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D,L-sulforaphane causes transcriptional repression of androgen receptor in human prostate cancer cells

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Abstract

D,L-sulforaphane (SFN), a synthetic analogue of cruciferous-vegetable derived L-isomer, inhibits growth of human prostate cancer cells in culture and *in vivo* and retards cancer development in a transgenic mouse model of prostate cancer. We now demonstrate that SFN treatment causes transcriptional repression of androgen receptor (AR) in LNCaP and C4-2 human prostate cancer cells at pharmacological concentrations. Exposure of LNCaP and C4-2 cells to SFN resulted in a concentration- and time-dependent decrease in protein levels of total AR as well as Ser210/213 phosphorylated AR. The SFN-mediated decline in AR protein level was accompanied by a decrease in intracellular as well as secreted levels of prostate specific antigen, an AR-regulated gene product. Decrease in AR protein level resulting from SFN exposure was not reversed in the presence of protein synthesis inhibitor cycloheximide. RT-PCR analysis revealed dose-dependent decrease in AR mRNA levels indicating transcriptional repression of this ligand-activated transcription factor. The SFN treatment inhibited AR promoter activity as revealed by luciferase reporter assay. Synthetic androgen (R1881)-stimulated nuclear translocation of AR was markedly suppressed in the presence of SFN in both cell lines. The SFN treatment also inhibited R1881-stimulated proliferation of LNCaP cells. Naturally occurring thio- (iberverin, erucin, and berteroin), but not the sulfonyl-analogues (cheirolin, erysolin, and alyssin sulfone), of SFN were also effective in reducing protein levels of AR in LNCaP cells. In conclusion, the present study demonstrates for the first time that SFN treatment causes transcriptional repression of AR and inhibition of its nuclear localization in human prostate cancer cells.

Keywords

Sulforaphane; Androgen receptor; Prostate cancer

Introduction

Observational studies suggest that dietary intake of cruciferous vegetables may be inversely associated with the risk of different malignancies including cancer of the prostate (1–4). For example, Kolonel et al. (2) observed an inverse association between intake of yellow-orange and cruciferous vegetables and the risk of prostate cancer in a multi-center case-control study. Anticarcinogenic effect of cruciferous vegetables is ascribed to organic isothiocyanates (ITCs) (5,6). Broccoli is a rather rich source of the ITC compound (–)-1-isothiocyanato-(4R)-(methylsulfinyl)-butane (L-SFN). The L-SFN and its synthetic analogue D,L-sulforaphane

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(SFN) have sparked a great deal of research interest because of their anti-cancer effects. For example, L-SFN and SFN were equipotent as inducers of quinone reductase activity in Hepa 1c1c7 hepatoma cells (7). The L-SFN was shown to cause transcriptional induction of Phase 2 enzymes in prostate cancer cells (8). The L-SFN or synthetic SFN offered protection against 9-10-dimethyl-1,2-benzanthracene-induced mammary cancer in rats, azoxymethane-induced colonic aberrant crypt foci in rats, and benzo[a]pyrene-induced forestomach cancer in mice (9–11). Dietary feeding of SFN and its *N*-acetylcysteine conjugate inhibited malignant progression of lung adenomas induced by tobacco carcinogens in A/J mice (12). Induction of phase 2 carcinogen inactivating enzymes is an important mechanism in prevention of chemically-induced cancers by SFN (5,6).

Recent studies, including those from our laboratory, have documented novel cellular responses to SFN exposure in cultured human cancer cells (13–27). The known cellular responses to SFN exposure in cultured cancer cells include cell cycle arrest, induction of apoptosis and autophagy, inhibition of histone deacetylase, protein binding, and sensitization of cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis (13–27). The mechanisms by which SFN causes growth arrest and apoptosis induction have been studied extensively in human prostate cancer cells (15–17,19–22,26,27). For example, we have reported a novel mechanism for SFN-induced G2/M phase cell cycle arrest in cultured prostate cancer cells involving checkpoint kinase 2-mediated phosphorylation of cell division cycle 25C leading to its sequestration in the cytosol (15). We also found that the SFN-induced apoptosis is selective towards prostate cancer cells and intimately linked to generation of reactive oxygen species (18,19). More recently we have demonstrated that the SFN-induced generation of reactive oxygen species is caused by inhibition of mitochondrial respiratory chain enzymes (20). Cellular effects downstream of reactive oxygen species generation in execution of SFN-induced apoptosis in human prostate cancer cells include activation of Bax and caspases (19, 26). Oral gavage of SFN to male nude mice significantly retarded the growth of PC-3 human prostate cancer xenografts *in vivo* (27). In addition, oral administration of SFN inhibited prostate cancer progression and pulmonary metastasis in a transgenic mouse model of prostate cancer without causing weight loss or any other side effects (28). The SFN-mediated inhibition of prostate cancer development and metastasis in transgenic mice correlated with suppression of cellular proliferation and augmentation of natural killer cell lytic activity (28).

Androgen receptor (AR), a member of the steroid receptor superfamily of ligand activated transcription factors, is critically involved in the initiation and progression of prostate cancer (29,30). Accordingly, novel strategies for blockade of AR signaling are desirable. Here, we report that SFN treatment decreases protein levels of AR by causing transcriptional repression. We also demonstrate that SFN treatment blocks synthetic androgen-stimulated nuclear translation of AR. Together, our preclinical observations (27,28, and the present study) merit clinical investigation of SFN for its efficacy against human prostate cancer.

Materials and Methods

Reagents

SFN and its naturally occurring thio- (iberiverin, erucin, and berteroin), sulfinyl- (iberin and alyssin), and sulfonyl-analogues (cheirolin, erysolin, and alyssin sulfone) were purchased from LKT Laboratories. Reagents for cell culture including fetal bovine serum (FBS) were from Invitrogen; phenol red free RPMI1640 medium was from Cellgro; charcoal-dextran stripped FBS (CSS) was from Hyclone; synthetic androgen R1881 was from Perkin-Elmer; and protein synthesis inhibitor cycloheximide (CHX), anti-actin and anti-tubulin antibodies were from Sigma. Antibody against prostate specific antigen (PSA) was from DakoCytomation; antibody specific for detection of Ser210/213 phosphorylated AR was from Imgenex; a kit for measurement of PSA levels was from R&D Systems; and a kit for quantification of cytoplasmic

histone-associated apoptotic DNA fragmentation was from Roche Applied Science. Antibody against AR and poly-(ADP-ribose) polymerase (PARP) were from Santa Cruz Biotechnology. The dual Luciferase Reporter Assay kit was from Promega.

Cell Culture and Treatments

The LNCaP and C4-2 cell lines were maintained in RPMI1640 medium supplemented with 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 0.2% glucose, 10% (v/v) FBS, and antibiotics. Prior to each experiment, the cells were trypsinized, washed twice with phosphate-buffered saline (PBS), plated in phenol-red free media containing 10% (v/v) CSS, and allowed to attach by incubation for 24 h at 37°C prior to drug treatment. This condition was followed in each experiment, except in the experiments designed to determine the effect of SFN treatment on AR protein level and cell viability/apoptosis in LNCaP cells cultured under regular 10% FBS. Stock solution of SFN and its analogues were prepared in dimethyl sulfoxide (DMSO) and equal volume of DMSO (final concentration 0.1%) was added to the controls.

Immunoblotting

After treatment with DMSO (control) or the desired concentration of SFN for specified time intervals, floating and attached cells were collected and lysed as described by us previously (31). Cell lysates were cleared by centrifugation at 14000 rpm for 20 min. The nuclear fractions from control cells and cells exposed to SFN in the absence or presence of R881 were prepared using a nuclear extraction kit from Pierce according to the manufacture's instructions. Lysate proteins were resolved by sodium-dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membrane was incubated with Tris-buffered saline containing 0.05% Tween 20 and 5% (w/v) nonfat dry milk and then exposed to the desired primary antibody. After treatment with appropriate secondary antibody, the immunoreactive bands were visualized using the enhanced chemiluminescence method.

Measurement of PSA Secretion

The LNCaP and C4-2 cells were treated with DMSO (control) and 10, 20 or 40 µmol/L SFN for 24 h. The medium was collected and used for measurement of secreted PSA levels using a kit from R&D Systems as suggested by the supplier.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA from LNCaP and C4-2 cells treated for 24 h with DMSO (control) or SFN was extracted using RNeasy mini kit (Qiagen) according to the manufacturer's protocol. The cDNA was synthesized using 1 µg of total RNA using superscript reverse transcriptase (Invitrogen) with Oligo dT primer. PCR was performed with specific primer (AR sense 5'-ATG GTG AGC AGA GTG CCC TA-3', antisense 5'-GTG GTG CTG GAA GCC TCT CCT-3') using following amplification conditions: 94°C for 2 min, followed by 28 cycles of 94°C for 1 min, 57°C for 1 min, and 68°C for 1 min. Human *GAPDH* primer was used as a loading control (Biomol)

Luciferase Reporter Assay

The LNCaP and C4-2 cells were transiently co-transfected with 2 µg pGL-AR-Luc plasmid and 0.2 µg pRL-CMV plasmid using Fugene6 transfection reagent. Twenty-four hours after transfection, the cells were treated with SFN for the desired time period, and harvested in reporter lysis buffer. The samples were centrifuged, and 20 µL aliquot was used for measurement of dual luciferase activity using a luminometer. The luciferase activity was normalized against protein concentration and expressed as a ratio of firefly luciferase to *Renilla* luciferase units.

Measurement of Cell Viability and Apoptosis

The effect of SFN treatment on cell viability was determined by trypan blue dye exclusion assay essentially as described by us previously (32). Briefly, cells in 1 mL of phenol-red free medium containing 10% CSS or under regular 10% FBS conditions were plated in 12-well plates and allowed to attach by incubation for 24 h. The medium was replaced with medium containing 20 $\mu\text{mol/L}$ SFN or 1 nmol/L synthetic androgen R1881, and the plates were incubated for an additional 24 h. At the end of the incubation, both floating and adherent cells were collected and used for trypan blue dye exclusion assay or cytoplasmic histone-associated DNA fragmentation assay (32).

Results

SFN Treatment Decreased Protein Levels of AR and PSA in LNCaP and C4-2 Cells

We determined the effect of SFN treatment on AR protein level by immunoblotting using androgen-responsive human prostate cancer cell line LNCaP and its androgen-independent variant (C4-2). The C4-2 cell line, which was generated through co-culture of parental LNCaP cells with human bone fibroblasts *in vivo* in castrated male athymic mice, displays elevated PSA expression and increased anchorage-independent growth in soft agar (33,34). As can be seen in Fig. 1A, a 24 h exposure to SFN in LNCaP and C4-2 cells, which were cultured for 24 h in phenol-red free medium supplemented with 10% CSS prior to drug exposure, resulted in a concentration-dependent decrease in AR protein levels. The SFN-mediated decline in total AR protein level was accompanied by suppression of phosphorylated AR and PSA protein levels in both cell lines (Fig. 1A). In time course kinetic studies using 20 $\mu\text{mol/L}$ SFN, the decline in protein levels of total AR, phospho-AR, and PSA was evident as early as 6 h of treatment (Fig. 1B).

To determine lowest effective dose of SFN, we carried out immunoblotting experiments using lysates from LNCaP cells cultured under conditions described for Fig. 1 and treated for 24 h and 48 h with DMSO (control) and 1, 2.5, or 5 $\mu\text{mol/L}$ SFN. As shown in Fig. 2A, the SFN-mediated decline in protein levels of AR, phospho-AR, and PSA was observed even at 1–2.5 $\mu\text{mol/L}$ concentration especially at the 48 h time point (Fig. 2A). Consistent with these results, SFN treatment resulted in a dose-dependent and statistically significant decrease in secreted levels of PSA into the culture media of both cell lines (Fig. 2B). These results indicated that SFN treatment decreased protein level of AR and suppressed its transcriptional activity as evidenced by a decline in intracellular as well as secreted levels of AR regulated gene product PSA.

SFN Treatment Suppressed Transcription of AR in LNCaP and C4-2 Cells

Next, we designed experiments to test whether the SFN-mediated decrease in AR protein level was caused by inhibition of protein synthesis. The SFN-mediated reduction in AR protein expression was maintained in the presence of protein synthesis inhibitor CHX in both LNCaP and C4-2 cells (Fig. 3). These results indicated that the decrease in AR protein level resulting from SFN exposure was not due to inhibition of protein synthesis.

Fig. 4A depicts effect of SFN treatment on AR message as determined by quantitative RT-PCR. Twenty-four hour exposure of LNCaP and C4-2 cells to SFN resulted in a marked and concentration-dependent decrease in AR mRNA levels in both cell lines. We determined the effect of SFN treatment on AR promoter activity using luciferase reporter assay and the results are shown in Fig. 4B. The pARLUC plasmid used in the present study (Ref. 35; a generous gift from Dr. William H. Walker, University of Pittsburgh, Pittsburgh, PA) is a modification of the pAR1.1LUC plasmid containing the proximal 1047 bp rat AR promoter fragment inserted upstream of the luciferase gene in the pGL2-enhancer vector (35). Exposure of LNCaP

and C4-2 cells to SFN for 24 h resulted in about 40–80% decrease in AR promoter activity. The SFN-mediated decrease in AR promoter activity was evident at each concentration of SFN tested in the preset study ($P < 0.05$ by one-way ANOVA followed by Dunnett's test). These results indicated that the SFN-mediated decline in AR protein level was due to repression of its promoter activity.

SFN Treatment Inhibited Androgen-stimulated Nuclear Translocation of AR

Because AR is a ligand-activated transcription factor, nuclear translocation of AR is critical for its transcriptional activity (29,30). As expected, nuclear level of AR was markedly increased in LNCaP and C4-2 cells in the presence of 1 nmol/L R1881 (2 h stimulation with R1881) as revealed by immunoblotting (Fig. 5A). The R1881-stimulated nuclear translocation of AR was markedly suppressed in the presence of 20 μ mol/L SFN (1 h pre-treatment with SFN followed by 2 h treatment with R1881 in the presence of SFN) in both cell lines (Fig. 5A). These results indicated that SFN was effective in blocking R1881-stimulated nuclear localization of AR.

SFN Treatment Inhibited Androgen-stimulated Growth of LNCaP Cells

We have shown previously that SFN treatment inhibits viability of LNCaP cells cultured under regular FBS conditions by causing apoptosis induction (26). Initially, we determined growth inhibitory and pro-apoptotic response to SFN exposure (24 h treatment) in LNCaP cells cultured under regular 10% FBS conditions or cultured for 24 h in phenol-red free medium supplemented with 10% CSS prior to drug exposure. As can be seen in the supplemental Fig. S1, the SFN treatment resulted in growth inhibition and apoptotic DNA fragmentation in a dose-dependent manner in LNCaP cells cultured in medium supplemented with regular FBS or CSS. Next, we determined the effect of SFN treatment (24 h exposure) on cell viability and apoptosis induction in the presence or absence of synthetic androgen R1881 using LNCaP cells cultured for 24 h in phenol-red free medium supplemented with 10% CSS. The R1881 treatment caused a nearly 2-fold increase in viability of LNCaP cells as judged by trypan blue dye exclusion assay (Fig. 5B). The viability of LNCaP cells was decreased by about 45% by a 24 h exposure to 20 μ mol/L SFN. The SFN-mediated suppression of LNCaP cell viability was maintained even in the presence of R1881. Consistent with these results, a 24 h exposure of LNCaP cells to 20 μ mol/L SFN resulted in increased cytoplasmic histone-associated apoptotic DNA fragmentation compared with DMSO-treated control in the presence of R1881 (Fig. 5C). These results indicated that SFN treatment inhibited R1881-stimulated growth of LNCaP cells by causing apoptosis.

We designed experiments to gain insights into the kinetics of SFN-mediated decline in AR and PSA protein levels and apoptosis induction using LNCaP cells (cultured in medium supplemented with 10% FBS) by immunoblotting for PARP. As can be seen in Fig. 5D, the SFN-mediated cleavage of PARP (an indicator of apoptosis) was not readily evident until 16 h of treatment whereas the decline in AR protein level resulting from SFN exposure was obvious at the 6 h time point.

Structure-Activity Relationship for Effect of SFN Analogues on AR Protein Level

We used naturally-occurring thio-, sulfinyl-, and sulfonyl-analogues of SFN to determine possible impact of oxidation state of sulfur and alkyl chain length on thioalkyl-ITC-mediated decline in AR protein level (please refer to supplemental Table S1 for chemical structures of the SFN analogues). As can be seen in Fig. 6, the thio-derivatives of SFN (iberverin, erucin, and berteroin) were highly effective in suppressing AR protein level regardless of the alkyl chain-length. For example, the propyl-, butyl-, and pentyl-thio analogues were more or less equally effective in reducing AR protein level (Fig. 6). On the other hand, the sulfonyl derivatives of SFN (cheirolin, erysolin, and alyssin sulfone) were either inactive or comparatively less effective than thio- or sulfinyl-derivatives in suppressing AR protein level.

Decline in AR protein level was also observed in cells exposed to other sulfinyl analogues (iberin and alyssin). These results suggested that the oxidation state of the sulfur, but not the alkyl chain length, might be a critical structural determinant in thioalkyl ITC-mediated suppression of AR protein level.

Discussion

Prostate cancer is a leading cause of cancer related deaths among men in the United States (36). Mechanism underlying pathogenesis of prostate cancer is not fully understood but age, race, dietary habits, and androgen secretion and metabolism are some of the risk factors associated with this malignancy (37). AR, a ligand activated transcription factor belonging to the steroid receptor superfamily, is critically involved not only in the development and maintenance of male reproductive organ but also in prostate cancer progression (29,30,38, 39). Moreover, AR is believed to be a major player in transition from hormone-sensitive to androgen-independent prostate cancer (29,40,41). Hormone ablation therapy is the main treatment for early stage prostate cancer. However, this treatment modality is palliative and often leads to incurable and highly aggressive hormone-refractory disease. Consequently, novel strategies to effectively eliminate AR signaling from prostate cancer are highly desirable for clinical management of this deadly disease. We have shown previously that SFN inhibits growth of human prostate cancer cells in culture by causing irreversible cell cycle arrest and apoptosis induction regardless of the androgen-responsiveness or the p53 status (15,19,26, 27). The present study extends these findings and now demonstrates that SFN treatment decreases protein level of AR in both androgen-responsive and androgen-independent prostate cancer cells. The SFN-mediated decline in AR protein level is not blunted in the presence of CHX. Instead, the SFN-mediated down-modulation of AR protein level correlates with reduction in AR message as judged by RT-PCR and inhibition of AR promoter activity as revealed by the luciferase reporter assay. Because AR protein down-regulating effect is conserved for thio-derivatives of SFN but lost for sulfonyl analogues regardless of the alkyl chain length, it is reasonable to conclude that the oxidation state of the sulfur atom influences anti-AR effect of SFN.

Ligand-free AR predominantly resides in the cytoplasm complexed with chaperone proteins including Hsp90 but in a conformational state receptive to ligand binding (29). Ligand-activated regulation of gene expression by AR is achieved by its nuclear translocation, dimerization, and binding to androgen response elements in the DNA of target genes (29,38). The present study indicates that SFN treatment inhibits transcriptional activity of AR. This conclusion is supported by the following observations: (a) R1881-mediated nuclear translocation of AR is markedly suppressed in the presence of SFN in both LNCaP and C4-2 cell lines; (b) SFN treatment results in a significant decrease in intracellular as well as secreted levels of AR regulated gene product PSA; and (c) SFN treatment inhibits R1881-stimulated growth of LNCaP cells in association with apoptosis induction.

An issue highly relevant to clinical application of SFN as an effective AR modulator relates to the plasma concentration of the agent. Even though pharmacokinetic parameters for SFN in humans are yet to be determined, this information is available in rodents. The SFN was detectable in the plasma after 1 h of oral gavage of rats with 50 μmol SFN (42). The maximal plasma concentration (C_{max}) of SFN was found to be about 20 $\mu\text{mol/L}$ after 4 h of oral gavage, and declined with a half-life of about 2.2 h (42). Hanlon et al. (43) have determined absolute bioavailability of SFN in rats treated with either a single intravenous dose of 2.8 $\mu\text{mol/kg}$ or single oral doses of 2.8, 5.6, and 28 $\mu\text{mol/kg}$ (43). The plasma profile of SFN following intravenous administration was best characterized by a two-compartment pharmacokinetic model (43). These investigators also showed that SFN was very well and rapidly absorbed with an absolute bioavailability of about 82%, which decreased at the higher doses (43). Our own

work has revealed that the Cmax of SFN in mice orally gavaged with 1 mg SFN is about 19 $\mu\text{mol/L}$ (28). The maximal plasma concentration of SFN and its cysteine conjugate varied between 0.65 and 0.82 $\mu\text{mol/L}$ in healthy volunteers who consumed a test meal of broccoli soup (150 mL) containing 100 g of broccoli (44). Lower plasma level of SFN in the human volunteer study could be attributed to differences in absorption and metabolism between humans and rodents or presence of ingredients in the broccoli soup affecting pharmacokinetics of SFN. Nonetheless, it is plausible that low micromolar concentrations of SFN required for AR protein suppression are achievable in humans.

Elegant work by Dou and colleagues has established that calpain-mediated AR degradation is intrinsic to the induction of apoptosis in prostate cancer cells in response to treatment with a variety of cancer chemotherapeutic agents including proteasomal inhibitor, topoisomerase inhibitor, DNA damaging agents, and docetaxel (45). We have previously determined the pro-apoptotic response to SFN exposure in PC-3 and LNCaP cells (26). We found that the LNCaP cell line, which is androgen-responsive and expresses wild-type p53, is relatively more sensitive to apoptosis induction by SFN compared with PC-3 cells (an androgen-independent human prostate cancer cell line lacking functional p53) (26). We showed further that the differential sensitivity of LNCaP and PC-3 cells to SFN-induced apoptosis was independent of p53 status (i.e., siRNA-based knockdown of p53 protein in LNCaP cells failed to significantly alter pro-apoptotic response to SFN), but explained by difference in kinetics of Bax activation resulting from SFN exposure (26). The SFN-mediated activation of Bax was much more robust in the LNCaP cell line than in PC-3 cells (26). Moreover, the SFN-mediated suppression of AR expression in the LNCaP cell line occurs earlier (~6 hours; present study) than onset of apoptosis (~16 hours; Ref. 26 and Fig. 5D). Based on these observations, we conclude that pro-apoptotic response to SFN is not dependent on AR signaling. However, inhibition of AR signaling as well as apoptosis induction is likely to contribute to overall anti-cancer effect of SFN.

SFN is known to trigger diverse anti-cancer responses in cultured cancer cells and therefore qualifies as a “promiscuous” agent (13–26). Promiscuity is not unique to the SFN because many known successful pharmaceutical agents (e.g., aspirin) as well as a number of promising dietary cancer chemopreventive agents (e.g., garlic constituent diallyl trisulfide) are promiscuous (46). Because pathogenesis of cancer is complex involving abnormalities in multiple cellular checkpoints and signal transduction pathways, promiscuity may be an advantageous attribute especially for cancer chemopreventive agents. We are reluctant in assigning preference on relative contribution of varied cellular responses (e.g., induction of phase 2 enzymes, modulation of AR signaling, apoptosis induction, inhibition of histone deacetylase, and autophagy) to overall cancer chemopreventive effect of SFN. However, dose-dependent distinction in some cellular responses to SFN exposure is discernible. For example, SFN-mediated induction of Phase 2 enzymes (7,8) as well as suppression of AR signaling (present study) is evident at 1–5 $\mu\text{mol/L}$ concentrations. On the other hand, higher concentrations of SFN (20–40 $\mu\text{mol/L}$) are required to elicit autophagic response, which serves to protect against apoptosis (22).

In conclusion the present study reveals that, analogous to certain other diet-derived agents (47), SFN is highly effective in reducing protein level of AR, androgen-stimulated nuclear translocation of AR, and transcriptional activity of AR in human prostate cancer cells. We also present evidence to indicate critical role of oxidation state of sulfur in thioalkyl-ITC-mediated decline in AR protein level. Results of the present study together with our previous preclinical observations (27,28) merit clinical investigation to determine efficacy of SFN against prostate cancer in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ITCs	isothiocyanates
L-SFN	(-)-1-isothiocyanato-(4 <i>R</i>)-(methylsulfinyl)-butane (L-SFN)
SFN	D,L-sulforaphane
AR	androgen receptor
FBS	fetal bovine serum
CSS	charcoal-dextran stripped FBS
CHX	cycloheximide
PSA	prostate specific antigen
PARP	poly-(ADP-ribose)-polymerase
PBS	phosphate-buffered saline
DMSO	dimethyl sulfoxide
RT-PCR	reverse transcriptase-polymerase chain reaction

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Figure 1.

Sulforaphane (SFN) treatment decreased levels of total androgen receptor (AR) protein, Ser210/213-phosphorylated AR and prostate specific antigen (PSA) protein in LNCaP and C4-2 cells. Immunoblotting for AR, p-AR, and PSA using lysates from LNCaP and C4-2 cells treated with the indicated concentrations of SFN for 24 h (**A**) and 20 μ M/L SFN for the indicated time periods (**B**). *Numbers on top of bands*, densitometric quantitation relative to corresponding control. Immunoblotting for each protein was performed at least twice using independently prepared lysates and the results were consistent.

Figure 2.

Sulforaphane (SFN) treatment suppressed secretion of prostate specific antigen (PSA) into the culture media of LNCaP and C4-2 cells. **A**, immunoblotting for androgen receptor (AR), p-AR, and PSA using lysates from LNCaP cells treated with DMSO (control) or the indicated concentrations of SFN for 24 h or 48 h. *Numbers on top of bands*, densitometric quantitation relative to corresponding control. **B**, levels of PSA secreted into the media of LNCaP and C4-2 cells following 24 h treatment of the cells with the indicated concentrations of SFN.

Columns, mean ($n=3$); *bars*, SE. *, $P<0.05$, significantly different compared with control by one-way ANOVA followed by Dunnett's test. Each experiment was repeated at least twice and the results were consistent.

Figure 3.

Sulforaphane (SFN)-mediated decrease in androgen receptor (AR) protein level was not rescued by protein synthesis inhibitor cycloheximide (CHX). Immunoblotting for AR using lysates from LNCaP and C4-2 cells treated with 20 μ mol/L SFN for the indicated time periods in the absence or presence of 10 μ mol/L CHX (30 min pretreatment). *Numbers on top of bands*, densitometric quantitation relative to corresponding control. Similar results were observed in two independent experiments.

Figure 4. Sulforaphane (SFN) treatment decreased *AR* mRNA level in LNCaP and C4-2 cells. **A**, *AR* and *GAPDH* (loading control) mRNA levels in LNCaP and C4-2 cells treated for 24 h with DMSO (control) or the indicated concentrations of SFN. *Numbers on top of bands*, quantitation relative to control. **B**, promoter activity of *AR*, determined by luciferase reporter assay, in LNCaP and C4-2 cells treated for 24 h with DMSO (control) or the indicated concentrations of SFN. *Columns*, mean ($n=3$); *bar*, SE. *, $P<0.05$, significantly different compared with DMSO-treated control by one-way ANOVA followed by Dunnett's test. Each experiment was repeated twice with similar results.

Figure 5.

Sulforaphane (SFN) treatment inhibited R1881-stimulated nuclear translocation of androgen receptor (AR) in LNCaP and C4-2 cells. **A**, immunoblotting for AR using nuclear and cytosolic fractions prepared from LNCaP and C4-2 cells after treatment with 20 μmol/L SFN (1 h pre-treatment) in the absence or presence of 1 nmol/L R1881 (2 h treatment with R1881 in the presence of SFN). The blots were stripped and re-probed with anti-PARP and anti-tubulin antibodies as loading controls for nuclear and cytosolic fractions, respectively. *Numbers on top of bands*, densitometric quantitation relative to DMSO-treated control. **B**, effect of SFN treatment (24 h exposure) on viability of LNCaP cells (cultured for 24 h in medium supplemented with 10% CSS prior to drug treatment) in the absence or presence of 1 nmol/L synthetic

androgen R1881. **C**, cytoplasmic histone-associated DNA fragmentation in LNCaP cells (cultured for 24 h in medium supplemented with 10% CSS prior to drug treatment) treated for 24 h with 20 $\mu\text{mol/L}$ SFN in the absence or presence of 1 nmol/L R1881. In panels B and C-*columns*, mean ($n=3$); *bars*, SE. Significantly different ($P<0.05$) compared with ^aDMSO-treated control and ^bR1881-treated cells by one-way ANOVA followed by Bonferroni's multiple comparison test. **D**, immunoblotting for AR, PSA, and PARP using lysates from LNCaP cells (cultured under 10% FBS conditions) treated for 6 h or 16 h with DMSO (control) or the indicated concentrations of SFN. Each experiment was repeated twice and the results were comparable.

Figure 6.

Effects of naturally-occurring analogues of sulforaphane (SFN) on protein levels of androgen receptor (AR) and/or prostate specific antigen (PSA) in LNCaP cell line. Immunoblotting for AR and/or PSA using lysates from LNCaP cells treated for 24 h with DMSO (control) or the indicated concentrations of SFN analogues. Chemical structures of the SFN analogues are shown in supplemental Table S1. *Numbers on top of bands*, densitometric quantitation relative to DMSO-treated control.