Prevention and Epidemiology

Chemoprevention of Prostate Cancer by D,L-Sulforaphane Is Augmented by Pharmacological Inhibition of Autophagy

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Abstract

There is a preclinical evidence that the oral administration of D,L-sulforaphane (SFN) can decrease the incidence or burden of early-stage prostate cancer [prostatic intraepithelial neoplasia (PIN)] and well-differentiated cancer (WDC) but not late-stage poorly differentiated cancer (PDC). Because SFN treatment induces cytoprotective autophagy in cultured human prostate cancer cells, the present study tested the hypothesis that chemopreventive efficacy of SFN could be augmented by the pharmacologic inhibition of autophagy using chloroquine (CQ). Incidence of PIN characterized by prostate weight of more than 1 g was significantly lower in the SFN þ CQ group than in control (P = 0.004), CQ group (P = 0.026), or SFN group (P = 0.002 by Fisher exact test). Average size of the metastatic lymph node was lower by about 42% in the SFN þ CQ group than in control (P = 0.043 by Wilcoxon test). On the other hand, the SFN þ CQ combination was not superior to SFN alone with respect to inhibition of incidence or burden of microscopic PIN or WDC. SFN treatment caused in vivo autophagy as evidenced by transmission electron microscopy. Mechanistic studies showed that prevention of prostate cancer and metastasis by the SFN þ CQ combination was associated with decreased cell proliferation, increased apoptosis, alterations in protein levels of autophagy regulators Atg5 and phospho-mTOR, and suppression of biochemical features of epithelial–mesenchymal transition. Plasma proteomics identified protein expression signature that may serve as biomarker of SFN þ CQ exposure/response. This study offers a novel combination regimen for future clinical investigations for prevention of prostate cancer in humans. Cancer Res 73(19): 5985–95. ©2013 AACR.

Introduction

Cruciferous vegetable intake in the diet is inversely associated with the risk of different malignancies including cancer of the prostate (1, 2), which is a leading cause of cancer-related deaths among men in the United States (3). Phytochemicals capable of eliciting cancer protective effect have now been isolated from different edible cruciferous vegetables including broccoli, watercress, and garden cress (4, 5). Sulforaphane [1-isothiocyanato-4-(methylsulfonyl)-butane] is one such small molecule of interest for prevention of cancers (5). Sulforaphane (SFN) occurs naturally as an L-isomer, but its synthetic D,L-analogue has been studied extensively for cancer prevention properties (5–10). Naturally occurring or synthetic SFN has exhibited cancer chemopreventive effects in chemically induced as well as oncogene-driven rodent cancer models (6–10). Talalay and colleagues were the first to report SFN-mediated inhibition of 9,10-dimethyl-1,2-benzanthracene-induced mammary cancer development in rats (6). Chemopreventive efficacy of SFN was subsequently established in other chemically induced rodent cancer models, including tobacco carcinogen–induced lung cancer (7), benzo[a]pyrene-induced stomach cancer (8), and azoxymethane-induced colonic aberrant crypt foci (9). Previous work from our own laboratory has revealed that oral administration of 6 μmol SFN 3 times per week results in significant inhibition of early-stage prostate carcinogenesis in transgenic adenocarcinoma of mouse prostate (TRAMP) mice without any side effects (10). Specifically, the incidence of prostatic intraepithelial neoplasia (PIN) and well-differentiated prostate cancer (WDC) was about 23% to 28% lower in the prostate of SFN-treated TRAMP mice than in controls (10). However, the incidence of poorly differentiated prostate cancer (PDC) was not affected by the SFN regimen used in our study (10). In another study, feeding of TRAMP mice with 240 mg of broccoli sprout/day resulted in a significant decrease in prostate tumor growth (11). We were the first to show in vivo efficacy of SFN against PC-3 human prostate cancer xenografts in male athymic mice (12).

Insight into the mechanisms by which SFN likely prevents cancer development continues to expand, albeit mostly from...
cellular studies. Nevertheless, the mechanisms potentially contributing to SFN-mediated chemoprevention include cell-cycle arrest (13), apoptosis induction (14, 15), inhibition of angiogenesis and histone deacetylase (16, 17), and suppression of oncogenic signaling pathways (e.g., NF-κB, STAT3, and androgen receptor; refs. 18–20). SFN is not a targeted agent as is the case for many other dietary cancer chemopreventive agents (21).

Previously, we made an exciting observation that exposure of cultured human prostate cancer cells (LNCaP and PC-3) to SFN resulted in induction of autophagy (22), which is an evolutionary conserved physiologic process for bulk degradation of macromolecules including organelles and considered a valid cancer therapeutic target (23, 24). We also found that autophagy was a protective mechanism against apoptosis induction by SFN (22). However, the in vivo significance of these observations was unclear. The present study addresses this gap in our knowledge using autophagy inhibitor chloroquine (CQ) and the TRAMP mouse model of prostate cancer.

Materials and Methods
Reagents and antibodies
SFN was synthesized as described previously (7). The purity of SFN was 98% as determined by high-performance liquid chromatography. Stock solution of SFN was stored at −20°C. SFN was diluted with PBS immediately before use. CQ was purchased from Sigma-Aldrich. Antibodies against T-antigen, synaptophysin, and E-cadherin were from BD Biosciences; antibodies against microtubule-associated protein 1 light chain 3 (LC3), androgen receptor (AR), and vimentin were purchased from Santa Cruz Biotechnology; anti-Ki-67 antibody was purchased from Dako-Agilent Technologies; and antibodies against p62, cleaved caspase-3, Atg5, and phospho-S2448-mTOR were purchased from Cell Signaling Technologies. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was conducted using ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (EMD Millipore). A kit for quantitation of VEGF in tumor lysates was purchased from R&D Systems.

Randomization and treatment of mice
Use of mice and their care were in accordance with the University of Pittsburgh Institutional Animal Care and Use Committee guidelines. Male TRAMP [(C57BL/6xFVB) F1] mice were obtained by crossing TRAMP female in the C57BL/6 background with male FVB mice. Transgene verification was conducted as described by Greenberg and colleagues (25). After transgene verification, 4-week-old male TRAMP mice were placed on AIN-93G diet (Harlan Teklad) for 1 week before the onset of treatments, and the mice were maintained on this diet throughout the experiment. Initially, as the mice became available from our breeding program, a total of 128 mice were placed into one of the following groups: control (n = 32), CQ alone (n = 32), SFN alone (n = 35), and SFN + CQ combination (n = 29). As summarized in Supplementary Table S1, some mice from each group were removed from the study due to a variety of reasons, including weight loss, hind limb paralysis, seminal vesicle invasion, and tumors at sites other than prostate. Final number of mice available for evaluations was: control (n = 28), CQ alone (n = 25), SFN alone (n = 28), and SFN + CQ (n = 25). Majority of the evaluable mice in each group were treated for 18 weeks but a fraction of mice from control (25%), CQ alone (16%), SFN alone (21%), and SFN + CQ combination group (8%) were sacrificed after 15 to 17 weeks of treatment due to large tumor burden. Nevertheless, these mice were included in the analysis. The mice of the control group received 0.1 mL PBS by oral intubation as well as intraperitoneal injection 3 times per week. The CQ alone group of mice were treated with 1.2 mg CQ (in 0.1 mL PBS) by intraperitoneal injection and 0.1 mL PBS by oral intubation 3 times per week. The SFN group of mice received 1 mg SFN (in 0.1 mL PBS) by oral intubation and 0.1 mL PBS by intraperitoneal injection 3 times per week. The SFN + CQ group of mice received 1 mg SFN (in 0.1 mL PBS) by oral intubation and 1.2 mg CQ (in 0.1 mL PBS) by intraperitoneal injection 3 times per week. Treatments were given on Monday, Wednesday, and Friday of each week. Body weights of the mice were recorded once weekly beginning at 5 weeks of age. The animals were sacrificed by CO2 inhalation followed by cervical dislocation. Blood was collected for separation of plasma, which was stored at −20°C. The prostate tissues were harvested, weighed, fixed in 10% neutral-buffered formalin, and sectioned at 4 to 5 μm thickness.

Histopathologic evaluations
Wholomount hematoxylin and eosin (H&E)-stained sections of prostate tissues were digitized by scanning and each section was blindly scored for microscopic PIN and WDC incidence and burden (affected area). The area of the lesions (in 2 dimensions) was calculated on histologic slides using on-screen drawing tool of the WebScope viewing software from Aperio. The mean PIN and WDC area were calculated from sum of the area in an animal and the average of area over all the animals for each group. Pathologic grading was consistent with the criteria defined in our previous studies (10, 26).

Immunohistochemistry
Briefly, prostate sections were quenched with 3% hydrogen peroxide and blocked with normal serum. The sections were then probed with the desired primary antibody (anti-synaptophysin, anti-T-antigen, anti-Ki-67, anti-LC3, anti-AR), washed with Tris-buffered saline and incubated with secondary antibody. Characteristic brown color was developed by incubation with 3,3'-diaminobenzidine. The sections were counterstained with Mayer's hematoxylin (Sigma) and examined under a Leica microscope. The images were analyzed with the use of ImageScope software (Aperio). Synaptophysin expression was analyzed using the membrane algorithm, LC3 expression was analyzed using the positive pixel algorithm, and nuclear algorithm was used to quantify Ki-67, T-antigen, and AR-positive cells.
Transmission electron microscopy

Transmission electron microscopy using prostate tissue was conducted for visualization and quantitation of autophagic vacuoles. Transmission electron microscopy was conducted essentially as described by us previously (26). Briefly, prostate tissues were immersion fixed in 2.5% electron microscopy grade glutaraldehyde in PBS overnight at 4°C, washed in PBS, and then post-fixed in 1% aqueous osmium tetroxide containing 0.1% potassium ferricyanide for 1 hour. Following 3 PBS washes, samples were dehydrated through a graded series of 30% to 100% ethanol. Propylene oxide (100%) was then infiltrated in a 1:1 mixture of propylene oxide and Poly/Bed 812 epoxy resin (Epon; Polysciences) for 1 hour. After several changes of 100% resin over 24 hours, samples were embedded in molds, cured at 37°C overnight, followed by additional hardening at 65°C for 2 days. Ultrathin (60 nm) sections were collected on 200 mesh copper grids, stained with 2% uranyl acetate in 50% methanol for 10 minutes, and then stained in 1% lead citrate for 7 minutes. Sections were imaged using JEOL 1011 transmission electron microscope at 80 kV fitted with a side-mount digital camera.

TUNEL assay

Prostate sections were deparaffinized, rehydrated, and then used to visualize apoptotic bodies by TUNEL staining. TUNEL staining was conducted according to the supplier’s instructions. Four to five randomly selected, nonoverlapping, and nonnecrotic fields were imaged to score TUNEL-positive cells.

Western blotting

Tumor samples from each group were processed for Western blotting as previously described (10). Western blotting was done as described previously (26).

Determination of VEGF levels

The levels of VEGF in tumor lysates from different groups were measured using a commercially available kit according to the supplier’s instructions. The results were normalized to protein concentration.

Plasma proteomics by 2-dimensional gel electrophoresis and mass spectrometry

Plasma proteomics was conducted by Applied Biomics. Randomly selected plasma samples (without complete knowledge of the histopathologic data) from control mice (n = 3), SFN alone–treated mice (n = 3), and mice treated with the SFN + CQ combination (n = 4) were used for plasma proteomics analyses essentially as described by us previously (26). Results are expressed as a ratio of abundance of a desired protein in the SFN or SFN + CQ group to the control group. Cluster analysis for protein abundance changes was conducted using a public bioinformatics tool [The Database for Annotation, Visualization and Integrated Discovery (DAVID), http://david.abcc.ncifcrf.gov/home.jsp; ref. 27]. The software uses a novel algorithm to measure the relationships among the annotation terms based on the degrees of their co-association genes to group the similar, redundant, and heterogeneous annotation contents from the same or different resources into annotation groups.

Statistical analysis

The association of incidence and treatment was examined by Fisher exact test. Comparisons of lymph node metastasis or prostate size between the 4 treatment groups were conducted using Wilcoxon 2-sample test. Area of PIN and WDC was analyzed by one-way ANOVA with Bonferroni multiple comparison adjustment. Statistical significance of differences in mechanistic correlates (immunohistochemical analyses, TUNEL-positive apoptotic bodies, and number of autophagic vacuoles) was determined using one-way ANOVA followed by Bonferroni adjustment. Statistical analyses were conducted using SAS version 9.2 or GraphPad Prism version 4.03. Difference was considered significant at P < 0.05.

Results

Effects of SFN and/or CQ treatments on PDC

We have shown previously that SFN treatment induces cytoprotective autophagy in cultured human prostate cancer cells (PC-3 and LNCaP) regardless of the AR or p53 status (22). The main objective of the present study was to determine the in vivo significance of these observations. We hypothesized that the chemopreventive efficacy of SFN against prostate cancer might be augmented by pharmacologic inhibition of autophagy using CQ, which disrupts lysosome acidification (28). As shown in Fig. 1A, the initial and final body weights of the mice were not affected by treatments with SFN and/or CQ when compared with control. Wet weight of the prostate is an indicator of tumor burden in the TRAMP model. The mean prostate weight of the mice treated with SFN + CQ (1.43 g) was lower by about 45% compared with control group (P = 0.02; Fig. 1B). The mean prostate weight of the mice of SFN + CQ group was also lower than SFN (2.74 g) or CQ alone group (2.47 g), but the difference did not reach statistical significance (Fig. 1B). Microscopic examinations of the wholemount H&E-stained sections from the prostate of mice revealed that PDC was the predominant pathology if the mean prostate weight was more than 1 g. We therefore computed percentage of mice with prostate weight more than 1 g. As shown in Fig. 1C, the percentage of mice with prostate weight more than 1 g was significantly lower in the SFN + CQ group (12%) than in control (50%), CQ alone (44%), and SFN alone group (54%; Fig. 1C). Collectively, these results indicated inhibition of PDC upon co-treatment with SFN and CQ, which was not observed with SFN alone administration.

Effects of SFN and/or CQ treatments on lymph node metastasis

Figure 2A depicts metastatic lymph node in a representative mouse each of the control group and the SFN + CQ treatment group. Lymph node metastasis incidence in the SFN + CQ–treated mice was lower (12%) than that of the
control group (36%), CQ alone group (36%), or the SFN alone group (36%; Fig. 2B). The size of the metastatic lymph node in the mice of SFN + CQ group was significantly lower (42% lower) than in the control group (Fig. 2C). These results indicated that treatment with SFN + CQ combination was associated with inhibition of lymph node metastasis, which was not observed by treatment with SFN alone.

Analyses of PIN and WDC incidence/burden

Figure 3A shows pathology associated with low-grade PIN (LG PIN), high-grade PIN (HG PIN), and WDC in representative TRAMP mice of the control group. Microscopic analysis of PIN and WDC was conducted in prostate of mice with mean prostate weight less than 1 g because PDC is the predominant pathology in mice with mean prostate weight more than 1 g. Neither SFN alone nor SFN + CQ combination was able to suppress incidence (Fig. 3B) or burden (affected area; Fig. 3C) of LG PIN or HG PIN. On the other hand, consistent with our previous observations (10), SFN treatment alone was able to significantly reduce area (79% decrease compared with control, \( P < 0.01 \)) of WDC compared with control (Fig. 3C). Furthermore, the area of the WDC in mice treated with SFN + CQ combination was about 69% lower than that of control mice (\( P < 0.01; \) Fig. 3C). Collectively, these results indicated that the effect of SFN + CQ combination was not superior to SFN alone with respect to the effect on PIN or WDC incidence and burden.
Effects of SFN and/or CQ treatments on neuroendocrine tumors

A small fraction of cells in the TRAMP tumor exhibit neuroendocrine differentiation (29). Figure 4A depicts synaptophysin-positive cells (a marker for neuroendocrine cells) in a representative mouse of each group. The number of synaptophysin-positive cells was not affected by SFN and/or CQ treatments when compared with control (Fig. 4A, bar graph). SFN treatment caused in vivo autophagy

Transmission electron microscopy was conducted for visualization and quantitation of autophagic vacuoles in the prostate of control mice and those treated with SFN and/or CQ (Fig. 4B). CQ impairs autophagic protein degradation by increasing intralysosomal pH (28, 30, 31). CQ treatment leads to accumulation of ineffective autophagosomes, which was also observed in the present study (Fig. 4B). It is important to point out that distinction between effective and ineffective autophagosomes is not possible by electron microscopy. Nevertheless, the number of autophagic vacuoles was significantly higher in the prostate of SFN-treated mice compared with control (Fig. 4B). Autophagy induction by SFN administration in vivo was confirmed by immunohistochemical analysis of LC3, which is a critical protein in autophagic machinery (32). Expression of LC3 was relatively higher in the prostate of mice treated with SFN alone and SFN + CQ compared with control but the difference did not reach statistical significance (Fig. 4C). Nevertheless, these observations provided in vivo evidence for SFN-induced autophagy.

Analysis of Ki-67 and AR expression and TUNEL-positive apoptotic bodies

We raised the question of whether SFN-mediated prevention of prostate cancer in TRAMP model was partly due to suppression of the transgene. Expression of T-antigen was not affected by SFN and/or CQ treatments (Fig. 5A). While the expression of Ki-67 (a marker of cell proliferation) was significantly lower in the tumors of SFN alone and SFN + CQ group than in control or CQ alone group (Fig. 5B), the AR expression did not differ significantly between.
groups (Fig. 5C). The TUNEL-positive apoptotic bodies were less in the prostate of control mice or those treated with CQ (Fig. 5D). The number of TUNEL-positive apoptotic bodies was increased upon treatment with SFN compared with CQ. The SFN + CQ combination was even more effective in increasing the number of TUNEL-positive cells but the difference was insignificant due to large variability.

We proceeded to determine the expression of p62, which is another marker of autophagy, and caspase-3 cleavage, which is an indicator of apoptosis, using tumor supernatants from 3 to 4 mice of each group. Two of 4 samples showed decreased intensity for p62 expression in the SFN + CQ group when compared with control (Supplementary Fig. S1). Cleavage of caspase-3 was very low in tumors of control and CQ alone groups as expected but was increased in SFN alone and SFN + CQ groups attesting to apoptosis induction by these treatments (Supplementary Fig. S1).

**Analysis of E-cadherin, vimentin, Atg5, phospho-mTOR, and VEGF proteins**

Epithelial–mesenchymal transition (EMT), biochemically characterized by loss of adherens junction protein E-cadherin concomitant with induction of vimentin, is implicated as a significant contributor to cancer progression as well as metastatic spread (33, 34). The expression of E-cadherin protein was either undetectable or very low in the tumors of control, CQ alone, and SFN alone treatment groups. On the other hand, the expression of E-cadherin was clearly visible in some tumors of mice from the SFN + CQ treatment group (Fig. 6A). Because of large variability in E-cadherin expression and less than
sufficient power because of a profound effect of the SFN + CQ combination on prostate cancer development, statistical analysis was not possible (Fig. 6B). Nevertheless consistent with the E-cadherin protein expression data, the level of vimentin protein was lower in the tumors of SFN + CQ group in comparison with other groups (Fig. 6A). Thus, potential inhibition of EMT in the tumor by the SFN + CQ combination treatment likely contributes to its anti-metastatic effect. Likewise, there was a trend of an increase in protein levels of autophagy regulator Atg5 in the tumors of SFN + CQ–treated mice compared with control or SFN treatment groups (Fig. 6A and B). mTOR is another protein implicated in regulation of autophagy (35). Protein level of phospho-mTOR (active form) was relatively lower in the tumors of SFN + CQ–treated mice than in the tumors of control mice. Finally, SFN + CQ combination also resulted in a modest decrease in protein levels of angiogenic cytokine VEGF (36) in the tumor in comparison with those of control and CQ groups of mice (Fig. 6C). These observations provide mechanistic insights into the chemopreventive effect of the SFN + CQ combination.

Changes in plasma protein abundance upon treatment with SFN and SFN + CQ

The cutoff criteria for spot selection and protein identification were at least 1.3-fold difference (increase or decrease) in abundance of different proteins in the plasma of mice from SFN + CQ group compared with control and P ≤ 0.10. Some of the spots meeting these criteria were abundant proteins (e.g., hemoglobin, macroglobulin, etc.) even though depletion of abundant proteins was conducted before the 2-dimensional
gel electrophoresis. The abundant proteins are not included in Table 1, which summarizes plasma protein abundance changes in response to treatment with SFN alone or SFN + CQ combination in comparison with control. Cluster analysis of the proteomics results indicated significant enrichment of proteins associated with proteasome, protease inhibitor family, and protein–lipid complex (results not shown).

Discussion

Our initial observations of autophagy induction by SFN treatment in cultured prostate cancer cells (22, 37) have since been confirmed by other investigators in different types of cancer cells (38–41). Majority of these studies support our conclusions that autophagy is a cytoprotective mechanism (38–40). For example, pharmacologic inhibition of autophagy using 3-methyl adenine or bafilomycin A1 augments SFN-induced apoptotic cell death in cultured human colon and breast cancer cells (38, 40). However, the in vivo significance of these findings was unclear. The present study is the first published report to show that (i) SFN administration causes in vivo autophagy as evidenced by transmission electron microscopy and immunohistochemical analyses of LC3 expression and (ii) inhibition of autophagy by an agent (CQ) already under clinical investigation as a chemotherapy sensitizer (30) increases efficacy of SFN for prevention of prostate cancer reflected by significant inhibition of PDC by the SFN + CQ combination, which is not observed with SFN treatment alone. These observations merit investigation of the SFN + CQ combination for prevention of prostate cancer in a clinical setting.

We have shown previously that the incidence of metastatic nodules in the lung, but not the lymph node metastasis, is decreased upon treatment with SFN alone (10).
Table 1. Plasma proteins altered by SFN or SFN + CQ administration in TRAMP mice

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Master no.</th>
<th>SFN/control</th>
<th>SFN + CQ/control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine (or cysteine) peptidase inhibitor, clade A, member 3K [Mus musculus]</td>
<td>83</td>
<td>0.600</td>
<td>1.1</td>
</tr>
<tr>
<td>Hemopexin precursor [Mus musculus]</td>
<td>195</td>
<td>0.590</td>
<td>−1.1</td>
</tr>
<tr>
<td>Hemopexin, partial [Mus musculus]</td>
<td>201</td>
<td>0.620</td>
<td>−1.1</td>
</tr>
<tr>
<td>Transferrin [Mus musculus]</td>
<td>212</td>
<td>0.500</td>
<td>1.2</td>
</tr>
<tr>
<td>Transferrin</td>
<td>229</td>
<td>0.950</td>
<td>1.0</td>
</tr>
<tr>
<td>Kininogen-1 isoform 1 precursor [Mus musculus]</td>
<td>277</td>
<td>0.850</td>
<td>1.0</td>
</tr>
<tr>
<td>Alpha-1-acid glycoprotein 1 precursor [Mus musculus]</td>
<td>529</td>
<td>0.640</td>
<td>1.7</td>
</tr>
<tr>
<td>Apolipoprotein A-I precursor [Mus musculus]</td>
<td>561</td>
<td>0.760</td>
<td>1.0</td>
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<tr>
<td>Sulfated glycoprotein-2 isoform 2 [Mus musculus]</td>
<td>766</td>
<td>0.960</td>
<td>1.0</td>
</tr>
<tr>
<td>Proteasome subunit alpha type-1 [Mus musculus]</td>
<td>929</td>
<td>0.780</td>
<td>1.0</td>
</tr>
<tr>
<td>Transthyretin precursor [Mus musculus]</td>
<td>989</td>
<td>0.500</td>
<td>−1.2</td>
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<tr>
<td>Unnamed protein product [Mus musculus]</td>
<td>1074</td>
<td>0.760</td>
<td>1.1</td>
</tr>
<tr>
<td>Proteasome subunit alpha type-4 [Mus musculus]</td>
<td>1033</td>
<td>0.740</td>
<td>1.1</td>
</tr>
<tr>
<td>Spermatogenesis-associated protein 22 OS = Mus musculus</td>
<td>1070</td>
<td>0.350</td>
<td>−1.3</td>
</tr>
</tbody>
</table>

The SFN + CQ combination regimen is not superior to SFN alone with respect to inhibition of PIN or WDC. Consistent with our previous observations (10), SFN alone is able to modestly and significantly inhibit WDC incidence and burden, respectively. However, effect on WDC incidence is abolished when SFN is combined with CQ. It is possible that SFN + CQ combination is more effective in inducing apoptosis in fully transformed cells (PDC) than in preneoplastic cells (e.g., PIN). This speculation is consistent with our earlier observations that normal human prostate epithelial cells are resistant to SFN-induced apoptosis (15). Relatively greater abundance of TUNEL-positive apoptotic cells and increased cleavage of caspase-3 in the tumors from mice treated with the SFN + CQ combination compared with SFN alone treatment group (present study) provides added support to this contention.

We have shown previously that SFN treatment causes transcriptional repression of AR in human prostate cancer cells, leading to downregulation of AR-regulated gene product prostate-specific antigen (20). The present study reveals that level of AR protein in vivo is not decreased upon treatment with SFN alone or SFN + CQ combination. It is possible that a more intense dosing regimen of SFN administration (i.e., daily administration or higher dose) is required to observe suppression of AR protein expression in vivo. At the same time, the possibility that SFN-mediated suppression of AR signaling is an in vitro phenomenon cannot be discarded.

Successful implementation of a cancer chemopreventive strategy is contingent upon systematic investigations starting with cellular studies to identify promising agents and to define the mechanism(s) underlying their cancer protective effect to animal-based studies focusing not only on in vivo bioavailability, safety, and efficacy assessments but also on identification of biomarker(s) predictive of tissue exposure, and possibly response, before translation in humans with a pilot biomarker modulation trial followed by larger studies with cancer incidence as the primary end point.
Identification of suitable biomarker(s) is particularly essential for the clinical development of chemopreventive agents as cancer incidence is too rigorous of an end point for malignancies with long latency such as prostate cancer. Plasma proteomics conducted in the present study provides novel leads that can be followed up to identify biomarkers associated with SFN + CQ exposure/response. Abundance change of some of these proteins seems unique to the SFN + CQ combination. For example, abundance of alpha 1 acid glycoprotein, which is an acute inflammatory biomarker that increases in various conditions including malignancy (43), is decreased after SFN + CQ treatment (2.6-fold decrease compared with control) but its level increases in the plasma of SFN alone treated mice. We observed decreased abundance of kininogen-1 isofrom 1 precursor and transthyretin precursor in the plasma of SFN + CQ–treated mice compared with control. Abundance of these proteins is not altered by SFN administration alone (present study). Interestingly, transthyretin and high molecular weight kininogen were found to be significantly enhanced in the sera of patients with prostate cancer compared with those of benign prostatic hyperplasia (44).

We found increased abundance of sulfated glycoprotein-2 isoform 2 in the plasma of SFN + CQ–treated mice compared with control. This protein is also known as clusterin and expressed as 3 forms with different subcellular localization (45). Expression of clusterin was shown to be downregulated during prostate cancer progression in the TRAMP model (46). Mice with homozygous or heterozygous deletion of clusterin exhibited PIN or differentiated carcinoma (47). Crossing of Mice with homozygous or heterozygous deletion of clusterin (45). Expression of clusterin was shown to be downregulated (47). At the same time, some studies have suggested oncogenic function for clusterin (48). Nevertheless, SFN + CQ combination restores plasma level of isoform 2 of clusterin, and this effect is not as pronounced upon treatment with SFN alone.

Men with prostate cancer suffer significant impairments in quality of life not only from the disease itself but also as a consequence of the treatments. Because the commonly associated risk factors for prostate cancer, including age, race, and genetic predisposition are not easily modifiable, novel approaches for prevention of this disease are desirable. Moreover, a clinically viable preventive intervention against prostate cancer is still lacking (49, 50). For example, increased incidence of high-grade tumors in the treatment arm in chemoprevention trials with 5α-reductase inhibitors hindered their broad acceptance as a preventive strategy (49, 50). The results of the present study provide preclinical in vivo evidence for prostate cancer preventive efficacy of a novel and clinically viable combination regimen to warrant investigations in a clinical setting.

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

Authors' Contributions
Conception and design: D. Desai, S.V. Singh
Development of methodology: E.R. Hahm, D. Desai, S. Amin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.R. Vyas, E.R. Hahm, J.A. Arlotti, S. Watkins, D. Beer-Stolz
Analysis and interpretation of data (e.g., statistical analysis, bios-statistics, computational analysis): A.R. Vyas, E.R. Hahm, J.A. Arlotti, D. Beer-Stolz, S. Amin, S.V. Singh
Writing, review and/or revision of the manuscript: A.R. Vyas, E.R. Hahm, D. Desai, S. Amin, S.V. Singh
Study supervision: S.V. Singh

Grant Support
This work was supported by the grant ROI CA115498-07 awarded by the National Cancer Institute. This research used the Animal Facility and Tissue and Research Pathology Facility supported in part by a grant from the National Cancer Institute at the NIH (P30 CA047904).

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Received March 15, 2013; revised June 27, 2013; accepted July 14, 2013; published OnlineFirst August 6, 2013.

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Chemoprevention of Prostate Cancer by d,l-Sulforaphane Is Augmented by Pharmacological Inhibition of Autophagy

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