

Epigenetics Reactivation of Nrf2 in Prostate TRAMP C1 Cells by Curcumin Analogue FN1

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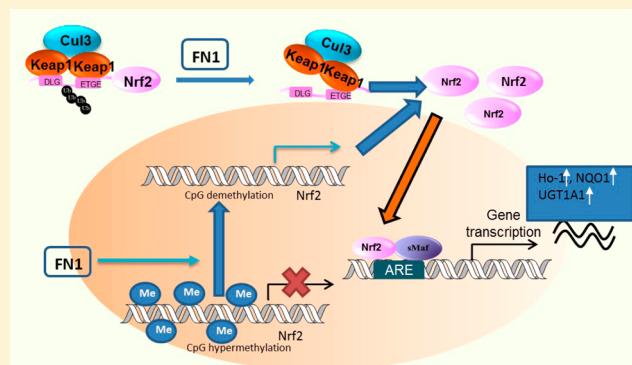
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ABSTRACT: It has previously been shown that curcumin can effectively inhibit prostate cancer proliferation and progression in TRAMP mice, potentially acting through the hypomethylation of the Nrf2 gene promoter and hence activation of the Nrf2 pathway to enhance cell antioxidant defense. FN1 is a synthetic curcumin analogue that shows stronger anticancer activity than curcumin in other reports. We aimed to explore the epigenetic modification of FN1 that restores Nrf2 expression in TRAMP-C1 cells. Stably transfected HepG2-C8 cells were used to investigate the effect of FN1 on the Nrf2- antioxidant response element (ARE) pathway. Real-time quantitative PCR and Western blotting were applied to study the influence of FN1 on endogenous Nrf2 and its downstream genes. Bisulfite genomic sequencing (BGS) and methylated DNA immunoprecipitation (MeDIP) were then performed to examine the methylation profile of the Nrf2 promoter. An anchorage-independent colony-formation analysis was conducted to examine the tumor inhibition activity of FN1. Epigenetic modification enzymes, including DNMTs and HDACs, were investigated by Western blotting. The luciferase reporter assay indicated that FN1 was more potent than curcumin in activating the Nrf2-ARE pathway. FN1 increased the expression of Nrf2 and its downstream detoxifying enzymes. FN1 significantly inhibited the colony formation of TRAMP-C1 cells. BGS and MeDIP assays revealed that FN1 treatment (250 nM for 3 days) reduced the percentage of CpG methylation of the Nrf2 promoter. FN1 also downregulated epigenetic modification enzymes. In conclusion, our results suggest that FN1 is a novel anticancer agent for prostate cancer. In the TRAMP-C1 cell line, FN1 can increase the level of Nrf2 and downstream genes via activating the Nrf2-ARE pathway and inhibit the colony formation potentially through the decreased expression of keap1 coupled with CpG demethylation of the Nrf2 promoter. This CpG demethylation effect may come from decreased epigenetic modification enzymes, such as DNMT1, DNMT3a, DNMT3b, and HDAC4.



INTRODUCTION

Nuclear factor erythroid-2 related factor 2 (Nrf2) is a key regulation factor of the phase II detoxifying enzymes that act as a defense system against oxidative stress. These enzymes include heme oxygenase-1 (HO-1), NAD[P]H/quinone oxidoreductase-1 (NQO1), superoxide dismutase (SOD), glutathione S-transferase (GST), and γ -glutamyl cysteine ligase (γ -GCL). These enzymes are mainly transcriptionally regulated by the antioxidant response element (ARE) and respond to the transcription factor Nrf2.³ Under normal conditions, Nrf2 is

bound to Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm and is degraded by the ubiquitin-proteasome pathway through the Keap1- and Cullin 3-based-E3/Rbx1 ligase complex. Under stress conditions or when activated by enhancers, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it binds to AREs of target protective genes and activates transcription to protect impairment from

Received: January 13, 2016

Published: March 18, 2016

oxidative stress, reactive carcinogenic metabolites, and carcinogenesis.^{4–6}

In the United States, prostate cancer (PCa) is the leading diagnosed noncutaneous male cancer subtype.⁷ Oxidative stress, which occurs when the reactive oxygen species (ROS) overwhelm the capacity of the antioxidant defense system, is one of the etiologic factors related to PCa. Epidemiological, experimental, and clinical studies have suggested an association between oxidative stress and risk of PCa development and progression.^{8–11} Excessive ROS induce DNA damage and mutation and cell and tissue damage, which could give rise to a variety of human pathogenesis, including cardiovascular, metabolic, inflammatory, and neurodegenerative diseases and cancer.^{12–14}

Epigenetic changes, mainly DNA methylation, histone modification, and microRNA regulation, are other hallmarks of PCa. DNA methylation is the most frequently studied of these changes.¹⁵ Among all of the related genes, hypermethylated Nrf2 has shown a close relationship to PCa carcinogenesis.^{16,17} We have previously reported that Nrf2 transcription is suppressed in the prostate tumors of transgenic adenocarcinoma mouse prostate (TRAMP) mice and tumorigenic TRAMP-C1 cells due to the hypermethylation of the first five CpGs in the Nrf2 promoter.^{16,17}

Accumulating evidence has shown that Nrf2 activation can defend against and prevent PCa carcinogenesis.^{18–20} Many dietary phytochemicals have beneficial effects and the ability to activate the Nrf2 signaling pathway. Isothiocyanates (cruciferous vegetables),²¹ organosulfur compounds (garlic and onions),²² polyphenols (green tea and spice turmeric),²³ and isoflavones (soy beans)²⁴ have been characterized as potent Nrf2 activators. These agents can stimulate various upstream kinases, interfere in the Keap1-Nrf2 interaction, and/or disturb cellular redox balance, all resulting in the activation of the Nrf2 pathway. Additionally, epigenetic modifications may contribute to the regulation of the transcription activity of Nrf2. Since DNA methylation is reversible by intervention with DNMT inhibitors, combined treatment with 5-azadeoxyctidine (5-aza) and Trichostatin A (TSA) can restore Nrf2 expression in TRAMP-C1 cells.¹⁷ In addition, a variety of bioactive nutrients (e.g., curcumin,²⁵ tocopherols,¹⁶ sulforaphane,^{26,27} and 3,3'-diindolylmethane (DIM))²⁸ modulate DNA methylation and/or histone modification, thereby effectively restoring Nrf2 expression.

In our previous study, curcumin showed a PCa chemopreventive effect through the epigenetic modification of the Nrf2 gene and the restoration of the Nrf2-mediated anti-oxidative stress cellular defense capability.²⁵ (3E,5E)-3,5-Bis(pyridin-2-methylene)-tetrahydrothiopyran-4-one (FN1) (Figure 1) is a newly synthesized curcumin analogue that is

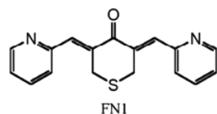


Figure 1. Chemical structure of (3E,5E)-3,5-bis(pyridin-2-methylene)-tetrahydrothiopyran-4-one (FN1).

substantially more potent than curcumin in inhibiting PCa cell growth.^{29,30} However, it is not quite clear by what route FN1 exerts chemopreventive function for prostate cancer. Here, we will examine FN1 in inhibiting proliferation and colony formation of TRAMP C1 cells, its effects in activating the Nrf2 pathway, and the underlining mechanisms.

MATERIALS AND METHODS

Reagents and Antibodies. Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/mL), versene, and trypsin-EDTA were supplied by Gibco (Grand Island, NY, USA). A Cell-Titer 96 Aqueous One Solution Cell Proliferation (MTS) Assay Kit was obtained from Promega (Madison, WI, USA). Platinum Taq DNA polymerase was purchased from Invitrogen (Grand Island, NY, USA). Tris-HCl precast gels, turbo transfer buffer, and PVDF membranes were obtained from Bio-Rad (Hercules, CA, USA). Tris-glycine-SDS running buffer and Super Signal enhanced chemiluminescent substrate were purchased from Boston BioProducts (Ashland, MA, USA) and Thermo (Rockford, IL, USA), respectively. Antibodies against Nrf2 (C-20), HO-1 (C-20), NQO1 (H-90), UGT1A1 (V-19), and actin (I-19) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The protease inhibitor cocktail, radioimmunoprecipitation (RIPA) buffer, and antibodies against HDACs (HDAC1, HDAC2, HDAC3, HDAC4, and HDAC6) were supplied by Cell Signaling Technology (Beverly, MA, USA). The anti-HDAC8 antibody was obtained from Proteintech Group (Chicago, IL, USA), and the anti-NQO1, -HDAC7, -DNMT3a, and -DNMT3b antibodies were from Abcam (Cambridge, MA, USA). Anti-DNMT1 was supplied by Novus Biologicals (Littleton, CO, USA). All other chemicals, unless otherwise noted, were obtained from Sigma (St. Louis, MO, USA).

Materials and Chemicals. Synthesized FN1 (purity >95%) was obtained from Kun Zhang's laboratory (Laboratory of Natural Medicinal Chemistry & Green Chemistry, Guangdong University of Technology, Guangzhou, China). Dimethyl sulfoxide (DMSO) (purity ≥99.7%), 5-aza (purity ≥97%), and TSA (purity ≥98%) were from Sigma (St. Louis, MO, USA).

Cell Culture. The human hepatocellular HepG2-C8 cell line was previously established by stable transfection with an ARE luciferase construct.³¹ The cells were cultured and maintained in DMEM supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. TRAMP-C1 cells were obtained from B. Foster (Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY, USA). Cells were cultured in DMEM (pH 7.0) containing 10% FBS at 37 °C in a humidified 5% CO₂ atmosphere, as described previously.¹⁷

Luciferase Reporter Activity Assay. The stably transfected HepG2-C8 cells expressing the ARE-luciferase vector were used to study the effects of FN1, curcumin, and sulforaphane (SFN) on the Nrf2-ARE pathway. The ARE-luciferase activity in the HepG2-C8 cells was determined using a luciferase assay kit in accordance with the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, HepG2-ARE-C8 cells (1.0×10^5 cells/well) were seeded in 12-well plates in 1 mL of medium containing 10% FBS, incubated for 24 h, and then treated with various concentrations of FN1, curcumin, and SFN samples. Afterward, the cells were lysed using the reporter lysis buffer, and 10 µL of the cell lysate supernatant was analyzed for luciferase activity using a Sirius luminometer (Berthold Detection System GmbH, Pforzheim, Germany). Normalization of the luciferase activity was performed based on protein concentrations, which were determined using a BCA protein assay (Pierce Biotech, Rockford, IL, USA). The data were obtained from three independent experiments and are expressed as the inducible fold change compared with the vehicle control.

MTS Assay. TRAMP-C1 cells were seeded in 96-well plates at a density of 1×10^3 cells per 100 µL of DMEM per well, incubated for 24 h, and treated with either 0.1% DMSO (control) or various concentrations of FN1 in DMEM containing 1% FBS for 1, 3, or 5 days. Series diluted FN1 samples were dissolved in DMSO (final concentration in the medium of <0.1%), and the medium was changed every 2 days. Cell viability was estimated with a CellTiter 96 Aqueous One Solution Cell Proliferation (MTS) assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR). TRAMP-C1 cells were seeded at a density of 1×10^5 cells in 10 cm dishes with 10% FBS/DMEM. After 24 h, the

cells were treated with DMEM medium containing 1% FBS with FN1 (50 nM, 100 nM, and 250 nM) or with 0.1% DMSO as a control. The treatment medium was changed every 2 days. After the 3-day treatment, the total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. mRNA expression levels were determined using first-strand cDNA as a template by quantitative real-time PCR (qPCR) with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) in an ABI7900HT system. The following primer sequences for Nrf2, HO-1, NQO1, and UGT1A1 were used: Nrf2, 5'-AGCAGGACTGGAG-AAGTT-3' (sense) and 5'-TTCTTTTCCAGCGAGGAGA-3' (antisense); HO-1, 5'-CCTCACTGGCAGGAAATCATC-3' (sense) and 5'-CCTCGTGGAGACGCTTACATA-3' (antisense); NQO1, 5'-AGCCCAGATATTGTGGCCG-3' (sense) and 5'-CTTTCAG-AATGGCTGCAC-3' (antisense); UGT1A1, 5'-GAAATTG-CTGAGGCTTGGCAGA-3' (sense) and 5'-ATGGGAG-CCAGAGTGTGATGAA-3' (antisense). β -Actin was used as an internal control with sense (5'-CGTTCAATACCCCCAGCCATG-3') and antisense (5'-ACCCCGTCACCAAGACTCC-3') primers.

Preparation of Protein Lysates and Western Blotting. TRAMP-C1 cells were seeded at a density of 1×10^5 cells in 10 cm dishes with 10% FBS/DMEM. After incubation for 24 h, the cells were treated with 0.1% DMSO as a control and FN1 (50 nM, 100 nM, and 250 nM) in DMEM containing 1% FBS. Following treatment for 3 days, the cells were washed with ice-cold PBS and harvested in ice-cold 1X RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing a protein inhibitor cocktail (Sigma). The protein concentrations of the cell lysates were measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Identical concentrations of protein (20 μ g) were subjected to 4 to 15% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA, USA) electrophoresis (SDS-PAGE) and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA and sequentially incubated with specific primary antibodies and HRP-conjugated secondary antibodies. The antibody-bound proteins were visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and measured with a Gel Documentation 2000 system (Bio-Rad).

Bisulfite Genomic Sequencing (BGS). TRAMP-C1 cells (1×10^5 cells per 10 cm dish) were treated with 0.1% DMSO as a control, FN1 (50 nM, 100 nM, and 250 nM), and a combination of 5-aza (500 nM) and TSA (100 nM) for 3 days. Genomic DNA was then extracted from the cells with a QIAamp DNA Mini kit (Qiagen, Valencia, CA, USA). Then, 500 ng of genomic DNA was subjected to bisulfite conversion with an EZ DNA Methylation-Gold Kit (Zymo Research Corp., Orange, CA, USA) according to the manufacturer's instructions. The converted DNA was amplified with Platinum Taq

DNA polymerase (Invitrogen, Grand Island, NY, USA) and primers that amplify the first five CpGs of the murine Nrf2 gene. The primer sequences were 5'-AGTTATGAAGTAGTAGTAAAAA-3' (sense) and 5'-AATATAATCTCATAAAACCCAC-3' (antisense). A TOPO TA Cloning kit (Invitrogen, Grand Island, NY, USA) was used to clone the PCR products into vector pCR4 TOPO. Plasmids containing PCR products from at least 10 colonies per treatment from three independent experiments were amplified and purified with a QIAprep Spin Miniprep kit (Qiagen), followed by sequencing (GeneWiz, South Plainfield, NJ, USA).

Methylated DNA Immunoprecipitation (MeDIP) Analysis. To verify the DNA methylation changes, methylated DNA was captured and quantified using methylated DNA immunoprecipitation coupled with quantitative real-time polymerase chain reaction analysis (MeDIP-qPCR), as described previously.²⁷ Briefly, the extracted DNA from treated cells was sonicated on ice using a Bioruptor sonicator (Diagenode Inc., Sparta, NJ, USA) to a fragment size of approximately 200–1000 base pairs. The fragmented DNA was denatured at 95 °C for 2 min. Methylated DNA was isolated by immunoprecipitation with anti-5'-methylcytosine antibody using a Methylamp Methylated DNA capture kit (Epigentek, Farmingdale, NY, USA) according to the manufacturer's manual. After final purification and elution, the methylation status was quantified by qPCR amplification of MeDIP-enriched DNA using the primer set 5'-GAGGTCACCACAAAC-ACGAAC-3' (forward) and 5'-ATCTCATAAGGCCACCTC-3' (reverse) to cover the DNA sequence of the first five CpGs of murine Nrf2. The enrichment of methylated DNA in each treatment was calculated according to the standard curve of the serial dilution of input DNA. The relative methylated DNA ratios were then calculated with the basis of the control as 100% of DNA methylation.

Anchorage-Independent Colony-Formation Assay. TRAMP-C1 cells (2×10^4 /ml) were suspended in 1 mL of basal medium Eagle (BME) containing 0.33% agar and plated over 3 mL of a solidified BME consisting of 0.5% agar and 10% FBS in 6-well plates in the presence of 50 nM, 100 nM, or 250 nM FN1. The cells were maintained at 37 °C in a 5% CO₂ incubator for 2 weeks. The cell colonies were imaged using a Nikon ACT-1 microscope (version 2.20; LEAD Technologies) and counted using ImageJ (version 1.48d; NIH, Bethesda, MD, USA).

Statistical Analyses. The data are presented as the mean \pm the standard deviation of three independent experiments. One-way analysis of variance (ANOVA) or unpaired Student's *t* test (SPSS 19.0, IBM, 2010, Chicago, IL, USA) were performed to identify significant differences between means (*p* < 0.05).

RESULTS

FN1 Induced ARE-Luciferase Reporter Activity. The relative fold changes of luciferase activity compared with HepG2-C8 cells which were stably transfected with the ARE-luciferase reporter vector are shown in Figure 2. FN1 induced a

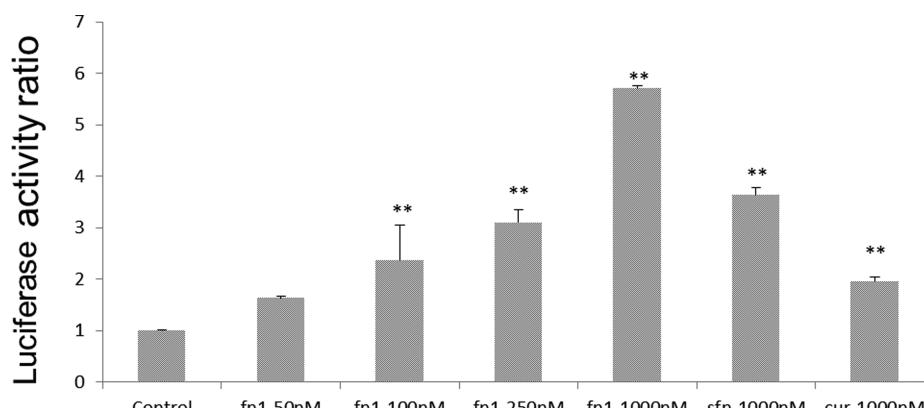


Figure 2. ARE-Luciferase activity assay of effects of FN1, curcumin, and SFN on the Hep-G2 C8 cell line. The BCA protein assay was determined to normalize the luciferase activity. The data obtained from three independent experiments expressed the inducible fold change compared with the vehicle control. *, *p* < 0.05; **, *p* < 0.01 comparing with control group.

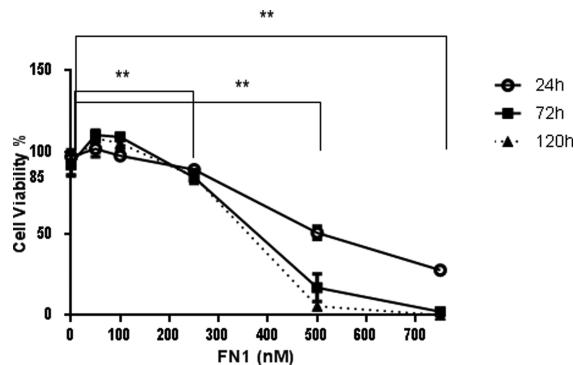


Figure 3. Cytotoxicity of FN1 against the TRAMP-C1 cell line. Cells were seeded in a 96-well plate for 24 h and then treated with various concentrations of FN1 for 1, 3, or 5 days. Cytotoxicity was determined by a MTS assay. The data are expressed as the means \pm SD ($n = 3$). **, $p < 0.01$ compared with the control group.

higher luciferase activity than the control in a dose-dependent manner from 50 to 250 nM which indicates that FN1 may possess the activation ability for those genes with an ARE sequence. When cells were treated with the same dosage (1000 nM), FN1 showed a greater effect than curcumin and SFN.

Cytotoxicity Measurement of FN1 by MTS. MTS measurement was performed to investigate the cytotoxicity of FN1 against TRAMP-C1 cells. Viability of TRAMP-C1 was found to be reduced by FN1 treatment in a time- and dose-dependent manner after 1, 3, and 5 days of treatment (Figure 3). The survival rate of TRAMP-C1 treated with FN1 at concentrations less than 250 nM were higher than 80%, which indicates a lower toxicity than the high-concentration groups. Hence, doses of 0, 50, 100, and 250 nM of FN1 were chosen for further epigenetic study on Nrf2 reactivation.

FN1 Enhanced Expression of Nrf2 and Its Downstream Antioxidant and Detoxifying Enzymes and Decreased Keap1 Expression. TRAMP is a well-studied prostate

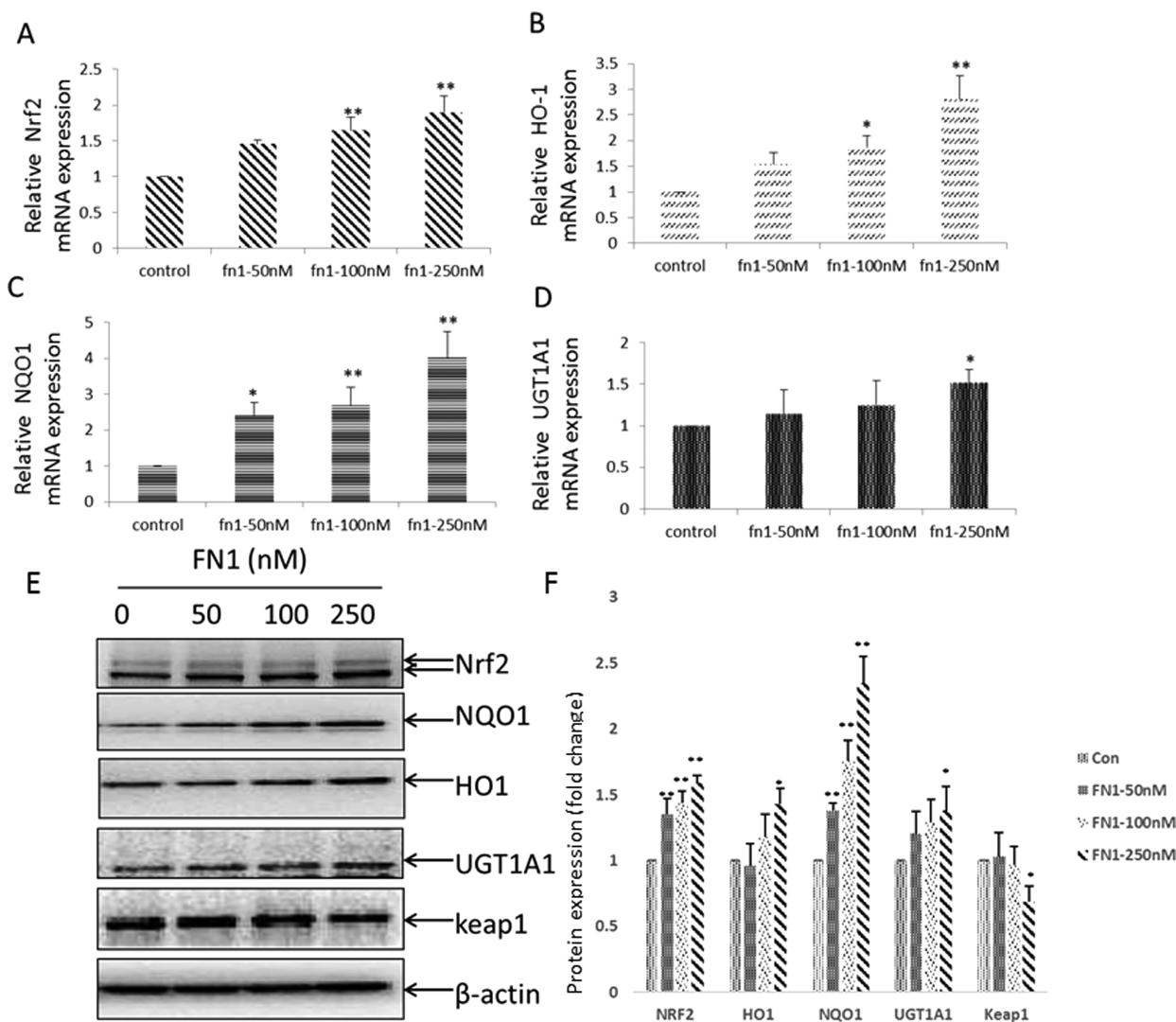


Figure 4. Effects of FN1 (50, 100, and 250 nM) on Nrf2 mRNA and the protein expression of Nrf2 target genes in TRAMP-C1 cells after the 3-day treatment were determined with real-time qPCR and Western blot. The graphical data are expressed as the means \pm SD from three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ comparing with control group. Relative endogenous mRNA expression of Nrf2 (A), HO-1 (B), NQO1 (C), and UGT1A1 (D) in TRAMP-C1 cells from three independent experiments after treatment by FN1 (50, 100, and 250 nM) for 3 days with β -actin as an internal control. (E) Effect of FN1 on the protein expression of Nrf2 target genes (HO-1, NQO1, and UGT1A1) and keap-1 in TRAMP C1 cells by FN1 (50, 100, and 250 nM) for 3 days. (F) The relative protein expression levels are quantified and compared based on the signal intensity of the corresponding bands from 3 independent experiments and normalized using β -actin for the total cellular protein level and are presented as the mean \pm SD.

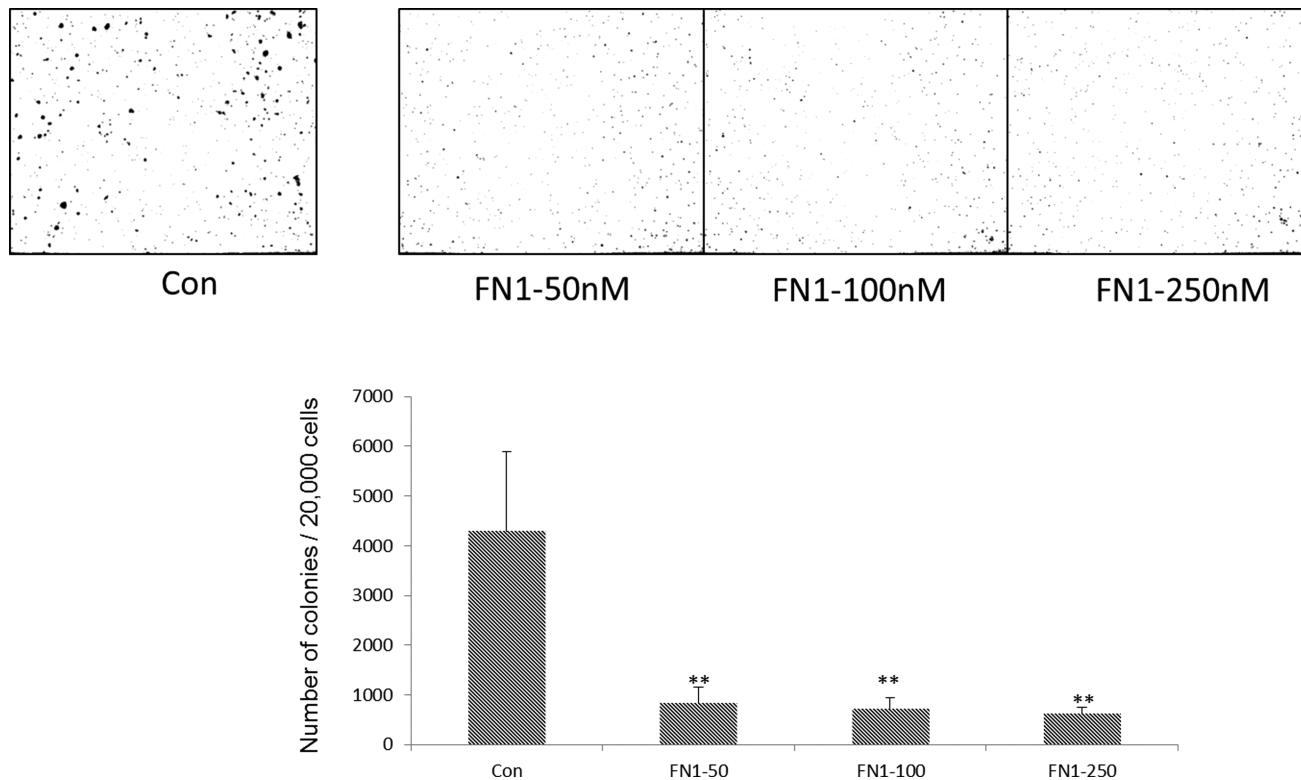


Figure 5. Inhibitory effect of FN1 on the anchorage-independent colony-formation of TRAMP-C1 cells. The colonies exhibiting anchorage-independent growth were counted under a microscope using ImageJ software. The graphical data are presented as the average of triplicate results from 3 independent experiments. **, $p < 0.01$ compared with the control group.

carcinogenesis model. In our previous experiments with TRAMP-C1, C3 cells, TRAMP prostate tumor samples, and wild type prostate samples, we have demonstrated that the highly methylated CpG region in Nrf2 promoters often comes with a decreased expression of Nrf2 gene and its protein and vice versa.¹⁷ Nrf2 is a vital regulating factor of antioxidant and detoxifying enzymes such as HO-1, NQO1, and UGT1A1.³² To test FN1 effects on Nrf2 and the downstream genes, we performed qPCR analysis to measure the mRNA level of Nrf2 and HO-1, NQO1, and UGT1A1 level in TRAMP-C1 cells after treating with FN1 for 3 days (Figure 4). As shown in Figure 4A, FN1 at 100 or 250 nM significantly upregulated the Nrf2 mRNA level ($p < 0.05$); FN1 at 100 or 250 nM significantly increased HO-1 mRNA expression (Figure 4B). FN1 at 50, 100, or 250 nM significantly increased NQO1 mRNA expression (Figure 4C) and FN1 at 250 nM significantly increased UGT1A1 mRNA compared with the controls (Figure 4D).

We then carried out Western blotting experiments to analyze their protein expression after the same treatment. FN1 (50–250 nM) enhanced the protein expression of Nrf2, and that enhancement correlates positively with the dose of FN1 (Figure 4E and F). Higher concentrations of FN1 also increased the protein expression of HO-1, NQO1, and UGT1A1. The above findings demonstrate that FN1 has the ability to enhance the expression of both mRNA and protein level of Nrf2 and that it mediated antioxidant and detoxifying genes in TRAMP-C1 cells. Figure 4E and F also indicate that the FN1 concentration has an inverse relationship with the expression of Keap1, which also may explain the activation of Nrf2.

FN1 Reduced Colony Formation in TRAMP-C1 cells. The anchorage-independent growth capacity of cells indicates their tumorigenicity.^{33,34} FN1 at concentrations of 50, 100, and

250 nM significantly suppressed the colony formation of TRAMP-C1 cells by 80.48%, 83.36%, and 85.66%, respectively (Figure 5). These results suggest that FN1 plays a vital role in suppressing anchorage-independent growth of TRAMP-C1 cells and has the potential for decreasing tumorigenicity.

FN1 Diminished the Methylation Ratio of the First Five CpG Regions in Nrf2 Promoter. It has been demonstrated that the hypermethylation ratio of the first five CpGs in the Nrf2 promoter region correlated inversely with expression of Nrf2.¹⁷ BGS assay was then used to determine if FN1 can induce hypomethylation to the CpG region. The high methylation rate (88.13%) of the promoter CpGs was found in the control group (3 day-treatment with 0.1% DMSO) (Figure 6A). Hypermethylation in the region was observed to reduce to 63.89%, 82.22%, and 73.55%, respectively, in 5-aza and the TSA group (3-day combination treatment of 5-aza (500 nM) and TSA (100 nM)), low dose FN1 group (100 nM for 3 days), and high dose FN1 group (3 days for 250 nM).

We proceeded with the eMeDIP-qPCR assay to verify the methylation profile from BGS. The first five CpGs of the Nrf2 promoter region from sonicated fragmented DNA after being captured was expanded by qPCR. It demonstrates that combination treatment of 5-aza (500 nM)-TSA (100 nM) or FN1 (250 μ M) induced the demethylation effect on the Nrf2 promoter region with statistical significance ($p < 0.01$) (Figure 6B). These results suggest that FN1 can induce hypomethylation in the Nrf2 promoter region, which may restore Nrf2 expression.

FN1 Downregulated Epigenetic Modification Enzymes. The effects of FN1 on epigenetic modification enzymes, including DNMTs and HDACs, were further examined to explore the epigenetic mechanism of FN1 in promoter demethylation and the induction of Nrf2 gene transcription. We found that FN1

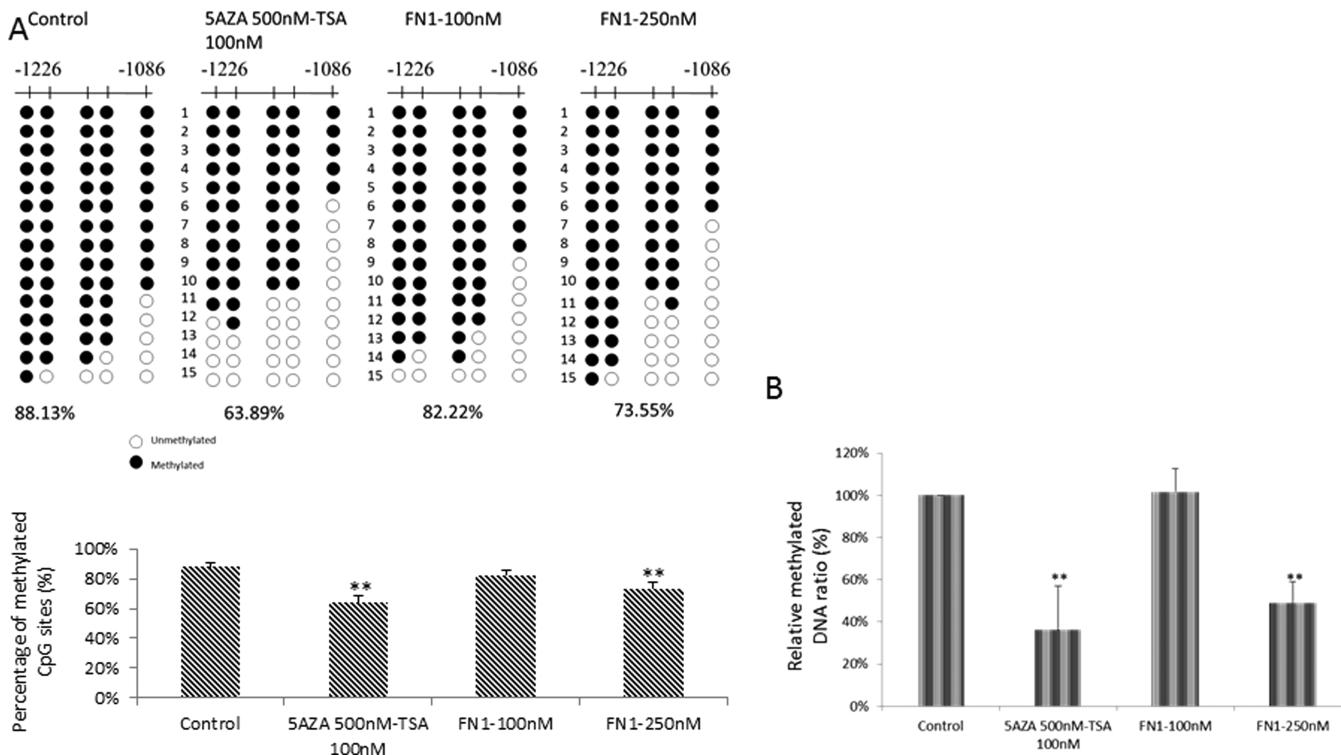


Figure 6. Methylation ratio of the first 5 CpGs of Nrf2 promoter region in TRAMP C1 cells after the 3-day treatment of FN1 (100 nM, 250 nM) and combination of 5-aza (500 nM) and TSA (100 nM) by BGS and MeDIP. The data are expressed as the means \pm SD of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with the control group. (A) BGS assay for the effect of FN1 on the methylation of the Nrf2 promoter regions in TRAMP C1 cells. Solid spots denote methylated CpGs, and hollow spots denote unmethylated CpGs. (B) MeDIP assay for FN1 on the methylation of Nrf2 Promoter regions in TRAMP-C1 cells. After sonication and denaturation, the genomic DNA was extracted and subjected to DNA immunoprecipitation. The Nrf2 gene with a methylated promoter in MeDIP-precipitated DNA was further analyzed by qPCR amplified by primers covering the first five CpGs in the Nrf2 promoter region. qPCR was performed to quantify the amount of MeDIP DNAs relative to their inputs. The relative methylated DNA ratio was evaluated in comparison with the control.

(50–250 nM) reduced the protein level of DNMT1, DNMT3a, and DNMT3b in TRAMP-C1 cells after 3 days of treatment (Figure 7A). In addition, FN1 has also reduced HDAC protein expression, especially HDAC4 ($p < 0.05$; Figure 7B).

■ DISCUSSION

PCa, a high-incidence and slow-progression disease, is typically diagnosed at the late stage of life. Hence, a modest delay in disease progression could have a significant impact on disease-related morbidity, mortality, and quality of life.³⁵ Natural phytochemicals, which have chemopreventive properties that delay the progress of carcinogenesis, have become as auspicious and practical approaches to deal with the increase of PCa.^{36–38} Curcumin, for example, has demonstrated its chemopreventive activity in preventing PCa.³⁷ FN1, a newly synthesized curcumin analogue, has shown anticarcinogenic effects against PC-3, Panc-1, and HT-29.²⁹ In our experiments, FN1 has shown to inhibit the proliferation of TRAMP-C1 cells, another tumorigenic prostate adenocarcinoma cell line. In addition, FN1 significantly inhibited the colony formation of TRAMP-C1 cells. Anchorage-independent colonies grown in soft agar indicate normal cell transformation or cancer cell tumorigenicity *in vitro*.^{34,39} In brief, FN1 has the ability to prevent prostate carcinogenesis *in vitro*.

Oxidative stress is believed to be mainly generated by the imbalance between ROS and cellular antioxidant defense capacity, which may be the cause of inflammation or PCa carcinogenesis.^{10,15,16} It has long been known that Nrf2 plays a vital role in defending cells against oxidative damage by regulating

antioxidant and detoxification enzymes, such as HO-1 (antioxidant), NQO1, and UGT1A1 (detoxification).^{32,40} Because of its protective properties, Nrf2 expression will normally be reduced in the initiation of carcinogenesis. In human PCa, Nrf2 levels were found to be extensively decreased though the analysis of 10 human PCa microarray data sets.⁴¹ Numerous dietary phytochemicals, such as curcumin,²⁵ indole-3-carbinol (I3C),⁴² tocopherols,¹⁶ and Z-ligustilide,⁴³ can inhibit prostate tumorigenesis by enhancing the expression of Nrf2 and its downstream phase II antioxidant and detoxification enzymes, HO-1, NQO1, and UGT1A1. In our experiments, we found that FN1, an analogue of curcumin, could enhance the expression of those genes transcriptionally and post-transcriptionally, which suggests that FN1 may also exert its chemoprevention effects via enhancing the Nrf2 pathway.

It has been widely accepted that epigenetic modifications are closely related to PCa initiation and progression.^{8,44–46} Hence, DNA methyltransferases (DNMTs) and histone deacetylases (HDACs), which induce DNA methylation and histone acetylation modification and cause gene expression silencing are becoming new targets for prostate cancer prevention and therapy.^{17,47–49} We previously reported that Nrf2 is epigenetically silenced by a high percentage of CpG methylation in the promoter region during TRAMP prostate carcinogenesis *in vivo* and *in vitro*.^{16,17,25} Many dietary compounds, such as apigenin, tanshinone IIA, and sulforaphane, were found to restore Nrf2 expression by epigenetic modification.^{27,50,51} Curcumin can cause DNA demethylation and histone modification to TRAMP-C1

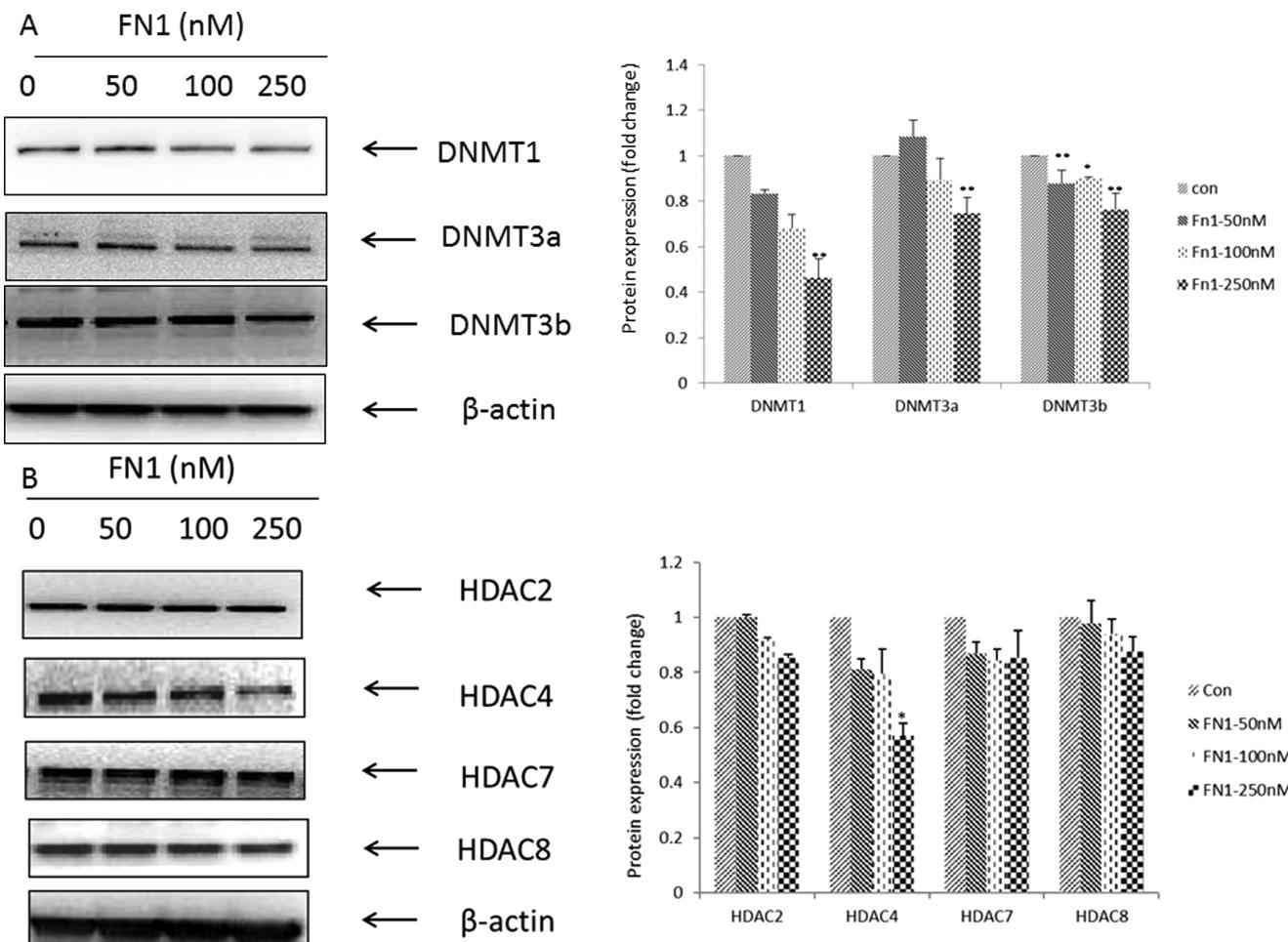


Figure 7. Effect of FN1 on DNMT protein expression (A) and protein expression of HDACs (B) in TRAMP-C1 cells. Cells ($1 \times 10^5/10 \text{ cm dish}$) were incubated with FN1 (50, 100, and 250 nM) for 3 days. The relative expression levels were quantified based on the signal intensity of the corresponding bands and normalized using β -actin. The graphical data are represented as the mean \pm SD from 3 independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with the control group.

cells and thus restore the silenced Nrf2 expression arising from promoter region hypermethylation.^{25,52} We were therefore interested in exploring whether the curcumin analogue FN1 can activate Nrf2 expression through the same route. In the BGS test, we found that FN1 treatment (250 nM) exhibited a demethylation effect on the first 5 CpGs in the Nrf2 promoter in TRAMP-C1 cells (Figure 6A). This demethylation effect was confirmed by the MeDIP assay, which indicated that the methylated DNA ratio of the same region was lower in TRAMP-C1 cells treated with FN1 (250 nM) than in control cells (Figure 6B). The protein levels of DNMT1, DNMT3a, and DNMT3b and HDACs in TRAMP-C1 cells treated with FN1 were compared with controls to reveal the underlying mechanism of Nrf2 demethylation. After FN1 treatment, we found that the protein levels of DNMT1, DNMT3a, and DNMT3b decreased significantly in a dose-dependent manner (Figure 7A). The protein levels of HDAC2, HDAC4, HDAC7, and HDAC8 were all reduced after FN1 treatment, although only the decrease of HDAC4 was statistically significantly different from the control.

In the modulation of the Nrf2 pathway, Keap1 can inactivate the function of Nrf2 by sequestering it in the cytoplasm and preventing it from entering the nucleus.⁵³ Keap1 also serves as a bridge between Nrf2 and ubiquitination ligase Cullin-3 to help induce Nrf2 degradation.³² ARE inducers can cause Nrf2

dissociation from Keap1, which helps Nrf2 to translocate into the nucleus and finally regulate the downstream antioxidant genes transcriptionally.⁵⁴ In our experiments, the protein expression of Keap1 was significantly reduced by treatment with 250 nM FN1 for 3 days (Figure 4E and F), which suggestss one of the other potential mechanisms of FN1 activation of the Nrf2 pathway.

Hyperactive proliferation and enhanced survival of cancer cells can be attributed to the elevated oxidative stress.⁵⁵ Oxidative stress and chronic inflammation, and chronic exposure to carcinogens and mutagens are crucial in the initiation of carcinogenesis.⁵⁶ Nrf2 pathway protects against oxidative stress and thus prevents carcinogenesis. In our previous *in vivo* study of treating TRAMP mice with broccoli sprouts, prostate tumorigenesis has been significantly inhibited via the activation of the Nrf2 pathway.⁵⁷

Anchorage-independent colony formation and growth are emblems of transformed cells.⁵⁸ 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced JB6 cell transformation and the soft agar anchorage-independent colony formation model is a well-studied skin carcinogenesis model. By comparing with Nrf2 knock-down cells and control cells, it indicates that Nrf2 plays a vital role in the TPA-induced JB6 cell transformation and that the epigenetic reactivation of the Nrf2 pathway could potentially contribute to the attenuation of JB6 cellular

anchorage-independent colony formation and inhibition of neoplastic transformation.^{26,51}

In addition, as we also found previously that prostate carcinogenesis in the TRAMP model *in vivo* and *in vitro* are highly correlated with suppressed expression of Nrf2, which mainly comes from epigenetically silence,^{16,17,25} it is very likely that hypermethylation in the first five CpG islands of Nrf2 is highly associated with the TRAMP cancer cell development and colony formation. Hence, the ability of FN1 in inhibiting the proliferation and colony formation of TRAMP C1 cells may probably be due to its capability in enhancing Nrf2 and its downstream antioxidant and detoxification enzymes by activation of the Nrf2-ARE pathway (Figures 2, 3, 4, and 5) through epigenetic reactivation of Nrf2, which includes DNA demethylation and histone modification effects (Figures 6 and 7) and inhibition of keap1 expression (Figure 4E and F). We will further investigate the above hypothesis with Nrf2-knockdown TRAMP cells in our future study.

In conclusion, our findings reveal that FN1, an analogue of curcumin, can inhibit growth and colony formation in TRAMP-C1 cells. FN1 can increase mRNA and protein level of Nrf2 and its downstream detoxifying and antioxidant enzymes, including HO-1, NQO1, and UGT1A1. Our results also indicate that FN1 can restore the silenced Nrf2 gene in TRAMP-C1 cells probably through demethylation of the Nrf2 promoter region and histone modifications. In addition, the keap1 level was reduced by FN1 treatment. The epigenetic regulation and inhibition of keap1 therefore may be the mechanisms of restoring Nrf2 and its downstream target genes. FN1 thus demonstrates its effectiveness and potential in inhibiting the initiation, progression, and development of PCa. FN1 is a novel cancer chemopreventive agent for the management of PCa. However, its *in vivo* efficacy and pharmacokinetics profile need further investigation.

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Funding

This work was supported in part by institutional funds and by R01AT007065 from the National Center for Complementary and Alternative Medicines (NCCAM) and the Office of Dietary Supplements (ODS).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We express our sincere gratitude to all of the members of Dr. Tony Kong's laboratory for their helpful discussions.

ABBREVIATIONS

Nrf2, nuclear factor erythroid-2 related factor 2; HO-1, heme oxygenase-1; NQO1, NAD[P] H:quinone oxidoreductase-1; SOD, superoxide dismutase; GST, glutathione S-transferase; γ -GCL, γ -glutamyl cysteine ligase; ARE, antioxidant response element; Keap-1, Kelch-like ECH-associated protein 1; PCa, prostate cancer; ROS, reactive oxygen species; DIM,

3,3'-diindolylmethane; FN1, (3E,5E)-3,5-bis(pyridin-2-methylene)-tetrahydrothiopyran-4-one; DMEM, Dulbecco's modified Eagle's medium; MEM, minimum essential medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; 5-aza, 5-azadeoxycytidine; TSA, trichostatin A; TPA, tetradecanoylephorbol-13-acetate; SFN, sulforaphane; BGS, bisulfite genomic sequencing; MeDIP, methylated DNA immunoprecipitation

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