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Sulforaphane Inhibits c-Myc-Mediated Prostate Cancer Stem-Like Traits

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Abstract

Preventive and therapeutic efficiencies of dietary sulforaphane (SFN) against human prostate cancer have been demonstrated *in vivo*, but the underlying mechanism(s) by which this occurs is poorly understood. Here, we show that the prostate cancer stem cell (pCSC)-like traits, such as accelerated activity of aldehyde dehydrogenase 1 (ALDH1), enrichment of CD49f+ fraction, and sphere forming efficiency, are attenuated by SFN treatment. Interestingly, the expression of c-Myc, an oncogenic transcription factor that is frequently deregulated in prostate cancer cells, was markedly suppressed by SFN both *in vitro* and *in vivo*. This is biologically relevant, because the lessening of pCSC-like phenotypes mediated by SFN was attenuated when c-Myc was overexpressed. Naturally-occurring thio, sulfinyl, and sulfonyl analogs of SFN were also effective in causing suppression of c-Myc protein level. However, basal glycolysis, a basic metabolic pathway that can also be promoted by c-Myc overexpression, was not largely suppressed by SFN, implying that, in addition to c-Myc, there might be another SFN-sensitive cellular factor, which is not directly involved in basal glycolysis, but cooperates with c-Myc to sustain pCSC-like phenotypes. Our study suggests that oncogenic c-Myc is a target of SFN to prevent and eliminate the onset of human prostate cancer.

Keywords

SULFORAPHANE; c-MYC; PROSTATE CANCER STEM CELLS; CHEMOPREVENTION

INTRODUCTION

The potential benefit of sulforaphane (SFN) occurring naturally as the L-isomer of a glucoraphanin precursor in edible cruciferous vegetables like broccoli, for chemoprevention of prostate cancer, is substantiated by preclinical data [Fahey et al., 2001; Singh et al., 2009; Keum et al., 2009; Singh and Singh, 2012]. Epidemiological studies also suggest an inverse association between dietary intake of cruciferous vegetables and the risk of prostate cancer [Kolonel et al., 2000]. Mechanistic studies using cultured prostate cancer cells have shown

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that SFN treatment not only causes induction of carcinogen-metabolizing enzymes, but inhibits processes and pathways relevant to cancer development [Brooks et al., 2001; Singh et al., 2004; Xu et al., 2005; Hahm and Singh, 2010]. SFN treatment induces apoptotic cell death in prostate cancer cells, whereas normal prostate epithelial cells are resistant to this effect [Choi and Singh, 2005; Singh et al., 2005]. We also showed previously that the *in vivo* growth of PC-3 human prostate cancer cells was significantly inhibited upon oral administration of SFN [Singh et al., 2004]. *In vivo* efficacy of SFN and SFN-containing broccoli sprout for chemoprevention of prostate cancer was demonstrated in Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) transgenic mice [Singh et al., 2009; Keum et al., 2009].

Demonstration of SFN's chemopreventive efficacy in the TRAMP model, coupled with its suppressive effect on androgen receptor (AR) expression and function, attracted interest in its clinical investigation [Myzak et al., 2006; Kim and Singh, 2009; Singh et al., 2009; Alumkal et al., 2015]. In a phase II study of men with recurrent prostate cancer ($n = 20$), oral administration of 200 $\mu\text{mol/day}$ of SFN-enriched broccoli sprout for 20 weeks resulted in <50% decline in serum prostate-specific antigen (PSA) levels in 35% of the patients [Alumkal et al., 2015]. Because of promising preclinical and clinical findings [Singh et al., 2009; Alumkal et al., 2015] it is important to continue to probe into the molecular mechanisms underlying cancer chemoprevention by SFN.

Recent developments in our understanding of prostate cancer biology suggest that eradication of the prostate cancer stem-like cell (pCSC) population might be necessary for prevention and treatment of this disease [Jaworska et al., 2015]. Previous studies have shown inhibition of pCSC self-renewal following SFN treatment [Kallifatidis et al., 2011; Labsch et al., 2014], but the underlying mechanism is unclear. The present study provides experimental evidence for c-Myc downregulation in SFN-mediated suppression of pCSC self-renewal. The overall rationale for the study stemmed from prior observations showing depletion of pCSC population by c-Myc silencing [Goodyear et al., 2009; Civenni et al., 2013]. Moreover, downregulation of c-Myc following SFN treatment has been documented in colon cancer cells [Kaminski et al., 2010; Zeng et al., 2011].

MATERIALS AND METHODS

ETHICS STATEMENT

Archived paraffin-embedded adenocarcinoma specimens from our previously published study in TRAMP mice were used to determine the *in vivo* effect of SFN administration on c-Myc protein levels [Vyas et al., 2013]. The use and care of mice were in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee guidelines.

REAGENTS

F-12K medium, fetal bovine serum and penicillin/streptomycin antibiotic mixture were purchased from Invitrogen-Life Technologies (now part of Thermo Fisher Scientific, Waltham, MA). RPMI 1640 medium was purchased from Cellgro-Mediatech (now part of Corning, Manassas, VA) whereas Dulbecco's Modified Eagle's Medium (DMEM) was from

Corning. SFN and its naturally-occurring analogs, including iberiverin, erucin, berteroin, iberin, alyssin, cheirolin, erysolin, and alyssin sulfone were purchased from LKT Laboratories (St. Paul, MN). Dimethyl sulfoxide (DMSO), cycloheximide, and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (St. Louis, MO). Stock solution of each compound was stored at -20°C and diluted in culture media before use. An antibody directed against total c-Myc was purchased from Cell Signaling Technology (Danvers, MA); anti phospho-c-Myc [T58] antibody was purchased from Santa Cruz Biotechnology (Dallas, TX); human-specific anti-phospho-c-Myc [S62] antibody was purchased from Abnova (Taoyuan City, Taiwan); and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from GeneTex (Irvine, CA). Chemiluminescence reagent was purchased from Perkin-Elmer (Waltham, MA). Plasmid for c-Myc was purchased from Addgene (Cambridge, MA). PE-Rat anti-human CD49f antibody (clone GoH3) was purchased from BD Biosciences (San Jose, CA). Kits for RNA isolation, RT² First Strand for cDNA synthesis, and RT² Profiler PCR array (human cancer stem cells) were purchased from Qiagen (Valencia, CA).

CELL LINES

LNCaP and PC-3 human prostate cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA) and cultured according to the supplier's instructions. The C4-2 cell line was obtained from UroCor (Oklahoma City, OK). The Myc-CaP cell line derived from prostate adenocarcinoma of a Hi-Myc transgenic mouse [Watson et al., 2005] was a kind gift from Dr Charles L. Sawyers (Memorial Sloan Kettering Cancer Center, New York, NY). The Myc-CaP cells were cultured in DMEM supplemented with 4.5 g/L glucose, L-glutamine, sodium pyruvate, 10% fetal bovine serum, and 1% penicillin/streptomycin antibiotic mixture. Each cell line was maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . The LNCaP, C4-2, and PC-3 cell lines used for the experiments described herein were authenticated in 2011–2012 and found to be of human origin and without any inter-species cross-contamination. Stocks of these cells were authenticated again in February 2015.

WESTERN BLOTTING

After treatment of desired cell lines with DMSO (solvent control), SFN or its analogs, cell lysates were prepared as described by us previously [Xiao et al., 2003]. Immunoreactive bands were detected with the use of enhanced chemiluminescence reagent. To determine the effect of SFN on c-Myc protein stability, PC-3 cells were treated with DMSO or 20 μM of SFN for 24 hours, and then incubated with 10 $\mu\text{g}/\text{mL}$ cycloheximide for 0, 30, 60, 90, and 120 minutes. After collection, the cells were subjected to immunoblot analysis for c-Myc protein.

IMMUNOFLUORESCENCE MICROSCOPY

For microscopic examination of c-Myc protein level by immunocytochemistry, LNCaP, PC-3 or Myc-CaP cells were cultured on coverslips in 12-well plates and processed for staining of c-Myc as described by us previously for other proteins [Vyas and Singh, 2014]. Images were captured at 100 $\times$ objective magnification.

QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (qRT-PCR)

Total RNA from DMSO-treated control cells, as well as SFN-treated cells, was isolated using RNeasy kit (Qiagen). First-strand cDNA was synthesized using Superscript Reverse Transcriptase (Invitrogen-Life Technologies) with oligo(dT)₂₀ primer. Primers for *c-Myc* were as follows: forward primer 5'-GCCACGTCTCCACACATCAG-3'; reverse primer 5'-TGGTGCATTTTCGGTTGTTG-3'. The quantitative PCR was done using 2× SYBR Green master mix (Applied Biosystems; now part of Thermo Fisher Scientific) with 15-second denaturation at 95°C, 60-second annealing at 60°C, and 30-second extension at 72°C for 40 cycles. The primers and amplification conditions for qRT-PCR for cancer stemness-related genes were as follows: *CD24* forward: CACGCAGATTTATTCCAGTGAAAC and *CD24* reverse: GACCACGAAGAGACTGGCTGTT (60°C annealing, 40 cycles); *DLL4* forward: AGGACCTCAAGGGTGACGAC and *DLL4* reverse: GAAGTTGAACAGCCCGAGTC (60°C annealing, 40 cycles); and *ITGA6* forward: ACGGTGTTTCCCTCAAAGAC and *ITGA6* reverse: GAAGAAGCCACACTTCCACA (55°C annealing, 40 cycles). Relative gene expression was calculated by the method described by Livak and Schmittgen [2001].

CELL VIABILITY AND APOPTOSIS ASSAYS

The effect of SFN and its analogs on cell viability was determined by trypan blue dye exclusion assay, essentially as described by us previously [Xiao et al., 2004]. The pro-apoptotic effect of SFN was determined by DAPI assay [Xiao et al., 2004]. Briefly, cells were treated with DMSO or SFN for 8 hours and fixed with paraformaldehyde. After washing with phosphate-buffered saline (PBS), cells were stained with DAPI (50 ng/mL) for 7 minutes. Nuclear condensation and fragmentation were examined under a fluorescence microscope at 100× objective magnification.

STABLE TRANSFECTION

PC-3 cells were stably transfected with empty pcDNA3.1 vector, or the same vector encoding c-Myc (6 µg of plasmid DNA), using Fugene6 transfection reagent (Promega, Madison, WI). The c-Myc overexpressing clones were selected by culture in medium supplemented with 800 µg/mL of G418 for > 2 months. Overexpression of c-Myc in stably transfected PC-3 cells was confirmed by immunoblotting.

COLONY FORMATION ASSAY

Four hundred cells were seeded in 6-well plates and allowed to attach during overnight incubation. Cells were then treated with DMSO or SFN. Drug-containing media were changed every third day, and the cultures were maintained for 10 days. The cells were rinsed with PBS, fixed with 100% methanol for 5 minutes, and stained with a 0.5% crystal violet solution in 20% methanol for 30 minutes at room temperature. The cells were rinsed with water and air-dried. Colonies of more than 50 cells were counted.

MEASUREMENT OF EXTRACELLULAR ACIDIFICATION RATE (ECAR)

The ECAR (a measure of glycolysis) after 24-hour treatment of empty vector-transfected cells and c-Myc-overexpressing PC-3 cells with DMSO or 10 µM SFN was measured in real

time using a Seahorse XF24 Extracellular Flux Analyzer essentially as described by us previously [Hahm et al., 2011]. The cell seeding density was 4×10^4 cells/well.

DETERMINATION OF ALDEHYDE DEHYDROGENASE 1 (ALDH1) ACTIVITY AND CD49f+ FRACTION

ALDH1 activity was determined using the ALDEFLUOR™ kit from STEMCELL Technologies (Vancouver, BC, Canada) as described by us previously [Kim et al., 2013]. Briefly, PC-3 cells were suspended in assay buffer containing ALDH1 substrate (BODIPY™-aminoacetaldehyde; BAAA) and incubated at 37°C for 30 minutes. As a control, half of the sample was transferred to a tube containing the ALDH1 inhibitor diethylaminobenzaldehyde (DEAB). Cells were re-suspended in assay buffer, and mixed with 1 µg/mL of propidium iodide. BAAA fluorescence was measured using a flow cytometer. For flow cytometric analysis of CD49f+ fraction, cells were treated with DMSO or SFN, and trypsinized. The cells were washed twice with PBS, and treated with 20 µL of PE-conjugated anti-CD49f antibody (BD Biosciences). Cells were incubated in the dark for 30 minutes at room temperature followed by washing with PBS. The CD49f-positive cells were analyzed using BD Accuri™ C6 flow cytometer.

PROSTATE SPHERE ASSAY

Cells were seeded in ultra-low attachment plates (Corning) at a density of 800–1000 cells/well in serum-free stem cell medium (DMEM:F12-Ham) containing 1% penicillin/streptomycin, B27 (1:50, Invitrogen-Life Technologies), 5 µg/mL insulin, 20 ng/mL epidermal growth factor (R&D Systems), 20 ng/mL basic fibroblast growth factor (STEMCELL Technologies) and 1% methyl cellulose (R&D Systems). SFN, at indicated concentrations, was added to the media on the day of cell seeding for the primary sphere assay. The primary spheres were removed from the plates, washed with PBS, gently pipetted, and then passed through a strainer to obtain single cells. Single cells from the primary spheres were then re-seeded in ultra-low attachment plates for the second generation sphere formation assay, without further treatment with SFN or DMSO.

RT² PROFILER PCR ARRAY

PC-3 cells stably transfected with empty vector (PC-3/V) or c-Myc plasmid (PC-3/Myc) were treated with 2.5 µM SFN or DMSO for 24 hours. Total RNA was extracted using the RNeasy kit, followed by reverse transcription with RT² First Strand kit. PCR array was performed according to the supplier's instructions (Qiagen). A panel of genes was selected, according to degree of statistical significance of the differential expression, for verification of microarray results.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry for total c-Myc was performed as described previously for other proteins [Powolny et al., 2011]. Briefly, antigen retrieval was performed using citrate buffer solution (pH 6). Staining was performed using an Autostainer Plus (Dako-Agilent Technologies, Carpinteria, CA). The c-Myc antibody (Santa Cruz Biotechnology, Dallas,

TX) dilution was 1:50. c-Myc protein expression was quantified using Aperio ImageScope software (Leica Biosystems), with the results expressed as H-score.

RESULTS

EFFECT OF SFN TREATMENT ON c-Myc PROTEIN LEVEL

We selected an androgen-sensitive cell line (LNCaP), an androgen-independent variant of LNCaP cells (C4-2), and an androgen-insensitive human prostate cancer cell line (PC-3) to determine the effect of SFN treatment on c-Myc protein level by western blotting (Fig. 1A). Level of total c-Myc protein was decreased upon SFN exposure in each cell line, albeit with different kinetics. For example, c-Myc protein suppression following SFN exposure was maximal at the 24-hour point in C4-2 cells, whereas the effect was transient in PC-3 cells, with maximum suppression detected at 16 hours (Fig. 1A). Similar to human prostate cancer cells, SFN treatment resulted in a marked decrease in c-Myc protein level in the Myc-CaP cell line derived from prostate adenocarcinoma of a Hi-Myc transgenic mouse (Fig. 1A). SFN-mediated suppression of c-Myc protein level in LNCaP, PC-3, and Myc-CaP cells was confirmed by immunocytochemistry (Fig. 1B). c-Myc protein was detected in both cytoplasmic and nuclear compartments in LNCaP cells whereas this protein was primarily localized in the nucleus of PC-3 and Myc-CaP cells (Fig. 1B). Nevertheless, a decrease in cytoplasmic and/or nuclear c-Myc protein level was clearly visible in each cell line after 24-hour treatment with SFN (Fig. 1B).

Phosphorylation at T58 and S62 is known to regulate c-Myc stability [Sears, 2004]. The effect of SFN treatment on levels of phospho-c-Myc was either inconsistent or cell line-specific (Fig. 1C). For example, the level of T58-phosphorylated c-Myc was decreased after treatment with SFN at both doses and at each time point in LNCaP cells (Fig. 1C). In contrast, SFN-treated PC-3 cells exhibited an increase in T58-phosphorylated c-Myc especially at the 20 μ M dose (Fig. 1C). The Myc-CaP cells showed a transient increase, followed by a decline in phospho-T58-c-Myc levels post-SFN exposure (Fig. 1C). S62-phosphorylated c-Myc was transiently affected by SFN treatment and this effect was inconsistent in replicate experiments. Collectively, these results indicate that a change in protein stability may not fully explain c-Myc protein suppression by SFN exposure. Consistent with this notion, stability of c-Myc protein was not affected by SFN treatment at least in the PC-3 cell line (Fig. 2A). On the other hand, SFN treatment caused a marked decrease in *c-Myc* mRNA levels in LNCaP, C4-2 and Myc-CaP cells (Fig. 2B), as revealed by qRT-PCR. Thus, a decrease in c-Myc protein level after SFN treatment is likely due to its transcriptional repression.

We determined cell viability (trypan blue dye exclusion assay) and apoptosis (DAPI assay) after 8-hour treatment of cells with SFN to rule out the possibility that c-Myc protein suppression was a reflection of reduced cell number and/or increased death. Cell viability inhibition following 8-hour exposure to SFN was minimal in PC-3, LNCaP, and C4-2 cells (Fig. 2C). Apoptosis induction after 8-hour treatment with SFN was highly variable in each cell line, but statistically not significant when compared to the solvent control (Fig. 2D). Based on these results, we conclude that c-Myc downregulation by SFN is not attributable to reduced viability/apoptosis at early time points. However, the possibility that c-Myc

downregulation after 24-hour treatment with SFN is partly due to reduced cell viability/apoptosis induction cannot be ruled out.

EFFECT OF NATURALLY-OCCURRING ANALOGS OF SFN ON c-Myc PROTEIN LEVEL

SFN analogs that differ in alkyl chain length (propyl, butyl, pentyl) and/or oxidation state of the sulfur (sulfinyl, sulfonyl) occur naturally in different plants [Fahey et al., 2001]. It was of interest to determine whether c-Myc protein suppression was optimal with the 4-methylsulfinyl side chain found in SFN. Initially, we determined the effect of SFN analogs (chemical formulae shown in Fig. 3A) on Myc-CaP cell viability by trypanblue dye exclusion assay and the results are shown in Fig. 3B. Forty eight-hour exposure to each test compound resulted in a dose-dependent decrease in Myc-CaP cell viability (Fig. 3B). However, erysolin and alyssin sulfone were relatively more effective than SFN in inhibiting Myc-CaP cell viability (Fig. 3B). Each analog was effective in reducing c-Myc protein level (Fig. 3C).

EFFECT OF SFN TREATMENT AND/OR c-Myc OVEREXPRESSION ON ECAR

c-Myc is the primary regulator of glycolysis in tumor cells, as many components of the glycolytic machinery are its transcriptional targets [Dang et al., 2009]. We used the Seahorse XF24 Flux Analyzer to determine whether ECAR (a measure of glycolysis) is affected by SFN treatment and/or c-Myc overexpression in PC-3 cells. Overexpression of c-Myc in stably transfected PC-3/Myc cells was confirmed by western blotting (Fig. 3D, *inset*). Mean basal ECAR was slightly higher in PC-3/Myc cells relative to empty vector-transfected (PC-3/V) control cells, but the difference was not statistically significant (Fig. 3D). Similarly, SFN treatment had no impact on basal ECAR in PC-3/V and PC-3/Myc cells (Fig. 3D). These results indicate that SFN treatment does not affect glycolysis in the PC-3 cell line. On the other hand, c-Myc overexpression partially counteracted inhibition of colony formation by low concentrations of SFN (Fig. 3E).

EFFECT OF SFN TREATMENT ON pCSC PHENOTYPE

A role for Myc in self-renewal and maintenance of pCSC has been suggested previously [Goodyear et al., 2009; Civenni et al., 2013]. For example, Myc is highly expressed in prostate cancer cells with pCSC phenotype (prostate spheres from PC-3 cells) [Civenni et al., 2013]. Therefore, it was of interest to determine whether the pCSC fraction is affected by SFN treatment and/or c-Myc overexpression. Initially, un-transfected PC-3 cells and Myc-CaP cells were used to optimize the inhibitory effect of SFN on pCSC markers, including ALDH1 activity and the CD49f+ fraction. Flow histograms of ALDH1 activity, in the absence or presence of its inhibitor DEAB, are shown in Fig. 4A. Quantitation of ALDH1 activity showed dose-dependent inhibition in PC-3 and Myc-CaP cells upon treatment with SFN (Fig. 4A). SFN-mediated inhibition of pCSC was confirmed by analysis of the CD49f+ fraction (Fig. 4B) and prostate sphere formation assay (Fig. 4C). Notably, SFN treatment resulted in a dose-dependent and statistically significant decrease in 2nd-generation spheres, even without further drug treatment of the 1st-generation spheres (Fig. 4C). Compared to PC-3, the Myc-CaP cell line showed an increased fraction of pCSC markers, as observed ~10% enrichment in ALDH1 activity. The drug concentration needed

for SFN-mediated inhibition of ALDH1 activity and sphere formation requires is markedly lower than that needed to induce apoptosis [Singh et al., 2004].

EFFECT OF c-Myc OVEREXPRESSION ON SFN-MEDIATED INHIBITION OF pCSC

As can be seen in Fig. 5A, c-Myc overexpression resulted in a marked increase in ALDH1 activity. ALDH1 activity was significantly decreased after SFN treatment of both PC-3/V and PC-3/Myc cells (Fig. 5B). In addition, c-Myc overexpression partially counteracted ALDH1 activity inhibition by SFN (Fig. 5B). The results for the CD49f+ (Fig. 5C) and prostate sphere analyses (Fig. 5D) were generally consistent, and revealed that c-Myc overexpression leads to: (a) an increase in the pCSC fraction, and (b) partial reduction in against SFN-mediated pCSC inhibition. For example, 2nd-generation prostate spheres were not detected with the 5 μ M dose in PC-3/V cells (Fig. 5D). On the other hand, a small number of 2nd-generation prostate spheres were seen with the same SFN dose in PC-3/Myc cells (Fig. 5D). These results indicate that c-Myc protein suppression likely contributed, at least in part, to inhibition of pCSC-like traits by SFN.

EFFECT OF SFN TREATMENT AND/OR c-Myc OVEREXPRESSION ON CANCER STEM CELL-RELATED GENES

To probe the molecular regulators that are downstream of c-Myc in SFN-mediated inhibition of pCSC, we compared expression of genes involved in cancer stem cell maintenance in PC-3/V and PC-3/Myc cells after 24-hour treatment with DMSO (solvent control) or SFN by qRT-PCR. The Venn diagram in Fig. 6A shows overlap of genes altered by c-Myc overexpression and/or SFN treatment relative to DMSO-treated PC-3/V cells (PC-3/V_DMSO). Table I lists genes for which expression was altered by c-Myc overexpression and/or SFN treatment. The magnitude of the gene expression change is shown by cluster analysis (Fig. 6B). A set of genes was identified using the cutoff of $P < 0.05$ (2-sided Student's *t* test) and fold-regulation change of ≥ 1.5 . The genes upregulated by c-Myc overexpression, at $P < 0.05$, included *CD24*, *DLL4*, *FOXA2*, *GSK3B*, *ITGA6*, *JAK2*, *TWIST2* and *ZEB2* (Fig. 6C). SFN treatment of PC-3/V cells (PC-3/V_SF) resulted in statistically significant downregulation of *CD24* (** $P < 0.06$), *ITGA6* (* $P < 0.05$), and *ZEB2* (* $P < 0.05$). Interestingly, SFN treatment was also able to reduce c-Myc-induced expression of some of these genes (Fig. 6C). Gene expression changes for *CD24*, *DLL4*, and *ITGA6* were confirmed by qRT-PCR, after 24-hour treatment of PC-3/V and PC-3/Myc cells with DMSO (control) and 2.5 or 5 μ M SFN (Fig. 7A). These results indicate that the mechanism of SFN-mediated inhibition of pCSC, downstream of c-Myc, likely involves multiple regulators.

EFFECT OF SFN ADMINISTRATION ON c-Myc PROTEIN LEVEL IN TRAMP ADENOCARCINOMA

We performed immunohistochemistry to determine whether SFN administration to TRAMP mice affects c-Myc protein expression in prostate adenocarcinoma. Fig. 7B depicts immunohistochemical images for c-Myc protein expression in TRAMP tumor sections. Although the expression of c-Myc protein in the tumor sections was not uniform, the mean H-score in non-necrotic adenocarcinoma from SFN-treated mice was about 43% lower in comparison with control. This difference was not significant, due to the large variability and

small sample size (Fig. 7C). Nevertheless, these results showed a trend for a decrease in c-Myc protein level in the tumors after SFN administration in TRAMP mice. Because a very mild regimen with 3 times/week SFN administration was employed in this study [Vyas et al., 2013], it is possible that a more intense schedule (e.g. daily treatment) is required to detect a significant decrease in c-Myc protein level *in vivo*.

DISCUSSION

Literature data suggest that c-Myc, which plays an important role in cellular metabolism and proliferation [Dang et al., 2009; Koh et al., 2010], is a valid target for prevention and treatment of prostate cancer. For example, overexpression of Myc has been shown to induce prostate intraepithelial neoplasia in association with Nkx3.1 loss in mouse luminal epithelial cells [Iwata et al., 2010]. The *c-Myc* oncogene is amplified in metastatic prostate cancer, and nuclear Myc overexpression seems to be an early event in prostate carcinogenesis [Jenkins et al., 1997; Gurel et al., 2008; Koh et al., 2010]. Moreover, molecular similarities exist in Myc-driven murine prostate cancer and the human disease [Zhang et al., 2000; Ellwood-Yen et al. 2003]. The present study reveals that c-Myc protein level is markedly decreased following SFN treatment in both androgen-sensitive (LNCaP) and androgen-independent (PC-3 and C4-2) human prostate cancer cells. In PC-3 cells, SFN-mediated downregulation of c-Myc appears to be transient, whereas in LNCaP and C4-2 cells, the effect is sustained for at least 24-hours post-treatment. Because Myc-CaP cells are also sensitive to growth inhibition and c-Myc downregulation by SFN, it is reasonable to postulate that c-Myc-driven prostate cancer is inhibited by this class of dietary bioactive compounds. Further work is needed to systematically explore this possibility.

The present study reveals that expression of *c-Myc* mRNA is decreased after treatment with SFN in LNCaP, C4-2, and Myc-CaP cells. Although the *c-Myc* promoter contains binding sites for several transcription factors, promoter deletion analysis has revealed a critical role for AP-1 and E2F1 in its transcriptional regulation [Elliott et al., 2001; Vartanian et al., 2011]. Because SFN treatment has been shown to activate both AP-1 and E2F1 in prostate cancer cells [Xu et al., 2006; Choi et al., 2007], their involvement in *c-Myc* mRNA suppression by SFN is highly unlikely. The possibility that SFN targets certain microRNA (e.g. let-7a) [Sampson et al., 2007] to suppress *c-Myc* expression cannot be excluded. Additional experimentation is necessary to explore this possibility.

SFN-mediated downregulation of c-Myc protein has been observed previously in human colon cancer cell lines [Kaminski et al., 2010; Zeng et al., 2011], but the functional relevance of this effect was not thoroughly studied. Because many glycolysis mediators are transcriptional targets of *c-Myc* [Dang et al., 2009], we suspected a decrease in glycolysis in SFN-treated cells. However, at least at a 10- μ M dose, basal ECAR is not affected in PC-3 cells after 24-hour SFN treatment. Additionally, c-Myc overexpression does not significantly increase ECAR in the PC-3 cell line. Consistent with literature data [Goodyear et al., 2009; Civenni et al., 2013], however, we found enrichment of the pCSC fraction when c-Myc is overexpressed, as evidenced by increases in ALDH1 activity, the CD49f+ fraction, and sphere forming efficiency. SFN-mediated inhibition of pCSC has been demonstrated previously [Kallifatidis et al., 2011; Labsch et al., 2014], but the mechanism for this

pharmacological effect was not explored. The present study reveals that c-Myc downregulation may be partly responsible for inhibition of pCSC by SFN. It also shows that c-Myc overexpression partially counteracts SFN-mediated inhibition of the pCSC phenotype and colony formation efficiency. Consistent with this observation, SFN reduces induction of cancer stemness-related genes in c-Myc-overexpressing PC-3/Myc cells. Furthermore, some of these genes are downregulated when PC-3/V cells, which do not overexpress c-Myc, are exposed to SFN.

It is interesting to note that the SFN-treated PC-3/V cells exhibited a trend toward decreased *TWIST2* expression (not statistically significant) when compared to the DMSO-treated control group. On the other hand, ~1000-fold increase in *TWIST2* expression was detected DMSO-treated PC-3/Myc cells, which overexpress c-Myc, when compared to DMSO-treated PC-3/V cells. Twist2 has been shown to contribute to breast cancer progression by promoting self-renewal of cancer stem-like cells [Fang et al., 2011], and more recently, to promote self-renewal of liver cancer stem-like cells by regulating CD24 [Liu et al., 2014]. Unlike certain other molecules (e.g., *CD24*, *DLL4*, *FOXA2*, *GSK3B*, and *JAK2*), however, *TWIST2* overexpression in PC-3/Myc cells is not reversed by SFN treatment. Our interpretation of these results is that while c-Myc overexpression undoubtedly increases *TWIST2* expression, it is not responsible for the inhibition of pCSC-like traits by SFN treatment.

We have previously shown that SFN treatment induces apoptotic cell death in prostate cancer cells [Singh et al., 2004; Singh et al., 2005]. SFN administration to TRAMP mice also resulted in an increased number of apoptotic bodies in prostate adenocarcinoma, but the difference from control was not found to be statistically significant [Singh et al., 2009]. In cellular systems, SFN-induced apoptosis is mediated by both intrinsic (mitochondria-mediated) and extrinsic (death receptor-mediated) caspase pathways [Singh et al., 2005]. It is interesting to note that overexpression of c-Myc sensitizes cancer cells to apoptosis induction by different stimuli [Hoffman and Liebermann, 2008]. The mechanism by which c-Myc overexpression influences apoptosis is quite complex, and involves both intrinsic and extrinsic caspase pathways. It would be interesting, in future, to determine whether the proapoptotic effect of SFN is affected by c-Myc overexpression.

In conclusion, the present study reveals that c-Myc protein is susceptible to downregulation by SFN treatment in both androgen-responsive and androgen-independent human prostate cancer cells. The primary underlying mechanism of SFN-mediated downregulation of c-Myc appears to be its transcriptional repression. The present study also demonstrates that SFN has the ability to eliminate the pCSC fraction, at least in part, by down regulating c-Myc.

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Fig. 1.

SFN treatment suppresses c-Myc protein level in prostate cancer cells. **A:** Western blotting for total c-Myc protein using lysates from prostate cancer cells after treatment with DMSO (solvent control) or SFN. Number above band indicates fold change in c-Myc protein level relative to DMSO-treated control after normalization for loading control (GAPDH). **B:** Immunocytochemistry for c-Myc protein after 24-hour treatment with DMSO (control) or 20 μM SFN (objective magnification 100×). **C:** Western blotting for phosphorylated c-Myc (T58 and S62) using lysates from prostate cancer cells after treatment with DMSO (control) or SFN. Each experiment was repeated 2–3 times and the results were generally consistent, except for large variability in levels of phosphorylated c-Myc in the C4-2 cells and total c-Myc in PC-3 cells.

Fig. 2.

SFN treatment decreases *c-Myc* mRNA level in prostate cancer cells. **A:** Effect of SFN treatment on c-Myc protein stability. The results shown are mean $\pm$ SD of 3 independent experiments. Statistical significance was determined by Student's *t* test. **B:** qRT-PCR for *c-Myc* mRNA expression after 24-hour treatment of cells with DMSO (control) or specified concentrations of SFN. Experiment was performed twice in triplicate and representative data from one experiment are shown as mean $\pm$ SD ($n = 3$). *Significantly different ($P < 0.05$) compared to DMSO-treated control by unpaired Student's *t* test. **C:** Viability of prostate cancer cells after 8-hour exposure to DMSO (solvent control) or the indicated concentrations of SFN. **D:** Apoptosis induction in prostate cancer cells after 8-hour treatment with DMSO or the indicated concentrations of SFN. For data in panels C and D, each experiment was performed twice in triplicate and combined data are shown as mean $\pm$ SD ($n = 6$).

*Significantly different ($P < 0.05$) compared to DMSO-treated control by one-way ANOVA with Dunnett's adjustment.

Fig. 3.

Naturally-occurring analogs of SFN decrease cell viability and c-Myc protein level in Myc-CaP cells. **A:** Chemical formulae of SFN and its analogs used in the present study. **B:** Effect of SFN analogs (48-hour treatment) on viability of Myc-CaP cells (trypan blue dye exclusion assay). Combined results from two independent experiments are shown as mean $\pm$ SD ($n = 6$). *Significantly different ($P < 0.05$) compared to DMSO-treated control by one-way ANOVA with Dunnett's adjustment. **C:** Western blots showing effect of SFN analogs (24-hour treatment) on c-Myc protein level in Myc-CaP cells. **D:** Basal ECAR in PC-3/V and PC-3/Myc cells after 24-hour treatment with DMSO (control) or 10 μM SFN. Combined results from two independent experiments are shown as mean $\pm$ SD ($n = 4$). Overexpression of c-Myc in PC-3/Myc cells was confirmed by western blotting as depicted in the *inset*. **E:** Colony formation in PC-3/V and PC-3/Myc cells after exposure to DMSO or SFN. Experiment was repeated twice and data from one experiment are shown. Data in each bar graph represent mean $\pm$ SD ($n = 3$). Statistical significance ($P < 0.05$) was tested by one-way ANOVA followed by Newman-Keuls multiple comparison test. *Significant compared to DMSO control for each cell type, and **significant ($P < 0.05$) between PC-3/V and PC-3/Myc cells.

Fig. 4.

SFN treatment inhibits self-renewal of pCSC. **A:** Representative flow histograms showing ALDH1 activity (BAAA fluorescence) and its quantitation (bar graph) in PC-3 cells or Myc-CaP cells after 72-hour (for PC-3) or 24-hour (for Myc-CaP) treatment with DMSO (control) or SFN. DEAB was used as a control (ALDH1 inhibitor). **B:** Quantitation of the CD49f+ population in PC-3 and Myc-CaP cells following 24-hour treatment with DMSO (control) or SFN. **C:** Representative images of 1st-generation prostate spheres after 2 days of treatment with DMSO (control) or SFN treatment (100 $\times$ magnification, scale bar = 100 μ m) and their quantitation (bar graphs). The 2nd-generation spheres were scored from single cells obtained from the 1st-generation spheres without further treatment (DMSO-control or SFN) 6 days after cell seeding. Each experiment was done at least twice, and the results from one such experiment are shown. The results shown are mean $\pm$ SD ($n = 3$). *Significantly different ($P < 0.05$) compared to control by one-way ANOVA with Dunnett's adjustment.

Fig. 5.

c-Myc overexpression partly attenuates SFN-mediated inhibition of pCSC. **A:** Representative flow histograms showing ALDH1 activity in PC-3/V and PC-3/Myc cells after 24-hour treatment with DMSO (control) or SFN. **B:** Quantitation of ALDH1 activity. **C:** The CD49f+ fraction in PC-3/V and PC-3/Myc cells after 24-hour treatment with DMSO (control) or SFN. **D:** Representative images of prostate spheres and their quantitation in PC-3/V and PC-3/Myc cells after 6 (1st-generation) or 12 days (2nd-generation) of DMSO (control) or SFN treatment (100 $\times$ magnification, scale bar = 100 μ m). Results in each bar graph represent mean $\pm$ SD ($n = 3$). Statistical significance of difference was tested by one-way ANOVA followed by Newman-Keuls multiple comparison test. *Significant ($P < 0.05$) compared to corresponding DMSO-treated control for each cell type. **Significant ($P < 0.05$) between PC-3/V and PC-3/Myc cells.

Fig. 6. Effect of SFN treatment and/or c-Myc overexpression on expression of cancer stem cell-related genes in PC-3/V and PC-3/Myc cells. **A:** Venn diagram showing overlap of gene expression modulation in different groups. **B:** Cluster diagram indicating changes in gene expression. **C:** Bar graph showing fold regulation of designated genes compared to that in the PC-3/V_DMSO control group. Significant compared to control (* $P < 0.05$; ** $P < 0.06$ by Student's t test). #Significant ($P < 0.05$) difference between the indicated groups (PC-3/Myc_DMSO vs. PC-3/Myc_SF_N).

Fig. 7.

c-Myc overexpression increases mRNA levels of cancer stem cell-related genes. **A:** qRT-PCR of *CD24*, *DLL4*, and *ITGA6* mRNA after 24-hour treatment with DMSO (control) or the specified concentrations of SFN. The results shown are mean $\pm$ SD ($n = 3$).

*Significantly different ($P < 0.05$) compared to corresponding DMSO-treated control or ** between PC-3/V and PC-3/Myc by one-way ANOVA with Newman-Keuls multiple comparison test. **B:** Immunohistochemical images for c-Myc protein expression in representative sections of prostate adenocarcinoma from a control and SFN-treated TRAMP mouse (40 $\times$ objective magnification, scale bar = 50 μ m). **C:** Quantitation of c-Myc protein staining in TRAMP tumors. The results shown are mean $\pm$ SD ($n = 5$). Statistical analysis was performed using Student's *t*-test.

Table I

Modulation of gene expression by SFN treatment (24 h) in PC-3/V and PC-3/Myc cells

Gene	PC-3/V_DMSO vs. PC-3/V_SFN		PC-3/V_DMSO vs. PC-3/Myc_DMSO		PC-3/Myc_DMSO vs. PC-3/Myc_SFN		PC-3/V_SFN vs. PC-3/Myc_SFN	
	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>	Fold
<i>Cancer Stem Cell Markers</i>								
<i>ABCB5</i>	0.44	1.30	0.74	0.92	0.50	1.20	0.71	0.85
<i>ALCAM</i>	0.53	1.07	0.004	1.51	0.96	0.99	0.003	1.40
<i>ALDH1A1</i>	0.35	0.36	0.35	0.27	0.56	1.07	0.56	0.81
<i>ATXN1</i>	0.76	1.06	0.89	0.98	0.50	0.86	0.31	0.80
<i>BMI1</i>	0.06	0.84	0.03	0.87	0.80	0.98	0.94	1.01
<i>CD24</i>	0.05	0.20	<0.001	3.60	<0.05	0.68	<0.001	12.00
<i>CD34</i>	0.35	4.50	0.34	1.70	0.76	1.79	0.35	0.68
<i>CD38</i>	0.35	24.15	0.97	1.13	0.21	1.29	0.35	0.06
<i>CD44</i>	0.62	1.07	<0.05	0.75	0.92	1.00	0.02	0.70
<i>ENG</i>	0.01	0.77	<0.05	0.76	0.07	0.73	0.03	0.72
<i>ETFA</i>	0.96	0.99	<0.001	0.71	0.56	1.06	0.08	0.76
<i>FLOT2</i>	0.49	0.94	0.24	0.89	<0.05	0.85	0.03	0.81
<i>GATA3</i>	0.08	0.70	0.34	0.82	0.52	0.80	0.98	0.93
<i>ITGA2</i>	1.00	1.03	0.06	1.48	0.40	0.87	0.13	1.25
<i>ITGA4</i>	0.37	0.62	0.83	10.79	0.38	0.66	0.03	11.51
<i>ITGA6</i>	0.01	0.87	<0.001	1.75	0.12	0.82	0.002	1.64
<i>ITGB1</i>	0.85	1.02	0.93	1.01	0.12	0.94	0.10	0.93
<i>KIT</i>	0.58	1.00	0.52	0.86	0.41	1.68	0.61	1.43
<i>MS4A1</i>	0.33	0.50	0.34	0.40	0.29	2.13	0.28	1.69
<i>MUC1</i>	0.03	0.63	0.15	0.77	0.35	0.78	0.95	0.95
<i>PECAM1</i>	0.90	0.90	0.55	1.10	0.70	0.87	0.77	1.06
<i>PROM1</i>	0.75	1.24	<0.001	8.08	0.01	0.48	0.08	3.11
<i>PTPRC</i>	0.96	0.71	0.26	0.55	0.36	1.72	0.72	1.34
<i>THY1</i>	0.45	0.96	0.39	0.88	0.24	1.41	0.31	1.30
<i>Proliferation</i>								
<i>EGF</i>	0.77	1.04	0.50	0.94	0.38	1.08	0.79	0.98
<i>ERBB2</i>	0.75	1.03	0.03	1.37	0.05	0.75	0.97	0.99
<i>KITLG</i>	0.66	1.05	<0.001	2.13	0.37	0.89	0.003	1.80
<i>LIN28B</i>	0.91	1.34	0.05	6.07	0.54	0.84	0.027	3.78
<i>NOS2</i>	0.41	1.18	0.15	1.70	0.83	1.32	0.46	1.91
<i>Self-Renewal</i>								
<i>BMP7</i>	0.24	0.72	0.02	0.15	0.06	3.72	0.35	0.77
<i>DNMT1</i>	0.31	0.90	0.71	1.02	0.003	0.84	0.53	0.96
<i>FGFR2</i>	0.11	0.38	0.44	1.19	0.13	0.48	0.83	1.50
<i>Pluripotency</i>								
<i>KLF4</i>	0.93	0.99	<0.001	2.75	0.62	0.97	<0.001	2.70

Gene	PC-3/V_DMSO vs. PC-3/V_SFN		PC-3/V_DMSO vs. PC-3/Myc_DMSO		PC-3/Myc_DMSO vs. PC-3/Myc_SFN		PC-3/V_SFN vs. PC-3/Myc_SFN	
	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>	Fold
<i>LIN28A</i>	0.68	0.88	0.38	0.77	0.93	0.94	0.55	0.82
<i>MYC</i>	0.72	1.04	0.03	1.26	0.39	1.06	0.09	1.29
<i>NANOG</i>	0.92	1.77	0.72	1.87	0.29	0.86	0.70	0.90
<i>POU5F1</i>	0.44	0.81	0.84	1.14	0.69	0.93	0.20	1.31
<i>SOX2</i>	0.49	1.18	0.18	1.55	0.86	1.04	0.99	1.36
<i>Asymmetric Division</i>								
<i>FOXP1</i>	0.55	0.92	0.01	1.75	0.06	0.72	0.03	1.37
<i>HDAC1</i>	0.21	0.92	0.002	1.47	0.11	0.86	0.002	1.37
<i>MYCN</i>	0.39	1.44	0.49	1.22	0.82	1.49	0.71	1.27
<i>SIRT1</i>	0.43	0.94	0.004	1.39	0.01	0.85	<0.001	1.26
<i>WNT1</i>	0.45	1.40	0.16	3.10	0.74	0.56	0.79	1.23
<i>Migration & Metastasis</i>								
<i>AXL</i>	0.31	0.91	0.001	1.42	0.37	0.90	0.02	1.40
<i>ID1</i>	0.30	0.86	0.004	1.29	0.26	0.92	0.02	1.38
<i>IL8</i>	0.10	1.29	0.17	0.89	0.07	1.13	0.08	0.77
<i>KLF17</i>	0.50	1.14	0.18	0.75	0.12	0.74	0.007	0.49
<i>PLAT</i>	0.46	0.89	0.06	0.54	0.86	0.93	0.22	0.56
<i>PLAUR</i>	0.77	1.03	0.37	1.10	0.51	1.09	0.27	1.17
<i>SNAI1</i>	0.42	1.16	0.98	1.03	0.37	0.90	0.12	0.80
<i>TWIST1</i>	0.19	0.90	0.06	1.19	0.02	0.86	0.03	1.14
<i>TWIST2</i>	0.20	0.57	<0.001	784.17	0.47	1.22	<0.001	1689.23
<i>ZEB1</i>	0.64	0.95	0.14	1.27	0.11	0.90	0.04	1.20
<i>ZEB2</i>	0.003	0.77	<0.001	0.70	0.35	1.09	0.89	0.98
<i>Loss of Stemness</i>								
<i>DACH1</i>	0.38	0.34	0.41	2.13	0.14	0.46	0.98	2.82
<i>FOXA2</i>	0.26	0.76	<0.001	4.50	<0.001	0.67	<0.001	3.94
<i>Signal Transduction Pathways</i>								
<i>Hippo Signalling</i>								
<i>LATS1</i>	0.35	0.90	0.58	0.95	0.19	0.88	0.44	0.93
<i>MERTK</i>	0.47	0.94	0.30	1.08	0.21	0.84	0.87	0.97
<i>SAV1</i>	0.08	0.85	<0.001	1.93	0.22	0.89	<0.001	2.02
<i>TAZ</i>	0.53	0.93	0.39	1.08	0.13	0.89	0.75	1.04
<i>WWC1</i>	0.53	0.95	0.27	1.17	0.77	0.99	0.02	1.23
<i>YAP1</i>	0.14	0.91	0.007	1.51	0.13	0.83	0.003	1.37
<i>Signal Transduction Pathways</i>								
<i>Hedgehog Signalling</i>								
<i>PTCH1</i>	0.49	0.91	0.06	1.56	0.25	0.77	0.17	1.32
<i>SMO</i>	0.60	0.93	0.14	0.75	0.20	0.75	0.02	0.61
<i>Notch Signalling</i>								
<i>DIL1</i>	0.65	1.20	0.33	2.21	0.29	0.55	0.61	1.01

Gene	PC-3/V_DMSO vs. PC-3/V_SF		PC-3/V_DMSO vs. PC-3/Myc_DMSO vs. PC-3/Myc_SF		PC-3/V_SF vs. PC-3/Myc_SF		PC-3/V_SF vs. PC-3/Myc_SF	
	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>	Fold
<i>DLL4</i>	0.52	0.58	<0.001	27.91	<0.001	0.28	<0.001	13.26
<i>JAG1</i>	0.35	0.86	0.04	1.42	0.43	0.88	0.06	1.44
<i>MAML1</i>	0.82	1.03	0.10	1.22	0.09	0.83	0.87	0.99
<i>NOTCH1</i>	0.89	1.00	0.06	1.24	0.85	1.01	0.02	1.25
<i>NOTCH2</i>	0.66	1.13	0.43	0.13	0.44	7.31	0.37	0.87
<i>WNT Signalling</i>								
<i>DKK1</i>	0.22	0.80	0.10	0.81	0.08	0.79	0.20	0.80
<i>EPCAM</i>	0.17	0.81	<0.001	1.97	0.12	0.79	0.003	1.91
<i>FZD7</i>	0.01	0.83	<0.05	0.53	<0.05	0.80	<0.05	0.51
<i>WNT1</i>	0.45	1.40	0.16	3.10	0.74	0.56	0.79	1.23
<i>PI3K/AKT/mTOR Signalling</i>								
<i>ABCG2</i>	0.24	0.69	<0.001	0.13	0.11	2.11	0.06	0.40
<i>GSK3B</i>	0.44	0.91	<0.001	1.66	0.003	0.90	<0.001	1.65
<i>STAT/NFκB Signalling</i>								
<i>IKBKB</i>	0.16	0.85	0.54	0.95	0.001	0.75	0.04	0.84
<i>JAK2</i>	0.28	0.88	0.001	1.89	0.003	0.68	<0.001	1.47
<i>NFKB1</i>	0.46	0.94	0.01	1.28	0.03	0.85	0.04	1.15