Sulfuraphane Enhances the Therapeutic Potential of TRAIL in Prostate Cancer Orthotopic Model through Regulation of Apoptosis, Metastasis, and Angiogenesis

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Abstract

Purpose: The purpose of this study was to examine the molecular mechanisms by which sulfuraphane enhances the therapeutic potential of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in prostate cancer.

Experimental Design: Cell viability and apoptosis assays were done by XTT and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay, respectively. Tumor-bearing mice were treated with vehicle, sulfuraphane, TRAIL, and sulfuraphane plus TRAIL. Markers of apoptosis, angiogenesis, and metastasis were measured by immunohistochemistry.

Results: Sulfuraphane enhanced the therapeutic potential of TRAIL in PC-3 cells and sensitized TRAIL-resistant LNCaP cells. Sulfuraphane-induced apoptosis in PC-3 cells correlated with the generation of intracellular reactive oxygen species (ROS), collapse of mitochondrial membrane potential, activation of caspase-3 and caspase-9, and up-regulation of DR4 and DR5. Sulfuraphane induced the expression of Bax, Bak, Bim, and Noxa and inhibited the expression of Bcl-2, Bcl-XL, and Mcl-1. The quenching of ROS generation with antioxidant N-acetyl-L-cysteine conferred significant protection against sulfuraphane-induced ROS generation, mitochondrial membrane potential disruption, caspase-3 activation, and apoptosis. Sulfuraphane inhibited growth of orthotopically implanted PC-3 tumors by inducing apoptosis and inhibiting proliferation and also enhanced the antitumor activity of TRAIL. Sulfuraphane up-regulated the expressions of TRAIL-R1/DR4, TRAIL-R2/DR5, Bax and Bak and inhibited the activation of nuclear factor-κB P13K/AKT and MEK/ERK pathways in tumor tissues. The combination of sulfuraphane and TRAIL was more effective in inhibiting markers of angiogenesis and metastasis and activating FOXO3a transcription factor than single agent alone.

Conclusions: The ability of sulfuraphane to inhibit tumor growth, metastasis, and angiogenesis and to enhance the therapeutic potential of TRAIL suggests that sulfuraphane alone or in combination with TRAIL can be used for the management of prostate cancer.

Prostate cancer is one of the major life-threatening diseases in most western countries. The incidence and mortality rates of prostate cancer have also rapidly increased in the past decade. Although patients with metastatic prostate cancer can benefit from androgen-ablation therapy at the initial stage, most patients die of hormone-refractory prostate cancer in only few years. Salvage cytotoxic therapy has been notoriously related to significant morbidity with little, if any, survival benefit. There is an urgent need to discover novel and effective chemopreventive agents using clinically relevant model of prostate cancer.

Sulfuraphane, a constituent of cruciferous vegetables, is a naturally occurring isothiocyanate with promising chemopreventive activity (1). Sulfuraphane possesses antioxidant, antiproliferative, and anticarcinogenic properties (2, 3). Sulfuraphane is effective in preventing chemically induced breast (4, 5), stomach (1), and colon (6) cancers in rats. Sulfuraphane inhibited the growth of established prostate cancer xenografts in mice by oral administration (7) and breast cancer by i.v. injection (8). Sulfuraphane and its metabolite mediate growth arrest and apoptosis in human cancer cells (3, 9–11). Epidemiologic studies have shown that people who eat cruciferous vegetables have reduced incidence of breast and prostate cancer. Test with animals have shown that feeding sulfuraphane reduced the frequency, size, and number of tumors. Sulfuraphane induces a phase II enzyme, thereby neutralizing carcinogens before they can damage DNA (14, 15). Sulfuraphane inhibits benzo[a]pyrene-DNA and 1,6-dinitropyrene-DNA adducts formation. Thus, sulfuraphane may be a potent biological inhibitor of human prostate cancer and thereby can reduce proliferative and invasive activities cancer cells.

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in a wide variety of transformed and cancer cells but has no effect on normal cells (16). Therefore, TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer prevention and treatment. TRAIL induces apoptosis by binding to TRAIL-R1 (DR4) and TRAIL-R2 (DR5) receptors (16), which are ubiquitously expressed in cancer cells. We have recently observed several TRAIL-resistant prostate, breast, and lung cancer cell lines (17–23). However, these resistant cells can be sensitized by down-regulation of constitutively active AKT and NF-κB or by pretreatment with chemotherapeutic drugs and irradiation (19–23). Recent studies have shown that sulforaphane can enhance the proapoptotic effects of TRAIL in hepatoma (24), osteosarcoma (25), and lung adenocarcinoma (26) in vitro. However, there are no studies to show the interactive effects of sulforaphane and TRAIL in vivo. It is therefore essential to show the efficacy of sulforaphane and TRAIL in a suitable mouse model before the clinical potential of this combination regimen is established. The study of the intracellular mechanisms of sulforaphane that control TRAIL sensitivity may enhance our knowledge of death receptor-mediated signaling and help to develop TRAIL and sulforaphane as cancer preventive and/or therapeutic agents.

FOXO subfamily of forkhead transcription factors include FOXO1α/FKHRL1, FOXO3α/FKHL11, and FOXO4/AFX (27). The phosphoinositide 3-kinase (PI3K) pathway, via activation of its downstream kinase AKT, phosphorylates each of the FOXO proteins (28). Inhibition of the PI3K pathway leads to dephosphorylation and nuclear translocation of active FKHRL1, FKHR, and AFX, which induce cells cycle arrest and apoptosis (29). FOXO transcription factors control several genes related to cell cycle and apoptosis such as cyclin-dependent kinase inhibitor p27/Kip1 (30), Bim (31, 32), Fas ligand (33), and Bcl-6 (34). Consequently, activation of the PI3K pathway serves to repress FOXO-mediated growth arrest and apoptosis. We have recently shown that inhibition of PI3K/AKT and MEK/extracellular signal-regulated kinase (ERK) pathways act synergistically to regulate antiangiogenic effects of EGCG through activation of FOXO transcription factors (35). Sulforaphane has been shown to down-regulate PI3K/AKT and NF-κB pathways in vitro. However, its effects on the activation of FOXO proteins have not been examined.

The objectives of our study are to examine the molecular mechanisms by which sulforaphane enhances the apoptosis-inducing potential of TRAIL in vitro and in vivo model of prostate cancer. We have shown that sulforaphane enhances the apoptosis-inducing potential of TRAIL through multiple mechanisms. In vitro, sulforaphane induces death receptors and proapoptotic members of Bcl-2 family, inhibits antiapoptotic Bcl-2 proteins, activates caspase(s), and enhances apoptosis-inducing potential of TRAIL. In vivo, sulforaphane inhibits growth of PC-3 cells orthotopically implanted in nude mice by inducing apoptosis and inhibiting tumor cell proliferation. It inhibits expression of proteins related to invasion, metastasis and angiogenesis, phosphorylation of AKT, ERK1/2, and FOXO3α, and activation of NF-κB. Our studies provide a strong preclinical evidence that sulforaphane either alone or in combination with TRAIL can be used to prevent and/or treat prostate cancer.

Materials and Methods

Reagents. Antibodies against CD31, factor VIII, vascular endothelial growth factor (VEGF), uridine kinase-type plasminogen activator receptor (uPAR), cyclooxygenase-2 (COX-2), transforming growth factor-β1 (TGF-β1), Bcl-2, Bcl-XL, Bax, Bak, TRAIL-R1/DR4, TRAIL-R2/DR5, Bid, PUMA, Noxa, Bim, and t-actin were purchased from Santa Cruz Biotechnology. Antibodies against hypoxia-inducible factor-1α, interleukin (IL)-6, and IL-8 were purchased from Abcam. Antibodies against membrane-type matrix metalloproteinase (MTI-MMP), MMP-2, MMP-7, and MMP-9 were purchased from Cell Signaling Technology. Enhanced chemiluminescence Western blot detection reagents were from Amersham Life Sciences. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay kit was purchased from EMD Biosciences/Calbiochem. TRAIL was purified as described elsewhere (36). Sulforaphane was purchased from LKT Laboratories. Anti-caspase-3, anti-caspase-8, anti-caspase-9, and anti-poly(ADP-ribose) polymerase antibodies were purchased from BD Biosciences/PharMingen. Kits for TUNEL and caspase-3 assays were purchased from EMD Biosciences/Calbiochem. Antibodies against TRAIL-R1/DR4, TRAIL-R2/DR5, DcR1, and DcR2 for flow cytometry were purchased from R&D Systems.

Cell culture. LNCaP and PC-3 cells were obtained from the American Type Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen) at 37°C in a humidified atmosphere of 95% air and 5% CO2. Primary mouse embryonic fibroblasts (MEF) derived from wild-type, Bax knockout (BaxΔC), Bak knockout (BakΔC), and Bax-Bak double-knockout (DKO) mice and immortalized by transfection with a plasmid containing SV40 genomic DNA were generously provided by Dr. Stanley J. Korsmeyer (Dana-Farber Cancer Institute) and Dr. Craig Thompson (University of Pennsylvania). These MEFs were used previously by us (37). MEFs were maintained in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 0.1 mmol/L nonessential amino acids, 0.1 mmol/L 2-mercaptoethanol, and 1% antibiotic-antimyotic (Invitrogen).

Western blot analysis. Western blots were done as described earlier (38). In brief, cells were lysed in radioimmunoprecipitation assay buffer containing 1× protease inhibitor cocktail, and protein concentrations were determined using the Bradford assay (Bio-Rad). Proteins were separated by 12.5% SDS-PAGE and transferred to membranes (Millipore) in a Tris (20 mmol/L) glycine (150 mmol/L), and methanol (20%) buffer at 55 V for 4 h at 4°C. After blotting in 5% nonfat dry milk in TBS, the membranes were incubated with primary antibodies at 1:1,000 dilution in TBS-Tween 20 overnight at 4°C and then secondary antibodies conjugated with horseradish peroxidase at 1:5,000 dilution in TBS-Tween 20 for 1 h at room temperature. Protein bands were visualized on X-ray film using an enhanced chemiluminescence system.

Caspase-3 assay. Cells (3 × 106 per well) were seeded in a 96-well plate with 200 μL culture medium. Approximately 16 h later, cells were treated with various doses of sulforaphane to induce apoptosis. Caspase-3 activity was measured as per manufacturer’s instructions (EMD Biosciences) with a fluorometer.

Measurement of mitochondrial membrane potential. Mitochondrial energization was determined by retention of JC-1 dye (Molecular Probes) as we described earlier (19). Briefly, drug-treated cells (5 × 105) were loaded with JC-1 dye (1 μg/mL) during the last 30 min of incubation at 37°C in a 5% CO2 incubator. Cells were washed in PBS twice. Fluorescence was monitored in a fluorometer using 570 nm excitation/595 nm emission for the J-aggregate of JC-1. Mitochondrial membrane potential (ΔΨm) was calculated as a ratio of the fluorescence of J-aggregate (aggregative phase) and monomer (membrane-bound) forms of JC-1.

Determination of reactive oxygen species. PC-3 cells were seeded in 96-well plates. After 16 h, cells were loaded with 5 μmol/L
CM-h2DCFDA dye for 30 min and treated with either resveratrol (10 or 20 μmol/L) or 0.05% DMSO for 0 to 360 min. Fluorescence was measured at excitation wavelength of 488 nm and emission wavelength of 515 nm using a fluorescence plate reader.

Orthotopic assays in nude mice. Athymic male nude mice (BALB/c nu/nu, 4-6 weeks old) were purchased from the National Cancer Institute. Nude mice were anesthetized, and prostate gland was exposed following a lower midline incision. Mice were inoculated with PC-3 cells (1 × 10^7 per 100 μL medium) into the dorsolateral lobe of the prostate capsule by means of a 21-gauge needle and a calibrated pushbutton syringe. The incision was closed by using a running suture of 5-0 silk. Tumor-bearing mice were randomized into four groups, and the following treatment protocols were implemented: group 1: vehicle control (0.1 mL normal saline containing 0.5% DMSO) administered by gavage, everyday 5 days a week throughout the duration of experiment; group 2: TRAIL (15 mg/kg) administered i.v. on days 2, 8, 15, and 22; group 3: sulforaphane (40 mg/kg in 0.1 mL normal saline containing 0.5% DMSO) administered by gavage, everyday 5 days a week throughout the duration of experiment; and group 4: sulforaphane plus TRAIL, sulforaphane (40 mg/kg in 0.1 mL normal saline containing 0.5% DMSO) administered by gavage, everyday 5 days a week throughout the duration of experiment, and TRAIL (15 mg/kg) administered i.v. on days 2, 8, 15, and 22.

Immunohistochemistry. Immunohistochemistry was done as described earlier (28, 29). In brief, tumor tissues were collected after 4 weeks of treatment, excised and fixed with 10% formalin, embedded in paraffin, and sectioned. Tissue sections were stained with various primary antibodies at room temperature for 4 h or at 4°C overnight. Subsequently, slides were washed three times in PBS and incubated with secondary antibody at room temperature for 1 h. Finally, alkaline phosphatase or hydrogen peroxide polymer-AEC chromagen substrate kits were used as per manufacturer’s instructions (Lab Vision). After washing with PBS, Vectashield (Vector Laboratories) mounting medium was applied and sections were coverslipped and imaged. TUNEL assays were done as per manufacturer’s instructions.

Fig. 1. Interactive effects of sulforaphane and TRAIL on cell viability, colony formation, and apoptosis. A, PC-3 and LNCaP cells were treated with various doses of sulforaphane (0-40 μmol/L) for 24 h followed by treatment with TRAIL for another 24 h. Cell viability was measured by XTT assay. B, PC-3 and LNCaP cells were seeded in 12-well plates in soft agar and treated with sulforaphane (0-40 μmol/L) in the presence or absence of TRAIL (25 nmol/L). At the end of 3 wk, number of colonies per field was counted by a microscope. C, PC-3 and LNCaP cells were treated with various doses of sulforaphane (0-40 μmol/L) for 24 h followed by treatment with TRAIL for another 24 h. Apoptosis was measured by TUNEL assay as per manufacturer’s instructions. *, **, ***; P < 0.05, significantly different from respective controls. #, %, significantly different between groups. SFN, sulforaphane.

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Statistical analysis. The mean and SD were calculated for each experimental group. Differences between groups were analyzed by one- or two-way ANOVA followed by Bonferroni’s multiple comparison tests using PRISM statistical analysis software (GraphPad Software). Significant differences among groups were calculated at \( P < 0.05 \).

Results

Sulforaphane enhances the antiproliferative and proapoptotic effects of TRAIL in prostate cancer. We first measured the interactive effects of sulforaphane and TRAIL on cell viability and apoptosis in androgen-independent PC-3 cells and androgen-dependent LNCaP cells. Sulforaphane inhibited cell viability in both PC-3 and LNCaP cells (Fig. 1A). PC-3 cells were sensitive to TRAIL, whereas LNCaP cells were resistant to TRAIL. Sulforaphane enhanced the apoptosis-inducing potential of TRAIL in PC-3 cells and sensitized TRAIL-resistant LNCaP cells.

We next sought to examine the interactive effects of sulforaphane and TRAIL on colony formation in soft agar assay. Sulforaphane inhibited colony formation of PC-3 and LNCaP cells in soft agar assay (Fig. 1B). Colonies formed by PC-3 cells were sensitive to TRAIL, whereas LNCaP colonies were resistant. Sulforaphane pretreatment enhanced the inhibitory effects of TRAIL on PC-3 colony formation and sensitized TRAIL-resistant LNCaP colonies. These data suggest that sulforaphane enhances the ability of TRAIL to inhibit colony growth.

Because sulforaphane enhances the antiproliferative and proapoptotic effects of TRAIL, we sought to examine the physiologic significance of TRAIL-death receptor pathway. The inhibition of death receptor pathway was achieved by transfecting prostate cancer cells with the dominant-negative FADD plasmid. We have shown previously that dominant-negative FADD blocked the interactions of TRAIL with histone deacetylase inhibitors, resveratrol, curcumin, ionizing radiation, or anticancer drugs (22, 39, 40). As before, sulforaphane induced apoptosis in both PC-3/neo and LNCaP/neo cells (Fig. 1C). Sulforaphane enhanced the apoptosis-inducing potential of TRAIL in PC-3/neo cells and sensitized TRAIL-resistant LNCaP/neo cells to undergo apoptosis. Transfection of dominant-negative FADD had no effect on sulforaphane-induced apoptosis in both PC-3 and LNCaP cells, whereas it inhibited TRAIL-induced apoptosis in PC-3 cells. The ability of sulforaphane to enhance TRAIL-induced apoptosis in PC-3 cells and sensitize TRAIL-resistant LNCaP cells...
was blocked by dominant-negative FADD. These data suggest the involvement of death receptor pathway in TRAIL-induced or sulforaphane plus TRAIL-induced apoptosis.

Sulforaphane induces expressions of death receptor TRAIL-R1/DR4 and TRAIL-R2/DR5 in LNCaP cells. We have shown recently that histone deacetylase inhibitors, curcumin, resveratrol, chemotherapeutic drugs, and γ-irradiation induce expression of death receptors DR4 and/or DR5 in leukemia, multiple myeloma, and breast and prostate cancer cells, so that successive treatment with TRAIL resulted in apoptosis in an additive or synergistic manner (20–22, 39–42). We therefore sought to examine whether sulforaphane induces sensitivity by up-regulating death receptor DR4 and/or DR5 expression. Treatment of PC-3 and LNCaP cells with sulforaphane resulted in an increased expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors but had no significant effects on the expression of decoy receptors DcR1 and DcR2 (Fig. 2A and B). These data suggest that up-regulation of death receptors DR4 and/or DR5 by sulforaphane may be one of the mechanisms to enhance the proapoptotic effects of TRAIL in PC-3 cells and sensitize TRAIL-resistant LNCaP cells.

Sulforaphane regulates expression of Bcl-2 family members and activation of caspase(s). We have shown recently that Bcl-2 family members are capable of regulating death receptor-mediated apoptosis when present at sufficient levels in cancer cells (43–46). To explore the underlying mechanisms by which sulforaphane enhances apoptosis in PC-3 cells, we first examined the possibility that sulforaphane might down-regulate the expression levels of these Bcl-2 family members. Most members of this family are targeted to mitochondria, which serve as a pivotal component of the apoptotic cell death machinery (47–49). Sulforaphane inhibited expression of antiapoptotic Bcl-2, Mcl-1, and Bcl-XL and induced expression of proapoptotic Bak, Bax, Bim, and NOXA in PC-3 cells (Fig. 3). Induction of Bak, Bax, Bim, and NOXA by sulforaphane suggests that these proteins may cause disruption of mitochondrial homeostasis, whereas the inhibition of antiapoptotic Bcl-2, Mcl-1, and Bcl-XL may facilitate the proapoptotic effects of TRAIL. Because sulforaphane enhances the apoptosis-inducing potential of TRAIL, we examined whether apoptosis occurred through activation of caspase-3 and caspase-9 (Fig. 3B). Sulforaphane induced caspase-3 and caspase-9 activities in PC-3 cells. Similarly, TRAIL induced caspase-3 and caspase-9 activities. Pretreatment of PC-3 cells with sulforaphane followed by TRAIL further enhanced caspase-3 and caspase-9 activities. These data suggest that the sulforaphane induces apoptosis through caspase-3 activation.

The mitochondrial outer membrane permeabilization is tightly regulated by members of the Bcl-2 family and involves the conformational change of proapoptotic family members such as Bax (50). Bax and Bak genes play a major role in apoptosis by regulating the release of mitochondrial proteins such as cytochrome c and Smac/DIABLO (37, 51). We have shown previously that Bax<sup>−/−</sup> and Bak<sup>−/−</sup> DKO MEFs were completely resistant to TRAIL- or curcumin-induced apoptosis (37, 38). To investigate the role of Bax and Bak in sulforaphane- and/or TRAIL-induced apoptosis, MEFs from wild-type, Bax<sup>−/−</sup>, Bak<sup>−/−</sup>, or Bak- and Bak-deficient (DKO) mice were used. Sulforaphane and TRAIL alone induced apoptosis in wild-type MEFs at 48 h (Fig. 3C). Sulforaphane- or TRAIL-induced apoptosis was significantly inhibited in Bax<sup>−/−</sup> MEFs or Bak<sup>−/−</sup> MEFs compared with wild-type MEFs. Interestingly, depletion of Bax and Bak genes significantly inhibited sulforaphane- or TRAIL-induced apoptosis in Bax and Bak DKO MEFs compared with single-knockout MEFs or wild-type MEFs. The combination of sulforaphane and TRAIL induced more apoptosis in wild-type MEFs than single agent alone. The DKO MEFs were significantly more resistant to apoptosis by combination of sulforaphane and TRAIL compared with wild-type MEFs, Bak<sup>−/−</sup> MEFs, or Bak<sup>−/−</sup> MEFs. These data suggest that deletion of either Bax or Bak did not completely block sulforaphane- and TRAIL-induced apoptosis, and both Bax and Bak genes are required for inducing cell death by sulforaphane and/or TRAIL.

Effects of sulforaphane on ΔΨ<sub>m</sub> reactive oxygen species production, caspase-3 activation, and apoptosis. The electrochemical gradient that is normally present across the inner mitochondrial membrane is lost (membrane depolarization)

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**Fig. 3.** Effects of sulforaphane on the expression of Bcl-2 family members and cleavage of caspases. A, prostate cancer PC-3 cells were treated with sulforaphane (0–40 μmol/L) for 48 h. The expression of Bak, Bax, Bim, NOXA, Bcl-2, Bcl-X<sub>L</sub>, and Mcl-1 was measured by the Western blotting. Anti-β-actin antibody was used as a loading control. B, interactive effects of sulforaphane and TRAIL on caspase-3 and caspase-9 activation. PC-3 cells were pretreated with sulforaphane (20 or 40 μmol/L) for 24 h followed by treatment with TRAIL (15 nmol/L) for another 24 h. Cleavage/activation of caspase-3 and caspase-9 was measured by the Western blotting. Anti-β-actin antibody was used as a loading control. C, involvement of Bak and Bak in sulforaphane and/or TRAIL-induced apoptosis. Wild-type MEFs, Bak<sup>−/−</sup> MEFs, Bak<sup>−/−</sup> MEFs, Bak<sup>−/−</sup> MEFs, Bax, and Bak DKO MEFs were treated with sulforaphane (20 μmol/L), TRAIL (25 nmol/L), or sulforaphane plus TRAIL for 48 h. Apoptosis was measured by TUNEL assay.
during apoptosis. On induction of mitochondrial outer membrane permeabilization, mitochondrial proteins are released to cytosol, which, in turn, activates caspase-3 and causes apoptosis. We therefore measure the ΔΨm, reactive oxygen species (ROS) production, caspase-3 activation, and apoptosis. Treatment of PC-3 cells with sulforaphane caused a drop in ΔΨm in a time-dependent manner (Fig. 4A).

Generation of ROS by oxidative damage play an important role in apoptosis (50). We have shown that chemopreventive agents such as resveratrol and curcumin induce apoptosis in cancer cells through generation of ROS (52–55). We therefore examined the involvement of ROS in sulforaphane-induced apoptosis. Treatment of PC-3 cells with sulforaphane resulted in ROS production, which increased over time, reached a maximum level at 120 min, and declined there after (Fig. 4B). N-acetyl-l-cysteine (NAC) is precursor of glutathione, an antioxidant. Pretreatment of PC-3 cells with NAC inhibited sulforaphane-induced ROS production. Because sulforaphane treatment caused a drop in ΔΨm and generated ROS, we next examined whether the drop in ΔΨm was due to generation of ROS by sulforaphane. Treatment of PC-3 cells with sulforaphane caused a drop in ΔΨm at 8 h. By comparison, NAC inhibited sulforaphane-induced drop in ΔΨm, suggesting that ROS generation may account for ΔΨm.

Because sulforaphane induced caspase-3 activity and apoptosis, we next examined whether generation of ROS was responsible for caspase-3 activation and apoptosis. Sulforaphane induced caspase-3 activity and apoptosis in PC-3 cells (Fig. 4C). Interestingly, sulforaphane-induced caspase-3 activity and apoptosis were inhibited by NAC. Together, these results suggest that sulforaphane-induced caspase-3 activity and apoptosis are mediated through generation of ROS.

We next examined whether the combination of sulforaphane and TRAIL had additive effects on ΔΨm (Fig. 4D). Sulforaphane and TRAIL had additive effects on ΔΨm.
and TRAIL alone caused a drop in $\Delta\Psi_m$ in PC-3 cells at 8 and 16 h. The combination of sulforaphane and TRAIL was more effective in dropping $\Delta\Psi_m$ than single agent alone. These data suggest that sulforaphane and TRAIL exert their proapoptotic effects through engagement of mitochondria.

Sulforaphane and TRAIL inhibit orthotopically implanted prostate tumor growth in nude mice. We first examined the interactive effects of sulforaphane and TRAIL on orthotopically implanted prostate tumor growth in nude mice. Treatment of nude mice with sulforaphane and TRAIL alone inhibited

Fig. 5. Sulforaphane enhances the antitumor activity of TRAIL in orthotopically implanted prostate tumor. A, PC-3 cells ($1 \times 10^6$ per 100 µL medium) were orthotopically implanted in prostate gland of BALB/c nude mice. Mice were treated with vehicle control (0.1 mL normal saline), TRAIL (15 mg/kg), sulforaphane (40 mg/kg), and sulforaphane plus TRAIL as described in Materials and Methods. After 4 wk, mice were euthanized, and prostate tumor weight was recorded. *, #, $P < 0.05$, significantly different from respective control. B, sulforaphane and TRAIL induce expression of DR4 and DR5 receptors. Tumor tissues obtained from above experiment on day 28 were subjected to immunohistochemistry using antibody against TRAIL-R1/DR4 or TRAIL-R2/DR5. DR4- or DR5-positive tumor cells were counted under a microscope. *, #, $P < 0.05$, significantly different from respective control. C, sulforaphane and TRAIL inhibit tumor cell proliferation and induce apoptosis. Top, tumor tissues obtained from above experiment on day 28 were subjected to immunohistochemistry using antibody against Ki-67 or proliferating cell nuclear antigen. TUNEL assay was done to examine the induction of apoptosis as per manufacturer's instructions (BD Biosciences). *, **, #, $P < 0.05$, significantly different from respective control. Bottom, regulation of Bcl-2 family members by sulforaphane and/or TRAIL. Tumor tissues obtained from above experiment on day 28 were subjected to immunohistochemistry using antibody against Bax, Bak, Bcl-2, and Bcl-XL. Bax, Bak, Bcl-2, and Bcl-XL positive tumor cells were counted under a microscope. *, **, #, $P < 0.05$, significantly different from respective control. D, regulation of active caspase-3 and caspase-8 by sulforaphane and/or TRAIL. Tumor tissues were fixed and immunohistochemistry was done using antibody against active caspase-3 and active caspase-8.
tumor growth (Fig. 5A). The combination of sulforaphane and TRAIL was more effective in inhibiting prostate tumor growth than single agent alone. These data suggest that sulforaphane can enhance the therapeutic potential of TRAIL in prostate cancer.

**Induction of DR4 and DR5 by TRAIL and/or sulforaphane in prostate tumors.** Because sulforaphane enhanced the antitumor activity of TRAIL, we sought to examine the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors in prostate tumor tissue. Immunohistochemistry of tumor tissues showed that TRAIL and sulforaphane alone induced DR4 and DR5 expression compared with control group (Fig. 5B). The combination of sulforaphane and TRAIL was more effective in inducing DR4 and DR5 expression than single agent alone. These data suggest that sulforaphane can enhance the apoptosis-inducing potential of TRAIL by up-regulating DR4 and DR5 expression.

**TRAIL and sulforaphane inhibit cell proliferation and induce apoptosis in orthotopically implanted prostate tumors.** Because tumor growth is determined by the balance of cell proliferation and apoptosis, mechanisms that promote cell survival or prevent apoptosis of cancer cells would favor the establishment of tumor colonies. Because sulforaphane and TRAIL inhibited tumor growth, we next sought to examine whether growth inhibition was due to inhibition of cell proliferation and induction of apoptosis. A very few apoptotic cells were found in the vascularized tumors derived from control mice. Immunohistochemistry of tumor tissues showed that TRAIL and sulforaphane alone inhibited tumor cell proliferation (less Ki-67 and proliferating cell nuclear antigen staining) and induced apoptosis (TUNEL staining) compared with control group (Fig. 5C). The combination of sulforaphane and TRAIL was more effective in inhibiting tumor cell proliferation and inducing apoptosis than single agent alone. These data suggest that sulforaphane can enhance the apoptosis-inducing potential of TRAIL by inhibiting cell proliferation and inducing apoptosis.

**In vivo regulation of Bcl-2 family members by sulforaphane and/or TRAIL.** Because sulforaphane and TRAIL inhibited tumor growth (Fig. 5A), the effect of the combination on the expression of Bcl-2 family members was examined. Immunohistochemistry of tumor tissues showed that the combination of sulforaphane and TRAIL inhibited the expression of Bcl-2, Bcl-xL, and Bcl-w, and increased the expression of Bax and Bad compared with control group (Fig. 5D). These data suggest that the combination of sulforaphane and TRAIL can enhance the apoptosis-inducing potential of TRAIL by inhibiting cell proliferation and inducing apoptosis.
tumor cell proliferation and induced apoptosis, we sought to examine the involvement of Bcl-2 family members (Bax, Bak, Bcl-2, and Bcl-XL) by immunohistochemistry in tumor tissues derived from in vivo experiment (Fig. 5C). Treatment of mice with sulforaphane or TRAIL enhanced the expression of proapoptotic Bax and Bak proteins and inhibited the expression of antiapoptotic Bcl-2 and Bcl-XL proteins in tumor tissues. By comparison, treatment of mice with a combination of sulforaphane and TRAIL significantly showed enhanced expression of Bax and Bak and reduced expression of Bcl-2 and Bcl-XL proteins than those mice treated with sulforaphane alone or TRAIL alone.

In vivo regulation of caspase-3 and caspase-8 activity. We next examined the interactive effects of sulforaphane and/or TRAIL on the activation of caspase-3 and caspase-8 in tumor tissues derived from control and treated mice. Sulforaphane induced significant caspase-3 activity, but its effect on caspase-8 activity was also slightly observed. TRAIL induced both caspase-3 and caspase-8 activities. By comparison, treatment of mice with the combination of sulforaphane and TRAIL resulted in higher caspase-3 and caspase-8 activities in tumor tissues than those mice treated with either agent alone.

In vivo regulation of angiogenesis by sulforaphane and/or TRAIL. Whether regression in tumor growth by sulforaphane was due to inhibition of angiogenesis, we analyzed the markers of angiogenesis by immunohistochemistry in tumor samples. Examination of tumor tissues by immunohistochemistry showed that control mice had increased VEGF-positive cells compared with sulforaphane- or TRAIL-treated mice (Fig. 6A). The combination of sulforaphane and TRAIL showed significantly less VEGF staining than single agent alone.

We next examined the effects of sulforaphane and/or TRAIL treatment on microvessel density by staining tissues with anti-CD31 antibody and anti-factor VIII antibody (markers of angiogenesis). Treatment of mice with sulforaphane or TRAIL caused an inhibition in microvessel density compared with control mice (Fig. 6A). The combination of sulforaphane with TRAIL further inhibited microvessel density compared with single agent alone.

In vivo regulation of MMP by sulforaphane and/or TRAIL. Elevated expression of MMP is associated with increased metastatic potential of tumor cells (56, 57). We therefore sought to examine the effects of sulforaphane on MT1-MMP, MMP-2, MMP-7, and MMP-9 on tumor tissues derived from tumor-bearing nude mice. Treatment of mice with sulforaphane or TRAIL resulted in inhibition of MT1-MMP, MMP-2, MMP-7, and MMP-9 expression than those of control or TRAIL-treated mice (Fig. 6B). The combination of sulforaphane and TRAIL was more effective in inhibiting MT1-MMP, MMP-2, MMP-7, and MMP-9 expression than single agent alone. These data suggest that sulforaphane and/or TRAIL can inhibit prostate cancer progression by inhibiting metastasis.

In vivo regulation of NF-κB and its gene products in tumor tissues derived from sulforaphane- and/or TRAIL-treated mice. NF-κB and its gene products play significant role in development and/or progression of malignancy by regulating the expression of genes involved in cell growth, differentiation, apoptosis, angiogenesis, and metastasis (58). We therefore measure the activation of NF-κB (phospho-p65-NF-κB) and the expression of its gene products TGF-β1, uPAR, IL-6, IL-8, HIF-1α, COX-2, and cyclin D1 (Fig. 6C). Sulforaphane and TRAIL alone inhibited constitutively active NF-κB as measured by using phospho-p65-NF-κB antibody. By comparison, the combination of sulforaphane and TRAIL further inhibited constitutively active NF-κB. These data suggest that sulforaphane and/or TRAIL can inhibit the activation of NF-κB and its gene products, which may play roles in tumor growth, metastasis and angiogenesis.

TGF-β1 and uPAR play important roles in prostate cancer progression (59, 60). Sulforaphane inhibited the expression of TGF-β1 and uPAR, whereas TRAIL treatment has no significant effect on the expression of TGF-β1 and uPAR (Fig. 6C). The combination of sulforaphane and TRAIL further inhibited the expression of TGF-β1 and uPAR.

NF-κB and proinflammatory cytokines are considered to have important roles in carcinogenesis (58). To better understand the relationship between cytokines and prostate cancer, we measured the expression of IL-6 and IL-8 in tissues derived from tumor-bearing mice (Fig. 6C). Sulforaphane and TRAIL alone inhibited the expression of IL-6 and IL-8. By comparison, the combination of sulforaphane and TRAIL further inhibited IL-6 and IL-8 expression.

HIF-1α is a transcriptional factor that activates multiple genes including VEGF in response to hypoxia and promotes neoangiogenesis (61). We therefore measured the expression of HIF-1α in tumor tissues (Fig. 6C). Sulforaphane and TRAIL alone inhibited the expression of HIF-1α. By comparison, the combination of sulforaphane and TRAIL further inhibited the expression of HIF-1α.

Because COX-2 is constitutively overexpressed in prostate carcinoma (62), we sought to examine the effects of sulforaphane and/or TRAIL on its expression. Sulforaphane and TRAIL alone inhibited COX-2 expression (Fig. 6C). By comparison, the combination of sulforaphane and TRAIL further inhibited COX-2 expression.

The D-type cyclins promote G1-S-phase cell cycle progression through activation of cyclin-dependent kinase 4/6. We therefore measured the effects of sulforaphane and/or TRAIL on the expression cyclin D1 (Fig. 6C). Sulforaphane and TRAIL alone inhibited COX-2 expression. By comparison, the combination of sulforaphane and TRAIL further inhibited COX-2 expression. These data suggest that sulforaphane and/or TRAIL can regulate tumor growth by inducing cell cycle arrest.

In vivo regulation of pAKT, pERK, and pFOXO3a in tumor tissues derived from sulforaphane- and/or TRAIL-treated mice. We have shown recently that the inhibition of PI3K/AKT and MEK/ERK pathways interact synergistically to inhibit angiogenesis through dephosphorylation/activation of FOXO transcription factors (35). We therefore measure the effects of sulforaphane and/or TRAIL on the phosphorylation of AKT, ERK1/2, and FOXO3a with phosphospecific antibodies in tumor tissues derived from nude mice (Fig. 6D). Treatment of mice with sulforaphane and TRAIL alone inhibited the phosphorylation of AKT, ERK1/2, and FOXO3a. By comparison, the combination of sulforaphane and TRAIL further inhibited the phosphorylation of AKT, ERK1/2, and FOXO3a in tumor tissues. These data suggest that sulforaphane and/or TRAIL can activate FOXO3a through dephosphorylation of AKT and ERK1/2 and may regulate FOXO-dependent gene transcription and apoptosis.
Discussion

We have shown that sulforaphane induces apoptosis in TRAIL-sensitive PC-3 cells and sensitizes TRAIL-resistant LNCaP cells in vitro through activation of multiple signaling pathways. Sulforaphane induces apoptosis by engaging mitochondria, which was evident by drop in ΔΨm and activation of caspase-3 and caspase-9. Inhibition of ROS inhibited sulforaphane-induced drop in ΔΨm, caspase-3 activation, and apoptosis. Sulforaphane induced expression of proapoptotic proteins (Bax, Bak, PUMA, Noxa, and Bim) and death receptors (TRAIL-R1/DR4 and TRAIL-R2/DR5) and inhibited expression of antiapoptotic proteins (Bcl-2 and Bcl-X.) Because these proteins regulate cell-intrinsic and/or cell-extrinsic pathways of apoptosis, they may be responsible for enhancing antiproliferative effects of TRAIL. Furthermore, sulfaphane inhibited the growth of PC-3 cells orthotopically implanted in nude mice by inducing apoptosis and inhibiting tumor cell proliferation, invasion, metastasis, and angiogenesis. Sulforaphane inhibited phosphorylation of AKT, ERK1/2, FOXO3A, and p65-NF-κB in tumor tissues. The combination of sulforaphane and TRAIL was more effective in inhibiting tumor growth, invasion, metastasis, and angiogenesis and inducing apoptosis than single agent alone.

The Bcl-2 family of proteins plays evolutionarily conserved and key regulatory roles in apoptosis. It comprised both prosurvival and proapoptotic proteins. Bcl-2 itself is a prosurvival member of the family and its aberrant expression may be responsible for enhancing antiproliferative effects of TRAIL. These DKO MEFs were resistant to TRAIL- or curcumin-induced apoptosis (37, 52). Our studies show that sulforaphane and TRAIL can engage cell-intrinsic pathway of apoptosis by regulating the expression of Bcl-2 family of proteins.

Cytokines are key mediators of inflammation that may relate to prostate cancer initiation and progression and that may be useful markers of prostatic neoplasias and related inflammation (63). TGF-β1, IL-6, and IL-8 act as survival factors inhibiting chemotherapy-induced apoptosis in androgen-independent PC-3 human prostate cancer cells (59, 60). TGF-β1 induces uPAR in prostate cancer PC-3, PC-3M, and PC-3MM2 cell lines (59). uPAR expression increases with metastatic passage in these cell lines and accompanies increased growth and motility responses in the presence of uPA (59). Clinical studies show that up-regulation of TGF-β1 in prostate cancer tissues and high urinary and serum levels of TGF-β1 are associated with enhanced tumor angiogenesis and tumor metastasis and with poor clinical outcomes (60, 64). Overexpression of TGF-β1 in prostate cancer cells enhances angiogenesis and metastasis, whereas down-regulation of TGF-β1 reduces tumorigenicity (65, 66). In our study, although TRAIL was ineffective, sulforaphane inhibited TGF-β1 expression in PC-3 tumor tissues. These data suggest that TGF-β1 is a potential target for novel therapies against prostate cancer, particularly advanced diseases.

MMP is up-regulated in many tumor types and has been implicated in tumor progression and metastasis (56, 57). MMP is critical for pericellular degradation of the extracellular matrix, thereby promoting tumor cell invasion and dissemination. Immunohistochemical data showed that sulforaphane and TRAIL alone inhibited markers of metastasis (uPAR, MT1-MMP, MMP-2, MMP-7, and MMP-9 staining). The inhibitory effects of sulforaphane on metastasis were further enhanced in the presence of TRAIL. These data suggest that sulforaphane either alone or in the presence of TRAIL can inhibit prostate cancer metastasis.

TRAIL induces apoptosis in cancer cells that express TRAIL-R1/DR4 and TRAIL-R2/DR5. We have shown that the chemotherapeutic drugs, irradiation, and chemopreventive agents enhance the apoptosis-inducing potential of TRAIL by up-regulating DR4 and/or DR5 expression (20–22, 40, 52, 53, 55, 67, 68). Similarly, our in vitro data show the up-regulation of DR4 and DR5 in PC-3 and LNCaP cells by sulforaphane. These in vitro data are in agreement with in vivo data where sulforaphane up-regulated the expression of DR4 and DR5 in prostate tumor tissues. These finding suggest that up-regulation of death receptors DR4 and DR5 by sulforaphane may be one of the mechanisms by which sulforaphane enhances the therapeutic potential of TRAIL.

NF-κB is responsible for the expression by regulating many genes for apoptosis, immune response, cell adhesion, differentiation, proliferation, angiogenesis, and metastasis (58). The function of NF-κB is inhibited by binding to NF-κB inhibitor (IκB), and imbalance of NF-κB and IκB has been associated with development of many diseases, including cancer. Prostate cancer cells have been reported to have constitutive NF-κB activity due to increased activity of the IκB kinase complex (58, 69). In prostate cancer cells, NF-κB may promote cell growth and proliferation by regulating expression of genes such as c-myc, cyclin D1, IL-6, Bcl-2, and Bcl-Xl (58, 70). NF-κB-mediated expression of genes, involved in angiogenesis (e.g., IL-6, IL-8, and VEGF), invasion, and metastasis (e.g., MMP-7 and MMP-9), may further contribute to the progression of prostate cancer. Constitutive NF-κB activity has also been shown in primary prostate cancer tissue samples and suggested to have prognostic importance for a subset of primary tumors. In the present study, sulforaphane and/or TRAIL inhibited the activation of NF-κB and its gene products such as VEGF, Bcl-2, Bcl-XL, cyclin D1, MT1-MMP, MMP-2, MMP-9, COX-2, IL-6, and IL-8 in PC-3 orthotopically implanted tumors. COX-2 is an immediate-early response gene that can be induced by a variety of tumor promoters, cytokines, growth factors, and hypoxia (62). These findings suggest that NF-κB may play a role in human prostate cancer development and/or progression, and sulforaphane and/or TRAIL can inhibit tumor growth, metastasis, and angiogenesis through regulation of NF-κB-regulated gene products.

Hypoxia occurs in most solid tumors as a result of inefficient vascular development and/or abnormal vascular architecture (61). Angiogenesis is dependent on the accumulation of HIFs, which are heterodimeric transcription factors of α and β subunits (61). The accumulation of HIFs under hypoxia allows them to activate the expression of many angiogenic genes and therefore initiates the angiogenic process. Intratumoral hypoxia correlates with poor prognosis and enhanced metastases formation. The HIFs regulate a large panel of genes that are
exploited by tumor cells for survival, resistance to treatment, and escape from a nutrient-deprived environment (61). Several studies have shown that in tumors HIF-1α mediates VEGF protein expression at the transcription level. In the present study, sulforaphane inhibited the expression of VEGF and HIF-1α, and their expressions were further enhanced in the presence of TRAIL. These data suggest that HIF-1α can mediate antiangiogenic effects of sulforaphane by inhibiting VEGF in prostate tumor tissues.

We have shown recently that inhibition of PI3K/AKT and Ras/MEK/ERK pathways interact synergistically to activate FOXO transcription factors that, in turn, inhibit angiogenesis (35). Inhibition of both of these pathways further enhances the antiangiogenic effects of EGCG (35). Similarly, in the present study, we have shown that sulforaphane can inhibit the phosphorylation of AKT and ERK1/2, which can inhibit the phosphorylation of FOXO3a transcription factor. Thus, the dephosphorylation/activation of FOXO3a by inhibition of Ras/MEK/ERK pathways could induce genes that play major roles in cell cycle, apoptosis, and angiogenesis.

In summary, our in vitro experiments have shown that sulforaphane enhances the apoptosis-inducing potential of TRAIL in PC-3 cells and sensitizes TRAIL-resistant LNCaP. Our data show that sulforaphane inhibits the growth of PC-3 tumors in nude mice through modulation of genes involved in cell cycle, apoptosis, metastasis, and angiogenesis. Sulforaphane induces death receptors, up-regulates proapoptotic members of Bcl-2 family (Bax and Bak), and inhibits antiapoptotic Bcl-2 proteins (Bcl-2 and Bcl-XL), markers of cell proliferation (proliferating cell nuclear antigen and Ki-67) and cell cycle (cyclin D1). Furthermore, sulforaphane can also inhibit the activation of NF-κB and its gene products (e.g., VEGF, Bcl-2, Bcl-XL, uPAR, cyclin D1, MTI-MMP, MMP-2, MMP-7, MMP-9, COX-2, IL-6, and IL-8), which play significant roles in apoptosis, invasion, metastasis, and angiogenesis. All these events will significantly contribute to the antiproliferative and antitumor activities of sulforaphane. In conclusion, our results showed that sulforaphane and TRAIL alone as well as their combination can be used for prevention and/or treatment of prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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