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Inhibition of AP-1 by Sulforaphane Involves Interaction with Cysteine in the cFos DNA-Binding Domain; Implications for Chemoprevention of UVB-Induced Skin Cancer

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Abstract

Sulforaphane (SFN) is an isothiocyanate derived from cruciferous vegetables which has been linked to decreased risk of certain cancers. Although the role of SFN in the induction of the transcription factor Nrf2 has been studied extensively, there is also evidence that inhibition of the transcription factor AP-1 may contribute to the chemopreventive properties of this compound. In this study, we show for the first time that SFN is effective at reducing the multiplicity and tumor burden of UVB-induced squamous cell carcinomas (SCCs) in a mouse model utilizing co-treatment with the compound and the carcinogen. We also show that SFN pretreatment is able to reduce the activity of AP-1 luciferase in the skin of transgenic mice after UVB. Chromatin immunoprecipitation analysis verified that a main constituent of the AP-1 dimer, cFos, is inhibited from binding to the AP-1 DNA binding site by SFN. EMSA analysis of nuclear proteins also show that SFN and diamide, both known to react with cysteine amino acids, are effective at inhibiting AP-1 from binding to its response element. Using truncated recombinant cFos and cJun we show that mutation of critical cysteines in the DNA binding domain of these proteins (Cys154 in cFos and Cys272 in cJun) results in loss of sensitivity to both SFN and diamide in EMSA analysis. Together, these data indicate that inhibition of AP-1 activity may be an important molecular mechanism in chemoprevention of SCC by SFN.

Keywords

Sulforaphane; AP-1; UVB; skin; chemoprevention

Introduction

Ultraviolet (UV) radiation is a known contributor to skin aging and carcinogenesis. Of the two types of UV light which penetrate our atmosphere, UVB (280–320 nm) and UVA (320–400 nm), UVB is much less abundant but exponentially more potent at inducing DNA damage and cell signaling events. The transcription factor Activator Protein-1 (AP-1) is known to be a key mediator of UV-induced non-melanoma skin cancer (NMSC), and particularly squamous cell carcinoma (SCC). AP-1 is composed of homodimers of Jun family members (cJun, JunD, JunB) or heterodimers of Jun and Fos family members (cFos, FosB, Fra1, Fra2). In cultured keratinocytes, UVB treatment leads to dramatic upregulation of cFos protein levels and an increase in cFos/JunD binding to the TPA-response element (TRE), a binding site in the

promoters of AP-1 target genes (1). Binding of AP-1 to the TRE can be prompted by a variety of stimuli and its activation regulates responses such as proliferation, apoptosis, and differentiation. Inhibition of AP-1 activity through genetic or pharmacological means has been shown to greatly reduce UVB-induced skin carcinogenesis in mice (2–4). AP-1 is therefore an intriguing target for chemoprevention of NMSC using topical agents.

Recent evidence indicates that UVB irradiation causes increased levels of reactive oxygen species (ROS) in the cell, which contributes to damage and signaling events. These ROS have been linked to UVB-induced AP-1 activation (5,6). Abate *et al.* demonstrated that DNA binding of the AP-1 dimer depends on a conserved lysine-cysteine-arginine (KCR) motif present in the DNA binding domain of each subunit of the transcription factor (7). The cysteine amino acid of this motif was shown to be the site of redox-mediated control of AP-1 TRE binding and therefore a major transcriptional control. Replacement of this cysteine with serine resulted in increased TRE binding and loss of sensitivity to thiol-reactive agents (7). Thus, the cysteine of the KCR motif must be maintained in a reduced state in order for AP-1 to bind DNA. Since many stimulants such as UVB actually increase oxidant levels in the cell, the reduction of the AP-1 DNA-binding cysteine is maintained by the redox-sensitive Ref-1 protein (8).

We are interested in characterizing the chemopreventive mechanisms of natural products which are potentially useful for preventing the development of SCCs. Sulforaphane (SFN) is an isothiocyanate compound found in cruciferous vegetables, especially broccoli and broccoli sprouts. SFN or SFN-containing extracts are effective at inhibiting lung adenocarcinomas (9), colon polyps (10), and skin cancer (11–13) in mouse models, but the molecular chemopreventive mechanism(s) employed by SFN are poorly understood. One major mechanism through which SFN mediates chemopreventive effects is through its ability to react with thiol groups such as those found on cysteine in target proteins. Interaction of SFN with cysteines in the Keap1 protein is thought to be responsible for activation of the Nrf2 transcription factor and therefore upregulation of antioxidant and detoxification genes in the cell (14,15). Cells treated with SFN also upregulate rate-limiting enzymes in the biosynthesis of the thiol-based antioxidant glutathione (GSH) and develop enhanced abilities to scavenge ROS or react to xenobiotic stress (16,17).

Recent work on the effects of SFN on AP-1 activity suggests that UVB-stimulated AP-1 might be inhibited by SFN due to changes in the redox potential of the cell (18). However, although SFN significantly reduces AP-1 luciferase activity induced by UVB treatment, this occurs independently of GSH levels in the cell (18). Therefore, we hypothesize that inhibition of AP-1 by SFN is dependent upon a chemical modification of the transcription factor by the compound, and not simply due to a change in cellular redox status. Although SFN is known to modify cysteines such as those known to be crucial for AP-1/DNA binding (7,14), the exact mechanism of its inhibition of AP-1 remains uncertain.

Materials and Methods

Reagents

R,S-Sulforaphane [SFN, 1-isothiocyanato-(4R,S)-(methylsulfinyl)butane] was purchased from LKT Laboratories (St. Paul, MN) and was diluted in acetonitrile or acetone from Sigma Aldrich (St. Louis, MO). Diamide (diazenedicarboxylic acid *bis*[*N,N*-dimethylamide]) was purchased from Sigma.

UVB skin carcinogenesis

SKH-1 hairless female mice were purchased from Charles River Laboratories (Kingston, NY) and housed in accordance with The University of Arizona Animal Care and Use Committee

standards. Mice were split into four groups of 20 each: UVB alone, Acetone+UVB, 1 μ mol SFN+UVB and 2.5 μ mol SFN+UVB. Mice were exposed to UVB using six FS40T12 UVB lamps (National Biological Corporation, Beachwood, OH) three times weekly for 25 weeks. Fluence was determined using a UVX radiometer (Ultraviolet Products, San Gabriel, CA). The UVB dose was initiated at 0.54 kJ/m² and increased 25% each week until the maximal dose of 1.65 kJ/m² was reached at week 5 and maintained for the remainder of the experiment. Mice were pretreated with drug or vehicle for one week prior to initiation of UVB exposure and then one hour prior to each irradiation. Tumors were measured weekly, and the experiment was terminated at week 25. Tumor burden was calculated by multiplying diameter by height in millimeters (19). Average tumor burden was calculated by dividing the sum of individual tumor burdens each week by the number of mice in the treatment group.

Mouse epidermal luciferase assay

SKH-1 mice expressing TRE-driven luciferase (AP-1 luciferase mice) (2,20) were separated into 2 groups of 10 sex and age-matched mice and pretreated with 0.3 μ moles SFN/ear or vehicle. This dosage approximates the exposure of 1 μ mol per back used in the above carcinogenesis experiment, given that the surface area of a mouse ear (front and back) is roughly 1/3 the size of the mouse back treatment area. Both ears of each mouse were pretreated four times (Monday, Wednesday, Friday and Monday) before an acute UVB treatment of 2.75 kJ/m² 48 hr later (Wednesday). Mice were sacrificed 48 hr post UVB and three 1.5mm punches were collected from each left ear, snap frozen and stored at -80 °C. Control ear punches were obtained from the right ear of each mouse one day before UVB irradiation. Ear punch samples were processed for luciferase assay as described previously (21).

Plasmid cloning and mutation

Full-length cFos (His-tagged) and cJun-expressing plasmids in a pET vector were generously provided by Dr. J. A. Goodrich of the University of Colorado. Truncated His-tagged cFos (tFos) and cJun (tJun) were created by PCR amplifying the sequence of interest and cloning back into pET19b at the BamHI and NdeI sites. A Stratagene Quickchange site-directed mutagenesis kit was used to specifically mutate cysteine 49 to serine on tFos and cysteine 58 to serine on tJun. Primer sequences and PCR reaction conditions are available upon request.

Recombinant protein expression and isolation

tFos and tJun proteins were expressed in *E. coli* BL21:DE3 (Promega, Madison, WI). Efficient expression of the cFos and tFos proteins requires co-transformation with a helper plasmid, pSBET (22). Proteins were isolated using Ni-NTA resin (Qiagen, Valencia, CA) under native conditions as described (QIAexpressionist handbook, June 2003) with optimization for recovery from inclusion bodies. Eluate fractions containing protein were pooled, concentrated, evaluated using SDS-PAGE and quantified using the BioRad Protein Assay (BioRad Laboratories, Hercules, CA).

Dialysis to form dimerized proteins

This protocol for forming tFos/tJun heterodimers (tAP-1) was based upon work by Ferguson and Goodrich (23). To form tAP-1, concentrated tFos protein was mixed in 2 molar excess with tJun protein. This 2:1 ratio, the fact that Fos proteins cannot form homodimers, and the fact that Fos/Jun interactions are more stable than Jun/Jun interactions, result in ideal conditions for heterodimer formation. Mixed proteins were denatured and renatured by sequential dialysis (>4 hr each) using Buffers Bi, Bii and Biii as described (23), followed by two exchanges of Final Buffer (25 mM sodium phosphate, pH 7.5, 1 mM DTT, 5% glycerol), one of which was overnight. Mutant dimers were formed using an identical protocol.

Chromatin Immunoprecipitation

Human keratinocyte HaCaT cells at 80% confluence were treated with 10 μ M SFN or vehicle (acetonitrile) in serum-free medium for 16–24 hr. Cells were then exposed to 250 J/m² UVB (FS20T12 bulbs, National Biological Corporation) and post-treated until harvest 6 hours later as described (24). All solutions/buffers used were based upon the Upstate Cell Signaling Technology EZ ChIP™ Kit (Billerica, MA). Samples containing 10⁶ cells were dispersed in 400 μ L SDS lysis buffer plus protease inhibitors, incubated on ice for 10 minutes, and stored at –80 °C prior to sonication. To quantify the chromatin, uncrosslinked DNA was treated with RNase A and Proteinase K before purification using a Qiaquick PCR Purification Kit (Qiagen, Valencia, CA).

Twenty-five μ g of sonicated chromatin in equal volumes of SDS lysis buffer were used for each immunoprecipitation (IP) including controls. These were diluted 10-fold using ChIP Dilution Buffer plus protease inhibitors and precleared using Protein A agarose/salmon sperm DNA blocked beads (Millipore, Billerica, MA) with rotation at 4 °C for 1 hr. Supernatants were divided into separate tubes for each IP. Aliquots of each tube were removed as “Input” control. Each IP tube received 8 μ g of the appropriate antibody (RNA Polymerase II: Upstate 05-623, cFos and IgG: Santa Cruz Biotechnologies sc-53 and sc-2027, respectively) or had no more additions (No Antibody control) and was rotated at 4 °C overnight. Next, 100 μ L of fresh beads were added to each tube and rotated at 4 °C for 2 hr. Pelleted beads were transferred to Handee spin cup columns (Pierce Biotechnology, Rockford IL) and washed as described in the kit. Chromatin was eluted from the beads by incubating 2 $\times$ with 250 μ L elution buffer. Eluted chromatin was uncrosslinked, subjected to RNase A and Proteinase K digestions, and purified using the Qiaquick PCR Purification kit. Cleaned immunoprecipitated DNA was eluted from the column using 50 μ L of nuclease-free H₂O.

Quantitative Real-Time PCR

ChIP products were tested for the presence of the TRE site found in the MMP-1 promoter using primers designed to detect the specified region from Applied Biosystems (Foster City, CA). These custom probes were based on the promoter sequence first described by Angel *et al.*, (25) and were confirmed against the human genome sequence. ChIP DNA (4 μ L) in triplicate were used for qPCR reactions using Applied Biosystems TaqMan Universal PCR Master Mix in an ABI Prism 7700 Sequence Detector (Applied Biosystems). Fold enrichment of the immunoprecipitated fragment was determined using the comparative C_t method, using the following equation: $2^{-(C_t \text{ IP} - C_t \text{ Input})}$. Fold enrichments for each experiment were normalized to the respective control sample. The GAPDH promoter was also amplified from input samples and from the RNA Polymerase II immunoprecipitation samples for each experiment using normal PCR primers and conditions supplied by the Upstate kit as a control (RNA Polymerase data not shown).

Electrophoretic Mobility Shift Assay (EMSA)

Two micrograms of recombinant heterodimers or 5 μ g of nuclear proteins were subjected to EMSAs using established protocols (2,18). Nuclear proteins were extracted from treated HaCaT human keratinocytes 12 hr after a dose of 250 J/m² UVB. Nuclear or recombinant proteins were mixed with either SFN, diamide or vehicle in a final volume of 10 μ L and incubated at 37 °C for 1 hr. Proteins were then mixed with 5 $\times$ Binding Buffer (50 mM Hepes pH 7.9, 250 mM KCl, 0.5 mM EDTA, 12.5 mM DTT, 50% glycerol, 2.5% Triton X-100), 1 μ g of Poly(dIdC):Poly(dIdC) and water to a final volume of 19 μ L. This was incubated on ice for 20 minutes, at which time 1 μ L of ³²P-labeled TRE probe (18) was added and the tubes were incubated at room temperature for 30 minutes. Products were loaded onto a 6% acrylamide, 0.25 $\times$ TBE, 2.4% glycerol nondenaturing gel. Finished gels were dried and exposed to film. All EMSAs displayed only one retention band in addition to the probe front.

Statistical Analysis

Primary analyses compared average tumor burden (diameter $\times$ height in millimeters) and tumor count (multiplicity) at week 25 in the 3 treatment groups (Acetone, 1 μ mol SFN, 2.5 μ mol SFN). These cross-sectional analyses among the three treatment groups used the Kruskal-Wallis Test. A nonparametric test for linear trend across treatment groups used Stata's `nptrend` command. For these primary analyses statistical significance was assessed at $p=0.05$. Two by two analyses used the Wilcoxon rank sum test. These post-hoc multiple comparisons used a bonferroni corrected p value of 0.025. For the luciferase assay, a two-tailed student's t -test was used to calculate significance between the fold induction of the Acetone versus SFN treated mice. The same analysis was used for comparison of the normalized quantitative real-time PCR data. In both cases, significance was defined as $p < 0.05$.

Results

SFN inhibits UVB-induced skin carcinogenesis in SKH-1 mice

Treatment of mouse back skin with SFN at either 1 μ mol/mouse or 2.5 μ mol/mouse produced marked reduction in tumor multiplicity and tumor burden, although there were no differences noted in tumor type between the groups. An overall test for differences in tumor multiplicity among the four experimental groups at week 25 using the Kruskal-Wallis test was borderline significant. ($p=0.06$). However, comparison of the acetone group to the 2.5 μ mol SFN group indicated a statistically significant difference at week 25 (58% fewer tumors with 2.5 μ mol SFN, $p=0.03$). A non-parametric test for linear relationship between tumor count and increasing SFN dose for weeks 15 to 25 was also statistically significant ($p=0.007$) (Figure 1A). Tumor burden was not different among the experimental groups at week 25. However, there was a statistically significant trend towards lower tumor burden in the groups treated with SFN ($p < 0.0001$) (Figure 1B). Thus, SFN treatment is effective at inhibiting tumorigenesis in this model, especially when using the higher dose of SFN.

SFN inhibits UVB-induced AP-1 luciferase in vivo

Full-thickness skin punch biopsies from the ears of AP-1 luciferase mice confirm significant activation of AP-1 luciferase by UVB ($p = 0.0002$) (data not shown). However, the 24-fold luciferase induction by UVB was significantly reduced to 13-fold with SFN pretreatment ($p = 0.01$) (Figure 2). Control punches taken 24 hr prior to UVB exposure show luciferase expression levels in both vehicle and SFN-treated ears to be very low overall (data not shown), in accordance with previous studies (21). Thus, SFN is effective at reducing AP-1 activation after UVB in mouse skin, but does not appear to influence baseline AP-1 activity. These data are in accordance with previous findings in cultured keratinocytes (18) and are the first to indicate that SFN can inhibit AP-1 activation *in vivo*.

SFN inhibits nuclear binding of cFos to the TRE

SFN is known to influence many factors in the cell which could affect UVB-induced AP-1 luciferase activation. To confirm that the inhibition of AP-1 noted in our previous results was due to inhibition of AP-1 binding to DNA in the cell, we performed chromatin immunoprecipitation (ChIP) assays using keratinocytes in culture. Quantitative PCR to amplify the TRE in the collagenase-1 (MMP-1) promoter, the same sequence as the promoter in the AP-1 luciferase construct, was performed. Our data show a nearly 2.5-fold activation of cFos DNA binding in cells treated with UVB ($p < 0.01$, Figure 3A), in agreement with previous observations using other assays (1). In addition, our data clearly indicate that SFN inhibits cFos from binding to the MMP-1 TRE after UVB exposure. The level of cFos binding is significantly reduced by SFN treatment when compared to the UVB-alone samples ($p < 0.01$). These results are supported by low levels of binding in all of the negative controls (No Antibody or IgG

controls), which are not affected by SFN or UVB treatment. PCR of the GAPDH promoter from each Input DNA sample confirms that the initial starting chromatin concentrations were equivalent (Figure 3B).

Both SFN and diamide, cysteine oxidizing agents, effectively block binding of nuclear AP-1 to the TRE in vitro

Nuclear extracts from cells either mock-irradiated or exposed to 250 J/m² UVB were incubated with SFN or diamide prior to the addition of TRE probe and EMSA analysis. The dose-dependent inhibition of AP-1 binding due to SFN exposure in vitro (Figure 4A) is typical of our previous results (18). At a dose of 1 mM SFN, the binding of nuclear AP-1 to the TRE was reduced to baseline levels. Diamide, another cysteine oxidizing agent, was also very effective at inhibiting the binding of AP-1 under the same conditions (Figure 4B).

Isolation and characterization of recombinant truncated cFos and cJun

PCR products encoding the beta-zip region of cFos and cJun were cloned into bacterial expression vectors encoding an N-terminal His tag to create tFos and tJun. These truncated proteins contain only two cysteine residues each—one in the DNA-binding domain (Cys154 for cFos, Cys272 for cJun) and one proximal to the C-terminus in the leucine zipper domain (Figure 5A). Recombinant proteins were purified and visualized on an SDS-PAGE gel (Figure 5B). After dimerization of tFos and tJun subunits to form tAP-1, EMSA analysis showed that these proteins bind tightly to the TRE. Preincubation of tAP-1 with cold wild type TRE oligos reduced binding to ³²P-labeled TRE in a dose-dependant manner, while cold mutant oligos did not (Figure 6A). The tAP-1 dimer therefore specifically binds to the TRE. This binding was inhibited by SFN pretreatment in a dose-dependent fashion, similar to that noted with nuclear extracts (Figure 6B). Thus, the recombinant tAP-1 protein reacts to the TRE and to SFN in a manner similar to that of its nuclear counterpart.

DNA-binding cysteine is required for SFN- or diamide-induced inhibition in vitro

To test for direct interaction of SFN with the DNA-binding cysteines in AP-1, we mutated the DNA-binding cysteines in tFos and tJun to serines, leaving the remaining cysteines near the C-terminus intact. Both wildtype and mutant heterodimers were then treated with SFN or diamide and analyzed for their ability to bind to the TRE via EMSA. As shown in Figure 6C, mutation of the DNA-binding cysteine resulted in loss of sensitivity to treatment with either of these oxidizing agents. In fact, although binding to the TRE is completely blocked by pretreatment of the wildtype dimer with 7 mM diamide, the mutant dimer is totally immune to this inhibition. The mutant form of tAP-1 is also completely resistant to inhibition by 1mM SFN.

Discussion

To our knowledge, this is the first study to report an inhibitory effect of SFN on UVB-induced skin carcinogenesis in mice exposed to both SFN and UVB simultaneously. Other reports have described the effect of SFN using chemically-induced mouse skin carcinogenesis (12,13), or a UVB model using a chemotherapeutic treatment protocol with broccoli extracts (i.e., UVB was stopped before the extract was applied) (11). All of these studies described a protective effect of SFN treatment. Therefore the current data corroborate previous chemopreventive reports and do so using purified SFN and a model of concurrent UVB/agent exposure which may be more relevant to human outcomes. SFN does not absorb light in the UV spectrum or produce a sunscreen effect (26). This provides us with a positive framework for utilizing SFN as a topical chemopreventive agent in conjunction with studies to identify molecular mechanisms of SFN chemopreventive effects in the skin.

Many of the molecular studies of SFN have focused on its effects on the Nrf2 transcription factor pathway (12,27,28). Nrf2 and its effector proteins help to protect cells from oxidative insults. While Nrf2 is implicated in protecting the skin against carcinogenic chemicals, its role in UV-induced carcinogenesis is unclear. In cell culture, different doses or wavelengths of UV can induce or reduce Nrf2 levels, depending on the cell type (29–31). Some have suggested that transient Nrf2 activation in the skin by electrophilic compounds (such as SFN) may be protective against tumorigenesis, but constitutive activation of Nrf2 may lead to malignant conversion (32). Protection from UV-induced carcinogenesis by SFN might also involve modulation of the inflammatory response by Nrf2 (33–37). However, a recent report discovered that although Nrf2 knockout mice had increased oxidative DNA damage, inflammation, and sunburn cell formation compared to wildtype mice after acute UVB exposure, chronic UVB treatment revealed no difference in the incidence rate or mean number of tumors between wildtype and Nrf2 KO mice (38). Therefore, the ability of SFN to inhibit UVB-induced NMSC may be due to factors other than Nrf2 stimulation.

The experiments reported here demonstrate for the first time the ability of SFN to inhibit AP-1 activity *in vivo*. Earlier luciferase assays and EMSAs indicated that SFN could regulate the DNA binding of AP-1 *in vitro* (18). In our transgenic mouse model we have successfully shown that SFN inhibits UVB-induced AP-1 luciferase activity in the skin. We have also confirmed through ChIP analysis that UVB causes increased binding of cFos to the TRE, and that this binding is inhibited by SFN. Although others have reported that SFN slightly increases AP-1 luciferase activity at low doses, our results do not indicate increased basal AP-1-luciferase activity with SFN pretreatment (data not shown) (39). These differences may be due to cell type-specific reactions. The data in Figures 2 and 3 support our hypothesis that inhibition of AP-1 may be a contributing factor to the ability of topical SFN to block UVB-induced SCC in mice.

SFN is known to bind to reactive thiol groups, especially cysteines, and may affect protein function through this mechanism (14). Diamide, another thiol oxidative agent, has been used previously to inhibit recombinant AP-1 binding *in vitro* (7). Treatment of nuclear extracts with either diamide or SFN showed dose-dependent inhibition of AP-1 DNA binding. Since both compounds interact with cysteines the inhibition of TRE binding is likely a result of cysteine oxidation of the AP-1 transcription factor. We have noted this reaction to SFN previously at the same dose levels (18), which are likely to be physiologically relevant (40). Although the doses needed for inhibition of AP-1 binding *in vitro* are higher than those used in luciferase assays, SFN is known to accumulate in the cell when added to culture media. Treatment of mouse hepatoma cells with micromolar concentrations of SFN yielded millimolar concentrations in cellular lysates (40). The data in Figure 4 suggest a cysteine-specific chemical reaction, since nuclear extracts were exposed to both of these oxidants in a test tube where transcriptional or translational input is minimal.

We next addressed the importance of the specific DNA binding cysteines of AP-1 (Cys154 in Fos and Cys272 in Jun) in the reaction with SFN and diamide. Mutations of the DNA binding domain of AP-1 can contribute to the oncogenic nature of AP-1 family members (41–43). We therefore purified His-tagged recombinant truncated forms of cFos and cJun consisting of the DNA-binding and leucine zipper (beta-zip) domains, modeling them after those used by Abate and colleagues (7). The truncated AP-1 proteins were able to dimerize and bind specifically to the TRE in a manner consistent with that observed using nuclear extracts. The fact that SFN could dose-dependently inhibit truncated AP-1 from binding to the TRE suggests functional similarity between the recombinant form and its endogenous counterpart. However, the complete lack of response to cysteine oxidation by either SFN or diamide when the DNA-binding cysteines are mutated supports our hypothesis: SFN chemically oxidizes Cys154 in Fos and Cys272 in Jun to inhibit binding of AP-1 to the TRE. The cysteine to serine mutation

creates a “permanently reduced” DNA binding domain, which is unaffected by SFN and leads to enhanced TRE binding (Figure 6C).

SFN and other isothiocyanates are gaining credibility as potential “natural” chemopreventive agents. These natural agents are present in our diet and are easily tolerated by our metabolism. Orally administered broccoli sprout extracts containing SFN have been safely tolerated by volunteers (44). Other groups are testing the efficacy of SFN or related compounds for possible use in humans to prevent hepatocarcinoma, breast cancer and NMSC (26–28,45), although to date there are no published studies regarding oral administration of SFN and the prevention of skin carcinoma. Due to the current focus on SFN-induced Nrf2 activation in chemoprevention, many of these studies justifiably turn to markers of Nrf2 activity in order to measure the potential efficacy of this compound. Many other molecular targets of SFN have been identified in the cell, including transcription factors such as NFκB, which may be subject to a similar form of thiol-mediated redox regulation as AP-1 (46). The results described here suggest that inhibition of AP-1 is also important to consider when studying the properties of SFN in human skin cancer chemoprevention trials.

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Figure 1. SFN inhibits UVB-induced SCC in mice

SKH-1 mice were pretreated with Acetone, 1 µmol SFN, 2.5 µmol SFN or nothing 1 hr prior to exposure to UVB three times a week for 25 weeks. Tumors were counted and measured weekly. The average tumors per mouse (multiplicity) in the 2.5 µmol group was significantly different than the acetone control at week 25 ($p=0.03$), and trend analysis from week 15 through 25 showed strong significant inhibition of multiplicity with SFN treatment (A, $p=0.007$). SFN treatment also resulted in a significantly lower trend in the average tumor burden for this group (B, $p=0.0001$).

Figure 2. Inhibition of UVB-induced AP-1 luciferase by SFN in mouse skin

AP-1 luciferase mouse ears were pretreated with acetone or SFN before acute treatment with UVB. Luciferase assays were performed on pre- and post-UV treated ear punches, and fold activation by UVB was calculated for each mouse. The statistically significant difference between acetone treated and SFN treated mice after UVB is marked as * ($p = 0.01$).

Figure 3. SFN inhibits cFos binding to the TRE in the nucleus

HaCaT keratinocytes were pre- and post-treated with acetonitrile or 10 μ M SFN before exposure to UVB and harvest for ChIP assay. Samples were immunoprecipitated with an antibody against cFos, normal IgG, or no antibody. Real time qPCR was performed using a probe specific for the TRE from the collagenase-1 promoter. Data are pooled from three independent experiments. The statistically significant difference between control versus UVB alone treated samples is marked as * ($p < 0.001$), while UVB alone versus SFN+UVB is marked as ** ($p = 0.0013$) (A). Input samples were used for amplification of the GAPDH promoter to show that each sample started with equivalent amounts of chromatin (B).

Figure 4. SFN and Diamide block AP-1 binding to the TRE in vitro

Nuclear extracts from mock treated or UVB treated cells were incubated with either SFN (A), diamide (B) or the appropriate vehicle control. They were then exposed to radiolabeled TRE probe and run on an EMSA gel. Both of these cysteine oxidants reduce the ability of AP-1 to bind to the TRE in a dose-dependent manner.

Figure 5. Structures and expression of recombinant tFos and tJun

The beta-zip domains of cFos and cJun were cloned into bacterial expression vectors. The domain structures of the resulting truncated proteins are shown in comparison to their full-length counterparts (A). Mutated tFos and tJun have the identical amino acid sequence as their wildtype forms, except that the cysteine in the DNA binding domain is replaced with serine. Recombinant tFos and tJun and their mutated forms were isolated using Ni-NTA resin and were visualized using SDS-PAGE and BioSafe Coomassie Blue staining (B).

Figure 6. Truncated recombinant AP-1 binds specifically to the TRE and is inhibited by SFN in a manner dependant upon the presence of the DNA-binding cysteine

Recombinant tFos and tJun were heterodimerized in order to form functional tAP-1. The binding specificity of tAP-1 was confirmed through EMSA analysis in which wild type cold competitor oligos successfully inhibited tAP-1 from binding to the labeled TRE in a dose-dependant fashion, but mutant competitors did not (A). tAP-1 was also incubated with SFN for 1 hr at 37° before being mixed with buffer and labeled probe. The ability of SFN to inhibit tAP-1 TRE binding in a dose-dependant fashion was therefore confirmed (B). Wildtype tAP-1 was then compared to mutant tAP-1 using EMSAs in which both dimers were pretreated with 1 mM SFN or 7 mM Diamide as above. Mutation of the DNA-binding cysteine in tAP-1 results

in a loss of activity of both SFN and diamide on the TRE binding response of these proteins (C).