

Augmentation of the anticancer effects of proteasome inhibitors by combination with sodium butyrate in human colorectal cancer cells

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Abstract. The ubiquitin-proteasome pathway plays a critical role in the degradation of cellular proteins and cell cycle control. As such, dysregulating the degradation of such proteins should have profound effects on tumor growth and should cause cells to undergo apoptosis. Proteasome inhibitors display marked anticancer tendencies in hematopoietic cells; however, this activity is much less potent against solid tumor malignant cells. In addition, it can mediate toxicities such as peripheral neuropathy and can induce primary/secondary resistances. These observations prompted us to investigate whether sodium butyrate (NaB), a natural product for inducing tumor cell apoptosis that exhibits a low degree of clinical toxicity, can enhance the anticancer effects of the proteasome inhibitors MG115, MG132, PSI-1, PSI-2 and epoxomicin in human colorectal cancer. Combinations of the proteasome inhibitors and NaB markedly inhibited the proliferation of human colorectal cancer cells in a dose- and time-dependent manner, particularly compared to single treatment with NaB and the proteasome inhibitors. A combination of the proteasome inhibitors and NaB produced additive and synergistic growth inhibitory effects. The human colorectal cancer cells treated with NaB, proteasome inhibitors, and their combinations exhibited growth arrest at the G₁, G₂-M/S, and G₁ or S/G₂-M phases of the cell cycle, respectively. NaB stimulated the conversion of procaspase-3 into caspase-3 and induced the cleavage of poly(ADP-ribose) polymerase and lamin-B, which are two hallmarks of apoptosis. NaB, the proteasome inhibitor epoxomicin, and their combination stimulated the release of cytochrome-c from mitochondria, which was most likely responsible for the activation of caspase-3. Combined

treatment with NaB and the proteasome inhibitors caused marked induction of apoptosis compared to single treatments of the proteasome inhibitors and NaB; the apoptotic signals were abolished by the inhibitors of caspase-3 activity. The proteasome inhibitors and their combinations with NaB counteracted the suppressive effect that was induced by NaB on the levels of p53. NaB, the proteasome inhibitors and their combinations decreased the levels of Bcl2, but increased the levels of Bax, p21^{Waf1} and p27^{Kip1}. Taken together, these results indicate a synergistic interaction between proteasome inhibitors and sodium butyrate that could present a new approach for the treatment of colorectal cancer.

Introduction

The ubiquitin-proteasome system (UPS) mediates the degradation of polyubiquitinated proteins and represents the main protein degradation pathway in eukaryotic cells (1). It is estimated that over 80% of intracellular proteins are degraded by the proteasome. By catalyzing limited or complete degradation of proteins, the UPS plays a role in many basic cellular processes, such as differentiation (2) proliferation (3), apoptosis (3), gene transcription (4), signal transduction (5), metabolic regulation (6), and immune surveillance (7), among others (8). Thus, the UPS is essential for the development and maintenance of all eukaryotic cells (9), implying that the UPS is also inevitably involved in pathophysiological processes that result in the development of many diseases, including autoimmune, neurodegenerative and rheumatoid diseases, viral infections, cachexia and cancer.

Based on the unique potential for cellular regulation via the UPS, several proteasome inhibitors have been developed. The possibility that proteasome inhibitors could be drug candidates was considered after studies demonstrated that they induced apoptosis in leukemia cell lines (10), including chemotherapy- and radiation-resistant chronic lymphocytic leukemia cells (11). This possibility was bolstered by findings that proteasome inhibitors preferentially induced apoptosis in transformed cells (11). Later, studies documented the efficacy of proteasome inhibition against preclinical models both as a single approach and in chemosensitization and overcoming resistance (12).

Proteasome inhibitors are targeted since they are extremely potent and selective. Due to their effect on the proteolysis of

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a wide array of cellular proteins, however, they share characteristics with general cytotoxic agents, such as vinflunine, straplatin, aurora kinase inhibitors and epothilones (13). In light of this, proteasome inhibitors have several important mechanisms of action, beyond their effects on NF κ B, that have been preclinically validated in cell line models (14). By interfering with the timely degradation of cyclins and other cell cycle regulatory proteins, proteasome inhibitors induce cell cycle arrest. Through their ability to stabilize pro-apoptotic proteins such as p53 and Bax while reducing levels of certain anti-apoptotic proteins such as Bcl2, they also induce a pro-apoptotic state. Other mechanisms include the induction of aggresome formation, endoplasmic reticulum stress and unfolded protein response (15).

Although proteasome inhibitors display marked anticancer effects in hematopoietic cells, they exhibit much less anticancer activity against solid tumor malignant cells, mediate toxicities such as peripheral neuropathy, and induce primary and secondary resistances. These observations prompted us to investigate whether sodium butyrate (NaB), a natural product of apoptosis that exhibits a low degree of clinical toxicity, can enhance the anticancer effect of the proteasome inhibitors MG115, MG132, PSI-1, PSI-2 and epoxomicin in human colorectal carcinoma.

Butyrate is a short 4-carbon fatty acid, one of three found in the mammalian colonic lumen (16). It is produced by anaerobic bacteria that ferment undigested dietary carbohydrates (mainly fiber) and protein (17). Butyrate has been considered to be a biological response modifier; that is, a reagent that has generally reversible effects on gene activation and growth control (18). Butyrate inhibits DNA synthesis, arrests actively proliferating cells of many tissue types (19), and induces differentiation in certain cell types. These observations have led investigators to propose that butyrate, a byproduct of microbial fermentation of fiber in the colonic milieu, may have an antiproliferative, and potentially an antineoplastic, effect on mucosal epithelial cells (20). Due to its growth inhibitory effects and low toxicity, butyrate was tested in this study in order to potentiate the anticancer effects of a group of proteasome inhibitors (MG115, MG132, PSI-1, PSI-2 and epoxomicin) towards solid tumor colorectal cancer cells. The effects of single and combined treatment with proteasome inhibitors and NaB on cell cycle progression, stabilization of p53 protein, induction of p53-inducible gene products, and the triggering of the apoptotic pathway were also investigated.

Materials and methods

Cell culture and reagents. Human colorectal cancer cell lines (SW48, SW1116, and SW837) were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Leibovitz's L-15 medium supplemented with 10% inactivated fetal bovine serum and 2 mM glutamine. The L-15 medium formulation was devised for use in a free gas exchange with atmospheric air. CO₂ is detrimental to cells when using this medium for cultivation. Goat polyclonal anti-human Bax, CPP32 and lamin B, rabbit polyclonal anti-human PARP and mouse monoclonal anti-human Bcl2, cytochrome-c, Bax, p53, p21^{Waf1}, p27^{Kip1} and β -actin were purchased from Santa Cruz Biotechnology

Inc. (Santa Cruz, CA, USA). MG115, MG132, PSI-1, PSI-2 and epoxomicin were purchased from Biomol Research Laboratories, Inc. (Plymouth, Meeting, PA, USA). Sodium butyrate and Ac-DEVD were obtained from the Sigma Chemical Co. (St. Louis, MO), and z-VAD-fmk was obtained from Bachem AG (Bubendorf, Switzerland). Stock solutions of z-VAD-fmk (100 mM) and Ac-DEVD (200 mM) were prepared in methanol and DMSO, respectively.

Time- and dose-dependent antiproliferative effects of proteasome inhibitors and NaB on human colorectal cancer cells. Human colorectal cancer cell lines (SW48, SW1116 and SW837) were plated (27×10^3 cells/well) into 96-well plates and treated with NaB (0.195–12.5 mM), MG115 (0.06–4.0 μ M), MG132 (0.06–4.0 μ M), PSI-1 (0.03–4.0 μ M), PSI-2 (0.03–4.0 μ M) and epoxomicin (7.8–62.5 nM) beginning 24 h after seeding the cells in culture. The final DMSO concentration in proteasome-treated cells was 0.1%; all control cultures were treated with the same 0.1% DMSO to control for any effect that DMSO may exert on the cells. Cell proliferation was determined at various time periods using an MTT assay.

Augmentation of the anticancer effects of proteasome inhibitors by their combination with NaB. The potential of butyrate to sensitize human colorectal cancer cells to proteasome inhibitors was determined as previously described (21). Colorectal cancer cell lines (SW48, SW1116 and SW837) were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non-CO₂ incubator. The cells were then treated with NaB, proteasome inhibitors (MG115, MG132, PSI-1, PSI-2 and epoxomicin), or combinations of NaB and each of the tested proteasome inhibitors. The experiment began 18 h after seeding the cells in culture; control cells were left untreated. Cell proliferation was determined at various time periods (1, 3, and 5 days) by an MTT assay. The concentrations of NaB and the proteasome inhibitors used for single treatment experiment were NaB (1.5–12.5 mM), MG115 (0.25–2.0 μ M), MG132 (0.25–2.0 μ M), PSI-1 (0.013–0.1 μ M), PSI-2 (0.375–3.0 μ M) and epoxomicin (3.0–12.0 nM). Meanwhile, the concentrations of the combined treatments were NaB and MG115 (1.56 mM/0.25 μ M, 3.125 mM/0.5 μ M, 6.25 mM/1.0 μ M, 12.5 mM/2.0 μ M), NaB and MG132 (1.56 mM/0.25 μ M, 3.125 mM/0.5 μ M, 6.25 mM/1.0 μ M and 12.5 mM/2.0 μ M), NaB and PSI-1 (1.56 mM/0.013 μ M, 3.125 mM/0.025 μ M, 6.25 mM/0.05 μ M, 12.5 mM/0.1 μ M), NaB and PSI-2 (1.56 mM/0.375 μ M, 3.125 mM/0.75 μ M, 6.25 mM/1.5 μ M, 12.5 mM/3.0 μ M), and NaB and epoxomicin (1.56 mM/3.0 nM, 3.125 mM/6 nM, 6.25 mM/9.0 nM and 12.5 mM/12.0 nM).

Assessment of the type of interaction between NaB and proteasome inhibitors in human colorectal cancer cells. Human colorectal cancer cell lines (SW48, SW1116 and SW837) were treated with NaB (1.56, 3.125, 6.25, 12.5 mM), MG115 (0.25, 0.5, 1.0, 2.0 μ M), MG132 (0.25, 0.5, 1.0, 2.0 μ M), PSI-1 (0.013, 0.025, 0.05, 0.1 μ M), PSI-2 (0.375, 0.75, 1.5, 3.0 μ M), epoxomicin (3.0, 6.0, 9.0, 12.0 nM), and the same combinations of NaB and proteasome inhibitors that were previously mentioned. The interactions of the tested combinations on

human colorectal cancer cell growth were determined as previously described (22,23), using the following formulas: $SF_{A+B} > (SF_A) \times (SF_B)$, antagonistic; $SF_{A+B} = (SF_A) \times (SF_B)$, additive; $SF_{A+B} < (SF_A) \times (SF_B)$, synergistic; where SF is the surviving fraction; A and B indicate the agent when used alone, and A+B refers to the agents when used in combination.

Cell cycle analysis. The cell cycle phase (G_0 - G_1 , S and G_2 -M) distribution of the human colorectal cancer cells (SW48, SW1116 and SW837) was evaluated by measuring the DNA content of nuclei that were labeled with propidium iodide via flow cytometry (23). The human colorectal cancer cells were plated (5×10^5 /well) into 24-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, the cells were treated with either NaB (3 mM), MG115 (1.0 μ M), MG132 (1.0 μ M), PSI-1 (0.1 μ M), PSI-2 (1.5 μ M), epoxomicin (12.0 nM), or with combinations of NaB and the proteasome inhibitors [NaB/MG115 (3.0 mM/1.0 μ M), NaB/MG132 (3.0 mM/1.0 μ M), NaB/PSI-1 (3.0 mM/0.1 μ M), PSI-2 (3.0 mM/1.5 μ M) and NaB/epoxomicin (3.0 mM/12.0 nM)] for 72 h. The tested cells were collected via trypsinization then washed with cold phosphate-buffered saline and counted with a cell counter. A sample of 5×10^5 cells/ml was processed using a DNA-Prep kit (Beckman and Coulter, FL, USA) and a DNA-Prep Epics workstation (Beckman and Coulter). During this process, the cell sample was treated with a cell membrane-premeabilizing agent, followed by the propidium iodide and RNase enzyme. The sample was then incubated at room temperature for at least 15 min before being analyzed by aligned flow cytometry (Epics XL, Beckman and Coulter). The percentage of cells in various cell cycle phases was calculated using the Phoenix statistical software package and advanced DNA cell cycle software (Phoenix Flow Systems, San Diego, CA, USA).

Acridine orange (AO) staining. Morphological evidence of apoptosis was obtained through the use of AO/ethidium bromide (EB) staining. After removal of the incubation medium, cells were rinsed and treated with an AO/EB solution (100 μ g/ml PBS of each dye), then examined by fluorescence microscopy and photographed. Viable cells appeared green with intact nuclei, while nonviable cells exhibited bright orange chromatin. Apoptosis was marked by the appearance of cell shrinkage with condensation and the fragmentation of nuclei; in addition, apoptotic cells were easily distinguished from necrotic cells since the latter appeared orange and had a normal nuclear structure.

DNA fragmentation analysis. A DNA fragmentation assay was also performed (24). Human colorectal cancer cells were plated (5×10^5 /well) into 24-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, the cells were treated for 72 h with NaB (3.0 mM), MG115 (1.0 μ M), MG132 (1.0 μ M), PSI-1 (0.1 μ M), PSI-2 (1.5 μ M), epoxomicin (12.0 nM), or the combinations of NaB and proteasome inhibitors that were previously mentioned. The cell pellets of SW48, SW1116 and SW837 cells were then lysed with 100 μ M of hypotonic buffer [10 mM Tris, (pH 8.0), 20 mM EDTA containing 0.5% Triton X-100] for 30 min at 4°C. After lysis, the intact chromatin (pellet) was separated from the DNA fragment (supernatant) by centrifugation for 15 min at 12,000 x g. The

supernatants containing fragmented DNA were precipitated overnight with 0.5 M NaCl and 50% isopropyl alcohol at -20°C. Pellets were recovered by centrifugation at 12,000 x g for 10 min, air dried, then re-suspended in 30 μ l of TE-buffer supplemented with 1 mg/ml RNase I at 37°C for 30 min and again with 2 mg/ml of proteinase K for another 1 h. A DNA sample was supplemented with 3 μ l of sample buffer (0.25% Bromophenol blue, 30% glyceric acid) and electrophoretically separated on a 1.0% agarose gel containing 0.1 μ g/ml ethidium bromide. DNA fragments were then visualized using ultra-violet transillumination.

Western blot analysis of cell cycle and apoptosis regulatory protein expression levels in human colorectal cancer cells treated with proteasome inhibitors, NaB, and their combinations. The expression levels of cell cycle and apoptosis regulatory proteins were determined as follows (24): $1-2 \times 10^6$ human colorectal cells were treated with NaB (3.0 mM), MG115 (1.0 μ M), MG132 (1.0 μ M), PSI-1 (0.1 μ M), PSI-2 (1.5 μ M), epoxomicin (12.0 nM), or a combination of NaB and each of the proteasome inhibitors. The samples were washed twice with ice-cold phosphate-buffered saline then lysed for 30 min with a solution composed of 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS (pH 7.4), then sonicated three times for 10 sec. The following protease inhibitors were added: 25 μ g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF, 25 μ g/ml leupeptin and 0.2 mM sodium PPI. Cell lysates were centrifuged at 15,000 x g for 20 min at 4°C; equivalent amounts of protein (60 μ g) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose for detection with antibodies. The primary antibodies used were: goat polyclonal anti-human Bax (1:500), CPP32 (1:1000), lamin B (1:1000), rabbit polyclonal anti-human PARP (1:2000), mouse monoclonal anti-human Bcl2 (1:1000), p53 (1:1000); p21^{Waf1} (1:500), p27^{Kip1} (1:500) and β -actin (1:1000). Visualization was performed using nitroblue tetrazolium and bromochloroindoyl-phosphate. The specificities of the antibodies used in this study were examined by testing their reactivities with unrelated antigens, such as bovine serum albumin (BSA). The blots were scanned, and the band intensities were determined using Master™ Total Lab Software v. 2.0 (Amersham Biosciences, UK). Expression of p53, p21^{Waf1}, p27^{Kip1}, and Bax and Bcl2 genes was calculated by setting the β -actin protein at 100% and calculating the expression of these genes in relation to this internal standard.

In vitro assay of caspase-3 activity. The catalytic activity of caspase-3 was measured using a colorimetric assay according to the manufacturer's (Calbiochem) instructions. The assay is based on spectrophotometric detection of the chromophore *p*-nitroanilide following cleavage from the labeled substrate of the enzyme DEVD-*p*-nitroanilide. Human colorectal cancer cells were treated with NaB (3.0 mM), epoxomicin (12 nM), or a combination of NaB (3.0 mM) and epoxomicin (12 nM) for 24 h, and then harvested by centrifugation at 1,000 x g for 10 min. The cells were then washed twice with ice-cold PBS. The cell pellet was re-suspended in 100 μ l of extraction buffer that contained 50 mM HEPES, 1 mM DDT, 0.1 mM EDTA, 10% glycerol and 0.1% CHAPS at a pH of 7.4. After 10 min

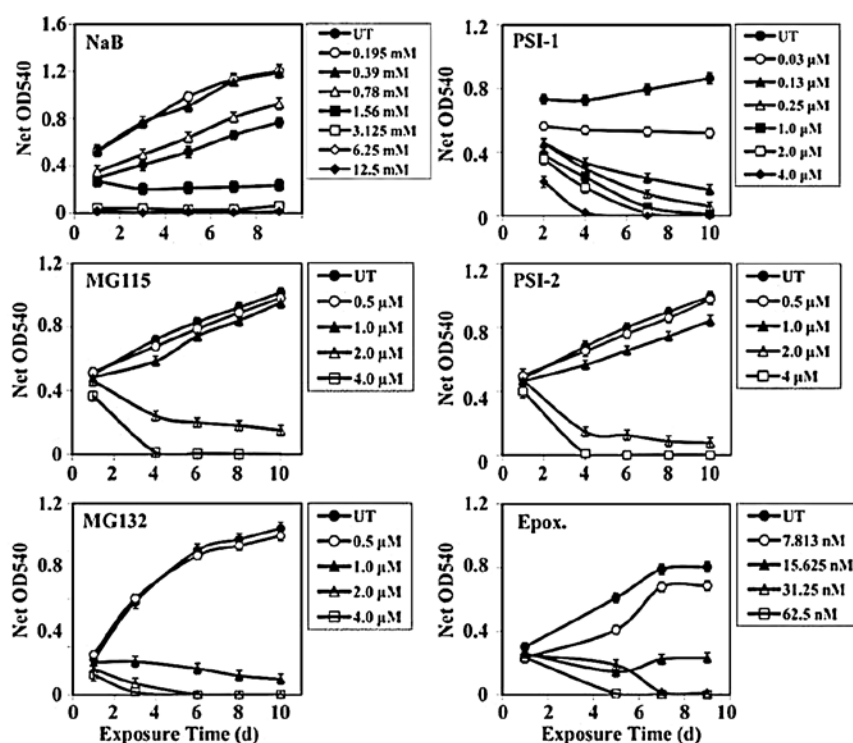


Figure 1. Time- and dose-dependent antiproliferative effects of proteasome inhibitors and NaB on human colorectal cancer cells. Human colorectal cancer SW837 cells were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, the cells were treated with various concentrations of NaB (0–12.5 mM), MG115 (0.06–4.0 μM), MG132 (0.06–4.0 μM), PSI-1 (0.03–4.0 μM), PSI-2 (0.03–4.0 μM) and epoxomicin (7.8–62.5 nM) for different periods of time. The cell growth was measured by an MTT assay.

of incubation on ice, the cells were centrifuged at $10,000 \times g$ at 4°C for 10 min, and the supernatants were removed and stored at -70°C . Proteolytic reactions were carried out in an assay buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1 CHAPS, 10 mM dithiothreitol, 0.1 mM EDTA and 10% glycerol] containing 20 μg of cytosolic protein extracts, and incubated at 37°C for 10 min. Thereafter, a freshly prepared colorimetric substrate was added to the mixtures, and the samples were mixed and recorded according to the manufacturer's instructions. Cells without drug treatment were used as controls. Enzyme activity was calculated as pmol/min, according to the formula provided by the manufacturer.

Inhibition of caspase-3 activity. Inhibition of caspases with DEVD cleavage activity was achieved using the inhibitors Ac-DEVD (500 μM) and z-VAD-fmk (100 μM) that were administered 1 h before the addition of either 3 mM NaB or 12 nM epoxomicin. Twenty-four hours after the addition of NaB or epoxomicin, the cells were harvested and analyzed for caspase-3 activity.

In vitro assay of cytochrome-c efflux from the mitochondria. Subcellular fractions were prepared as described by Yang *et al* (25). The human colorectal cells ($1\text{--}2 \times 10^6$) were treated with NaB (3.0 mM), epoxomicin (12.0 nM), or a combination of NaB (3.0 mM) and epoxomicin (12.0 nM). The cells were washed twice in PBS and re-suspended in a lysis buffer [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EGTA, 1 mM EDTA, 1 mM DTT and 0.1 mM phenylmethylsulfonyl fluoride] containing 250 mM sucrose. The

cells were homogenized with 20 strokes of Teflon homogenizers, and the homogenates were centrifuged at $750 \times g$ for 10 min at 4°C ; the resulting supernatants were centrifuged at $10,000 \times g$ for 30 min. The mitochondrial pellets were re-suspended in the same buffer. The cytosolic fraction was obtained after centrifugation at $100,000 \times g$ for 1 h at 4°C . For the immunoblotting analysis, equal amounts of protein (50 μg) were subjected to a 10% SDS-PAGE. After being transferred to a nitrocellulose filter, the filter was probed using the mouse monoclonal anti-human cytochrome-c antibody (1:1000).

Statistical analysis. The data were recorded as the mean \pm SE and were analyzed by SPSS (version 10, SPSS Inc.). One-way analysis of variance was performed using the ANOVA procedure. Significant differences between the means of percentage growth inhibition were determined by least significant difference (LSD), and differences were considered statistically significant at $P < 0.05$.

Results

Time- and dose-dependent antiproliferative effect of proteasome inhibitors and NaB on human colorectal cancer cells. Human colorectal cancer SW837 cells treated with various concentrations of NaB or the proteasome inhibitors, MG115, MG132, PSI-1, PSI-2 and epoxomicin, showed marked time- and dose-dependent growth inhibition (Fig. 1). Treatment of SW837 cells with low concentrations of NaB (0.195–0.78 mM) had no effect on their growth. But when SW837 cells were treated with 1.56 mM NaB, higher growth inhibition (mean

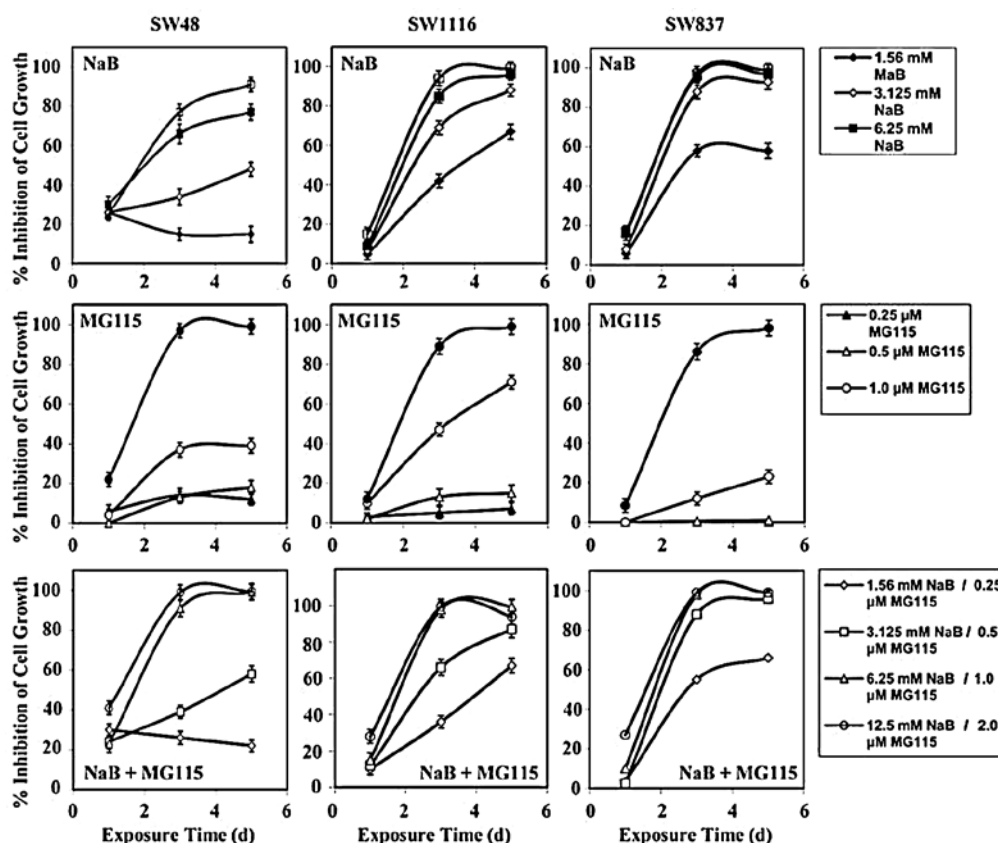


Figure 2. Enhancement of the anticancer effects of proteasome inhibitor MG115 with NaB on human colorectal cancer cells. Human colorectal cancer cells, SW48, SW1116 and SW837 were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (1.56–12.5 mM) and MG115 (0.25–2.0 μM) or with the combinations of NaB/MG115 (1.56 mM/0.25 μM ; 3.125 mM/0.5 μM ; 6.25 mM/1.0 μM ; 12.5 mM/2.0 μM), starting 18 h after seeding the cells in culture. Control cells were left untreated or treated with vehicle (DMSO at a final concentration 0.1%).

percentage growth inhibition, $51.2 \pm 10\%$) was observed. Marked growth inhibition (mean, $96 \pm 0.95\%$) was noted when the SW837 cells were treated with even higher concentrations of NaB (3.125–12.5 mM) (Fig. 1).

The growth of SW837 cells was slightly affected (mean, $10 \pm 2\%$) after treatment with proteasome inhibitor MG115 (0.5–1.0 μM). Treatment of SW837 cells with higher concentrations of MG115 (2.0–4.0 μM) produced $17.5 \pm 0.9\%$ growth inhibition after 24 h; however, a much higher growth inhibition ($87 \pm 5\%$) was noticed after 96–240 h of treatment with MG115 (2.0–4.0 μM) (Fig. 1).

The growth of SW837 cells was slightly affected (mean, $26 \pm 9\%$) after 24 h of treatment with 1.0–4.0 μM of MG132. A marked growth inhibitory effect (mean, $92 \pm 3\%$) was observed with longer periods of treatment (72–240 h) with the same range of MG132 concentrations (1.0–4.0 μM) (Fig. 1).

Treatment of SW837 cells with 0.03 μM PSI-1 inhibited their growth by $31 \pm 4\%$. Increasing concentrations of PSI-1 (0.13–0.25 μM) produced a higher growth inhibition ($65 \pm 7\%$). An even higher growth inhibition (mean, $83 \pm 6\%$) was obtained with higher concentrations of PSI-1 (1.0–4.0 μM) (Fig. 1).

The growth of SW837 cells was slightly affected (mean, $9 \pm 2\%$) after treatment with PSI-2 at 0.5–1.0 μM . However, the growth of SW837 cells was dramatically inhibited (mean, $93 \pm 3\%$) after 96–240 h of treatment with higher concentrations of PSI-2 (2.0–4.0 μM) (Fig. 1).

Treatment of SW837 cells with a low concentration of epoxomicin (7.8 nM) slightly affected (mean, $21 \pm 4\%$) their growth throughout 24–240 h of treatment. A similar degree of inhibition (mean, $22.8 \pm 3\%$) was obtained when SW837 cells were treated with higher concentrations (15.6–250 nM) for 24 h. However, a marked growth inhibition (mean, $92 \pm 3\%$) was obtained when SW837 cells were exposed to 15.6–250 nM of epoxomicin for 120–216 h (Fig. 1).

Augmentation of the antimitogenic effect of proteasome inhibitors by combination with NaB in human colorectal cancer cells

Augmentation of the anticancer effect of proteasome inhibitor MG115 by combination with NaB in colorectal cancer cells.

Treatment of human colorectal cancer SW48 cells with NaB, MG115, and their combination inhibited their growth by 26.8 ± 2 , 8.0 ± 0.97 and $29.75 \pm 0.8\%$, respectively, after 24 h of treatment (Fig. 2). The differences in growth inhibition between the combined treatment and single treatment with NaB ($P=0.899$) and MG115 ($P=0.314$) were statistically non-significant.

Treatment of SW48 with NaB, MG115, and their combination inhibited their growth by 47.5 ± 3 , 38 ± 4 and $63 \pm 3.8\%$, respectively, after 72 h of treatment. Although the combined treatment netted a higher growth inhibition than single treat-

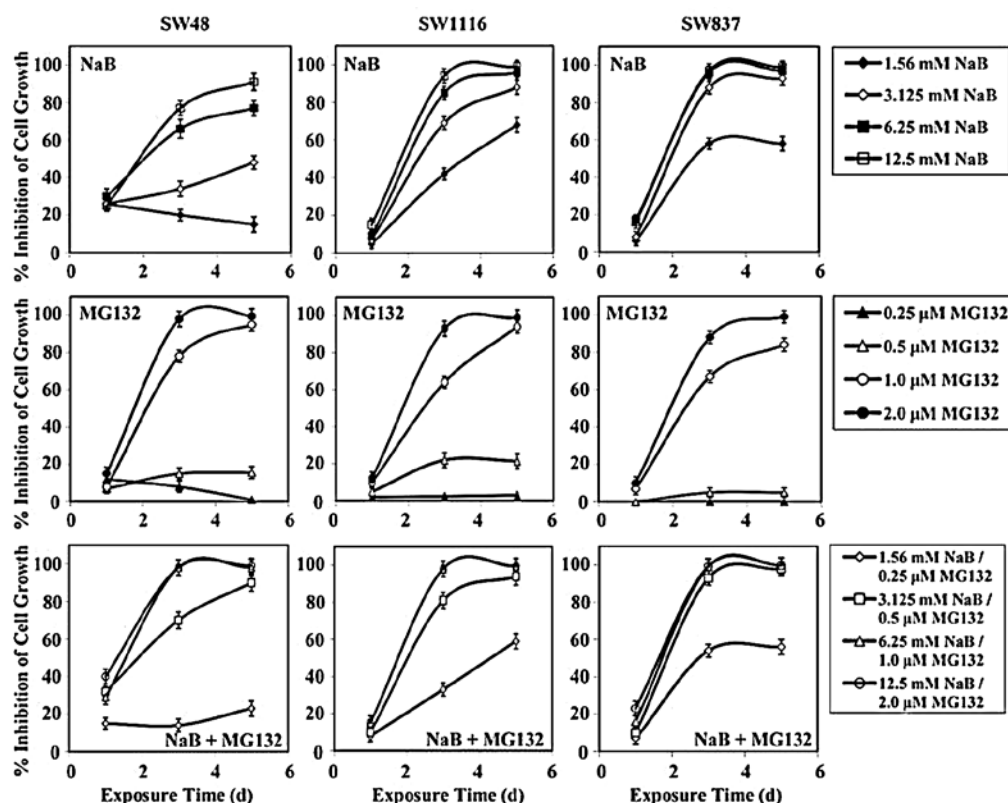


Figure 3. Enhancement of the anticancer effects of proteasome inhibitor MG132 with NaB on human colorectal cancer cells. Human colorectal cancer cells, SW48, SW1116 and SW837, were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (1.56–12.5 mM) and MG132 (0.25–2.0 μM) or with the combinations of NaB/MG132 (1.56 mM/0.25 μM ; 3.125 mM/0.5 μM ; 6.25 mM/1.0 μM ; 12.5 mM/2.0 μM), starting 18 h after seeding the cells in culture. Control cells were left untreated or treated with vehicle (DMSO at a final concentration 0.1%).

ment with NaB ($P=0.418$) or MG115 ($P=0.303$), the differences in growth inhibition were non-significant. The combined treatment of SW48 with NaB and MG115 netted a higher growth inhibition (mean percentage growth inhibition, $70 \pm 3.1\%$) than single treatment with NaB (mean percentage growth inhibition, $58 \pm 5\%$) and MG115 (mean percentage growth inhibition, $42 \pm 3\%$) after 120 h of treatment. The differences in growth inhibition of SW48 between combined treatment and single treatment with NaB ($P=0.584$) and MG115 ($P=0.206$) were statistically non-significant.

Treatment of SW1116 cells with NaB, MG115, and their combination had very little effect on their growth after 24 h of treatment (Fig. 2). However, higher growth inhibition was observed when SW1116 cells were treated for a longer period (72 h) with NaB (mean, $72.5 \pm 2.3\%$), MG115 (mean, $39 \pm 4\%$), and the combination of NaB and MG115 (mean, $75 \pm 3\%$). The combined treatment produced a significant growth inhibition effect ($P=0.05$) on SW1116, as compared to single treatment with MG115. However, the difference in growth inhibition of SW1116 exerted by combined treatment, as compared to that produced by NaB, was statistically non-significant ($P=0.9$). Treatment of SW1116 with the combination of NaB and MG115 for an even longer period (120 h) produced marked growth inhibition (mean, $87 \pm 14\%$), as compared to that produced by treatment with MG115 (mean, $43 \pm 5\%$). The difference between the growth inhibition produced by combined treatment and MG115 was statistically very significant ($P=0.024$). Treatment of SW1116 with NaB for 120 h gave comparable

growth inhibition (mean, $87.7 \pm 14\%$) to that produced by combined treatment for 120 h (Fig. 2).

Treatment of SW837 cells with NaB, MG115, and their combination for 24 h produced very small growth inhibitory effects (mean, $6 \pm 0.4\%$) (Fig. 2). However, after 72 h of treatment, NaB markedly inhibited the growth of SW837 cells (mean, $84 \pm 2\%$). On the other hand, MG115 produced a moderate growth inhibition (mean, $31 \pm 4\%$). The growth inhibition of SW837 cells (mean, $85 \pm 2\%$) produced by the combination of NaB and MG115 was comparable to that produced by NaB. Inhibition of SW837 cells by the combination of NaB and MG115 was statistically very significant ($P=0.004$), as compared to the inhibition produced by single treatment with MG115 for 72 h. Treatment of SW837 cells with NaB for 120 h produced growth inhibition (mean, $90 \pm 2\%$) comparable to that produced by single treatment with NaB (mean, $87 \pm 2\%$). In contrast, MG115 produced moderate growth inhibition (mean, $31 \pm 5\%$) after 120 h of treatment. Inhibition of SW837 cell growth produced by the combination of NaB and MG115 was statistically very significant ($P=0.002$), as compared to that produced by MG115 after 120 h of treatment.

Augmentation of the antimetogenic effect of proteasome inhibitor MG132 by combination with NaB in colorectal cancer cells. Treatment of human colorectal cancer SW48 cells for 24 h with NaB, MG132, and their combination inhibited cell growth by 27 ± 2 , 11 ± 3 and $29 \pm 56\%$, respectively (Fig. 3).

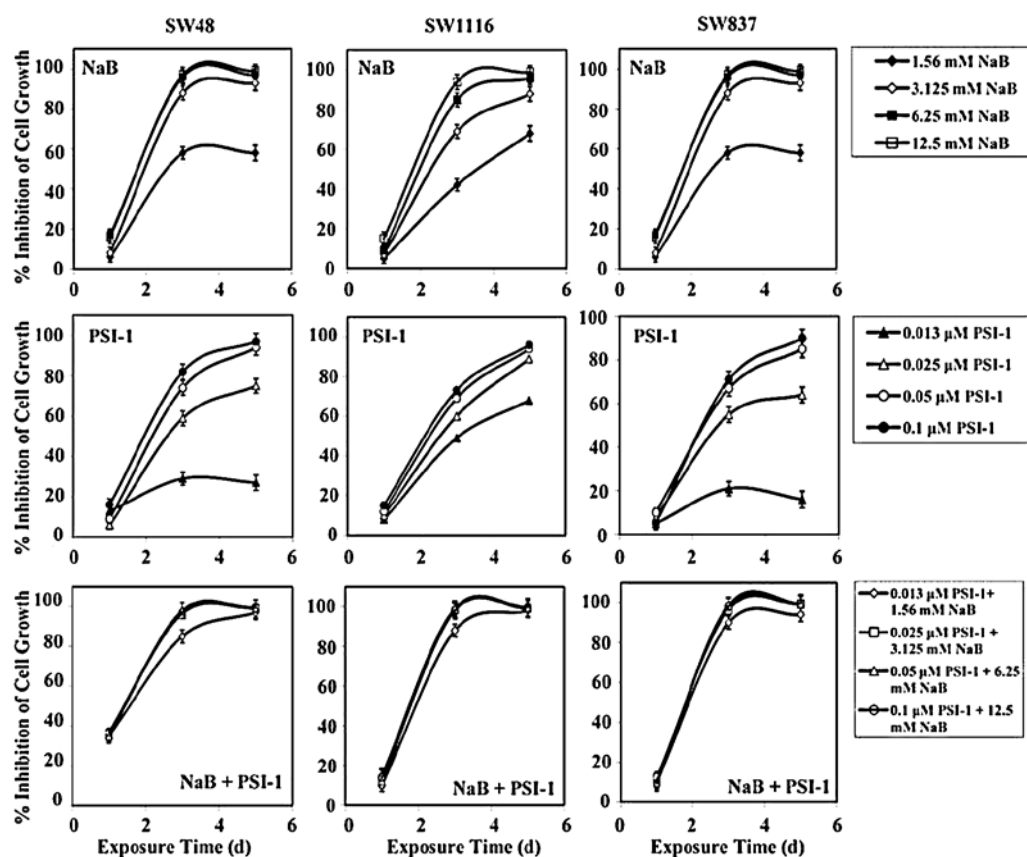


Figure 4. Enhancement of the anticancer effects of proteasome inhibitor-1 with NaB on human colorectal cancer cells. Human colorectal cancer cells, SW48, SW1116 and SW837, were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (1.56–12.5 mM) and PSI-1 (0.013–0.1 μM) or with the combinations of NaB/PSI-1 (1.56 mM/0.013 μM ; 3.125 mM/0.025 μM ; 6.25 mM/0.05 μM ; 12.5 mM/0.1 μM), starting 18 h after seeding the cells in culture. Control cells were left untreated or treated with vehicle (DMSO at a final concentration 0.1%).

The differences in the growth inhibition produced by the combination of NaB and MG132 compared to that produced by NaB ($P=0.923$) or MG132 ($P=0.428$) were statistically non-significant. After 72 h of treatment with NaB, MG132, or their combination, the growth of SW48 cells was inhibited by 48 ± 3 , 50 ± 5 and $70 \pm 4\%$, respectively (Fig. 3). Although the combination of NaB and MG132 produced a higher growth inhibition than single treatment with NaB ($P=0.34$) and MG132 ($P=0.39$), the differences were non-significant. Additionally, combined treatment produced a higher growth inhibition (mean, $78 \pm 4\%$), as compared to single treatment of NaB (mean, $58 \pm 3\%$) and MG132 (mean, $55 \pm 5\%$) after 120 h of treatment. However, the differences in growth inhibition between combined treatment and NaB ($P=0.4$) and MG132 ($P=0.33$) were statistically non-significant after 120 h of treatment.

Treatment of SW1116 cells with NaB, MG132, and their combination had very little effect on the growth of SW1116 cells after 24 h of treatment (Fig. 3). However, after 72 h of treatment, the combination of NaB and MG132 produced a higher growth inhibition (mean, $78 \pm 3\%$) than single treatment with MG132 (mean, $46 \pm 4\%$). This difference in growth inhibition was statistically non-significant ($P=0.1$). NaB had similar growth inhibition (mean, $73 \pm 2\%$) to that produced by combined treatment (Fig. 3). After 120 h of treatment, the combination of NaB and MG132 produced a higher growth inhibition (mean, $88 \pm 2\%$) than single treatment with MG132

(mean, $54 \pm 5\%$). The difference in SW1116 growth inhibition was statistically significant ($P=0.05$). NaB produced comparable growth inhibition (mean, $88 \pm 4\%$) to that produced by combined treatment (Fig. 3).

The growth of human colorectal cancer cells SW837 was slightly affected when treated with NaB, MG132, or their combination for 24 h (Fig. 3). However, the growth of SW837 cells was markedly affected by the combination of NaB and MG132 (mean, $86 \pm 2\%$) after 72 h of treatment. NaB produced growth inhibition (mean, $84 \pm 2\%$) similar to that of the combined treatment. However, much less growth inhibition was observed when SW837 cells were treated for 72 h with MG132 (mean, $40 \pm 4\%$). The difference in the growth inhibition of SW837 after treatment with the combination of NaB and MG132 and single treatment with MG132 was statistically significant ($P=0.022$). Treatment of SW837 cells with the combination of NaB and MG132 for 120 h produced a marked growth inhibition (mean, $89 \pm 2\%$). NaB produced growth inhibition of SW837 (mean, $87 \pm 2\%$) similar to that produced by the combination of NaB and MG132 after 120 h of treatment. Much less growth inhibition was obtained after treatment with MG132 (mean, $47 \pm 5\%$) for 120 h. The difference in the growth inhibition of SW837 cells produced by the combination of NaB and MG132 was statistically significant ($P=0.037$), when compared to single treatment with MG132.

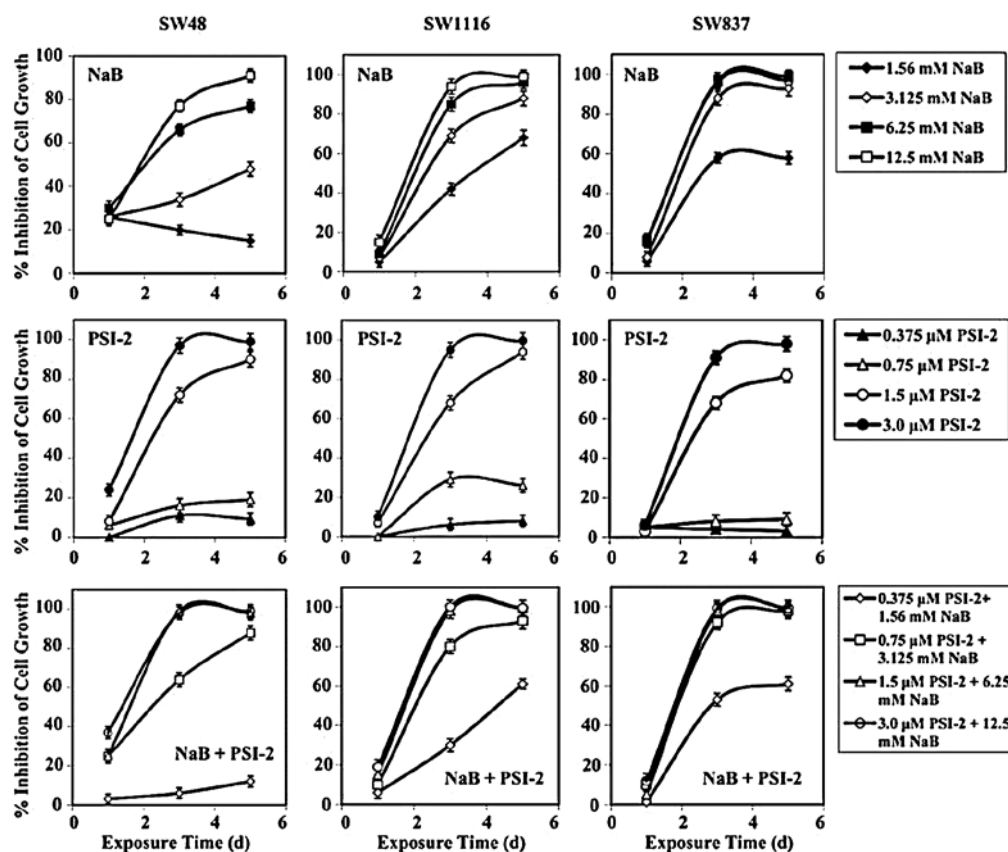


Figure 5. Enhancement of the anticancer effects of proteasome inhibitor-2 with NaB on human colorectal cancer cells. Human colorectal cancer cells, SW48, SW1116 and SW837, were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (1.56–12.5 mM) and PSI-2 (0.375–3.0 μM) or with the combinations of NaB/PSI-2 (1.56 mM/0.375 μM ; 3.125 mM/0.75 μM ; 6.25 mM/1.5 μM ; 12.5 mM/3.0 μM), starting 18 h after seeding the cells in culture. Control cells were left untreated or treated with vehicle (DMSO at a final concentration 0.1%).

Augmentation of the antimitogenic effect of proteasome inhibitor PSI-1 by combination with NaB. Treatment of SW48 cells with the combination of NaB and PSI-1 for 24 h produced a growth inhibition of $36 \pm 0.8\%$, while single treatment with NaB and PSI-1 produced a growth inhibition of 27 ± 2 and $11 \pm 4\%$, respectively. After 72 h of treatment, the combination of NaB and PSI-1 produced a greater inhibition of SW48 growth (mean, $94 \pm 6\%$) than single treatment with NaB (mean, $48 \pm 3\%$; $P=0.003$) and PSI-1 (mean, $61 \pm 2\%$; $P=0.03$). Treatment of SW48 with the combination of NaB and PSI-1 for 120 h produced a marked growth inhibition (mean, $99 \pm 1\%$), as compared to single treatment with NaB (mean, $58 \pm 3\%$; $P=0.008$) and PSI-1 (mean, $73 \pm 3\%$; $P=0.085$) (Fig. 4).

Treatment of human colorectal cancer cells SW1116 with NaB, PSI-1, and their combination for 24 h had no effect on their proliferation. However, after 72 h of treatment, SW1116 cell growth was markedly inhibited (mean, $96 \pm 7\%$), as compared to single treatment with NaB (mean, $73 \pm 2\%$) and PSI-1 (mean, $63 \pm 1\%$). The differences in the growth inhibition produced by the combination of NaB and PSI-1 after 72 h of treatment and NaB ($P=0.005$) or PSI-1 ($P=0.001$) were statistically very significant. After 120 h, the combined treatment produced a slightly higher growth inhibition (mean, $99 \pm 1.0\%$) than NaB (mean, $88 \pm 4\%$) or PSI-1 (mean, $87 \pm 3\%$) (Fig. 4).

The growth of human colorectal cancer SW837 cells was slightly affected after 24 h of treatment with NaB (mean, $12 \pm 1.0\%$), PSI-1 (mean, $1.3 \pm 0.4\%$), and their combination (mean, $3.0 \pm 0.4\%$) (Fig. 4). The combination of NaB and PSI-1 produced a marked growth inhibition of SW837 (mean, $96.0 \pm 4.0\%$), as compared to that produced by PSI-1 (mean, $54.0 \pm 2.0\%$) after 72 h of treatment. The difference in SW837 growth inhibition produced by combined treatment and single treatment with PSI-1 was statistically very significant ($P=0.001$). The combined treatment produced a higher growth inhibition of SW837 than NaB (mean, 84.0 ± 2.0) after 72 h of treatment. However, the difference in SW837 growth inhibition between these treatments was statistically non-significant ($P=0.322$). Similar results were obtained when SW837 cells were treated for 120 h. The combined treatment produced greater significant growth inhibition (mean, $98.0 \pm 3.0\%$), as compared to that produced by PSI-1 (mean, $64.0 \pm 3.0\%$, $P=0.006$). Additionally, the combined treatment produced higher SW837 growth inhibition, as compared to that produced by single treatment with NaB (mean, $87.0 \pm 2.0\%$). However, the difference in SW837 growth inhibition between these treatments was statistically non-significant ($P=0.322$) (Fig. 4).

Augmentation of the antimitogenic effect of PSI-2 by combination with NaB. Treatment of human colorectal

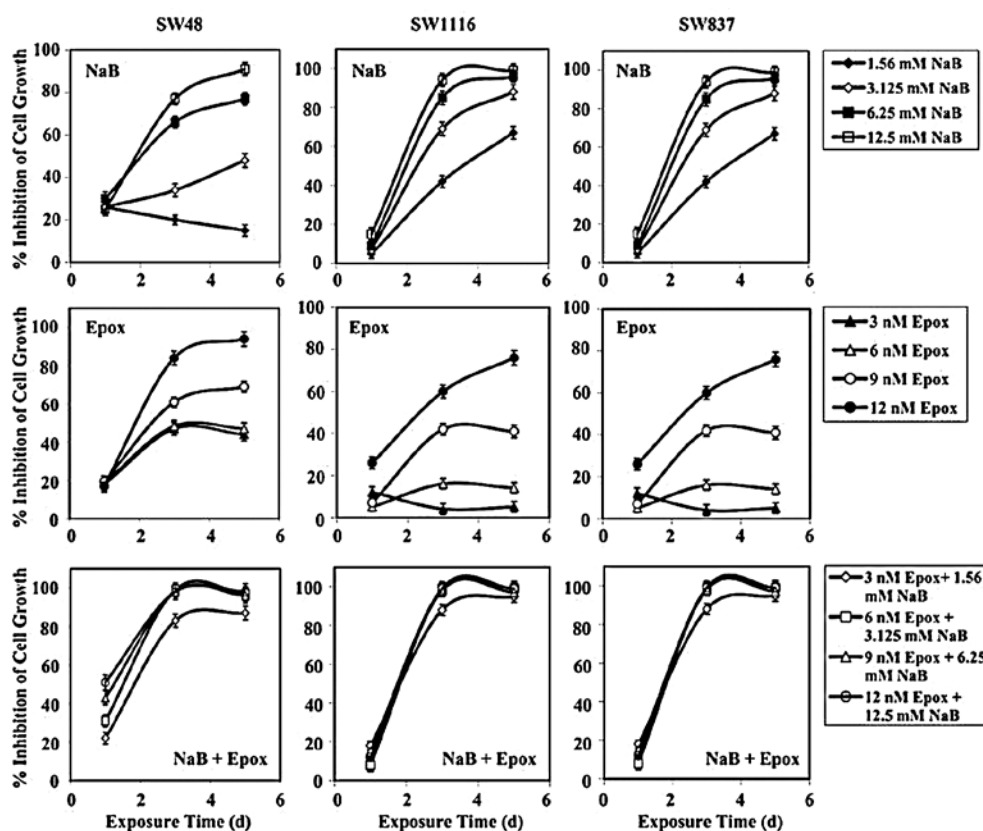


Figure 6. Enhancement of the anticancer effects of proteasome inhibitor epoxomicin with NaB on human colorectal cancer cells. Human colorectal cancer cells, SW48, SW1116 and SW837, were plated (27×10^3 cells/well) into 96-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (1.56–12.5 mM) and epoxomicin (3.0–12.0 nM) or with the combinations of NaB/epoxomicin (1.56 mM/3.0 nM; 3.125 mM/6 nM; 6.25 mM/9.0 nM; 12.5 mM/12.0 nM), starting 18 h after seeding the cells in culture. Control cells were left untreated or treated with vehicle (DMSO at a final concentration 0.1%).

cancer SW48 cells with the combination of NaB and PSI-2 produced comparable growth inhibition (mean, $31 \pm 5\%$) to that produced by NaB (mean, $27 \pm 2\%$) after 24 h of treatment. The combined treatment produced higher growth inhibition than that produced by PSI-2 (mean, $10 \pm 1\%$) after 24 h of treatment. However, this difference in growth inhibition was statistically non-significant ($P=0.376$) (Fig. 5). After 72 h of treatment, the combination of NaB and PSI-2 produced higher SW48 growth inhibition (mean, $68 \pm 4\%$) than that produced by NaB (mean, $47 \pm 3\%$) or PSI-2 (mean, $49 \pm 4\%$). The differences in the SW48 growth inhibition produced by combined treatment and single treatment with NaB ($P=0.381$) and PSI-2 ($P=0.416$) were statistically non-significant (Fig. 5). Similar results were obtained when SW48 cells were treated with NaB and PSI-2 (mean, $73 \pm 4\%$), NaB (mean, $58 \pm 3\%$), or PSI-2 (mean, $54 \pm 5\%$) for 72 h (Fig. 5). The differences in SW48 growth inhibition produced by combined treatment and NaB ($P=0.525$) or PSI-2 ($P=0.434$) were statistically non-significant.

Human colorectal cancer cells SW1116 were not affected by exposure to NaB, PSI-2, or their combination for 24 h (Fig. 5). The combination of NaB and PSI-2 produced a marked growth inhibition of SW1116 cells after 72 h of treatment (mean, $77 \pm 3\%$) that was comparable to the growth inhibition produced by single treatment with NaB (mean, $73 \pm 2\%$). The combined treatment produced higher but statistically non-significant growth inhibition of SW1116 than that

produced by treatment with PSI-2 (mean, $50 \pm 4\%$; $P=0.155$) (Fig. 5). The growth of SW1116 cells was markedly inhibited when the cells were exposed for 120 h to the combination of NaB and PSI-2 (mean, $89 \pm 2\%$) or NaB (mean, $88 \pm 13\%$). Exposure of SW1116 cells to PSI-2 for 120 h inhibited their growth (mean, $55 \pm 5\%$). Although the combination of NaB and PSI-2 markedly inhibited the growth of SW1116, as compared to single treatment with PSI-2, the difference in growth inhibition was statistically non-significant ($P=0.083$).

Treatment of SW837 cells with NaB, PSI-2 or their combination for 24 h exerted very little effect (2–12%) on their growth (Fig. 5). However, after 72 h, single treatment with NaB and the combination of NaB and PSI-2 produced a marked comparable growth inhibition of 84 ± 2 and $85 \pm 2\%$, respectively, of the SW837 cells (Fig. 5). Moreover, exposure of SW837 cells to PSI-2 for 72 h produced much lower growth inhibition (mean, $42 \pm 4\%$) than that produced by NaB ($P=0.029$) or the combination of NaB and PSI-2 ($P=0.026$). Exposure of SW837 cells to NaB, PSI-2, or their combination for 120 h produced a similar growth inhibition to that observed when these cells were treated for 72 h. NaB and the combination of NaB and PSI-2 produced growth inhibition of 87 ± 2 and $89 \pm 2\%$, respectively (Fig. 5). Meanwhile, single treatment with PSI-2 produced a growth inhibition of $47 \pm 5\%$ after 120 h of treatment. The differences in SW837 growth inhibition produced by PSI-2 and NaB or the combination of

NaB and PSI-2 were statistically significant, as indicated from P-values of 0.041 and 0.031, respectively.

Augmentation of the antimitogenic effect of epoxomicin by combination with NaB. Treatment of SW48 cells with the combination of NaB and the proteasome inhibitor epoxomicin produced a higher growth inhibition (mean, $37 \pm 8\%$) than that produced by NaB (mean, $27 \pm 2\%$; $P=0.542$) or epoxomicin (mean, $17 \pm 3\%$; $P=0.134$) after 24 h of treatment (Fig. 6). However, the differences in growth inhibition were statistically non-significant (Fig. 6). After 72 h of treatment, the combination of NaB and epoxomicin produced a marked growth inhibition (mean, $95 \pm 8\%$), as compared to single treatment with NaB (mean, $48 \pm 3\%$) and epoxomicin (mean, $60 \pm 2\%$). The differences in SW48 growth inhibition produced by combined treatment with NaB and epoxomicin and that produced by single treatment with NaB ($P=0.001$) and epoxomicin ($P=0.014$) were statistically significant. Similar results were obtained when SW48 cells were treated for 120 h with the combination of NaB and epoxomicin (mean, $94 \pm 6\%$), as compared to single treatment with NaB (mean, $58 \pm 3\%$) and epoxomicin (mean, $64 \pm 2\%$). The differences in SW48 growth inhibition produced by combined treatment and single treatment with NaB ($P=0.01$) and epoxomicin ($P=0.027$) were statistically significant.

The growth of SW1116 cells was slightly affected after 24 h of treatment with epoxomicin (mean, 13%) or the combination of NaB and epoxomicin (mean, 12.75%). Meanwhile, NaB exhibited no effect on SW1116 proliferation (Fig. 6). Marked SW1116 growth inhibition was observed after 72 h of treatment with the combination of NaB and epoxomicin (mean, $96 \pm 5\%$). The differences in the growth inhibition produced by combined treatment and single treatment with NaB (mean, $73 \pm 2\%$; $P=0.05$) or epoxomicin (mean, $31 \pm 5\%$; $P=0.001$) were statistically very significant. The combination of NaB and epoxomicin produced a slightly higher SW1116 growth inhibition than that produced by NaB alone (mean, $88 \pm 14\%$). However, the growth inhibition of SW1116 produced either by the combination of NaB and epoxomicin or NaB alone was much greater than that produced by epoxomicin alone (mean, $34 \pm 3\%$) after 120 h of treatment. The differences in the growth inhibition produced by either the combination or NaB and that produced by epoxomicin were statistically very significant ($P=0.001$).

The combined treatment with NaB and epoxomicin produced a higher growth inhibition of SW837 cells (mean, $30 \pm 1.2\%$) than that produced by NaB (mean, $12 \pm 0.6\%$) and epoxomicin (mean, $17.5 \pm 7\%$) alone after 24 h of treatment. The difference in growth inhibition produced by combined treatment was statistically significant ($P=0.05$), as compared to that produced by NaB alone. However, this difference was statistically non-significant ($P=0.177$), as compared to that produced by epoxomicin alone (Fig. 6). After 72 h, the combined treatment produced a much higher growth inhibition of SW837 (mean, $98 \pm 3\%$) than that produced by single treatment with epoxomicin (mean, $21 \pm 2\%$; $P=0.001$). Although the combined treatment produced a higher growth inhibition than that produced by NaB alone (mean, $84 \pm 2\%$), the difference in growth inhibition was statistically non-significant ($P=0.177$). Similar results were obtained after 120 h of treatment; the

growth of SW837 was markedly suppressed after treatment with the combination of NaB and epoxomicin (mean, $99.0 \pm 1\%$), as compared to that produced by epoxomicin alone (mean, $19 \pm 2\%$; $P=0.001$). The combined treatment produced a higher growth inhibition than that produced by NaB alone (mean, $87 \pm 2\%$). However, the difference in growth inhibition was statistically non-significant ($P=0.194$).

Analysis of the combined effects of proteasome inhibitors and NaB on human colorectal cancer cells. The effects produced by treating human colorectal cancer cells SW48, SW1116 and SW837 with various combinations of NaB and the proteasome inhibitors (MG115, MG132, PSI-1, PSI-2 and epoxomicin) were observed as previously described (22,23). The results of three independent experiments are summarized in Table I.

Various combinations of NaB and MG115 ($6.25 \text{ mM}/1.0 \mu\text{M}$ and $12.5 \text{ mM}/2.0 \mu\text{M}$) had additive or synergistic anti-proliferative effects on SW48 and SW1116 after 72 and 120 h of treatment. All the tested combinations exhibited additive or synergistic anti-proliferative effects on SW837 after 120 h of treatment (Table I).

Various combinations of NaB and MG132 ($3.125 \text{ mM}/0.5 \mu\text{M}$, $6.25 \text{ mM}/1.0 \mu\text{M}$ and $12.5 \text{ mM}/2.0 \mu\text{M}$) exhibited synergistic or additive antiproliferative effects on the SW48, SW1116 and SW837 cells after 120 h of treatment. Additionally, these combinations had additive antiproliferative effects on SW837 after 72 h of treatment. The combinations of NaB and MG132 ($6.25 \text{ mM}/1.0 \mu\text{M}$ and $12.5 \text{ mM}/2.0 \mu\text{M}$) had additive antiproliferative effects on SW48 and SW1116 after 72 h of treatment.

All the tested combinations of NaB and PSI-1 exhibited additive or synergistic antimitogenic effects on the human colorectal cancer cells after 72 and 120 h of treatment (Table I). All of the tested combinations of NaB and PSI-2 except 1.56 mM NaB and $0.375 \mu\text{M}$ PSI-2 produced additive or synergistic antimitogenic effects on SW48, SW1116 and SW837 after 120 h of treatment (Table I). The same combinations had an additive antiproliferative effect on SW837 after 72 h of treatment. All of the combinations of NaB and epoxomicin exhibited synergistic or additive antiproliferative effects on SW48, SW1116 and SW837 after 72 and 120 h of treatment (Table I).

These results indicate that the combinations of NaB and the tested proteasome inhibitors can be ranked with respect to their antimitogenic effects in the following order: NaB + epoxomicin > NaB + PSI-1 > NaB + PSI-2 \equiv Na + MG115 \equiv NaB + MG132. The antiproliferative activities and the type of combined treatment were time-, dose- and cell line-dependent.

Cell cycle phase distribution of human colorectal cancer cells treated with NaB, proteasome inhibitors, and their combinations. FACS analysis showed that treatment of the human colorectal cancer cells with 3 mM NaB resulted in the accumulation of cells in the G_1 phase (82.7%) with a corresponding decrease in the number of cells in G_2/M (2.61%) and S (14.7%) phases (Fig. 7). Meanwhile, treatment with $1.0 \mu\text{M}$ MG115, $1.0 \mu\text{M}$ MG132, $0.1 \mu\text{M}$ PSI-1, $1.5 \mu\text{M}$ PSI-2, or 12 nM epoxomicin resulted in the accumulation of cells in the S phase (55.5 , 33.7 , 41.5 , 42.7 and 32% , respectively) and the

Table I. Analysis of the combined effects of proteasome inhibitors and sodium butyrate on human colorectal cancer cell lines.

Single and combined treatment of NaB and proteasome inhibitors	Combined interaction of proteasome inhibitors and NaB in human colorectal cancer cell lines:								
	SW48			SW1116			SW837		
	1 d	3 d	5 d	1 d	3 d	5 d	1 d	3 d	5 d
1.56 mM NaB + 0.25 μ M MG115	ant	ant	ant	ant	ant	ant	ant	ant	add
3.125 mM NaB + 0.5 μ M MG115	ant	ant	ant	ant	ant	ant	ant	ant	syn
6.25 mM NaB + 1.00 μ M MG115	ant	add	syn	ant	syn	syn	ant	add	syn
12.5 mM NaB + 2.00 μ M MG115	ant	add	add	ant	add	syn	ant	add	add
1.56 mM NaB + 0.25 μ M MG132	ant	ant	ant	ant	ant	ant	ant	ant	ant
3.125 mM NaB + 0.5 μ M MG132	ant	ant	syn	ant	ant	add	ant	add	syn
6.25 mM NaB + 1.00 μ M MG132	ant	add	add	ant	add	add	ant	add	add
12.5 mM NaB + 2.00 μ M MG132	add	add	syn	ant	add	add	ant	add	add
1.56 mM NaB + 0.013 μ M PSI-1	ant	add	syn	ant	add	syn	ant	syn	syn
3.125 mM NaB + 0.025 μ M PSI-1	ant	syn	syn	ant	syn	add	ant	add	syn
6.25 mM NaB + 0.05 μ M PSI-1	ant	add	add	ant	add	add	ant	add	add
12.5 mM NaB + 0.10 μ M PSI-1	ant	add	add	ant	add	add	ant	add	add
1.56 mM NaB + 0.375 μ M PSI-2	ant	ant	ant	ant	ant	ant	ant	ant	ant
3.125 mM NaB + 0.750 μ M PSI-2	ant	ant	add	ant	ant	add	ant	add	syn
6.25 mM NaB + 1.500 μ M PSI-2	ant	add	add	ant	add	add	ant	add	add
12.5 mM NaB + 3.000 μ M PSI-2	ant	add	add	ant	add	add	ant	add	add
1.56 mM NaB + 3.000 nM Epox	ant	syn	syn	ant	syn	syn	ant	syn	syn
3.125 mM NaB + 6.000 nM Epox	ant	syn	syn	ant	syn	syn	ant	syn	syn
6.25 mM NaB + 9.000 nM Epox	ant	syn	add	ant	syn	add	ant	syn	syn
12.5 mM NaB + 12.000 nM Epox	ant	add	add	ant	add	add	ant	add	add

Data are based on 3 independent experiments. ant, antagonistic; add, additive; syn, synergistic. d, day.

G₂ phase (15.2, 36, 44.3, 29.9 and 45.7%, respectively) with a corresponding decrease in the number of cells in the G₁ phase (29.0, 29.7, 14.1, 27.4 and 22.3%, respectively).

Treatment of the human colorectal cancer cells with the combination of 3 mM NaB and 1.0 μ M MG115 or MG132 resulted in the accumulation of cells in the G₁ phase (79.8 or 75.5%, respectively) and the G₂ phase (6.73 or 14.4%, respectively) with a corresponding decrease in the number of cells in the S phase (13.5 or 10%, respectively) (Fig. 7). On the other hand, the combination of 3 mM NaB and 1.0 μ M PSI-1, 1.5 μ M PSI-2, or 12 nM epoxomicin resulted in the accumulation of cells in the G₂ phase (45, 92.7 or 88%, respectively) with a corresponding decrease in the number of cells in the G₁ phase (55, 7.29 or 12%, respectively) and the S phase (0%) (Fig. 7).

The effects of NaB, proteasome inhibitors, and their combinations on apoptosis in human colorectal cancer cells. The agarose gel electrophoresis of DNA extracted from the untreated and treated (with NaB, proteasome inhibitors and their combinations) human colorectal cancer cells exhibited a distinct ladder pattern, which is the hallmark of apoptosis. Our results clearly demonstrated that treatment with NaB, the proteasome inhibitors, or their combinations induced

apoptotic involution. The extent of apoptosis in the human colorectal cancer cells treated with the combination of NaB and the proteasome inhibitors was more pronounced, as compared to that observed in the cells treated with either NaB or proteasome inhibitors (Fig. 8).

To investigate the type of cell death induced by NaB, the proteasome inhibitors, and their combinations, cells were treated with AO/EB, which allows for the identification of viable, apoptotic, and necrotic cells based on color and appearance. Staining with AO/EB of the human colorectal cancer cells treated for 24 h with 3 mM NaB exhibited ~50% orange-stained cells. The apoptotic effect appeared earlier in cells treated with proteasome inhibitors or the combination of NaB and proteasome inhibitors. In fact, 30 and 50% of apoptotic cells were observed after 8 h of incubation with the proteasome inhibitors and their combinations with NaB, respectively. The maximum effect was reached at 24 h when 50 and 80% of cells showed signs of apoptosis with the proteasome inhibitors and the combination of NaB and the proteasome inhibitors (data not shown).

Effects of NaB and epoxomicin on the activities of caspases and on cytochrome-c release. Caspase-3, a cysteine protease,

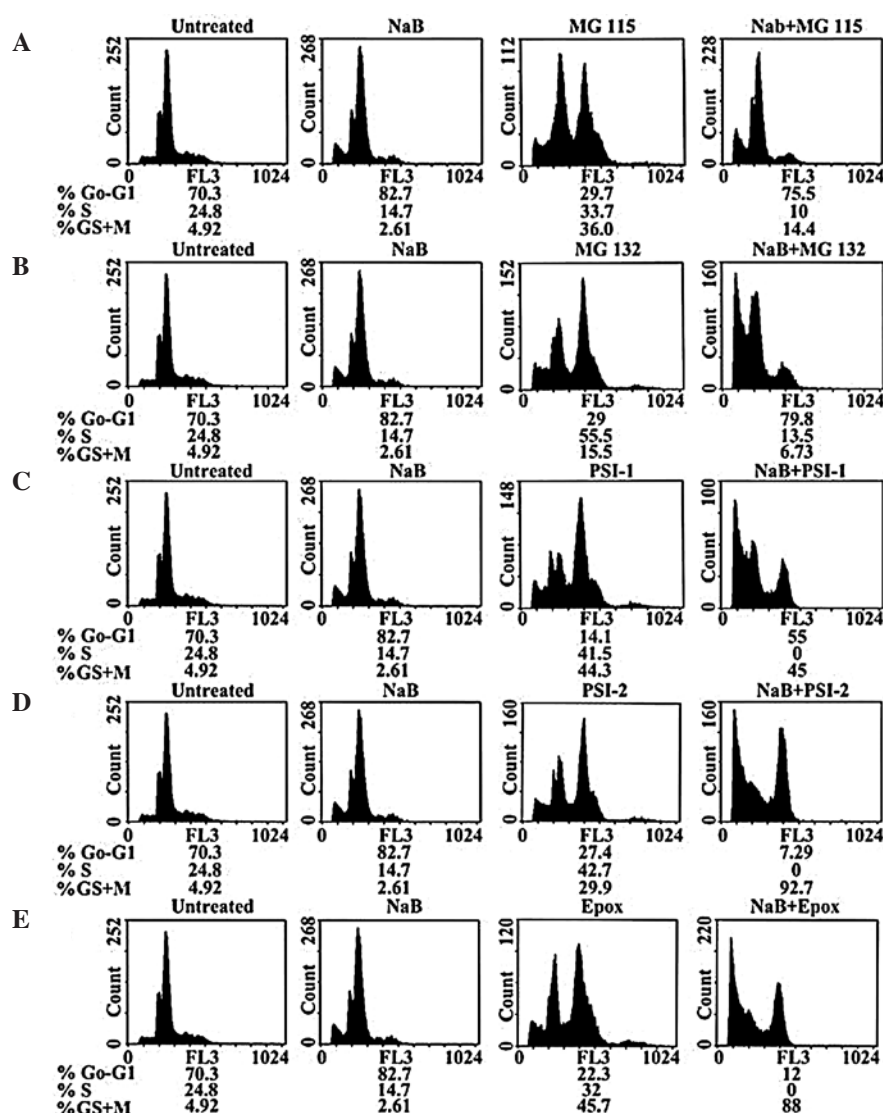


Figure 7. Cell cycle distribution of human colorectal cancer cells treated with NaB, proteasome inhibitors and their combinations. Human colorectal cancer cells were plated (5×10^5 cells/well) into 24-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (3 mM), MG115 (1.0 μM), MG132 (1.0 μM), PSI-1 (0.1 μM), PSI-2 (1.5 μM) and epoxomicin (12 nM) or treated with the combinations NaB/MG115 (3 mM/1.0 μM), NaB/MG132 (3 mM/1.0 μM), NaB/PSI-1 (3 mM/0.1 μM), NaB/PSI-2 (3 mM/1.5 μM) and NaB/epoxomicin (3 mM/12 nM) for 72 h. At least duplicate samples were analyzed and 20,000 events were scored for each sample. The vertical axis represents the relative number of events and the horizontal axis represents the fluorescence intensity. The percentage of cells in different cell cycle phases was calculated using Phoenix statistical software package.

is present in cells as an inactive pro-enzyme. Activation of this form requires cleavage at specific aspartate sites to produce subunits of M_r 17,000 Da and M_r 12,000 Da. The active enzyme takes part in the execution phase of apoptosis (26).

In this study, NaB induced the activation of caspase-3 in human colorectal cancer cells. The direct estimation of caspase-3 activity showed a 3-fold increase after exposure to 3 mM NaB. The effect was inhibited by both z-VAD-fmk and Ac-DEVD (Fig. 9A). Exposure of colorectal cancer cells to epoxomicin enhanced the activity of caspase-3, and z-VAD-fmk (100 μM) was capable of suppressing the effect exerted by epoxomicin on caspase activity (Fig. 9A).

The activation of caspase-3 was also shown by means of Western blot analysis using a primary antibody that recognizes both the M_r 32,000 Da pro-enzyme and the M_r 12,000 Da subunit of the active enzyme. As shown in Fig. 9B, in the cells

treated with 3 mM NaB, the p12 subunit increased, whereas that of the pro-enzyme diminished at the same time. The addition of z-VAD-fmk suppressed the production of the p12 subunit induced by NaB, providing evidence that the activation of caspase-3 was stimulated by caspase activities inhibited by z-VAD-fmk (Fig. 9B).

To demonstrate the proteolytic activity of caspase in cells treated with NaB, we studied the fragmentation of two nuclear proteins, PARP and lamin B, which are cleaved by caspase activities (27). PARP is a protein of M_r 16,000 Da that is associated with chromatin and is cleaved in a number of cell death systems by cysteine proteases to yield two fragments of M_r 24,000 Da. Western blot analysis revealed that treatment of the human colorectal cancer cells with 3 mM NaB induced the proteolytic cleavage of the M_r 116, Da PARP protein to yield the characteristic M_r 85,000 Da

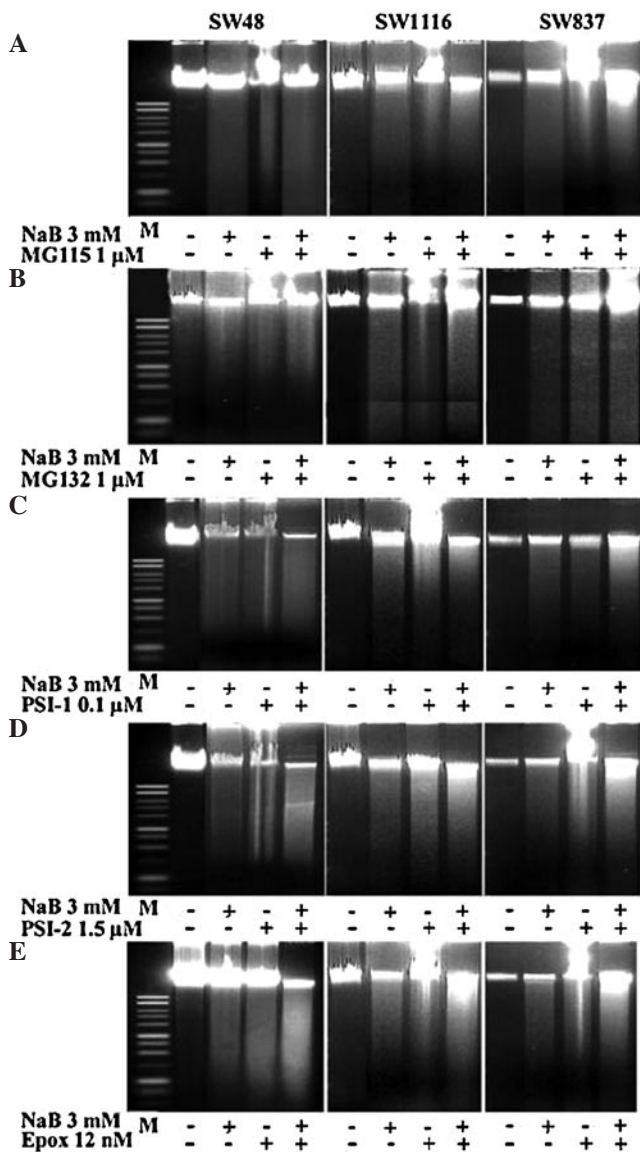


Figure 8. Assessment of apoptosis in human colorectal cancer cells treated with NaB, proteasome inhibitors and their combinations. Human colorectal cancer cells were plated (5×10^5 cells/well) into 24-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (3 mM), MG115 (1.0 μM), MG132 (1.0 μM), PSI-1 (0.1 μM), PSI-2 (1.5 μM) and epoxomicin (12 nM) or treated with the combinations NaB/MG115 (3 mM/1.0 μM), NaB/MG132 (3.0 mM/1.0 μM), NaB/PSI-1 (3.0 mM/0.1 μM), NaB/PSI-2 (1.5 μM) and NaB/epoxomicin (3.0 mM/12.0 nM) for 72 h. DNA fragments were extracted and analyzed on 1% agarose gel.

fragment (Fig. 9B). Additionally, NaB (3.0 mM) induced the cleavage of lamin B. The effects on both the cleavage of PARP and lamin B were suppressed by the addition of z-VAD-fmk (Fig. 9B). Similar results were obtained when human colorectal cancer cells were treated with epoxomicin (12.0 nM) and the combination of NaB (3 mM) and epoxomicin (12.0 nM) (data not shown).

Mitochondria play a decisive role in apoptosis (27), functioning as integrators of different pro-apoptotic signaling pathways. In response to these signals, mitochondria activate mega-channels (also called permeability transition pores) that are present between the inner and outer mitochondrial membranes. Bcl2, a fundamental death antagonist protein,

inhibits mega-channel opening, whereas Bax, a death antagonist protein, facilitates the opening of mega-channels (28). The increase in the content of agonists and the concomitant decrease in the content of antagonists stimulate the release of cytochrome-c from the mitochondria, with the consequent activation of caspase activities. As shown in Fig. 9C, treatment with 3 mM NaB, 12 nM epoxomicin, and their combination for 72 h seemed to favor the release of cytochrome-c from the mitochondria as the mitochondrial level of cytochrome-c appeared diminished and the cytosolic level increased.

Differential expression of p53, p21^{Waf1}, p27^{Kip1}, Bax and Bcl2 proteins in human colorectal cancer cells treated with NaB, proteasome inhibitors and the combinations of NaB and proteasome inhibitors. Western blot analysis (Fig. 10A and B) revealed that treatment of the human colorectal cancer cells with NaB or proteasome inhibitors differentially decreased the levels of Bcl2 and concomitantly increased the levels of Bax. Moreover, the combinations of NaB with each of the tested proteasome inhibitors modified the suppressive effects of NaB and proteasome inhibitors on Bcl2 levels and enhanced the enhancing effect on Bax levels (Fig. 10A and B).

Studies from several laboratories have suggested that deregulation of cell progression may be involved in the initiation of apoptosis (29). In addition, it has been reported that the ubiquitin-proteasome pathway plays an essential role in the regulation of several important cell cycle proteins, including p53 (30) and the cyclin-dependent kinase inhibitors p21^{Waf1} (31) and p27^{Kip1} (32).

After treatment with NaB, proteasome inhibitors (MG115, MG132, PSI-1, PSI-2 and epoxomicin), and their combinations, the protein levels of p53, p21^{Waf1} and p27^{Kip1} were examined by Western blotting. It is well known that ubiquitin-proteasome systems play an important role in the degradation of many short-lived proteins, including p53 (33). As shown in Fig. 10C and D, treatment of the human colorectal cancer cells with 3.0 mM NaB lowered the level of p53 by $\sim 50\%$ with respect to the control. Since p53 is cleaved by the ubiquitin-proteasome complex, the inhibition of this activity would be expected to stabilize the p53 protein. To investigate this possibility, the effect of proteasome inhibitors MG115, MG132, PSI-1, PSI-2 and epoxomicin on the p53 level was determined. Clear differential enhancement in the levels of p53 was observed after 72 h exposure to 1.0 μM MG115, 1.0 μM MG132, 1.0 μM PSI-1, 1.5 μM PSI-2 or 12.0 nM epoxomicin (Fig. 10C and D).

Notably, when human colorectal cancer cells were treated with the combinations of each of these proteasome inhibitors and NaB, the proteasome inhibitors counteracted the decreasing effects of NaB on p53. In this way, in the colorectal cancer cells exposed for 72 h to combined treatment, the levels of p53 were higher than those in the control and were comparable to the levels found in the cells treated with the proteasome inhibitors alone (Fig. 10C and D).

It is well known that p21^{Waf1}, a transcriptional target of p53, plays a role in the control of the cell cycle. Moreover, it has been reported that NaB is capable of stimulating p21^{Waf1} expression in a p53-independent manner (34). The results presented in this study showed that in the human colorectal cancer cells, NaB increased the level of the p21^{Waf1} protein. Treatment of cancer cells with the rest of the proteasome inhibitors and their combi-

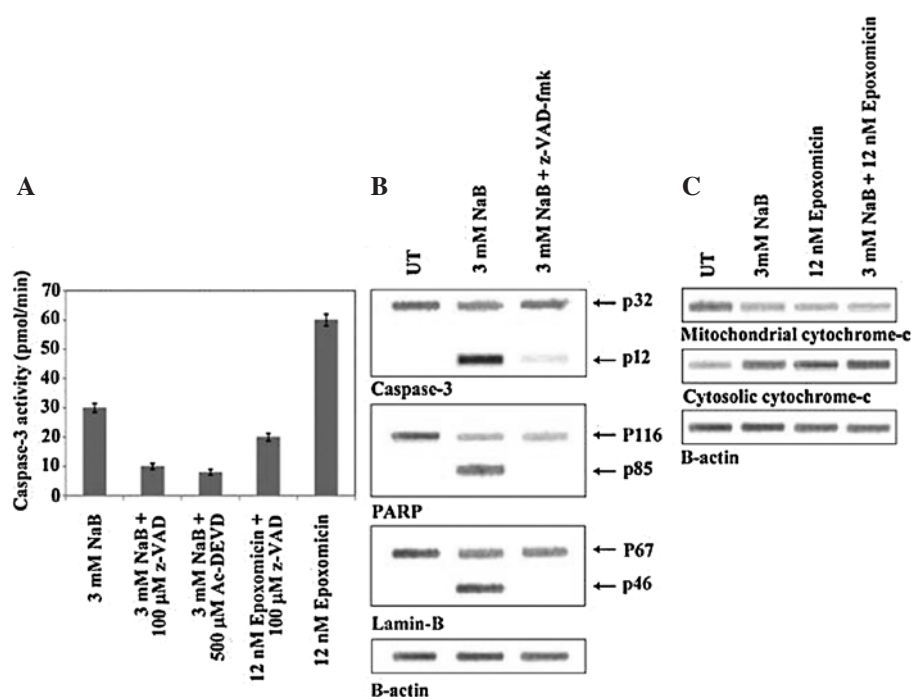


Figure 9. Analysis of caspase-3, PARP, lamin B and cytochrome-c in human colorectal cancer cells treated with NaB, epoxomicin and their combination. (A) Cells were treated with 3.0 mM NaB or 12.0 nM epoxomicin in the absence or presence of 100 or 500 μ M Ac-DEVD, and caspase-3 activity was measured using a colorimetric assay according to the manufacturer's instructions. Values are the mean \pm SE from three separate experiments. (B) Cell lysates of human colorectal cancer cells treated with 3.0 mM NaB in the absence or presence of 100 μ M z-VAD-fmk were resolved by 10% SDS-PAGE, immunoblotted, and detected using specific antibodies as described in Materials and methods. (C) Cells were treated with 3.0 mM NaB, 12.0 nM epoxomicin or their combination for 24 h. Mitochondrial and cytosolic fractions were resolved by SDS-PAGE, proteins were immunoblotted and detected using specific antibodies.

nations with NaB for 72 h differentially increased the levels of p21^{Waf1} depending upon the type of proteasome inhibitor and its combination with NaB. Similar to p21^{Waf1}, treatment with NaB, proteasome inhibitors and their combinations resulted in increased expression of p27^{Kip1} (Fig. 10C and D).

Discussion

The proteasome is a multi-catalytic protease complex that is responsible for the ubiquitin-dependent turnover of cellular proteins (35). It is estimated that over 80% of intracellular proteins are degraded by proteasomes. Besides carrying out protein turnover, the ubiquitin proteasome pathway plays an essential role in regulating protein levels during the cell cycle, during apoptosis, in response to cellular stress (i.e. DNA damage or hypoxia), and during intracellular signal transduction (36).

Clinical validation of the proteasome as a therapeutic target in oncology has been provided by the boronic acid bortezomib, a dipeptide (36). Bortezomib has proven to be effective as a single agent in multiple myeloma (37) and against some forms of non-Hodgkin's lymphoma (38); however, despite the clinical success of bortezomib, a significant fraction of patients remain refractory to treatment (37,38). Furthermore, a number of side effects, including cardiotoxicity (39,40), painful peripheral neuropathy (41) and thrombocytopenia (42), have restricted treatment of bortezomib to a biweekly dosing schedule that allows for the full recovery of proteasome activity between doses (43). In addition, drug resistance ultimately emerges in all tumors that initially respond to proteasome inhibitors (44).

The induction of apoptosis has been recognized as an effective tool in the therapeutic treatment of many types of tumors, and apoptosis can be triggered by a number of drugs, such as proteasome inhibitors. However, the efficacy of treatment with apoptotic drugs is limited by their toxicity and cell resistance (45). Sodium butyrate, a natural product that is capable of inducing apoptosis in a number of tumor cells, exhibits a low degree of clinical toxicity (46) and may therefore be of particular importance in tumor therapy. With this in mind, the aim of the current study was to investigate whether sodium butyrate enhances the anticancer effects of proteasome inhibitors MG115, MG132, PSI-1, PSI-2 and epoxomicin in human colorectal cancer carcinoma.

The results obtained from this study demonstrated that single treatment of human colorectal cancer cells with NaB and proteasome inhibitors MG115, MG132, PSI-1, PSI-2 or epoxomicin inhibited cell growth in a time- and dose-dependent manner (Fig. 1). Moreover, the combinations of proteasome inhibitors and NaB markedly inhibited the proliferation of the human colorectal cancer cells in a dose- and time-dependent manner (Figs. 2-6); the combinations of proteasome inhibitors and NaB produced additive and synergistic growth inhibitory effects (Table I). Our results are consistent with those reported by Giuliano *et al* who found that the treatment of retinoblastoma Y79 cells with NaB progressively reduced cell viability, and after 72 h the number of cells decreased by approximately 70% (47). Our results are also consistent with those obtained by Kim *et al* who demonstrated that MG132 inhibited the cellular growth of HCT116 cells in a time- and dose-dependent manner (48). Similar to those reported by Fan *et al* our results

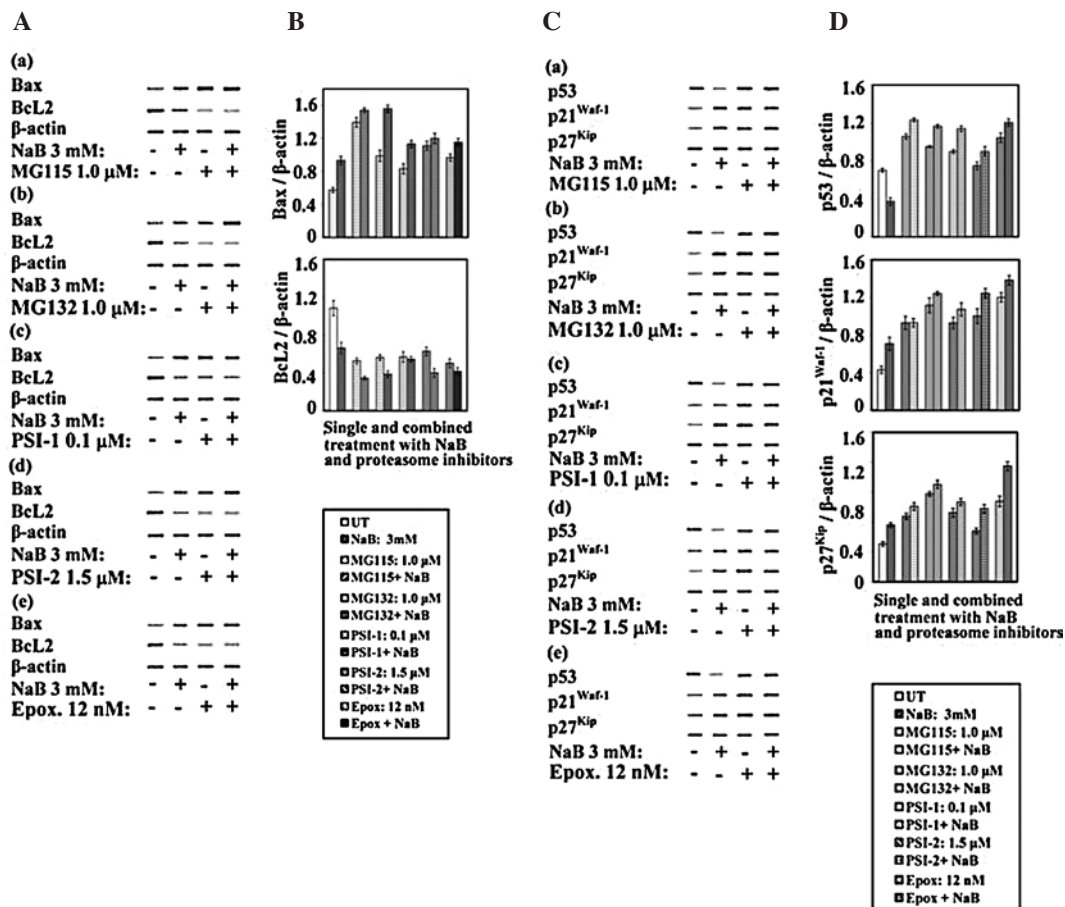


Figure 10. Expression of cell cycle and apoptosis regulatory proteins in human colorectal cancer cells treated with NaB, proteasome inhibitors and their combinations. Human colorectal cancer cells were plated (1×10^6 cells/well) into 24-well plates and incubated at 37°C in a non- CO_2 incubator. After 18 h, cells were treated individually with NaB (3.0 mM), MG115 (1.0 μM), MG132 (1.0 μM), PSI-1 (0.1 μM), PSI-2 (1.5 μM) and epoxomicin (12.0 nM) or treated with the combination of NaB/MG115 (3.0 mM/1.0 μM), NaB/MG132 (3.0 mM/1.0 μM), NaB/PSI-1 (3.0 mM/0.1 μM), NaB/PSI-2 (1.5 μM) or NaB/epoxomicin (3.0 mM/12.0 nM) for 72 h. Cell extracts of untreated and NaB-, proteasome inhibitor- or the combinations of NaB and proteasome inhibitor-treated colorectal cancer cells were analyzed for the expression of Bax, Bcl2, p21^{Waf1}, p27^{Kip1}, p53 and β -actin by Western blot analysis. The blots (A and C) were scanned, and the intensities of the bands were determined (B and D).

showed that MG132 inhibited the growth of human colorectal cell lines in a dose- and time-dependent manner (24). Similar results were recently reported by Wu *et al* who found that MG-132 significantly suppressed the proliferation of colon cancer SW1116 and HT-29 cells (49). However, our results contradicted those obtained by Denlinger *et al* who found that non-small cell lung cancer cell lines treated with NaB were resistant to NaB-induced apoptosis (50).

Flow cytometric analysis showed that NaB induced an arrest of human colorectal cancer cells at the G_1 -phase. Our results are similar to those reported by Abramova *et al* who showed that NaB induced G_1 -S arrest in rat embryo fibroblast cells that were transformed with the oncogenes E1A and c-Ha-Ras (51). On the other hand, these results contradict the observation of other investigators (47,52). In the present study, the treatment of colorectal cancer cells with proteasome inhibitors resulted in the accumulation of cells in the S phase and G_2 phase. Meanwhile, the combinations of NaB and MG115 or MG132 induced an arrest of the colorectal cancer cells in the G_1 phase and G_2 phase. Conversely, the combinations of NaB and PSI-1, PSI-2 or epoxomicin growth arrested colorectal cancer cells in the G_2 phase (Fig. 7).

Coordinated proteolytic activity of the ubiquitin-proteasome system has proven to be crucial to the normal progression of the cell cycle. In this study, the levels of p21^{Waf1} and p27^{Kip1} were markedly increased following treatment with the combinations of NaB and each of the tested proteasome inhibitors (Fig. 10C and D). Other researchers have demonstrated the accumulation of p21^{Waf1} and p27^{Kip1} in different cell lines treated with different proteasome inhibitors (24,53). Our results are also in agreement with those reported recently by Wu *et al* (49) who demonstrated that MG-132 activated bone morphogenetic protein (BMP) signaling, which manifested as an increase in the up-regulation of p21^{Waf1} and p27^{Kip1} expression as well as Smad 1/5/8 phosphorylation. Knockdown of the BMP receptor II abolished Smad 1/5/8 phosphorylation, the induction of p21^{Waf1} and p27^{Kip1}, and the inhibition of cell proliferation that was induced by MG132. These findings suggest that proteasome inhibition suppresses gastric cancer cell proliferation via activation of BMP signaling, demonstrating a novel aspect of proteasome function in the regulation of colon cancer cell proliferation (49). Overexpression of p27^{Kip1}, as reported in this study, can directly result in apoptosis (54). Recently, Timmerbeul *et al*

demonstrated that p27^{Kip1} stabilization prevents progression from adenomatous polyps to invasive intestinal cancer (55). Proteasome-induced cytotoxicity could potentially result from several events, including the stabilization and deregulated function of cyclins, CDK inhibitors, tumor suppressor proteins, and a large number of other proteins that are associated with cell cycle progression (33). Inhibiting the degradation of key cell cycle regulatory proteins causes a disparity in the proliferative signals and eventually leads to apoptosis.

In the present study, the human colorectal cancer cells exhibited a distinct ladder pattern that is indicative of apoptosis following treatment with NaB, proteasome inhibitors, or their combinations. The extent to which apoptosis occurred in colorectal cancer cells treated with the combinations of NaB and the proteasome inhibitors was more pronounced than it was when produced by a single treatment with NaB or the proteasome inhibitors (Fig. 8). These results are consistent with those reported by other researchers (24,47,48). Quite recently, similar results were obtained using a combination of the proteasome inhibitor bortezomib and thiazol antibiotics, which synergistically induced apoptosis in prostate cancer cells (56). Also, the combination of the novel proteasome inhibitor NPI-0052 and lenalidomide induced apoptosis in multiple myeloma cell lines and in multiple myeloma cells (57). Moreover, Matondo *et al* investigated the induction of apoptosis by proteasome inhibitors in several human myeloid leukemia (AML) cell lines and in primary cells from patients, and found that various AML subtypes may present different responses to proteasome inhibitors and that the amount of 20S proteasome in AML cells may be predictive of the cellular response to these inhibitors (58).

Members of the ICE family of cysteine proteases are present as inactive zymogens within the cell and are processed at the onset of apoptosis into enzymatically active heterodimer complexes (59). Three of these caspases, including caspase-3, -6 and -7, have been implicated in the execution phase of apoptosis (60). Caspase-3 was shown to cleave a wide range of cytoplasmic and nuclear proteins (60); caspase-7 is closely related to caspase-3 and has the same synthetic substrate specificity *in vitro* (61). PARP cleavage has been widely used as an indicator of the activation of caspase-3 or of closely related enzymes with similar substrate specificity (62). Similarly, caspase-7 is also involved in the cleavage of PARP during apoptosis (63). In the present study, butyrate or tested proteasome inhibitor-induced apoptosis was preceded by the activation of caspase-3, which was clearly observed at 24 h of treatment by direct measurement of its activity (Fig. 9A). Western blot analysis also demonstrated the conversion of procaspase-3 into the active form of the enzyme (Fig. 9B). Activation of caspase-3 was accompanied by degradation of PARP with the production of the M_r 85 kDa fragment (Fig. 9B). The observation that NaB also provokes the degradation of lamin B, a substrate of caspase-6 (64), suggests that other caspases besides caspase-3 could be involved in butyrate-induced apoptosis. All of these events preceded the appearance of morphological signs that were observed in a large percentage of cells after a 48-h treatment.

Members of the Bcl2 gene family are major regulators of programmed cell death. Bcl2 is a potent inhibitor of apoptosis, while Bax, one of the Bcl2 family genes, promotes apop-

toxis. Bax can dimerize with Bcl2, and the regulation of the Bcl2-Bax heterodimer determines whether a cell will continue to grow or undergo apoptosis (65). Bcl2, located on the outer mitochondrial membrane, inhibits cytochrome-*c* release, thereby blocking apoptosis (66); conversely, Bax induces caspase activation and apoptosis via cytochrome-*c* release (34). The present study tested whether NaB, epoxomicin, or their combination is capable of inducing changes in the levels of cytochrome-*c* that is present in both the mitochondria and cytosol. The results shown in Fig. 9C showed that after 24 h of treatment, cytochrome-*c* was released from the mitochondria under the influence of NaB, epoxomicin, and their combination. Such an event could be responsible for the production of the apoptosome that, with the consequent activation of caspase-9, activates caspase-3 (67). The release of cytochrome-*c* was induced in the colorectal cancer by a decrease in the level of the anti-apoptotic factor Bcl2 as well as concomitant enhancement in the level of the pro-apoptotic factor Bax in the human colorectal cancer cells treated with NaB, proteasome inhibitors, and their combinations (Fig. 10A and B).

It has been ascertained that 26S proteasome inhibitors are capable of triggering apoptosis in rapidly dividing cells (30); such an effect has been correlated with the ability of proteasome inhibitors to increase the intracellular levels of many short-lived factors (32). However, it has been suggested that apoptosis that is induced by proteasome inhibitors can be a consequence of the activation of c-Jun NH2-terminal kinase (68), an enzyme that is involved in the initiation of programmed cell death.

The results presented in this study clearly indicate that proteasome inhibitors MG115, MG132, PSI-1, PSI-2 and epoxomicin induced apoptosis (Fig. 8), together with a marked increase in the level of the short-lived protein p53 (Fig. 10C and D). Expression of the p53 protein is controlled largely by mdm-2/hdm-2-mediated ubiquitylation and is degraded via the proteasome. It therefore stands to reason that proteasome inhibitors will result in the accumulation of p53 in cells that contain the wild-type protein. Furthermore, our results demonstrated that the tested proteasome inhibitors increased the level of Bax protein (Fig. 10A and B), a factor that seems to be involved in the p53-mediated response as it favors the release of cytochrome-*c* from the mitochondria into the cytosol and the consequent activation of caspase-3. Our results are in agreement with those reported by Williams and McConkey (69) who found that bortezomib stabilized p53 and induced its nuclear translocation in human LNCaP prostate cancer cells (69). In addition, bortezomib activated p53 downstream target genes, including p21, Fas, and Bax and transfection with the human papilloma virus E6 protein, which blocked p53, attenuated bortezomib-induced cell death (69).

The results reported in this study demonstrate that NaB decreases the levels of p53, most likely through the activation of the 26S proteasome. Also, the finding that the tested proteasome inhibitors induced apoptosis and concomitantly increased the levels of p53 suggests a possible synergistic interaction between NaB and the tested proteasome inhibitors in the induction of apoptosis in human colorectal cancer cell lines. This postulate was confirmed by our results, which illustrate the synergistic relationship between NaB and proteasome inhibitors on apoptosis. These effects are likely a result of the

inhibition of proteasome activity by the proteasome inhibitors with the suppression of the stimulatory action of butyrate; consequently, the cells were prevented from decreasing their levels of p53. The presence of adequate amounts of p53 most likely enhances the susceptibility of the cells to apoptosis induction. A synergistic effect was reported by Medina *et al* (70) who observe that cells primed with butyrate were rendered highly susceptible to apoptosis by staurosporine, an agent that causes mitochondrial release of cytochrome-*c*.

In the present study, when the colorectal cancer cells were simultaneously exposed to the proteasome inhibitors and NaB, Bax protein levels increased markedly compared to single treatments. Bax protein is the product of a gene that represents a transcriptional target of p53. In addition, Bcl2 levels in these cells were down-regulated. Our results concur with those reported in another study (71). Meanwhile, Zhu *et al* did not find any changes in the expression of Bcl2 or Bcl-XL in human colorectal, lung and ovarian cancer cells that were treated with bortezomib (72).

The increase in the Bax levels and the concomitant decrease in the Bcl2 levels could have been responsible for the enhanced release of cytochrome-*c* from the mitochondria, with the consequent activation of caspase-3 and the induction of apoptosis. Our results agree with Goldstein *et al* who show that the release of cytochrome-*c* during apoptosis via GFA-tagged cytochrome-*c* was rapid, complete and kinetically invariant (73). Also, our results are in agreement with those reported by Zhu *et al* who found that cytochrome-*c* release was detectable in as little time as 3 h, and that a dramatic cytochrome-*c* release was detected at 18 h following bortezomib treatment (72).

The ability of butyrate to influence cell function is associated with its regulation of gene expression, which is often attributed to its inhibition of histone deacetylase (74). This results in the hyperacetylation of histones and the enhancement of the accessibility of transcription factors to nucleosomal DNA (75). However, it is likely that butyrate has other intracellular targets, including hyperacetylation of nonhistone proteins, alteration of DNA methylation, selective inhibition of histone phosphorylation, and modulation of intracellular kinase signaling (74). This multiplicity of effects may underlie the ability of butyrate to modulate gene expression and impact the key regulators of apoptosis and the cell cycle.

We therefore conclude that the synergistic interactions between NaB and proteasome inhibitors MG115, MG132, PSI-1, PSI-2, or epoxomicin are closely correlated with the increased levels of the pro-apoptotic factor p53 and subsequent p21^{Waf1} transactivation, resulting in cell cycle arrest. Increases in p53 activity also results in the up-regulation of Bax and induced apoptosis. Combined treatment with NaB and proteasome inhibitors may also directly induce the up-regulation of both Bax and p27^{Kip1}; accumulation of p27^{Kip1} contributes to both apoptosis and cell cycle arrest.

The synergistic apoptotic effects of NaB and proteasome inhibitors may open new and interesting perspectives in the therapeutic strategy for the treatment of human colorectal cancer. It is of interest that the proteasome inhibitors seem to behave as apoptotic agents only in rapidly dividing cells while protecting quiescent cells from apoptosis that may be triggered by many different compounds (30). Due to this particular behavior, proteasome inhibitors may represent

a new alternative in the treatment of certain proliferative diseases. In addition, the association between a proteasome inhibitor and other effective agents of apoptosis could induce a clear effect at lower concentrations of the compounds, as is shown in this study, thereby reducing the toxicity of the therapeutic treatment.

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