Epigenetics reactivation of Nrf2 in Prostate TRAMP C1 Cells by curcumin analog FN1

Wenji Li[a,b,#], **Doug Pung**[a,b,c,#], **Zheng-Yuan Su**[a,b], **Yue Guo**[a,b,c], **Chengyue Zhang**[a,b,c], **Anne Yuqing Yang**[a,b,c], **Xi Zheng**[d], **Zhi-Yun Du**[e], **Kun Zhang**[f], and **Ah-Ng Tony Kong**[a,b,*]

[a]Center for Phytochemical Epigenome Studies, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

[b]Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

[c]Graduate Program in Pharmaceutical Sciences, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, USA

[d]Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey,164 Frelinghuysen Road, Piscataway, NJ 08854, USA

[e]Allan H. Conney Laboratory for Anticancer Research, Guangdong University of Technology, Guangzhou, P.R. China

[f]Laboratory of Natural Medicinal Chemistry & Green Chemistry, Guangdong University of Technology, Guangzhou, China

Abstract

It has previously been demonstrated that curcumin is effective against prostate cancer growth and progression in TRAMP mice, potentially acting through the epigenetic modification of the Nrf2 gene and the subsequent induction of the Nrf2-mediated anti-oxidative stress cellular defense pathway. FN1 is a synthetic curcumin analog that shows stronger anti-cancer activity than curcumin. The purpose of this study was to investigate a potential epigenetic effect of FN1 that restores Nrf2 gene expression in TRAMP-C1 cells. Stably transfected HepG2-C8 cells were used to investigate the effect of FN1 on the Nrf2-ARE pathway. Real-time quantitative PCR and western blotting were used 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 assay was conducted to test the tumor inhibitory effect of FN1. Epigenetic modification enzymes, including DNMTs and HDACs, were investigated by western blotting. Luciferase reporter assay indicated FN1 was more potent than curcumin in activating the Nrf2-ARE pathway. FN1 increased the mRNA and protein expression of Nrf2 and downstream

[*]Correspondence should be addressed to: Professor Ah-Ng Tony Kong, Rutgers, The State University of New Jersey, Ernest Mario School of Pharmacy, Room 228, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA, kongt@pharmacy.rutgers.edu, Phone: 848-445-6369/8, Fax: 732-445-3134.
[#]Equal contribution

genes, such as HO-1, NQO1, and UGT1A1. FN1 significantly inhibited the colony formation of TRAMP-C1 cells. BGS and MeDIP assays revealed that FN1 treatment (250 nM for 3 days) decreased the level of CpG methylation of the Nrf2 promoter. FN1 also downregulated epigenetic modification enzymes. In conclusion, our results suggest that FN1 is a novel anti-cancer agent for prostate cancer. FN1 can activate the Nrf2-ARE pathway, inhibit the colony formation of TRAMP-C1 cells and increase the expression of Nrf2 and downstream genes 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.

Graphical Abstract
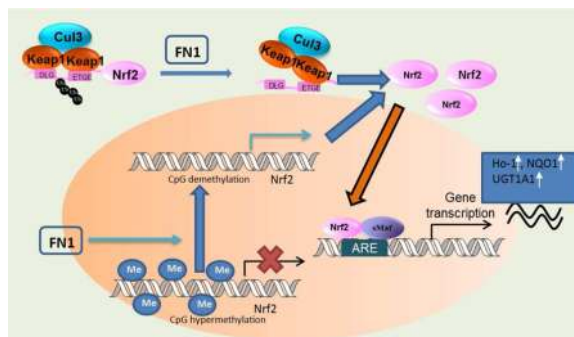
Keywords

Prostate cancer; TRAMP; Nrf2; Epigenetics; FN1

Introduction

Nuclear factor erythroid-2 related factor 2 (Nrf2) is a key regulator 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).[1, 2] 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 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 close relationship to PCa carcinogenesis.[16, 17] We have previously reported that Nrf2 transcription is suppressed in the prostate tumors of TRAMP mice and tumorigenic TRAMP-C1 cells due to the hypermethylation of selected CpGs in 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-azadeoxycytidine (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 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) (Fig 1) is a newly synthesized curcumin analog that is 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 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% CO_2 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. Afterwards, 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 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′-AGCAGGACTGGAGAAGTT-3′ (sense) and 5′-TTCTTTTTCCAGCGAGGAGA-3′ (antisense); HO-1, 5′-CCTCACTGGCAGGAAATCATC-3′ (sense) and 5′-CCTCGTGGAGACGCTTTACATA-3′ (antisense); NQO1, 5′-AGCCCAGATATTGTGGCCG-3′ (sense) and 5′-CTTTCAGAATGGCTGGCAC-3′ (antisense); UGT1A1,5′-GAAATTGCTGAGGCTTTGGGCAGA-3′ (sense) and 5′-ATGGGAGCCAGAGTGTGTGATGAA-3′ (antisense). β-Actin was used as an internal control with sense (5′-CGTTCAATACCCCAGCCATG-3′) and antisense (5′-ACCCCGTCACCAGAGTCC-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 1× 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 μg) 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 Femtov 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′-AATATAATCTCATAAAACCCCAC-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′-GAGGTCACCACAACACGAAC -3′ (forward) and 5′-ATCTCATAAGGCCCCACCTC-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% CO_2 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 ± 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 cells transfected with the ARE-luciferase reporter vector are shown in Fig 2. FN1 induced a higher luciferase activity than the control in a dose-dependent manner at concentrations ranging from 50 to 250 nM. When cells were treated with the same dosage (1000 nM), FN1 showed a greater effect than curcumin and SFN.

Cytotoxicity of FN1 against TRAMP1 C1 Cells

To examine the cytotoxicity of FN1, we analyzed the cell viability of TRAMP-C1 cells treated with FN1 using an MTS assay. The results showed that FN1 treatment decreased cell viability in a time- and dose-dependent manner after 1, 3, and 5 days of treatment (Fig 3). The viability of the cells 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 selected for subsequent studies of epigenetic modifications of the Nrf2 gene promoter.

Expression of Nrf2 and the Nrf2-Mediated Downstream Genes and Keap1

We previously showed that the mRNA and protein expression of Nrf2 are inversely related to the methylation ratio of the Nrf2 gene promoter region in TRAMP prostate tumors, tumorigenic TRAMP-C1 cells, nontumorigenic TRAMP-C3 cells, and normal prostate tissues.[17] Nrf2 is a key regulator of antioxidant and detoxifying enzymes such as HO-1, NQO1, and UGT1A1.[32] In this study, qPCR was used to quantify the transcriptional level of Nrf2 and Nrf2-mediated enzymes in TRAMP-C1 cells following treatment with FN1 for 3 days (Fig 4). As shown in Fig 4A, FN1 at 100 or 250 nM significantly increased the level of Nrf2 mRNA expression ($p < 0.05$); FN1 at 100 or 250 nM significantly increased HO-1 mRNA expression (Fig 4B). FN1 at 50, 100, or 250 nM significantly increased NQO1 mRNA expression (Fig 4C), and FN1 at 250 nM significantly increased UGT1A1 mRNA compared with the controls (Fig 4D).

The protein levels of Nrf2, HO-1, NQO1, and UGT1A1 in TRAMP-C1 cells treated with FN1 were evaluated by western blotting. FN1 (50–250 nM) increased the protein expression level of Nrf2 in a dose-dependent manner (Fig 4E and 4F). Higher concentrations of FN1 induced higher levels of HO-1, NQO1, and UGT1A1. These results suggest that FN1 has the potential to increase the levels of both mRNA and protein expression of Nrf2 and thereby induce the expression of Nrf2-mediated antioxidant detoxifying enzymes in tumorigenic TRAMP-C1 cells. Fig 4E and 4F 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 (Fig 5). These results suggest that FN1 plays an important role in suppressing anchorage-independent growth of TRAMP-C1 cells and has potential for decreasing tumorigenicity.

FN1 Decreased the Methylated CpG Ratio in the Nrf2 Gene Promoter Region

Transcriptional activation of Nrf2 is suppressed when the first five CpGs within the Nrf2 gene promoter are hypermethylated.[17] In this study, we performed bisulfite sequencing to determine whether FN1 can demethylate these five CpGs. Hypermethylation of the five CpGs (88.13% methylation) was observed in TRAMP-C1 cells treated with 0.1% DMSO as a control after 3 days (Fig 6A). The methylation level was decreased (to 63.89%, 82.22%, or 73.55%) when cells were treated with 5-aza (500 nM) and TSA (100 nM) or FN1 (100 nM, 250 nM), respectively, for 3 days. In the MeDIP analysis, the Nrf2 promoter region containing the first five CpGs from enriched methylated DNA after immunoprecipitation was amplified. The results of qPCR (Fig 6B) demonstrate that treatment of 5-aza (500 nM) and TSA (100 nM) or FN1 (250 μM) significantly decreased the ratio of methylated DNA containing the first five CpGs of the Nrf2 promoter ($p < 0.01$). These results indicate that FN1 can reverse the CpG methylation status of the Nrf2 gene promoter, which may restore Nrf2 expression.

FN1 Downregulated Epigenetic Modification Enzymes in TRAMP-C1 Cells

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

Discussion

PCa, a high-incidence and slow-progression disease, is typically diagnosed in a 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 emerged as promising and cost-effective approaches to reduce the incidence and morbidity of PCa.[36–38] Curcumin, for example, has been shown to have cancer chemopreventive activity in preventing PCa.[37] FN1, a newly synthesized curcumin analog, has shown anti-carcinogenic 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. This stress causes alterations of proteins, nucleic acids, and lipids, which may further lead to inflammation or PCa carcinogenesis.[10, 15, 16] It has long been known that Nrf2 plays a vital role in protecting cells from oxidative stress by regulating phase II antioxidant and detoxification enzymes, such as HO-1 (antioxidant), NQO1, and UGT1A1 (detoxification).[32, 40] Due to 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 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 analog of curcumin, could enhance the mRNA and protein expression of those genes, which suggests that FN1 may also exert its chemoprevention effects via enhancing Nrf2 pathway.

It has been widely accepted that epigenetic modifications, including DNA methylation and histone modification, 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 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 hypermethylation of the first five CpG islands during prostate cancer development in TRAMP mice and TRAMP-C1 cells.[16, 17, 25] Many dietary compounds, such as apigenin, tanshinone IIA, and sulforaphane were found to restore Nrf2 and its downstream antioxidant and detoxification enzymes by epigenetic modification.[27, 50, 51] Curcumin has been shown to restore the epigenetically silenced Nrf2 gene through DNA demethylation and histone modification in TRAMP-C1 cells.[25, 52] We were therefore interested in exploring whether the curcumin analog FN1 can activate Nrf2 expression though the same route. In the bisulfite sequencing 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 (Fig 6A). This hypomethylation effect was confirmed by the MeDIP assay, which showed that the unmethylated DNA ratio of the first 5 CpGs in Nrf2 promoter was lower in TRAMP-C1 cells treated with FN1 (250 nM) than in control cells (Fig 6B). The protein expression 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 (Fig 7A). The protein expression levels of HDAC2, HDAC4, HDAC7 and HDAC8 were all reduced after FN1 treatment, although only the decrease of HDAC4 was statistical significantly different from control.

In the modulation of 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] Exposure of cells to ARE inducers may result in the dissociation of Nrf2 from Keap1, and this process facilitates the translocation of Nrf2 to the nucleus, eventually resulting in the transcriptional regulation of target genes.[54] In our experiments, the protein expression of Keap1 was significantly reduced by treatment with 250 nM FN1 for 3 days (Fig 4E and 4F), which suggests 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 sprout, prostate tumorigenesis has been significantly inhibited via the activation of the Nrf2 pathway.[57]

Anchorage-independent colonies formation and growth in soft agar are hallmarks of transformed cells in vitro.[58] 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced JB6 cell transformation and 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 Nrf2 plays an important role in the TPA-induced JB6 cell transformation and the epigenetic reactivation of the Nrf2 pathway could potentially contribute to the attenuation of JB6 cellular anchorage-independent colonies formation and inhibition of neoplastic transformation.[26, 51]

In addition, as we also found previously that prostate cancer development in TRAMP mice and TRAMP-C1 cells 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 cells development and colony formation. Hence the ability of FN1 in inhibiting the proliferation and colony formation of TRAMP C1 cells may probably due to its function in increasing the expression of Nrf2 and its downstream antioxidant and detoxification enzymes by activation Nrf2-ARE pathway (Fig 2, 3, 4 and 5) through epigenetically reactivation of Nrf2, which includes DNA demethylation and histone modification effects (Fig 6 and 7) and inhibition of keap1 expression (Fig 4E and 4F). We will further investigate the above hypothesis with Nrf2-knockdown TRAMP cells in our future study.

In conclusion, our findings reveal that FN1, an analog of curcumin, can inhibit growth and colony formation in TRAMP-C1 cells and can increase mRNA and protein expression of Nrf2 and its downstream phase II detoxifying and antioxidant enzymes, including HO-1, NQO1, and UGT1A1. Our results also indicate that FN1 is an epigenetic regulator that restores the silenced Nrf2 gene in TRAMP-C1 cells through demethylation of the Nrf2 promoter and histone modifications. In addition, the keap1 level was reduced by FN1 treatment. The epigenetic regulation and inhibition of keap1 may be the mechanisms driving the induction of Nrf2 and its downstream target genes. FN1 is thus effective at inhibiting the initiation, progression and development of PCa in the TRAMP model. FN1 is a novel potential cancer chemopreventive agent for the management of PCa. Its in vivo efficacy and pharmacokinetics profile needs further investigation.

Acknowledgments

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 ECHassociated 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 Medium
MEM	Minimum essential medium
FBS	fetal bovine serum
DMSO	Dimethyl sulfoxide
5-aza	5-azadeoxycytidine
TSA	Trichostatin A
TPA	tetradecanoylphorbol-13-acetate
SFN	sulforaphane
BGS	Bisulfite Genomic Sequencing
MeDIP	Methylated DNA Immunoprecipitation

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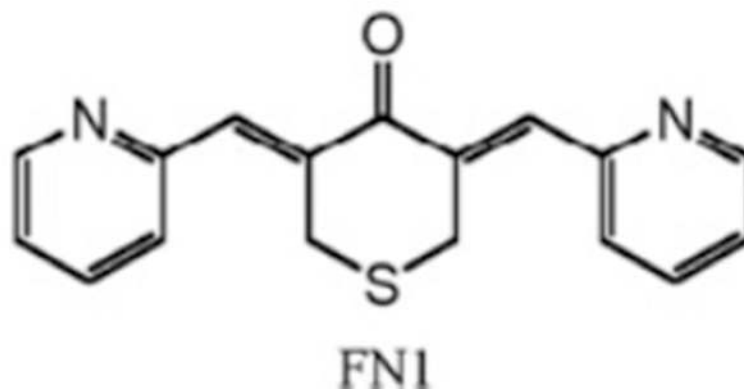
Fig 1.
Chemical structure of (3E,5E)-3,5-Bis(pyridin-2-methylene)-tetrahydrothiopyran-4-one (FN1)
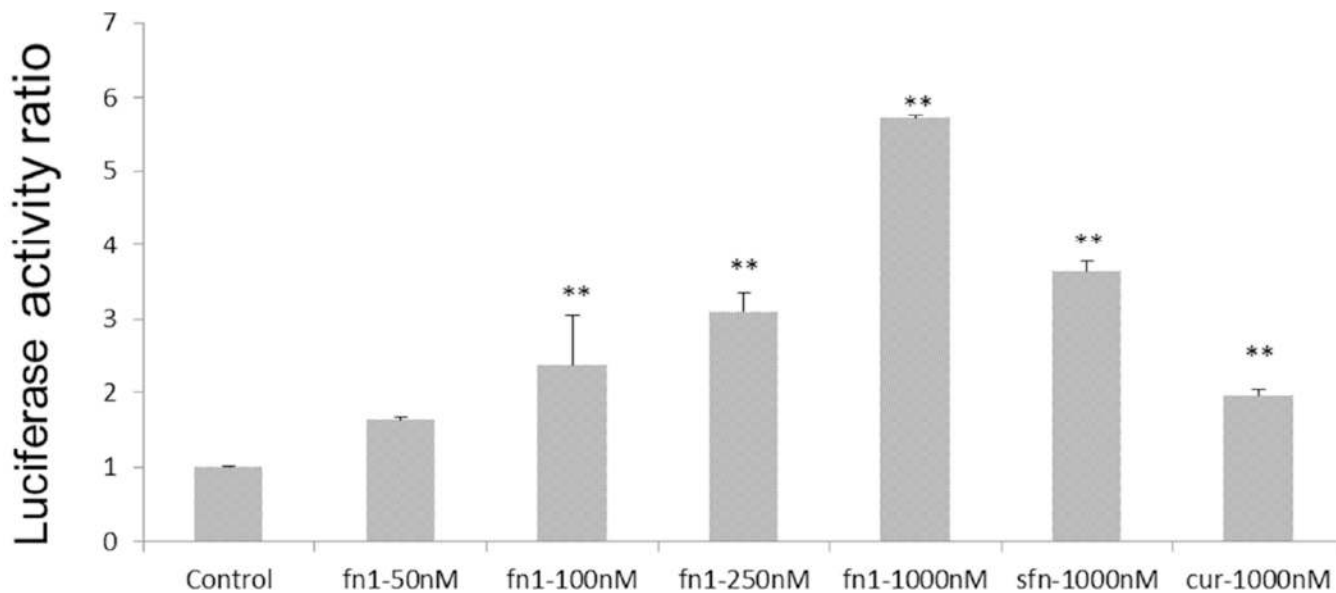
Fig. 2.
ARE-Luciferase activity assay of effects of FN1, curcumin and SFN on the Hep-G2 C8 cell line. The BCA protein assay was determined to normalize the luciferase activity. The data obtained from three independent experiments expressed the inducible fold change compared with the vehicle control. *, $p<0.05$; **, $p<0.01$ comparing with control group.
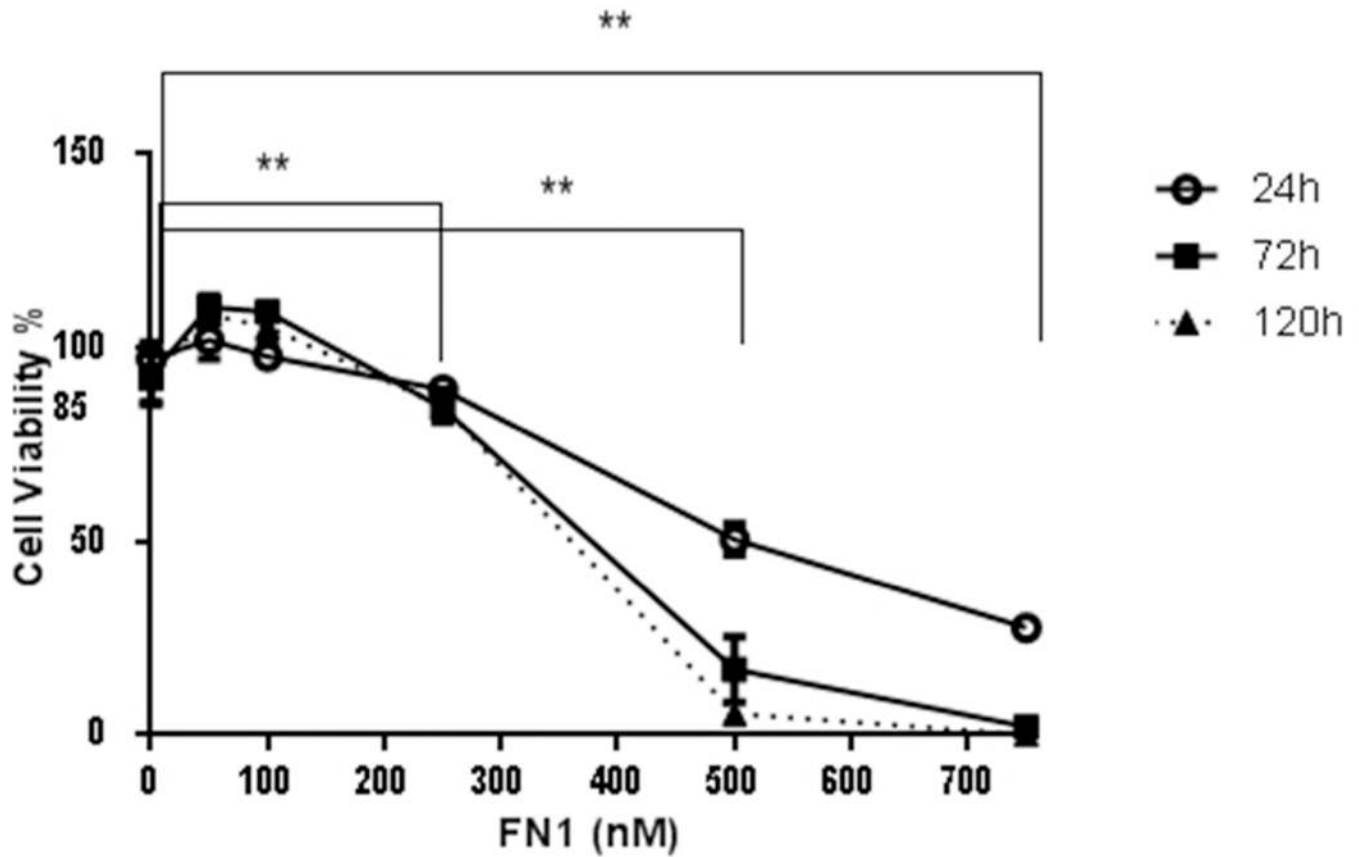
Fig 3.
Cytotoxicity of FN1 against the TRAMP-C1 cell line. Cells were seeded in a 96-well plate for 24 h and then incubated in medium with various concentrations of FN1 for 1, 3, or 5 days as described in Materials and Methods. Cell viability was determined using an MTS assay. The data are expressed as means ± SD (n = 3). **, $p < 0.01$ compared with the control group

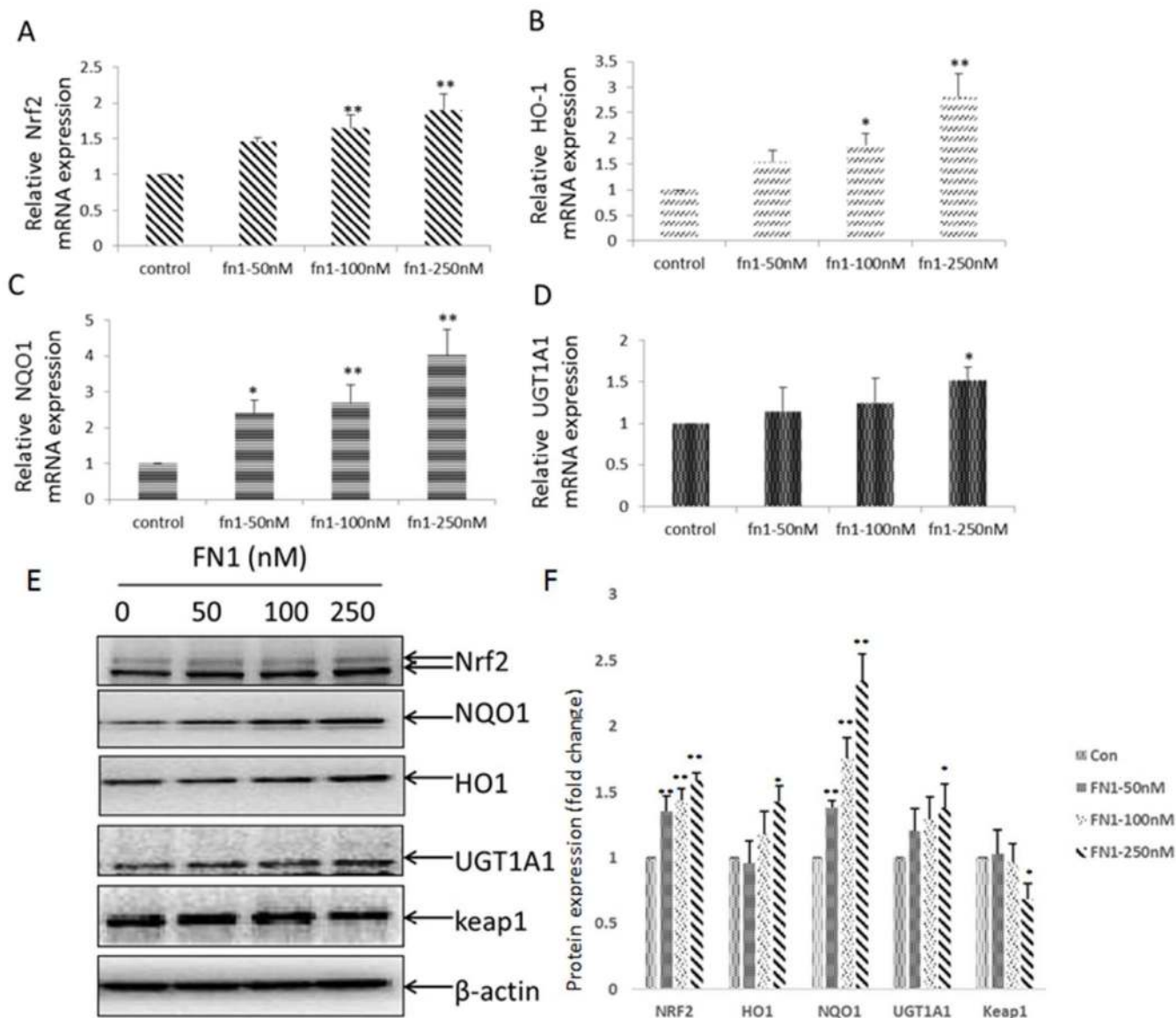**Fig 4.**
Effects of FN1 (50, 100 and 250 nM) on Nrf2 mRNA and protein expression of Nrf2 target genes in TRAMP-C1 cells after 3-day treatment were determined with real-time qPCR and Western blot. The graphical data are expressed as means ± SD from three independent experiments. *, pπ.05 and **, p<0.01 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 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 ± SD.
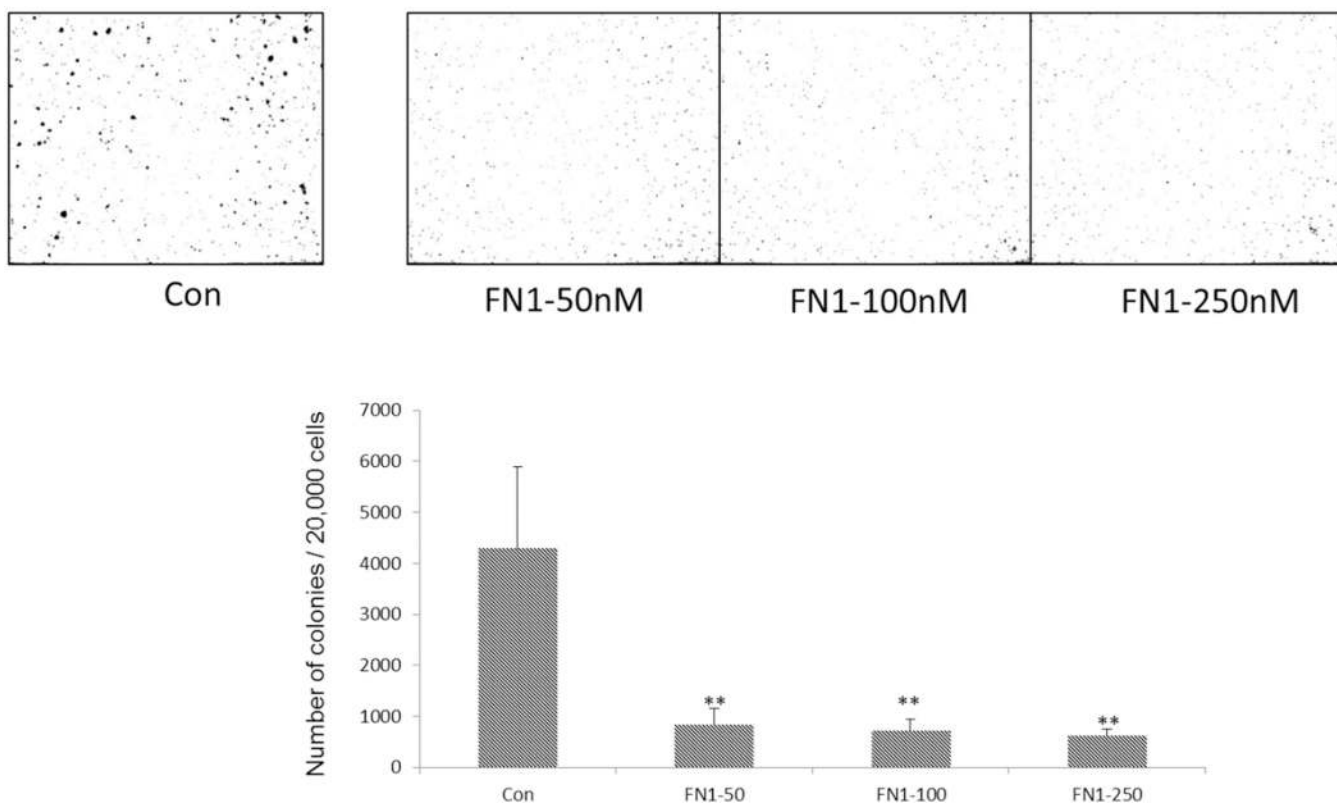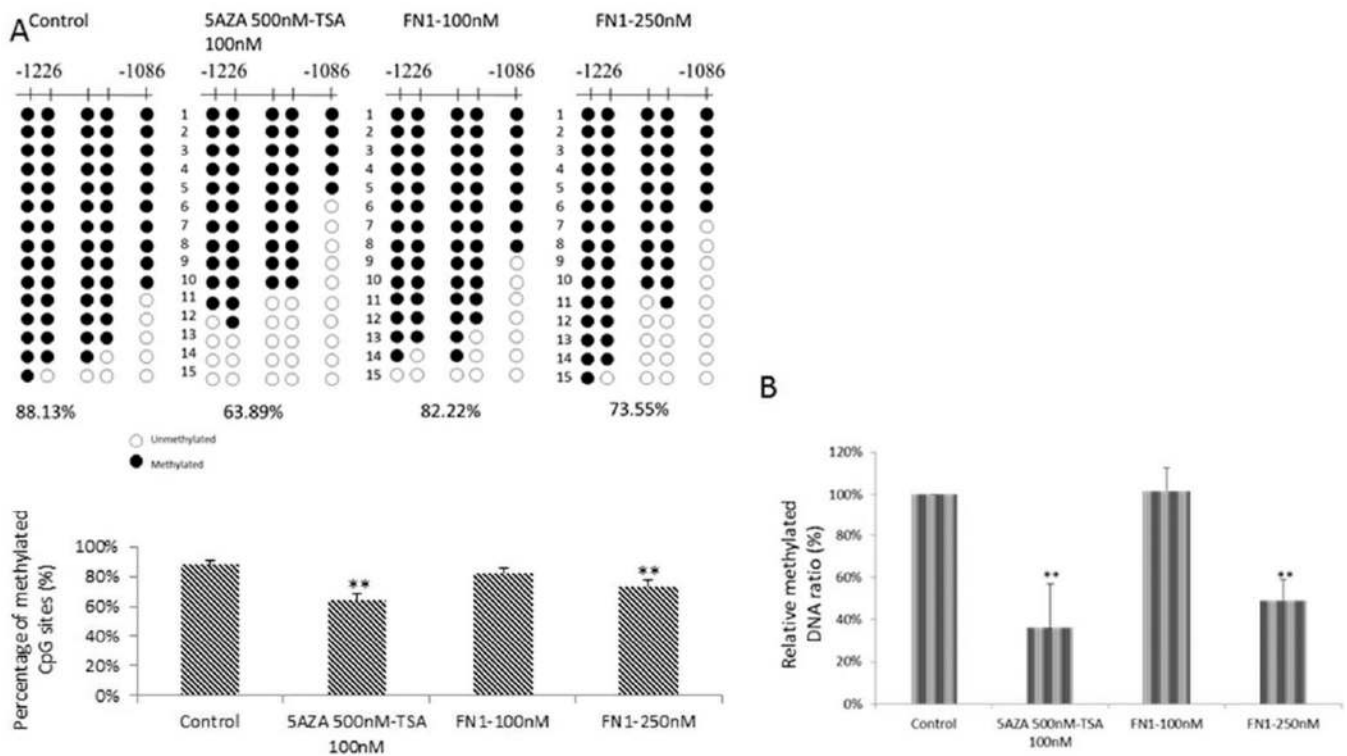
Fig 5.
Inhibitory effect of FN1 on the Anchorage-Independent Colony-Formation of TRAMP-C1 cells. The colonies exhibiting anchorage-independent growth were counted under a microscope using ImageJ software. The graphical data are presented as the average of triplicate results from 3 independent experiments. **, $p < 0.01$ compared with the control group.

Fig 6.
Methylation ratio of the first 5 CpGs located at positions −1226 to −1086 from the translation start site of Nrf2 promoter region in TRAMP C1 cells after 3-day treatment of FN1 (100nM, 250nM) and combination of 5-aza (500 nM) and TSA (100 nM) by BGS and MeDIP. The data are expressed as means ± SD of three independent experiments.*. $p < 0.05$ and **, $p < 0.01$ comparing with control group. (A) BGS assay for effect of FN1 on methylation of the Nrf2 promoter regions in TRAMP C1 cells. Filled dots indicate methylated CpGs, and empty dots indicate 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 using primers that covered the DNA sequence containing the first five CpGs in the Nrf2 gene promoter region. qPCR was performed to quantify the amount of MeDIP DNAs relative to their inputs by calculating the standard curve of ΔCT values from a serial dilution of the inputs. The relative methylated DNA ratio was evaluated in comparison with the control (defined as 100% MeDIP DNA).
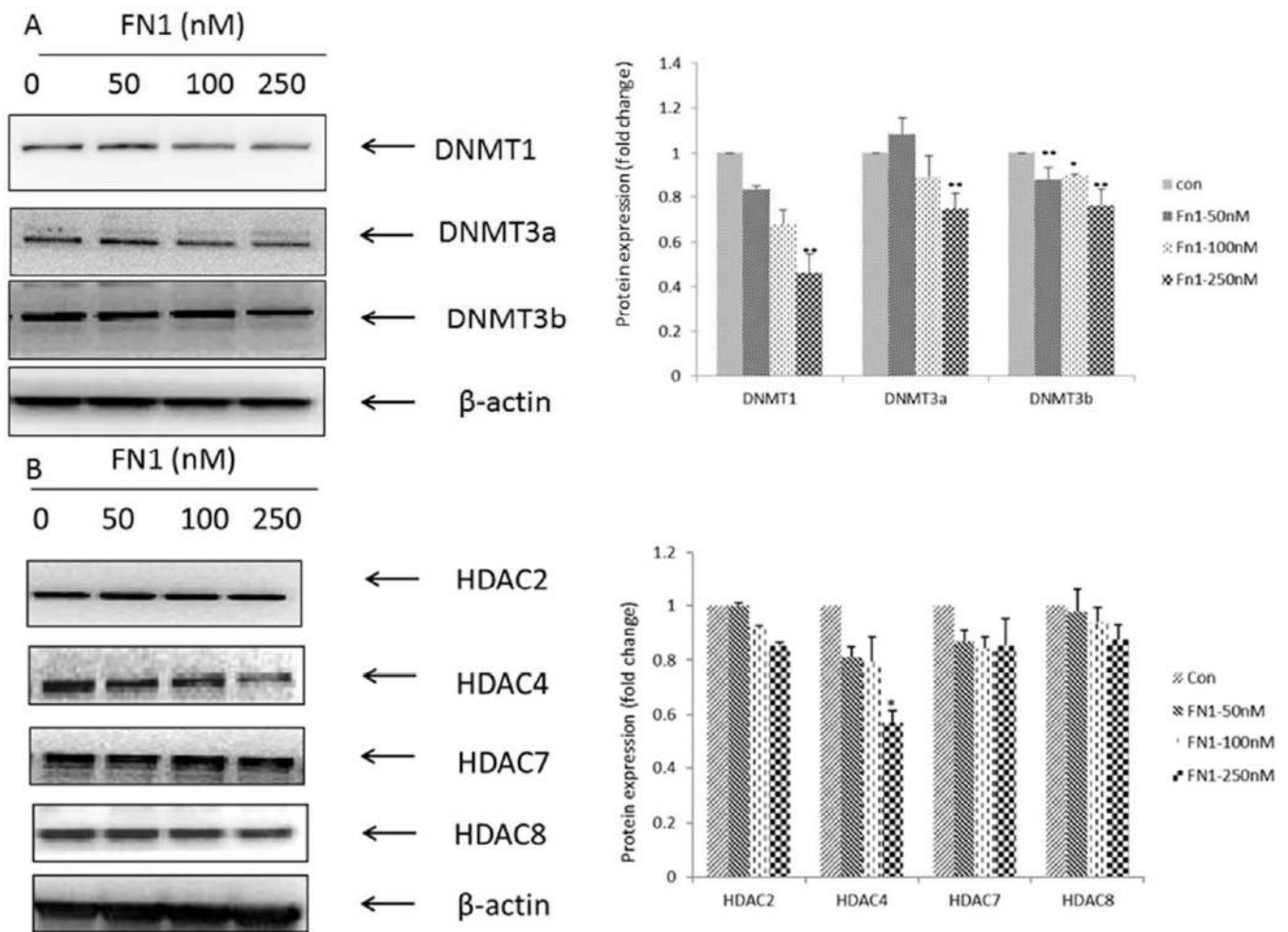
Fig 7.
Effect of FN1 on DNMT protein expression (A) and protein expression of HDACs (B) in
TRAMP-C1 cells. Cells (1×10^5/10-cm dish) were incubated with FN1 (50, 100, and 250
nM) for 3 days. The relative expression levels were quantified based on the signal intensity
of the corresponding bands and normalized using β-actin. The graphical data are represented
as the mean ± SD from 3 independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared
with the control group.