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Research Paper

Synergistic Effects of a Combination of Dietary Factors Sulforaphane and (−) Epigallocatechin-3-gallate in HT-29 AP-1 Human Colon Carcinoma Cells

Sujit Nair,[1] Vidya Hebbar,[2] Guoxiang Shen,[2] Avantika Gopalakrishnan,[2] Tin Oo Khor,[2] Siwang Yu,[2] Changjiang Xu,[2] and Ah-Ng Kong[2,3]

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Purpose. The objective of this study was to investigate combinations of two chemopreventive dietary factors: EGCG 20 μM (or 100 μM) and SFN (25 μM) in HT-29 AP-1 human colon carcinoma cells.
Methods. After exposure of HT-29 AP-1 cells to SFN and EGCG, individually or in combination, we performed AP-1 luciferase reporter assays, cell viability assays, isobologram analyses, senescence staining, quantitative real-time PCR (qRT-PCR) assays, Western blotting, and assays for HDAC activity and hydrogen peroxide. In some experiments, we exposed cells to superoxide dismutase (SOD) or Trichostatin A (TSA) in addition to the treatment with dietary factors.
Results. The combinations of SFN and EGCG dramatically enhanced transcriptional activation of AP-1 reporter in HT-29 cells (46-fold with 25 μM SFN and 20 μM EGCG; and 175-fold with 25 μM SFN and 100 μM EGCG). Isobologram analysis showed synergistic activation for the combinations with combination index, CI<1. Interestingly, co-treatment with 20units/ml of SOD, a free radical scavenger, attenuated the synergism elicited by the combinations (2-fold with 25 μM SFN and 20 μM EGCG; and 15-fold with 25 μM SFN and 100 μM EGCG). Cell viability assays showed that the low-dose combination decreased cell viability to 70% whereas the high-dose combination decreased cell viability to 40% at 48 h, with no significant change in cell viability at 24 h as compared to control cells. In addition, 20 μM and 100 μM EGCG, but not 25 μM SFN, showed induction of senescence in the HT-29 AP-1 cells subjected to senescence staining. However, both low- and high-dose combinations of SFN and EGCG attenuated the cellular senescence induced by EGCG alone. There was no significant change in the protein levels of phosphorylated forms of ERK, JNK, p38, and Akt-Ser473 or Akt-Thr308. Besides, qRT-PCR assays corroborated the induction of the luciferase gene seen with the combinations in the reporter assay. Relative expression levels of transcripts of many other genes known to be either under the control of the AP-1 promoter or involved in cell cycle regulation or cellular influx–efflux such as cyclin D1, cMyc, ATF-2, Elk-1, SRF, CREB5, SLCO1B3, MRP1, MRP2 and MRP3 were also quantified by qRT-PCR in the presence and absence of SOD at both 6 and 10 h. In addition, pre-treatment with 100 ng/ml TSA, a potent HDAC inhibitor, potentiated (88-fold) the synergism seen with the low-dose combination on the AP-1 reporter transcriptional activation. Cytoplasmic and nuclear fractions of treated cells were tested for HDAC activity at 2 and 12 h both in the presence and absence of TSA, however, there was no significant change in their HDAC activity. In addition, the H_2O_2 produced in the cell system was about 2 μM for the low-dose combination which was scavenged to about 1 μM in the presence of SOD.
Conclusion. Taken together, the synergistic activation of AP-1 by the combination of SFN and EGCG that was potentiated by HDAC inhibitor TSA and attenuated by free radical scavenger SOD point to a possible multifactorial control of colon carcinoma that may involve a role for HDACs, inhibition of cellular senescence, and SOD signaling.

KEY WORDS: AP-1; colon cancer; dietary factors; EGCG; isothiocyanate; sulforaphane.

INTRODUCTION

Colorectal cancer (cancer of the colon or rectum), according to the Centers for Disease Control and Prevention (CDC) (1), is the second leading cause of cancer-related deaths and the third most common cancer in men and in women in the United States. In addition, the National Cancer Institute (NCI)'s Surveillance Epidemiology and End Results (SEER) Statistics Fact Sheets (2) show that, based on rates from 2001–2003, 5.56% of men and women born today will be diagnosed with cancer of the colon and rectum during their lifetime, i.e., 1 in 18 men and women in the United States are at a lifetime

[1] Graduate Program in Pharmaceutical Science, Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA.
[2] Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, USA.
[3] To whom correspondence should be addressed (e-mail: kongT@rci.rutgers.edu)

ABBREVIATIONS: AP-1, activator protein; EGCG, (−) epigallocatechin-3-gallate; HDAC, histone deacetylase; MAPK, mitogen-activated protein kinase; qRT-PCR, quantitative real-time PCR; SFN, sulforaphane; SOD, superoxide dismutase; TSA, Trichostatin A.

risk of developing colorectal cancer. Since colorectal cancer is initiated in colonic crypts, a succession of genetic mutations or epigenetic changes can lead to homeostasis in the crypt being overcome, and subsequent unbounded growth (3). Using mathematical models of tumorigenesis through failure of programmed cell death or differentiation, it was predicted (4) that exponential growth in cell numbers does sometimes occur, usually when stem cells fail to die or differentiate. At other times, exponential growth does not occur, instead, the number of cells in the population reaches a new, higher equilibrium which may explain many aspects of tumor behavior including early premalignant lesions such as cervical intraepithelial neoplasia (4). The development of colon cancer results from the sequential accumulation of activating mutations in oncogenes, such as *ras*, and inactivating mutations, truncations, or deletions in the coding sequence of several tumor suppressor genes, including *p53* and *adenomatous polyposis coli* (*APC*), together with aberrant activity of molecules controlling genomic stability (5,6).

Epidemiological studies have revealed an inverse correlation between the intake of cruciferous vegetables and the risk of certain types of cancer (7,8). It has also been reported (9) that because elevated vegetable consumption has been associated with a lower risk of colorectal cancer, vegetables may have a stronger role in preventing the progression of adenomas to carcinomas rather than in preventing the initial appearance of adenomas. Isothiocyanates are a chemical class of compounds that are not naturally present in cruciferous vegetables, such as broccoli and cauliflower, but are nevertheless generated from hydrolysis of secondary metabolites known as glucosinolates by the enzyme myrosinase during the process of vegetable crushing or mastication (10). Also, they may be produced in the intestines where resident microflora can promote the hydrolysis of glucosinolates to isothiocyanates (11). Sulforaphane (SFN), an isothiocyanate compound found at high levels in broccoli and broccoli sprouts, is a potent inducer of phase 2 detoxification enzymes and inhibits tumorigenesis in animal models (12). Indeed, sulforaphane has been implicated in a variety of anti-carcinogenic mechanisms including effects on cell cycle checkpoint controls and cell survival and/or apoptosis in various cancer cells (12), however, epidemiological studies indicate (13) that the protective effects in humans may be influenced by individual genetic variation (polymorphisms) in the metabolism and elimination of isothiocyanates from the body. Recently, we reported (14) that SFN induces hemoxygenase-1 (HO-1) by activating the antioxidant response element (ARE) through the induction of Nrf2 protein in HepG2 cells and that overexpression of all four p38 mitogen-activated protein kinase (MAPK) isoforms negatively regulated the constitutive and inducible ARE-dependent gene expression. Myzak *et al.* (12) have also reported inhibition of histone deacetylase (HDAC) as a novel mechanism of chemoprotection by SFN.

Green tea polyphenol (−) epigallocatechin-3-gallate (EGCG) is noted to suppress colonic tumorigenesis in animal models and epidemiological studies. The water-extractable fraction of green tea contains abundant polyphenolic compounds, in which EGCG is the major constituent (>50% of polyphenolic fraction) (15). It has been reported that EGCG, when administered to rats, inhibited azoxymethane-induced colon tumorigenesis (16), and also blocked the formation of 1,2-dimethylhydrazine-induced colonic aberrant crypt foci (17), which is a typical precursor lesion of chemical-initiated colon cancer. Recently (18), EGCG was reported to inhibit inflammation-associated angiogenesis by targeting inflammatory cells, mostly neutrophils, and also inhibit the growth of the highly angiogenic Kaposi's sarcoma tumor cells (KS-Imm) in nude mice. We have observed (19) that EGCG treatment causes damage to mitochondria, and that c-*Jun* N-terminal kinase (JNK) mediates EGCG-induced apoptotic cell death in HT-29 human colon cancer cells. EGCG is also reported (20,21) to inhibit DNA methyltransferase with demethylation of the CpG islands in the promoters, and to reactivate methylation-silenced genes such as p16INK4a, retinoic acid receptor beta, O6-methylguanine methyltransferase, human mutL homolog 1, and glutathione S-transferase-pi in human colon cancer HT-29 cells, esophageal cancer KYSE 150 cells, and prostate cancer PC3 cells. These activities could be enhanced by the presence of HDAC inhibitors or by a longer-term treatment (21).

Transcription factor activator protein-1 (AP-1) is a redox-sensitive transcription factor that senses and transduces changes in cellular redox status and modulates gene expression responses to oxidative and electrophilic stresses presumably *via* sulfhydryl modification of critical cysteine residues found on this protein and/or other upstream redox-sensitive molecular targets (22). In budding yeast, the transcription factor Yap1 (yeast AP1), which is a basic leucine zipper (bZip) transcription factor, confers the cellular response to redox stress by controlling the expression of the regulon that encodes most yeast antioxidant proteins (23). AP-1 is responsive to low levels of oxidants resulting in AP-1/DNA binding and an increase in gene expression. AP-1 activation is typically due to the induction of JNK activity by oxidants resulting in the phosphorylation of serine 63 and serine 73 in the c-*Jun* transactivation domain (24–26). With high concentrations of oxidants, AP-1 is inhibited and gene expression is impeded. Inhibition of AP-1/DNA interactions is attributed to the oxidation of specific cysteine residues in c-*Jun*'s DNA binding region, namely cysteine 252 (24,27). Indeed, for AP-1, a nuclear pathway to reduce the Cys of the DNA-binding domain is apparently distinct from the upstream redox events that activate the signaling kinase pathway (28).

The fate of cancer chemopreventive strategies relies largely on the ability to maximally exploit the intrinsic anti-carcinogenic potential of chemopreventive agents, both singly and in combination, without incurring undue toxicity. Targeting multiple signal transduction pathways involved in different stages of carcinogenesis by use of combinatorial approaches would ideally empower the clinician to better manage or delay the progression of the disease. Given the abundance of literature on the multifarious anti-carcinogenic mechanisms of SFN and EGCG, we investigated the combinations of these two dietary factors and the role(s) mediated by redox transcription factor AP-1 in modulating the anti-cancer potential of this putative chemopreventive combination for the management of colon cancer.

MATERIALS AND METHODS

Cell Culture and Reagents. Human colon carcinoma HT-29 cells were stably transfected with an Activator Protein (AP-1) luciferase reporter construct, and are referred to as HT-29 AP-1 cells. The cells were cultured in Minimum

Essential Medium (MEM) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin–Streptomycin. Twelve hours prior to experimental treatments, the cells were exposed to MEM containing 0.5% FBS. Sulforaphane (SFN) was obtained from LKT Labs, (−) epigallocatechin-3-gallate (EGCG), superoxide dismutase (SOD) were obtained from Sigma-Aldrich Co., and Trichostatin A (TSA) was obtained from Biomol. SFN, EGCG and TSA were dissolved in dimethylsulfoxide (DMSO, Sigma), whereas SOD was dissolved in 1X phosphate-buffered saline (PBS).

Reporter Gene Assays. HT-29 AP-1 cells were seeded in six-well culture plates and treated in duplicate with dimethylsulfoxide (control), 20 μM EGCG, 100 μM EGCG, 25 μM SFN, 20 μM EGCG+25 μM SFN, or 100 μM EGCG+25 μM SFN for 24 h. Thereafter, the supernatant medium was aspirated on ice, cells were washed thrice with ice-cold 1X PBS, treated with 1X Luciferase Reporter Lysis Buffer (Promega) and subjected to one cycle of snap freeze-thaw at −80°C. Cell lysates were harvested with sterile RNAse-free and DNAse-free cell scrapers into microcentrifuge tubes that were immediately placed on ice. They were then centrifuged at 4°C for 10 min at 13,000×*g* and returned to ice. Twenty microliters of supernatant solution was analyzed for relative luciferase activity using a Sirius Luminometer (Berthold Detection Systems). The relative luciferase activities were normalized by protein concentrations of individual samples as described below.

Protein Assays. Protein concentrations of samples were determined by the bicinchonic acid-based BCA Protein Assay Kit (Pierce) according to the manufacturer's instructions using a 96-well plate. Standard curves were constructed using bovine serum albumin (BSA) as a standard. The sample readings were obtained on a μQuant microplate reader (Bio-tek Instruments, Inc.) at 560 nm.

Western Blotting. HT-29 AP-1 cells were subjected to treatment with different dietary factors for one or 2 h and harvested on ice with either 1X Whole Cell Lysis Buffer or 1X MAPK Buffer. Protein (20 μg) was boiled with sample loading buffer containing β-mercaptoethanol and loaded onto 18-well Criterion Pre-cast gels (Bio-Rad) with Precision Plus Dual Color Protein Marker (Bio-Rad). Electrophoresis was performed at 200 V and semi-dry transfer of each gel was effected onto a polyvinylidene difluoride (PVDF) membrane in an electroblotter at 130 mA for 1.5 h. The membranes were then blocked with 5% BSA in TBST for 1 h, washed thrice for 10 min each with 1X TBST, and incubated with primary antibodies against ERK, JNK, p38, AKT Ser473, AKT Thr308, and Actin (Cell Signaling Inc.) in 3% BSA in TBST (1:2,000 antibody dilutions, except beta-actin which was 1:1,000) for 1 h at room temperature with gentle agitation. After further three washes with 1X TBST, the membranes were incubated with appropriate secondary antibodies in 3% BSA in TBST (1:2,000 for P-ERK and P-JNK, 1:5,000 for P-p-38 and beta-actin, and 1:10,000 for phosphorylated forms of Akt) overnight at 4°C with gentle rocking. The following day, the membranes were washed again thrice with 1X TBST and treated with ECL chemiluminescence reagent (Pierce) and visualized using a Bio-Rad Imaging Station. The protein expression was normalized against that of actin as a control.

Cell Viability Assays. The cell viability assays were performed in 24-well cell culture plates using MTS Assay Kit (Promega) according to the manufacturer's instructions. Cell viability was determined at both 24 and 48 h after treatment with dietary factors. The absorbance readings were obtained on an μQuant microplate reader (Bio-tek Instruments, Inc.) at recommended wavelength of 490 nm.

RNA Extraction and Assessment of RNA Integrity. HT-29 AP-1 cells were subjected to treatment with different dietary factors in triplicate for 6 or 10 h. RNA was harvested using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA integrity was assessed using formaldehyde gels in 1× MOPS buffer and RNA concentration was determined by the 260/280 ratio on a DU 530 UV/Visible spectrophotometer (Beckman).

Quantitative Real-time PCR Assays. Several genes of interest including luciferase gene as well as genes known to be either under the control of the AP-1 promoter or involved in cell cycle regulation or cellular influx–efflux such as cyclin D1, cMyc, ATF-2, Elk-1, SRF, CREB5, MDR1, SLCO1B3, MRP1, MRP2 and MRP3 were selected for quantitative real-time PCR analyses both in the presence or absence of SOD treatment. Beta-actin served as the "housekeeping" gene. The specific primers for these genes listed in Table I were designed by using Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and were obtained from Integrated DNA Technologies, Coralville, IA. The specificity of the primers was examined by a National Center for Biotechnology Information Blast search of the human genome. For the real-time PCR assays, briefly, after the RNA extraction and assessment of RNA integrity, first-strand cDNA was synthesized using 4 μg of total RNA following the protocol of SuperScript III First-Strand cDNA Synthesis System (Invitrogen) in a 40 μl reaction volume. The PCR reactions based on SYBR Green chemistry were carried out using 100 times diluted cDNA product, 60 nM of each primer, and SYBR Green master mix (Applied Biosystems, Foster City, CA) in 10 μl reactions. The PCR parameters were set using SDS 2.1 software (Applied Biosystems, Foster City, CA) and involved the following stages: 50°C for 2 min, 1 cycle; 95°C for 10 min, 1 cycle; 95°C for 15 s→55°C for 30 s→72°C for 30 s, 40 cycles; and 72°C for 10 min, 1 cycle. Incorporation of the SYBR Green dye into the PCR products was monitored in real time with an ABI Prism 7900HT sequence detection system, resulting in the calculation of a threshold cycle (C_T) that defines the PCR cycle at which exponential growth of PCR products begins. The carboxy-X-rhodamine (ROX) passive reference dye was used to account for well and pipetting variability. A control cDNA dilution series was created for each gene to establish a standard curve. After conclusion of the reaction, amplicon specificity was verified by first-derivative melting curve analysis using the ABI software; and the integrity of the PCR reaction product and absence of primer dimers was ascertained. The gene expression was determined by normalization with control gene beta-actin.

Hydrogen Peroxide Assays. The levels of hydrogen peroxide in the cell-free medium was ascertained by the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes/Invitrogen) according to the manufacturer's instructions. Briefly, a working solution of 100 μM Amplex Red reagent and 0.2 U/ml HRP was prepared, of which 50 μl was added to each microplate well containing the positive control

Table I. Oligonucleotide Primers used for Quantitative Real-time PCR (qRT-PCR)

Gene Name	GenBank Accession No.	Forward Primer	Reverse Primer
Photinus pyralis (firefly) luciferase	M15077	5′-TCATTCTTCGCCAAAAGCACT-3′	5′-GCTTCCCCGACTCTTTCG-3′
Homo sapiens cyclin D1	NM_053056	5′-CCCGCACGATTTCATTGAAC-3′	5′-CACAGAGGGCAACGAAGGTC-3′
Homo sapiens c-myc binding protein (MYCBP)	NM_012333	5′-CTGCTTCGCCTAGAACTGGC-3′	5′-TGAGGTGGTTCATACTGAGCAAG-3′
Homo sapiens activating transcription factor 2 (ATF2)	NM_001880	5′-GGTCATGGTAGCGGATTGGT-3′	5′-GCTGGAGAAGCCGGAGTTTC-3′
Homo sapiens ELK1, member of ETS oncogene family (ELK1)	NM_005229	5′-TGGACCCATCTGTGACGCT-3′	5′-GAATTCACCACCATCCCGTG-3′
Homo sapiens serum response factor (c-*fos* serum response element-binding transcription factor, SRF)	NM_003131	5′-TCAACTCGCCAGACTCTCCA-3′	5′-CTCCGACACCTGGTAGGTGAG-3′
Homo sapiens cAMP responsive element binding protein 5 (CREB5)	NM_182898	5′-GACGAGGATCCGGACGAGA-3′	5′-TCCAATGACATCACCCAGACC-3′
Homo sapiens solute carrier organic anion transporter family, member 1B3 (SLCO1B3)	NM_019844	5′-GCTTTGCACTGGGATCTCTGT-3′	5′-TCCAACCCAACGAGAGTCCT-3′
Homo sapiens ATP-binding cassette, sub-family C (CFTR/MRP), member 1 (ABCC1)	NM_004996	5′-GCGCTGGCTTCCAACTATTG-3′	5′-AGGGCTCCATAGACGCTCAG-3′
Homo sapiens ATP-binding cassette, sub-family C (CFTR/MRP), member 2 (ABCC2)	NM_000392	5′-GTCATGATCTGCATGGCCAC-3′	5′-TGGCGGAGGTAGACACATAA-3′
Homo sapiens ATP-binding cassette, sub-family C (CFTR/MRP), member 3 (ABCC3)	NM_003786	5′-GGTCAGCACCCCTGCAGAT-3′	5′-TGGAATCAGCAAGACCATGAAA-3′
Homo sapiens cytoplasmic beta-actin	M10277	5′-CCCAGCCATGTACGTTGCTA-3′	5′-CAGTGTGGGTGACCCCGT-3′

(10 μM H_2O_2), negative control (1X Reaction Buffer without H_2O_2) and test samples. The fluorescence signal was measured on a FLx-800 microplate fluorescent reader (Biotek Instruments, Inc.) at excitation wavelength of 560 nm and emission wavelength of 590 nm.

HDAC Activity Assays. Cytoplasmic and nuclear fractions of cells treated with dietary factors, both in the presence and absence of 100 ng/ml TSA, were extracted using the NePer extraction kit (Pierce). The HDAC activity was determined using a Fluor-de-Lys HDAC Fluorescent Activity Assay Kit (Biomol) according to the manufacturer's instructions. Briefly, incubations were performed at 37°C for 10 min with HeLa nuclear cell extracts containing known HDAC activity that were provided by the manufacturer. The HDAC reaction was initiated by the addition of Fluor-de-Lys substrate. After 10 min, the reaction was quenched by adding the Fluor-de-Lys Developer, and the mixture was incubated for another 10 min at ambient temperature. The fluorescence signal was measured using a FLx-800 microplate fluorescent reader (Bio-tek Instruments, Inc.) at excitation wavelength of 360 nm and emission wavelength of 460 nm.

Senescence Staining. HT-29 AP-1 cells were grown on cover slips and treated with DMSO (control), individual dietary factors, or combinations of dietary factors. The X-gal-based staining was performed using the Senescence Assay Kit (Sigma-Aldrich Co.) according to the manufacturer's instructions. Bluish-green stain was positive for senescence-associated beta-galactosidase activity. The slides were fixed and images were obtained using a Nikon Eclipse E600 microscope (Micron-Optics, Cedar Knolls, NJ) equipped with DXM 1200 Nikon Digital Camera.

Statistical Analyses. Data are expressed as mean ± standard deviation, and comparisons among treatment groups were made using one-way analysis of variance (ANOVA) followed by a post hoc test for multiple comparisons—the Tukey's Studentized Range Honestly Significant Difference (HSD) test. In all these multiple comparisons, $P<0.05$ was considered statistically significant. When only two groups of treatment means were evaluated, we employed paired, two-tailed Student's t-test ($P<0.01$ was considered significant) or paired, one-tailed Student's t-test ($P<0.05$ was considered significant) as indicated in the text where applicable. Statistical analyses were performed using SAS 9.1 software (SAS Institute Inc, NC) licensed to Rutgers University.

RESULTS

Transactivation of AP-1 Luciferase Reporter by Combinations of SFN and EGCG

As shown in Fig. 1, treatment of HT-29 AP-1 cells for 24 h with either SFN 25 μM, EGCG 20 μM or EGCG 100 μM individually resulted in about fivefold induction of AP-1 luciferase activity as compared to control cells that were treated with DMSO. Surprisingly, a low-dose combination of SFN 25 μM+EGCG 20 μM elicited a dramatic induction of AP-1 luciferase activity (over 45-fold). In addition, a high-dose combination of SFN 25 μM+EGCG 100 μM further

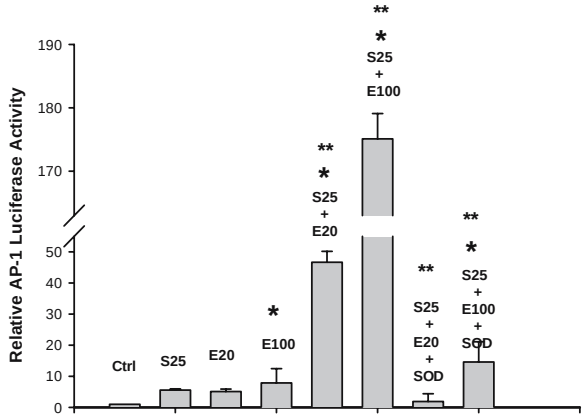

Fig. 1. Transactivation of AP-1 luciferase reporter by combinations of SFN and EGCG, and attenuation by SOD. HT-29 AP-1 cells were seeded in six-well plates and treated with individual dietary factors or with combinations of SFN and EGCG, as indicated, in the absence or presence of 20 U/ml SOD. The AP-1 luciferase activity was measured relative to vehicle control (DMSO) after 24 h of incubation and normalized against protein concentration. Values represent mean ± standard deviation for three replicates, and are representative of seven independent experiments. *P<0.05, significantly different from vehicle control (Ctrl); **P<0.05, significantly different from each other.

potentiated the induction of AP-1 luciferase activity to about 175-fold. We also investigated the effects of pre-treatment on the induction of AP-1 luciferase activity. In these experiments (data not shown), we first pre-treated the HT-29 AP-1 cells for 6 h with EGCG (20 or 100 μM), then washed off the EGCG thrice with phosphate-buffered saline (PBS) and treated the cells with SFN 25 μM for an additional 18 h before assaying for luciferase activity. Alternatively, we also pre-treated the cells with SFN 25 μM for 6 h before washing with PBS as above and treating with EGCG (20 μM or 100 μM) for an additional 18 h. It was observed that there was no significant difference in induction of AP-1 luciferase activity in these pre-treatment experiments (data not shown) as compared to when the two agents were co-treated as shown in Fig. 1. This enabled us to rule out any physicochemical interaction between the two agents in cell culture when co-treated that may have otherwise produced any experimental artifacts in the luciferase assay. Hence, since the effects of the combinations when co-treated were not physicochemical, but potentially modulated at a mechanistic level, we continued all our experiments by co-treating both agents together for 24 h for ease of experimentation without confounding variables.

SOD Attenuates the Synergism Elicited by Combinations of SFN and EGCG

Since EGCG is known to produce oxidative stress (19), we also investigated whether the effects of the SFN and EGCG combinations on AP-1 luciferase induction were mediated, in part, by the free radical scavenger SOD. Accordingly, we also co-treated the combinations with 20 U/ml of SOD before assaying for AP-1 luciferase activity. Interestingly, the co-treatment with SOD significantly attenuated the induction observed with the SFN + EGCG combinations in the HT-29 AP-1 cells. The relative luciferase activity of the SFN 25 μM+EGCG 20 μM combination (over 45-fold) was attenuated to about twofold in the presence of SOD; whereas the relative luciferase activity of the SFN 25 μM+EGCG 100 μM combination (175-fold) was attenuated to about 15-fold in the presence of SOD as shown in Fig. 1 indicating that SOD signaling may play a role in modulation of AP-1 luciferase activity by these chemopreventive combinations.

Isobologram Analyses and Combination Indices for the Combinations of SFN and EGCG

In order to confirm the synergistic interaction observed in the luciferase assays with the combinations of SFN and EGCG, we performed isobologram analyses as reported by Zhao *et al.* (29). Twenty-five combinations of SFN (2.5, 5, 7.5, 10, 12.5 μM) with EGCG (2.5, 5, 7.5, 10, 12.5 μM) were tested in addition to the SFN 25 μM+EGCG 20 μM combination. Nine of these combinations that showed same effect as individual agents in terms of fivefold induction of AP-1 luciferase activity were selected for isobologram analyses. Under the conditions of the analyses, all the combinations tested showed synergistic interaction in the isobologram analyses as shown in Fig. 2. This confirmed the synergistic nature of the interaction between the combinations and indicated that lower doses of SFN with EGCG would also be able to elicit synergistic transactivation of the AP-1 luciferase reporter although to a lesser degree. In addition, as reported by Zhao *et al.* (29), we evaluated the combination indices that were generated by these combinations in these analyses, and it was observed that, in conformity with the general consensus, all the synergistic combinations had a value of combination index <1 (ranging from 0.325 to 0.7, data not shown) which further confirmed the synergistic interaction between the combinations of SFN with EGCG.

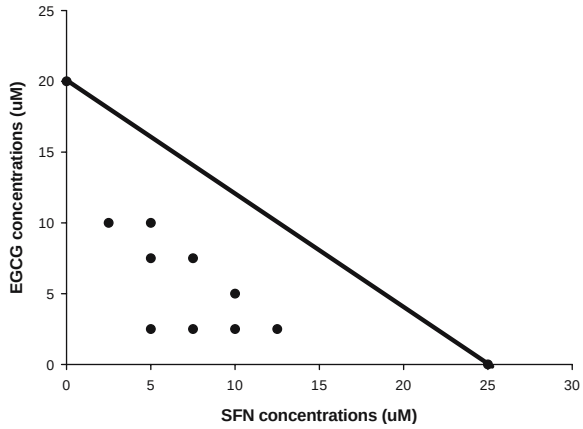

Fig. 2. Isobologram analyses of synergy between combinations of SFN and EGCG. Several combinations of individual dietary factors SFN and EGCG were analyzed for synergy by the method of isobologram analysis as described elsewhere (29) and were confirmed as synergistic. Data points are described by concentrations (in μM) of SFN and EGCG reflected on x- and y-axes respectively, and are representative of three independent experiments. The corresponding combination indices ranged from 0.325 to 0.7 (data not shown) which further confirmed the synergy between the combinations of SFN and EGCG.

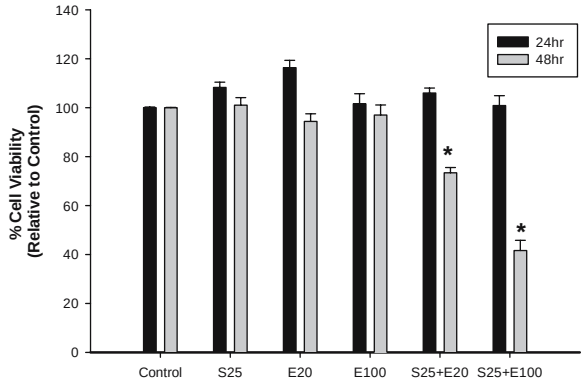

Fig. 3. Viability of the HT-29 AP-1 cells with the combinations of SFN and EGCG. HT-29 AP-1 cells were treated with individual dietary factors or with combinations of SFN and EGCG for 24 or 48 h as indicated and treated with MTS assay reagent to ascertain cell viability. Values represent mean ± standard deviation for six replicates, and are representative of three independent experiments. **P*<0.05, significantly different from control.

Viability of the HT-29 AP-1 Cells with the Combinations of SFN and EGCG

In order to ascertain the effects of the combinations of SFN and EGCG on the cell viability of the HT-29 AP-1 cells, we used the MTS assay with treatment durations of 24 and 48 h. As shown in Fig. 3, there was no significant change in cell viability at 24 h between the combination treatments and the individual agent treatments relative to the control cells showing that the doses used were non-toxic to the cells at 24 h. At 48 h, however, the viability of cells treated with combinations comprising SFN 25 μM+EGCG 20 μM and SFN 25 μM+EGCG 100 μM decreased to 70 and 40% respectively relative to control cells indicating that the low-dose combination of SFN 25 μM+EGCG 20 μM may be more appropriate to pursue in longer duration *in vitro* studies or potential *in vivo* studies without seemingly toxic effects *a priori,* and at the same time not compromising on the synergistic efficacy elicited by the combination of these two chemopreventive agents.

Inhibition of EGCG-Induced Senescence by the Combinations of SFN and EGCG

Since EGCG is known to inhibit telomerase and induce senescence in leukemic cells (30), we also investigated the effects of the combinations of SFN and EGCG on senescence-associated beta-galactosidase activity by a standard staining procedure as described in Materials and Methods. As shown in Fig. 4, HT-29 AP-1 cells, when cultured on cover slips and exposed to individual treatment of EGCG (20 or 100 μM) for 24 h, showed induction of senescence which was not observed in the case of SFN 25 μM. Interestingly, on combining EGCG with SFN, the EGCG-associated senescence was attenuated. Both combinations comprising SFN 25 μM+EGCG 20 μM, and, SFN 25 μM+EGCG 100 μM attenuated the cellular senescence induced by EGCG suggesting that the synergistic effects of the combination on AP-1 transactivation may also be potentially mediated in part *via* inhibition of cellular senescence pathways.

Temporal Gene Expression Profiles Elicited by Combinations of SFN and EGCG and Attenuation by SOD

We performed quantitative real-time PCR (qRT-PCR) experiments with primers (Table I) for the luciferase gene to

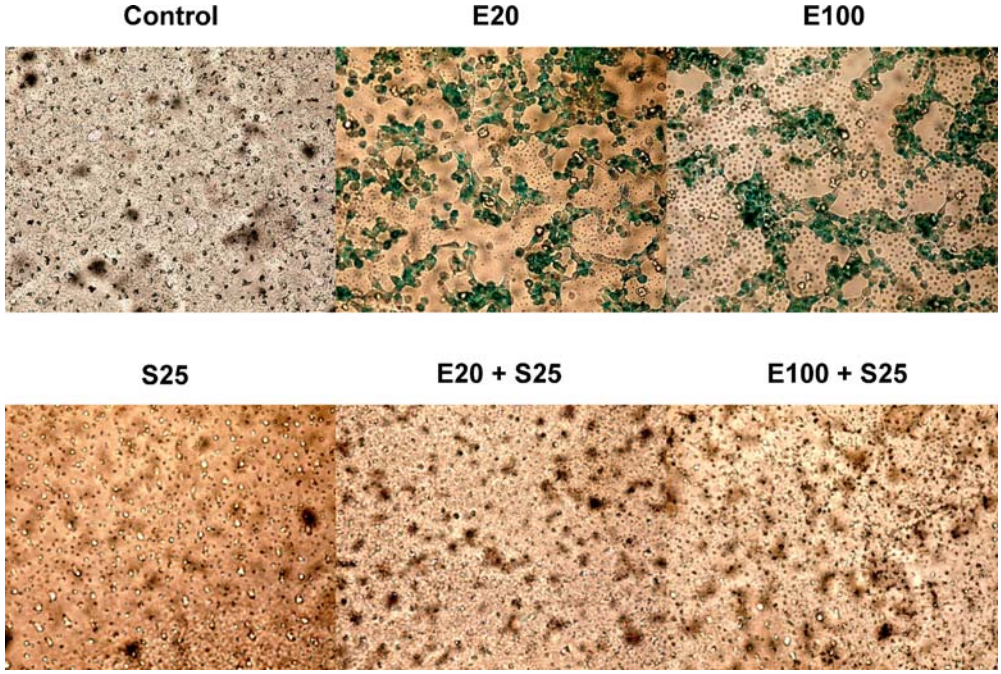

Fig. 4. Inhibition of EGCG-induced senescence by the combinations of SFN and EGCG. HT-29 AP-1 cells were cultured on cover slips and treated with individual dietary factors or with combinations of SFN and EGCG for 24 h. The cells were then fixed and subjected to a histochemical stain for β-galactosidase activity following which they were examined microscopically for senescence. Images are representative of three independent experiments.

Table II. Temporal Gene Expression Profiles Elicited by Combinations of SFN and EGCG

	Luc 6 h	Luc 10 h	CyD1 6 h	CyD1 10 h	cMyc 6 h	cMyc 10 h	ATF-2 6 h	ATF-2 10 h	Elk-1 6 h	Elk-1 10 h	SRF 6 h	SRF 10 h	CREB5 6 h	CREB5 10 h	SLCO 6 h	SLCO 10 h	MRP1 6 h	MRP1 10 h	MRP2 6 h	MRP2 10 h	MRP3 6 h	MRP3 10 h
Control (DMSO)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
S25	2.78± 0.16	2.3± 0.10	1.09± 0.15	0.89± 0.23	1.03± 0.31	1.28± 0.34	2.64± 0.18	1.43± 0.22	2.84± 0.10	1.32± 0.26	1.26± 0.35	0.88± 0.2	0.32± 0.06	0.15± 0.04	0.36± 0.10	0.40± 0.15	1.63± 0.19	0.93± 0.11	2.71± 0.24	2.30± 0.28	2.37± 0.32	0.84± 0.16
E20	1.16± 0.22	1.78± 0.29	1.71± 0.31	1.32± 0.19	1.08± 0.24	1.17± 0.17	1.07± 0.16	1.10± 0.27	1.12± 0.13	1.11± 0.17	0.49± 0.09	1.16± 0.11	0.46± 0.20	0.10± 0.03	1.47± 0.22	1.27± 0.16	2.11± 0.28	1.39± 0.21	1.79± 0.16	1.85± 0.21	1.59± 0.25	1.07± 0.10
E100	6.74± 0.54	2.73± 0.25	3.30± 0.47	1.22± 0.16	1.04± 0.09	0.70± 0.15	1.36± 0.25	0.70± 0.14	1.25± 0.2	0.78± 0.14	1.02± 0.23	0.97± 0.14	0.48± 0.18	0.32± 0.21	1.48± 0.18	2.02± 0.31	2.49± 0.37	1.89± 0.19	1.26± 0.15	1.85± 0.27	0.86± 0.19	0.65± 0.23
E20+S25	7.60± 0.78	3.17± 0.37	0.94± 0.26	0.53± 0.14	1.40± 0.23	1.40± 0.37	2.34± 0.35	1.48± 0.23	1.43± 0.31	0.87± 0.15	0.56± 0.12	0.44± 0.07	0.25± 0.13	0.52± 0.19	0.64± 0.23	0.22± 0.11	1.07± 0.17	0.81± 0.05	3.34± 0.25	4.75± 0.68	1.34± 0.14	0.75± 0.08
E100+S25	16.41± 0.98	3.43± 0.41	0.90± 0.32	0.52± 0.22	1.23± 0.17	0.94± 0.22	1.68± 0.16	0.81± 0.25	1.42± 0.15	0.67± 0.21	0.71± 0.16	0.42± 0.13	0.21± 0.06	0.17± 0.08	0.46± 0.16	0.24± 0.09	1.22± 0.33	0.77± 0.12	2.87± 0.20	4.56± 0.74	1.24± 0.06	0.61± 0.11

HT-29 AP-1 cells were treated for 6 or 10 h with individual dietary factors or combinations of SFN and EGCG as indicated. RNA was extracted, transcribed into cDNA after ascertaining RNA integrity, and quantitative real-time PCR assays were performed using beta-actin as the housekeeping gene. Values represent mean±standard deviation for three replicates of each gene, and are representative of two independent experiments.

Table III. Temporal Gene Expression Profiles Elicited by Combinations of SFN and EGCG in the Presence of SOD

	Luc 6 h	Luc 10 h	CyD1 6 h	CyD1 10 h	Cmyc 6 h	cMyc 10 h	ATF-2 6 h	ATF-2 10 h	Elk-1 6 h	Elk-1 10 h	SRF 6 h	SRF 10 h	CREB5 6 h	CREB5 10 h	SLCO 6 h	SLCO 10 h	MRP1 6 h	MRP1 10 h	MRP2 6 h	MRP2 10 h	MRP3 6 h	MRP3 10 h
Control (DMSO)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
S25+SOD	3.18± 0.24	3.61± 0.34	0.83± 0.11	1.10± 0.20	1.18± 0.12	0.63± 0.16	1.91± 0.26	1.33± 0.31	0.81± 0.12	1.26± 0.34	0.60± 0.15	1.50± 0.27	0.25± 0.12	0.46± 0.15	0.83± 0.19	0.63 ±0.10	1.07± 0.21	2.42± 0.32	3.17± 0.26	5.44± 0.38	0.57± 0.15	1.31± 0.22
E20+SOD	0.66± 0.14	1.57± 0.28	1.15± 0.05	2.10± 0.26	1.07± 0.13	1.50± 0.19	1.20± 0.15	0.98± 0.17	1.09± 0.16	1.05± 0.13	1.11± 0.10	1.23± 0.19	0.20± 0.09	0.76± 0.11	1.23± 0.22	1.25± 0.27	1.69± 0.18	1.45± 0.31	3.39± 0.34	1.50± 0.18	1.68± 0.23	1.67± 0.31
E100+SOD	3.24± 0.35	3.97 0.21	0.60± 0.17	0.87± 0.11	0.42± 0.15	0.41± 0.10	0.62± 0.16	0.30± 0.12	0.37± 0.15	0.41± 0.17	1.24± 0.33	1.88± 0.45	0.07± 0.03	0.20± 0.08	0.68± 0.16	0.45± 0.12	0.69± 0.24	0.89± 0.18	5.35± 0.29	0.80± 0.12	0.35± 0.18	0.74± 0.24
E20+S25+SOD	2.21± 0.15	1.58± 0.13	0.80± 0.19	2.01± 0.25	0.86± 0.22	1.14± 0.17	1.99± 0.25	1.43± 0.18	1.57± 0.25	1.21± 0.19	1.12± 0.24	1.37± 0.15	0.12± 0.07	0.54± 0.16	0.72± 0.21	0.64± 0.19	2.26± 0.26	3.46± 0.35	6.96± 0.48	5.38± 0.35	1.69± 0.13	1.52± 0.20
E100+S25+ SOD	6.93± 0.36	1.67± 0.23	0.32± 0.14	1.83± 0.27	0.38± 0.16	0.86± 0.09	0.38± 0.21	0.94± 0.29	1.08± 0.15	1.51± 0.23	0.82± 0.14	1.65± 0.25	0.22± 0.13	0.71± 0.20	0.49± 0.18	0.48± 0.15	1.61± 0.14	3.20± 0.20	1.61± 0.23	7.04± 0.28	0.60± 0.15	1.35± 0.28

HT-29 AP-1 cells were co-treated for 6 or 10 h with 20 U/ml SOD and individual dietary factors or combinations of SFN and EGCG as indicated. RNA was extracted, transcribed into cDNA after ascertaining RNA integrity, and quantitative real-time PCR assays were performed using beta-actin as the housekeeping gene. Values represent mean±standard deviation for three replicates of each gene, and are representative of two independent experiments.

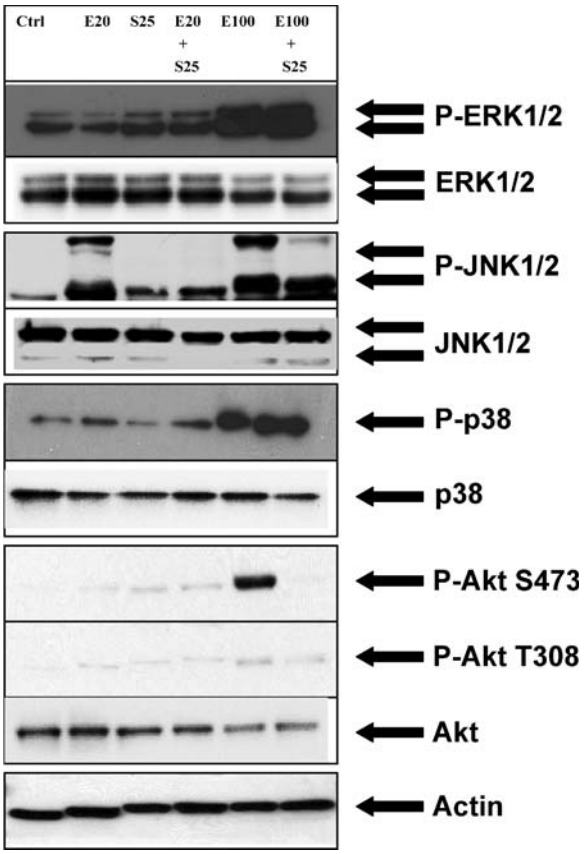

Fig. 5. Protein expression with the combinations of SFN and EGCG. HT-29 AP-1 cells were treated with individual dietary factors or with combinations of SFN and EGCG as indicated for 1 h. Protein was harvested using a MAPK lysis buffer for phosphorylated MAPK or with a whole cell lysis buffer for other proteins. The proteins were immunoblotted using specific antibodies as indicated using actin as the control. Blots are representative of three independent experiments.

corroborate the synergism elicited with the combinations of SFN and EGCG in the luciferase protein assay with mRNA levels in qRT-PCR. As shown in Table II, the temporal expression (at 6 and 10 h) of luciferase gene in qRT-PCR assays was significantly higher ($P<0.01$ at 6 h by a two-tailed, paired Student's t-test; and $P<0.05$ at 10 h by a one-tailed, paired Student's t-test) for the combinations of SFN and EGCG as compared to individual dietary factor treatments in consonance with our data in the luciferase protein assays. The treatment means for all the treatment groups at a specific time point (6 or 10 h) were significantly different from each other ($P<0.05$ by ANOVA and post hoc Tukey's test for multiple comparisons to detect significantly different means). In addition, co-treatment with SOD attenuated the synergism elicited with the combinations of SFN and EGCG at both 6 and 10 h as shown in Table III which also further validated our luciferase data. We also determined by qRT-PCR the relative expression levels of transcripts of many genes that were known to be either under the control of the AP-1 promoter or involved in cell cycle regulation or cellular influx-efflux such as cyclin D1, cMyc, ATF-2, Elk-1, SRF, CREB5, SLCO1B3, MRP1, MRP2 and MRP3 in the absence of SOD (Table II) and in the presence of SOD (Table III).

The low- and high-dose combinations of SFN and EGCG in this study elicited the downregulation of positive cell cycle regulator cyclin D1 expression (Table II) that was further decreased by SOD (Table III) as compared with individual dietary factors. There was, however, no appreciable change in expression of cell proliferation-related cMyc except for its downregulation in the high-dose combination in the presence of SOD. In addition, transcription factors/coactivators that are known to be under the control of the AP-1 promoter such as activating transcription factor (ATF-2), Ets-like transcription factor (Elk-1), serum response factor (SRF) and cyclic AMP response element binding protein 5 (CREB5) were also studied in the absence and presence of SOD (Tables II and III respectively). Interestingly, the expression of ATF-2 with the low-dose combination of SFN and EGCG was similar to that of the SFN-only treatment. The activation of Elk-1 that was observed in our qRT-PCR studies (Table II) was similar for the combinations as well as individual agents which complemented our results for phosphorylated ERK1/2 protein (Fig. 5). Interestingly, the low-dose combination of SFN and EGCG inhibited the transcriptional activation of SRF as compared to individual dietary factors; this inhibition was reversed on co-treatment with SOD at both time points. The combinations had no effect on transcriptional expression of CREB5 as compared to individual agents. Since exogenous stress can potentially stimulate the influx–efflux machinery of cells, we also investigated some key transporter genes (Table II). In this study, we observed the induction of the SLCO1B3 gene, which encodes for the organic anion transporter protein OATP1B3, by EGCG-alone treatments which was reversed by treatment with the combinations of SFN and EGCG (Table II). Interestingly, the combinations of SFN and EGCG greatly induced the expression of the efflux transporter MRP2 as shown in Table II. In addition, the expression of influx transporters MRP1 and MRP3 was lower for the combination-treated cells as compared to the individual agent-treated cells.

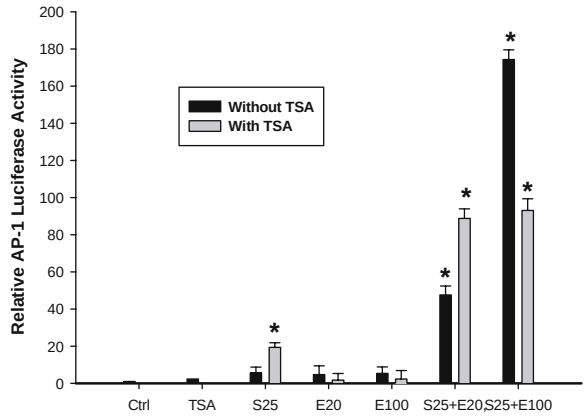

Fig. 6. HDAC inhibitor Trichostatin A potentiates the synergism elicited by the low-dose combination of SFN and EGCG. HT-29 AP-1 cells were seeded in six-well plates and pre-treated with 100 ng/ml TSA for 4 h. Thereafter, they were additionally treated with individual dietary factors or with combinations of SFN and EGCG as indicated for another 24 h. The AP-1 luciferase activity was measured relative to vehicle control (DMSO) and normalized against protein concentration. Values represent mean ± standard deviation for three replicates, and are representative of three independent experiments. * $P<0.05$, significantly different from vehicle control (Ctrl).

Protein Expression with the Combinations of SFN and EGCG

We investigated the effects of the combinations on protein expression of major mitogen-activated protein kinase (MAPK) pathway members including ERK, JNK and p38 as well as the Akt pathway (Fig. 5). Interestingly, although the expression of c-*Jun* N-terminal kinase (JNK) was greater for the combinations relative to the control, it was not greater than the JNK expression of individual agents, suggesting that the SFN+EGCG combination-mediated activation of AP-1 reporter may occur by cellular mechanisms that are exclusive of JNK activation. Similarly, there was no significant change in protein expression of extracellular signal regulated kinase (ERK), p38 or Akt Ser and Akt Thr for the combination-treated cells as compared to the individual agent-treated cells.

HDAC Inhibitor Trichostatin A Potentiates the Synergism Elicited by the Low-Dose Combination of SFN and EGCG

Since inhibition of histone deacetylase (HDAC) has been reported (12) as a novel mechanism of chemoprotection by the isothiocyanate SFN, we investigated the effects of HDAC inhibitor Trichostatin A (TSA) on the transactivation potential of the combinations by pre-treatment of HT-29 AP-1 cells with 100 ng/ml TSA for 4 h followed by treatment with dietary factors for 24 h. Interestingly, as shown in Fig. 6, pre-treatment with HDAC inhibitor TSA potentiated (about 20-fold) the transactivation of the AP-1 luciferase reporter by SFN 25 μM alone, suggesting that activation of AP-1 luciferase activity in this cell system may possibly relate to HDAC inhibition. Similarly, a strong potentiation of AP-1 transactivation (about 88-fold) was also observed with the low-dose combination of SFN 25 μM+EGCG 20 μM suggesting that maximal transcriptional activation of the AP-1 reporter genes may potentially be achieved by combining TSA with this synergistic combination. On the other hand, transactivation by both EGCG 20 μM and EGCG 100 μM was not potentiated by TSA inhibition. Interestingly, the high-dose combination of SFN 25 μM+EGCG 100 μM attenuated (about 93-fold) the synergism elicited with this combination which may be attributed to toxicity caused by exposure to TSA in addition to the high-dose combination.

Cytoplasmic and Nuclear HDAC Activity Assays for the Combinations of SFN and EGCG

HDAC activity assays for cytoplasmic and nuclear fractions of cells treated with dietary factors were conducted as described under Materials and Methods at both 2 and 12 h after treatment, as well as after first pre-treating the cells with 100 ng/ml TSA before treatment with dietary factors for 2 or 12 h. Interestingly, there was no significant change in HDAC activity of cytoplasmic or nuclear fractions of dietary factor-treated cells relative to vehicle control (data not shown).

Reactive Oxygen Species and SOD may Modulate AP-1 Transactivation by the Combinations of SFN and EGCG

Since EGCG is known to induce oxidative stress, we investigated the potential role of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) along with the free radical scavenger SOD in modulating the transcriptional events through the AP-1-responsive reporter. Accordingly, we performed assays for H_2O_2 in cell-free media using an assay kit as described in Materials and Methods. Consistent with the ability of EGCG to induce oxidative stress, we quantified a production of 2 μM H_2O_2 at 15 min in HT-29 AP-1 cells treated with 20 μM EGCG alone. Presence of SFN in the SFN 25 μM+EGCG 20 μM combination did not affect the amount of H_2O_2 produced by EGCG. However, co-treatment with 20 U/ml of SOD decreased the amount of H_2O_2 detected at 15 min by half to 1 μM, which result was consistent in the case of both EGCG 20 μM alone or SFN 25 μM+EGCG 20 μM as shown in Fig. 7. The amount of H_2O_2 detected decreased in a generally time-dependent manner thereafter for most dietary factor treatments. In addition, about 4 μM H_2O_2 was produced at 15 min in HT-29 AP-1 cells treated with 100 μM EGCG alone or SFN 25 μM+EGCG 100 μM. However, co-treatment with 20 U/ml SOD was not able to significantly scavenge the H_2O_2 produced at the high dose of EGCG or by the high-dose combination of SFN 25 μM+EGCG 100 μM.

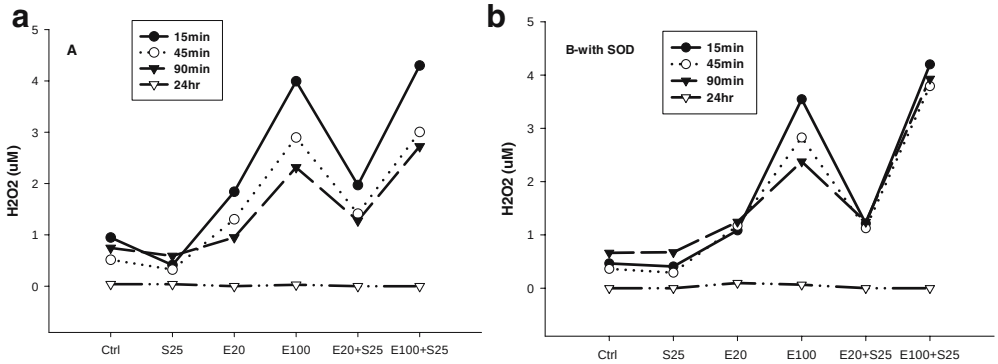

Fig. 7. Reactive oxygen species and SOD may modulate AP-1 transactivation by the combinations of SFN and EGCG. HT-29 AP-1 cells were treated with individual dietary factors or with combinations of SFN and EGCG as indicated both in the absence (**a**) and presence (**b**) of co-treatment with 20 U/ml SOD. Cell-free media were tested for H_2O_2 levels using only H_2O2 (10 μM) as a positive control in a fluorescence-based assay. For clarity of presentation, only mean values of three replicates are shown that are representative of three independent experiments.

DISCUSSION

Colorectal cancer has a natural history of transition from precursor to malignant lesion that spans, on average, 15–20 years, providing a window of opportunity for effective interventions and prevention (31). Data accumulating in recent years have suggested that aspirin, non-steroidal anti-inflammatory drugs, and selective cycloxygenase (COX-2) inhibitors all have a potential to reduce both colorectal cancer and colorectal adenomas (32), however, issues of safety and therapeutic indices have come up as barriers to the use of some of these agents. Many dietary phytochemicals exhibit beneficial effects to health including prevention of diseases such as cancer. Mammalian, including human, cells respond to these dietary phytochemicals by "non-classical receptor sensing" mechanism of electrophilic chemical-stress typified by "thiol modulated" cellular signaling events primarily leading to gene expression of pharmacologically beneficial effects, but sometimes unwanted cytotoxicity (22). Indeed, with the ultimate goal of preventing cancer, science has advanced greatly in better understanding cancer biology as also in identifying chemotherapeutic/chemopreventive agents that would inhibit or delay the progression of this disease. However, the need to maximally exploit the preventive or therapeutic efficacy of agents without incurring toxicity to normal cells remains challenging. A combinatorial approach to cancer therapy/prevention is being widely recognized as an alternative strategy to potentially improve treatment success rates. Recently (33), a Phase I Trial of sorafenib in combination with IFN α-2a was conducted in patients with unresectable and/or metastatic renal cell carcinoma or malignant melanoma. Besides, combination therapy of an orthotopic renal cell carcinoma model using intratumoral vector-mediated costimulation and systemic interleukin-2 was recently reported (34). Interestingly, Adhami *et al.* (35) recently reported combined inhibitory effects of green tea polyphenols and selective COX-2 inhibitors on the growth of human prostate cancer cells both *in vitro* and *in vivo*. Our laboratory has been studying two groups of dietary phytochemical cancer chemopreventive compounds (isothiocyanates and polyphenols) (36,37), which are effective in chemical-induced as well as genetically-induced animal carcinogenesis models (38,39). We decided to pursue the current study on colon cancer prevention by examining the biologic modulation via AP-1 which is a group of dimeric transcription factors composed of Jun, Fos, and ATF family proteins (40). Notably, in the present study, we investigated the combinations of two dietary factors—isothiocyanate SFN and green tea polyphenol EGCG—and the role(s) mediated by redox transcription factor AP-1 in modulating the anti-cancer potential of this putative chemopreventive combination in stably transfected HT-29 AP-1 human colon carcinoma cells.

We investigated the transactivation of the AP-1 luciferase reporter in our colon cancer cell system by individual treatments with SFN and EGCG and with combinatorial treatments of these two dietary factors (Fig. 1). Indeed, both low-dose and high-dose combinations of SFN and EGCG synergistically induced transactivation of the AP-1 reporter as compared to individual dietary factors. This observation correlated with a corresponding trend of luciferase gene induction in the quantitative real-time PCR (qRT-PCR) assays (Table II). We also confirmed the synergistic interaction in the luciferase assays by testing various combinations of SFN and EGCG by isobologram analyses and determining combination index values as reported by Zhao *et al.* (29) as shown in Fig. 2. Studies in genetically modified mice and cells have highlighted a crucial role for AP-1 in a variety of cellular events involved in normal development or neoplastic transformation causing cancer (41). Both gain- and loss-of-function studies have revealed specific roles for individual AP-1 components in cell proliferation, differentiation, apoptosis, and other biological processes (40). Recently, Maurer *et al.* (42) observed that in tumors with long necessary follow-up, such as colorectal cancer, early-risk predictors would be needed, and provided first evidence for early prognostic relevance of transcription factors including AP-1 differentially bound to the promoter of the invasion-related gene u-PAR, and their molecular inducers, in colorectal cancer. The synergistic transcriptional activation of the AP-1 reporter that we observe with the combinations of SFN and EGCG in the present study may be seen in the light of the above evidence that point to a singular role for AP-1 mediated transcriptional control of potentially critical genes mediating cancer initiation and progression. This translates into potentially greater efficacy, of the combination of SFN and EGCG in chemoprevention of cancer. Interestingly, co-treatment with free radical scavenger SOD attenuated the synergism elicited by the combinations. This observation also corroborated with a corresponding attenuation of luciferase gene transcript in the qRT-PCR assays (Table III). Indeed, assays for H_2O_2 in cell-free media (Fig. 7) revealed that SOD co-treatment decreased the amount of H_2O_2 noted with the low-dose combination of SFN and EGCG by half. Taken together, these above observations point to a potential role for SOD signaling in modulating the pharmacologic activity of the combination of SFN and EGCG. Additional studies are necessary to better understand and delineate the specific pathway cross-talk with AP-1 signaling.

The downregulation of cyclin D1 by the SFN and EGCG combinations may be related to the intrinsic ability of SFN to induce G1 cell cycle arrest in HT-29 cells as we have reported earlier (43). ATF-2 can form a heterodimer with c-*Jun* and controls the induction of c-*Jun* in an AP-1 independent manner, however, both ATF-2 and c-*Jun* can be activated by c-Jun N-terminal kinases (JNK) (44). The absence of major ATF-2 activation with the combinations in our study may thus also relate to the absence of JNK activation that we observed with the combinations as compared to individual dietary factors (Fig. 5). Biochemical studies have indicated that Elk-1 is a good substrate for ERKI/ERK2 *in vitro*, and that the kinetics of its modification correlated well with MAPK activation *in vivo* (45). Thus, the limited transcriptional (Table II) and translational (Fig. 5) activation of Elk-1 and ERK1/2 respectively that we see in this study may potentially be inter-related, although additional biochemical studies will be necessary to substantiate this hypothesis. Because the influx-efflux machinery of cells could be potentially turned on by exogenous stress, we also investigated some key transporter genes (Table II). Using Hagenbuch and Meier's (46) new nomenclature, the gene encoding for the organic anion transporter protein OATP1B3 (old name

OATP8) is known as the SLCO1B3 (old nomenclature SLC21A8). OATP1B3 has been shown to be expressed in various human cancer tissues as well as in different tumor cell lines derived from gastric, colon, pancreas, gallbladder, lung and brain cancers (46). The induction of SLCO1B3 that we observed with EGCG-alone treatments which was reversed by treatment with the combinations of SFN and EGCG (Table II) may be relevant since the intracellular, pharmacologically active concentration of any drug is the balance between uptake and neutralizing pathways, either by biotransformation or extrusion from the targeted cells (47), although the pathobiological significance of expression of this gene is not yet fully understood (46). Interestingly, the combinations of SFN and EGCG greatly induced the expression of the efflux transporter MRP2 (Table II) but downregulated the expression of influx transporters MRP1 and MRP3. Since the combination treatment of SFN and EGCG would impose exogenous stress on the cellular environment, the induction of MRP2 may be related to a cellular defense response purported to increase the excretion/efflux of the xenobiotics or their metabolites.

Cell senescence is broadly defined as the physiological program of terminal growth arrest, which can be triggered by alterations of telomeres or by different forms of stress (48). Although senescent cells do not proliferate, they remain metabolically active and produce secreted proteins with both tumor-suppressing and tumor-promoting activities (48). Besides apoptosis, cell proliferation could, thus, be limited by senescence (49). In fact, it seems that activation of the senescence program and consequent permanent growth arrest significantly contributes to the loss of the clonogenic capacity of tumor cells and probably to tumor regression after anticancer therapy (49,50). EGCG is known to inhibit telomerase and induce senescence in leukemic cells (30) and we were able to confirm this in HT-29 AP-1 cells as shown in Fig. 4. Interestingly, the combinations of SFN and EGCG inhibited the EGCG-induced senescence (Fig. 4) of HT-29 AP-1 cells. Recently (39), we demonstrated that ApcMin/+ mice fed with SFN-supplemented diet developed significantly less and smaller polyps with higher apoptotic and lower proliferative indices in their small intestine, in a SFN dose-dependent manner. SFN also regulated different sets of genes involving apoptosis, cell growth/maintenance and inflammation in the small intestinal polyps of ApcMin/+ mice (51). SFN also induced G (1) phase cell cycle arrest in HT-29 cells (43). We have also shown that EGCG treatment causes damage to mitochondria, and induces apoptotic cell death (19). Thus, the inhibition of cellular senescence we observed with the combinations of SFN and EGCG may relate to the ability of SFN and EGCG to activate apoptotic pathways that predominate over the senescence pathways induced by EGCG. The viability (Fig. 3) of the HT-29 AP-1 cells at 48 h was about 70 and 40% with the low- and high-dose combinations respectively since the low-dose combination was not toxic to the cells as compared with the high-dose combination of SFN and EGCG.

The effects of the combinations on protein expression (Fig. 5) of major mitogen-activated protein kinase (MAPK) pathway members including ERK, JNK and p38 as well as the Akt pathway was not dramatic as compared to individual agents, suggesting that the SFN+EGCG combination-mediated activation of AP-1 may occur by cellular mechanisms that are exclusive of JNK activation. Since inhibition of histone deacetylase (HDAC) has been reported (12) as a novel mechanism of chemoprotection by the isothiocyanate SFN, we investigated the effects of HDAC inhibitor Trichostatin A (TSA) on the transactivation potential of the combinations. Interestingly, TSA potentiated (Fig. 6) the synergism elicited by the low-dose combination of SFN and EGCG leading us to speculate whether the strong AP-1 induction may relate to HDAC inhibition. However, there was no significant change in HDAC activity of cytoplasmic or nuclear fractions of dietary factor-treated cells (data not shown). Indeed, maximal transcriptional activation of the AP-1 reporter genes may potentially be achieved by combining TSA with the synergistic low-dose combination of SFN and EGCG. In contrast, the synergism elicited with the high-dose combination was attenuated by TSA (Fig. 6) which may be attributed to toxicity caused by exposure to the high-dose combination together with TSA. Further empirical and heuristic studies are necessary to elucidate the exact biochemical mechanisms and the nature of potential cross-talk between AP-1 and HDAC from a physiological perspective.

We have previously reported (52) that the peak plasma concentration (Cmax) achievable with SFN in rats was 20 μM after oral administration. In addition, we have reported (53) that SFN 50 μM was toxic to HepG2 C8 cells, whereas SFN 25 μM was suboptimal in its efficacy. Since a desirable objective of using combinatorial approaches is to reduce the dose of the administered agents thereby reducing toxic side-effects, our dose selection of 25 μM of SFN for the current study was guided by its proximity to the observed Cmax and its suboptimal effectiveness in eliciting transcriptional effects as compared to higher doses of SFN. Besides, in most studies, the concentrations needed to observe the activities of EGCG typically range from 1 to 100 μM; these are, in reality, concentrations that exceed those found in rodent and human plasma by 10- to 100-fold (54,55). However, the uptake of EGCG in HT-29 cells has also been shown to be concentration-dependent in the range of 20–600 μM (54). In addition, we have also previously reported (19) that EGCG inhibited HT-29 cell growth with an IC50 of approximately 100 μM. Accordingly, we elected to test two doses of EGCG (20 and 100 μM) in the current study in combination with the 25 μM dose of SFN.

In summary, it is necessary to evolve and to justify alternative strategies to develop agents that modulate multiple targets simultaneously with the aim of enhancing efficacy or improving safety relative to agents that address only a single molecular target. Combinatorial approaches to cancer chemoprevention lend themselves to the cause of maximally exploiting the intrinsic anti-carcinogenic potential of known dietary factors with already proven beneficial effects individually. Taken together, the synergistic activation of the AP-1 reporter that was potentiated by HDAC inhibitor TSA and attenuated by free radical scavenger SOD point to a possible multifactorial control of colon carcinoma that may involve a role for HDACs, inhibition of cellular senescence, and SOD signaling. Future studies to delineate the complex regulation in biological systems, as well as *in vivo* studies, would be useful in elucidating the

effects of combining dietary factors SFN and EGCG to better appreciate the pharmacological benefits of this synergy in cancer prevention.

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