

Sequential treatment of HPV E6 and E7-expressing TC-1 cells with bortezomib and celecoxib promotes apoptosis through p-p38 MAPK-mediated downregulation of cyclin D1 and CDK2

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Abstract. Interruption of the cell cycle is accompanied by changes in several related molecules that result in the activation of apoptosis. The present study was performed to verify the apoptotic effects of sequential treatment with bortezomib and celecoxib in TC-1 cells expressing the human papillomavirus (HPV) E6 and E7 proteins. In TC-1 cells sequentially treated with bortezomib and celecoxib, apoptosis was induced through decreased expression of signal transducer and activator of transcription-3 (STAT3), cyclin D1 and cyclin-dependent kinase (CDK) 2, which are major regulators of the G0/G1 cell cycle checkpoint. In addition, increased levels of p21, CHOP, BiP and p-p38 MAPK were identified in these cells. The treatment-induced apoptosis was effectively inhibited by treatment with SB203580, an inhibitor of p-p38. Moreover, the growth of tumors sequentially treated with bortezomib and celecoxib was retarded compared to the growth of tumors exposed to a single treatment with either bortezomib or celecoxib *in vivo*. We demonstrated that sequential treatment with bortezomib and celecoxib induced apoptosis via p-p38-mediated G0/G1 cell cycle arrest and endoplasmic reticulum (ER) stress. Sequential treatment with these two drugs could therefore be a useful therapy for cervical cancer.

Introduction

Human papillomavirus (HPV) infection is a major cause of cervical cancer, one of the most common cancers in women worldwide (1,2). HPV DNA integration into the host genome results in constitutive expression of the viral oncoproteins E6 and E7, which deregulate cell cycle control through interactions with the tumor-suppressor protein p53 and pRb-type oncoproteins (3). The HPV E6 protein induces the degradation of p53, thereby inhibiting p53-dependent signaling and contributing to tumorigenesis (4). The HPV E7 protein binds to the retinoblastoma family of proteins (pRb, p107 and p130) and prevents G1 arrest in response to a variety of antiproliferative signals (5).

pRb is important for progression of the cell cycle from the G1 to the S phase, which is regulated by the cyclin-dependent kinases (CDKs) cyclin D-CDK4/6 (6) and cyclin E-CDK2 (7). Cyclin and CDK inhibitors, such as p16^{INK4A} and p21^{CIP1}, regulate CDK activity. p21^{CIP1} activation by p53 inhibits the formation of cyclin E-CDK2 complexes and triggers cell cycle arrest in the G1 phase (8). Therefore, many tumors exhibit increased cyclin D1 or CDK4 levels, and a loss of p16^{INK4A}, Rb and p53. A previous study demonstrated that the cell cycle regulator proteins cyclin D1 and cyclin E1 are overproduced in cervical cancer cells (9). When TC-1 cells, which are immortalized mouse lung epithelial cells, were transduced with HPV E6 and E7, p53 and Rb expression in the TC-1 cells was reduced as a result of degradation mediated by interaction with HPV16 E6 and E7 (10). Combining bortezomib treatment with DNA vaccines was found to result in improved immunity against TC-1 cells in mice compared to monotherapy, and treatment with bortezomib increased TC-1 tumor cell apoptosis (11). However, the present study did not identify the mechanism of apoptosis since cyclin-dependent kinase inhibitors (CKIs), such as p21, are transcriptionally activated primarily by p53 during the G1 phase.

Bortezomib reversibly inhibits the 26S proteasome, preventing the degradation of pro-apoptotic proteins related to

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the regulation of cell proliferation and survival. Many previous studies have shown that bortezomib decreases NF- κ B and STAT3 activation as well as activation of their downstream target genes, ICAM-1, MCP-1 and cyclin D1 (12,13). For these reasons, many clinical trials are in progress to test the effect of bortezomib alone or in combination with chemo/radiation to treat various NF- κ B-dependent solid tumor models. Cyclooxygenase-2 (COX-2) is known to play an important role in carcinogenesis and cancer progression (14). Several studies have shown that COX-2 is overexpressed in cervical intraepithelial neoplasia, but not in normal cervical tissue (15,16). The HPV16 E6 and E7 oncoproteins contribute to carcinogenesis by increasing COX-2 transcription by activating the EGFR-Ras-MAP kinase pathway (17). COX-2 and Ki-67 expression levels were found to be downregulated and neoangiogenesis was inhibited in cervical cancer patients treated with celecoxib (400 mg) twice daily (18). Moreover, celecoxib induced apoptosis in various cancer cell lines via a COX-2-independent mechanism, which may involve inhibition of NF- κ B and STAT3 phosphorylation (19). From these results, we predicted that celecoxib, a specific inhibitor of COX-2, may have an important role in cancer treatment in various malignancies when combined with bortezomib.

We, therefore, investigated whether bortezomib treatment acts synergistically with celecoxib to increase therapeutic efficacy against TC-1 cells, in an HPV16 E6- and E7-expressing cervical cancer model. We assessed various combinations of bortezomib and celecoxib in TC-1 cells to determine the most effective cancer therapy with drugs *in vitro* and *in vivo*.

Materials and methods

Cells, reagents and animals. Bortezomib (provided by Janssen Korea) was prepared as a 7.8 mM stock in PBS and stored at -20°C . Celecoxib (Toronto Research Chemicals Inc., Toronto, Ontario, Canada) was prepared as a 200 mM stock in DMSO and stored at -20°C . Antibodies to STAT3, p21, cyclin D1, CHOP and β -actin, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit/mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to CDK2, CDK4, survivin, p38, p-p38, Bid, Bcl-2, Bax and caspase-3, -8 and -9 were purchased from Cell Signaling Technology (Beverly, MA, USA). An antibody to COX-2 was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). An antibody to BiP was purchased from Abcam (Cambridge, UK). SB203580 was purchased from Calbiochem (Darmstadt, Germany). C57BL/6 mice were acquired from the Chung-Ang Laboratory Animal Service (Seoul, Korea). All procedures were performed according to the approved protocols and in accordance with the recommendations of the Ethics Committee of the College of Medicine, Inje University and Chung-Ang University for the proper use and care of laboratory animals.

Cell culture conditions and cell viability assay. The TC-1 cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone), 1% penicillin-streptomycin, 1% glutamine, 1% MEM-non essential amino acids, and 1% sodium pyruvate (Life Technologies, Grand Island, NY, USA) in a humidified incubator supplied with 5% CO_2 at 37°C (10). TC-1 cells were

seeded into 96-well plates at a density of 5×10^3 cells/well. The cells were pre-treated with bortezomib for 6 h and then treated with celecoxib for an additional 18 h. For comparison, TC-1 cells were treated either with bortezomib or celecoxib alone, or co-treated for 24 h. Finally, TC-1 cells pre-exposed to celecoxib for 6 h were then treated with bortezomib for 18 h. Cell viability was determined by an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay using the Vybrant MTT cell assay kit (Molecular Probes, Eugene, OR, USA). The analysis was performed according to the manufacturer's instructions. The absorbance at 560 nm was measured using a microplate reader (Molecular Probes).

Western blot analysis for apoptosis-related proteins and cell cycle regulators. TC-1 cells were seeded into 24-well plates at a density of 3×10^4 cells/well. The TC-1 cells were then treated with bortezomib and celecoxib for the indicated times. After the cells were pelleted and resuspended in lysis buffer, 40 μg of total cell protein per sample was subjected to 8-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA) for immunoblotting. The primary antibodies used were: anti-Bip, anti-CDK2, anti-CDK4, anti-CHOP, anti-CyclinD1, anti-cleaved caspase-3, anti-caspase-8, anti-caspase-9, anti-p38, anti-p53, anti-p21, anti-Stat3 (1:1,000); and anti-Cox-2, anti-Bid, anti-Bax, anti-Bcl-2 and anti- β -actin (1:2,000). The primary antibodies were detected using horseradish peroxidase-conjugated goat anti-rabbit (1:2,000) or goat anti-mouse (1:10,000) secondary antibodies. Western blot analysis was performed using standard techniques, and the bands were visualized with the ECL system (Amersham Pharmacia Biotech, UK).

Flow cytometric analysis of apoptosis and the cell cycle. TC-1 cells were treated with bortezomib and celecoxib singly, in combination, and sequentially for 12 h. The cells were then analyzed using an Annexin V-FITC detection kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. The TC-1 cells were harvested and fixed in cold 70% ethanol at 4°C overnight. The next day, the cells were stained with a 50 $\mu\text{g}/\text{ml}$ propidium iodide (PI) solution (Sigma, St. Louis, MO, USA) and 500 μl 10 $\mu\text{g}/\text{ml}$ RNase A (Sigma) for 30 min at 37°C in the dark. The cells were analyzed immediately using a FACSCalibur flow cytometer (Becton-Dickinson, San Diego, CA, USA).

In vivo tumor treatment using bortezomib and celecoxib. C57BL/6 mice (5-6 per group) were challenged with 5×10^4 TC-1 tumor cells by subcutaneous (s.c.) injection for the *in vivo* tumor treatment experiment. By day 8, the average tumor size was ~ 90 -100 mm^3 . The mice were then divided into the following groups: control (no treatment), single treatment, co-treatment, and sequential treatment with bortezomib (0.5 mg/kg) and celecoxib (5 mg/kg). Drug treatment was administered via intraperitoneal (i.p.) injection. The sequential treatment group was administered bortezomib (day 8, 10, 12 and 14) followed by celecoxib (day 9, 11, 12 and 14) or celecoxib (day 8, 10, 12 and 14) followed by bortezomib (day 9, 11, 12 and 14) daily. The mice in the co-treatment groups

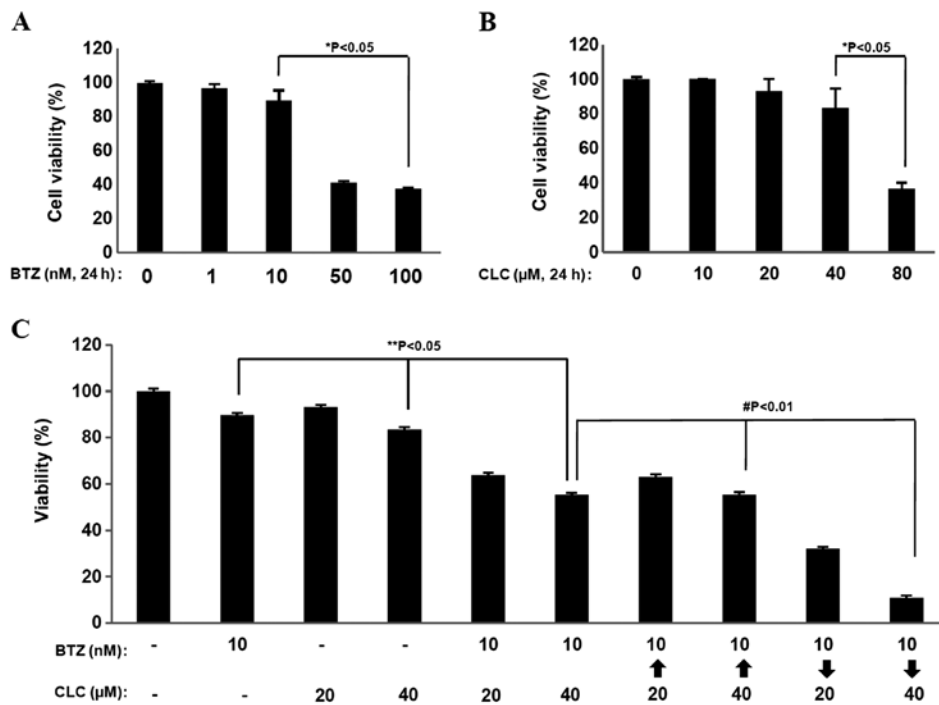


Figure 1. Effect of bortezomib and celecoxib on TC-1 cell viability. Survival of TC-1 cells was determined by MTT assay after 24 h of culture in the presence of increasing concentrations of bortezomib (BTZ) (A) and celecoxib (CLC) (B) ($P < 0.05$; low dose vs. high dose). (C) Viability of TC-1 cells sequentially treated with bortezomib and celecoxib was assessed at the indicated concentrations. The cells were pre-incubated with bortezomib for 6 h and then treated with celecoxib for 18 h ($^{*}P < 0.05$, difference between bortezomib or celecoxib treatment and co-treatment; $^{#}P < 0.01$, difference between co-treatment or CLC→BTZ and BTZ→CLC).

were injected with the appropriate drug four times every other day (day 8, 10, 12 and 14). The tumor size was measured at intervals of approximately two days. At the end of the treatment, the mice were sacrificed, and the tumors were collected and weighed.

Statistics. The mean value for cell viability or mean fluorescence intensity for the apoptosis assay with standard error of the mean (SEM) from representative experiments is shown in the figures. Data were confirmed by at least three independent experiments. All data shown are representative of at least two different experiments. The data were compared by analysis of variance (one-way ANOVA). Statistical significance was defined by a P-value of < 0.05 .

Results

Sequential treatment with bortezomib and celecoxib increases apoptosis via an intrinsic pathway in TC-1 cells. Treatment with bortezomib (BTZ) or celecoxib (CLC) significantly reduced cell viability at relative high doses (50–100 nM of bortezomib and 80 μM of celecoxib) based on MTT assays ($P < 0.05$) (Fig. 1A and B). However, co-treatment with relatively low doses of BTZ (10 nM) and CLC (20 and 40 μM) induced significant levels of cell death in the MTT assays compared to single treatment ($P < 0.05$, individual drug treatment vs. co-treatment) (Fig. 1C). Notably, the effects of combination treatment with BTZ (10 nM) and CLC (40 μM) on cell viability showed strong differences depending on the treatment order ($P < 0.01$, sequential drug treatment vs.

co-treatment). When TC-1 cells were treated with bortezomib and then celecoxib (BTZ→CLC), cell viability was significantly reduced compared to the group treated simultaneously with BTZ and CLC or the group treated with celecoxib and then bortezomib (CLC→BTZ) ($P < 0.01$, co-treatment or CLC→BTZ treatment vs. BTZ→CLC treatment) (Fig. 1C). The number of Annexin V-positive TC-1 cells increased after sequential treatment with bortezomib and celecoxib ($P < 0.001$, BTZ or CLC treatment vs. BTZ→CLC treatment) (Fig. 2A). Furthermore, production of pro-apoptotic protein Bax increased, whereas production of the anti-apoptotic protein Bcl-2 decreased after pre-treatment with bortezomib followed by treatment with celecoxib. Bid and caspase-8 levels, which are related to the extrinsic apoptotic pathway, did not change after bortezomib and celecoxib treatment, while levels of cleaved caspase-3 and -9 increased in the TC-1 cells that were sequentially treated with bortezomib and celecoxib (Fig. 2B and C). These results suggest that sequential treatment with relatively low doses of bortezomib and celecoxib may effectively induce TC-1 cell death through intrinsic apoptosis.

The apoptosis of TC-1 cells sequentially treated with bortezomib and celecoxib is mediated by STAT3, cyclin D1, CDK2 and ER stress. Co-treatment with bortezomib and celecoxib has been reported to induce apoptosis via production of the ER stress-related proteins, glucose-regulated protein 78 (GRP78/BiP) and CCAAT/enhancer binding protein homologous transcription factor (CHOP/GADD153), but not through inhibition of COX-2 production (20). Our results are consistent with these earlier studies, including the presence of increased COX-2

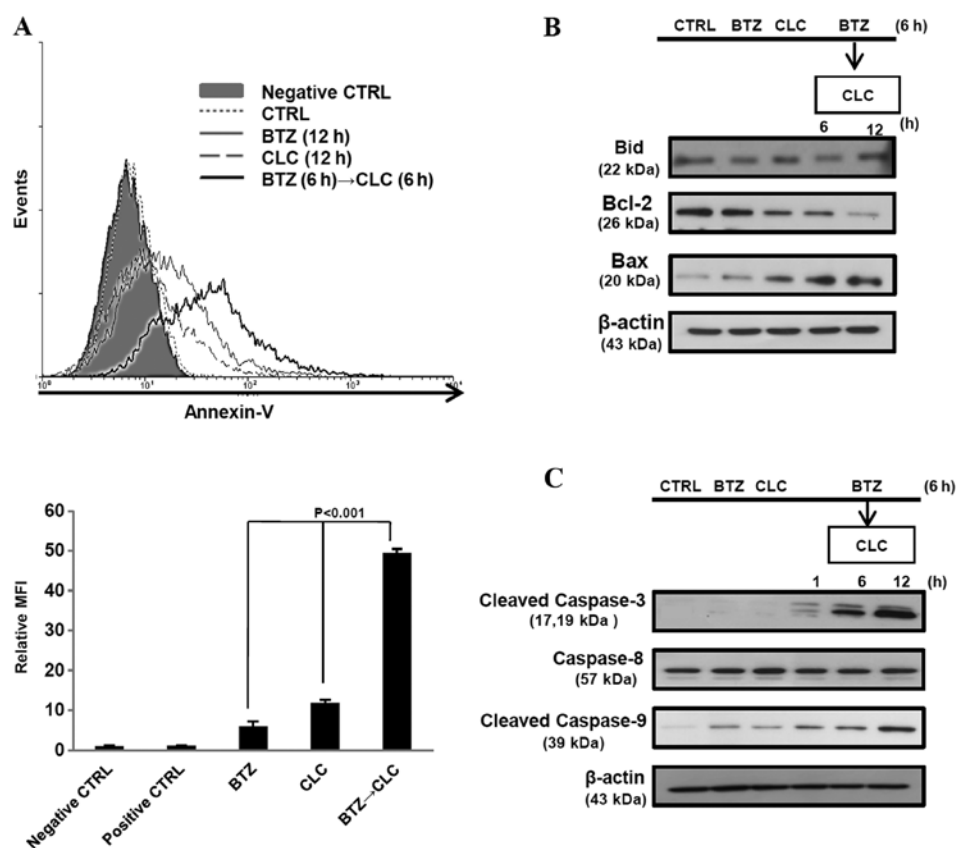


Figure 2. Effects of sequential bortezomib and celecoxib treatment on the induction of apoptosis and the expression of apoptosis-related proteins in TC-1 cells. (A) The apoptosis rate after incubation with a single drug or combined sequential treatment with bortezomib (10 nM) and celecoxib (40 μ M) was measured by Annexin V staining and flow cytometric analysis. The mean fluorescence intensity (MFI) of the apoptosis rate is also shown (P < 0.001, difference between individual drug treatments and sequential bortezomib). (B) Expression of apoptosis-related Bcl-2 family proteins and (C) cleaved caspase-3 or -9 and caspase-8 proteins was detected by western blotting. BTZ, bortezomib; CLC, celecoxib.

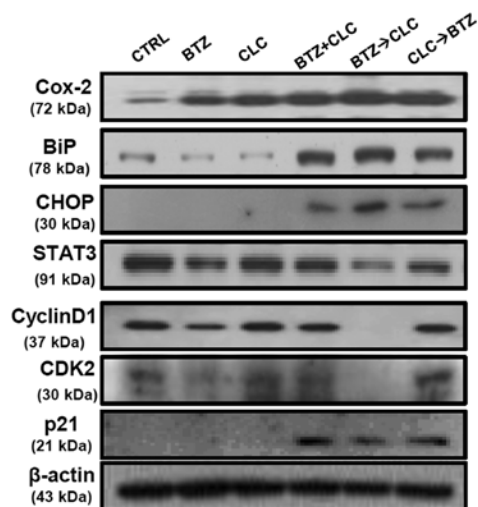


Figure 3. Mechanisms underlying the synergistic effect of sequential bortezomib and celecoxib treatment on apoptosis. Lysates were prepared from TC-1 cells treated with one or both drugs (BTZ, 10 nM; CLC, 40 μ M) and immunoblotted using antibodies against ER stress-related proteins (BiP and CHOP) and G0/G1 cell cycle-related proteins (STAT3, cyclin D1, CDK2 and p21). BTZ, bortezomib; CLC, celecoxib.

levels after combined treatment with bortezomib and celecoxib (Fig. 3). However, the significant production of BiP and

CHOP is not sufficient to explain the high levels of apoptosis in TC-1 cells since TC-1 cells co-treated with bortezomib and celecoxib or sequential celecoxib and bortezomib treatment also exhibited elevated BiP and CHOP protein levels (Fig. 3). STAT3 and cyclin D1 expression was significantly decreased in the TC-1 cells sequentially treated with bortezomib and celecoxib compared to the levels in the TC-1 cells co-treated with bortezomib and celecoxib or treated with a single drug. p21 production was not significantly upregulated in the TC-1 cells after single treatment with bortezomib or celecoxib. Co-treatment with bortezomib and celecoxib or sequential celecoxib and bortezomib treatment also enhanced p21 production in the p53-degraded TC-1 cells, while the levels of the cell cycle regulator proteins cyclin D1 and CDK2 did not change at all (Fig. 3). Notably, we observed that in the TC-1 cells sequentially treated with bortezomib and celecoxib, p21 production increased in a time-dependent manner and was associated with the downregulation of STAT3, cyclin D1, and CDK2, but not CDK4. BiP and CHOP levels were also significantly increased in the TC-1 cells sequentially treated with bortezomib and celecoxib in a time-dependent manner (Fig. 4A). In addition, we found that apoptosis in TC-1 cells sequentially treated with bortezomib and celecoxib was associated with cell cycle arrest in the G0/G1 phase, and the apoptosis rate increased in a time-dependent manner (Fig. 4B). These results suggest that the apoptosis of TC-1 cells sequentially treated with bortezomib

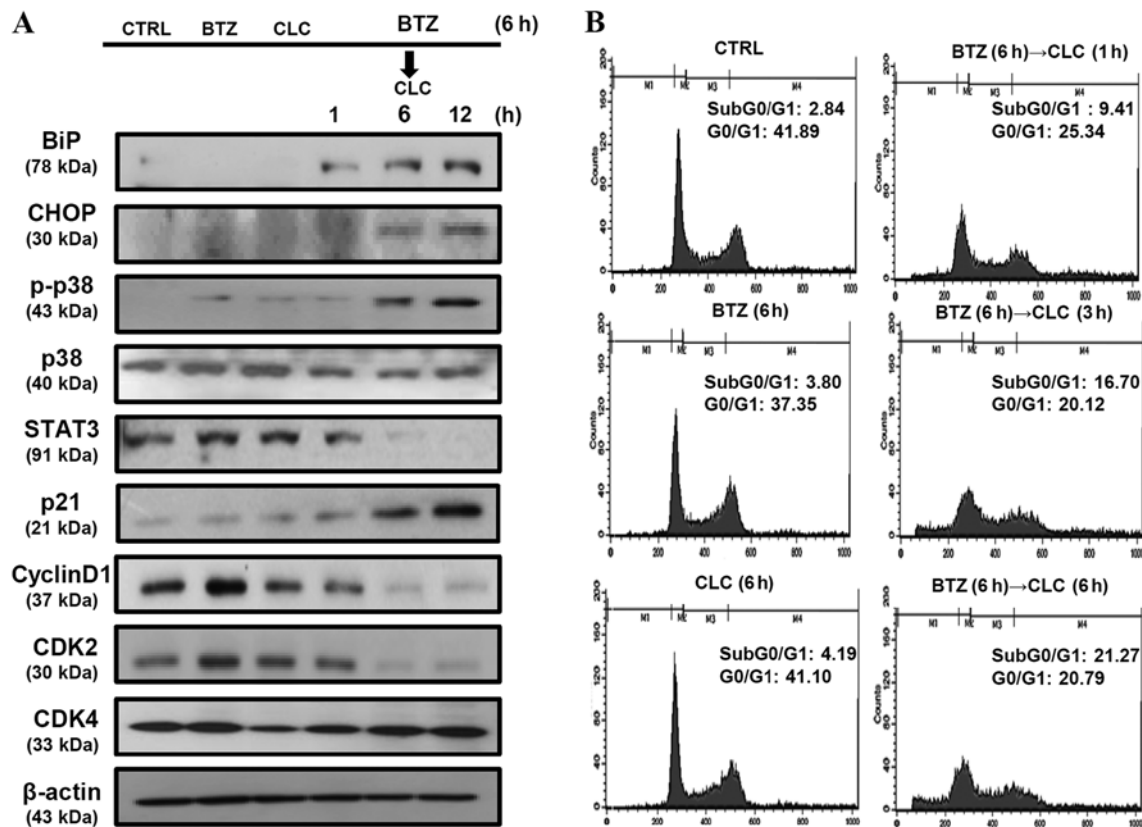


Figure 4. Sequential bortezomib and celecoxib treatment induces ER stress and cell cycle arrest in TC-1 cells. (A) Changes in expression of ER stress-related proteins (BiP and CHOP) and G0/G1 cell cycle-related proteins (p38 MAPK, p21, STAT3, cyclin D1, CDK2 and CDK4) after sequential treatment with bortezomib (10 nM) and celecoxib (40 μ M) in TC-1 cells. (B) After incubation with combined sequential treatment, the cell cycle profile was analyzed by PI staining and FACS analysis. BTZ, bortezomib; CLC, celecoxib.

and celecoxib may be related to cell cycle regulation as well as ER stress.

Sequential treatment with bortezomib and celecoxib enhances TC-1 cell apoptosis via activation of p38 MAPK and inhibition of the cell cycle. p-p38 MAPK activation has been reported to mediate cell cycle arrest via an increase in p21 and p27 production and a decrease in the production of G1-specific cyclins (cyclin A, D1 and D3) and CDK4 and CDK6. Moreover, activation of p-p38 MAPK is associated with caspase activation as well as ER stress-induced apoptosis (21). We found that sequential treatment with bortezomib and celecoxib increased the expression of BiP, CHOP, p21 and phosphorylated p38 MAPK, but not unphosphorylated p38 MAPK, in a time-dependent manner in TC-1 cells (Fig. 4A). Next, we investigated the effects of activated p-p38 MAPK on BiP, CHOP, p21, STAT3, cyclin D1 and CDK2 using SB203580, an inhibitor of p-p38, in the TC-1 cells sequentially treated with bortezomib and celecoxib. When p-p38 was inhibited in these cells, production of STAT3, cyclin D1, CDK2 and anti-apoptotic protein Bcl-2 was restored. In contrast, p21, Bax, cleaved caspase-3 and -9 were downregulated after treatment with SB203580 in these cells. However, SB203580 did not have an effect on BiP and CHOP production in the TC-1 cells sequentially treated with bortezomib and celecoxib (Fig. 5A and B). The rate of apoptosis was also significantly decreased when p-p38 MAPK was inhibited by treatment with SB203580 ($P < 0.001$, BTZ→CLC treatment vs. BTZ→CLC treatment after

exposure to SB203580) (Fig. 5C). These results suggest that sequential treatment with bortezomib and celecoxib may induce apoptosis in TC-1 cells through a p-p38-mediated increase in p21 expression and cell cycle arrest, rather than through ER stress.

Sequential treatment with bortezomib and celecoxib retards tumor growth in TC-1 cell-inoculated mice. Finally, we determined whether sequential treatment with bortezomib and celecoxib inhibits the growth of TC-1 cells *in vivo*. C57BL/6 mice were challenged with an s.c. injection of TC-1 cells (5×10^4 /mouse) 1 week prior to treatment with bortezomib or celecoxib or sequential treatment (Fig. 6A). We observed that the growth of TC-1 tumors sequentially treated with bortezomib and celecoxib was retarded compared to tumors co-treated with bortezomib and celecoxib or sequentially treated with celecoxib and bortezomib ($P < 0.05$, co-treatment or CLC→BTZ treatment vs. BTZ→CLC treatment) (Fig. 6B and C). Therefore, our data suggest that sequential treatment with bortezomib and celecoxib has greater therapeutic anti-tumor effects than each single treatment alone or any other bortezomib and celecoxib combination.

Discussion

The proteasome inhibitor bortezomib is currently used to treat various malignancies (11,22), and the selective COX-2 inhibitor celecoxib has been used widely for chemoprevention (23).

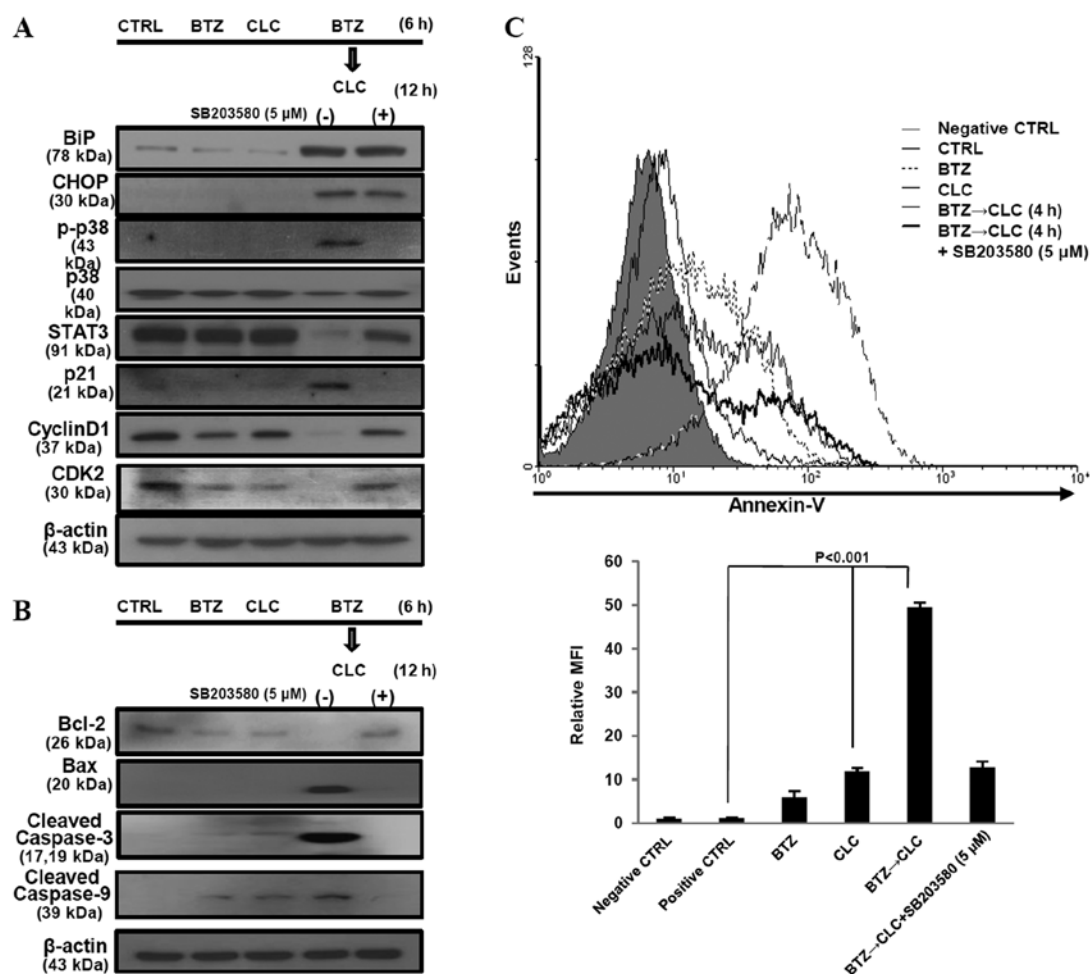


Figure 5. Effect of sequential bortezomib and celecoxib treatment on the induction of apoptosis via p-p38 MAPK-mediated cell cycle arrest in TC-1 cells. (A) The effect of SB203580 (5 μ M), a p-p38 MAPK inhibitor, on the expression of ER stress-mediated proteins and G0/G1 cell cycle-related proteins, as determined by western blotting. (B) The effect of SB203580, a p-p38 MAPK inhibitor, on the expression of apoptosis-related Bcl-2 family proteins and the caspase cascade was detected by western blotting. (C) The reduced rate of apoptosis induced by SB203580 before sequential treatment with bortezomib and celecoxib was measured by Annexin V (A-V) staining and flow cytometric analysis. The mean fluorescence intensity (MFI) of the apoptosis rate is also shown. ($P < 0.001$, difference between sequential bortezomib and celecoxib treatments and sequential bortezomib and celecoxib treatments after exposure to SB203580). BTZ, bortezomib; CLC, celecoxib.

In the present study, we demonstrated that pre-treatment with bortezomib followed by treatment with celecoxib enhanced apoptotic cell death through p38 MAPK-dependent dysregulation of cell cycle-related proteins including cyclin D1, CDK2 and p21, as well as ER stress in the HPV E6 and E7 protein-expressing TC-1 cervical cancer model. The growth of tumors sequentially treated with bortezomib and celecoxib was retarded compared to tumors exposed to a single treatment with bortezomib or celecoxib in mice challenged with TC-1 cells.

Recent studies have shown that induction of apoptotic death by bortezomib is triggered via a c-Jun NH2-terminal kinase (JNK)-dependent mechanism and the accumulation of unfolded or misfolded proteins, which results in ER stress (24,25). Bortezomib is thought to induce cell death by stimulating the expression of CHOP and other pro-apoptotic components (26). Celecoxib has shown potent anticancer activity in various animal tumor models. However, the pro-apoptotic effects of celecoxib are not entirely understood, as normal or upregulated levels of COX-2 have been detected in

various cancers after treatment (27-29). We also observed that TC-1 cells were overall slightly less sensitive to bortezomib or celecoxib at relatively low doses, and detected increased COX-2 levels after co-treatment or sequential treatment with bortezomib and celecoxib. Notably, sequential treatment with bortezomib (10 nM) and celecoxib (40 μ M) effectively induced apoptosis, compared to co-treatment or sequential treatment with celecoxib and bortezomib.

The intrinsic apoptotic pathway involves dysregulation of pro- and anti-apoptotic mitochondrial proteins, and the extrinsic apoptotic pathway is triggered by the binding of tumor necrosis factor family death ligands to their appropriate death receptors (DRs) on the cell surface, followed by caspase-8 cleavage (30). Caspase-8 and Bid, a pro-apoptotic BH3 family member, are then cleaved by the death ligands. Expression of STAT3 is correlated with the production of high levels of anti-apoptotic gene products in cancer (31). Similarly, our results showed that sequential treatment with bortezomib and celecoxib increased the production of intrinsic apoptotic-related molecules, such as Bax and the cleaved form

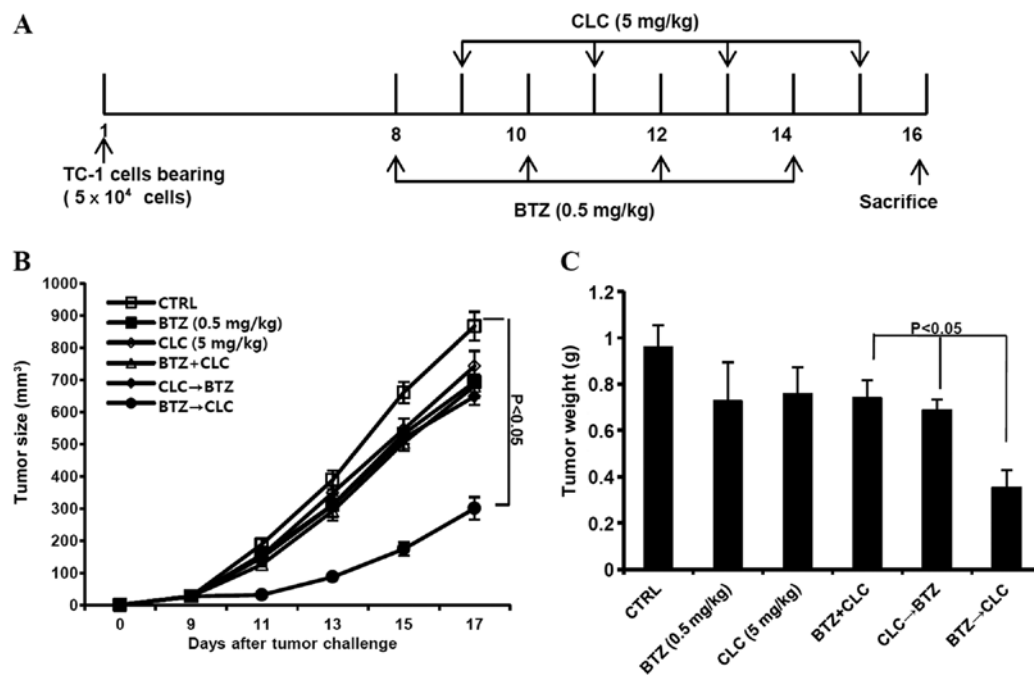


Figure 6. Inhibition of tumor growth by sequential bortezomib and celecoxib treatment *in vivo*. (A) The experimental design. (B and C) C57BL/6 mice (n=5 per group) were challenged with TC-1 cells 1 week prior to initiating sequential bortezomib and celecoxib treatment. Each treatment group was treated via i.p. injection as indicated at day 8 after tumor challenge. Tumor size was measured at intervals of about two days. At the end of the treatment, the mice were sacrificed, and the tumors were collected and weighed ($P < 0.05$, difference between co-treatment or CLC→BTZ and sequential BTZ→CLC treatment). The data are from one representative experiment that was performed three times. BTZ, bortezomib; CLC, celecoxib.

of caspase-9, while it decreased levels of Bcl-2 and did not affect the levels of Bid and caspase-8.

STAT3 plays an important role in tumor progression by promoting cell growth and angiogenesis as well as immune evasion and inflammation. Constitutive activation of STAT3 had been reported in many cancer cell types, including cervical cancer cells (32). Activation of STAT3 has an effect on the expression of cell cycle regulator genes as well as the expression of Bcl-2 and Bax. STAT3 production also affects tumor growth through control of transient G1 to S phase molecules, particularly cyclin D1, which is a G0/G1 cell cycle-related protein (33). It has been reported that cyclin D1-dependent repression of STAT3 may induce apoptosis (34). Transition through G1 into S phase requires activation of cyclin D through the binding of CDK4 (4). In the next phase, cyclin E is activated by CDK2 binding (5), and cell cycle progression is regulated by CKIs such as p21. p21 in particular, when activated by p53, binds to the cyclin-CDK complex and subsequently blocks progression of the cell cycle at the G0/G1 checkpoint (35). Many studies have demonstrated that STAT3 overproduction in tumor cells is related to tumor survival (30), and decreased cyclin D1 and CDK2 levels are known to induce apoptosis in various cancer cells (36,37). A previous study has shown that withaferin A, the active component of the medicinal plant *Withania somnifera*, significantly downregulates the expression of HPV E6 and E7 proteins in CaSki cells and restores the p53 pathway. Withaferin A also decreases the levels of STAT3 (38). Our results showed that STAT3, cyclin D1, and CDK2 levels were decreased in TC-1 cells sequentially treated with bortezomib and celecoxib. Moreover, increased levels of p21 led to cell cycle arrest, despite the lack of a concurrent upregulation in p53 expression. These results indicate that apoptosis in TC-1

cells sequentially treated with bortezomib and celecoxib may be mediated by decreased production of STAT3, cyclin D1 and CDK2 in the G1 phase even in p53-deficient cancer cells.

We detected an increase in p21 protein expression with all combined treatment regimens, which correlates with previous research indicating that p21 is positively regulated by CHOP under conditions of ER stress (39). The ER maintains a balance between protein synthesis and degradation (40). BiP is a major component of the pro-survival pathway in the unfolded protein response (UPR) and plays an important role in the ER in protein folding and assembly (41,42). BiP dissociates from the three ER transmembrane receptors (PERK, ATF-6 and IRE-1 α), leading to their activation and triggering the UPR. The activated PERK pathway induces downstream CHOP expression, triggering apoptosis (43). CHOP is usually produced at very low levels in the cytosol, but is produced at high levels in response to ER stress, leading to the induction of CHOP and its accumulation in the nucleus (39). p38 MAPK has been reported to promote the ER stress response (44) and cell cycle arrest in the G1 phase (45). In the present study, we observed an increased expression of CHOP and phosphorylated p38 (p-p38) after co-treatment with bortezomib and celecoxib in TC-1 cells. Notably, our results also showed that the apoptosis of TC-1 cells sequentially treated with bortezomib and celecoxib was more closely related to cell cycle arrest and p-p38 levels than to increased CHOP expression. SB203580, an inhibitor of p-p38, affects the expression of G0/G1 cell cycle molecules, cyclin D1 and CDK2, but has no effect on CHOP. These results suggest that sequential treatment with bortezomib and celecoxib in p53-degraded cancer cells induces apoptosis through p38 MAPK-dependent cell cycle arrest rather than ER stress.

A previous clinical trial showed that the combination of bortezomib and celecoxib can be used to treat several types of advanced solid tumors without dose-limiting toxicities, although disease progression was reported in most of the patients (46). Sequential treatment with bortezomib and celecoxib demonstrated the most effective antitumor effects *in vivo* study. Unfortunately, tumor growth was not completely inhibited by sequential treatment with bortezomib and celecoxib. Based on these results, further studies are needed to clarify related apoptotic pathways and new target molecules for the development of a combined anticancer chemotherapy.

The present study supports the concept that apoptosis induced by ER stress is enhanced by treatment with a combination of bortezomib and celecoxib. In addition, our results suggest that the sequential administration of bortezomib and celecoxib may be an effective strategy for inducing apoptosis in p53-degraded cancer cells via p38-mediated cell cycle regulation.

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