Sulindac Sulfide-induced Apoptosis Involves Death Receptor 5 and the Caspase 8-dependent Pathway in Human Colon and Prostate Cancer Cells

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ABSTRACT

Sulindac is the most extensively investigated clinically relevant chemopreventive nonsteroidal anti-inflammatory drug. Sulindac sulfide is one of the major metabolites of sulindac that is believed to mediate its antitumorigenic effects by inducing apoptosis. Recent evidence suggests that sulindac sulfide engages the mitochondrial pathway involving caspase-9 and Bax to mediate its apoptotic effects [Zhang et al., Science (Wash. DC), 290: 989–992, 2000]. In this report, we demonstrate that sulindac sulfide also engaged the membrane death receptor (DR) pathway to mediate apoptosis. Sulindac sulfide up-regulated DR5 and activated the proximal caspase 8 in various different colon and prostate cancer cell lines. Sulindac sulfide specifically up-regulated the DR5 levels but had no effect on the levels of other DRs including DR4, Fas, and tumor necrosis factor receptor 1. To further delineate the role of DR5 in sulindac sulfide-induced apoptosis, we used JCA-1 prostate cancer cells that are deficient in mounting a Fas and tumor necrosis factor receptor 1-dependent apoptotic response but are proficient in mediating DR5-dependent apoptosis. JCA-1 cells were stably transfected with dominant-negative Fas-associated death domain to block the flow of apoptotic signals originating from the endogenous DR5, and sulindac sulfide-induced apoptosis was investigated. Our results indicated that by blocking the DR5-dependent apoptotic pathway, dominant-negative Fas-associated death domain did indeed inhibit sulindac sulfide-induced apoptosis. Furthermore, exogenous tumor necrosis factor-related apoptosis-inducing ligand, the ligand for DR5, also potentiated sulindac sulfide-induced apoptosis in all of the cell lines tested, thereby further supporting the involvement of DR5 in sulindac sulfide-induced apoptosis. Thus, our results demonstrate that sulindac sulfide also engages the membrane DR pathway involving DR5 and proximal caspase 8 to induce apoptosis.

INTRODUCTION

NSAIDs1 are well known for their analgesic, antipyretic, and anti-inflammatory effects. NSAIDs are believed to mediate their effects by inhibiting two COX isoenzymes, COX-1 and COX-2 (reviewed in Ref. 1). Both COX-1 and COX-2 catalyze the formation of prostaglandins from arachidonic acid, and whereas COX-1 is constitutively expressed, COX-2 is an inducible enzyme (reviewed in Ref. 1). A number of studies have demonstrated that the regular use of NSAIDs can reduce the risk of colon cancer (reviewed in Refs. 1–3). There are also reports suggesting beneficial effects of NSAIDs in the prevention of lung, esophageal, and stomach cancers (reviewed in Ref. 4). In fact, the National Cancer Institute is currently sponsoring clinical trials to investigate the efficacy of COX-2-selective NSAIDs in the prevention and treatment of colon, skin, and bladder cancer. The mechanisms by which the NSAIDs mediate their chemopreventive and antitumorigenic effects remain less well understood but may be multifaceted in nature. Evidence suggests that the chemopreventive effects of NSAIDs could be attributed to their apoptosis-inducing potential. For example, sulindac is the most extensively investigated clinically relevant chemopreventive NSAID that reduces the number and size of the colorectal tumors in genetically susceptible humans and animals (5–7). Sulindac sulfide and sulindac sulfone are the two major metabolites of sulindac; sulindac sulfide is COX selective, whereas sulindac sulfone is believed to lack COX-inhibitory activity (8). Both sulindac metabolites have been reported to induce apoptosis in various different cell types (8–11). Despite the fact that NSAIDs are important cancer chemopreventive agents that induce apoptosis, relatively little is known about the molecular and biochemical mechanisms of NSAID-induced apoptosis. Clearly, more studies are needed to fully elucidate the molecular and biochemical pathways of NSAID-induced apoptosis.

A wealth of information supports the notion that apoptosis is controlled via two major apoptotic pathways, one that originates at the membrane and another that involves the mitochondria (reviewed in Refs. 12–14). The apoptotic pathways originating at the membrane involve membrane DRs such as Fas, TNF-R1, DR3, DR4, and DR5 (reviewed in Refs. 12–14). These DRs are activated by their respective ligands and engage the intracellular apoptotic machinery involving adaptor molecules and proximal as well as distal (executioner) caspases. TNF-α, Fas ligand, and Apo3L are ligands for TNF-R1, Fas, and DR3, respectively, whereas TRAIL (also known as Apo2L) activates DR4 and DR5 (reviewed in Refs. 12–14). Agonist antibodies or overexpression also activates these DRs, albeit in a ligand-independent manner. Ligand-dependent or -independent activation of DRs results in the recruitment of the intracellular adaptor molecules that further engage the intracellular proximal caspase 8 at the receptor site (reviewed in Refs. 12–14). For example, activated TNF-R1 and DR3 sequentially recruit the intracellular adaptor molecules TRADD, FADD, and proximal caspase 8, which results in the flow of apoptotic signals further downstream. The DR Fas does not interact with TRADD but directly recruits FADD to activate the proximal caspase 8 (reviewed in Refs. 12–14). Although evidence from FADD knock-out cells suggests that FADD may not be absolutely required for DR4-mediated apoptosis (15), a number of recent studies demonstrate that both DR4 and DR5 independently interact with FADD to engage and activate proximal caspase 8 (16–20).

The apoptotic events engaging the mitochondrial pathway affect mitochondrial permeability and the release of cytochrome c from the mitochondria into the cytosol (reviewed in Ref. 21). In the presence of ATP/ADP, cytosolic cytochrome c interacts with apaf1 and procaspase 9, which results in the activation of caspase 9 and the downstream caspases (reviewed in Ref. 21). A large body of evidence suggests that Bcl-2 or Bcl-xL inhibits the release of cytochrome c from the mitochondria and consequently blocks apoptosis induced by a variety of agents. By contrast, Bax and Bid, the proapoptotic members of the Bcl-2 family, are believed to mediate their apoptotic effects by increasing the release of cytochrome c from the mitochondria (reviewed in Ref. 21).

In a recent study, Zhang et al. (11) have reported that the NSAIDs

References

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5. The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; DR, death receptor; TNF, tumor necrosis factor; TNF-R1, tumor necrosis factor receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; COX, cyclooxygenase; FADD, Fas-associated death domain; FADD-DN, dominant-negative FADD; TRADD, TNF-R-associated death domain; RT-PCR, reverse transcription-PCR.
sulindac sulfide and indomethacin mediate apoptosis by engaging the mitochondrial pathway involving caspase 9 and Bax in human colon cancer cells. They further demonstrated that the disruption of the BAX gene in human colon cancer cells abrogated the apoptosis mediated by these NSAIDs (11). We undertook this study to investigate whether NSAID-mediated apoptosis also involves membrane DRs and proximal caspase 8. Here we report, for the first time, that the NSAID sulindac sulfide also engages DR5 and proximal caspase 8 to mediate apoptosis in human colon and prostate cancer cells.

MATERIALS AND METHODS

Materials. Sulindac sulfide and sulindac sulfone were provided by Merck (Rahway, NJ). Recombinant TNF-α was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-human DR5 antibody was purchased from Alexis Biochemicals (San Diego, CA), antihuman pro-caspase 8 antibody was purchased from PharMingen (San Diego, CA), antihuman FADD and antihuman Fas antibodies were purchased from Transduction Laboratories (Lexington, KY), and anti-human β-actin antibody was purchased from Sigma Chemical Co. Anti-TNF-R1 antibody and recombinant DR5-Fc were obtained from R&D Systems (Minneapolis, MN). Dr. Avi Ashkenazi (Genentech, San Francisco, CA) kindly provided TRAIL.

Cell Lines and Cell Culture. HT29 and HCT116 human colon cancer cells and DU145, JCA-1, and TSU-Pr1 human prostate cancer cells were regularly maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA). MCF7, MDA-MB-231, and DU145 cells growing in 60-mm plates were exposed to sulindac sulfide (125 μM) and 48 h later for apoptosis detection as described in the Materials and Methods.

Analysis for Apoptosis. Cells exhibiting morphological features of apoptosis were detected as described previously (22, 23). Briefly, floating and adherent cells were fixed with methanol, stained with 4',6-diamidino-2-phenylindole solution, and examined under a fluorescent microscope using a 420 nm filter. The apoptotic cells exhibiting morphological features of apoptosis including chromatin condensation and nuclear fragmentation were counted in 5–10 randomly selected fields. Approximately 300–1000 nuclei were examined for each sample, and the results were expressed as the number of apoptotic nuclei divided by the total number of nuclei counted.

Cell Lines and Cell Culture. HT29 and HCT116 human colon cancer cells and DU145, JCA-1, and TSU-Pr1 human prostate cancer cells were regularly maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (Gemini Bioproducts, Calabasas, CA).

RESULTS AND DISCUSSION

Fig. 1A illustrates the morphological features of sulindac sulfide-induced apoptosis in one representative JCA-1 human prostate cancer cell line. Sulindac sulfide-treated prostate cancer cells exhibit char-
The sulfone metabolite had no effect on DR5 expression when used at an equimolar concentration (data not shown). At higher concentrations, however, sulindac sulfone did regulate DR5 expression, but the effect was less pronounced than that noted with lower concentrations of sulindac sulfide. A representative Northern blot in Fig. 2B illustrates the effects of 400 and 500 μM sulindac sulfone on DR5 regulation in HCT116 and JCA-1 cells, respectively. Weak induction of DR5 by 600 μM sulindac sulfone was also noted in DU145 cells (data not shown). DR4 is the second DR that is activated by TRAIL. Consistent with previous reports (22, 25), we also detected multiple transcripts of the DR4 gene in these cell lines (Fig. 2C). However, unlike DR5, DR4 expression was not regulated by sulindac sulfide (Fig. 2C), although the DU145 prostate cancer cell line exhibited a modest increase in DR4 mRNA expression (Fig. 2C); the significance of this change on the overall DR4 levels remains to be investigated. Next, we investigated the effects of sulindac sulfide on TRAIL (the DR5 ligand) gene expression. Because the TRAIL cDNA probe cross-hybridized with several nonspecific transcripts, Northern blotting was not informative. RT-PCR was therefore used to investigate the expression of TRAIL and its regulation by sulindac sulfide in these cells. All cell lines were found to express TRAIL mRNA, albeit differentially, but sulindac sulfide did not regulate its expression. Fig. 2D shows representative results indicating the lack of effect of sulindac sulfide on TRAIL mRNA regulation in HT29 human colon cancer cells. To ensure that these results were not due to a PCR artifact, we confirmed the DR5 mRNA induction using similar RT-PCR conditions (data not shown).

To determine whether sulindac sulfide-induced DR5 mRNA up-regulation was coupled with similar changes at the protein levels, Western blot analyses were performed using commercially available antihuman DR5 antibodies. Our results indicated that sulindac sulfide also enhanced DR5 protein levels (Fig. 2E). It is noteworthy that the two-band pattern of DR5 protein is consistent with the previously described two-band pattern of DR5 and Caspase 8 activation by sulindac sulfide.

Fig. 2. A, representative Northern blots show that sulindac sulfide up-regulates DR5 gene expression in human prostate and colon cancer cells. Cells were treated with vehicle (DMSO) alone as a control (C) or with sulindac sulfide (125 μM for HCT116 cells and 200 μM for the rest of the cell lines) for the indicated time periods (in h). Total RNA was extracted, and Northern blot analyses were performed by standard methods. A human DR5 cDNA probe was used to detect DR5 expression. Ethidium bromide staining of the gels shows RNA integrity and comparable loading. B, representative Northern blots show that sulindac sulfone up-regulates DR5 gene expression in human prostate and colon cancer cells. Cells were treated with vehicle (DMSO) alone as a control (C) or with sulindac sulfone (100 μM for HCT116 cells and 500 μM for ICA-1 cells) for 20 or 40 h. Total RNA was extracted, and Northern blot analyses were performed by standard methods. Ethidium bromide staining of the gels shows RNA integrity and comparable loading. C, representative Northern blot showing the effect of sulindac sulfide on DR4 gene expression in JCA-1, DU145, and HCT116 human prostate and colon cancer cells. Cells were treated with sulindac sulfide as described in A, and Northern analysis was performed by standard methods using a human DR4 cDNA probe. D, RT-PCR analysis of TRAIL and β-actin expression in sulindac sulfide-treated HT29 human colon cancer cells. Cells were treated with vehicle (DMSO) alone as a control or with sulindac sulfide (SD), and RNA was extracted and reverse-transcribed into cDNA. The indicated dilutions of cDNA were used for PCR amplifications as described in “Materials and Methods” and analyzed on an ethidium bromide-stained agarose gel. E, representative Western blots showing that sulindac sulfone up-regulates the DR5 protein levels in DU145 and HCT116 cells. Cells were treated with vehicle (DMSO) alone as a control (C) or with sulindac sulfide (200 μM for DU145 cells and 125 μM for HCT116 cells) for the indicated time periods (in h). Western blot analyses were performed using a polyclonal anti-DR5 antibody (top panels). The same blots were probed with anti-β-actin antibody to demonstrate comparable loading (middle panels). The specificity of the anti-DR5 antibody was confirmed using 10 ng of purified DR5-Fc recombinant protein (R&D Systems) as a positive control, which was analyzed along with 50 μg of total protein from untreated DU145 cells (bottom panels). The two-band pattern of the endogenous DR5 protein is consistent with previously published results (26).
published results on DR5 protein (26). Furthermore, the specificity of the anti-DR5 antibody was confirmed by using the recombinant DR5 protein as a positive control (Fig. 2E).

Previous studies have demonstrated that DRs can be activated in ligand-dependent and -independent manners (reviewed in Ref. 12). The ligand-independent activation occurs via two mechanisms: (a) by agonist antibody; or (b) when the levels of DRs are increased through endogenous induction or exogenous overexpression. Exogenously overexpressed DR5, without concomitant increases in the levels of its ligand, has been shown to induce apoptosis (24, 27–29). Up-regulation of endogenous DR5 by several genotoxic and nongenotoxic agents is also associated with induction of apoptosis (22). Our results indicate that sulindac sulfide-induced apoptosis is coupled with DR5 induction without changes in the expression of its ligand, TRAIL (Fig. 2D). It is therefore possible that sulindac sulfide may activate DR5 to induce cell death in a ligand-independent manner. Alternatively, or coupled with this possibility, sulindac sulfide, by increasing the levels of endogenous DR5, may sensitize these cells to pre-existing TRAIL that is known to exist as a soluble as well as a type II cell surface protein. Our results suggest that this could be a possibility because these cells do express TRAIL mRNA (Fig. 2D; data not shown).

Caspase 8 is one of the most proximal caspases activated by signals originating at the membrane DRs including DR5. We reasoned that if sulindac sulfide up-regulation of DR5 results in activation of this membrane DR (regardless of whether it is ligand dependent or independent), then caspase 8 should also be activated in these cells. We therefore investigated the effects of sulindac sulfide on caspase 8 activation, and the results presented in Fig. 3A indicate that sulindac sulfide did indeed activate caspase 8 in all of the cell lines in which it also induced DR5 expression. Sulindac sulfone did not induce caspase 8 activation at an equimolar concentration (Fig. 3A); however, at a higher concentration (400–600 μM), some activation of caspase 8 was observed, but the effect was less pronounced than that noted with sulindac sulfide (data not shown). Caspase 8 (like other caspases) exists in an inactive form as a pro-caspase, and its activation involves cleavage of the pro-form into smaller active subunits. Thus, the decrease in the level of pro-caspase 8 as shown in Fig. 3A reflects its activation (30–33). The same blots were probed with antiactin antibody to demonstrate comparable loading of the samples in each lane (Fig. 3A). To further confirm caspase 8 activation, we performed a caspase 8 activity assay. Ac-IETD-AFC caspase 8 fluorogenic substrate (PharMingen) was used to measure caspase 8 activity in the lysates of untreated and sulindac sulfide-treated DU145 cells. The representative results shown in Fig. 3B indicate that the cleavage of Ac-IETD-AFC, representing caspase 8 activity, increased in sulindac sulfide-treated cells within approximately 15 h. Together, these results demonstrate that caspase 8 is activated during apoptosis induced by sulindac sulfide. We further reasoned that if caspase 8 activation was critical for sulindac sulfide-induced apoptosis, then caspase 8 inhibitor should block/inhibit sulindac sulfide-mediated apoptosis. Therefore, we used a cell-permeable caspase 8 inhibitor, z-IETD-FMK (R&D Systems), to test its effect on sulindac sulfide-induced apoptosis. As shown in Fig. 3C, caspase 8 inhibitor clearly inhibits sulindac sulfide-induced apoptosis.
Caspase 8 is predominantly activated by signals originating at the membrane DRs. It was possible that sulindac sulfide, in addition to activating DR5, might also activate other DRs. We therefore investigated whether sulindac sulfide also regulates other DRs. Representative results in Fig. 3D indicate that both cell lines did not exhibit detectable Fas and TNF-R1, and sulindac sulfide did not enhance their levels. The lysates of Jurkat and human endothelial cells serving as positive controls for Fas and TNF-R1, respectively, were used on the same membranes to confirm that the antibodies were working (Fig. 3D). It is possible that DU145 and HCT116 cells may exhibit very low levels of Fas and TNF-R1 that cannot be detected by Western blotting. DR3 is expressed primarily by cells and tissues of lymphoid origin (reviewed in Refs. 12 and 13); Northern blot analyses revealed that untreated or sulindac sulfide-treated cells also did not exhibit detectable DR3 mRNA (data not shown). Therefore, these results suggest that sulindac sulfide may predominantly engage DR5, but not Fas, TNF-R1, and DR3, to mediate its apoptotic effects.

Recent studies demonstrate that DU145 and JCA-1 human prostate cancer cells are resistant to Fas ligand-induced apoptosis and thus have a defective Fas-mediated apoptotic signaling pathway (Ref. 34 and the references therein). To further investigate whether these two cell lines harbor intact TNF-R1 and/or DR5-dependent apoptotic pathways, we treated these cells with TNF-α, the TNF-R1 ligand, and with TRAIL, the DR5 ligand. Our results indicated that both the DU145 and JCA-1 cell lines are resistant to TNF-α (data not shown) but sensitive to TRAIL-induced apoptosis (see below). These results suggest that, in addition to a defective Fas pathway, both of these cell lines also harbor a defective TNF-R1-dependent apoptotic pathway. However, both of these cell lines are proficient in mounting a TRAIL-mediated DR5-dependent apoptotic response. These results, coupled with our other findings that these cells do not express DR3 and that sulindac sulfide does not regulate DR4 (Fig. 2C), strongly implicate DR5 as the predominant DR that is regulated and activated during sulindac sulfide-mediated apoptosis.

To further investigate the contribution of DR5 in sulindac sulfide-induced apoptosis, we established stable transfectants expressing FADD-DN in JCA-1 cells. We chose JCA-1 cells and FADD-DN for the following reasons: (a) JCA-1 cells do not express DR3, and they harbor defective Fas and TNF-R1 pathways (Ref. 34 and this study); thus, sulindac sulfide may not engage these three DRs to mediate apoptosis in these cells; (b) JCA-1 cells do express DR4 and DR5 and are sensitive to TRAIL-mediated cell death; however, sulindac sulfide regulates DR5 but not DR4, and thus DR5 is likely to be the main DR mediating the apoptotic effects of this NSAID; and (c) FADD-DN has been shown to block/inhibit apoptosis induced by all of the known DRs including DR5 (15–19). Our results suggest that the other DRs including Fas, TNF-R1, DR3, and DR4 may not be involved in sulindac sulfide-mediated apoptosis. Thus, if DR5 plays an important role in sulindac sulfide-induced apoptosis, then the interruption of DR5-dependent apoptotic signal transduction by FADD-DN should inhibit/block cell death induced by this NSAID.

JCA-1 cells were transfected with expression vector carrying FADD-DN cDNA; control cells were transfected with the same vector without the FADD-DN cDNA insert. The exogenous FADD-DN lacks the death effector domain and thus migrates faster than the endogenous full-length FADD when analyzed on SDS-PAGE. As shown in Fig. 4A, several independently isolated clones express exogenous FADD-DN, whereas vector only-transfected cells do not. We selected...
Evidence also suggests that certain apoptic caspases 7, 6, and 3 without engaging the mitochondrial pathway to mediate its apoptotic effects. Our current findings demonstrate that sulindac sulfide engages the mitochondrial pathway involving caspase 9 and suggests the apoptotic pathway. A recent study (11) has demonstrated that sulindac sulfide, by increasing the DR5 (but not DR4) levels, appears to regulate the DR5 levels. These results further suggest that sulindac sulfide, by increasing the DR5 (but not DR4) levels, appears to sensitize these cells to killing by TRAIL.

In this study, we have presented evidence that sulindac sulfide mediates apoptosis by engaging the membrane DR DR5-dependent apoptotic pathway. A recent study (11) has demonstrated that sulindac sulfide engages the mitochondrial pathway involving caspase 9 and Bax to mediate its apoptotic effects. Our current findings demonstrate that this NSAID also engages the membrane DR pathway to mediate apoptosis. Evidence suggests that, in general, signals originating at the membrane DRs are transduced via adaptor molecules FADD, TRADD, and proximal caspase 8 to the executioner caspases such as caspases 7, 6, and 3 without engaging the mitochondrial pathway (reviewed in Refs. 12–14). Evidence also suggests that certain apoptotic signals can directly engage the intracellular apoptotic machinery in a DR-independent manner and involve the mitochondrial and postmitochondrial pathways to induce apoptosis (reviewed in Refs. 21 and 30). Recent studies, however, indicate the existence of a cross-talk between the apoptotic signals originating at the membrane DRs and mitochondria (35, 36). Bid is an intracellular molecule that bridges the apoptotic interactions between the DRs Fas/TNF-R1 and the mitochondria (35, 36). Whether Bid and/or other as yet unidentified molecules can also bridge such a cross-talk between the apoptotic signals originating at DR4/DR5 and mitochondria is a possibility that remains to be investigated. Zhang et al. (11) have demonstrated that mitochondrial and postmitochondrial events appear to be important in sulindac sulfide-mediated apoptosis because deletion of Bax completely blocked sulindac sulfide-induced apoptosis in HCT116 human colon cancer cells. In our studies, we have demonstrated that sulindac sulfide appears to engage DR5 and proximal caspase 8. Although caspase 8 inhibitor and FADD-DN clearly inhibited sulindac sulfide-induced apoptosis, they did not completely block apoptosis induced by this NSAID in the human prostate cancer cells tested. Based on these findings, it is likely that sulindac sulfide would also engage other apoptotic pathways such as the mitochondrial pathway to fully execute apoptosis. Whether DR5 and caspase 8 activation resides upstream or downstream or is independent of mitochondrial events in sulindac sulfide-mediated apoptosis is an important issue that requires further investigation. Additional studies are also needed to explore the role of Bid in mediating interactions between DR5 and mitochondrial pathways during sulindac sulfide-mediated apoptosis.

Our results also indicate that the sulfone metabolite of sulindac did not regulate DR5, activate caspase 8, or induce apoptosis when tested at equimolar concentrations. However, at higher concentrations, sulindac sulfone did induce apoptosis and selectively up-regulate DR5 expression, but its effects were less pronounced than those noted with sulindac sulfide. Sulindac sulfide is known to inhibit COX activity, whereas sulindac sulfone does not (8). Therefore, it is possible that sulindac sulfide regulation of DR5 could occur independent of its effect on COX activity. Regardless of COX dependence or independence, the sulindac sulfide regulation of DR5 is a novel mechanism by which this NSAID may mediate its anticancer effects. In conclusion, we have shown that sulindac sulfide, an important clinically relevant NSAID, mediates apoptosis by engaging DR5, and its effects can be potentiated with TRAIL. TRAIL has shown significant promise as a novel anticancer agent that selectively kills tumor cells and is well tolerated by animals (37). Recently, however, TRAIL has been shown to be more toxic to human hepatocytes than to animal hepatocytes (38). Thus, hepatic toxicity may limit the utility of TRAIL as a single anticancer agent. Based on our findings that TRAIL and sulindac sulfide exhibit synergy to mediate apoptosis, we propose that a combination of lower doses of TRAIL and sulindac sulfide (and perhaps other NSAIDs) could be a novel therapeutic strategy against prostate cancer, colon cancer, and other types of malignancies.

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