

Sulindac sulfide–induced apoptosis is enhanced by a small-molecule Bcl-2 inhibitor and by TRAIL in human colon cancer cells overexpressing Bcl-2

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Abstract

Sulindac is a nonsteroidal anti-inflammatory drug (NSAID) that induces apoptosis in cultured colon cancer cells and in intestinal epithelia in association with its chemopreventive efficacy. Resistance to sulindac is well documented in patients with familial adenomatous polyposis; however, the molecular mechanisms underlying such resistance remain unknown. We determined the effect of ectopic Bcl-2 expression upon sulindac-induced apoptotic signaling in SW480 human colon cancer cells. Sulindac sulfide activated both the caspase-8-dependent and mitochondrial apoptotic pathways. Ectopic Bcl-2 attenuated cytochrome *c* release and apoptosis induction compared with SW480/neo cells. Coadministration of sulindac sulfide and the small-molecule Bcl-2 inhibitor HA14-1 increased apoptosis induction and enhanced caspase-8 and caspase-9 cleavage, Bax redistribution, and cytochrome *c* and second mitochondria-derived activator of caspase release. Given that sulindac sulfide activated caspase-8 and increased membrane death receptor (DR4 and DR5) protein levels, we evaluated its combination with the endogenous death receptor ligand tumor necrosis factor–related apoptosis-inducing ligand (TRAIL). Coadministration of sulindac sulfide and TRAIL cooperatively enhanced apoptotic signaling as effectively as did HA14-1. Together, these data indicate that HA14-1 or TRAIL can enhance sulindac sulfide–induced apoptosis and represent novel strategies for circumventing Bcl-2-mediated apoptosis resistance in human colon cancer cells. [Mol Cancer Ther 2005;4(10):1475–83]

Introduction

Epidemiologic studies have consistently shown that nonsteroidal anti-inflammatory drugs (NSAID) taken regularly

can reduce the incidence of colorectal cancer (reviewed in ref. 1). NSAIDs also exert potent chemopreventive effects in animal models of colon cancer and the NSAID sulindac was shown to regress established colorectal polyps relative to placebo in patients with familial adenomatous polyposis (FAP) (1, 2). However, resistance to sulindac was observed in FAP patients as shown by an increase in adenoma number after 6 months of continuous drug treatment (2) and by cancer development in the retained rectum during sulindac chemopreventive therapy (3–5). Therefore, strategies to circumvent resistance to NSAIDs are needed to increase their chemopreventive and antitumor efficacy. Sulindac is metabolized *in vivo* to its sulfide and sulfone derivatives of which only the sulfide inhibits COX (COX-1 and COX-2) enzymes that catalyze the conversion of arachidonic acid to prostaglandins (6). Whereas COX enzymes are the best-defined targets of NSAIDs (7), both COX-dependent and COX-independent mechanisms contribute to their antitumor effects (reviewed in ref. 8).

Sulindac mediates its antitumor effects by inducing apoptosis. Sulindac has been shown to induce apoptosis in cultured colon cancer cell lines (9, 10) and in colonic epithelia from animal models and humans where it accompanies intestinal tumor prevention or regression (11–14). To date, the molecular and biochemical mechanism of apoptosis induction by sulindac remains incompletely understood. Apoptosis can be initiated at the level of the mitochondria (i.e., intrinsic pathway; ref. 15), or the membrane death receptors (Fas, tumor necrosis factor-R1 [TNF-R1], DR4, and DR5; i.e., extrinsic pathway; ref. 16). Mitochondrial events include alterations in mitochondrial membrane permeability and release of proapoptotic proteins into the cytosol, including cytochrome *c* (17), second mitochondria-derived activator of caspases (Smac)/DIABLO (18), and Omi/HtrA2 (19). Smac/DIABLO and Omi/HtrA2 bind to and neutralize inhibitor of apoptosis (IAP) proteins and thereby allow caspase activation to proceed. XIAP is the most potent IAP and functions as an endogenous inhibitor of effector caspase-9, caspase-7, and caspase-3 (20). It is well established that the antiapoptotic proteins Bcl-2 and Bcl-x_L block cytochrome *c* release, whereas proapoptotic Bax and Bid promote cell death (15). Bcl-2 has been shown to protect tumor cells from diverse and structurally unrelated cytotoxic drugs (15, 21). It is established that crosstalk can occur between the membrane death receptors and the mitochondria that is mediated by caspase-8-dependent cleavage of the proapoptotic Bcl-2 family member, Bid (22).

Defective mitochondrial apoptotic signaling may underlie clinical resistance to sulindac. Sulindac sulfide–induced apoptosis has been shown dependent upon intact

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Bax proteins because homozygous deletion of the *Bax* gene in HCT116 colon cancer cells suppressed apoptosis induction by this NSAID (23). Bcl-2 proteins may also confer resistance to sulindac-induced apoptosis. In cultured colon cancer cells, exogenous prostaglandin E₂ and forced COX-2 expression were shown to up-regulate Bcl-2 levels (24–27). Strategies to circumvent Bcl-2-mediated resistance have been developed and include small-molecule inhibitors of Bcl-2 such as HA14-1 (28). HA14-1 (M_r 409,000) binds in the hydrophobic groove of Bcl-2 to displace BH3-only proteins and thereby disrupt its antiapoptotic function (28). In human acute myeloid leukemia and myeloma cell lines overexpressing Bcl-2, HA14-1 induced apoptosis in association with a decrease in mitochondrial membrane potential and activation of caspase-9 and caspase-3 (29, 30). HA14-1 also increased chemosensitivity to cytotoxic drugs in these cell types.

A potential strategy to overcome defective mitochondrial apoptotic signaling in human cancer cells may be to trigger the extrinsic apoptotic pathway or to combine drugs that trigger both the extrinsic and intrinsic pathways. We (27) and others (31) have shown that sulindac sulfide can up-regulate membrane DR5 expression in human colon and prostate cancer cells, suggesting that it may enhance TRAIL-mediated apoptosis. TRAIL is a DR4, DR5 ligand that induces apoptosis in multiple malignant cell types while sparing most normal cells (16, 32–35). Recombinant human TRAIL preparations are available that closely resemble the endogenous soluble ligand (34–36) and have entered clinical trials.

In this report, we determined whether ectopic Bcl-2 expression can confer resistance to sulindac sulfide-induced apoptosis and evaluated potential strategies for reversal of Bcl-2-mediated resistance in SW480 human colon cancer cells. One such strategy involves the use of the small-molecule Bcl-2 inhibitor HA14-1. We also determined whether sulindac sulfide acts cooperatively with TRAIL to enhance apoptotic signaling.

Materials and Methods

Cell and Drug Treatments

The SW480 human colon cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 5% fetal bovine serum, 2 mmol/L L-glutamine, and antibiotics. Cells were grown in a monolayer and maintained at 37°C in a humidified atmosphere including 5% CO₂. SW480 cells or stable Bcl-2 transfectants, described below, were seeded at a density of 3 × 10⁶ cells per 100-mm dish in medium for protein isolation experiments. After 48 hours, medium was aspirated and replaced with medium containing drugs. Recombinant human TRAIL comprised of a 6× histidine-tag at the NH₂-terminal end of the extracellular domain of human TRAIL (Thr⁹⁵ to Gly²⁸¹) was obtained from R&D Systems (Minneapolis, MN). A second formulation of recombinant human TRAIL was obtained from Calbiochem (San Diego, CA)

and used in cell viability experiments. TRAIL was dissolved in 1× PBS and then diluted in medium for experiments. Other drugs used included sulindac sulfide (Sigma Chemical, St. Louis, MO); HA14-1 (Calbiochem); and the caspase inhibitors Z-VAD-FMK (pan specific), Z-IETD-FMK (caspase-8 specific), and Z-LEHD-FMK (caspase-9 specific; all from R&D Systems). After drug treatment, both floating and attached cells were harvested for subsequent analysis. All other reagents were purchased from Sigma (St. Louis, MO).

Transfection of Bcl-2 cDNA

SW480 cells (3 × 10⁵ in 2 mL of Leibovitz's L-15 medium) were plated in 6-well Costar (Corning, Inc., Corning, NY) tissue culture plates. Twenty-four hours later, cells were stably transfected with vector alone (PC3-neo) or with the PC3-Bcl-2 plasmid (a gift of Dr. Timothy J. McDonnell, University of Texas M.D. Anderson Cancer Center, Houston, TX), as previously described (35). Transfection was done with LipofectAMINE reagent (Life Technologies, Inc., Rockville, MD), according to the manufacturer's instructions. Positive transfectants were selected in medium containing 500 µg/mL gentamicin (Life Technologies). Cell lines were established from individual colonies using cloning cylinders.

Western Blotting

For experiments using whole cell lysates, cells were washed with cold PBS immediately before lysate collection in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% NP10, 150 mmol/L NaCl, and 1 mmol/L EDTA, which was augmented with protease inhibitors at the time of addition to cells. The samples were rocked at 4°C for 20 minutes and centrifuged at 14,000 rpm for 10 minutes to remove cellular debris. The protein concentration in the cell lysates was measured using a Bradford protein assay kit (Bio-Rad, Richmond, CA). Protein concentrations were equalized and were then diluted in SDS-PAGE loading buffer. Samples were maintained at 4°C, boiled for 5 minutes, and separated on 10% or 12% polyacrylamide gels. Proteins were then transferred electrophoretically to Immobilon-P PVDF membranes (Millipore, Billerica, MA). Membranes were blocked with I-Block (Applied Biosystems, Foster City, CA) overnight at 4°C and incubated at room temperature with polyclonal antibodies against Bid (R&D Systems), caspase-9 and caspase-3 (BD PharMingen, San Diego, CA), DR4 and DR5 (ProSci, Inc., Poway, CA), and Omi/HtrA2 (a gift from Dr. Emad Alnemri, Thomas Jefferson University). Blots were also incubated with monoclonal antibodies against Bcl-2 and Smac (Upstate Biotechnology, Lake Placid, NY), β-actin and Bax (Sigma), caspase-3, caspase-8, cytochrome *c*, and XIAP (BD PharMingen). The anti-BID antibody used detects human BID and the COOH-terminal portion of recombinant BID generated by caspase-8-mediated cleavage. Tyrosine Tubulin (ICN Biomedicals, Aurora, OH) was used as a marker of the cytosolic fraction and COX IV (Molecular Probes, Eugene, OR) for the mitochondrial fraction, as described below. Blots were washed with PBS containing 0.1% Tween 20 for

15 minutes each and incubated with a secondary antibody. The signal was detected by the Western-Star chemiluminescent detection kit (Applied Biosystems) using alkaline phosphatase detection. Scanning densitometry was done using the AlphaEaseFC software package (Alpha Innotech Corp., San Leandro, CA) and protein expression was quantified relative to β -actin.

Subcellular Fractionation

Cells were plated in serum-containing medium 48 hours before treatment. For subcellular fractionation of SW480 cells and Bcl-2 transfectants, cytoplasmic fractions were collected using a modification of the method described by Leist et al. (37). Briefly, after treatment for the designated time periods, cells were collected in $1\times$ PBS and lysed in permeabilization buffer (210 mmol/L D-mannitol, 70 mmol/L sucrose, 10 mmol/L HEPES, 5 mmol/L sodium succinate, 200 μ mol/L EGTA, 0.15% bovine serum albumin, 80 μ g/mL digitonin) on ice. The lysate was subjected to centrifugation at $13,000 \times g$ to pellet nuclei as well as the heavy membrane fraction and the supernatant (cytoplasm-enriched fraction) was collected. Protein from subcellular fractionation was quantified, separated on SDS-PAGE, and immunoblotted as described above.

Annexin V Binding

SW480 cells (parental and recombinant human Bcl-2 transfectants) were plated at a density of 2×10^5 cells per well in a six-well plate 2 days before experimentation. Cells were washed with $1\times$ PBS and treated for 5 or 72 hours. After treatment, adherent and floating cells were collected. Pelleted cells were washed twice with $1\times$ PBS and resuspended in $1\times$ Annexin Binding Buffer (BD Pharmingen) to a concentration of 1×10^6 cells/mL. A 200- μ L aliquot was taken and mixed with Annexin V-FITC (Alexis Biosciences, San Diego, CA) and propidium iodide for

20 minutes at room temperature. After incubation, the cell solution was augmented to 500 μ L with Annexin Binding Buffer and the results were read using a Becton Dickinson FACSCalibur bench-top flow cytometer (San Jose, CA). All samples were run in triplicate and results were expressed as mean values \pm SD.

Results

Sulindac Sulfide Engages Both Caspase-8-Dependent and Mitochondrial Apoptotic Pathways

SW480 cells were chosen for study as they are representative of human colorectal cancers in that they contain biallelic mutations in the *APC* gene and carry mutated *p53* alleles (38). We determined the effect of sulindac sulfide treatment upon membrane DR4 and DR5 protein expression in SW480 cells by immunoblotting. Sulindac sulfide was found to induce DR4 or DR4 and DR5 proteins at 20 μ mol/L and 120 μ mol/L dosages, respectively (Fig. 1). At the lower dose of sulindac sulfide, DR4 induction was seen at 12 hours. The higher dosage induced DR4 and DR5 expression with maximal induction seen at 12 to 24 hours. Sulindac sulfide treatment activated caspase-8 as shown by a reduction in the level of procaspase-8 and cleaved Bid (Fig. 2A). Crosstalk with the mitochondrial apoptotic pathway is evidenced by sulindac sulfide-induced release of cytochrome *c*, Smac/DIABLO, and Om/HtrA2 into the cytosol (Fig. 2A). Smac/DIABLO release was maximal at the 4-hour time point, whereas the relative expression of cytochrome *c* was maximal at 24 hours. This finding suggests that Smac release precedes that of cytochrome *c* at least in SW480 cells. The sulindac sulfide-mediated reduction in Bax expression is consistent

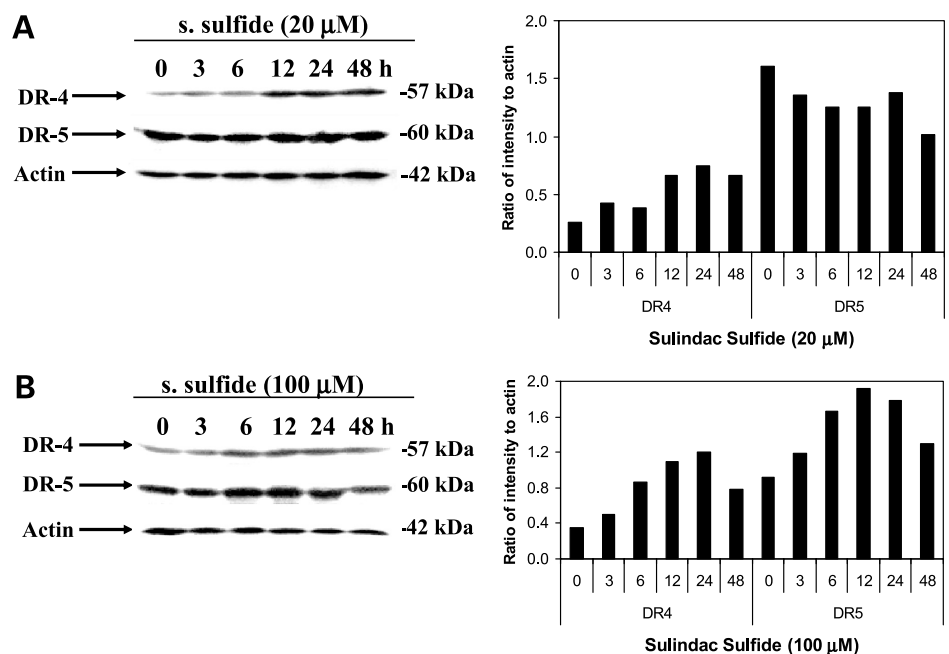


Figure 1. Effect of sulindac sulfide (*s. sulfide*) treatment upon DR4 and DR5 expression. Incubation of SW480 cells with sulindac sulfide for the indicated doses and times induces DR4 (A) or DR4 and DR5 (B) protein levels. Protein expression was measured in whole cell lysates by immunoblotting. Expression was quantified relative to β -actin by scanning densitometry.

with its redistribution to the mitochondria. Furthermore, sulindac was found to cleave XIAP downstream of mitochondria consistent with its inactivation (Fig. 2A). Sulindac sulfide induced dose-dependent apoptosis in SW480 cells as shown by Annexin V-FITC binding (Fig. 3A).

Ectopic Bcl-2 Expression Attenuates Sulindac Sulfide-Induced Apoptosis that Is Reversed by HA14-1

We determined whether ectopic expression of Bcl-2, a known inhibitor of cytochrome *c* release (17), can confer resistance to sulindac sulfide-induced apoptosis. SW480

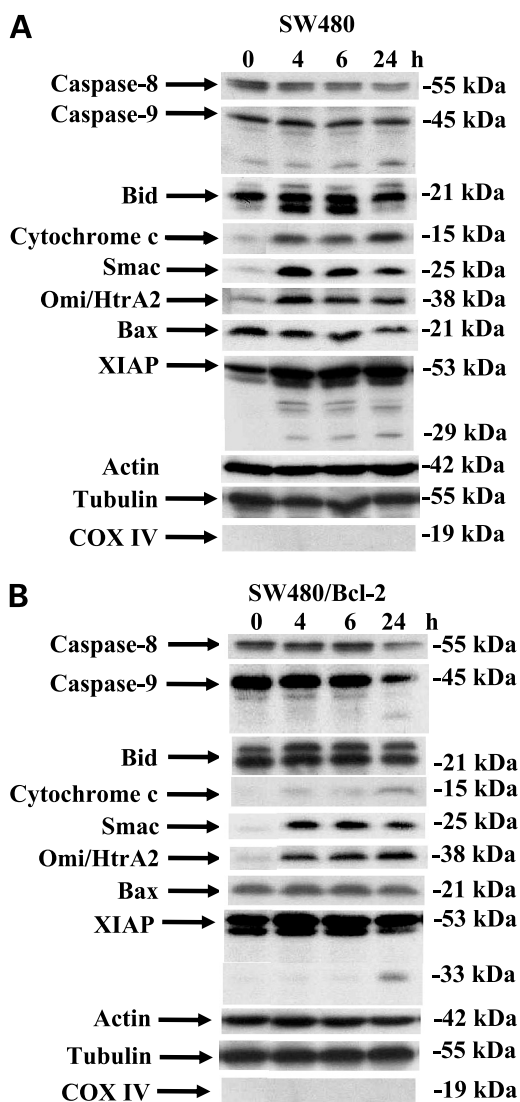


Figure 2. Sulindac sulfide activates procaspase-8 and the mitochondrial pathway as indicated by the cytosolic release of proapoptotic proteins. **A**, SW480 human colon carcinoma cells were incubated with 100 $\mu\text{mol/L}$ sulindac sulfide for the indicated times. After isolation of the cytoplasm-enriched cell fractions, protein expression was determined by immunoblotting. **B**, SW480 cells with ectopic Bcl-2 expression were treated as above. Tubulin and COXIV are markers for the cytosolic and mitochondrial fractions, respectively.

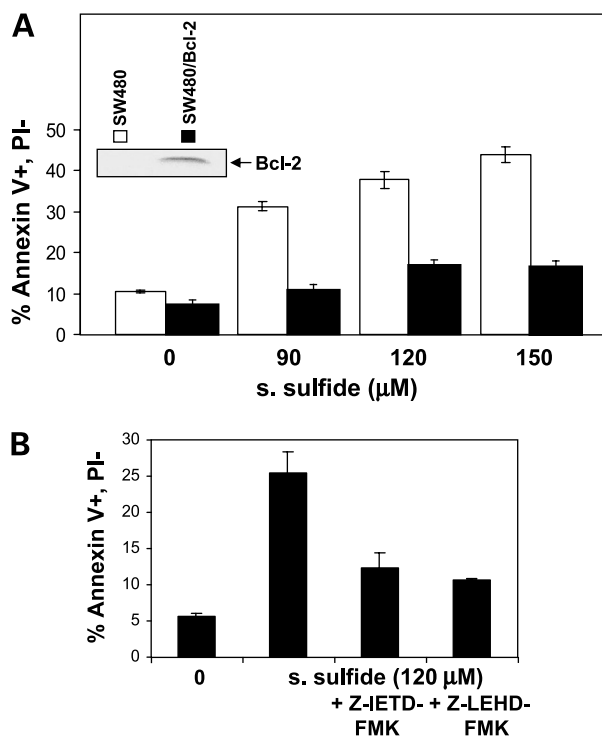


Figure 3. Ectopic Bcl-2 expression protects SW480 cells from sulindac sulfide-induced apoptosis. **A**, SW480/neo (open column) and Bcl-2 transfectants (closed column, inset) were incubated with increasing doses of sulindac sulfide for 72 h. Cells were then collected and assayed for Annexin V-FITC binding and propidium iodide (PI) staining by fluorescence-activated cell sorting analysis; Annexin V-positive, PI-negative cells were considered apoptotic. **B**, selective inhibitors of caspase-8 (Z-IETD-FMK) and caspase-9 (Z-LEHD-FMK) attenuate sulindac sulfide (120 $\mu\text{mol/L}$)-induced Annexin V-FITC binding in SW480/Bcl-2 cells. Cells were incubated with sulindac sulfide for 72 h in the presence or absence of a caspase inhibitor. Columns, mean values of triplicate determinations; bars, \pm SD.

cells overexpressing Bcl-2 (SW480/Bcl-2) showed an \sim 3-fold reduction in sulindac sulfide-induced apoptosis relative to SW480/neo cells (Fig. 3A). Ectopic Bcl-2 expression was found to delay sulindac sulfide-mediated caspase-9 activation. After an apoptotic stimulus, caspase-9 and caspase-3 cleavage have been shown to modulate caspase-8 activation consistent with a feedback amplification loop (39–41). Bid phosphorylation was suggested in SW480/Bcl-2 cells, indicating a posttranslational modification compared with SW480/neo cells (Fig. 2B). Bid phosphorylation has been shown to confer insensitivity to caspase-8 cleavage in human tumor cell lines (42). In SW480/Bcl-2 cells, sulindac sulfide-induced release of cytochrome *c* was strongly inhibited, but no inhibition of Smac and Omi/HtrA2 release was observed (Fig. 2B). In SW480/Bcl-2 cells, sulindac sulfide was shown to cleave XIAP downstream of the mitochondria. Bcl-2 delayed XIAP cleavage in these cells compared with SW480/neo cells (Fig. 2B).

We determined the effect of selective inhibitors of caspase-8 (IETD) and caspase-9 (LEHD) upon sulindac

sulfide-induced apoptosis. As shown in Fig. 3B, both caspase inhibitors were found to significantly attenuate sulindac sulfide-induced Annexin V-FITC binding, confirming that this NSAID activates both major apoptotic pathways.

In an attempt to reverse Bcl-2-mediated resistance, we used the small-molecule Bcl-2 inhibitor, HA14-1 [4 ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate]. HA14-1 alone induced apoptosis only at the highest dose evaluated (40 $\mu\text{mol/L}$) in SW480/Bcl-2 cells relative to vehicle-treated cells (Fig. 4A). HA14-1 dosages exceeding 25 $\mu\text{mol/L}$ have been shown to exhibit a loss of selectivity for Bcl-2 (29). In SW480/Bcl-2 cells, coadministration of HA14-1 (20 $\mu\text{mol/L}$) and sulindac sulfide enhanced apoptosis induction compared with sulindac sulfide alone (Fig. 4A). In contrast, HA14-1 did not augment sulindac-induced apoptosis in SW480/neo cells with very low-to-absent Bcl-2 protein expression, as previously reported by our laboratory (43). We then determined whether HA14-1 could reverse the antiapoptotic effects of Bcl-2. SW480/

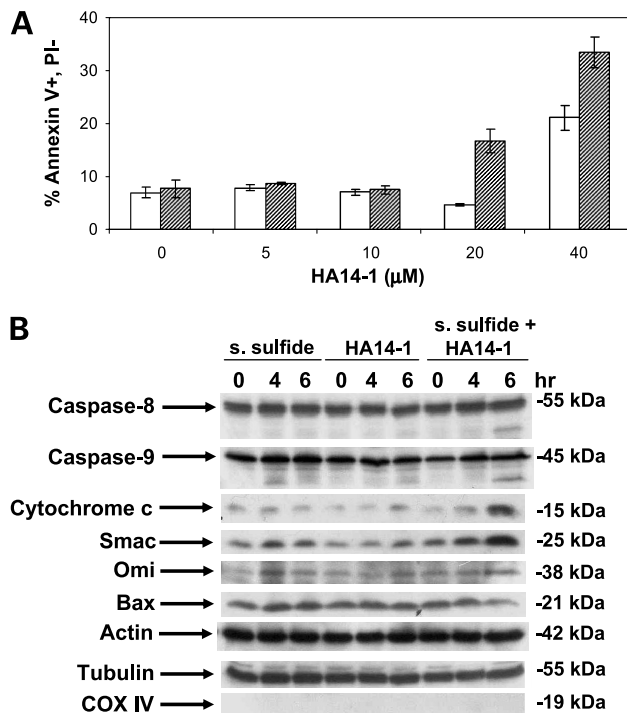


Figure 4. Small-molecule Bcl-2 inhibitor, HA14-1, enhances sulindac sulfide-induced apoptosis and overcomes Bcl-2-mediated resistance to apoptotic signaling. **A**, SW480/Bcl-2 cells were incubated with increasing concentrations of HA14-1 for 72 h in the absence (*open columns*) or presence (*hatched columns*) of sulindac sulfide (100 $\mu\text{mol/L}$). Apoptosis was determined using the Annexin-V-FITC/PI binding assay. A non-apoptotic dose of HA14-1 (20 $\mu\text{mol/L}$) augments sulindac sulfide-induced apoptosis. **B**, coadministration of HA14-1 (20 $\mu\text{mol/L}$) and sulindac sulfide (100 $\mu\text{mol/L}$) enhances apoptotic signaling in SW480/Bcl-2 cells compared with treatment with sulindac (100 $\mu\text{mol/L}$) or HA14-1 (20 $\mu\text{mol/L}$) alone. Cell fractionation and immunoblotting were done as described in Materials and Methods.

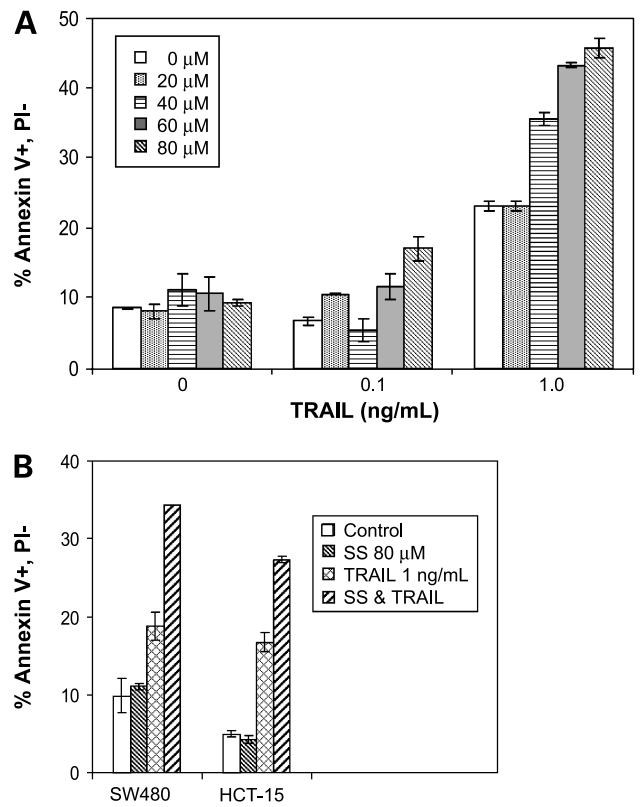


Figure 5. **A**, incubation of parental SW480 cells with increasing doses of sulindac sulfide (SS) and TRAIL for 5 h produces a cooperative increase in apoptosis compared with either drug alone. **B**, similar results are seen in HCT-15 human colon cancer cells where sulindac was previously found to increase DR5 mRNA and protein expression (26).

Bcl-2 cells were treated with sulindac sulfide, HA14-1, or their combination for 0, 4, and 6 hours and analysis of caspase activation and release of proapoptotic mitochondrial proteins was determined by immunoblotting (Fig. 4B). Coadministration of sulindac sulfide and HA14-1 cleaved caspase-8 and caspase-9, whereas either drug alone failed to activate these caspases after a 6-hr incubation period. These data suggest that Bcl-2 can suppress procaspase-8 cleavage through a feedback amplification loop (39, 40, 44). Furthermore, functional inhibition of Bcl-2 by HA14-1 also enhanced caspase-9 activation and markedly enhanced the cytosolic release of cytochrome *c* and Smac/DIABLO. The combination of HA14-1 and sulindac sulfide also reduced cytosolic Bax expression consistent with its redistribution to the mitochondria (Fig. 4B). Taken together, these data indicate that sulindac sulfide-induced apoptosis in SW480 cells requires a mitochondrial amplification step that is inhibited by Bcl-2 yet can be restored by HA14-1.

Sulindac Sulfide Enhances TRAIL-Mediated Apoptosis

Based upon our finding that sulindac sulfide treatment of SW480 cells can induce DR4 and DR5 expression, we determined whether this NSAID could enhance TRAIL-mediated apoptosis. SW480/neo cells were

incubated with increasing doses of sulindac sulfide (0–80 $\mu\text{mol/L}$) alone and in combination with low doses of TRAIL (0.1, 1.0 ng/mL; Fig. 5A). Sulindac sulfide at a dose of ≥ 40 $\mu\text{mol/L}$ enhanced apoptosis induction by 1 ng/mL of TRAIL. We also evaluated the combination of sulindac sulfide and TRAIL in the HCT-15 human colon cancer cell line given our previous finding that sulindac sulfide can increase DR5 mRNA and protein expression in these cells (27). When coadministered with TRAIL, sulindac sulfide significantly increased Annexin V-FITC labeling compared with either drug alone in HCT-15 and SW480 cells (Fig. 5B). We then determined whether sulindac sulfide could enhance TRAIL-induced apoptosis in SW480 cells with ectopic Bcl-2 expression. Bcl-2 has been shown by ourselves (43) and others (45–47) to attenuate TRAIL-mediated apoptosis. These data indicate the need to overcome the mitochondrial block imposed by Bcl-2 to enhance TRAIL efficacy. In SW480/Bcl-2 cells, coadministration of sulindac sulfide and TRAIL produced a cooperative apoptotic response (Fig. 6A). In an attempt to elucidate the molecular basis for the cooperative proapoptotic effect, we examined their effects upon apoptotic signaling in SW480/Bcl-2 cells. Coadministration of sulindac sulfide and TRAIL enhanced cleavage of procaspase-8 and caspase-9 and also increased the release of cytochrome *c* and Omi/HtrA2 into the cytosol (Fig. 6B). This drug combination also markedly decreased Bax expression consistent with its redistribution to the mitochondria. Together, these data indicate that sulindac sulfide engages both the extrinsic and intrinsic apoptotic pathways to sensitize SW480 colon cancer cells to TRAIL-mediated apoptosis. The combination of sulindac sulfide and TRAIL overcame Bcl-2-mediated apoptosis resistance, as did HA14-1 (Fig. 4B), suggesting that sulindac can increase TRAIL efficacy in Bcl-2-overexpressing colorectal cancers.

Discussion

Elucidating mechanisms of apoptosis resistance to sulindac and their circumvention have important implications for chemoprevention and treatment of established neoplasms. We found that sulindac sulfide up-regulates DR4 and DR5 protein expression and can activate procaspase-8 to engage the extrinsic pathway. Crosstalk with the intrinsic pathway was evidenced by activation of Bid and release of cytochrome *c*, Smac/DIABLO, and Omi/HtrA2 into the cytosol. Smac/DIABLO and Omi/HtrA2 promote caspase activating by binding to and degrading IAPs (18–20). Suppression of Omi/HtrA2 by small interfering RNA has been shown to reduce cell death in response to TRAIL and etoposide (48). Sulindac sulfide was also found to cleave caspase-9 and to inactivate XIAP. XIAP binds caspases -9, -7, -3 and studies indicate that down-regulation of XIAP enhances caspase activation and tumor cell apoptosis (49). The finding that sulindac sulfide engages both the extrinsic and intrinsic apoptotic pathways in SW480 cells is further supported by the ability of selective inhibitors of caspase-8 or caspase-9 to attenuate sulindac-induced apoptosis.

Bcl-2 proteins are overexpressed in a majority of colorectal adenomas, including diminutive tumors (50, 51). Dysregulated Bcl-2 localizes to dysplastic epithelial cells compared with normal colonic crypts where it is confined to the stem cell region (50, 51). Strategies to overcome Bcl-2-mediated apoptosis resistance have the potential to significantly increase the efficacy of chemopreventive agents, as well as cancer therapy. In a recent report, celecoxib was found to induce apoptosis in an apoptosome-dependent yet Bcl-2-independent pathway in lymphoma cell lines (52). To determine whether Bcl-2 is a critical determinant of sulindac resistance, we ectopically expressed Bcl-2 in SW480 human colon cancer cells. Sulindac sulfide-induced apoptosis was attenuated in SW480/Bcl-2 cells that also showed

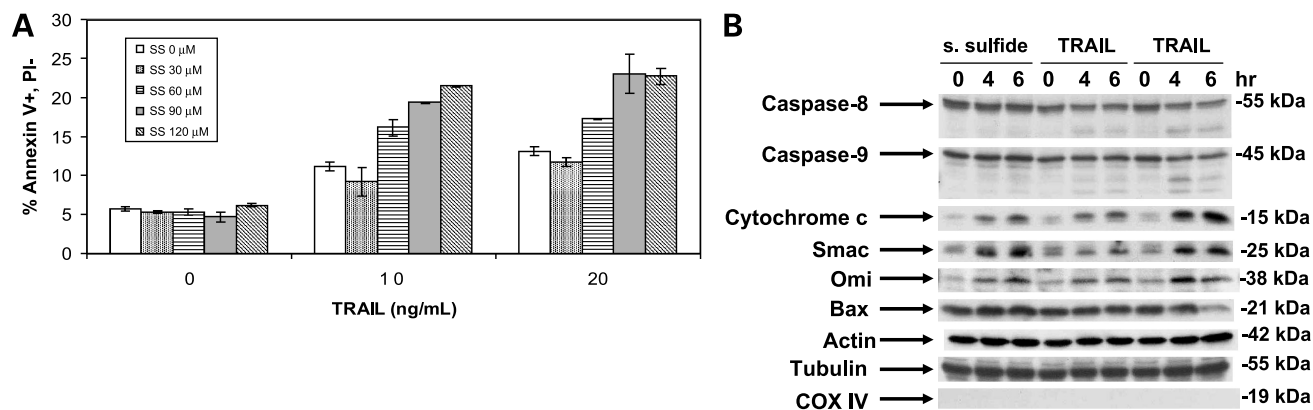


Figure 6. Sulindac sulfide and TRAIL cooperatively increase apoptosis and enhance apoptotic signaling in SW480/Bcl-2 cells. **A**, SW480/Bcl-2 were incubated for 5 h with increasing doses of sulindac combined with TRAIL. Apoptosis was measured using an Annexin V-FITC, propidium iodide (PI) binding assay (**B**). Coadministration of sulindac and TRAIL enhanced apoptotic signaling compared to either drug alone. SW480/Bcl-2 cells were treated with sulindac sulfide (100 $\mu\text{mol/L}$), TRAIL (10 ng/mL), or their combination for indicated times. Cells were then fractionated and cytoplasm-enriched extracts were collected and analyzed by immunoblotting.

reduced cytochrome *c* release and delayed caspase-9 and XIAP cleavage compared with SW480/neo cells. Inhibition of XIAP cleavage by Bcl-2 would therefore result in a delay or inhibition of caspase activation as is shown here for caspase-9. Bcl-2 did not inhibit Smac/DIABLO nor Omi/HtrA2 release into the cytosol. Phosphorylation of Bid was suggested in Bcl-2-overexpressing cells indicating a posttranslational modification. Phosphorylation of murine and human Bid have been reported and in the case of murine Bid, this was shown to occur by casein kinases I and II with identification of the specific phosphorylation sites (42). Bid phosphorylation has been shown to render tumor cells insensitive to caspase-8 cleavage and to Fas-mediated apoptosis (42).

In an effort to reverse Bcl-2-mediated apoptosis resistance, we used the small-molecule Bcl-2 inhibitor HA14-1 that interacts at the BH3-binding site of Bcl-2 to inhibit its function (28). Incubation of SW480/Bcl-2 cells with a nonapoptotic dose of HA14-1 enhanced sulindac sulfide-induced annexin V binding. Coadministration of HA14-1 and sulindac sulfide cleaved caspase-8 and caspase-9 compared with the NSAID alone (Fig. 4B). The ability of HA14-1 to enhance caspase-8 cleavage by sulindac sulfide suggests that Bcl-2 exerts an inhibitory effect upon caspase-8 activation consistent with the presence of a feedback amplification loop. Specifically, effector caspase-3 can serve as a feedback loop by cleaving caspase-8 to amplify the apoptotic signal (39, 44). Caspase-9 has also been shown to activate caspase-8 in a caspase-3-dependent manner (39, 40). In this regard, an inhibitor of caspase-9 was found to reduce caspase-8 activation and vice versa in ovarian cancer cells (41). Therefore, enhancement of sulindac sulfide-induced caspase-9 activation by HA14-1 may enhance caspase-8 activation. These data further support the observation that caspase-8 can be activated upstream and downstream of mitochondria as shown after TRAIL (47, 53) and CD95(APO-1/Fas) treatment in type II cells (54). Coadministration of HA14-1 and sulindac sulfide reduced Bax expression consistent with its redistribution to the mitochondria thereby enabling cytochrome *c* release. The addition of HA14-1 also enhanced Smac release. Of note, Bcl-2 levels in human colon cancer cells have been shown to be unaffected by sulindac treatment (55).

Consistent with these data, suppression of Bcl-2 using RNA interference produced abundant p53-dependent apoptosis in colon cancer cells even in the absence of cytotoxic drugs (56). In addition to Bcl-2, resistance to sulindac-induced apoptosis can occur secondary to mutational inactivation of *Bax* in colon cancer cells (23). Furthermore, mutations in codon 12 of the *K-ras* oncogene were shown to confer resistance to sulindac in transformed enterocytes (57).

We previously reported that forced COX-2 expression transcriptionally repressed DR5 expression and inhibited the effects of TRAIL upon caspase activation and cell viability in HCT-15 colon cancer cells. In these cells, sulindac sulfide induced DR5 expression levels (27).

Sulindac sulfide was found to up-regulate DR4 and DR5 protein levels in SW480 cells, suggesting that this drug may enhance TRAIL-mediated apoptosis. Accordingly, we evaluated the combination of sulindac sulfide and TRAIL in SW480/Bcl-2 cells in an effort to overcome the mitochondrial block. Engagement of the extrinsic pathway by TRAIL is a promising and novel strategy for treatment of tumor cells, particularly those with defective mitochondrial apoptotic signaling (16). As shown here, coadministration of sulindac sulfide and low doses of TRAIL increased Annexin V binding to a greater extent than did either drug alone in both SW480/neo and SW480/Bcl-2 cells, as well as in HCT-15 colon cancer cells. Sulindac sulfide increases DR5 mRNA and protein levels in HCT-15 cells as previously shown by our laboratory (43). Coadministration of sulindac sulfide and TRAIL in SW480/Bcl-2 cells enhanced cleavage of caspase-8 and caspase-9; increased release of cytochrome *c*, and Omi/HtrA2; and reduced cytosolic Bax, compared with either drug alone. These findings are highly relevant to most human tumor cells that require a Bcl-2 regulated mitochondrial amplification step after a death receptor stimulus (58). TRAIL enhanced sulindac sulfide-induced apoptotic signaling as effectively as did HA14-1, suggesting an alternative and novel approach to circumventing Bcl-2-mediated resistance. Perhaps other NSAIDs can increase TRAIL sensitivity in Bcl-2-overexpressing human tumor cells. Thus, the use of sulindac in cancer therapy may largely reside in its ability to sensitize tumor cells for apoptosis. Sulindac may also enable a reduction in the dose of TRAIL used clinically. Such a strategy is attractive in that NSAIDs are relatively nontoxic agents compared with cytotoxic chemotherapy.

Our results provide a biological rationale for the combination of sulindac sulfide and TRAIL. We used doses of sulindac sulfide that have been shown to exert relevant biological effects upon colon cancer growth and apoptosis (1, 9, 10). Importantly, sulindac sulfide is concentrated in the colonic epithelium to levels that are at least 20-fold higher than those achieved in serum (59). SW480 cells carry mutated *p53* (49), as do HCT-15 cells. Death receptor-mediated apoptosis occurs independently of *p53* (16), and therefore, modulation of death receptor expression levels, as shown here for sulindac sulfide, may be an effective approach in colorectal cancers where the majority carry *p53* mutations.

In conclusion, ectopic Bcl-2 expression confers resistance to sulindac sulfide-induced apoptotic signaling that can be circumvented by use of the small-molecule Bcl-2 inhibitor HA14-1. Alternatively, Bcl-2-mediated resistance can be reversed by coadministration of sulindac sulfide and TRAIL, which cooperatively enhanced apoptotic signaling. The mechanism for this effect seems to be up-regulation of death receptor expression with increased caspase-8 activation and crosstalk with the mitochondrial pathway. These novel strategies for enhancing apoptotic signaling in Bcl-2-overexpressing tumor cells have important implications for both chemoprevention and treatment of established colorectal neoplasms.

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