Synergistic Effect of Histone Deacetylase Inhibitors FK228 and m-Carboxycinnamic Acid Bis-Hydroxamide with Proteasome Inhibitors PSI and PS-341 against Gastrointestinal Adenocarcinoma Cells

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ABSTRACT

Purpose: We investigated whether the histone deacetylase inhibitors m-carboxycinnamic acid bis-hydroxamide (CBHA) and a bicyclic depsipeptide, FK228, can enhance the anticancer effect of the proteasome inhibitors PSI and PS-341 in gastrointestinal carcinoma cells.

Experimental Design: The anticancer effect of CBHA or FK228 and PSI or PS-341 was evaluated by cell death, caspase-3 activity, externalization of phosphatidylserine and DNA fragmentation, and colony formation assay. Expression of apoptosis-related molecules and cell cycle regulatory molecules, as well as phosphorylation of p38 were investigated by immunoblots. Generation of reactive oxygen species (ROS) was detected by intracellular oxidation of 5-(and-6)-carboxy-2,7'-dichlorodihydrofluorescein diacetate.

Results: CBHA or FK228 plus PSI or PS-341 synergistically induced apoptosis in human colon cancer DLD-1 and gastric MKN45 carcinoma cell lines. CBHA or FK228, but not 5-fluorouracil, plus PS-341 strongly decreased plating efficiency of DLD-1 cells. FK228 elicited ROS generation, and the free radical scavenger l-N-acetylcysteine inhibited the synergistic anticancer effect of combined therapy. In addition, l-N-acetylcysteine inhibited the combined therapy-mediated elevation of a proapoptotic BH3-only protein Bim expression, phosphorylation of H2AX, and accumulation of 8-hydroxydeoxyguanosine.

Conclusions: FK228 or CBHA and PSI or PS-341 synergistically induce apoptosis in DLD-1 and MKN45 cells depending on ROS-mediated signals. Our data suggest that a combination of FK228 or CBHA with PSI or PS-341 may be a valuable therapy against gastrointestinal adenocarcinoma cells.

INTRODUCTION

The proteasome is an essential enzyme complex for the nonlysosomal, ATP-dependent proteolytic pathway, which catalyzes the rapid degradation of a variety of short-lived functional proteins. This pathway is pivotal in cell cycle progression, cytokine-mediated transcription, and apoptosis (1–3). It is also essential for the rapid elimination of impaired proteins, which arise from oxidation stress, heat damage, and transcription of mutant genes (4, 5). The N-benzyloxycarbonyl-Ile-Glu(O-tert-buty)-Ala-leucinal, PSI, and a dipeptide boronic acid analog, PS-341, specifically inhibit the chymotrypsin-like activity of the proteasome, probably because of tight binding to the NH₂-terminal catalytic site, and, consequently, function as highly specific nuclear factor κB inhibitors (6–8). They represent anticancer agents against a variety of malignant cells; for example, they strongly inhibit cell cycle progression and induce apoptosis not only in vitro but also in vivo. PS-341 has been shown recently to overcome drug resistance in human myeloma cells and to be highly effective in such patients (9, 10). Although PSI and PS-341 display marked anticancer activity in hematopoietic cells, they possess much less anticancer activity against solid tumor malignant cells. For instance, 0.1 μM PS-341 almost completely inhibits cell cycle progression of multiple myeloma cells but does not affect significantly that of non-small cell lung cancer cells (7, 11). Thus far, the molecular mechanism(s) for their anticancer effect remains unknown, although there have been many reports suggesting the mechanism. PS-341 causes p53, p21WAF1, MDM2, MDM3, cyclin A, and cyclin B to accumulate and induces the phosphorylation of Bcl-2, resulting in G2-M-phase arrest and apoptosis (7, 11, 12). More importantly, PS-341 initiates generation of reactive oxygen species (ROS), and this effect may be critical in initiating PS-341-mediated apoptosis (13).

Histone deacetylase (HDAC) inhibitors hyperacetylate histones and increase transcription activity in selected genes through a relative increase of acetyltransferase activity due to inactivation of HDAC (14, 15), and, importantly, they strongly induce apoptosis (16, 17). Although molecular mechanism(s) for their biological actions remain obscure, transcriptional activation of selected genes may be crucial for cell cycle arrest and apoptosis induction. For instance, they strongly increase expression of p21WAF1 in various cell types (18). Thus, we hypothe-
sized that HDAC inhibitors and proteasome inhibitors cooperatively function against various cancer cells. In this article, we report that the HDAC inhibitors m-carboxycinnamic acid bis-hydroxamide (CBHA; Ref. 19) and FK228 (20), but not 5-fluorouracil (5-FU), can augment proteasome inhibitors PSI and PS-341-induced cell death in gastrointestinal MKN45 and DLD-1 adenocarcinoma cells. They synergistically induce expression of a BH3-only protein Bim (21) and p21, as well as phosphorylation of p38. In addition, these synergistic effects are sensitive to the free radical scavenger l-N-acetylcysteine (l-NAC; Ref. 22), suggesting that the synergistic effect may be linked to ROS-mediated stress and subsequent accumulation of DNA damage, probably in concert with Bim-mediated apoptotic signals.

MATERIALS AND METHODS

Cell Culture. Human gastric MKN45 and colorectal DLD1 adenocarcinoma cells, obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), were grown in RPMI 1640 supplemented with 10% FCS. To evaluate cell viability, cells were mixed with the same volume of 0.4% trypan blue solution, and their ability to exclude the dye was immediately examined.

Reagents and Antibodies. Two different HDAC inhibitors, CBHA and FK228, were obtained from Calbiochem-Novabiochem Co. (Darmstadt, Germany) and kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan), respectively. The l-NAC was purchased from Sigma. The anti-Bcl-XL and p21WAF antibodies were purchased from BD PharMingen (San Diego, CA). The anti-Bim, p27, and HSC70 antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-p38, phospho-p38, and acetyl-histone H4 antibodies were from Cell Signaling (Beverly, MA). The anti-phospho-H2A.X antibody was from Upstate (Charlottesville, VA).

Annexin V Binding Analysis. As an early marker of apoptosis, annexin V binds to negatively charged phosphatidylserines. Cells (1 × 10⁶) were incubated with green fluorescent protein-annexin V for 30 min, washed, and incubated addition-

![Figure 1](synergistic-effect-of-hdac/proteasome-inhibitors.png)

Fig. 1 Synergistic effect of m-carboxycinnamic acid bis-hydroxamide (CBHA) or FK228 with PSI in DLD-1 cells. A, histone hyperacetylation. Cells were incubated with the indicated concentrations of CBHA (μm) or FK228 (nm). After 24 h, histones were separated on acid-urea-acrylamide gels (stained in 0.25% Coomassie Blue), and the arrows indicate unacetylated histone H4, whereas their acetylation is shown as super-shifted bands. After 12 h, lysates were harvested to evaluate acetylation of histone H4 (Ac-H4) by Western blots. B, HSC70 protein levels show the same amount of protein loaded in each lane. C, CBHA or FK228 and PSI synergistically induced cell death. After treatment with 1 μm CBHA, 5 nm FK228, or 10 μm 5-fluorouracil (5-FU) in the presence (●) or absence (▲) of 10 nm PSI for 2 days, cell death was evaluated by trypan blue exclusion assay. D, caspase-3 activity. After incubation with 1 μm CBHA or 10 μm 5-FU in the presence (●) or absence (▲) of 10 nm PSI for 36 h, caspase-3 activity was measured. In C and D, columns display the data from three separate experiments; bars, ±SD.
ally with propidium iodide for 15 min following the manufacturer’s protocol (MBL, Nagoya, Japan). Cells were analyzed for cellular fluorescence by fluorescence-activated cell sorter flow cytometry (Becton Dickinson, Mountain View, CA) using CellQuest Software.

**DNA Fragmentation Assay.** As described previously (23), low molecular weight genomic DNA extracted with the lysis buffer (0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris-HCl (pH 7.4)) was treated with 400 μg/ml RNase A and proteinase K for 1 h at 37°C, isopropanol precipitated, and subjected onto 1.0% agarose gels. The gels were stained with 1 μg/ml ethidium bromide.

**Western Blotting.** Cells were lysed by adding 200 μl of radioimmunoprecipitation assay buffer (100 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% NP40, and 50 mM Tris-HCl (pH 7.2)), and protein concentration of these lysates was evaluated using a protein assay (Bio-Rad, Melville, NY). The lysates (80 μg/lane) were separated by 10%–15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 20 V for 70 min. After incubation in Block Ace (Dainippon Pharmacia Co., Tokyo, Japan) overnight at 4°C, the membranes were incubated with primary antibodies overnight at 4°C. After washing with the washing buffer (140 mM NaCl, 25 mM Tris-HCl (pH 7.8), and 0.05% Tween 20), they were incubated with the corresponding peroxidase-linked secondary antibodies (Amersham, Piscataway, NJ) for 1 h at room temperature. Signals were developed by a standard enhanced chemiluminescence method following the manufacturer’s protocol (Amersham).

**Histone Preparation and Gel Fractionation.** As described previously (24), cells (5 × 10^6) were washed and lysed in ice-cold lysis buffer [10 mM Tris (pH 8), 50 mM NaCl, 0.1% Triton X-100, 10 mM MgCl2, 8.6% sucrose, and 0.5 mM DTT]. Nuclei were collected by centrifugation for 5 min at 6000 rpm in a microcentrifuge and incubated with 0.4 M sulfuric acid. Soluble fractions were precipitated by addition of 10 volumes of acetone and incubation at −20°C. Histone acetylation was evaluated by fractionating histones on acid/urea/acylamide gels,

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**Fig. 2** FK228 augments strongly PSI-induced apoptosis in DLD-1 cells. A, dose-response analysis for the effect of PSI on survival fraction in the presence (●) or absence (□) of 5 nM FK228 at 48 h-incubation. FK228 decreased the LD50 of PSI from 25 to 4.8 nM. B, time course analysis. After incubation with 5 nM FK228 plus 10 nM PSI for the indicated hours, cell death was evaluated by trypan blue exclusion assay. C, DNA fragmentation. Low molecular weight DNA was prepared at 48 h after incubation with or without 10 nM PSI and/or 5 nM FK228. Molecular weight marker (kb) is shown. D, annexin V staining assay. Cells were incubated with or without 10 nM PSI and/or 10 nM FK228 for 42 h and additionally incubated with green fluorescent protein-annexin V plus propidium iodide. Numbers indicate percentage positive of total cells. E, caspase-3 activity was measured at 12 (□) or 24 (■) h after the indicated treatment (10 nM PSI and/or 5 nM FK228). Each column displays the data from three separate experiments; bars, ±SD.
which were stained in 0.25% Coomassie Blue/10% acetic acid/40% methanol.

**Reverse Transcription-PCR.** Total RNA of DLD1 cells was extracted with TRIzol (Life Technologies, Inc., Rockville, MD). Bim cDNAs were amplified from 0.5 μg total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) with oligodeoxythymidines 12–18 and TOPO TA Cloning kit (Invitrogen). The following primer pairs were used for reverse transcription-PCR: glyceraldehyde-3-phosphate dehydrogenase, 5′-cgaccactttgtcaagctca-3′, and 5′-aggggtctacatggcaactg-3′; and Bim, 5′-atggcaaagcaaccttctga-3′ and 5′-tcaatgcattctccacacca-3′.

**ROS Detection.** After treatment, cells were incubated with 5 μM 5(and 6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate C-400 (Invitrogen) for 30 min, after which, they were washed and incubated additionally with complete medium for 4 h. ROS generation was determined using a FACScan flow cytometer.

**Colony Assay.** For colony formation assay, 3 × 10³ cells were seeded into 60-mm plates and cultured with the indicated treatment for 2 days, washed out, incubated additionally for 8 days, and the number of colonies (>50 cells) were counted. The data represent the mean ± SD of three independent experiments. Survival fraction was defined as number of colonies divided by the number of plated cells.

**Confocal Microscopy.** DLD-1 cells were cultured on BSA-coated coverslips overnight and exposed to the indicated treatment for 24–48 h. Cells were washed with cold PBS and, thereafter, fixed with cold methanol for 10 min. After blocking nonspecific binding capability by 5% BSA, sections were incubated with anti-phospho-H2AX polyclonal and anti-8-hydroxydeoxyguanosine (8-OHdG) monoclonal N45.1 (Japan Institute for the Control of Aging, Fukuroi, Japan) antibodies for 30 min. After washing with PBS, sections were incubated with Alexa Fluor 488 goat antirabbit IgG and Alexa Fluor 594 goat antimouse IgG antibodies (Invitrogen) for 30 min. Confocal imaging was performed using a ZEISS Pascal laser-scanning microscope.

**RESULTS**

**FK228 and CBHA Augmented PSI-Induced Apoptosis in DLD-1 Cells.** To identify agents that enhance the anticancer effect of PSI, we first screened a variety of chemical agents and determined that several HDAC inhibitors strongly enhanced PSI-induced cell death (data not shown). HDAC inhibitors increase acetylation of histones by inhibiting deacetylase activity, and we first determined the concentrations of HDAC inhibitors required for distinct histone hyperacetylation in DLD-1 cells. Exposure to the HDAC inhibitors, CBHA (19),
or a bicyclic depsipeptide FK228 (20) for 24 h clearly increased histone acetylation at >2 μM and 5 nM concentrations, respectively (Fig. 1A). We confirmed additionally hyperacetylation of histone H4 by Western blot analysis, showing that the hyperacetylation was apparent after 12 h treatment with >0.5 μM CBHA or 1 nM FK228 (Fig. 1B). Although these reagents alone at these concentrations did not induce clearly cell death, they augmented strongly PSI-induced cell death, and 10 nM PSI alone induced only slightly cell death (Fig. 1C). The observed cell death with combination therapy exceeded the calculated additive effect. For example, calculated survival rate = 0.94 ± 0.01 (survival rate of FK228 treatment) × 0.92 ± 0.01 (survival rate of PSI treatment) and calculated apoptosis rate = 1 – 0.94 × 0.92 = 0.14. However, their combination caused a much greater apoptosis rate, 0.71 ± 0.07, indicating that combination therapy has synergistic anticancer activity. In addition, 1 μM CBHA augmented strongly PSI-induced caspase-3 activity, but CBHA alone did not (Fig. 1D). In contrast, 10 μM 5-FU combined with 10 nM PSI, neither augmented cell death nor caspase-3 activity (Fig. 1, C and D). Thus, CBHA and FK228 augment selectively PSI-mediated apoptosis in DLD-1 cells.

As shown in Fig. 1C, FK228 enhanced PSI-induced cell death much more strongly than CBHA. Thus, we investigated additionally their synergistic effects on DLD1 cells. Dose-response analysis demonstrated that the LD₅₀ for PSI was 26 nM in the absence of FK228 but only 5 nM in its presence (Fig. 2A). Time course analysis revealed that cell death first became apparent 36 h after treatment with 10 nM PSI plus 5 nM FK228 and affected ~80% of total cells at 60 h (Fig. 2B). Although the combined treatment exhibited extensive DNA fragmentation at 48 h (Fig. 2C), agents used alone did not induce distinct DNA fragmentation. Importantly, annexin V binding assay revealed that the agents administered individually induced minimal apoptosis in DLD-1 cells, whereas the combination produced 24.6% apoptosis but far less necrosis (5.1%) at 42 h (Fig. 2D). It is noted also that the combined treatment had increased already caspase-3 activity at 24 h, although cell death was not apparent yet (Fig. 2E). These results indicate that CBHA or FK228-mediated enhanced cell death in PSI-treated DLD-1 is followed by caspase-3 activation and annexin V binding, suggesting a cooperative function in apoptosis.

CBHA/FK228 and PSI Synergistically Induced Apoptosis in MKN45 Cells. Having demonstrated that FK228 and CBHA augment strongly PSI-mediated apoptosis in DLD-1 cells, we examined their effect in other cell types. In gastric carcinoma MKN45 cells at 24 h-incubation, histone hyperacetylation was clearly induced by 2 μM CBHA or 25 nM FK228 (Fig. 3A). After treatment with 4 μM CBHA or 50 nM FK228 alone for 48 h, no apparent cell death was observed; however, they both strongly augmented 10 nM PSI-induced cell death (Fig. 3B). Time course experiments showed that the CBHA-mediated effect became apparent 36 h after treatment (Fig. 3C). A dose-response analysis of CBHA demonstrated that its augmented effect required >2 μM of CBHA and strengthened in a dose-dependent manner (Fig. 3D). We next investigated whether the augmented cell death arose from increased apoptosis. At 24 h, 4 μM CBHA or 50 nM FK228 with 10 nM PSI induced some cell death (Fig. 3C), but had already increased caspase-3 activity (Fig. 3E) and induced extensive DNA fragmentation in MKN45 cells (Fig. 3F). Any of the drugs used alone had far less effect
on caspase-3 activity and DNA fragmentation. Thus, as in DLD-1 cells, CBHA or FK228 with PSI synergistically induced apoptosis in MKN45 cells, suggesting that the combined treatment may have strong proapoptotic effects in these gastrointestinal cancer cells.

**Combination of FK228/CBHA with PS-341 Synergistically Induced Apoptosis.** PSI specifically inhibits proteasome activity and may modify a variety of intracellular signals through mostly accumulation of selected proteins. If our findings are linked to inhibition of proteasome functions, FK228 and CBHA are believed to similarly augment proapoptotic activity of other proteasome inhibitors. A proteasome inhibitor, PS-341, has been used clinically recently as an anticancer agent in patients with multiple myeloma (9). We tested whether FK228 and CBHA can augment PS-341-mediated apoptosis in DLD-1 and MKN45 cells. In DLD-1 cells, CBHA or FK228, but not 5-FU, dramatically augmented PS-341-induced cell death (Fig. 4A) and caspase-3 activation (Fig. 4B). Dose-response curves clearly demonstrate that 10 nM FK228 extensively augmented PS-341-mediated cell death at >10 nM concentrations of PS-341, which alone only faintly induced cell death (Fig. 4C). Similarly, CBHA or FK228 strongly augmented PS-341-induced cell death (Fig. 4D) and caspase-3 activation in MKN45 cells (Fig. 4E). Thus, any of these combinations (CBHA or FK228 plus PSI or PS-341) synergistically induce apoptosis in DLD-1 and MKN45 cells, suggesting cooperative functions of these HDAC and proteasome inhibitors in apoptotic pathways.

**Combined Treatment Strongly Inhibited Colony Formation of DLD-1 Cells.** The observed effects of CBHA and FK228 suggest that combination therapy might reduce tumor progression. We determined the effect of FK228 or CBHA with or without PSI or PS-341 on the colony formation of DLD-1 cells. DLD-1 cells were treated with either a combination or a single reagent for 48 h, washed, and incubated additionally to allow colony formation. Combined treatment (CBHA or FK228 and PS-341) inhibited significantly colony-forming ability, and at least 75% fewer colonies were detected in each of the three independent plates tested (P < 0.01). Any of them used alone or the combination of 5-FU with PS-341 caused a slight delay of colony formation (Fig. 5).

**Fig. 5** Colony formation of DLD-1 cells. Cells were seeded in 60-mm culture dishes (3000 cells/dish) and treated with the indicated drugs, i.e., 0.5 μM CBHA, 2.5 nM FK228, or 2.5 μM 5-fluorouracil (5-FU), in the absence or presence of 10 nM PS-341 or 10 nM PSI for 48 h. After washing, cells were incubated additionally for 8 days, and colonies were counted. Columns display the data from three separate experiments; bars, ±SD. Representative results are shown in the bottom panels.

**Fig. 6** Combination therapy-mediated modulation of signaling molecules. A, cell cycle regulating molecules. DLD-1 cells were incubated with the indicated reagents, i.e., 10 nM FK228, 1 μM CBHA, 10 nM PS-341, and/or 10 μM 5-fluorouracil (5-FU) for 24 h. B, p38 mitogen-activated protein kinase. DLD-1 cells were incubated with the indicated reagents, i.e., 10 nM FK228, PS-341, PSI, and/or 2 μM SB203580 for 24 h. C, up-regulation of Bim expression. DLD-1 and MKN45 cells were incubated with 1 μM and 4 μM CBHA, respectively, in the presence or absence of 10 nM PS-341 for 24 h. In A–C, total cell lysates were analyzed by Western blots. Bcl-xl, p38, or HSC70 protein levels show the same amount of protein loaded in each lane. D, increase of Bim mRNA transcripts. Bim cDNAs were amplified from total RNA of DLD-1 cells treated with 1 μM CBHA in the presence or absence of 10 nM PS-341 for 24 h using reverse transcription-PCR technique. As a control experiment, reverse transcription-PCR was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) paired primers. Molecular weight marker (bases) is shown. PCR products were sequenced and confirmed as human Bim cDNAs.
colony formation but did not reduce significantly colony numbers compared with control groups ($P = 0.4$; Fig. 5). We confirmed also that another combination (CBHA and PSI) therapy reduced similarly colony formation ($P < 0.01$), implying that these combinations (FK228 or CBHA and PSI or PS-341 but not 5-FU and PS-341) are potentially potent anticancer treatments against gastrointestinal carcinoma cells.

**Combined Treatment Strongly Increased Bim Expression.** To explore the molecular mechanism(s) by which FK228 or CBHA and PSI or PS-341 cooperatively induce apoptosis, we investigated first whether combined therapy affected cell cycle regulatory proteins. These inhibitors are known to induce p21WAF accumulation (11, 25). In DLD-1 cells, p21WAF expression was clearly elevated by FK228 or CBHA alone, whereas the effect of PS-341 was considerably less pronounced (Fig. 6A). Combined treatments (FK228 or CBHA + PS-341) caused a slight additional increase in p21 expression. In contrast, 5-FU alone or its combination with PS-341 had no significant effect on p21 expression. PS-341 alone increased p27 expression, but there was no additional effect on its expression in cells treated with its combination with FK228 or CBHA (Fig. 6A). Thus, an increase of p21WAF expression appears to be associated primarily with the augmented effect of combination therapy.

We next investigated whether combination treatment affects stress-related mitogen-activated protein kinase signaling molecules (26). Combined treatments markedly increased p38 mitogen-activated protein kinase phosphorylation, but single agents had only a small effect (Fig. 6B). Thus, p38 activation may contribute also to cooperative apoptosis in combined treatment. This p38 activation may arise from activation of p38$\alpha$ or p38$, because a p38$\alpha$ inhibitor, SB203580, did not strongly inhibit the combination therapy-induced p38 activation (27). We found also that combination therapy did not affect extracellular signal-regulated kinase and c-Jun NH$_2$-terminal kinase activity extensively (data not shown), suggesting their marginal effect on augmented cell death.

We next monitored the expression of apoptosis-related molecules, such as Bid, Bax, Bad, Bmf, Bcl-2, FLIP, and IAP-1, in cells treated with FK228 or CBHA. Consistent with previous reports that HDAC inhibitors do not significantly modulate expression of proapoptotic molecules (28), these molecules were barely detectable or not affected by FK228, CBHA, PS-

![Fig. 7](http://example.com/image.png) Reactive oxygen species (ROS)-mediated DNA damage in combination therapy-treated DLD-1 cells. A, ROS generation. After incubation with 10 nM FK228 and/or PS-341 for 40 h, cells were exposed to 10 $\mu$M C400 for 30 min, incubated additionally for 3 h, and thereafter, ROS generation was measured. B, inhibitory effect of L-N-acetylcysteine (L-NAC) on combination therapy-mediated cell death. After incubation with 10 nM FK228, PS-341, and/or PSI in the presence (●) or absence (○) of 10 nM L-NAC for 48 h, cell death was evaluated by trypan blue exclusion assay. In A and B, each column displays the data from three separate experiments; bars, ±SD. C, effect of L-NAC on Bim expression. After treatment with 10 nM FK228, PS-341, and/or PSI in the presence or absence of 10 nM L-NAC for 24 h, total cell lysates were analyzed by Western blots. D, phosphorylation of H2AX. After incubation with the indicated reagents (10 nM FK228, PS-341, and/or 10 nM L-NAC) for 24 h, cells were fixed at 30 min with methanol at $-20^\circ$C and immunostained by antiphosphorylated H2AX antibody. Images were obtained by confocal laser microscopy. Bar, 10 $\mu$M.
341, and/or PSI in MKN45 and DLD-1 cells (data not shown). Therefore, it is unlikely that these apoptosis-related molecules are central to the synergistic effects of combination treatments. However, we found that CBHA increased Bim expression in both MKN45 and DLD-1 cells at 24 h and increased additionally its expression when combined with PS-341 (Fig. 6C). CBHA alone or CBHA plus PS-341 also increased Bim mRNA expression (Fig. 6D), thereby suggesting a possible transcriptional activation in elevating Bim proteins. These data implicate Bim in combination therapy-mediated augmented cell death.

**The L-NAC Antagonized Proapoptotic Activity of Combination Therapy.** PS-341 and a HDAC inhibitor, MS-275, were found recently to induce ROS generation, which is suggested to be crucial in their anticancer functions (29, 30). Therefore, ROS generation may be necessary for the synergistic effect of combined therapy. Indeed, we detected increased ROS generation in DLD-1 cells treated with FK228 or FK228 plus PS-341 (Fig. 7A). Furthermore, the free radical scavenger L-NAC substantially suppressed augmented cell death in DLD-1 cells treated with FK228 plus PS-341 or PSI (Fig. 7B), indicating that ROS generation is crucial for their synergistic effects. We investigated additionally the effects of L-NAC on several signals linking combination therapy. The L-NAC barely or only slightly inhibited p21 accumulation and augmented p38 phosphorylation, whereas it extensively inhibited the enhanced Bim expression in the treated cells (Fig. 7C). Furthermore, H2AX phosphorylation was extensively detected in DLD-1 cells receiving combination therapy, but far less phosphorylation was observed in cells treated individually. More importantly, L-NAC almost completely blocked the observed H2AX phosphorylation (Fig. 7, C and D). Because H2AX phosphorylation is an initial event linking to DNA damage (31), this may reflect the increase of intracellular ROS levels. To explore this possibility, we investigated additionally whether H2AX phosphorylation was accompanied by accumulation of 8-OHdG, which is specifically generated by oxidative DNA damage (32, 33). Confocal microscopy revealed that H2AX phosphorylation was tightly associated with accumulation of 8-OHdG and was clearly inhibited by L-NAC (Fig. 8). These data suggest strongly that ROS-mediated oxidative DNA damage may, at least partially, explain the synergistic anticancer effects of FK228 and PS-341.

**DISCUSSION**

We demonstrate that FK228 or CBHA combined with PSI or PS-341 synergistically induce apoptosis in human gastroin-
testinal DLD-1 and MKN45 carcinoma cells. These inhibitors are considered to be promising anticancer agents against hematological malignant cells. For example, PS-341 is used clinically in multiple myeloma, whereas FK228 is in Phase II clinical trials as an agent against T-cell leukemia cells (34–36). However, they exhibit weaker anticancer effects against gastrointestinal carcinoma cells and are still under investigation regarding whether they can be useful for those cancer cells. Our findings support their use in combination therapy against adenocarcinoma cells.

We show that their synergistic effects are specific, because both FK228 and CBHA enhance PSI or PS-341-mediated pro-apoptotic activity, but 5-FU does not, suggesting that they act cooperatively to induce cell death in DLD-1 and MKN45 cells. To clarify the molecular mechanism(s) for its action, we investigated several signals linking apoptosis and cell cycle progression. Combination of FK228 with PS-341 synergistically increased p21 accumulation, p38 phosphorylation, and Bim expression compared with individually treated cells. The increase in Bim expression may be especially important for augmented cell death, because l-NAC substantially inhibited augmented cell death, whereas p21 accumulation and p38 phosphorylation were barely affected. However, it is possible that the apparent synergistic effect of combination therapy actually represents a mechanistic change in the effects of single agent. In addition, we demonstrated that l-NAC inhibited partly but clearly both augmented cell death and oxidative DNA damage as indicated by accumulation of 8-OHdG. Thus, ROS-mediated signals may contribute also to the augmented cell death probably independently of Bim-mediated signals. It has been demonstrated that PS-341 induces ROS generation when it causes cell death (29). Here, we demonstrate that FK228 alone, but not PS-341, at a subtoxic concentration clearly increases ROS generation in DLD-1 cells. However, combined treatment had no synergistic effect on ROS generation, and thus, ROS generation may be mostly because of FK228 actions. Because FK228 alone did not induce apparent cell death, the elevated ROS may be insufficient to induce cell death. This suggests that PS-341 may have additional actions that augment death signals. In understanding the PS-341-mediated contribution to augmented cell death, it is of interest that PS-341 alone increased slightly 8-OHdG accumulation (Fig. 8), although it did not definitely induce ROS generation (Fig. 7). This allows us to consider that PS-341 may affect DNA repair functions. Consistent with this idea, a recent study showed that PS-341 down-regulates expressions of several molecules involved in base-excision repair to protect from oxidative DNA damage such as 8-OHdG DNA and uracil-DNA glycosylases (37).

Importantly, combination therapy augmented clearly cell death in two different carcinoma cells, colonic DLD-1 and gastric MKN45. This suggests that combination therapy may be effective in at least these cancer cell types. Relevant to this is a recent article reporting a similar synergistic effect of PS-341 with two HDAC inhibitors, SAHA and sodium butyrate, in leukemia cells (38). These accumulating data imply that combination therapy may become a central strategy against a broader array of cancer cell types.

In our studies, FK228 is more potent in DLD-1 cells than in MKN45 cells, but CBHA has comparable potential in both cells. This suggests that HDAC inhibitors affect differently cancer cells in a cell type-specific manner. To date, at least 11 isoforms and their alternative splicing variants have been identified (39, 40), but it remains largely unknown how they are regulated and what their precise functions are. In this regard, it is crucial to clarify these unsolved questions and to determine their sensitivity to each HDAC inhibitor to discover more appropriate combination therapy.

Histone hyperacetylation generally activates transcription of several selected genes. In this article, we show that FK228 or CBHA alone increases slightly Bim expression and increases additionally its expression in concert with PSI or PS-341. This may, at least partially, explain the mechanism by which combined treatment synergistically induces apoptosis in MKN45 and DLD1 cells. In fact, overexpression of Bcl-2 or Bcl-XL strongly inhibited combination therapy-mediated cell death (data not shown). Thus, Bim-mediated mitochondrial damage may be important for augmented cell death. However, it remains unknown how Bim is induced, although because its induction was clearly inhibited by l-NAC, the induction must depend on ROS generation. It is of great interest how ROS generation and histone hyperacetylation are linked to Bim induction.

FK228 is the most promising HDAC inhibitor, because systemic administration can achieve biologically active serum concentrations according to Phase I clinical trials (34). Phase II trials are ongoing, although several dose-limiting toxicities occurred in some cases, for example, fatigue, nausea, transient thrombocytopenia, and neutropenia. PS-341 has been used clinically for myeloma patients and exhibits a strong anticancer activity. Importantly, the concentrations of FK228 and PS-341 used in our study are comparable with the levels in plasma at therapeutic doses. The mean maximum plasma concentrations of FK228 and PS-341 at maximum tolerated doses are 472.6 ng/ml (Ref. 34; ~0.6 μM) and 509 ng/ml (Patient information from MILLENNIUM; 1.3 μM), respectively. In this regard, PS-341 and FK228 appear to be the most promising combination. Additional investigation of this novel combination therapy in patients with adenocarcinoma and hematological malignancies may demonstrate high potency and provide a new anticancer strategy.

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REFERENCES

6. Traenckner EB, Wilk S, Baueerle PA. A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated
form of I kappa B-alpha that is still bound to NF-kappa B. EMBO J 1994;13:5433–41.
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