

# Chemoprevention of Familial Adenomatous Polyposis by Natural Dietary Compounds Sulforaphane and Dibenzoylmethane Alone and in Combination in *Apc*<sup>Min/+</sup> Mouse

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## Abstract

**Cancer chemopreventive agent sulforaphane (SFN) and dibenzoylmethane (DBM) showed antitumorigenesis effects in several rodent carcinogenesis models. In this study, we investigated the cancer chemopreventive effects and the underlying molecular mechanisms of dietary administration of SFN and DBM alone or in combination in the *Apc*<sup>Min/+</sup> mice model. Male *Apc*<sup>Min/+</sup> mice (12 per group) at age of 5 weeks were given control AIN-76A diet, diets containing 600 ppm SFN and 1.0% DBM, or a combination of 300 ppm SFN and 0.5% DBM for 10 weeks. Mice were then sacrificed, and tumor numbers and size were examined. Microarray analysis, Western blotting, ELISA, and immunohistochemical staining were done to investigate the underlying molecular mechanisms of cancer chemopreventive effects of SFN and DBM. Dietary administrations of SFN and DBM alone or in combination significantly inhibited the development of intestinal adenomas by 48% ( $P = 0.002$ ), 50% ( $P = 0.001$ ), and 57% ( $P < 0.001$ ), respectively. Dietary administration of 600 ppm SFN and 1.0% DBM also reduced colon tumor numbers by 80% ( $P = 0.016$ ) and 60% ( $P = 0.103$ ), respectively, whereas the combination of SFN and DBM treatment blocked the colon tumor development ( $P = 0.002$ ). Both SFN and DBM treatments resulted in decreased levels of prostaglandin E<sub>2</sub> or leukotriene B<sub>4</sub> in intestinal polyps or apparently normal mucosa. Treatments also led to the inhibition of cell survival and growth-related signaling pathways (such as Akt and extracellular signal-regulated kinase) or biomarkers (such as cyclooxygenase-2, proliferating cell nuclear antigen, cleaved caspases, cyclin D1, and p21). In conclusion, our results showed that both SFN and DBM alone as well as their combination are potent natural dietary compounds for chemoprevention of gastrointestinal cancers. [Cancer Res 2007;67(20):9937–44]**

## Introduction

Colorectal cancer is one of the leading causes of cancer-related mortality in the Western countries and the third most common

cancer in both men and women in the United States. In 2006, an estimated 148,610 new cases were diagnosed, and 55,170 deaths from colorectal cancer will occur in the United States (1). Although most colorectal cancers are sporadic (90%), early *Apc* mutation was also found in 50% to 80% of cases. The mutation of *Apc* gene will cause the dysregulation of Wnt signaling pathway in which elevated  $\beta$ -catenin levels in nucleus lead to the transcription of many cell proliferation-related genes (2). The constitutive activation of the Wnt/ $\beta$ -catenin/Tcf4 signaling pathway resulting from the mutation of *Apc* is therefore believed to be a major cause of the development of human colorectal cancer.

Epidemiologic studies indicated that the incidence of colon cancer is inversely correlated with the consumption of fruits and vegetables (3). Many dietary phytochemicals have been investigated for their colon cancer chemopreventive effects using rodent colorectal cancer models, such as the *Apc*<sup>Min/+</sup> mouse, which mimics the rapid development of adenomatous polyps that affect human familial adenomatous polyposis (FAP) and sporadic colorectal cancer patients with early *Apc* mutation. Sulforaphane (SFN) is one of the major isothiocyanates found as its glucosinolate precursor glucoraphanin in cruciferous vegetables, such as broccoli, and has been shown to possess strong anticancer effect (4). Our recent short-term SFN chemoprevention study in *Apc*<sup>Min/+</sup> mice also showed that it could strongly inhibit the intestinal neoplasia development in this model (5). Dibenzoylmethane (DBM) is a minor constituent of licorice that has shown strong antimutagenic activity. DBM could inhibit 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast tumorigenesis in mice (6, 7) and DMBA-induced mammary carcinogenesis in rats (8). DBM could also induce human colorectal carcinoma cells COLO 205 apoptosis through coordinative regulation of cyclin D3, Bcl-X<sub>L</sub>, Bax, and cytochrome *c* release *in vitro* (9).

In this study, we further investigated the long-term chemopreventive efficacy of SFN at dose of 600 ppm in the *Apc*<sup>Min/+</sup> mice model based on our previous results (5). In addition, we also examined the colorectal cancer chemopreventive effect of DBM for the first time in this study. DBM was given at dose of 1.0% in diet based on its previous mammary gland tumor chemoprevention study (7). The selection of SFN and DBM as well as their combination in this study is based on their potential different mechanisms in inhibition of human colon cancer cell growth illustrated in previous *in vitro* studies (10). SFN could inhibit human colon carcinoma HT-29 cell growth by inducing G<sub>1</sub> cell cycle arrest and apoptosis, probably through the regulation of mitogen-activated protein kinases (MAPK), without affecting the Akt pathway (10). On the other hand, our preliminary studies of DBM in HT-29 cells showed that DBM could significantly inhibited

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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cancer cell growth by inhibiting Akt pathway activity. Therefore, a combination treatment of SFN and DBM at half of the single dose was included to investigate their potential synergistic effect on colon cancer chemoprevention in this model. The *in vivo* mechanisms of chemoprevention of adenomatous lesions in the *Apc*<sup>Min/+</sup> mouse model by SFN and DBM were investigated by examining their effects on tumor development, arachidonic acid metabolism, cell survival signaling pathways, cell growth and apoptosis, as well as global gene expression profiles in intestinal polyps and apparently normal mucosa.

## Materials and Methods

**Animal and treatment protocol.** Male *Apc*<sup>Min/+</sup> mice were purchased from The Jackson Laboratory at age of 4 weeks. Treatments were started after 1 week of acclimatization. Total of 48 *Apc*<sup>Min/+</sup> mice was randomly divided into four groups. Group 1 was given the control AIN-76A diet (Research Diets), group 2 was given 600 ppm SFN-supplemented AIN-76A diet, group 3 was given 1% DBM-supplemented AIN-76A diet, and group 4 was given AIN-76A diet supplemented with 300 ppm SFN and 0.5% DBM. Mice were housed at Rutgers Animal Facility under 12-h light/dark cycles with free access to water and food. Mice were checked daily for signs of illness and fresh diet was given daily during the 10-week treatment period.

**Tissue processing and tumor scoring.** All mice were euthanized by CO<sub>2</sub> after treatment, blood was collected immediately by heart puncture, and plasma was separated by centrifugation and stored at -80°C until further analysis. Processing of small intestine, colon, and tumor scoring were carried out as described previously (5). Briefly, the total number and size of polyps on intestinal segments and colon was examined under an Olympus SZH10 stereo dissecting microscope. After that, a small part of small intestine segment was collected for drug concentration measurement, total RNA extraction, and histopathology/immunohistochemical staining. The counting and characterization of all tumors were done blinded with respect to the treatment group. All immunohistochemical determinations were made with 400-fold magnification with a light microscope. These examinations were also done blinded with respect to the treatment group. The rest of the small intestine was snap frozen in liquid nitrogen and stored at -80°C until further Western blotting analysis.

**Quantification of SFN and DBM in plasma and small intestine using liquid chromatography/mass spectrometry.** Plasma and small intestine concentrations of SFN and its major glutathione conjugate metabolite SFN-GSH in *Apc*<sup>Min/+</sup> mice after 10 weeks of consumption of AIN-76 diets containing 300 and 600 ppm of SFN were measured as described previously (5). DBM concentrations in plasma and small intestine were also analyzed using similar liquid chromatography/mass spectrometry (LC/MS) methods. Briefly, a 50- $\mu$ L plasma samples were added to internal standard curcumin at a final concentration of 50 ng/mL. The spiked plasma samples were extracted twice with 3 volumes of ethyl acetate/methanol (95:5, v/v). After centrifugation, the organic phase was transferred to a clean tube and evaporated to dryness using nitrogen gas. The samples were then reconstituted in acetonitrile/water (50:50, v/v) and filtered through a 0.45- $\mu$ m spin filter, and 20  $\mu$ L of supernatant were subjected to LC/MS analysis. For small intestine samples, samples were first completely homogenized in 2 volumes (w/v) of water using a tissue homogenizer followed by 30 s of sonification. DBM in the homogenates was extracted using the ethyl acetate/methanol method described above. The high-performance liquid chromatography and LC/MS system were same as described in the SFN analysis except an analytic Synergi Max-RP C12 150  $\times$  2.0 mm column (Phenomenex) was used for the analysis.

**Measurement of prostaglandin E<sub>2</sub> and leukotriene B<sub>4</sub> levels in small intestine.** Frozen intestinal polyps and normal mucosa were quickly excised from the small intestines under frozen condition and then homogenized using a Polytron in an ice-cold homogenizing buffer containing protease inhibitors (0.2 mmol/L phenylmethylsulfonyl fluoride, 2.5  $\mu$ g/mL of pepstatin, aprotinin, and leupeptin), 10  $\mu$ mol/L nordihydroguaiaretic acid [a lipoxygenase (LOX) inhibitor], and 10  $\mu$ mol/L indometh-

acin [a cyclooxygenase (COX) inhibitor; ref. 11]. The homogenates were then centrifuged at 14,000  $\times$  g for 10 min at 4°C, and the supernatant was aliquoted and stored at -80°C. The concentration of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) was measured using an EIA kit (Cayman Chemical) according to the manufacturer's protocol.

**Apoptosis and proliferation immunohistochemical staining.** The apoptotic cells were detected using an ApopTag *In situ* Apoptosis Detection kit (Chemicon). The assay was done according to the manufacturer's manual. After deparaffinization, the tissue sections were incubated with proteinase K for 15 min at room temperature. The sections were then incubated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 h, washed thrice with PBS, and incubated with antidigoxigenin conjugate in a humidified chamber at room temperature for 30 min. The color was developed by incubating the sections with peroxidase substrate and then counterstained with hematoxylin for 30 s. For detection of proliferative cells, proliferating cell nuclear antigen (PCNA) antibody (1:50; Dako) was used. The assay was done following the manufacturer's protocols. The scoring of apoptotic and proliferative cells was done at  $\times$ 400. A positive control slide of rat mammary glands provided by the manufacturer was used as positive control for the *in situ* apoptosis detection assay. For the PCNA staining, small intestinal crypt cells were used as an internal positive control.

**Western blotting analysis.** Intestinal polyps and normal mucosa were excised from the small intestines from individual *Apc*<sup>Min/+</sup> mice and pooled together based on the treatment group. To prepare total cell lysate, about 30 to 50 polyps were incubated with 2 mL lysis buffer in an ice-cold French Douncer for 15 min before homogenization by 40 strokes. The homogenates were cleared by centrifugation at 14,000  $\times$  g for 10 min at 4°C. The supernatants were further diluted and protein concentrations were measured by bicinchoninic acid kit (Pierce). A total amount of 30  $\mu$ g protein was resolved on precasted SDS-PAGE gels (Bio-Rad), and Western blotting was done as described earlier (5).

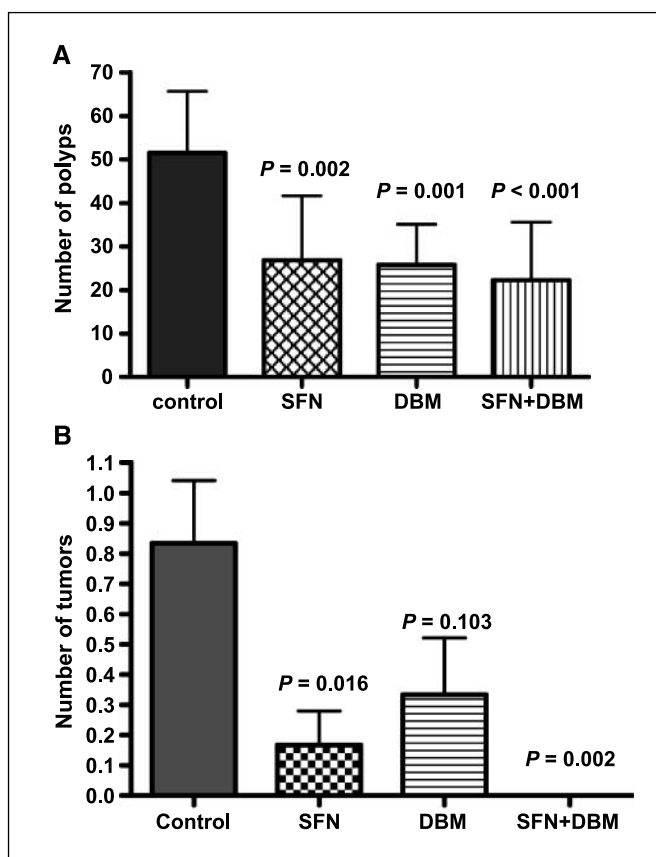
**Total RNA preparation and microarray analysis.** Total RNA was extracted from intestinal polyps and normal mucosa samples collected from individual *Apc*<sup>Min/+</sup> mice as described previously (5). After examining the quality and concentration of RNA samples, an equal amount of total RNA from individual mouse within the same group was pooled together and submitted to the DNA Core Expression Facility of University of Medicine and Dentistry of New Jersey for microarray analysis. Affymetrix Mouse Genome 430 2.0 Array was used to analyze the global gene expression patterns in the *Apc*<sup>Min/+</sup> mice polyps and normal mucosa samples from all the groups (total of eight microarrays was used). Genes that were induced or inhibited >2-fold by treatments comparing with control were identified using GeneSpring 6.0 software. To further validate the microarray results, quantitative real-time PCR was used to examine the effect of drug administration on individual genes.

**Quantitative real-time PCR for microarray data validation.** To verify microarray data, several genes [including housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)] from different categories were chosen for quantitative real-time PCR analyses. The specific primers for these genes were designed by using PrimerExpress software (Applied Biosystems). The procedure of real-time PCR was described previously (12). The gene expression changes compared with control sample were determined after normalization with the corresponding *GAPDH* expression level. The correlation between corresponding microarray data and real-time PCR data was validated by using Spearman rank correlation method.

**Statistical analysis.** One-way ANOVA analysis and post hoc Tukey's test were used to compare mean value between different groups. For simple group means analysis, two-tailed *t* test was used.

## Results

**Dietary administration of SFN and DBM alone or in combination for 10 weeks significantly inhibited intestinal polyposis without affecting body weight in *Apc*<sup>Min/+</sup> mice.** At age of 15 weeks, mice in the control group developed an average of 51.5 intestinal polyps (Fig. 1A). Dietary consumption of 600 ppm SFN and 1.0% DBM alone or in combination at half dose for



**Figure 1.** Dietary administration of 600 ppm SFN, 1.0% DBM, or combination of 300 ppm SFN and 0.5% DBM in diet for 10 wk significantly inhibited intestinal (A) and colon (B) tumor development in *Apc<sup>Min/+</sup>* mice. One-way ANOVA and post hoc Tukey's test were used to compare the means between treated groups and control group ( $n = 12$ ).  $P$  values were shown for each treated group.

10 weeks reduced the intestinal polyps formation by 48% ( $P = 0.002$ ), 50% ( $P = 0.001$ ), and 57% ( $P < 0.001$ ), respectively (Fig. 1A). Interestingly, the number of large-sized polyps ( $>2$  mm) was more significantly decreased than the number of small-sized polyps by all treatments (Table 1). Dietary administration of 600 ppm SFN and 1.0% DBM also inhibited colon tumor numbers by 80% ( $P = 0.016$ ) and 60% ( $P = 0.103$ ), respectively; whereas the combination of SFN and DBM treatment completely blocked the colon tumor development ( $P = 0.002$ ; Fig. 1B). No significant body weight change nor noticeable signs of toxicity were observed in treatment groups compared with control group (data not shown) during the treatments.

**Plasma and small intestine concentrations of SFN and DBM in treated *Apc<sup>Min/+</sup>* mice.** LC/MS analysis indicated a dose-dependent plasma and tissue concentrations of SFN and DBM in *Apc<sup>Min/+</sup>* mice. As shown in Table 2, mean plasma concentration of SFN in 600 ppm-treated group was 68 ng/mL (0.384  $\mu$ mol/L), which is about twice of that in the 300 ppm treatment group. The concentration of SFN-GSH, which is the major metabolite of SFN, was 347 ng/mL (0.717  $\mu$ mol/L) and 274 ng/mL (0.566  $\mu$ mol/L) in the 600 and 300 ppm group, respectively. Mean plasma concentration of DBM was 145 ng/mL (0.647  $\mu$ mol/L) and 338 ng/mL (1.509  $\mu$ mol/L) in the combinatorial and 1.0% treatment group, respectively. In small intestine, mean tissue concentration of SFN was 0.8 and 2.7 ng/mg protein in combinatorial treatment and 600 ppm group, respectively; therefore, in small intestine,

concentrations of SFN were also lower than concentrations of its metabolite SFN-GSH (6.8 and 15.5 ng/mg protein in 300 and 600 ppm group, respectively). Mean tissue concentration of DBM was 198 ng/mg protein in combination group and 424 ng/mg protein in 1.0% group, which were much higher than concentrations of SFN and SFN-GSH in the tissue.

**Effects of dietary administration of SFN and/or DBM on arachidonic acid metabolism.** As shown in Fig. 2A and B, arachidonic acid metabolism pathway metabolites PGE<sub>2</sub> and LTB<sub>4</sub> levels were elevated significantly in intestinal polyps comparing with that in the normal-appearing mucosa (normal mucosa). Ten weeks of dietary administration of SFN alone at 600 ppm resulted in a slightly increase of PGE<sub>2</sub> levels in both intestinal polyps and normal mucosa but strongly decreased the level of LTB<sub>4</sub> in both type of tissues. Dietary administration of DBM alone dramatically decreased both PGE<sub>2</sub> and LTB<sub>4</sub> in intestinal tumors. DBM treatment also decreased PGE<sub>2</sub> level dramatically in normal mucosa, whereas LTB<sub>4</sub> level was inhibited to a much less extent in normal mucosa. Combinatorial treatment with half dose of SFN and DBM achieved similar inhibition effects on PGE<sub>2</sub> and LTB<sub>4</sub> levels as the DBM 1% treatment.

COX-2 expression level was also much higher in intestinal polyps than that in the normal-appearing mucosa (Fig. 2C). Coordinate with the down-regulation of PGE<sub>2</sub> levels in small intestine, the expression of COX-2 was also strongly inhibited by DBM treatments. Although SFN alone did not change PGE<sub>2</sub> levels significantly, the expression of COX-2 was slightly inhibited by SFN treatment. Whereas LTB<sub>4</sub> levels were significantly decreased by both SFN and DBM treatments in tumor and normal mucosa, the expression of 5-LOX was almost unaffected by both treatments.

**Dietary administration of SFN and DBM significantly altered phosphorylation of Akt and MAPKs.** As shown in Fig. 3A, the basal phosphorylation level of Akt at Ser<sup>473</sup> was higher in the polyps than that in the normal-appearing mucosa. Long-term treatment of SFN, DBM, or in combination significantly down-regulated the phosphorylation of Akt at Ser<sup>473</sup> in polyps, and the inhibition was more significant in the normal-appearing mucosa. However, inhibition of Akt phosphorylation did not disturb the  $\beta$ -catenin signaling pathway, which is downstream of Akt pathway, as evidenced by the unchanged expression level of  $\beta$ -catenin, E-cadherin, and the phosphorylation of glycogen synthase kinase 3 $\beta$  kinase (data not shown) by all treatments. Interestingly, phosphorylation of extracellular signal-regulated kinase (ERK) 2 (42 kDa) was inhibited by treatments in polyps but not in normal-appearing mucosa. Similarly, c-Jun NH<sub>2</sub>-terminal

**Table 1.** Effects of 10-wk SFN, DBM, or combination of SFN and DBM dietary administration on intestinal tumor size in *Apc<sup>Min/+</sup>* mice ( $n = 12$ )

Treatment	$n$	Tumor size (mm)			Total, $n$ (%)
		$<1.0$ , $n$ (%)	$1.0-1.9$ , $n$ (%)	$\geq 2.0$ , $n$ (%)	
Control	12	56 (9.1)	350 (56.6)	212 (34.3)	618 (100)
SFN (600 ppm)	12	48 (14.9)	206 (64.0)	68 (21.1)	322 (100)
DBM (1.0%)	12	63 (20.3)	205 (66.1)	42 (13.6)	310 (100)
SFN + DBM	12	56 (21.0)	179 (67.0)	32 (12.0)	267 (100)

**Table 2.** Plasma and tissue concentration of SFN, SFN-GSH, and DBM in *Apc*<sup>Min/+</sup> mice after 10 wk of drug administration (*n* = 9)

	Plasma (ng/mL)			Intestine (ng/mg protein)		
	SFN	SFN-GSH	DBM	SFN	SFN-GSH	DBM
SFN	68 ± 29	347 ± 268		2.7 ± 0.2	15.5 ± 7.7	
SFN + DBM	32 ± 10	274 ± 386	145 ± 54	0.8 ± 0.5	6.8 ± 2.9	198 ± 166
DBM			338 ± 143			424 ± 117

kinases (JNK) were inhibited in polyps but slightly activated in normal-appearing mucosa. The phosphorylation of p38 was not changed by SFN treatment in both tissues but was activated by DBM treatment in the polyps. The combination of SFN and DBM decreased the expression of phosphorylated p38 in normal mucosa.

#### Dietary administration of SFN and DBM significantly altered proteins involved in cell cycle regulation and apoptosis.

SFN treatment significantly increased the expression of cyclin-dependent kinase inhibitor p21 in polyps but to a less extent in normal mucosa (Fig. 3B). The decreased induction of p21 in the combination group by SFN might be explained by the fact that DBM treatment alone slightly inhibited the expression of p21 in both polyps and normal mucosa. DBM treatment dramatically decreased the expression of cyclin D1, whereas SFN treatment increased the expression of cyclin D1. SFN treatment alone strongly activated caspase-3 and caspase-7; interestingly, activation of caspase-3 by SFN only occurred in intestinal polyps but not in normal mucosa. Both SFN and DBM treatments strongly induce the expression of Bax. These observations were further supported by PCNA staining and *in situ* apoptosis detection assay. Percentage of PCNA-positive cells, representing cells under proliferation, was significantly reduced in the treated mice (Supplementary Fig. S1A). In contrast, the percentage of apoptotic cells was significantly increased in mice treated with SFN alone or in combination with DBM using ApopTag *In situ* Apoptosis Detection kit, although the increased level was not significant after DBM treatment alone (Supplementary Fig. S1B).

#### Quantitative real-time PCR validation of microarray data.

Nine genes from different categories (Supplementary Table S1) were selected to confirm the microarray data using quantitative real-time PCR analyses. Expression values for each gene were normalized by the values of corresponding *GAPDH* gene in the same sample and the ratios of treated/control were calculated. The value of expression change of each gene obtained in microarray analysis correlated very well to those obtained from real-time PCR assay (Supplementary Table S1) as verified by the Spearman correlation ( $r^2 = 0.957$ ; Fig. 4).

**Gene expression profiles in tumor and normal-appearing mucosa after DBM and SFN treatment.** Using GeneSpring software, lists of genes that were induced or inhibited >2-fold by treatment both in intestinal polyps and normal-appearing mucosa were identified (Supplementary Table S2). Among these genes, glutathione metabolism-related genes, such as glutamate-cysteine ligase catalytic subunit (*Gclc*) and modifier subunit (*Gclm*), glutathione *S*-transferases (GST; *Gsta* and *Gstm*), glutathione synthetase (*Gss*), and glutathione reductase 1 (*Gsr*), were all highly induced by all treatments in both tumor and normal-appearing mucosa. Interestingly, glutathione peroxidase (*Gpx*) genes were

inhibited by the treatments. Several other antioxidant and phase II detoxification genes, such as heme oxygenase 1 (*HO-1*), microsomal epoxide hydrolase 1 (*Ephx1*), thioredoxin reductases (*Txnrd*), and UDP-glucuronosyltransferase (*Ugt2b5*) were also induced highly by treatments in normal-appearing mucosa and to a less extent in intestinal tumors. DBM alone or in combination with SFN dramatically induce the expression of many cytochrome *P450* genes in apparently normal mucosa and to a lesser extent in intestinal polyps. In addition to these drug metabolism and detoxification genes, all treatments also altered the expression of genes involved in cell proliferation and signal transduction. For example, Eph receptors were differentially regulated by both treatments in tumor and normal-appearing mucosa. Many cytokine or cytokine signaling pathway-related genes, such as interleukins (*Il*), insulin (*Insl5*), and insulin-like growth factor binding proteins (*Igfbp*), were suppressed in treated tumor and normal-appearing mucosa. Wnt signaling pathway-related gene WNT1-inducible signaling pathway protein 1 (*Wisp1*) was also suppressed by both treatments. In addition to genes regulated by both SFN and DBM, cluster of genes specifically regulated by each compound were also identified. As shown in Supplementary Table S2, chemokine ligand (*Cxcl*) gene expression was suppressed by DBM treatment, whereas expression of chemokine receptor genes (*Ccr*) was only inhibited by SFN treatment. DBM alone or in combination also strongly inhibited the expression of colon cancer biomarker clusterin gene (*Clu*) in the normal-appearing mucosa. Many isoforms of G protein-coupled receptors (*Gpr*) were also down-regulated by DBM treatments. Among the kinase genes being regulated, SFN treatment specifically inhibited the expression of p21-activated kinase 2 and 3 (*Pak2* and *Pak3*), whereas many MAPKs (*Map2k6*, *Map2k7*, *Map3k1*, and *Map4k1*) and protein kinase C genes (*Prkc*) were regulated by DBM-related treatment.

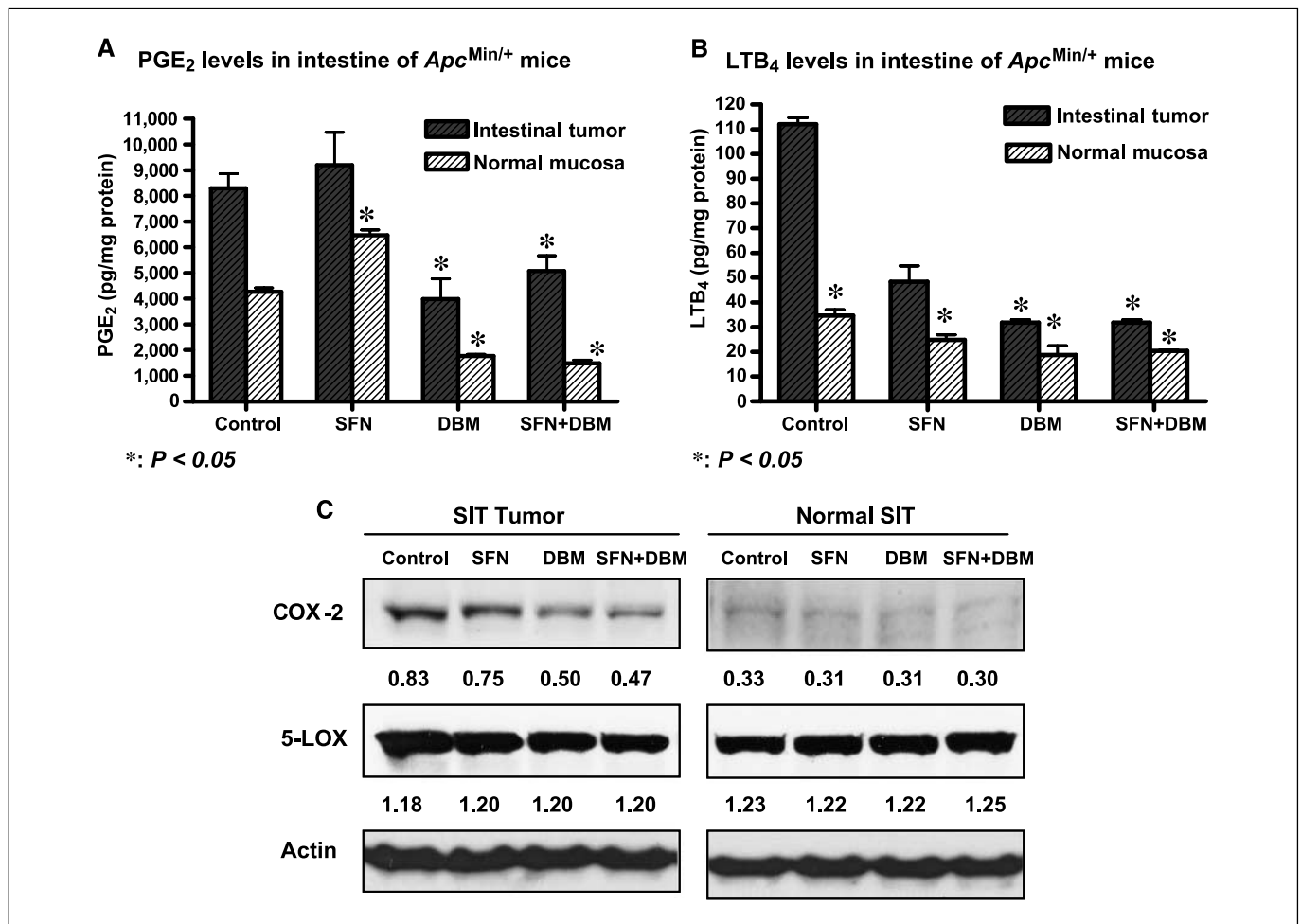
## Discussion

Dietary consumption of SFN for 3 weeks at doses of 300 and 600 ppm resulted in significant inhibition of intestinal polyposis by 25% and 48% in the *Apc*<sup>Min/+</sup> mouse model in our previous study (5). In this study, long-term (10 weeks) dietary consumption of SFN at dose of 600 ppm also achieved 50% tumor growth inhibition, indicating that longer and earlier treatment with SFN did not improve its colon cancer chemopreventive effects in *Apc*<sup>Min/+</sup> mice. Consumption of DBM at dose of 1.0% in the diet resulted in 50% intestinal tumor growth inhibition in this study. This is the first time to show that DBM has colon cancer chemopreventive effect *in vivo*. Under current experimental conditions, each mouse (body weight, ~20 g) consumed ~3 g of diet daily; therefore, consumption of SFN at dose of 600 ppm and DBM at dose of

1.0% in diets was equivalent to a daily dose of 90 mg/kg for SFN and 1,500 mg/kg for DBM. Because 50% of tumor growth inhibition is generally considered to be effective in cancer chemotherapy or chemopreventive studies in animal models, the above doses actually could be considered as the minimum efficacious dose for SFN and DBM because 50% intestinal tumor growth inhibition was both achieved. Comparing their minimum efficacious doses also suggested that SFN is a much more potent colon cancer chemopreventive agent than DBM in this model. Although combination treatment with only half dose of SFN and DBM (300 ppm and 0.5%, respectively) also resulted in a potent inhibition of intestinal polyposis (57% inhibition), the improvement of efficacy by combinatorial treatment is not statistically different from the individual treatments. Therefore, whether the combinatorial effect of SFN and DBM on intestinal tumor growth is additive or synergistic can only be characterized by individual and combination treatments of SFN and DBM at more dose levels. Although the inhibitory effect of combinatorial treatment is not statistically higher than that of the individual treatments, it still suggests the feasibility of achieving similar or even stronger anticancer effects using lower-dose combination of SFN or DBM. This might be an important and

practical approach in clinical treatments when a high dose of single chemopreventive agent is not feasible to achieve. As supported by LC/MS drug concentration analysis, the combinatorial treatment group achieved higher tumor inhibition effect with much lower plasma and tissue concentrations of SFN and DBM than that in the individual treatment groups.

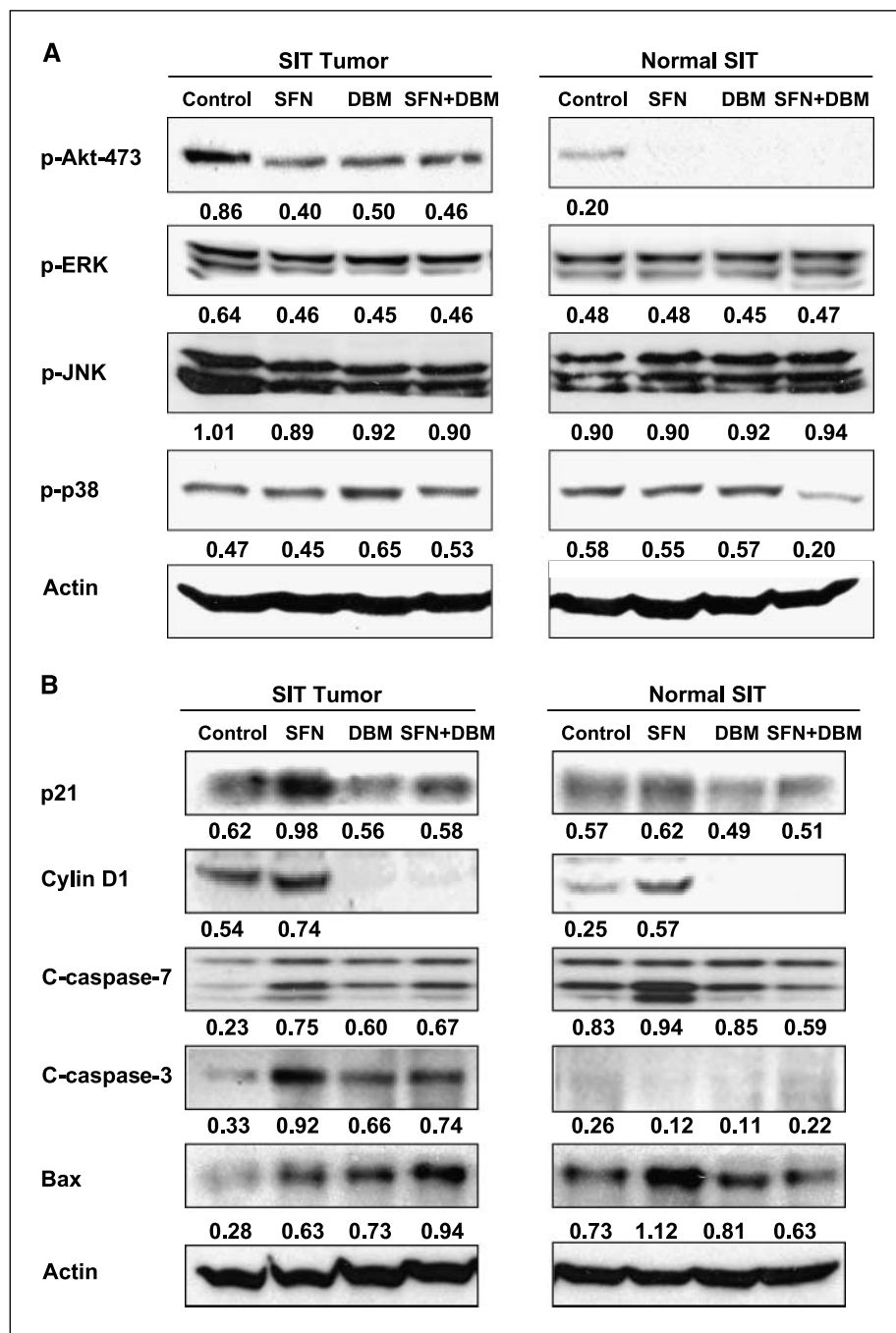
PGE<sub>2</sub> and LTB<sub>4</sub> are two major metabolites generated from arachidonic acid metabolism via COX-dependent and 5-LOX-dependent pathways. Increased production of PGE<sub>2</sub> was found in the intestinal adenomas of FAP patients and in colon cancer tissues (13). PGE<sub>2</sub> levels were also found to be increased ~2-fold in intestinal polyps compared with that in normal-appearing mucosa in this study. A previous report suggested that increased PGE<sub>2</sub> level generated by COX-2 up-regulation could accelerate the intestinal polyposis through activation of prostaglandin receptor EP2-mediated cellular events (14). Several other studies also suggested that PGE<sub>2</sub> promotes colon carcinoma growth and invasion through phosphatidylinositol 3-kinase (PI3K)/Akt signaling or by activation of a Ras-MAPK signaling cascades (15, 16). In this study, the down-regulation of PGE<sub>2</sub> levels in both apparently normal mucosa and intestinal polyps by DBM and combination treatment was



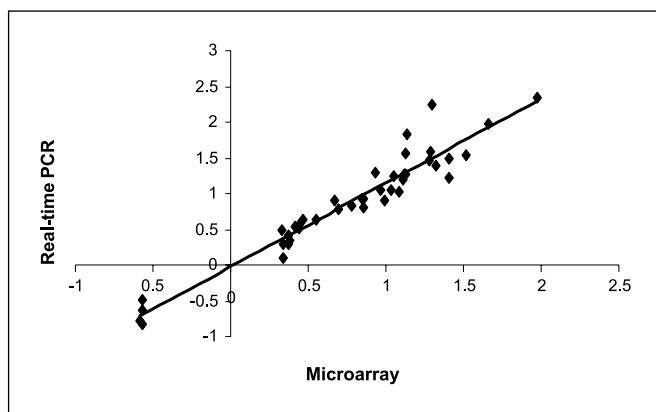
**Figure 2.** Effects of dietary administration of 600 ppm SFN, 1.0% DBM, and combination of 300 ppm SFN and 0.5% DBM in AIN-76A diet on arachidonic acid metabolism in both intestinal tumors and normal-appearing mucosa of *Apc*<sup>Min/+</sup> mice. **A**, both DBM and combinatorial treatments significantly decreased PGE<sub>2</sub> levels in intestinal tumors and normal-appearing mucosa, whereas SFN treatment increased the level of PGE<sub>2</sub> slightly. **B**, LTB<sub>4</sub> levels were significantly decreased by all treatments and in a much more extent in the intestinal tumors than in the normal-appearing mucosa. **C**, decreased intestinal PGE<sub>2</sub> and LTB<sub>4</sub> levels were associated with down-regulation of COX-2 but not 5-LOX enzyme expression.

correlated with the inhibition of COX-2 expression. Furthermore, the down-regulation of genes involved in PI3K/Akt pathway (such as *Pik3ap1*, *Pik3ca*, and *Pik3cg*) and Ras signaling pathways (*Rab14*, *Rab30*, *Rab38*, and *Rit2*) shown by microarray analysis strongly suggests that DBM and in combination with SFN can inhibit intestinal tumorigenesis by inhibiting the COX-2/PGE<sub>2</sub>-mediated multiple signaling pathways. Down-regulation of LTB<sub>4</sub> levels by both DBM and SFN treatments may also contribute to their antitumorigenesis because LTB<sub>4</sub> could stimulate proliferation of human colon carcinoma cells *in vitro* (17, 18) and its concentration in tumors was found to be 3-fold of that in apparently normal mucosa in this study. Surprisingly, the 5-LOX expression was almost unaffected by all treatments. However, both DBM and

combination treatment strongly induced the LTB<sub>4</sub> 12-hydroxydehydrogenase (*Ltb4dh*) gene in both polyps and apparently normal mucosa, which is the key enzyme to metabolize LTB<sub>4</sub> (19), and increased expression of this enzyme can result in decreased level of LTB<sub>4</sub> *in vivo*. This result is also consistent with recent reports that many cancer chemopreventive reagent can induce the metabolism of LTB<sub>4</sub> (20). Furthermore, the strong induction of cytochrome *P450* genes and epoxide hydrolases (*Ephx1* and *Ephx2*) by both SFN and DBM treatments may shift the arachidonic acid metabolism more toward the epoxygenase pathway that forms the epoxyeicosatrienoic acid and dihydroxyacids, which will also result in decreased level of both PGE<sub>2</sub> and LTB<sub>4</sub>. On the other hand, the induction of many phase II detoxification (GST) and



**Figure 3.** Regulation of cellular survival signaling pathways and proteins by dietary administration of 600 ppm SFN, 1.0% DBM, and combination of 300 ppm SFN and 0.5% DBM in *Apc<sup>Min/+</sup>* mice. **A**, effects of SFN, DBM, and combinatorial treatments on phosphorylation levels of Akt and MAPKs, including ERK1/2, JNK, and p38 in intestinal tumor and normal-appearing mucosa in *Apc<sup>Min/+</sup>* mice. **B**, dietary administration of SFN, DBM, or combination of SFN and DBM significantly altered proteins involved in cell cycle regulation and apoptosis.



**Figure 4.** Correlation of microarray and real-time PCR data. Log<sub>10</sub>-transformed fold of changes of genes measured by microarray from different samples were plotted against the corresponding log<sub>10</sub>-transformed values obtained by quantitative real-time PCR analysis. Data obtained from the two different approaches were highly correlated with Spearman correlation coefficient  $r^2 = 0.957$ .

antioxidant genes (*HO-1*, *Ephx1*, *Txnrd*, etc.) by both SFN and DBM treatment will increase the cellular defense mechanisms against electrophiles, reactive oxygen species, or inflammatory mediators, including those generated during arachidonic acid metabolism or lipid peroxidation process. The decreased cellular oxidative stress by SFN and DBM through the above mechanism may create a microenvironment that can slow or inhibit the development of intestinal polyps from normal-appearing mucosa cells with genetic *Apc* mutation in this animal model.

Mutation of *Apc* gene in *Apc*<sup>Min/+</sup> mouse leads to the dysregulation of Wnt signaling pathway, which is responsible for the development of multiple intestinal neoplasia in this model (21). Down-regulation of this pathway by increasing the expression of E-cadherin to decrease nuclear  $\beta$ -catenin level was suggested as one of the cancer chemopreventive mechanisms of (–)-epigallocatechin-3-gallate in this model (11). However, both SFN and DBM treatments did not change the level of E-cadherin in both polyps and apparently normal mucosa nor did the  $\beta$ -catenin level in nucleus (data not shown). This result suggests that inhibition of intestinal polyposis in *Apc*<sup>Min/+</sup> by SFN and DBM may not through the regulation of nuclear  $\beta$ -catenin level. On the other hand, Akt kinase, which is a downstream target of numerous receptor tyrosine kinases and showed elevated phosphorylation at Ser<sup>473</sup> in polyps compared with that in apparently normal mucosa in this study, was strongly inhibited by both SFN and DBM treatments. In agreement with this event, several PI3K-related genes (*Pik3ap1*, *Pik3ca*, *Pik3cg*, and *Pik3c2a*) were down-regulated by SFN and/or DBM treatments as shown by microarray analysis. Genetic abrogation of c-Jun NH<sub>2</sub>-terminal phosphorylation or gut-specific conditional c-Jun inactivation reduced tumor number/size and prolonged life span in *Apc*<sup>Min/+</sup>

mice. As the JNK and *Apc*/ $\beta$ -catenin pathways were viewed as two distinct pathways activated by Wnt signal transduction (22), the specific inhibition of JNK by SFN and DBM in intestinal tumor might be the result of down-regulation of Wnt signaling pathway without affecting the  $\beta$ -catenin pathway. Furthermore, the inhibition of cell proliferation and growth-related ERK1/2 by both DBM and SFN treatments might also contribute to their tumor inhibitory effects.

Both SFN and DBM could induce cell cycle arrest and apoptosis in various cancer cells (9, 23, 24). In this study, SFN strongly induced the expression of p21, which is in accordance with our previous studies (10, 25). Interestingly, DBM strongly inhibited cyclin D1 expression but did not induce p21, suggesting distinct mechanisms and potentially synergistic effects between these two compounds in regulating cell growth. Immunohistochemical results also showed that both SFN and DBM could inhibit the expression of PCNA in intestinal polyps, and stronger inhibition was also observed in the combination treatment. SFN treatment also resulted in the activation of caspases, including caspase-7 and caspase-3, whereas DBM treatment has lesser effect in inducing apoptosis. It is interesting to point out that caspase-3 was only activated in the intestinal polyps but not in apparently normal mucosa, suggesting induction of tissue-specific apoptotic effect by SFN. The efficacy of both compounds to induce cell apoptosis was further verified by *in situ* apoptosis detection assay. All these observations suggested that SFN and DBM may exert its cancer chemoprevention effects through induction of cell cycle arrest and/or apoptosis.

In summary, the present study shows for the first time that dietary administration of SFN and DBM alone or in combination significantly suppressed colorectal tumorigenesis in the *Apc*<sup>Min/+</sup> mice model. We also showed that their cancer chemopreventive effects may involve mechanisms including alteration of arachidonic acid metabolism, inhibition of cellular survival signaling pathway such as Akt and MAPKs, and induction of cell growth inhibition and apoptosis. The gene expression profiles elicited by these treatments further support these potential mechanisms and provide new molecular insights on their *in vivo* pharmacologic effects. Taken together, our results suggest that the combination of these relatively nontoxic and low-cost dietary phytochemical compounds will be beneficial in preventing common malignancies, such as colorectal cancers, particularly in high-risk FAP patients.

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## Chemoprevention of Familial Adenomatous Polyposis by Natural Dietary Compounds Sulforaphane and Dibenzoylmethane Alone and in Combination in *Apc*<sup>Min/+</sup> Mouse

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