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Epigenetic Reactivation of Nrf2 in Murine Prostate Cancer TRAMP C1 Cells by Natural Phytochemicals Z-Ligustilide and Radix *Angelica Sinensis* via Promoter CpG Demethylation

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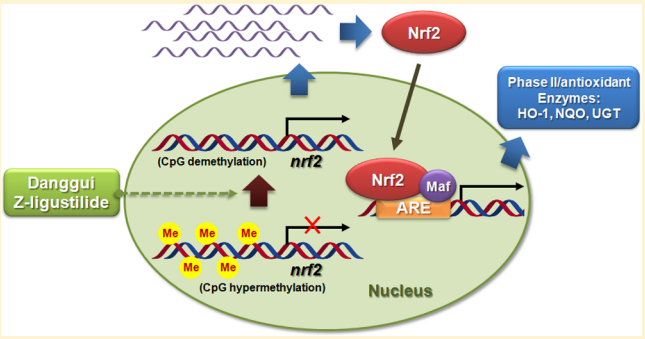
ABSTRACT: Cancer development has been linked to epigenetic modifications of cancer oncogenes and tumor suppressor genes; in advanced metastatic cancers, severe epigenetic modifications are present. We previously demonstrated that the progression of prostate tumors in TRAMP mice is associated with methylation silencing of the Nrf2 promoter and a reduced level of transcription of Nrf2 and Nrf2 target genes. Radix *Angelicae Sinensis* (RAS; Danggui) is a medicinal herb and health food supplement that has been widely used in Asia for centuries. Z-Ligustilide (Lig) is one of the bioactive components of RAS. We investigated the potential of Lig and RAS to restore Nrf2 gene expression through epigenetic modification in TRAMP C1 cells. Lig and RAS induced the mRNA and protein expression of endogenous Nrf2 and Nrf2 downstream target genes, such as HO-1, NQO1, and UGT1A1. Bisulfite genomic sequencing revealed that Lig and RAS treatment decreased the level of methylation of the first five CpGs of the Nrf2 promoter. A methylation DNA immunoprecipitation assay demonstrated that Lig and RAS significantly decreased the relative amount of methylated DNA in the Nrf2 gene promoter region. Lig and RAS also inhibited DNA methyltransferase activity in vitro. Collectively, these results suggest that Lig and RAS are able to demethylate the Nrf2 promoter CpGs, resulting in the re-expression of Nrf2 and Nrf2 target genes. Epigenetic modifications of genes, including Nrf2, may therefore contribute to the overall health benefits of RAS, including the anticancer effect of RAS and its bioactive component, Lig.



INTRODUCTION

Epigenetic modifications, including DNA methylation, histone modification, nucleosome remodeling, and micro RNA silencing, appear to contribute to many diseases, including cancer. DNA methylation is a major form of epigenetic transcriptional control that often occurs at the regulatory promoter regions of genes and plays an important role in maintaining DNA stability and integrity in mammals.[1,2] Hypermethylation of CpG islands usually causes gene silencing, and demethylation often leads to gene reactivation.[3] Cancer development is usually associated with hypermethylation of the promoters of a number of critical cancer-related genes, particularly tumor suppressor genes, resulting in epigenetic inactivation in many cancers, including prostate cancer.[4−6] DNA methyltransferases (DNMT) and histone deacetylases (HDAC) play important roles in the inactivation of various critical genes in many human prostate cancer cell lines, and 5-azadeoxycytidine (5-aza, a DNMT inhibitor) and trichostatin A (TSA, an HDAC inhibitor) have been shown to reduce the level of cell proliferation.[7] Certain naturally occurring phytochemicals have recently been shown to exhibit a cancer chemopreventive effect via epigenetic alterations and inhibition of tumor growth. For example, epigallocatechin 3-gallate

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(EGCG), a green tea polyphenol, has been reported to inhibit DNMT and histone acetyltransferase (HAT) activity.[8,9] Sulforaphane from cruciferous vegetables such as broccoli, broccoli sprouts, and cabbage displays inhibitory effects on DNMT and HDAC.[10] Genistein from soybean, lycopene from tomatoes, curcumin from turmeric, and resveratrol from red grapes all possess DNMT inhibitory activity.[11−14]

Oxidative stress and reactive carcinogenic metabolites induce genetic mutations, genomic instability, neoplastic transformation, and, ultimately, carcinogenesis.[15−19] Nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2) is a key regulator of the induction of phase II detoxifying and antioxidation enzymes via transcriptional control of the antioxidant response element (ARE), resulting in protection from oxidative stress, reactive carcinogenic metabolites, and carcinogenesis.[20,21] When cells are exposed to oxidative stress, electrophilic compounds, or dietary cancer chemopreventive compounds, Nrf2 is released from the anchor protein Kelch-like ECH-associated protein-1 (Keap1) in the cytoplasm and translocates to the nucleus.[22] Cancer chemopreventive compounds can regulate Nrf2, leading to the induction of phase II detoxifying enzymes such as NAD(P)H:quinine oxidoreductase-1 (NQO1) and antioxidation enzymes such as heme oxygenase 1 (HO-1).[23] We previously observed that the development of prostate tumors in TRAMP mice is accompanied by a gradual loss of expression of Nrf2 and its downstream target genes, including NQO1, HO-1, UDP-glucuronosyltransferases (UGT), glutathione *S*-transferases (GST), and glutathione peroxidase (GPx).[24,25] Interestingly, curcumin, a dietary chemopreventive compound that prevents prostate tumorigenesis in TRAMP C1 mice, demethylates the first five CpGs of the Nrf2 promoter in TRAMP C1 cells and induces re-expression of Nrf2 and NQO1.[13]

Radix *Angelicae Sinensis* (RAS), known as "Danggui", is the dried root of *Angelica sinensis* and has been used as an edible and medicinal herb for centuries in Asia. The biological activities of Danggui have been investigated, including its anti-inflammatory,[26] immunomodulatory,[27] anticancer,[28] antiangiogenic,[29] antioxidative,[30] neuroprotective,[31] and liver and kidney protective effects.[32,33] Some major active components such as phthalides, organic acids, and polysaccharides have been isolated and identified.[27,34,35] Z-Ligustilide (Lig), a characteristic phthalide compound (Figure 1), is the major constituent

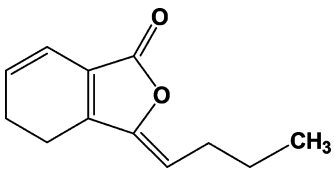

Figure 1. Chemical structure of Z-ligustilide (Lig).

of the lipophilic extract of RAS. The reported pharmacological activities of Lig include anticancer,[36,37] anti-inflammatory,[38] antihepatotoxic,[39] and neuroprotective effects.[40] RAS dietary supplements are potential chemopreventive agents because lipophilic extracts of RAS strongly induce detoxification enzymes such as NQO1.[39] Lig promotes the activities of GPx and superoxide dismutase (SOD) and thereby reduces oxidative stress in brain tissues.[41] Lig has also been reported to activate Nrf2 and induce NQO1 activity via targeting of the cysteine residues of human Keap1.[39] However, the possible chemopreventive and/or therapeutic effect of Danggui or Lig in prostate cancer through epigenetic modifications of the Nrf2 gene remains unknown. The aim of this study was to investigate the demethylation potential of Lig and a supercritical CO_2 extract of RAS to restore the expression of Nrf2 in murine tumorigenic prostate cancer TRAMP C1 cells. We demonstrated that RAS and Lig demethylate the CpG of the Nrf2 promoter and induce the re-expression of Nrf2 and Nrf2-mediated target genes.

MATERIALS AND METHODS

Materials and Chemicals. Z-Ligustilide (Lig) was purchased from ChromaDex (Irvine, CA). Radix *Angelicae Sinensis* (RAS) was extracted with supercritical carbon dioxide (CO_2) using a supercritical fluid extractor (HA220-50-01) in the laboratory of Q. Wu (Beijing University of Chinese Medicine). The content of Lig (62.3%) in RAS was measured by high-performance liquid chromatography (HPLC). Dimethyl sulfoxide (DMSO), 5-azadeoxycytidine (5-aza), and trichostatin A (TSA) were from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and the trypsin/EDTA solution were from Gibco Laboratories (Grand Island, NY).

Cell Culture. TRAMP C1 cells were obtained from B. Foster (Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, NY). Cells were cultured in DMEM (pH 7.0) containing 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere as described previously.[42,43]

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 Lig or RAS in DMEM containing 1% FBS for 1, 3, or 5 days. The 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) according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qPCR). TRAMP C1 cells were cultured in 6 cm dishes at a density of 1×10^4 cells/dish. After 24 h, the cells were treated with medium containing 1% FBS and various concentrations of Lig or RAS. The treatment medium was changed after 2 days. After 3 days, the total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from total RNA using a SuperScript III First-Strand Synthesis System (Invitrogen, Grand Island, NY) according to the manufacturer's instructions. mRNA expression levels were determined using first-strand cDNA as the template by quantitative real-time PCR (qPCR) with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) in an ABI7900HT system. The following sequences of the primers for Nrf2, HO-1, NQO1, and UGT1A1 were used: Nrf2, 5′-AGCAGGACTGGAGAAGTT-3′ (sense) and 5′-TTCTTTTTCC-AGCGAGGAGA-3′ (antisense); HO-1, 5′-CCTCACTGGCAGGAA-ATCATC-3′ (sense) and 5′-CCTCGTGGAGACGCTTTACATA-3′ (antisense); NQO1, 5′-AGCCCAGATATTGTGGCCG-3′ (sense) and 5′-CCTTTCAGAATGGCTGGCAC-3′ (antisense); UGT1A1, 5′-GAAATTGCTGAGGCTTTGGGCAGA-3′ (sense) and 5′-ATG-GGAGCCAGAGTGTGTGATGAA-3′ (antisense). β-Actin was used as an internal control with sense (5′-CGTTCAATACCCCAGCCA-TG-3′) and antisense (5′-GACCCCGTCACCAGAGTCC-3′) primers.

Preparation of Protein Lysates and Western Blotting. After incubation for 24 h, TRAMP C1 cells (1×10^4 cells per 6 cm dish) were treated with 0.1% DMSO as a control, Lig (50 μM), or RAS (8.5 μg/mL) in DMEM containing 1% FBS. The medium was changed after 2 days. 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) containing a protein inhibitor cocktail (Sigma). The protein concentrations of the cell lysates were measured using the bicinchoninic acid (BCA) method (Pierce,

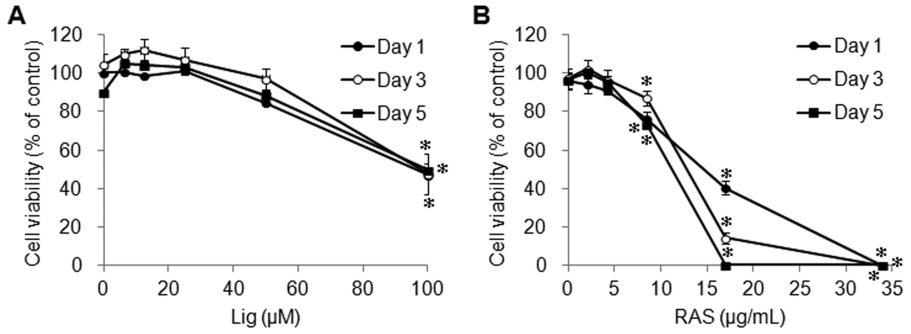

Figure 2. Effect of Lig and RAS on the growth of TRAMP C1 cells. Cells were seeded in DMEM on a 96-well plate for 24 h and then incubated in medium with various concentrations of Lig (A) or RAS (B) for 1, 3, or 5 days as described in Materials and Methods. Cell viability was determined using an MTS assay. The data are expressed as means ± SD (n = 3). Asterisks indicate significant (P < 0.05) decreases in cell viability in comparison with control.

Rockford, IL). Identical concentrations of protein were subjected to 4 to 15% SDS−polyacrylamide gel (Bio-Rad, Hercules, CA) electrophoresis (SDS−PAGE) and then transferred to PVDF membranes (Millipore, Billerica, MA). 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) and measured with a Gel Documentation 2000 system (Bio-Rad). Anti-Nrf2, anti-HO-1, anti-NQO-1, anti-UGT1A1, and anti-β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Bisulfite Genomic Sequencing (BGS). TRAMP C1 cells (1×10^4 cells per 6 cm dish) were treated with 0.1% DMSO as a control, Lig (50 μM), RAS (8.5 μg/mL), or 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), and bisulfite conversion of the genomic DNA was performed with an EZ DNA Methylation-Gold Kit (Zymo Research Corp., Orange, CA) according to the manufacturer's instructions as described previously.[13,42] The converted DNA was amplified with Platinum Taq DNA Polymerase (Invitrogen, Grand Island, NY) and primers that amplify the first five CpGs (between positions −1226 and −1086 with the translation initiation site defined as position 1) of the murine Nrf2 gene.[42] The sequences of the primers were 5′-AGTTATGAAGTAG-TAGTAAAAA-3′ (sense) and 5′-AATATAATCTCATAAAACCCC-AC-3′ (antisense). A TOPOTM TA Cloning kit (Invitrogen, Grand Island, NY) 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).

Methylation DNA Immunoprecipitation (MeDIP) Analysis. MeDIP analysis was performed with a MagMeDIP kit (Diagenode, Denville, NJ) according to the manufacturer's instructions as described previously.[13,44] Briefly, the extracted DNA from treated cells was sonicated on ice to approximately 100−500 bp. The fragmented DNA was denatured at 95 °C for 3 min and subjected to immunoprecipitation overnight at 4 °C. After incubation, the DNA on magnetic beads with an antibody was isolated for use in regular PCR and qPCR assays. The primers, 5′-TGAGATATTTTGCACATCCGATA-3′ (sense) and 5′-ACTCTCAGGGTTCCTTTACACG-3′ (antisense), were used to cover the DNA sequence of the first five CpGs of murine Nrf2. For regular PCR, MeDIP and input DNA were amplified using Platinum PCR SuperMix (Invitrogen, Carlsbad, CA). The PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light. For qPCR, the enriched MeDIP DNA content was calculated on the basis of the calibration of the serial dilution of input DNA, and the relative methylated DNA ratios were calculated on the basis of the control, which was defined as 100% methylated DNA.

In Vitro Methylation Assay. This assay was adapted from the work of Brueckner et al.,[45] with slight modifications. The 850 bp fragment from position −444 to 401 relative to the initiation codon of the promoter region of the human $p16^{Ink4a}$ gene was used as the substrate DNA. The methylation reaction was performed at 37 °C in a final volume of 20 μL containing 100 ng of substrate DNA with various concentrations of Lig or RAS and 4 units of M.SssI methylase (New England Biolabs, Frankfurt, Germany) for 1 h. The unmethylated DNA was digested by 30 units of BstUI (New England Biolabs) at 60 °C for 1 h. The digested DNA was analyzed by 2% agarose gel electrophoresis. The methylated DNA (larger fragments) and unmethylated DNA (smeared fragments of <850 bp) bands were visualized with a Gel Documentation 2000 system (Bio-Rad) and quantified with Quantity One. The inhibition of methylation by Lig or RAS was calculated on the basis of the density of methylated DNA on an agarose gel compared with that of methylated DNA that was exposed to M.SssI methylase in the absence of Lig or RAS. Stock solutions of Lig and RAS were prepared in 100% DMSO and subsequently diluted with DMSO. The final concentration of DMSO was 0.1% in the reaction system. Lig (0 μM) and RAS (0 μg/mL) treatments were used as DMSO-containing controls (final concentration of 0.1%). The concentration of Lig or RAS required to inhibit methylation by 50% (IC_{50}) was determined by interpolation from the dose−response curve.

Statistical Analysis. Data are presented as means ± the standard deviation of three independent experiments. One-way analysis of variance (ANOVA) and Duncan's multiple-comparison test (SAS Institute Inc., Cary, NC) were performed to determine statistically significant differences among the means (P < 0.05).

RESULTS

Lig and RAS Exhibit Cytotoxicity against TRAMP1 C1 Cells. To examine the cytotoxicity of Lig and RAS, we analyzed the cell viability of TRAMP C1 cells following Lig or RAS treatment using an MTS assay. The results showed that Lig or RAS treatment decreased the cell viability in a dose-dependent manner 1, 3, and 5 days after treatment (Figure 2). Low concentrations of Lig (<50 μM) or RAS (<4.25 μg/mL) were much less toxic than higher concentrations of Lig (100 μM) or RAS (8.5−34 μg/mL). Because the viability of cells treated with 50 μM Lig or 8.5 μg/mL RAS was greater than 70%, these doses were selected for subsequent studies of epigenetic modifications of the Nrf2 gene promoter.

Lig and RAS Increase the Level of mRNA and Protein Expression of Nrf2 and Nrf2-Mediated Downstream Genes. We previously demonstrated that the mRNA and protein levels of Nrf2 are inversely correlated with the methylation ratio of the Nrf2 gene promoter region in TRAMP prostate tumors, tumorigenic TRAMP C1 cells,

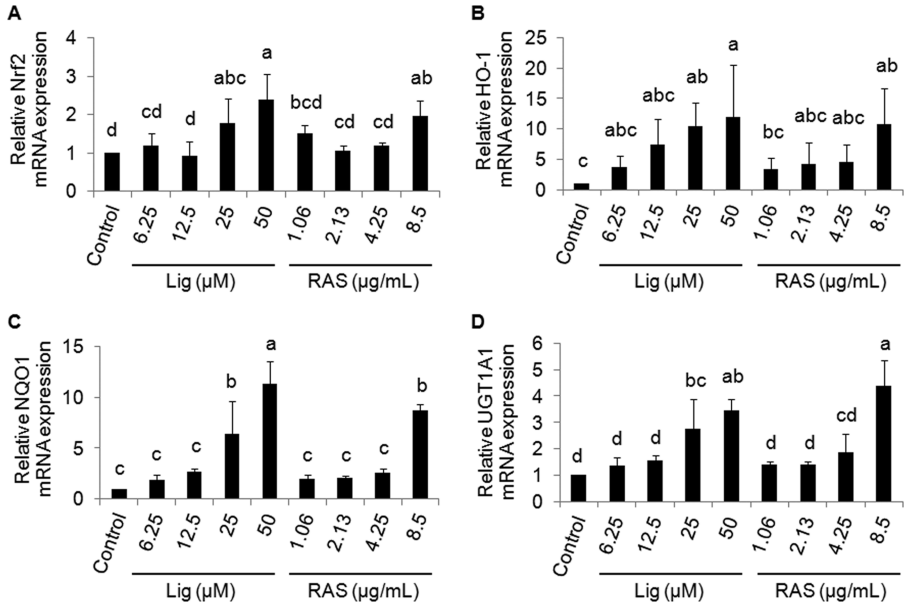

Figure 3. Effect of Lig and RAS on the relative endogenous mRNA expression of Nrf2 (A), HO-1 (B), NQO1 (C), and UGT1A1 (D) in TRAMP C1 cells. Cells were incubated for 3 days with various concentrations of Lig (6.25, 12.5, 25, and 50 μM) or RAS (1.06, 2.13, 4.25, and 8.5 μg/mL). RNA was extracted in three independent experiments. The data are expressed as means ± SD. β-Actin was used as an endogenous housekeeping gene. Data not sharing the same letter (a, b, c, or d) are significantly different from one another ($P < 0.05$).

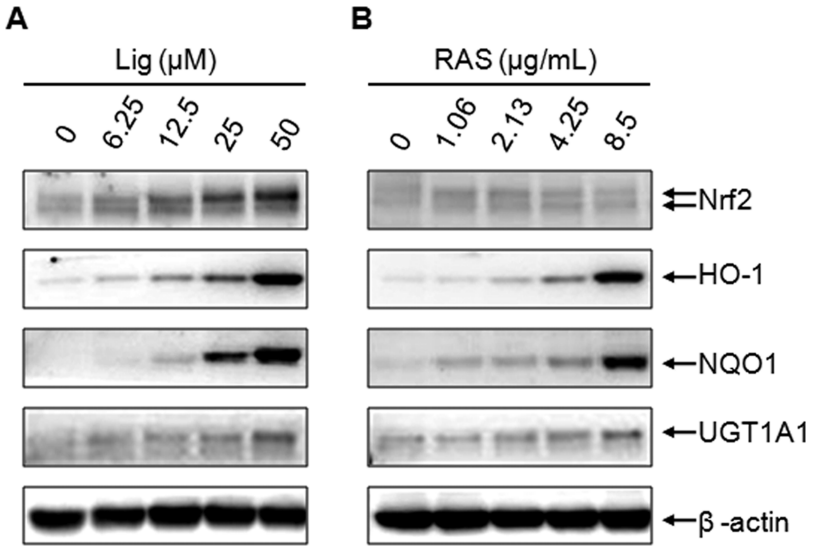

Figure 4. Effect of Lig (A) and RAS (B) on the protein expression of Nrf2, HO-1, NQO1, and UGT1A1 in TRAMP C1 cells. Cells were incubated with various concentrations of Lig (6.25, 12.5, 25, and 50 μM) or RAS (1.06, 2.13, 4.25, and 8.5 μg/mL) for 3 days. Cellular proteins were resolved by 4 to 15% SDS−PAGE and Western blotting as described in Materials and Methods. The protein expression of Nrf2 and Nrf2-mediated enzymes, including HO-1, NQO1, and UGT1A1, was detected by hybridization with specific antibodies. β-Actin was used a control to ensure loading and transfer of equal amounts of protein.

nontumorigenic TRAMP C3 cells, and normal prostate tissues.[42] Nrf2 is a key regulator of antioxidant and detoxifying enzymes such as HO-1, NQO1, and UGT1A1.[25,46] In the study presented here, we used qPCR to quantify the transcriptional level of Nrf2 and Nrf2-mediated enzymes in TRAMP C1 cells following treatment with Lig or RAS for 3 days (Figure 3). The results indicate that both Lig (25 or 50 μM) and RAS (8.5 μg/mL) significantly increased the level of Nrf2 mRNA expression ($P < 0.05$) (Figure 3A) and induced HO-1, NQO1, and UGT1A1 mRNA in comparison with the controls (Figure 3B−D). It also shows that Lig (50 μM) treatment had a larger effect on NQO1 mRNA expression than RAS (8.5 μg/mL) treatment; however, there was no statistically significant difference in the effectiveness between these two treatments in the induction of Nrf2, HO-1, and UGT1A1 gene expression. The protein levels of Nrf2, HO-1, NQO1, and UGT1A1 in TRAMP C1 cells treated with Lig or RAS were evaluated by Western blotting. Lig (6.25−50 μM) increased the level of protein expression of Nrf2 in a dose-dependent manner, while RAS (1.06−8.5 μg/mL) slightly increased the level of Nrf2 expression (Figure 4). Higher concentrations of Lig and RAS induced higher levels of HO-1, NQO1, and UGT1A1. These results suggest that Lig and RAS have the potential to increase the levels of both mRNA and protein expression of Nrf2 and

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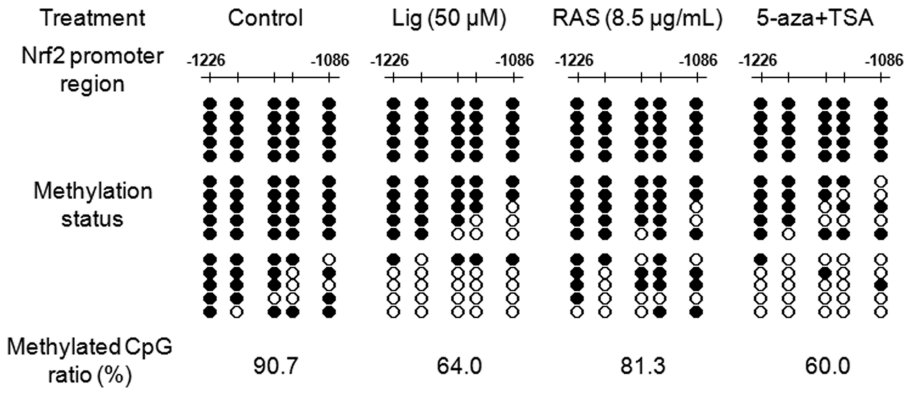

Figure 5. Effect of Lig and RAS on methylation of the Nrf2 promoter regions in TRAMP C1 cells. Cells were treated with Lig (50 μM) or RAS (8.5 μg/mL) for 3 days, and genomic DNA was extracted from the cells. For the 5-aza (500 nM) and TSA (100 nM) combination treatment (positive control), TSA was added 20 h before the cells were harvested. The methylation patterns of the first five CpGs located at positions −1226 to −1086 from the translation start site, which was defined as position 1 in the Nrf2 gene promoter, were determined by bisulfite genomic sequencing (BGS) as described in Materials and Methods. Filled dots indicate methylated CpGs, and empty dots indicate unmethylated CpGs. To estimate the percentage of methylated CpGs, the number of methylated dots was counted and divided by the total number of dots in the Nrf2 gene promoter region. At least 10 clones from three independent experiments were analyzed, and 15 clones were selected on the basis of the relative frequency of methylation during each treatment.

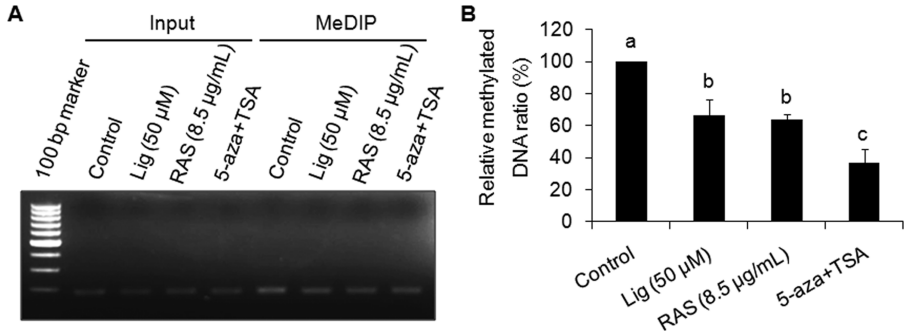

Figure 6. MeDIP (methylation DNA immunoprecipitation) assay for analyzing the effect of Lig and RAS on the methylation of Nrf2 promoter regions in TRAMP C1 cells. Cells were treated with Lig (50 μM) or RAS (8.5 μg/mL) for 3 days, and genomic DNA was extracted from the cells. For the 5-aza (500 nM) and TSA (100 nM) combination treatment (positive control), TSA was added 20 h before the cells were harvested. After sonication and denaturation, the genomic DNA was extracted and subjected to DNA immunoprecipitation using a MagMeDIP kit as described in Materials and Methods. The Nrf2 gene with a methylated promoter in MeDIP-precipitated DNA was further analyzed by standard PCR and qPCR using primers that covered the DNA sequence containing the first five CpGs in the Nrf2 gene promoter region. (A) Standard PCR was performed to compare the immunoprecipitated DNAs with their inputs. A representative result from three independent experiments is shown. (B) qPCR was performed to quantify the amount of MeDIP DNAs relative to their inputs. The relative amount of MeDIP DNA was calculated on the basis of 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). The data are expressed as means ± SD of three independent experiments. Data not sharing the same letter (a, b, c, or d) are significantly different from one another ($P < 0.05$).

thereby induce the expression of Nrf2-mediated antioxidant and detoxifying enzymes in tumorigenic TRAMP C1 cells.

Lig and RAS Decrease the Methylated CpG Ratio in the Nrf2 Gene Promoter Region. We previously demonstrated that the transcriptional activation of Nrf2 is significantly suppressed when the first five CpGs within the Nrf2 gene promoter are hypermethylated.[42] In the study presented here, we performed bisulfite sequencing to determine whether Lig and RAS demethylate these five CpGs. Hypermethylation of these five CpGs (90.7% methylation) was observed in TRAMP C1 cells treated with 0.1% DMSO as a control after 3 days (Figure 5). The methylation level was decreased (to 64.0, 81.3, or 60.0% of the control value) when cells were treated with Lig (50 μM) or RAS (8.5 μg/mL) or cotreated with 5-aza (500 nM) and TSA (100 nM), respectively, for 3 days. We performed MeDIP analysis, in which methylated DNA fragments are enriched via immunoprecipitation with an anti-methylcytosine (mecyt) antibody that binds specifically to methylated cytosine.[47] The Nrf2 promoter region containing the first five CpGs from enriched methylated DNA after immunoprecipitation was amplified (Figure 6). The results of regular PCR (Figure 6A) and qPCR (Figure 6B) demonstrate that Lig (50 μM), RAS (8.5 μg/mL), or cotreatment with 5-aza (500 nM) and TSA (100 nM) significantly decreased the ratio of methylated DNA containing the first five CpGs of the Nrf2 promoter ($P < 0.05$). These results indicate that Lig and RAS can reverse the CpG methylation status of the Nrf2 gene promoter, which drives the transcriptional re-expression of Nrf2 (see Figure 3).

Lig and RAS Inhibit the Activity of DNA Methyltransferase in Vitro. DNA methylation is one of the key epigenetic mechanisms in the regulation of gene expression in eukaryotes. DNA methyltransferases (DNMT) such as DNMT1, -3a, and -3b are the major enzymes that transfer and maintain a methyl group at the fifth carbon of cytosine residues within CpG islands in DNA.[48] In addition to DNA

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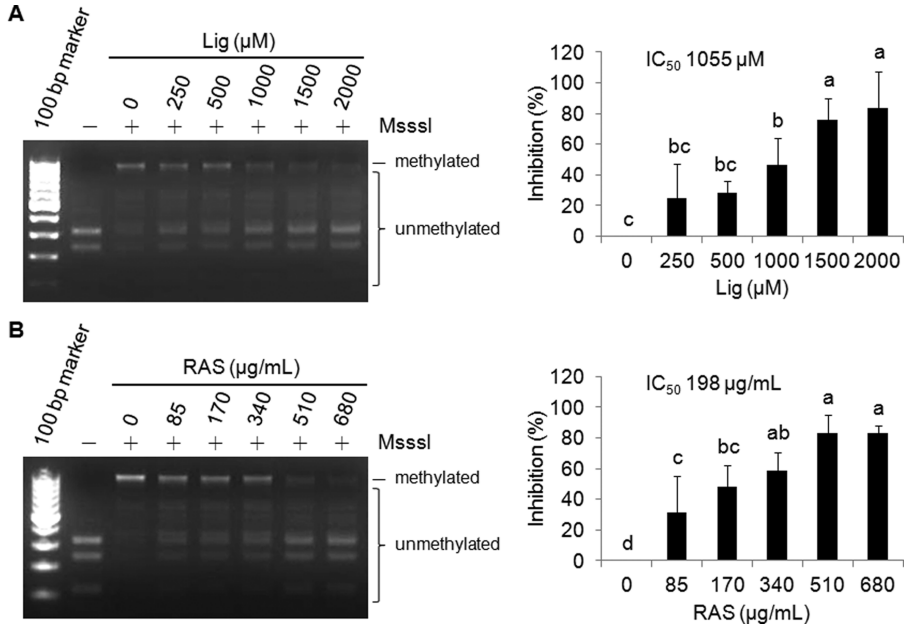

Figure 7. Inhibitory effect of Lig (A) and RAS (B) on purified recombinant DNA methyltransferase activity. Substrate DNA was incubated with various concentrations of Lig or RAS and purified M.SssI methylase at 37 °C for 1 h. The BstUI restriction enzyme was then added to digest the unmethylated DNA at 60 °C for 1 h. The bands of methylated DNA were visualized by ethidium bromide staining and documented with a Gel Documentation 2000 system (Bio-Rad). The inhibition of methylation by Lig or RAS was calculated by comparison with the level of DNA methylation catalyzed by M.SssI in the absence of Lig or RAS. The data are expressed as means ± SD of three independent experiments. Data not sharing the same letter (a, b, c, or d) are significantly different from one another ($P < 0.05$).

methylation, histone modifications that affect chromatin structure are closely associated with gene regulation and carcinogenesis.[49] Histones can be posttranslationally modified by acetylation, which is reversed by histone deacetylases (HDACs).[50] We analyzed the mRNA expression of DNMTs using qPCR and the protein expression of DNMTs by Western blotting in TRAMP C1 cells. Our results indicated that Lig (0−50 μM) and RAS (0−8.5 μg/mL) did not affect either the mRNA or protein expression levels of DNMT1, DNMT3a, and DNMT3b in comparison with control cells (data not shown). There were also no significant differences in the protein expression of HDACs (HDAC1−HDAC5 and HDAC8) between the control and Lig (0−50 μM) or RAS (0−8.5 μg/mL) treatments (data not shown). We examined the inhibitory effect of Lig and RAS on DNA methyltransferase activity using the purified recombinant CpG methylase M.SssI, which is significantly structurally similar to the DNMT1 catalytic domain and displays strong in vitro DNA methylation activity. A substrate DNA from the promoter region of the human $p16^{Ink4a}$ gene (850 bp PCR product) was incubated with M.SssI, and the unmethylated DNA was recognized and digested into smaller fragments by the restriction enzyme BstUI (Figure 7). Lig and RAS inhibited M.SssI activity in a dose-dependent manner, with IC_{50} values of 1055 μM and 198 μg/mL, respectively (Figure 7A,B). These findings suggest that Lig and RAS are able to block the DNA methyltransferase activity of M.SssI in vitro.

DISCUSSION

Previous studies have shown that Radix *Angelicae Sinensis* (Danggui) possesses anticancer activity.[28,36,37] For example, a chloroform extract of Danggui suppressed growth and induced apoptosis in glioblastoma multiforme brain tumor cells in vitro and in vivo.[28,51] It has also been demonstrated that Z-ligustilide (Lig), one of the major lipophilic phthalides in Danggui, displays cytotoxic effects against human chronic myelogenous K562, murine leukemia L1210, and colon cancer HT-29 cell lines.[36,37] Moreover, an ethanol extract of Danggui protected Neuro 2A cells from β-amyloid peptide (Aβ)-induced toxicity, decreased the reactive oxygen species (ROS) and lipid peroxidation levels, and increased glutathione (GSH) levels.[40] Lig was shown to reduce the extent of H_2O_2-induced rat adrenomedullary chromaffin PC12 cell death through anti-oxidant and anti-apoptotic mechanisms.[52] In ICR mice with forebrain ischemic injury, Lig reduced the level of lipid peroxidation and induced the activities of antioxidant enzymes, including GPx and SOD, in brain tissues.[41] These studies suggested that Danggui might have cancer chemopreventive potential accompanied by an ability to reduce cellular oxidative stress as well as the mechanism of neurological and cardiovascular protection.

In this study, the yield of CO_2 supercritical fluid Danggui extract (RAS) is 0.93 g/100 g containing 62.3% Lig determined by HPLC. Additionally, in Asian countries such as China, the recommended dosage is 10 g of Danggui per day in the clinic.[53] It has also been reported that the bioavailability of Lig is ∼2.6% via oral administration.[54] This information suggested that there might be 1.5 mg of Lig in the bloodstream after oral administration of 10 g of Danggui or 93 mg RAS that would contain 58 mg Lig. We previously demonstrated that RAS and Lig induce ARE luciferase activity in HepG2-C8 cells in a dose-dependent manner (unpublished data). Lig (100 μM) and a supercritical CO_2 extract of Danggui (8.5−34 μg/mL) displayed cytotoxic effects against murine prostate cancer TRAMP C1 cells in this study (Figure 2). We further investigated the chemopreventive activity of RAS and Lig through the epigenetic reactivation of Nrf2 and the induction of antioxidant and detoxification enzymes. The results show that

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both Lig (6.25−50 μM) and RAS (1.06−8.5 μg/mL) increase the levels of mRNA and protein expression of Nrf2 and induce the expression of Nrf2-targeted antioxidant and detoxification enzymes, including HO-1, NQO1, and UGT1A1, in TRAMP C1 cells in a dose-dependent manner (Figures 3 and 4). In our previous study of prostate tumorigenesis in TRAMP mice, we found that a progressive loss of the mRNA expression of antioxidant and detoxification enzymes such as NQO1, UGT, GST, and HO-1 and their upstream regulator Nrf2 was caused by epigenetic CpG methylation of the Nrf2 promoter.[24,37] A γ-tocopherol-rich mixed tocopherol diet has also been reported to inhibit the progression of prostate cancer and restores the expression of Nrf2 and its target genes, UGT, GST, GPx, and HO-1.[37] In addition, previously we have also shown that lower mRNA and protein levels of Nrf2 are correlated with the hypermethylation ratio of the Nrf2 gene promoter region in TRAMP prostate tumors and tumorigenic TRAMP C1 cells as compared to nontumorigenic TRAMP C3 cells and normal prostate tissue with the hypomethylated Nrf2 gene promoter.[42] Thus, Lig and RAS are potential chemopreventive agents against prostate cancer through Nrf2-mediated antioxidant and detoxification pathway in which Nrf2 gene expression might be regulated by epigenetic modification.

Prostate cancer is one of the most frequently diagnosed cancers. Advanced metastatic human prostate cancer has an extremely high lethality rate, and there is a growing body of evidence that epigenetic modification is a major driver of prostate cancer.[5] Hypermethylation of CpG islands located on the promoters of various tumor suppressor genes inhibits transcription initiation of these genes in prostate cancer.[5,55,56] DNA methyltransferases (DNMT) are important enzymes that cause the epigenetic inactivation of critical cancer-related genes in human prostate cancer cells.[7] We previously demonstrated that certain dietary compounds restore Nrf2 and its downstream antioxidant and detoxification enzymes, resulting in a cancer chemopreventive effect.[20,24,25] Nrf2 is epigenetically silenced during prostate cancer development in TRAMP mice, and hypermethylation of the first five CpG islands of the Nrf2 gene promoter was observed in prostate tumors from TRAMP mice and in TRAMP C1 cells.[42] In the study presented here, we demonstrated by bisulfite sequencing that treatment with Lig (50 μM) or RAS (8.5 μg/mL) decreased the methylated CpG ratio in the Nrf2 gene promoter region in TRAMP C1 cells (Figure 5). The MeDIP assay revealed that the unmethylated DNA ratio was lower in TRAMP C1 cells treated with Lig (50 μM) or RAS (8.5 μg/mL) than in control cells (Figure 6). To understand the mechanism of the DNA demethylation effect of Lig and RAS, we assessed the expression of DNMT1, DNMT3a, and DNMT3b in TRAMP C1 cells treated with Lig or RAS. No significant differences were observed in the expression of DNMT1, DNMT3a, and DNMT3b (mRNA and protein levels) or HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, and HDAC8 (protein level) upon either Lig or RAS treatment versus the control (data not shown). By contrast, in vitro Lig or RAS treatment inhibited the activity of the CpG methylase M.SssI in a dose-dependent manner (Figure 7). Such inhibition required a very large dose, as has been frequently observed in previous studies.[13,45,57] Lig and RAS may decrease the methylated ratio of CpG islands in the Nrf2 promoter region through either suppression of DNMT activity or other epigenetic mechanisms, such as histone modification by methylation, acetylation, or phosphorylation, thus restoring Nrf2 expression in TRAMP C1 cells. Further studies are needed to elucidate the detailed epigenetic mechanism.

In conclusion, our results show that Lig and RAS increase the levels of mRNA and protein expression of Nrf2 through demethylation of the Nrf2 gene promoter in TRAMP C1 cells. The restoration of Nrf2 may be one of the mechanisms driving the induction of Nrf2 downstream target genes, such as phase II detoxifying and antioxidative stress enzymes, including HO-1, NQO1, and UGT1A1. Thus, Lig and RAS are novel potential cancer chemopreventive agents for the treatment of prostate cancer.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

5-aza, 5-azadeoxycytidine; ARE, antioxidant response element; DNMT, DNA methyltransferase; EGCG, epigallocatechin 3-gallate; GPx, glutathione peroxidase; GST, glutathione *S*-transferase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HO-1, heme oxygenase 1; HPLC, high-performance liquid chromatography; Keap1, Kelch-like ECH-associated protein-1; Lig, Z-ligustilide; MeDIP, methylation DNA immunoprecipitation; NQO1, NAD(P)H:quinine oxidoreductase-1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; qPCR, quantitative real time-PCR; RAS, supercritical CO_2 extract of Danggui; SOD, superoxide dismutase; TRAMP, transgenic adenocarcinoma of mouse prostate; TSA, trichostatin A; UGT1A1, UDP-glucuronosyltransferase 1A1

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