Sulforaphane Inhibits Constitutive and Interleukin-6–Induced Activation of Signal Transducer and Activator of Transcription 3 in Prostate Cancer Cells

Eun-Ryeong Hahm and Shivendra V. Singh

Abstract

D,L-Sulforaphane (SFN), a synthetic analogue of broccoli-derived L-isomer, inhibits viability of human prostate cancer cells and prevents development of prostate cancer and distant site metastasis in a transgenic mouse model. However, the mechanism underlying the anticancer effect of SFN is not fully understood. We now show that SFN inhibits constitutive and interleukin-6 (IL-6)–inducible activation of signal transducer and activator of transcription 3 (STAT3), which is an oncogenic transcription factor activated in many human malignancies, including prostate cancer. Growth-suppressive concentrations of SFN (20 and 40 µmol/L) decreased constitutive (DU145 cells) and IL-6–induced (DU145 and LNCaP cells) phosphorylation of STAT3 (Tyr^{705}) as well as its upstream regulator Janus-activated kinase 2 (Tyr$^{1007/1008}$). Exposure of DU145 and LNCaP cells to SFN resulted in suppression of (*a*) IL-6–induced transcriptional activity of STAT3 as judged by luciferase reporter assay and (*b*) nuclear translocation of phospho-STAT3 as revealed by immunofluorescence microscopy. Levels of many STAT3-regulated gene products, including Bcl-2, cyclin D1, and survivin, were also reduced in SFN-treated cells. The IL-6–mediated activation of STAT3 conferred partial but marked protection against SFN-induced apoptosis as evidenced by cytoplasmic histone-associated DNA fragmentation and cleavage of poly(ADP-ribose) polymerase and procaspase-3. Furthermore, knockdown of STAT3 protein using small interfering RNA resulted in a modest yet statistically significant increase in SFN-induced apoptotic DNA fragmentation in DU145 cells. Suppression of STAT3 activation was also observed in cells treated with naturally occurring analogues of SFN. In conclusion, the present study indicates that inhibition of STAT3 partially contributes to the proapoptotic effect of SFN. *Cancer Prev Res; 3(4); 484–94. ©2010 AACR.*

Introduction

Epidemiologic studies suggest that dietary intake of cruciferous vegetables may lower the risk of different malignancies, including prostate cancer (1–3). Antineoplastic activity of cruciferous vegetables is attributed to isothiocyanates, which are released by myrosinase-mediated hydrolysis of corresponding glucosinolates (4). Broccoli is a rich source of (−)-1-isothiocyanato-(4*R*)-(methylsulfinyl)-butane (L-SFN). The L-SFN and its synthetic derivative D,L-sulforaphane (SFN) have attracted a great deal of research inquiry because of their anticancer effects. For example, the L-SFN and SFN are equipotent inducers of quinone reductase activity in Hepa 1c1c7 hepatoma cells

(5). The L-SFN or synthetic SFN has been shown to prevent chemically induced cancer in experimental animals, including 9,10-dimethyl-1,2-benzanthracene–induced mammary cancer in rats (6), azoxymethane-induced colonic aberrant crypt foci in rats (7), and benzo[*a*]pyrene-induced forestomach cancer in mice (8). Dietary feeding of SFN and its *N*-acetylcysteine conjugate inhibited malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice (9).

In addition to prevention of chemically induced cancer in experimental animals, SFN treatment inhibits growth of human cancer cells in culture and *in vivo* (10–12). For example, growth of PC-3 human prostate cancer xenografts in athymic mice was inhibited significantly by oral or dietary administration of SFN (13, 14). Oral administration of SFN inhibited incidence and/or burden of prostatic intraepithelial neoplasia and well-differentiated prostate cancer as well as pulmonary metastasis multiplicity in a transgenic mouse model in association with augmentation of natural killer cell activity (15). Mechanistic knowledge explaining anticancer effect of SFN continues to expand, but the SFN-mediated suppression of cancer cell proliferation correlates with G$_2$-M–phase cell cycle arrest (10, 11, 16), apoptosis induction (10, 12, 17), inhibition of histone deacetylase

Authors' Affiliation: Department of Pharmacology and Chemical Biology, and University of Pittsburgh Cancer Institute, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Corresponding Author: Shivendra V. Singh, 2.32A Hillman Cancer Center Research Pavilion, University of Pittsburgh Cancer Institute, 5117 Centre Avenue, Pittsburgh, PA 15213. Phone: 412-623-3263; Fax: 412-623-7828; E-mail: singhs@upmc.edu.

*American Association for **Cancer Research***

(18), and protein binding (19). Mechanistic details of some of these cellular responses have been well described. For example, the SFN-induced G_2-M–phase cell cycle arrest in human prostate cancer cells is caused by checkpoint kinase 2–mediated Ser^{216} phosphorylation of cell division cycle 25C (16). We also found that the SFN-induced apoptosis is selective toward cancer cells and intimately linked to production of reactive oxygen species (17, 20, 21). The SFN has been shown to inhibit activity of mitochondrial respiratory chain enzymes, leading to production of reactive oxygen species (21). The SFN-mediated suppression of androgen receptor signaling in prostate cancer cells has also been shown recently (22).

Signal transducer and activator of transcription 3 (STAT3) is an oncogenic transcription factor implicated in development and progression of various malignancies, including prostate cancer (23). STAT3 is constitutively activated in a variety of cancers, including breast and prostate cancers, melanoma, multiple myeloma, and leukemia (23–25). The present study shows that SFN treatment inhibits constitutive and interleukin-6 (IL-6)–inducible activation of STAT3 in human prostate cancer cells.

Materials and Methods

Reagents

The SFN and its naturally occurring thio (iberverin, erucin, and berteroin), sulfinyl (iberin and alyssin), and sulfonyl (cheirolin, erysolin, and alyssin sulfone) analogues were purchased from LKT Laboratories. Stock solution of SFN and its analogues was prepared in DMSO and diluted with fresh medium immediately before use. An equal volume of DMSO (final concentration of <0.1%) was added to controls. Cell culture reagents, including medium, fetal bovine serum, and antibiotic mixture, were purchased from Life Technologies, whereas IL-6 was obtained from Sigma. Antibodies against phospho-STAT3 (pSTAT3; Tyr^{705}), phospho–Janus-activated kinase 2 (pJAK2; $Tyr^{1007/1008}$), total STAT3, total JAK2, cleaved caspase-3, and cleaved poly(ADP-ribose) polymerase were obtained from Cell Signaling; antibodies against Mcl-1 and cyclin D1 were from Santa Cruz Biotechnology; anti–Bcl-2 antibody was from DakoCytomation; and anti-survivin antibody was from Novus Biologicals. The pSTAT3 (Tyr^{705}) antibody used for immunofluorescence microscopy was from Santa Cruz Biotechnology. The STAT3-targeted small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology. A control nonspecific siRNA (UUCUCCGAACGUGUCACGUdTdT) was from Qiagen. The sequence of the STAT3 siRNA is not revealed by the supplier. SYTOX Green and Alexa Fluor 568 goat anti-rabbit antibody were from Invitrogen.

Cell culture

Prostate cancer cell lines DU145, LNCaP, PC-3, and CWR22Rv1 were purchased from the American Type Culture Collection. Cell line authentication was done by analysis of known genetic differences (e.g., expression of androgen receptor and p53, and analysis of androgen responsiveness). The DU145, LNCaP, and PC-3 cells were cultured as described by us previously (20, 26). The CWR22Rv1 cells were cultured as recommended by the supplier.

Immunoblotting

Lysates from control and SFN-treated prostate cancer cells, and prostate tumor supernatants from control and SFN-treated transgenic adenocarcinoma of mouse prostate (TRAMP) mice were prepared as described by us previously (15, 27). Lysate/supernatant proteins were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membrane. Immunoblotting was done as described by us previously (15, 27).

Reverse transcription-PCR

Total cellular RNA was extracted from control and SFN-treated LNCaP and DU145 cells using RNeasy kit (Invitrogen). Complementary DNA was synthesized from 1 to 2 μg of total RNA with reverse transcriptase and oligo(dT)$_{20}$. Reverse transcription-PCR was carried out using High Fidelity Taq polymerase (Invitrogen), gene-specific primers, and cDNA. The primers for JAK2 were 5′-GAT-GAGCAAGCTTTCTCACAAGC-3′ (forward) and 5-GCATGGCCCATGCCAACTGTTT-3′ (reverse). The primers for GAPDH were 5′-TGATGACATCAAGAAGGTGGT-GAAG-3′ (forward) and 5′-TCCTTGGAGGC-CATGTGGGCCAT-3′ (reverse). Amplification conditions for JAK2 were 28 cycles at 94°C for 45 s, 58°C for 45 s, and 68°C for 45 s. Amplification conditions for GAPDH included 25 cycles at 94°C for 30 s, 55°C for 30 s, and 68°C for 30 s. The PCR products were resolved by 2% agarose gel prestained with ethidium bromide and visualized under UV illuminator.

Luciferase assay

Luciferase assay was done to determine the effect of SFN treatment on constitutive (DU145) and IL-6–induced (DU145 and LNCaP) transcriptional activity of STAT3. Briefly, cells ($5 × 10^4$ per 12-well plates) were plated and allowed to attach by overnight incubation at 37°C. The cells were then cotransfected with 1 μg of pSTAT3-Luc plasmid encoding STAT3-responsive element and 0.1 μg of pRL-CMV plasmid using Fugene6 (Roche Applied Science) according to the manufacturer's recommendations. The pSTAT3-Luc plasmid was kindly provided by Dr. Bharat B. Aggarwal (University of Texas M.D. Anderson Cancer Center, Houston, TX; ref. 28). Twenty-four hours after transfection, the cells were treated with DMSO or SFN (20 or 40 μmol/L) for 12 h before stimulation with 10 ng/mL IL-6 for 12 h in the presence of SFN. Luciferase activity was determined using a luminometer and normalized against protein concentration.

Analysis of STAT3 dimerization

Protein extracts from control and SFN-treated DU145 cells were prepared as described by Shin et al. (29).

Proteins were resolved by 4% nondenaturing gel electrophoresis. Proteins were transferred onto polyvinylidene fluoride membrane and immunoblotted with the anti-STAT3 or anti-actin antibody as described above.

Immunocytochemistry for nuclear localization of pSTAT3

The effect of SFN treatment on nuclear localization of pSTAT3 was determined by immunocytochemistry. Briefly, DU145 or LNCaP cells (1×10^5) were plated on coverslips, allowed to attach by overnight incubation, and exposed to DMSO (control) or SFN for 12 h. STAT3 activation was achieved by exposure to 10 ng/mL IL-6 for 15 min at the end of incubation period. The cells were fixed with 2% paraformaldehyde for 1 h at room temperature and permeabilized with 0.05% Triton X-100 for 5 min. The cells were then incubated with PBS supplemented with 0.5% bovine serum albumin and 0.15% glycine for 1 h followed by overnight incubation with anti-pSTAT3 antibody at 4°C. The cells were then treated with 2 μg/mL of Alexa Fluor 568–conjugated secondary antibody (Molecular Probes) for 1 h at room temperature. After washing with PBS, the cells were counterstained with 0.5 μmol/L SYTOX Green for 3 min at room temperature. Subsequently, the cells were mounted and observed under a Leica DC300F fluorescence microscope at ×100 magnification.

RNA interference

The DU145 cells were seeded in six-well plates and transfected at 50% confluency with 100 nmol/L of control siRNA or STAT3-targeted siRNA using Oligofectamine according to the manufacturer's instructions. Twenty-four hours after transfection, the cells were treated with DMSO (control) or specified concentrations of SFN for 24 h. The cells were then collected and processed for immunoblotting of total STAT3 and analysis of cytoplasmic histone-associated DNA fragmentation. Cytoplasmic histone-associated DNA fragmentation assay was done using a kit from Roche Applied Sciences according to the manufacturer's instructions.

Results

SFN treatment suppressed STAT3 phosphorylation in prostate cancer cells

The STAT3 activation is caused by phosphorylation at Tyr[705] (23, 24). Initially, we did immunoblotting for pSTAT3 (Tyr[705]) using lysates from a panel of prostate cancer cell lines (CWR22Rv1, DU145, PC-3, and LNCaP) to determine constitutive activation of this transcription factor. Constitutive pSTAT3 was detectable only in the DU145 cell line with loading of up to 80 μg of the lysate protein (Fig. 1A). A 30-minute exposure of serum-starved (12-hour starvation) cells to 10 ng/mL IL-6, a known activator of STAT3 (30, 31), resulted in a robust increase in Tyr[705] phosphorylation of STAT3 in DU145 and LNCaP cells (Fig. 1A, lanes 2 and 4). A modest increase in pSTAT3

level following IL-6 treatment was also observed in the CWR22Rv1 cell line but not in the PC-3 cells (Fig. 1A). Total STAT3 protein was detectable in every cell line except PC-3 (Fig. 1A). We selected DU145 and LNCaP cells to determine the effect of SFN treatment on constitutive and inducible activation of STAT3. As can be seen in Fig. 1B, SFN treatment reduced levels of pSTAT3 in DU145 cells in a concentration- and time-dependent manner. For example, the level of pSTAT3 was reduced by about 80% to 90% by a 24-hour exposure of DU145 cells to 20 and 40 μmol/L SFN (Fig. 1B). The SFN-mediated suppression of Tyr[705] phosphorylation of STAT3 was not explained by a decrease in its protein level. Phosphorylation of STAT3 is mediated by different kinases, including JAK2 (23, 30, 31). We did immunoblotting for pJAK2 (Tyr[1007/1008]) to determine if SFN-mediated inhibition of STAT3 activation was due to suppression of JAK2 phosphorylation. The level of pJAK2 was also reduced in a concentration- and time-dependent manner on exposure of DU145 cells to SFN, especially at the 12- and 24-hour time points (Fig. 1B). Unlike STAT3, however, suppression of JAK2 phosphorylation by SFN treatment correlated with a marked decrease in its protein level (Fig. 1B). We did reverse transcription-PCR to test whether SFN-mediated decline in JAK2 protein level was due to its reduced transcription. The level of *JAK2* mRNA, after correction for *GAPDH* control, was decreased by approximately 70% to 90% in DU145 and LNCaP cells treated for 24 hours with 40 μmol/L SFN (results not shown). These results indicated that SFN treatment reduced transcription of *JAK2*.

Next, we tested the possibility whether SFN treatment affected IL-6–induced activation of STAT3. For these experiments, DU145 and LNCaP cells were treated with 40 μmol/L SFN for 24 hours and stimulated with 10 ng/mL IL-6 for the indicated time periods at the end of incubation period. The IL-6–induced phosphorylation of JAK2 as well as STAT3 was suppressed in both DU145 (Fig. 1C) and LNCaP cells (Fig. 1D). However, the LNCaP cell line (Fig. 1D) was markedly more sensitive to SFN-mediated inhibition of IL-6–inducible JAK2 and STAT3 phosphorylation as well as downregulation of total JAK2 protein expression compared with DU145 cells (Fig. 1C). The results were comparable if the cells were cotreated with SFN and IL-6 for 24 hours (data not shown). Collectively, these results indicated that SFN treatment inhibited constitutive as well as IL-6–induced phosphorylation of STAT3 by reducing the levels of total JAK2 and pJAK2.

SFN treatment inhibited STAT3-dependent luciferase activity in prostate cancer cells

We did luciferase reporter assay to determine the effect of SFN treatment on transcriptional activity of STAT3. In DU145 cells, a 24-hour treatment with 40 μmol/L SFN resulted in a statistically significant decrease in STAT3-dependent luciferase activity (~58% inhibition compared with DMSO-treated control; Fig. 2A). As expected, transcriptional activity of STAT3 was modestly increased in the presence of IL-6 (10 ng/mL, 12-hour exposure in the

Fig. 1. A, immunoblotting for pSTAT3 and total STAT3 using lysates from CWR22Rv1 (lane 1), DU145 (lane 2), PC-3 (lane 3), and LNCaP (lane 4) cells without or after treatment with 10 ng/mL IL-6 for 30 min in serum-starved cells (12-h starvation). B, immunoblotting for pSTAT3, total STAT3, pJAK2, and total JAK2 using lysates from DU145 cells treated for 6, 12, or 24 h with DMSO (control) or SFN (20 or 40 µmol/L). Immunoblotting for pSTAT3, total STAT3, pJAK2, and total JAK2 using lysates from DU145 (C) and LNCaP (D) cells treated for 24 h with 40 µmol/L SFN in the presence or absence of 10 ng/mL IL-6. Cells were stimulated with IL-6 at the end of incubation period. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein level. Numbers on top of the immunoreactive bands represent change in protein level relative to corresponding DMSO-treated control. Each experiment was repeated at least twice, and representative data from one such experiment are shown.
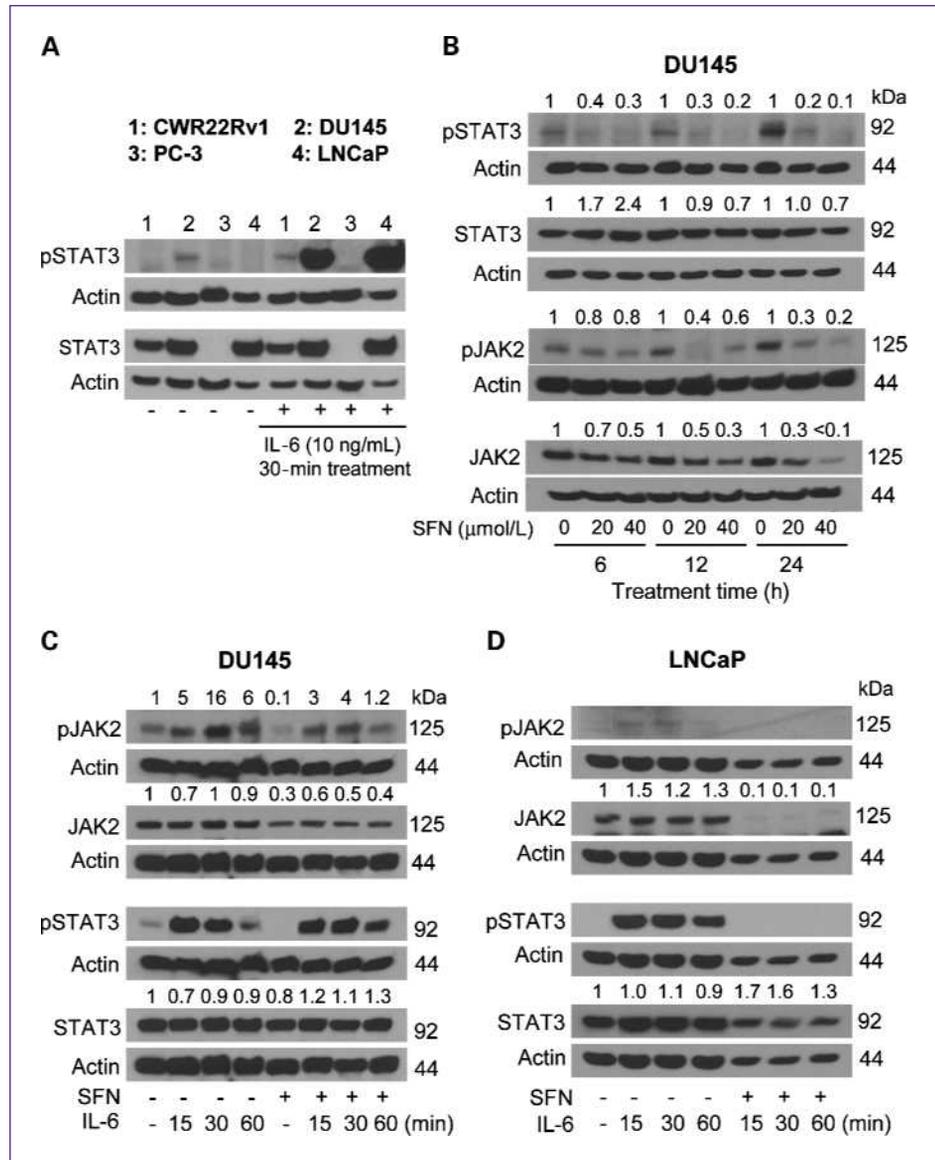
presence of SFN). The IL-6–mediated increase in STAT3-associated luciferase activity was completely abolished in the presence of SFN (Fig. 2A). Consistent with immunoblotting data (Fig. 1D), the IL-6–stimulated STAT3-dependent luciferase activity in LNCaP cells was completely abolished by a 24-hour exposure to 20 and 40 µmol/L SFN (Fig. 2B). These results indicated that SFN treatment inhibited constitutive and IL-6–induced transcriptional activity of STAT3 in DU145 and LNCaP cells.

SFN treatment diminished dimerization and nuclear localization of STAT3

Phosphorylation at Tyr[705] results in homodimerization of STAT3 or heterodimerization with other STATs, which enables nuclear translocation of STAT3 for binding to specific sequences of target genes (23, 28, 31). We designed

experiments using DU145 cells to determine whether SFN treatment affected homodimerization and nuclear translocation of STAT3. As shown in Fig. 2C, electrophoresis followed by immunoblotting revealed time-dependent reduction in level of STAT3 homodimer in DU145 cells on treatment with 40 µmol/L SFN.

Figure 3A depicts immunostaining for constitutive pSTAT3 (red fluorescence) and SYTOX Green–associated nuclear staining (green fluorescence) in DU145 cells following 12-hour treatment with DMSO (control) or the indicated concentrations of SFN. In DU145 cells, pSTAT3 was predominantly localized in the nucleus as evidenced by yellow-orange staining due to merging of red and green fluorescence. Nuclear level of pSTAT3 was decreased markedly in cells treated with 20 and 40 µmol/L SFN (Fig. 3A). As expected, a 15-minute exposure of DU145 cells to

10 ng/mL IL-6 resulted in increased nuclear level of pSTAT3 as evidenced by more intense yellow-orange staining (Fig. 3B) compared with unstimulated DU145 cells (Fig. 3A). The IL-6–stimulated nuclear localization of pSTAT3 was also inhibited in the presence of SFN in both DU145 (Fig. 3B) and LNCaP (Fig. 3C) cells at each concentration. Nuclear staining for pSTAT3 in unstimulated LNCaP cells was practically undetectable (results not shown), which is consistent with the immunoblotting data (Fig. 1A). These results showed inhibition of constitutive and IL-6–induced dimerization and/or nuclear localization of pSTAT3 in the presence of SFN in DU145 and LNCaP cells.

SFN treatment altered levels of STAT3-regulated gene products in prostate cancer cells

STAT3 is known to regulate expression of various genes involved in cell proliferation and apoptosis, including Mcl-1, Bcl-2, cyclin D1, and survivin (23, 28, 31). To test whether SFN-mediated suppression of STAT3 activation resulted in downregulation of its target gene products, we did immunoblotting for above-mentioned proteins using lysates from DU145 and LNCaP cells treated with DMSO (control) and SFN. The levels of Mcl-1, Bcl-2, cyclin D1, and survivin proteins were altered on treatment with SFN in both cell lines, albeit with different kinetics or cell line specificity. The SFN-mediated downregulation of Bcl-2 and cyclin D1 protein levels was clearly evident in both cell lines, especially at the 12- to 24-hour time points (Fig. 4A and B). The SFN treatment decreased protein level of survivin at 6- to 12-hour time points in DU145 cells (Fig. 4A) and at 24-hour time point in the LNCaP cell line (Fig. 4B). We did additional immunoblotting analyses to test whether expression of the above proteins was regulated by IL-6/STAT3 in DU145 and LNCaP cells. As can be seen in Fig. 4C, expression of Mcl-1, Bcl-2, and cyclin D1 proteins was increased by stimulation with IL-6. The effect on survivin protein level was inconsistent (results not shown). The IL-6–stimulated induction of Mcl-1, Bcl-2, and cyclin D1 protein expression was attenuated to varying extent in the presence of SFN (Fig. 4C). These results indicated that the SFN-mediated suppression of STAT3 activation was accompanied by downregulation of some of its target gene products.

IL-6–induced activation of STAT3 conferred protection against SFN-induced apoptosis

To test biological significance of SFN-mediated suppression of STAT3 activation in our model, we determined apoptosis induction by SFN in LNCaP cells treated for 24 hours with 20 and 40 μmol/L in the presence or absence of 10 ng/mL IL-6 (24-hour treatment). As shown in Fig. 5A, the SFN treatment resulted in a concentration-dependent and statistically significant increase in cytoplasmic histone-associated DNA fragmentation, which is a highly sensitive and reliable technique for quantitation of apoptotic cell death. The apoptotic DNA fragmentation resulting from SFN treatment was partially but significantly

abrogated in IL-6–stimulated cells (Fig. 5A). Consistent with these results, SFN-mediated cleavage of poly(ADP-ribose) polymerase and caspase-3 was markedly inhibited in the presence of IL-6 in LNCaP cells (Fig. 5B).

Effect of STAT3 knockdown on SFN-induced apoptosis in DU145 cells

We determined the effect of STAT3 protein knockdown on SFN-induced apoptosis using DU145 cells. The level of STAT3 protein was reduced by ~70% on transient transfection of DU145 cells with STAT3-targeted siRNA in comparison with cells transfected with a control nonspecific siRNA (Fig. 5C, inset). Knockdown of STAT3 protein in DU145 cells resulted in a modest yet statistically significant increase in SFN-induced cytoplasmic histone-associated DNA fragmentation (Fig. 5C). Collectively, these results
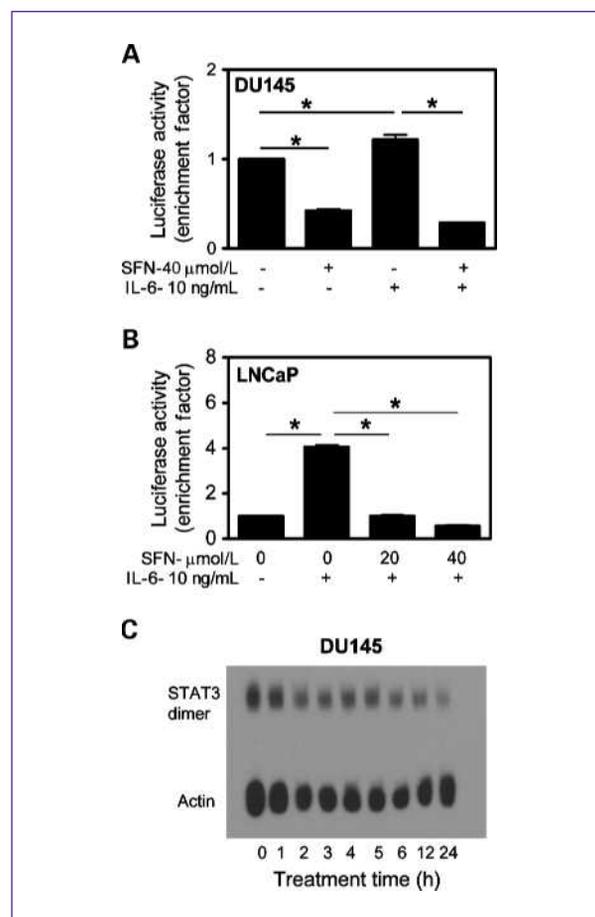

Fig. 2. Effect of 24-h treatment with the indicated concentrations of SFN on STAT3-associated luciferase activity in DU145 (A) and LNCaP (B) cells with or without 12-h stimulation with 10 ng/mL IL-6. Columns, mean ($n = 3$); bars, SE. *, $P < 0.05$, significantly different between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. C, analysis for STAT3 dimerization in DU145 cells treated with 40 μmol/L SFN for the indicated time periods. Numbers on top of the immunoreactive bands represent fold change in STAT3 dimer level relative to control. Each experiment was repeated at least twice, and representative data from one such experiment are shown.
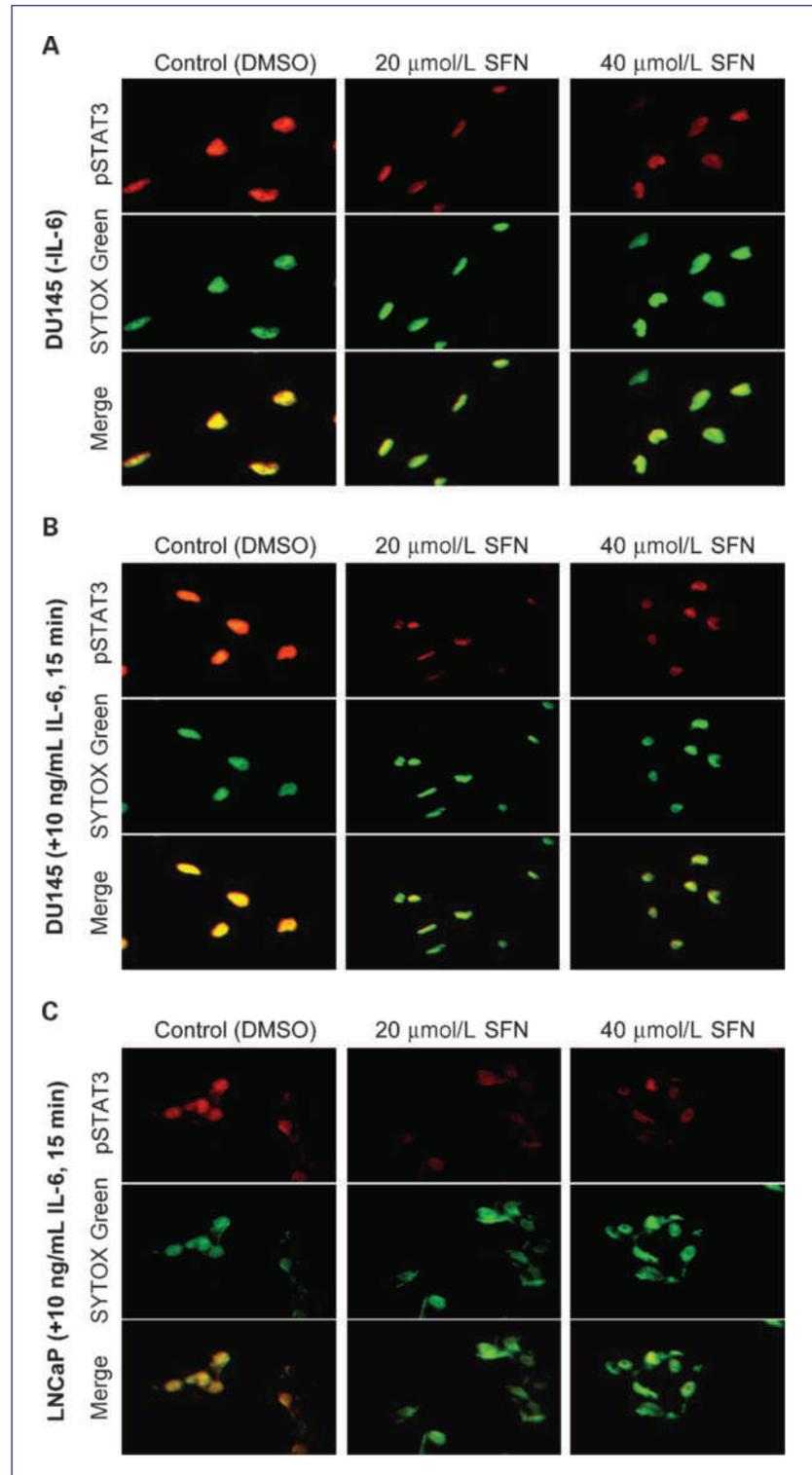
Fig. 3. DU145 (A and B) and LNCaP (C) cells were treated with SFN for 12 h. For stimulation, cells were exposed to IL-6 for 15 min at the end of incubation period. The staining for pSTAT3 and nuclei is indicated by red and green fluorescence, respectively. Each experiment was repeated twice, and representative data from one such experiment are shown.

indicated that STAT3 activation conferred partial protection against SFN-induced apoptosis in prostate cancer cells. It is also clear that the DU145 cell line is markedly more resistant to SFN-induced apoptotic DNA fragmentation (Fig. 5C) compared with LNCaP cells (Fig. 5A). Because STAT3 protein knockdown resulted in only modest increase in SFN-induced apoptosis in DU145 cells, our interpretation is that differences in constitutively active STAT3

levels between these cell lines may only partially account for differential sensitivity of these cells to SFN-induced apoptosis. At the same time, the possibility that complete knockdown of STAT3 protein results in a greater sensitization of DU145 cells to SFN-induced apoptosis cannot be fully discarded.

Effect of oral SFN administration on levels of total STAT3, total JAK2, pSTAT3, and pJAK2 in prostate tumors of TRAMP mice

We have shown previously that oral administration of SFN retards development of prostate cancer in TRAMP mice (15). We used the prostate tumor tissues harvested from representative control and SFN-treated TRAMP mice to determine effect on total STAT3/JAK2 and pSTAT3/pJAK2 levels. As shown in Fig. 5D, the prostate tumors

from SFN-treated TRAMP mice exhibited a decrease in levels of pSTAT3 (~20% decrease), total STAT3 (~30% decrease), and total JAK2 (~70% decrease) compared with controls. Expression of pJAK2 was not detectable in the prostate tumors of either control or SFN-treated TRAMP mice (results not shown). These results indicated that SFN administration reduced levels of total STAT3/JAK2 and pSTAT3 in prostate tumors of TRAMP mice.

Structure-activity relationship for effect of SFN analogues on pSTAT3 and pJAK2

We used naturally occurring thio, sulfinyl, and sulfonyl analogues of SFN (refer to Table 1 for chemical formula of the analogues) to determine possible effect of oxidation state of sulfur and alkyl chain length on thioalkyl isothiocyanate–mediated inhibition of STAT3/JAK2 phosphorylation.
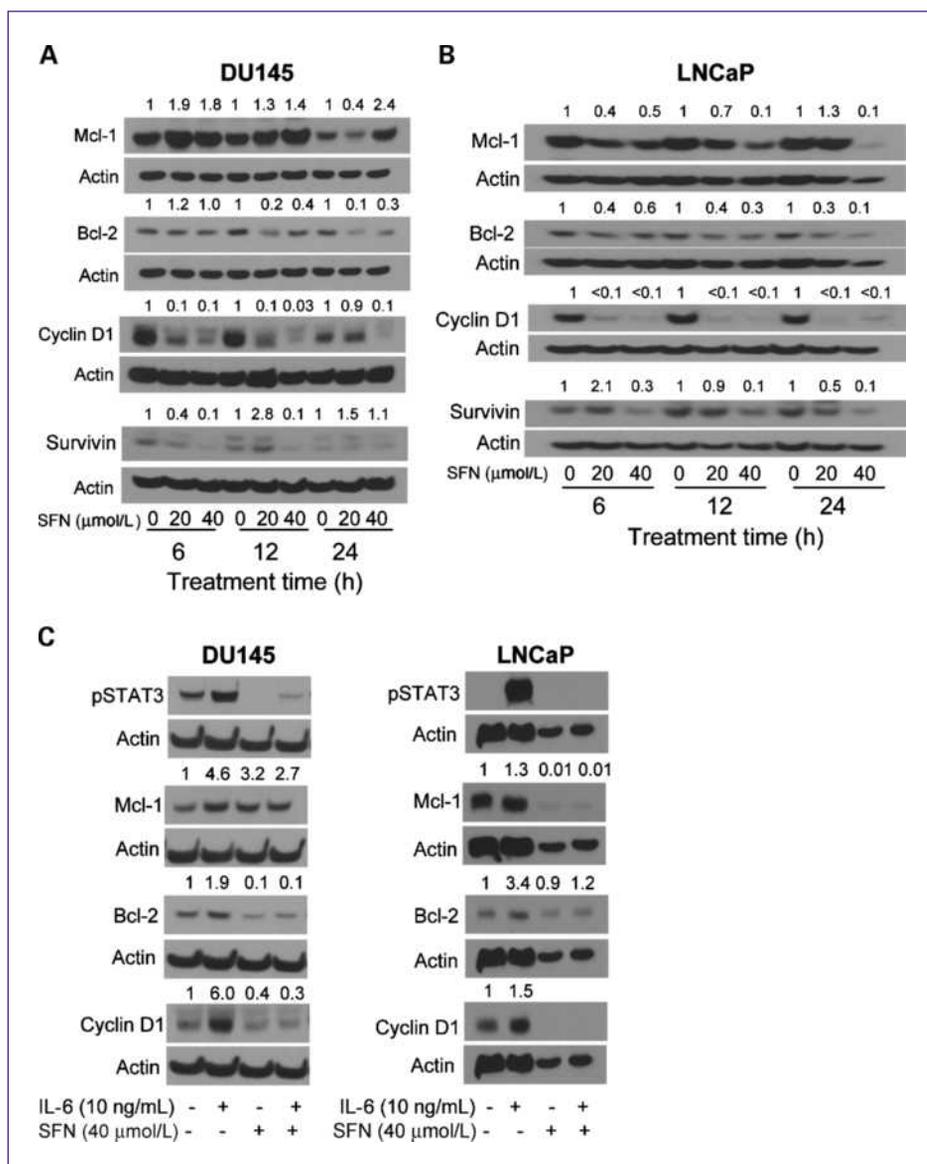

Fig. 4. Immunoblotting for Mcl-1, Bcl-2, cyclin D1, and survivin using lysates from DU145 (A) and LNCaP (B) cells treated for 6, 12, or 24 with DMSO (control) or the indicated concentrations of SFN. C, immunoblotting for pSTAT3, Mcl-1, Bcl-2, and cyclin D1 using lysates from DU145 and LNCaP cells treated for 24 h with 40 μmol/L SFN with or without 1-h stimulation (at the end of the 24-h incubation period) with 10 ng/mL IL-6. The blots were stripped and reprobed with anti-actin antibody to normalize for differences in protein level. Numbers on top of the immunoreactive bands represent changes in protein levels relative to DMSO-treated control. Each immunoblotting experiment was repeated at least twice, and representative data from one such experiment are shown.
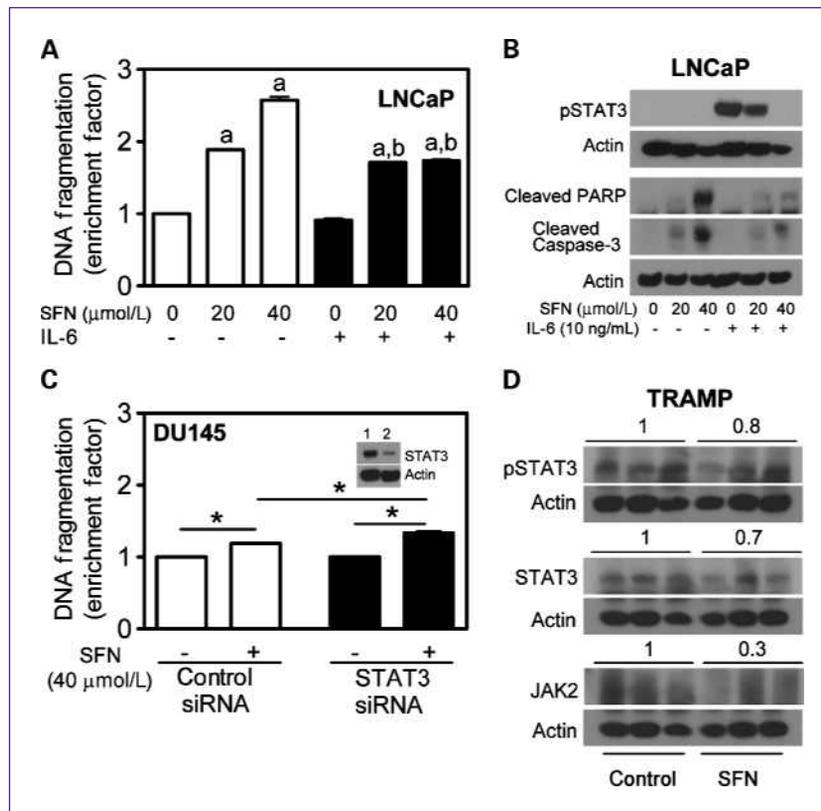
Fig. 5. A, analysis of cytoplasmic histone-associated DNA fragmentation in LNCaP cells following 24-h cotreatment with the indicated concentrations of SFN and 10 ng/mL IL-6. Columns, mean (n = 3); bars, SE. Significantly different (P < 0.05) compared with respective DMSO-treated control (a) and DNA fragmentation in the absence of IL-6 stimulation (b) by one-way ANOVA followed by Bonferroni's multiple comparison test. B, immunoblotting for pSTAT3, cleaved poly(ADP-ribose) polymerase (PARP), and cleaved caspase-3 using lysates from LNCaP cells following 24-h treatment with the indicated concentrations of SFN in the presence or absence of 10 ng/mL IL-6 (24-h treatment). C, analysis of cytoplasmic histone-associated DNA fragmentation in DU145 cells transiently transfected with a control nonspecific siRNA or STAT3-targeted siRNA and treated for 24 h with DMSO (control) or 40 μmol/L SFN. Inset, immunoblotting for total STAT3 in cells transiently transfected with the nonspecific siRNA (lane 1) and the STAT3-targeted siRNA (lane 2). Columns, mean (n = 3); bars, SE. *, P < 0.05, significantly different between the indicated groups by one-way ANOVA followed by Bonferroni's multiple comparison test. Each experiment was repeated at least twice, and representative data from one such experiment are shown. D, immunoblotting for total STAT3/JAK2 and pSTAT3 using supernatants from three representative prostate tumors harvested from control and SFN-treated TRAMP mice (15).

As can be seen in Fig. 6, inhibition of JAK2 and STAT3 phosphorylation as well as JAK2 downregulation was also observed in cells treated for 24 hours with SFN analogues. Although a clear structure-activity relationship was not discernible, suppression of STAT3 phosphorylation at 20 μmol/L concentration was more pronounced for SFN compared with other compounds. In general, the sulfonyl derivatives (cheirolin, erysolin, and alyssin sulfone) were relatively weak suppressors of STAT3 phosphorylation compared with SFN despite potent inhibition of JAK2 phosphorylation. These results suggest that the sulfonyl derivatives might activate other kinases (e.g., Src) to counteract inhibition of JAK2 phosphorylation.

Discussion

STAT3, a nuclear transcription factor belonging to the seven-member STAT gene family of transcription factors, plays an important role in promotion of growth and pro-

gression of prostate cancer (24, 25, 32–35). STAT3 is constitutively active in clinical human prostate cancer (33, 35). For example, Dhir et al. (33) observed significantly higher levels of constitutively active STAT3 in both prostate carcinomas and the matched normal prostate tissues adjacent to tumors compared with the normal prostates from donors without prostate cancer. However, no correlation was noted between STAT3 activation and Gleason grade or serum prostate-specific antigen levels (33). Several reports also indicate that STAT3 promotes proliferation and inhibits apoptosis in cultured prostate cancer cells (24, 36). Moreover, STAT3 is implicated in transition of hormone-sensitive prostate cancer to androgen-independent state (also referred to as "castration resistant" or "hormone refractory"; refs. 25, 36), which is highly aggressive and resistant to chemotherapy (37). Accordingly, agents that are relatively safe but could suppress activation of STAT3 are highly attractive for both prevention of prostate cancer and treatment of castration-resistant disease. The

Table 1. Chemical formula of the SFN analogues used

Chemical name	Common name	Chemical formula
3-(Methylthio)-propyl isothiocyanate	Iberverin	$CH_3\text{-}S\text{-}(CH_2)_3\text{-}N{=}C{=}S$
4-(Methylthio)-butyl isothiocyanate	Erucin	$CH_3\text{-}S\text{-}(CH_2)_4\text{-}N{=}C{=}S$
5-(Methylthio)-pentyl isothiocyanate	Berteroin	$CH_3\text{-}S\text{-}(CH_2)_5\text{-}N{=}C{=}S$
3-(Methylsulfinyl)-propyl isothiocyanate	Iberin	$CH_3\text{-}SO\text{-}(CH_2)_3\text{-}N{=}C{=}S$
4-(Methylsulfinyl)-butyl isothiocyanate	Sulforaphane	$CH_3\text{-}SO\text{-}(CH_2)_4\text{-}N{=}C{=}S$
5-(Methylsulfinyl)-pentyl isothiocyanate	Alyssin	$CH_3\text{-}SO\text{-}(CH_2)_5\text{-}N{=}C{=}S$
3-(Methylsulfonyl)-propyl isothiocyanate	Cheirolin	$CH_3\text{-}SO_2\text{-}(CH_2)_3\text{-}N{=}C{=}S$
4-(Methylsulfonyl)-butyl isothiocyanate	Erysolin	$CH_3\text{-}SO_2\text{-}(CH_2)_4\text{-}N{=}C{=}S$
5-(Methylsulfonyl)-pentyl isothiocyanate	Alyssin sulfone	$CH_3\text{-}SO_2\text{-}(CH_2)_5\text{-}N{=}C{=}S$

present study shows that SFN, a promising cancer chemopreventive agent, inhibits both constitutive and IL-6–induced activation of STAT3 in human prostate cancer cells regardless of their androgen responsiveness. The SFN-mediated suppression of STAT3 activation in human prostate cancer cells is evident at pharmacologically relevant concentrations based on our own pharmacokinetic data. We have shown previously that gavage with a single dose of 6 μmol SFN results in peak plasma concentration of ~19 μmol/L in mice (15).

Activation of STAT3 entails phosphorylation on a critical tyrosine residue (Tyr[705]), which is mediated by different kinases, including JAKs, Rac1, and Src (23, 28, 31, 38).

The IL-6–mediated activation of STAT3 is regulated by JAKs through cytoplasmic domain of gp130 of the IL-6 receptor (39). The present study reveals that SFN-mediated inhibition of STAT3 activation in prostate cancer cells is accompanied by suppression of constitutive as well as IL-6–inducible Tyr[1007/1008] phosphorylation of JAK2. The time course kinetics of SFN-mediated suppression of pJAK2 mirrors that of pSTAT3 inhibition. The mechanism by which SFN inhibits JAK2 phosphorylation seems to involve transcriptional repression of *JAK2*, leading to a reduction in its protein level. Inhibition of STAT3 activation in DU145 cells by a naturally occurring structural analogue of SFN (phenethyl isothiocyanate) was reported
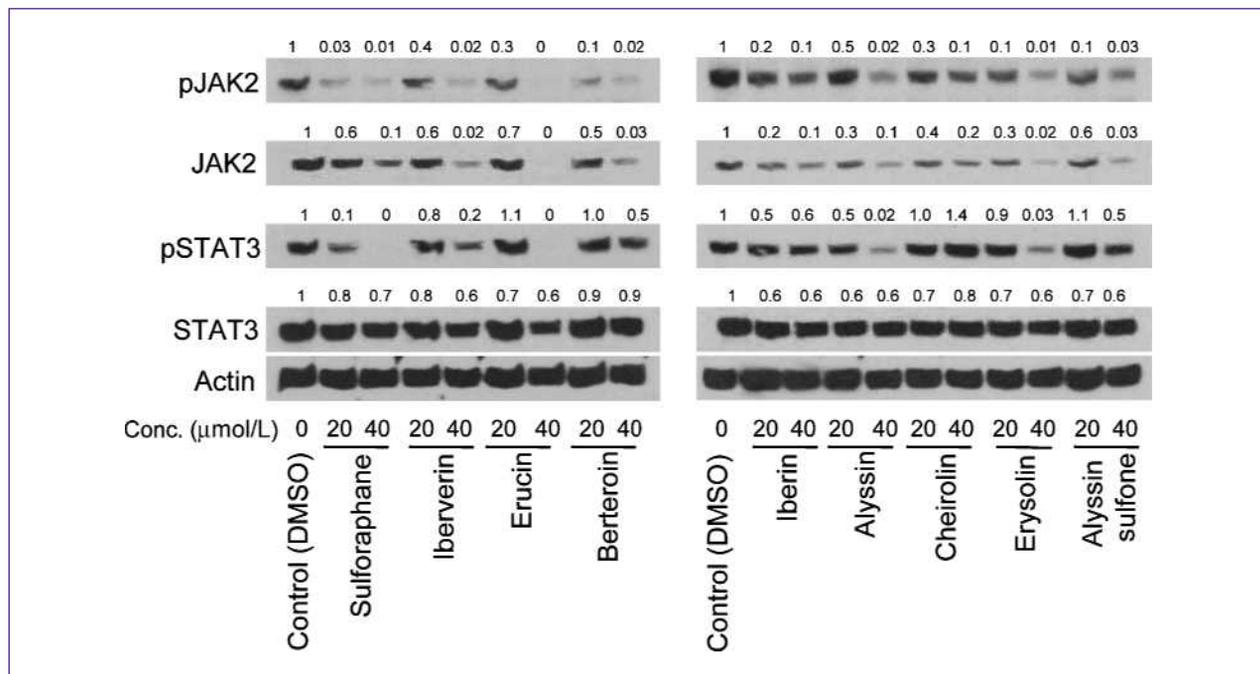

Fig. 6. Immunoblotting for total JAK2, total STAT3, pJAK2, and pSTAT3 using lysates from DU145 cells treated for 24 h with DMSO (control) or the indicated concentrations of SFN analogues. Numbers on top of the immunoreactive bands represent changes in protein levels relative to DMSO-treated control. Immunoblotting experiment was repeated two to three times with each analogue using independently prepared cell lysates. The results were consistent at the 40 μmol/L dose but somewhat variable at the lower concentration.

recently during completion of our work (40). However, unlike our results, the phenethyl isothiocyanate–mediated suppression of STAT3 activation was not due to a decrease in level of JAK2 protein (40). These observations underscore mechanistic differences between structurally related SFN and phenethyl isothiocyanate. Recent studies from our laboratory have documented additional unique mechanistic differences between SFN and phenethyl isothiocyanate. For example, SFN causes autophagy that serves to protect prostate cancer cells against apoptosis (41). On the other hand, autophagy induction seems to contribute to the overall cell death caused by phenethyl isothiocyanate (42).

The Tyr^{705} phosphorylation of STAT3 causes its homodimerization (28, 31). The STAT3 dimer translocates to the nucleus for binding to specific DNA sequences in the promoter of target genes (28, 31). The present study indicates that the SFN-mediated inhibition of STAT3 phosphorylation suppresses its transcriptional activity as evidenced by (a) inhibition of constitutive and IL-6–inducible STAT3-dependent luciferase reporter activity in the presence of SFN, (b) a reduction in level of constitutive STAT3 homodimer in SFN-treated DU145 cells, (c) a decrease in constitutive and IL-6–stimulated nuclear translocation of pSTAT3 on treatment with SFN, and (d) suppression of many STAT3-regulated gene products (e.g., cyclin D1 and survivin) in SFN-treated DU145 and LNCaP cells.

The present study also addresses the question of whether suppression of STAT3 activation contributes to apoptotic cell death resulting from SFN exposure. The IL-6–stimulated activation of STAT3 confers partial yet significant protection against SFN-induced apoptosis in both LNCaP (Fig. 5A) and DU145 cells (results not shown). Knockdown of STAT3 results in only modest increase in SFN-induced apoptosis in DU145 cells, which could be attributed to incomplete knockdown of the STAT3 protein. Alternatively, the possibility that suppression of STAT3 only partially accounts for SFN-induced apoptosis in prostate cancer cells is also likely. To this end, we have shown previously that SFN treatment causes generation of reactive oxygen species, leading to activation of mitochondria-mediated apoptosis in PC-3 and LNCaP human prostate cancer cells (20, 21).

In conclusion, the present study provides experimental evidence to indicate that cancer chemopreventive agent SFN causes suppression of constitutive as well as IL-6–induced activation of STAT3 in human prostate cancer cells irrespective of their androgen responsiveness. Moreover, the IL-6–mediated activation of STAT3 activation confers partial yet statistically significant protection against SFN-induced apoptosis.

Disclosure of Potential Conflicts of Interest

Acknowledgments

Grant Support

References

1. Verhoeven DT, Goldbohm RA, van Poppel G, Verhagen H, van den Brandt PA. Epidemiological studies on *Brassica* vegetables and cancer risk. Cancer Epidemiol Biomarkers Prev 1996;5:733–48.

2. Kolonel LN, Hankin JH, Whittemore AS, et al. Vegetables, fruits, legumes and prostate cancer: a multiethnic case-control study. Cancer Epidemiol Biomarkers Prev 2000;9:795–804.

3. Ambrosone CB, McCann SE, Freudenheim JL, Marshall JR, Zhang Y, Shields PG. Breast cancer risk in premenopausal women is inversely associated with consumption of broccoli, a source of isothiocyanates, but is not modified by GST genotype. J Nutr 2004;134:1134–8.

4. Hecht SS. Inhibition of carcinogenesis by isothiocyanates. Drug Metab Rev 2000;32:395–411.

5. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A 1992;89:2399–403.

6. Zhang Y, Kensler TW, Cho CG, Posner GH, Talalay P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. Proc Natl Acad Sci U S A 1994;91:3147–50.

7. Chung FL, Conaway CC, Rao CV, Reddy BS. Chemoprevention of colonic aberrant crypt foci in Fischer rats by sulforaphane and phenethyl isothiocyanate. Carcinogenesis 2000;21:2287–91.

8. Fahey JW, Haristoy X, Dolan PM, et al. Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of *Helicobacter pylori* and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A 2002;99:7610–5.

9. Conaway CC, Wang CX, Pittman B, et al. Phenethyl isothiocyanate and sulforaphane and their *N*-acetylcysteine conjugates inhibit malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice. Cancer Res 2005;65:8548–57.

10. Gamet-Payrastre L, Li P, Lumeau S, et al. Sulforaphane, a naturally occurring isothiocyanate, induces cell cycle arrest and apoptosis in HT29 human colon cancer cells. Cancer Res 2000;60:1426–33.

11. Jackson SJT, Singletary KW. Sulforaphane: a naturally occurring mammary carcinoma mitotic inhibitor, which disrupts tubulin polymerization. Carcinogenesis 2004;25:219–27.

12. Xu C, Shen G, Chen C, Gelinas C, Kong AN. Suppression of NF-κB and NF-κB-regulated gene expression by sulforaphane and PEITC through IκBα, IKK pathway in human prostate cancer PC-3 cells. Oncogene 2005;24:4486–95.

13. Singh AV, Xiao D, Lew KL, Dhir R, Singh SV. Sulforaphane induces caspase-mediated apoptosis in cultured PC-3 human prostate cancer cells and retards growth of PC-3 xenografts *in vivo*. Carcinogenesis 2004;25:83–90.

14. Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E. Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med 2007;232:227–34.

15. Singh SV, Warin R, Xiao D, et al. Sulforaphane inhibits prostate

carcinogenesis and pulmonary metastasis in TRAMP mice in association with increased cytotoxicity of natural killer cells. Cancer Res 2009;69:2117–25.

16. Singh SV, Herman-Antosiewicz A, Singh AV, et al. Sulforaphane-induced G$_2$/M phase cell cycle arrest involves checkpoint kinase 2 mediated phosphorylation of Cdc25C. J Biol Chem 2004;279: 25813–22.

17. Choi S, Singh SV. Bax and Bak are required for apoptosis induction by sulforaphane, a cruciferous vegetable derived cancer chemopreventive agent. Cancer Res 2005;65:2035–43.

18. Myzak MC, Hardin K, Wang R, Dashwood RH, Ho E. Sulforaphane inhibits histone deacetylase activity in BPH-1, LNCaP and PC-3 prostate epithelial cells. Carcinogenesis 2006;27:811–9.

19. Mi L, Wang X, Govind S, et al. The role of protein binding in induction of apoptosis by phenethyl isothiocyanate and sulforaphane in human non-small lung cancer cells. Cancer Res 2007;67:6409–16.

20. Singh SV, Srivastava SK, Choi S, et al. Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species. J Biol Chem 2005;280:19911–24.

21. Xiao D, Powolny AA, Antosiewicz J, et al. Cellular responses to dietary cancer chemopreventive agent D,L-sulforaphane in human prostate cancer cells are initiated by mitochondrial reactive oxygen species. Pharmaceut Res 2009;26:1729–38.

22. Kim SH, Singh SV. D,L-Sulforaphane causes transcriptional repression of androgen receptor in human prostate cancer cells. Mol Cancer Ther 2009;8:1946–54.

23. Aggarwal BB, Kunnumakkara AB, Harikumar KB, et al. Signal transducer and activator of transcription-3, inflammation, and cancer: how intimate is the relationship? Ann NY Acad Sci 2009;1171:59–76.

24. Barton BE, Karras JG, Murphy TF, Barton A, Huang HF. Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: direct STAT3 inhibition induces apoptosis in prostate cancer lines. Mol Cancer Ther 2004;3:11–20.

25. Tam L, McGlynn LM, Traynor P, Mukherjee R, Bartlett JM, Edwards J. Expression levels of the JAK/STAT pathway in the transition from hormone-sensitive to hormone-refractory prostate cancer. Br J Cancer 2007;97:378–83.

26. Herman-Antosiewicz A, Xiao H, Lew KL, Singh SV. Induction of p21 protein protects against sulforaphane-induced mitotic arrest in LNCaP human prostate cancer cell line. Mol Cancer Ther 2007;6: 1673–81.

27. Xiao D, Srivastava SK, Lew KL, et al. Allyl isothiocyanate, a constituent of cruciferous vegetables, inhibits proliferation of human prostate cancer cells by causing G$_2$/M arrest and inducing apoptosis. Carcinogenesis 2003;24:891–7.

28. Ahn KS, Sethi G, Sung B, Goel A, Ralhan R, Aggarwal BB. Guggulsterone, a farnesoid X receptor antagonist, inhibits constitutive and inducible STAT3 activation through induction of a protein tyrosine phosphatase SHP-1. Cancer Res 2008;68:4406–15.

29. Shin DS, Kim HN, Shin KD, et al. Cryptotanshinone inhibits constitutive signal transducer and activator of transcription 3 function through blocking the dimerization in DU145 prostate cancer cells. Cancer Res 2009;69:193–202.

30. Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway. Biochem J 1998;334:297–314.

31. Darnell JE, Jr. STATs and gene regulation. Science 1997;277:1630–5.

32. Mora LB, Buettner R, Seigne J, et al. Constitutive activation of Stat3 in human prostate tumors and cell lines: direct inhibition of Stat3 signaling induces apoptosis of prostate cancer cells. Cancer Res 2002;62:6659–66.

33. Dhir R, Ni Z, Lou W, DeMiguel F, Grandis JR, Gao AC. Stat3 activation in prostatic carcinomas. Prostate 2002;51:241–6.

34. Huang HF, Murphy TF, Shu P, Barton AB, Barton BE. Stable expression of constitutively-activated STAT3 in benign prostatic epithelial cells changes their phenotype to that resembling malignant cells. Mol Cancer 2005;4:2.

35. Horinaga M, Okita H, Nakashima J, Kanao K, Sakamoto M, Murai M. Clinical and pathologic significance of activation of signal transducer and activator of transcription 3 in prostate cancer. Urology 2005;66: 671–5.

36. Lee SO, Lou W, Hou M, de Miguel F, Gerber L, Gao AC. Interleukin-6 promotes androgen-independent growth in LNCaP human prostate cancer cells. Clin Cancer Res 2003;9:370–6.

37. Feldman BJ, Feldman D. The development of androgen-independent prostate cancer. Nat Rev Cancer 2001;1:34–45.

38. Yu CI, Meyer DJ, Campbell GS, et al. Enhanced DNA binding activity of a Stat3-related protein in cells transformed by the Src oncoprotein. Science 1995;269:81–3.

39. Ni Z, Dyer K, Tweardy DJ, Gao AC. Interleukin-6 induces prostate cancer cell growth accompanied by activation of STAT3 signaling pathway. Prostate 2000;42:239–42.

40. Gong A, He M, Vanaja DK, Yin P, Karnes RJ, Young CYF. Phenethyl isothiocyanate inhibits STAT3 activation in prostate cancer cells. Mol Nutr Food Res 2009;53:878–86.

41. Herman-Antosiewicz A, Johnson DE, Singh SV. Sulforaphane causes autophagy to inhibit release of cytochrome *c* and apoptosis in human prostate cancer cells. Cancer Res 2006;66:5828–35.

42. Bommareddy A, Hahm ER, Xiao D, et al. Atg5 regulates phenethyl isothiocyanate-induced autophagic and apoptotic cell death in human prostate cancer cells. Cancer Res 2009;69:3704–12.

American Association
AACR for Cancer Research

Cancer Prevention Research

Sulforaphane Inhibits Constitutive and Interleukin-6–Induced Activation of Signal Transducer and Activator of Transcription 3 in Prostate Cancer Cells

Eun-Ryeong Hahm and Shivendra V. Singh

Updated version	Access the most recent version of this article at: doi:10.1158/1940-6207.CAPR-09-0250

Cited articles	This article cites 42 articles, 25 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/3/4/484.full#ref-list-1
Citing articles	This article has been cited by 5 HighWire-hosted articles. Access the articles at: http://cancerpreventionresearch.aacrjournals.org/content/3/4/484.full#related-urls

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions	To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/3/4/484. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.