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Combined Effects of Sulindac and Suberoylanilide Hydroxamic Acid on Apoptosis Induction in Human Lung Cancer Cells

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ABSTRACT

Histone deacetylase (HDAC) inhibitors represent a promising group of anticancer agents. Treatment of cancer cells with HDAC blockers, such as suberoylanilide hydroxamic acid (SAHA), leads to the activation of apoptosis-promoting genes. To enhance pro-apoptotic efficiency, SAHA has been used in conjunction with radiation, kinase inhibitors, and cytotoxic drugs. In the present study, we show that at the suboptimal dose of 250 μM, sulindac [2-[6-fluoro-2-methyl-3-[(4-methylsulfinylphenyl)methylidene]inden-1-yl]-acetic acid] significantly enhances SAHA-induced growth suppression and apoptosis of A549 human non–small cell lung cancer cells, primarily via enhanced collapse of the mitochondrial membrane potential, release of cytochrome *c*, and caspase activation. Furthermore, sulindac/SAHA cotreatment induced marked down-regulation of survivin at both the mRNA and protein levels and stimulated the production of reactive oxygen species (ROS), which were blocked by the antioxidant *N*-acetyl-L-cysteine. Overexpression of survivin was associated with reduced sulindac/SAHA-induced apoptosis of A549 cells, whereas suppression of survivin levels with antisense oligonucleotides or small interfering RNA further sensitized cells to sulindac/SAHA-induced cell death. Our results collectively demonstrate that sulindac/SAHA-induced apoptosis is mediated by ROS-dependent down-regulation of survivin in lung cancer cells.

Despite ongoing efforts of clinicians to find effective treatments for lung cancer, the leading cause of tumor-related mortality, its incidence and associated death rates continue to rise (Sandler and Dubinett, 2004). Although combination chemotherapy constitutes a major part of the treatment program for patients with inoperable lung cancer, improvements in treatment efficacy, even with newly developed anticancer agents, have been unsatisfactory to date (Rigas, 1998). Recent efforts have focused on identifying a novel combination of anticancer agents with nonoverlapping mechanisms of action to obtain optimal efficacy and reduced toxicity (Rigas and Lara, 2005).

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Aberrant histone acetyltransferase or histone deacetylase (HDAC) activity is reported in numerous cancers. Histone acetyltransferase inactivation is associated with oncogenesis, and abnormal HDAC activity is associated with the transcriptional repression of specific tumor suppressor genes, thus contributing to tumor formation (Karagiannis and El-Osta, 2006). In view of these findings, several HDAC inhibitors have been developed as potential cancer-targeting therapeutics. These compounds induce differentiation, cell growth, cell cycle arrest, and, in certain cases, apoptosis, in numerous transformed cell lines, both in culture and in vivo (Kelly et al., 2002; Marks et al., 2004; Karagiannis and El-Osta, 2006). Several HDAC inhibitors are currently in clinical trials. Promising anticancer effects at well-tolerated doses have been observed in phase I and II trials, particularly with suberoylanilide hydroxamic acid (SAHA) (Kelly et al.,

ABBREVIATIONS: HDAC, histone deacetylase; SAHA, suberoylanilide hydroxamic acid; NSCLC, non–small cell lung cancer; siRNA, small interfering RNA; ROS, reactive oxygen species; NSAID, nonsteroidal anti-inflammatory drug; NAC, *N*-acetyl-l-cysteine; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; PI, propidium iodide; DCFH-DA, dichlorofluorescein diacetate; MMP, mitochondrial transmembrane potential; RT-PCR, reverse transcription-polymerase chain reaction; IAP, inhibitor of apoptosis protein; XIAP, X-linked inhibitor of apoptosis protein.

2003; O'Connor et al., 2006; Duvic et al., 2007). However, non–small cell lung cancer (NSCLC) is resistant to HDAC inhibitors, such as trichostatin A, sodium butyrate, and SAHA (Mayo et al., 2003; Rundall et al., 2005). In a preliminary phase I clinical trial, patients with solid tumor malignancies, including NSCLC, were treated with the HDAC inhibitor pivaloyloxymethyl butyrate (Pivanex). It is noteworthy that the drug was well tolerated by patients, but little incidental clinical response was observed (Patnaik et al., 2002).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are effective agents for the treatment of various cancer types. NSAIDs inhibit cell cycle progression and induce apoptosis in several cancer cells (Piazza et al., 1997; Subbegowda and Frommel, 1998; Grösch et al., 2001). To date, several molecular mechanisms underlying the proapoptotic effects of NSAIDs have been identified. The biochemical mechanism generally ascribed to this effect is the inhibition of cyclooxygenase enzymes, which catalyze the initial step in prostaglandin synthesis (Thun et al., 2002; Dannenberg and Subbaramaiah, 2003). However, NSAIDs exert proapoptotic effects on cancer cells containing neither cyclooxygenase-1 nor cyclooxygenase-2, indicating that other mechanisms are additionally involved, such as reactive oxygen species (ROS) production and inhibition of nuclear factor κB-mediated signals (Tegeder et al., 2001; Seo et al., 2007). The chemopreventive mechanism of NSAIDs is currently a subject of controversy, and it is uncertain whether sulindac can be used as an effective anticancer agent. Recent studies show that anticancer drugs such as cisplatin, paclitaxel, or docetaxel, in combination with sulindac metabolites, synergistically inhibit lung cancer growth (Soriano et al., 1999; Bunn et al., 2002; Jones et al., 2005). Moreover, sulindac enhances the potency of other chemotherapeutic agents, including arsenic trioxide (Jin et al., 2006).

In the present study, we examined the combined effects of sulindac and SAHA in a lung carcinoma cell line. Sulindac further promoted SAHA-induced apoptosis of A549 cells by down-regulating survivin expression. Thus, although sulindac itself is a chemopreventive agent, it may additionally be applied to enhance the effects of the anticancer agent SAHA via promotion of ROS production.

Materials and Methods

Cell Cultures and Reagents. A549 and NCI-H460 human NSCLC cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in the recommended growth medium (Invitrogen, Carlsbad, CA). Sulindac and SAHA were purchased from Calbiochem (San Diego, CA) and Alexis Corporation (Läufelfingen, Switzerland), respectively. *N*-Acetyl-l-cysteine (NAC) and indomethacin (1-(4-chlorobenzoyl)-5-methoxy-2-methyl-3-indoleacetic acid) were purchased from Sigma-Aldrich (St. Louis, MO). Celecoxib (4-[5-(4-methylphenyl)-3-(trifluoromethyl)pyrazol-1-yl]benzenesulfonamide) and mefenamic acid (2-[(2.3-dimethylphenyl)amino]benzoic acid) were kind gifts from Dr. Hong Sung-Hee (Korea Institute of Radiological & Medical Sciences, Republic of Korea). Anti-cytochrome *c* and anti-XIAP antibodies were purchased from BD Pharmingen (San Diego, CA), the anti-poly(ADP-ribose) polymerase antibody was from Cell Signaling Technology (Danvers, MA), and antibodies against survivin, myc, cIAP1, cIAP2, caspase 9, and β-actin were acquired from Santa Cruz Biotechnology (Santa Cruz, CA).

Measurement of Cell Viability. Cell viability was determined by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) to a colored product. Cells were treated with the specified drugs, and the medium was exchanged with serum-free medium containing 1 mM MTT. After 4 h at 37°C, cells were solubilized in dimethyl sulfoxide. The amount of formazan, the converted form of MTT, was determined by measuring absorbance at 570 nm.

Analysis of Apoptosis. Apoptosis was determined using an annexin V-fluorescein isothiocyanate/propidium iodide (PI) kit (BD Pharmingen). In brief, cells were washed with ice-cold phosphate-buffered saline and resuspended in binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Cells were incubated with 5 μl each of annexin V-fluorescein isothiocyanate and PI and analyzed with a FACScan flow cytometer (BD Biosciences).

Measurement of Caspase Activity. Caspase 3/7 activities were assessed using the CaspaTag Caspase Activity Kit (Millipore Bioscience Research Agents, Temecula, CA), according to the manufacturer's instructions. This kit uses a cell-permeable and noncytotoxic carboxyfluorescein-labeled fluoromethyl ketone peptide blocker of caspases 3/7, FAM-DEVD-FMK, that covalently binds to a reactive cysteine residue on the large subunit of the active caspase heterodimer, inhibiting enzymatic activity and producing green fluorescence. Thus, the green fluorescent signal directly corresponds to the amount of active caspase present in the cell at the time of reagent addition. Fluorescein-conjugated caspase substrate was added directly to the cell suspension, incubated for 1 h at 37°C under 5% CO_2, and protected from light. After washing, labeled live cells were detected by flow cytometry.

Measurement of the Mitochondrial Transmembrane Potential. Loss of mitochondrial transmembrane potential (MMP) was measured using an ApoAlert mitochondrial membrane sensor kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. In brief, after drug treatment, cells were incubated with Mitosensor for 15 min at 37°C. After centrifugation, cells were resuspended in washing buffer and analyzed with the fluorescence channel by flow cytometry. Results are presented as mean values of the stained cell histogram.

Detection of ROS. ROS in cells were measured using dichlorofluorescein diacetate (DCFH-DA; Calbiochem), an oxidation-sensitive fluorescent probe. After incubation with the specified drugs, cells were stained with 20 μM DCFH-DA for 30 min at 37°C in the dark and analyzed using a FACScan (BD Biosciences) to determine the ROS level.

Subcellular Fractionation. Cytosolic and mitochondrial fractions were prepared using a cytosol/mitochondria fractionation kit from Calbiochem. In brief, cells treated with sulindac or SAHA under various conditions were washed in ice-cold phosphate-buffered saline and resuspended in ice-cold cytosol extraction buffer. After homogenization using an ice-cold Dounce tissue grinder, the preparation was centrifuged at 700*g* for 10 min. The supernatant was transferred, centrifuged at 10,000*g* for 30 min, and kept as the cytosolic fraction. Pellets were resuspended in mitochondrial extraction buffer and centrifuged at 12,000*g* for 2 min. The supernatant was collected as the mitochondrial fraction. All samples were stored at −20°C until use.

Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH). An aliquot of total RNA (2 μg) was transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Next, cDNA (2 μl) was amplified using *Taq* polymerase (Invitrogen). The following primers were used: 5′-GGACCACCG-CATCTCTAC-3′ and 5′-CAGCCTTCCAGCTCCTTG-3′ for survivin, and 5′-GGATTCCTATGTGGGCGACAG-3′ and 5′-CGCTCGGTGAG-GATCTTCATG-3′ for β-actin (Jin et al., 2006). Polymerase chain reaction was performed at 95°C for 5 min (first denaturation), and then 32 cycles were run at 95°C for 30 s (denaturation), 55°C for 30 s (annealing), and 72°C for 30 s for survivin and β-actin.

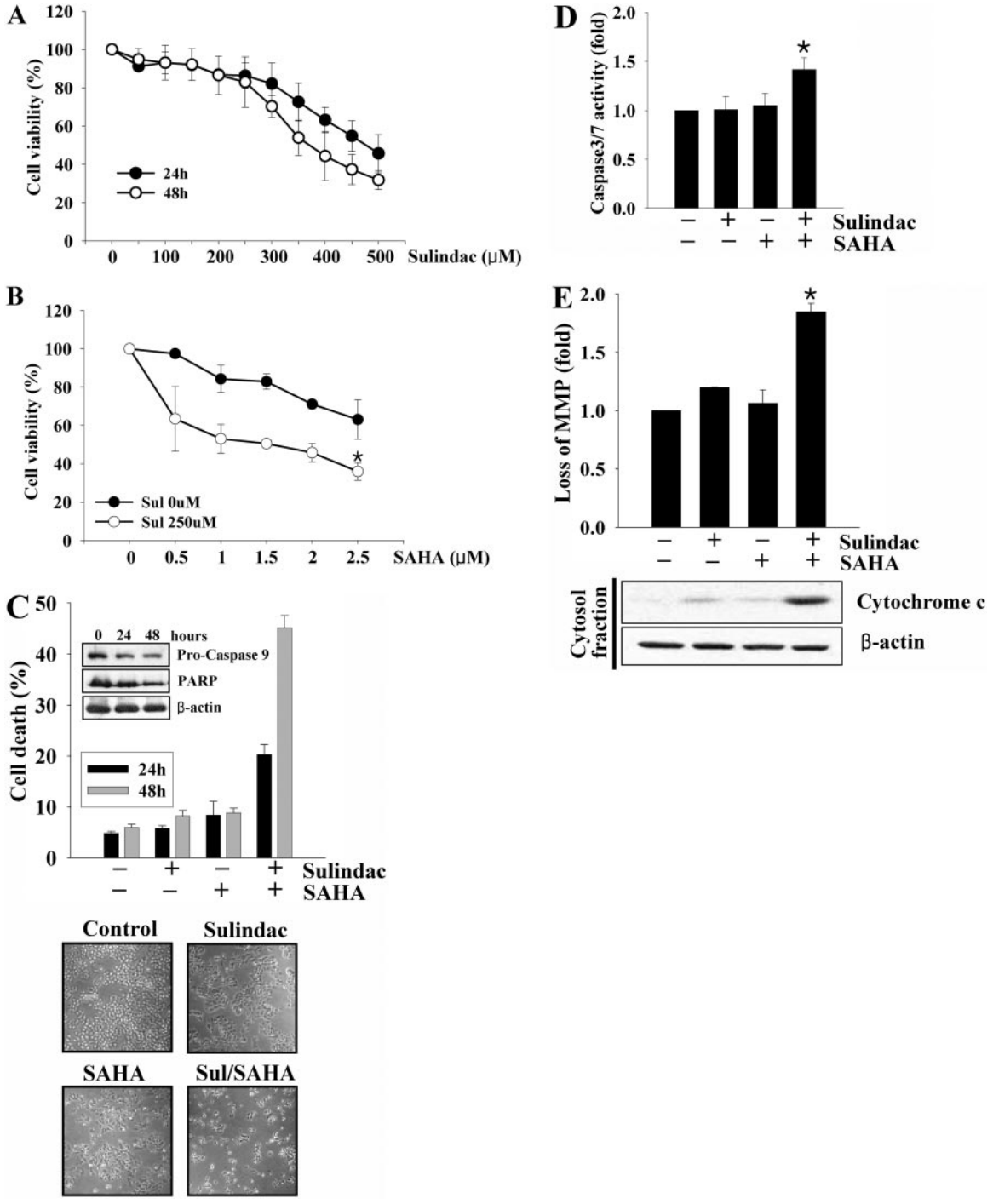

Fig. 1. Effects of the sulindac/SAHA combination therapy on apoptosis in A549 cells. A combination of sulindac and SAHA exerts synergistic cytotoxic effects. Sulindac/SAHA induced cell death through mitochondrial injury and caspase activation. A and B, MTT assay. Cells were treated with 0 to 500 μM sulindac for 24 or 48 h (A) followed by treatment with SAHA alone or a combination of 250 μM sulindac and SAHA at the indicated concentrations for 48 h. B, after treatment with MTT, data were acquired as absorbance of solubilized formazan. The viability of control cells was set at 100%, and survival relative to the control is presented. $*$, $P < 0.05$ versus the 2.5 μM SAHA-treated groups. C, apoptosis assay and morphology. Cells were treated with 250 μM sulindac and 2.5 μM SAHA, either alone or in combination. Apoptosis was measured as the percentage of cells with PI (+) or annexin V (+)using flow cytometry. Cell morphology was observed after 48-h incubation with the drug combination. At 24 and 48 h, cells were lysed and subjected to Western blot analysis using the indicated primary antibodies (inlet). Equal protein loading was confirmed by Western blotting for β-actin. D, caspase activity assay. Cells were treated for 24 h as described in C. Caspase activity was detected by staining with fluorescein isothiocyanate-labeled caspase inhibitors followed by flow cytometry. $*$, $P < 0.05$ versus the untreated groups. E, measurement of the MMP. Cells were treated as described for 48 h in C. MMP was analyzed by staining with Mitosensor followed by flow cytometry. At 48 h, cells were separated into cytosolic and mitochondrial fractions as described under *Materials and Methods* and analyzed by Western blotting. Equal protein loading in the cytosolic fractions was confirmed by Western blotting for β-actin. $*$, $P < 0.05$ versus the untreated groups. Each value represents means $\pm$ S.D. of three independent experiments. Immunoblots are representative of at least two independent experiments.

Western Blotting. Cells were harvested and lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS supplemented with protease inhibitor cocktail) (Roche, Mannheim, Germany). Concentrations of each lysate were determined with the Bradford assay. Equal amounts of protein (20–50 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were incubated with the relevant primary antibodies, followed by horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were visualized with enhanced chemiluminescence reagents (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK).

Transfection. Myc-tagged survivin was a kind gift from Dr. Jin Q. Cheng (Department of Pathology, University of South Florida College of Medicine, Tampa, FL). Antisense primers were synthesized by Bioneer (Seoul, Republic of Korea) in the form of phosphorothioate oligodeoxynucleotides. The sequences of survivin antisense and control primers were 5′-CCCAGCCTTCCAGCTCCTTG-3′ (Kuo et al., 2004) and 5′-GGAGCCAGGGGGGAGCAGGG-3′, respectively. Survivin siRNA was acquired from Santa Cruz Biotechnology.

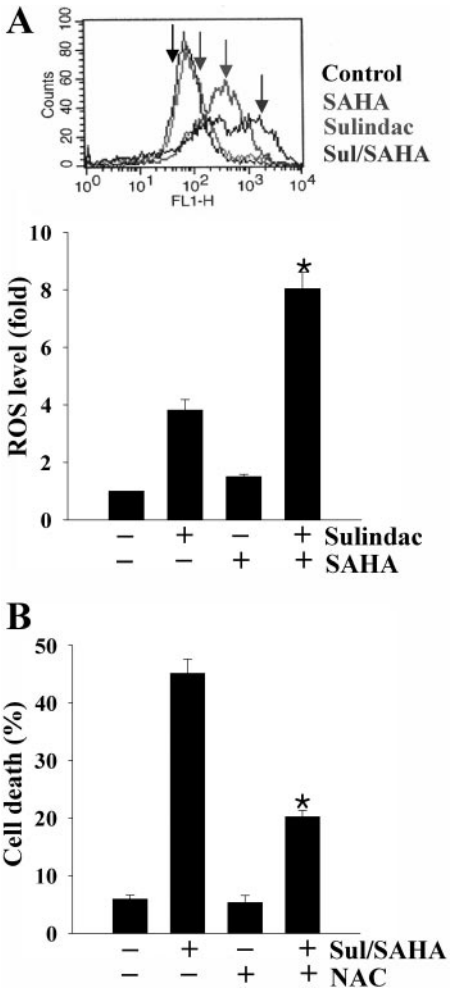

Fig. 2. Involvement of ROS in the anticancer effects of sulindac/SAHA. ROS were generated after sulindac/SAHA treatment. NAC blocked sulindac/SAHA induced cell death. A, ROS detection. Cells were treated for 24 h, as described in Fig. 1C. ROS levels were measured using DCFH-DA followed by flow cytometry. Data were presented as means of histograms and visualized as fold values using graphs compared with control. *, $P < 0.05$ versus the untreated group. B, apoptosis assay. Cells were treated with 250 μM sulindac and 2.5 μM SAHA in the presence or absence of 2 mM NAC for 48 h. *, $P < 0.05$ versus the sulindac/SAHA-treated groups. Each value represents the mean ± S.D. of three independent experiments.

A549 cells were transfected with 50 nM antisense oligodeoxynucleotides or 1 μg of vector or 100 nM siRNA using LipofectAMINE 2000 (Invitrogen) in 1 ml of serum-free medium for 5 h at 37°C in a CO_2 incubator, according to the manufacturer's recommendations. Next, 500 μl of 20% fetal bovine serum was added without removing the transfection mixture, followed by incubation for an additional 12 h. After transfection, cells were subjected to apoptosis, as described above.

Statistical Analysis. Data are presented as means ± S.D. Comparisons between groups were performed using the paired Student's *t* test. Asterisks (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$) represent statistical significance.

Results

Anticancer Effects of Combined Sulindac and SAHA. Because previous studies by our group have shown that sulindac exerts cytotoxic effects on cancer cells (Seo et al., 2007), we performed the MTT assay to determine the minimum cytotoxic dose of sulindac in the A549 lung cancer cell line. As shown in Fig. 1A, sulindac is cytotoxic at concentra-

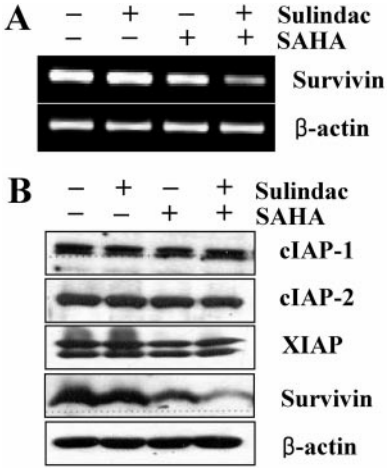

Fig. 3. Effects of the sulindac/SAHA combination on IAP protein expression. The mRNA and protein levels of survivin, but not cIAP1, cIAP2, or XIAP, were down-regulated in sulindac/SAHA-treated A549 cells. RT-PCR (A) and Western blotting (B). Cells were treated for 24 (A) and 48 h (B) as described in Fig. 1C. RT-PCR was accomplished as described under *Materials and Methods*. Equal mRNA and protein loading were confirmed using β-actin. RT-PCR data and immunoblots are representative of at least two independent experiments.

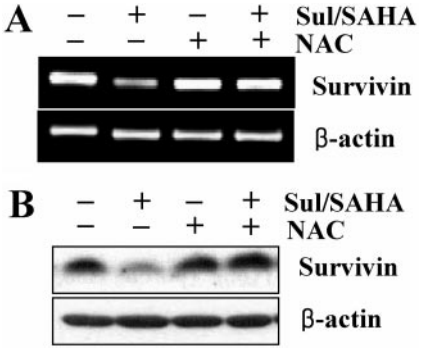

Fig. 4. Inhibition of survivin down-regulation by NAC in sulindac/SAHA-treated A549 cells. NAC prevented survivin down-regulation by sulindac/SAHA at both the mRNA and protein levels. RT-PCR (A) and Western blotting (B). Cells were treated for 24 (A) and 48 h (B), as described for Fig. 2B. Equal mRNA and protein loading were confirmed using β-actin. RT-PCR and immunoblots are representative of at least two independent experiments.

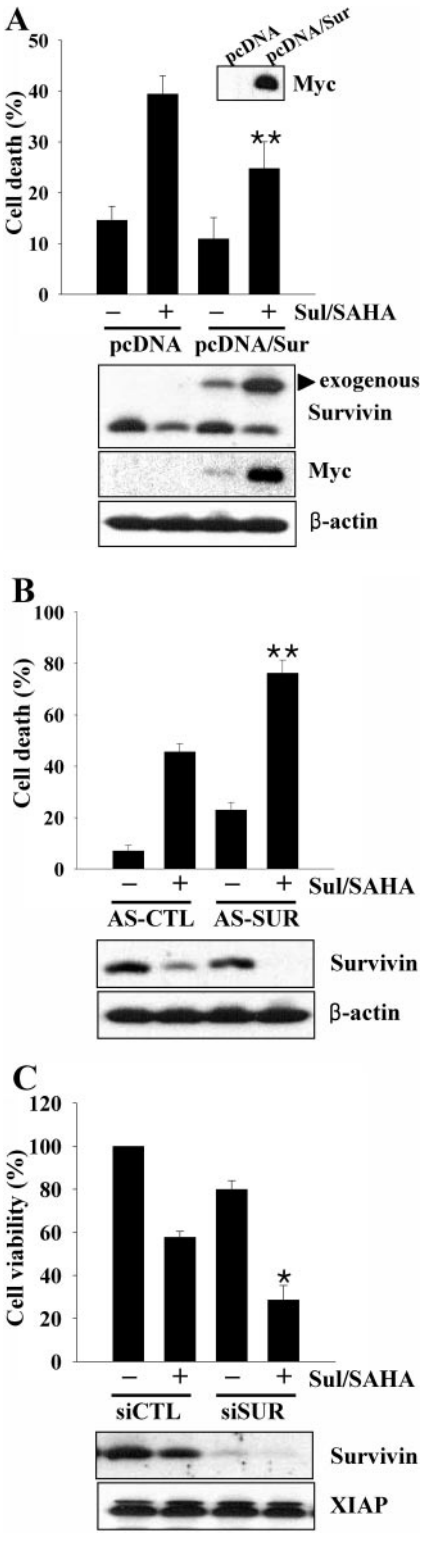

Fig. 5. The role of survivin in sulindac/SAHA-induced cell death. Survivin overexpression rescues A549 cells from sulindac/SAHA-induced death, and reduction of survivin levels using antisense oligonucleotides confers resistance to cell death. A, apoptosis assay. Cells were transiently transfected with the pcDNA vector encoding Myc-tagged survivin. The extent of overexpression was determined by Western blot analysis at 24 h after transfection (inset). Wild-type (pcDNA) and survivin-overexpressing cells (pcDNA/Sur) were treated without (−) or with (+) 250 μM sulindac and 2.5 μM SAHA for 48 h. Cells were lysed and subjected to Western blot analysis using the survivin antibody. Equal protein loading was confirmed by Western blotting for β-actin. **, $P < 0.01$ versus the pcDNA-transfected and sulindac/SAHA-treated groups. B, apoptosis assay. Cells were transfected with antisense primers using the Lipofectamine kit. The extent of protein expression interference was determined using Western blot analysis. Antisense control-transfected cells (AS-CTL) and antisense survivin-transfected cells (AS-SUR) were treated without (−) or with (+) 250 μM sulindac and 2.5 μM SAHA for 48 h. Cells were lysed and subjected to Western blot analysis using the survivin antibody. Equal protein loading was confirmed by Western blotting for β-actin. **, $P < 0.01$ versus the antisense control transfected and sulindac/SAHA treated groups. C, MTT assay. Cells were transfected with siRNA using the Lipofectamine kit. Control siRNA (siCTL) transfected cells and survivin siRNA (siSUR) transfected cells were treated without (−) or with (+) 250 μM sulindac and 2.5 μM SAHA for 48 h. Cells were lysed and subjected to Western blot analysis using the survivin antibody. The specificity of target protein expression interference was confirmed by Western blotting for XIAP. *, $P < 0.05$ versus the control siRNA transfected and sulindac/SAHA-treated groups. Data are presented as means ± S.D. of three independent experiments. Immunoblots are representative of at least two independent experiments.

tions at or greater than 250 μM for 24 and 48 h. Consequently, we examined the combined effects of 250 μM sulindac and increasing concentrations of SAHA. In the presence of sulindac, SAHA-induced cytotoxicity was significantly enhanced. For instance, incubation of A549 cells with 2.5 μM SAHA alone for 48 h decreased cell viability to 63.1%, whereas cotreatment with 250 μM sulindac resulted in 35.9% viability (Fig. 1B), indicating that sulindac sensitizes human lung cancer cells to SAHA-induced cytotoxicity. Next, we investigated whether the observed sensitization effect is associated with potentiation of apoptosis. Annexin V/PI staining revealed that the combination of sulindac and SAHA induced synergistic, time-dependent cell death (Fig. 1C). Consistent with the Annexin V/PI staining data, sulindac and SAHA exerted a similar synergistic effect on caspase activation (Fig. 1C, inset, and D) and MMP depolarization (Fig. 1E). It seems that treatment with the drug combination triggers a higher degree of apoptosis than either drug alone.

Role of Reactive Oxygen Species in Sulindac/SAHA-Induced Cell Death. To explore the molecular mechanism underlying the augmented effects of sulindac and SAHA, we evaluated the generation of ROS after drug exposure using the fluorescent indicator DCFH-DA. Despite several controversial results regarding ROS generation in NSAID-treated cells, sulindac enhanced DCFH-DA-detectable ROS levels significantly, whereas SAHA induced ROS to a lower extent in our experiments. It is interesting that the sulindac/SAHA combination further increased ROS generation relative to either drug alone (Fig. 2A). To determine whether elevated ROS participates in apoptosis induced by the drug combination, cells were incubated with the free radical scavenger, NAC, before treatment. NAC markedly inhibited combination therapy-induced cell death, as evaluated with Annexin V/PI staining (Fig. 2B). Our results imply that elevated ROS is necessary for the potentiation of cell death.

Down-Regulation of Survivin by the Sulindac/SAHA Combination. Next, we examined the expression of the antiapoptotic gene, *survivin*, in A549 cancer cells after treatment. As shown in Fig. 3, treatment of A549 cells with sulindac and SAHA resulted in a significant decrease in the survivin protein level relative to treatment with either drug alone but did not affect the expression of other IAP family members (cIAP1, cIAP2, and XIAP). Furthermore, reverse-transcription polymerase chain reaction (RT-PCR) analysis disclosed decreased survivin mRNA levels in A549 cells cotreated with sulindac and SAHA. Our data suggest that

these drugs modulate survivin expression at the transcriptional level.

Role of ROS in Survivin Down-Regulation. To gain further insight into the relationship between ROS generation and survivin down-regulation, we examined the effects of NAC on survivin expression in the presence of sulindac and SAHA. As shown in Fig. 4, NAC markedly attenuated the ability of sulindac/SAHA to suppress survivin protein and mRNA levels, indicating that this down-regulation of survivin is mediated by ROS.

Survivin Down-Regulation Leads to Sulindac/SAHA-Induced Cell Death. Finally, we examined the effects of the drugs in cells transiently transfected with a plasmid encoding Myc-survivin. Expression of Myc-survivin in transiently transfected cells, which normally display approximately 50% transfection efficiency in our experimental settings (data not shown), was confirmed by Western blot analysis with an anti-Myc antibody (Fig. 5A, inset). As shown in Fig. 5A, treatment of A549 cells overexpressing myc-survivin with the sulindac/SAHA combination for 48 h resulted in decreased apoptosis compared with control cells. Based on these results, we propose that ectopic expression of survivin in A549 cells may aid in overcoming sulindac/SAHA-induced apoptosis to a certain level. The role of survivin in sulindac/SAHA-induced apoptosis was further investigated using antisense oligonucleotides and siRNA. Cells transfected with the survivin antisense oligonucleotides displayed significantly elevated death, compared with control oligonucleotide-treated cells (Fig. 5B). Moreover, treatment with a combination of survivin antisense oligonucleotides and sulindac/SAHA promoted cell death to a greater extent than with a combination of control oligonucleotides and sulindac/SAHA. Combined treatment with survivin siRNA and sulindac/SAHA also resulted in a greater extent of cell death than combined treatment with control siRNA and sulindac/SAHA (Fig. 5C). It is interesting that the exogenous survivin level is very high in cells treated with sulindac and SAHA (Fig. 5A). The results indicate that survivin counteracts apoptosis induced by sulindac and SAHA in A549 cells, and cell death induced by this drug combination is due, at least in part, to survivin down-regulation.

Anticancer Effects of Combined Sulindac and SAHA on Another Human Lung Cancer Cell Line. To determine whether the effects of sulindac and SAHA are cell type-specific, we examined the effects of sulindac and SAHA combination on apoptosis induction and survivin down-regulation in another human lung cancer cell line, H460. Cotreatment with sulindac and SAHA resulted in a synergistic induction of apoptosis (Fig. 6, A and B) and a significant decrease in the survivin protein level relative to treatment with either drug alone in H460 cells (Fig. 6C). These data indicate that the anticancer mechanisms of sulindac/SAHA in lung cancer cells are not cell line-specific.

Effects of Celecoxib and SAHA on Cell Death. Celecoxib, a well known NSAID, is in clinical trials to evaluate its chemopreventive and therapeutic effects against a broad spectrum of epithelial malignancies, including lung cancers, either as a single agent or in combination with other agents. The antitumor activity of celecoxib is possibly associated with its ability to induce apoptosis in a variety of cancer cells (Thun et al., 2002). On the basis of the finding that cotreatment with sulindac and SAHA down-regulates survivin gene expression, we examined the effects of celecoxib and SAHA on survivin expression and cell death. As shown in Fig. 7A, treatment with celecoxib or SAHA alone did not significantly affect the percentage of apoptotic cells, even after 48 h incubation. In contrast, celecoxib plus SAHA strongly stimulated apoptosis and induced a considerable decrease in the survivin protein level than either drug alone. Although these results were similar to those obtained with sulindac plus SAHA, the synergistic induction of cell death and survivin down-regulation were observed at lower concentrations of celecoxib (30 μM) compared with sulindac (250 μM). Thus, it seems that down-regulation of survivin is an essential step in cancer cell apoptosis by specific chemopreventive agents and that cotreatment with SAHA plus sulindac or celecoxib has a synergistic effect on apoptosis induction via augmenting this decrease in survivin expression. Furthermore, we observed the effects of additional NSAIDs, including mefenamic acid and indomethacin, on ROS production and induction of apo-

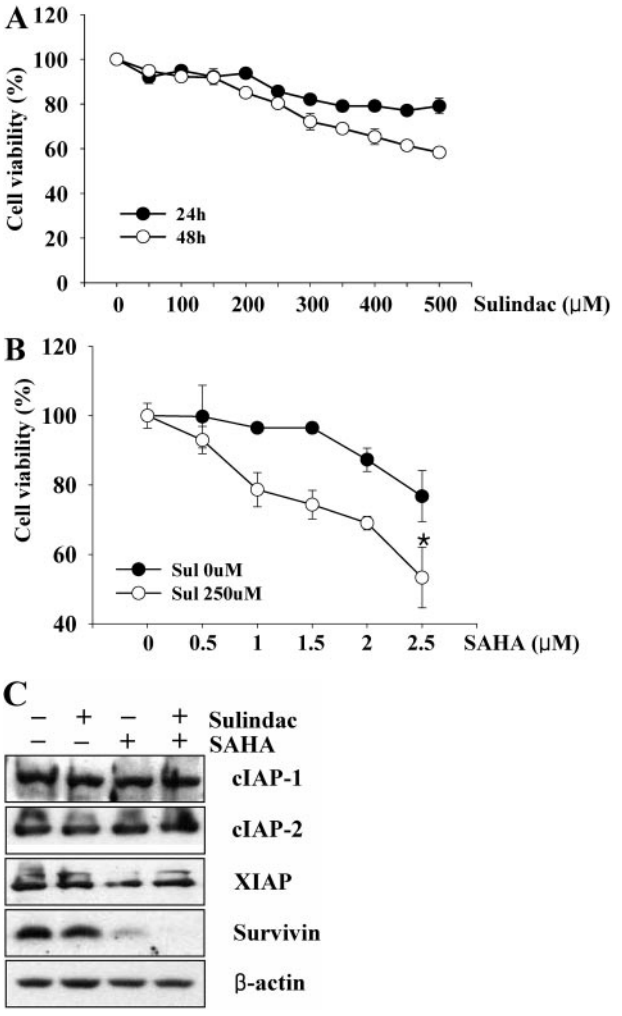

Fig. 6. Anticancer effects of combined sulindac and SAHA on another human lung cancer cell line, H460 cells. A, combination of sulindac and SAHA exerts synergistic cytotoxic effects. A and B, MTT assay. Cells were treated with 0 to 500 μM sulindac for 24 or 48 h (A) followed by treatment with SAHA alone or a combination of 250 μM sulindac and SAHA at the indicated concentrations for 48 h (B). $*$, $P < 0.05$ versus the 2.5 μM SAHA-treated groups. C, Western blotting. Cells were treated with 250 μM sulindac and 2.5 μM SAHA, either alone or in combination for 48 h. Equal protein loading was confirmed using β-actin. Immunoblots are representative of at least two independent experiments.

ptosis when combined with SAHA. ROS production was increased in cells treated with mefenamic acid and indomethacin, and cotreatment of mefenamic acid or indomethacin with SAHA showed enhanced production of ROS. In addition, cotreatment of mefenamic acid or indomethacin with SAHA also showed a synergistic cytotoxicity (Fig. 7, B and C).

Discussion

Apoptosis is an important mechanism by which anticancer agents induce cancer cell death. Both sulindac and SAHA are promising anticancer therapeutics with independent pathways of action that are currently in clinical trials. Sulindac acts through inhibition of Cox-2, whereas SAHA seems to induce the expression of specific target genes to stimulate growth arrest, differentiation, or apoptosis of malignant cells. Here, we demonstrate that a combination of sulindac and SAHA has a greater-than-additive effect on apoptosis of NSCLC cells than either agent alone. These findings indicate that a combination of these two agents can be used to kill lung cancer cells more effectively with minimal side effects, thus providing a rationale for combining these and other similar reagents for the clinical treatment of lung cancer.

To test whether combination effects of sulindac and SAHA on induction of apoptosis are dependent on Cox-2, we investigated the role of Cox-2 in sulindac/SAHA-induced apoptosis using siRNA of Cox-2. Combined treatment with Cox-2 siRNA and sulindac/SAHA did not change the level of survivin expression and cell death value (data not shown). Therefore, sulindac and SAHA combination might exert their cytotoxic antitumor effects via the down-regulation of survivin in a Cox-2-independent manner. Moreover, to examine the role of HDAC in sulindac/SAHA-induced survivin down-regulation, we observed the survivin level after treated with siRNA of HDAC1. The results showed that the survivin protein level was decreased in cells treated with siRNA of HDAC1. Combined treatment with HDAC1 siRNA and sulindac/SAHA resulted in a greater extent of down-regulation of survivin (data not shown). From these data, we might speculate that HDAC1, at least in part, plays a role in the expression of survivin.

Inhibitors of apoptosis may contribute to cancer promotion, persistence of unchecked mutations, and resistance to chemotherapy (Thompson, 1995). Previous studies have shown that survivin, although absent in human adult differentiated tissues, is expressed in transformed cell lines, cancers in vivo, and fetal tissues (Li et al., 1999). In lung cancer, high survivin expression is associated with poor patient prognosis and tumor resistance to chemotherapy and radiotherapy (Lu et al., 2004; Singhal et al., 2005). In several other experimental systems, down-regulation of survivin expression (e.g., with antisense or siRNA approaches) results in elevated basal apoptosis and substantially increased sensitivity of tumor cells to killing by chemotherapeutic drugs or ionizing radiation (Altieri, 2003; Lu et al., 2004; Rödel et al., 2005; Fuessel et al., 2006). Hence, targeting survivin may lead to selective inhibition of tumor growth and increased lung cancer cell apoptosis while sparing normal cells. In the current study, we show that a combination of sulindac and SAHA triggers a more marked decrease in the levels of survivin protein and mRNA than either drug alone. Moreover, down-regulation of survivin by antisense oligonucleotides in combination with sulindac/SAHA treatment induced apoptosis to a greater degree in NSCLC cells compared with either agent alone. Overexpression of survivin by transfection with an Myc-tagged survivin vector abrogated sulindac/SAHA-induced apoptosis to a certain degree. Therefore, we propose that survivin down-regulation is mechanistically linked with sulindac/SAHA-induced apoptosis.

The ability of sulindac to potentiate the anticancer activity of SAHA is intimately linked to ROS generation. Several

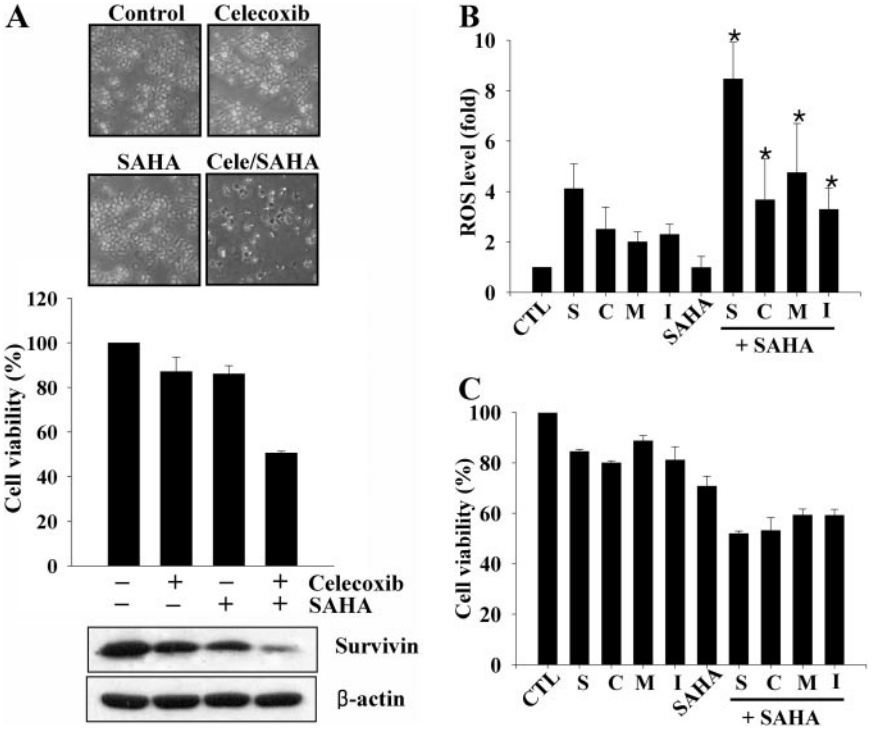

Fig. 7. Effects of the celecoxib/SAHA combination on cell death. Celecoxib and SAHA exerts synergistic cytotoxic effects. A, MTT assay and morphology. Cells were treated with 30 μM celecoxib and 2.5 μM SAHA either alone or in combination for 48 h. The viability of control cells was set at 100%, and survival relative to the control is presented. Cells were lysed and subjected to Western blot analysis for confirmation of survivin expression. Equal protein loading was confirmed by Western blotting for β-actin. B, ROS detection. Cells were treated with each drug in the presence or absence of 2.5 μM SAHA for 24h. (S, 250 μM sulindac; C, 30 μM celecoxib; M, 100 μM mefenamic acid; I, 50 μM indomethacin) *, $P < 0.05$ versus the untreated groups. C, MTT assay. Cells were treated with each drugs in the presence or absence of 2.5 μM SAHA for 48 h. Each value represents mean ± S.D. of three independent experiments. Immunoblots are representative of at least two independent experiments.

reports establish that increased ROS generation plays a critical role in cell death induced by SAHA and other HDAC inhibitors. Sulindac alters the cellular redox status (Minami et al., 2005; Rahmani et al., 2005; Ungerstedt et al., 2005; Seo et al., 2007). We observed a modest increase in cellular ROS generation upon treatment with sulindac alone. However, a combination of sulindac and SAHA induced a marked increase in ROS generation over that achieved by either single agent. Furthermore, elevated ROS generation is required for maximal cell death by these agents, because treatment with the antioxidant NAC dramatically reduced their efficacy. It is interesting that several studies suggest that disruption of the cellular redox status is an important component of the mechanism underlying the synergy between HDAC inhibitors and other anticancer agents.

Our results collectively indicate that targeting survivin with a sulindac/SAHA combination is an effective novel approach for the prevention and/or treatment of NSCLC. Moreover, down-regulation of survivin by sulindac/SAHA is a useful strategy for chemosensitization of NSCLC cells to standard therapies. However, further in-depth investigations are essential to establish the cause-and-effect relationship between survivin gene regulation and sulindac/SAHA-induced cell death of NSCLC in animal and human models.

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