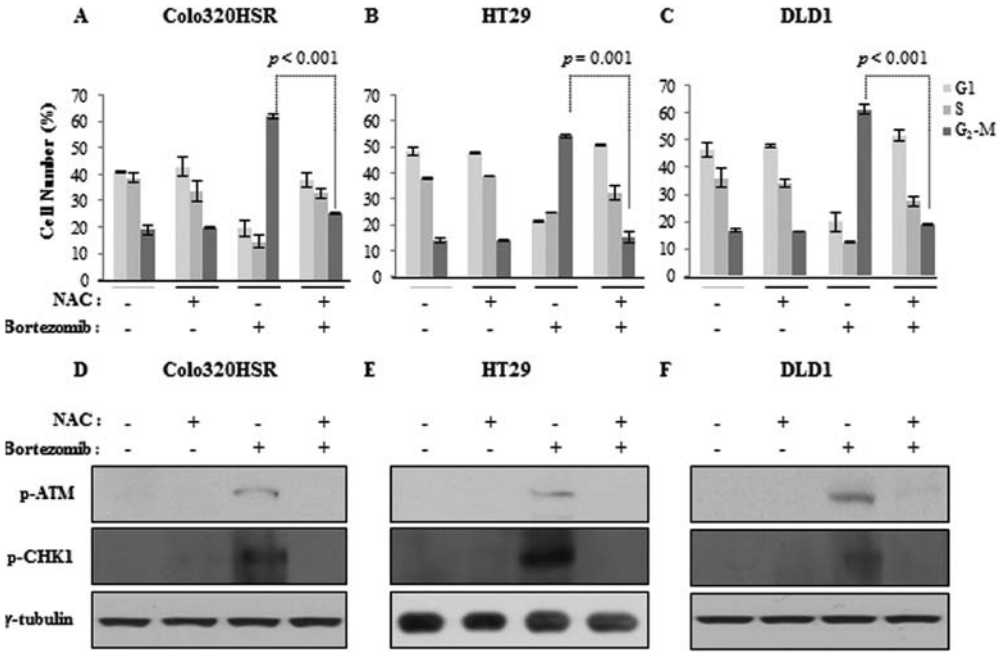

Figure 6. Production of ROS is necessary for bortezomib-induced G_2-M arrest. (A-C) Colo320HSR, HT29 and DLD1 cells, where indicated, were pre-treated with 5 mM NAC for 1 h and then treated with bortezomib for 24 h, washed and incubated with 25 μM of DCF-DA for 30 min. Finally, cells were washed with PBS, trypsinized and collected in 1 ml of PBS for FACS analysis. (D-F) Cell lysates were prepared for immunoblot analysis with antibodies to p-ATM and p-CHK1. γ-tubulin was used as a loading control.

In the present study, we also demonstrated that intracellular concentrations of ROS increased after bortezomib treatment and could be blocked by NAC, which detoxifies ROS, suggesting that ROS are responsible for DNA damage induced by bortezomib in these human colon cancer cells. The relationship between proteasome inhibition and increased ROS generation is not clearly understood. Mechanisms have recently been suggested to include generation of superoxide anion radicals, interference with the mitochondrial electron transport system, and endoplasmic reticulum stress (24,25,39).

In conclusion, bortezomib shows antitumour activity against human colon cancer cell lines by inducing G_2-M cell cycle arrest, which is associated with increased production of intracellular ROS resulting from proteasome inhibition. DNA damage from ROS activates the ATM-CHK1 pathway, which plays a key role in G_2-M cell cycle arrest. This study has identified a chain of events in human colon cancer cells with different *KRAS* genotypes following exposure to bortezomib. Insight has been provided into the possible targets for colon cancer treatments, particularly those with mutated KRAS; however, further studies are required to determine whether the mechanisms identified are common to the diverse spectrum of primary and metastatic colorectal tumours. The findings presented here could also inform clinical trials of combination chemotherapy containing bortezomib and contribute to further in-depth study of predictive or prognostic biomarkers for patients with mCRC.

Acknowledgements

This study was supported by grants from the Korea Health 21 R&D Project, Ministry of Health and Welfare and Family Affairs, Korea (A062254 and A102059).

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