Sulindac Enhances the Proteasome Inhibitor Bortezomib-Mediated Oxidative Stress and Anticancer Activity

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Abstract

Purpose: The nonsteroidal antiinflammatory drug sulindac is a promising chemopreventive agent against colon cancer. Here, we address whether sulindac enhances the anticancer effects of the proteasome inhibitor bortezomib (PS-341) in colon cancer cells.

Experimental Design: The synergistic effects of sulindac with bortezomib were evaluated by cell death, colony formation assay, DNA fragmentation, and tumor progression of DLD-1 xenografts. Reactive oxygen species (ROS) generation was detected using carboxy-H2DCFDA or dihydroethidium. Oxidative stress was evaluated by heme oxygenase-1 induction and stress-activated mitogen-activated protein kinases p38 and c-Jun-NH2-kinase phosphorylation. Oxidative DNA damage was evaluated by histone H2AX phosphorylation and accumulation of 8-hydroxy-2'-deoxyguanosine.

Results: Sulindac and its metabolites enhanced the anticancer effects of bortezomib in DLD-1 and BM314 colon cancer cells. Sulindac induced ROS generation and enhanced bortezomib-mediated oxidative stress and subsequent DNA damage. Their combined effects were highly sensitive to free radical scavengers L-N-acetylcysteine and α-tocopherol, but were much less sensitive to a p38 inhibitor SB203580.

Conclusion: Sulindac synergistically augments the anticancer effects of bortezomib primarily through cooperative ROS generation and oxidative DNA damage, thereby representing a novel combination therapy against colon cancer.

Nonsteroidal antiinflammatory drugs (NSAID) exert chemopreventive effects in colon cancer. Human epidemiological studies, animal models, and in vitro experiments reveal that NSAIDs reduce the risk of colorectal cancer, and induce regression of colorectal adenomas in patients with familial adenomatous polyposis (1–5). Importantly, NSAIDs inhibit cell cycle progression and induce apoptosis in several cancer cells (6–8). To date, several molecular mechanisms underlying the proapoptotic effects of NSAIDs have been suggested. Reduced prostaglandin synthesis by inhibiting cyclooxygenase activity may be crucial for the mechanism. However, NSAIDs show proapoptotic effects against a colon cancer cell line HCT-15 expressing no COX-1 or COX-2 (9), and against embryo fibroblasts carrying neither COX-1 nor COX-2 genes (10). Thus, their effect rather involves other mechanisms, such as overproduction of ceramide and inhibition of nuclear factor κB–mediated signals (11–13). Although there are many suggestions, scientists are still arguing on how NSAIDs function as chemopreventive agents, and it remains uncertain whether sulindac can be an anticancer agent.

The proteasome is an essential enzyme complex for the ATP-dependent proteolytic pathway, which catalyzes the rapid degradation of many short-lived functional proteins and plays pivotal roles in cell cycle progression, cytokine-mediated transcription, and apoptosis (14). Inhibition of this pathway strongly induces cell cycle arrest and apoptosis in a variety of malignant cells. A dipeptide boronic acid analogue, bortezomib, a specific proteasome inhibitor, shows strong anticancer activity and is approved for clinical use in human myeloma cells (15–17). Although bortezomib displays marked proapoptotic effects in hematological malignant cells, it possesses fewer effects on solid malignant tumor cells. For instance, 0.1 μmol/L of bortezomib almost completely inhibits cell cycle progression of multiple myeloma cells, but the same dose does not significantly affect that of non–small cell lung cancer cells (15, 18). To address this issue, combination therapies have been employed (19, 20). Bortezomib accumulates p53, p21WAF1, MDM2, cyclin A, and cyclin B, phosphorylates Bcl-2 and inhibits nuclear factor κB signals, resulting in G2-M phase arrest and apoptosis (15, 18, 21). Bortezomib also triggers generation of reactive oxygen species (ROS), which is now considered to be critical in initiating bortezomib-mediated apoptosis (22).

In this article, we report that sulindac and its metabolites enhanced bortezomib-mediated anticancer effects in human...
colon cancer cell lines and the combination strongly inhibited tumor progression of DLD-1 xenografts. They cooperatively induced ROS generation, oxidative stress, and oxidative DNA damage, and the augmented death was sensitive to free radical scavengers. Although sulindac is considered as a chemo-preventive agent, our findings outline a new concept for its use as an enhancer of the anticancer agent bortezomib through ROS-mediated signals.

Materials and Methods

Cell culture and reagents. Human colon DLD-1 and BM314 cancer cells, obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), were grown in RPMI 1640 supplemented with 10% fetal calf serum. To evaluate viability, cells were mixed with the same volume of 0.4% trypan blue solution, and immediately examined to determine whether they could exclude the dye. For 4’,6-diamidino-2-phenylindole (DAPI) staining assay, both floating and adherent cells were mixed, fixed in 70% ethanol for 10 minutes, stained with DAPI (0.5 μg/mL PBS) for 10 minutes, and chromatin fluorescence was observed under UV light using a confocal microscope (R2100AG2, Bio-Rad, Hercules, CA). Sulindac, sulindac sulfide, sulindac sulfone, NS-398, t-NAc, α-tocopherol and 5-fluorouracil (5-FU) were purchased from Sigma (St. Louis, MO). SB203580 was from Calbiochem (San Diego, CA).

Tumor xenografts in nude mice and in vivo combination therapy. DLD-1 cells were injected s.c. (2 × 106 cells) in the back of 4- to 5-week-old athymic nude mice. Tumor volumes were determined from caliper measurements of tumor length (L) and width (W) according to the formula (L × W2)/2. When tumor volumes reached a minimal size of 60 ± 10 mm3 (~7 days after cell injection), mice (n = 6, each group) were given 0.6 mmol/L sulindac in drinking water (~3 μmol daily) and/or 100 μL of bortezomib (16 μmol/L) was intratumor-injected twice a week. Tumor size was measured every week until sacrifice, and tumor weight was measured at 3 weeks after treatment began. Tumors were fixed in neutral-buffered formalin, embedded in paraffin, sectioned (5 μm) and stained with H&E. Tumors were also embedded in Tissue-Tek OCT, frozen in liquid nitrogen, and sectioned into 5 μm slices. H2AX phosphorylation was evaluated by confocal microscopy. Our institute approved these animal protocols.

Annexin V binding analysis. Cells (1 × 106) were incubated with green fluorescent protein-annexin V for 30 minutes, washed, further incubated with propidium iodide for 15 minutes following the manufacturer’s protocol (MBL, Nagoya, Japan), and analyzed for cellular fluorescence by FACScan flow cytometry (Becton Dickinson, Mountain View, CA) using CellQuest Software.

Western blots. Cell lysates were fractionated by 10% to 15% SDS-PAGE. The following antibodies were used for Western blots with a standard enhanced chemiluminescence method (Amersham, Piscataway, NJ): e-Jun-NH2-kinase (JNK) and hs70 (Santa Cruz Biotechnol- ogy, Santa Cruz, CA) p38 and phospho-p38 (Cell Signaling, Beverly, MA); phospho-JNK (Promega, Madison, WI); phospho-H2AX (Upstate Biotechnical, Lake Placid, NY); and heme oxygenase-1 (HO-1; BD Bioscience PharMingen, San Jose, CA).

Reactive oxygen species detection. Cells were incubated with 10 μmol/L 5-, (and -6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate, carboxy-H2DCFDA (Molecular Probes, Eugene, OR) for 30 minutes, after which they were washed, and further incubated for 3 to 4 hours. Alternatively, cells were incubated for 60 minutes with 10 μmol/L dihydroethidium (Sigma) and thereafter washed with PBS. ROS generation was determined using a FACScan flow cytometer. Carboxy-H2DCFDA-detectable ROS (FL1) and dihydroethidium-detectable superoxide anion (FL2) fluorescent signals were displayed as histograms. We also calculate ratios of their signals (versus control data) by mean fluorescence intensity.

Colonony assay. Cells (3 × 106) were seeded onto 60 mm plates and cultured with the indicated treatment for 9 days and the number of colonies (>25 cells) were counted. The data represent the mean ± SD of three independent experiments. Survival fraction was defined as the number of colonies divided by the number of plated cells.

Confocal microscopy. DLD-1 cells were cultured on bovine serum albumin–coated coverslips overnight, and treated as indicated for 24 hours. After fixing with cold methanol for 20 minutes, sections were soaked into 5% bovine serum albumin, and incubated with anti-phospho-H2AX polyclonal, anti-8-hydroxy-2-deoxyguanosine monoclonal N43.1 (Japan Institute for the Control of Aging, Fukuroi, Japan) or rabbit IgG antibodies for 30 minutes. Sections were thereafter incubated with Alexa Fluor 488 goat anti-rabbit IgG and/or Alexa Fluor 594 goat anti-mouse IgG antibodies (Molecular Probes) for 30 minutes. Confocal imaging was done using a ZEISS Pascal laser-scanning microscope.

Statistical analysis. Statistical differences between two groups were calculated using Student’s t test or Student-Newman-Keuls test. One-way ANOVA was used to determine statistical differences between multiple groups by performing SPSS program version 10.1 (San Rafael, CA). P < 0.05 was considered statistically significant.

Results

Anticancer effect of combined sulindac and bortezomib. Because bortezomib is less effective in solid tumors, identification of its combination partner may improve its efficacy in these cancers. Based on this idea, we first investigated whether sulindac and its metabolites augment the bortezomib-mediated anticancer effect. In DLD-1 cells, 20 nmol/L bortezomib alone induced limited cell death, whereas its combination with sulindac, sulindac sulfide, or sulindac sulfone clearly enhanced anticancer effect. In DLD-1 cells, 20 nmol/L bortezomib alone induced limited cell death, whereas its combination with sulindac, sulindac sulfide, or sulindac sulfone clearly enhanced cell death (Fig. 1A). Because individual treatment with each NSAID showed no toxic or anticancer effects, combination therapy strongly augmented cell death. Indeed, the observed cell death with combination therapy exceeded the calculated additive effect. For example, calculated survival rate = 0.83 ± 0.3 (survival rate of bortezomib treatment) × 0.94 ± 0.02 (survival rate of sulindac treatment) and calculated death rate = 1 – 0.83 × 0.94 = 0.22. However, the combination caused a much greater death rate, 0.76 ± 0.6, indicating that the combination has synergistic anticancer activity. Similarly, other sulindac metabolites also caused synergistic effects on cell death. In contrast, a COX-2 specific inhibitor, NS-398, and an anticancer agent, 5-FU, showed no distinct enhancing effect. Annexin V and DAPI staining assays revealed that combination of sulindac with bortezomib strongly increased apoptosis compared with their individual treatment (Fig. 1B and C). Furthermore, a colony formation assay revealed that concentrations required for a distinct anticancer effect of combination therapy were as little as 4 nmol/L bortezomib and 20 μmol/L sulindac, and the combination of 8 nmol/L bortezomib with 80 μmol/L sulindac almost completely inhibited colony formation (Fig. 1D). Importantly, these biologically active concentrations are comparable to those achieved in serum in clinical use.

Anticancer effect of combination therapy against DLD-1 xenografts. We next explored whether the combination therapy is actually effective in nude mice. Based on several of our pilot studies, DLD-1 xenografts were treated with bortezomib (6 × 1.6 mmol) and/or sulindac (~3 μmol daily) once the tumor xenografts reached a minimal size of 60 ± 10 mm3 (~7 days after cell inoculation). Treatment with bortezomib alone had no distinct effect, and sulindac alone resulted in a partial tumor growth delay compared with control tumors, whereas combined therapy significantly suppressed tumor growth (Fig. 2A). On day
21, the mean tumor size and weight of the combined therapy group were significantly reduced compared with the other groups (P < 0.01 versus control, bortezomib alone or sulindac alone; Fig. 2B and C). Although sulindac alone exhibited a tendency toward tumor inhibition, it could not reduce the progression significantly [P = 0.074 (size) and P = 0.067 (weight) versus control]. Importantly, there were no apparent phenotypic abnormalities in all mice related to treatment. Representative H&E staining of liver, kidney, and stomach from control or combined therapy groups are shown in Fig. 2D. In addition, there was no distinct difference in body weight among the four groups (Fig. 2E).

**Nonsteroidal antiinflammatory drug–mediated reactive oxygen species generation.** To explore the molecular mechanism for the augmented effects of sulindac on bortezomib, we first investigated whether sulindac induces ROS generation. Because bortezomib induces apoptosis primarily mediated by an increase of ROS generation (22), sulindac may affect this activity. Although there have been several controversial results regarding ROS generation in NSAIDs-treated cells (23–25), we found that sulindac and its metabolites greatly elevated carboxy-H2DCFDA-detectable ROS levels (top left), whereas a COX-2 selective inhibitor (NS-398) and an anticancer agent (5-FU) induced much less ROS generation (top right) in DLD-1 cells (Fig. 3A). Time course experiments showed that the sulindac-mediated ROS generation was first detectable as early as 6 hours after exposure, peaked at 18 hours and declined gradually (bottom left), and the ROS levels increased in a dose-dependent manner (bottom right). In contrast, carboxy-H2DCFDA unlabelled cells exhibited no distinct signals (data not shown). This ROS generation may be cyclooxygenase-independent, because sulindac sulfone, having no inhibitory effect on cyclooxygenase enzymatic activity, induced extensive ROS generation (top left, Fig. 3A).

**Roles of reactive oxygen species generation and oxidative stress in combination therapy.** We also monitored superoxide anion (O2•−) generation by dihydroethidium, which selectively
detects \( \cdot O_2^\bullet \) (26). Treatment with 0.5 mmol/L sulindac or 20 nmol/L bortezomib for 36 hours clearly increased ROS generation (Fig. 3B), confirming ROS generation by sulindac or bortezomib in DLD-1 cells. It is noted that bortezomib-induced ROS (dihydroethidium-detectable) generation was greater than sulindac-induced ROS.

Although sulindac alone transiently increased ROS generation, bortezomib gradually increased ROS levels at 24 to 48 hours (top, Fig. 3C). At 36 hours, there was a distinct increase of ROS generation in combination-treated cells compared with individually treated cells (bottom), suggesting that combination therapy may maintain ROS at higher levels for longer. We next monitored expression of oxidative stress-inducible HO-1 (27). Although the level cannot directly link to the strength of oxidative stress, individual treatment with sulindac or bortezomib clearly induced HO-1 expression, but no synergistic effect was observed at 12 hours. At 24 hours after exposure, sulindac-induced HO-1 expression slightly decreased, but combination therapy further increased the expression (Fig. 3D). This enhanced expression of HO-1 was similarly observed in other combination therapy–treated cells, i.e., sulindac sulfide or sulfone with bortezomib. However, combination of NS-398 or 5-FU with bortezomib failed to increase HO-1 expression, and rather suppressed it (top, Fig. 3E). These data suggest that sulindac and bortezomib cooperatively increase intracellular oxidative stress in DLD-1 cells.

We next investigated whether combination therapy activates stress-activated mitogen-activated protein kinases. Previous reports suggest that oxidative stress introduces positive signals toward the mitogen-activated protein kinase p38 activation, and the activation is crucial for HO-1 induction (28). Bortezomib alone clearly induced JNK, but much less p38 phosphorylation. Although sulindac barely activated p38 and JNK, its combination with bortezomib clearly induced p38 phosphorylation (bottom, Fig. 3E). Thus, combination therapy preferentially augmented p38 activation, which may at least partially explain the reason why combination therapy further induced HO-1 expression.

We further investigated whether elevated ROS is crucial for combination therapy–induced cell death. To explore this, the free radical scavenger l-N-acetylcysteine (l-NAC) was incubated prior to the combination treatment. l-NAC almost completely inhibited the combination therapy–induced cell death and apoptosis evaluated by annexin V staining assay (Fig. 4A and B), implying that elevated ROS is necessary for the augmented death. l-NAC also reduced the enhanced ROS generation, although it did not completely block that (top, Fig. 4C). Interestingly, l-NAC almost completely inhibited bortezomib-mediated ROS (bottom) but barely affected sulindac-mediated ROS generation (data not shown). These data suggest that bortezomib-mediated ROS generation is sensitive to l-NAC, but sulindac-mediated ROS generation is not.

**Fig. 2.** Combination therapy–mediated tumor suppression. A, tumor progression of DLD-1 xenografts. DLD-1 xenografts were untreated (●), treated with bortezomib (▲), sulindac (▼) or both (●●) and evaluated by measurement of tumor volume every week. ***, P < 0.01 by ANOVA statistical analysis comparing four groups. After 3 weeks, mice were killed, and tumor volume (B) and tumor weight (C) were measured. D, histologic analysis. For each group, histology of at least four samples from the liver, kidney, stomach, and colon was analyzed after HE staining. Bars, 100 μm. E, body weights of all mice were measured at days 1 and 21. BC, and E, each group has six mice; columns, mean; bars, ± SE; **, P < 0.01 comparing other groups.
We next investigated the effect of a p38 inhibitor, SB203580, on the combination therapy-mediated augmented cell death because combined treatment strongly augmented p38 phosphorylation. The SB203580 treatment substantially inhibited p38 activation (Fig. 4D), but only slightly inhibited the augmented cell death (Fig. 4E), suggesting that ROS generation is primarily important but activation of p38 may be marginal for the augmented cell death.

Oxidative stress-mediated DNA damage. We next explored whether combination therapy causes DNA damage. H2AX phosphorylation is tightly linked to DNA damage (29, 30), and combination therapy strongly induced H2AX phosphorylation along with strong HO-1 induction, but far less phosphorylation was observed in cells treated individually, and both of them were highly sensitive to l-NAC (Fig. 5A). Confocal microscopy revealed that combined treatment induced H2AX phosphorylation more extensively than individual treatment (top, Fig. 5B). Importantly, most cells with increased phosphorylation of H2AX exhibited distinct accumulation of 8-hydroxy-2'-deoxyguanosine (bottom, Fig. 5B). Because 8-hydroxy-2'-deoxyguanosine is specifically generated by oxidative DNA damage (31), the increased DNA damage may be mostly caused by oxidative stress. This is strongly supported by the data that both events were almost completely inhibited by l-NAC.
We also found an increased phosphorylation of H2AX in xenografts receiving combination therapy (Fig. 5C).

Anticancer effect of combination therapy in BM314 cells. To address whether combination therapy is effective in other colon cancer cells, we tested their effect in a colon cancer BM314 cell line. Combination therapy strikingly augmented loss of mitochondrial membrane potential and cell death (Fig. 6A and B). In addition, the therapy strongly activated both p38 and JNK along with increased HO-1 expression (Fig. 6C). As observed in DLD-1 cells, these events were distinctly suppressed by t-NAC, indicating a pivotal role of oxidative stress in their augmented cell death.

We finally investigated effects of another free radical scavenger α-tocopherol on sulindac-induced ROS generation. The lipid soluble α-tocopherol prevents cellular membranes from lipid peroxidation (32). Interestingly, α-tocopherol, but not t-NAC, reduced the sulindac-induced ROS generation, although t-NAC more strongly reduced the bortezomib-induced ROS (Fig. 6D). In addition, α-tocopherol plus t-NAC almost completely blocked the bortezomib-induced ROS generation, and more strongly inhibited the combination therapy–mediated ROS generation than individual treatment (Fig. 6E), and most strikingly inhibited combination-induced apoptotic death (Fig. 6F).

In addition, we also observed similar anticancer effect of combination therapy in another colon cancer COLO201 cell line, i.e., augmented cell death, ROS generation, and HO-1 expression, and these events were clearly inhibited by t-NAC (data not shown). Taken together, NSAIDs and bortezomib may represent an oxidative stress-mediated combination therapy against a broad array of colon cancer cells.
Discussion

We have shown that sulindac and its metabolites strongly induce ROS generation in two colon cancer cells and clearly augment the anticancer effect of bortezomib. Although there are controversial data regarding ROS generation in sulindac-treated cells (23–25), we clearly show that they transiently but strongly induce ROS generation in colon cancer cells. Importantly, the combination therapy strongly inhibited tumor progression of DLD-1 xenografts, and its potential is strengthened because both drugs are clinically available and their effective concentrations are comparable to those used clinically.

We present that induction of ROS generation may be crucial in the sulindac-mediated anticancer effect. Sulindac dramatically elevates ROS generation 6 hours after exposure, but the elevation declines after 18 hours, and the time course is strikingly different from that of bortezomib-mediated ROS generation, which gradually increases after 24 hours incubation. In addition, they exhibited different sensitivity to l-NAC. These data suggest that they induce ROS generation probably via different molecular mechanisms. Recently, mitochondrial megachannel is suggested to be a source of bortezomib-mediated ROS generation (33). However, cyclosporin A, which inhibits the mitochondrial electron transport chain (34), could not affect sulindac-mediated ROS generation (data not shown). Thus, the involvement of mitochondrial channel may be marginal for the molecular mechanism(s) by which sulindac induces ROS generation. We found that α-tocopherol, but not l-NAC, distinctly inhibited sulindac-induced ROS generation, whereas l-NAC more strongly inhibited bortezomib-induced ROS generation. Considering their different antioxidant functions, i.e., l-NAC increases intracellular content of glutathione and alters redox balance, whereas α-tocopherol protects membranes from lipid peroxidation (32), our data suggest that sulindac may primarily affect membranes and increase lipid peroxidation-mediated ROS.

![Fig. 5. Combination therapy – mediated oxidative DNA damage in DLD-1 cells. A, effect of l-NAC on HO-1 induction. Cells were harvested after incubation with 0.5 mmol/L sulindac, 20 nmol/L bortezomib, or both in the presence or absence of l-NAC for 20 hours and subjected to Western blot analysis using anti-HO-1 antibody or anti-phospho H2AX antibodies. Hsc70 protein levels show the same amount of protein loaded in each lane. B, histone H2AX phosphorylation and 8-hydroxy-2′deoxyguanosine accumulation in cells incubated with 20 nmol/L bortezomib and/or 0.5 mmol/L sulindac in the presence or absence of l-NAC for 24 hours. Immuno-staining images of DLD-1 cells with anti-phospho H2AX (green) antibody alone (top) or with anti-phospho H2AX (green) and 8-hydroxy-2′deoxyguanosine (red) antibodies (merged pictures; bottom) were obtained by confocal laser microscopy. C, histone H2AX phosphorylation in xenografts. Frozen tissue sections of xenograft tumors treated as described in Fig. 2 were immunostained with anti-phospho H2AX antibody. B and C, no signals were detected after incubation with rabbit IgG (1 μg/mL) instead of specific antibodies as shown in sulindac/bortezomib (N). Bar, 20 μm.](https://www.aacrjournals.org/doi/10.1158/1078-0432.CCR-05-0027)
We showed that at 36 hours, combination therapy increased ROS levels compared with individual treatment, suggesting that the combination may maintain ROS at higher levels. Indeed, the combination further strongly induced HO-1 expression, activated stress-activated mitogen-activated protein kinases, and DNA damage, and these events were profoundly inhibited by L-NAC. However, it remains uncertain how these oxidative stresses confer their proapoptotic effects. Because inhibition of p38 activation failed to rescue the augmented cell death, oxidative DNA damage seems to be primarily important. In this context, it is noted that bortezomib alone increased phosphorylation of histone H2AX more strongly than sulindac alone. Consistently, bortezomib induced dihydroethidium-detectable ROS and HO-1 more greatly than sulindac.

In conclusion, our study identifies sulindac and its metabolites as strong ROS-inducing agents, and shows their synergistic effects on anticancer activity of bortezomib in colon cancer cells. Importantly, sulindac sulfone also induces extensive ROS generation, although it has no inhibitory effect on cyclooxygenase (35). This suggests that sulindac sulfone may induce far less mucosal damage and become an appropriate partner for bortezomib. Because combination therapy depends upon ROS generation, this is entirely different from conventional chemotherapies targeting DNA synthesis or cell division. Considering that cancer cells are under increased oxidative stress (36), our findings facilitate the development of improved anticancer strategies, especially against colon cancer cells.

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References


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