Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable

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Abstract

A recent study reported that broccoli consumption is linked to a lowered risk of colon cancer and that the protective effect is especially evident in individuals with a glutathione S-transferase (GST) M1 null genotype. As GSTs facilitate the conjugation of isothiocyanates (ITC) resulting in their excretion as N-acetylcysteine (NAC) conjugates via the mercapturic acid pathway, it has been suggested that the isothiocyanates in broccoli may play a role in protection against human colon cancer (7). Sulforaphane (SFN) is the predominant ITC found in broccoli, and it has been found to inhibit carcinogen-induced mammary gland tumorigenesis (8), colonic aberrant crypt foci (9,10), stomach tumors (11) and lung cancer (12) in rats/mice. Here, we investigated the chemopreventive efficacy of SFN in preventing the development of adenomas in the ApcMin/+ mouse model. ApcMin/+ mice were fed with two different doses of SFN in diet for 3 weeks. At the end of the study, differences of adenoma multiplicities and sizes, apoptotic and proliferative indices among treatment groups were compared. To elucidate the underlying mechanism, the expression levels of β-catenin and cyclin-D1 were examined. These biomarkers are important downstream targets for the Wnt signaling pathway and have been previously shown to be highly expressed in the adenomas of ApcMin/+ mice (13). Additionally, we also compared the expression levels of phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular signal-regulated kinases (p-ERKs) and c-MYC (3,4). The ApcMin/+ mouse has an autosomal dominant heterozygous nonsense mutation of the mouse Apo gene at codon 850 (5), homologous to the human germ line and somatic APC mutations. The ApcMin/+ mouse model is unique in that tumors appear spontaneously in the gastrointestinal tract, rather than as a result of induction by a carcinogen. This model is particularly advantageous for testing chemopreventive agents targeted against early-stage lesions because scores of adenomas grow to a grossly detectable size within a few months on a defined genetic background (6). As ApcMin/+ mice develop adenomas as a result of inactivation of the same tumor suppressor gene known to be involved in the pathogenesis of most colon cancer in humans, experiments with this model are likely to be relevant to the design of human chemoprevention clinical trials (6).

Introduction

The adenomatous polyposis coli (APC) gene has been directly implicated in the development of human colon cancer by both germ line and somatic mutation (1). Loss of APC function is the initiating event in both familial polyposis as well as in the vast majority of sporadic colon cancers. In fact, APC mutations have been identified in the earliest histologically identifiable lesions of the adenoma–carcinoma sequence termed aberrant crypt foci (2). APC function has recently been linked to the WNT signal transduction pathway, where it normally functions to target β-catenin for degradation. Truncation mutation of APC gene increases cytoplasmic accumulation and nuclear translocation of β-catenin. In the nucleus, the transcription factor, T cell factor/lymphoid enhancer factor (TCF/LEF), will be transactivated by β-catenin leading to an increase expression of genes that regulate cell proliferation and apoptosis such as cyclin-D1 and c-MYC (3,4). The ApcMin/+ mouse has an autosomal dominant heterozygous nonsense mutation of the mouse Apo gene at codon 850 (5), homologous to the human germ line and somatic APC mutations. The ApcMin/+ mouse model is unique in that tumors appear spontaneously in the gastrointestinal tract, rather than as a result of induction by a carcinogen. This model is particularly advantageous for testing chemopreventive agents targeted against early-stage lesions because scores of adenomas grow to a grossly detectable size within a few months on a defined genetic background (6). As ApcMin/+ mice develop adenomas as a result of inactivation of the same tumor suppressor gene known to be involved in the pathogenesis of most colon cancer in humans, experiments with this model are likely to be relevant to the design of human chemoprevention clinical trials (6).

A recent study reported that broccoli consumption is linked to a lowered risk of colon cancer and that the protective effect is especially evident in individuals with a glutathione S-transferase (GST) M1 null genotype. As GSTs facilitate the conjugation of isothiocyanates (ITC) resulting in their excretion as N-acetylcysteine (NAC) conjugates via the mercapturic acid pathway, it has been suggested that the isothiocyanates in broccoli may play a role in protection against human colon cancer (7). Sulforaphane (SFN) is the predominant ITC found in broccoli, and it has been found to inhibit carcinogen-induced mammary gland tumorigenesis (8), colonic aberrant crypt foci (9,10), stomach tumors (11) and lung cancer (12) in rats/mice. Here, we investigated the chemopreventive efficacy of SFN in preventing the development of adenomatous lesions in the ApcMin/+ mouse model. ApcMin/+ mice were fed with two different doses of SFN in diet for 3 weeks. At the end of the study, differences of adenoma multiplicities and sizes, apoptotic and proliferative indices among treatment groups were compared. To elucidate the underlying mechanism, the expression levels of β-catenin and cyclin-D1 were examined. These biomarkers are important downstream targets for the Wnt signaling pathway and have been previously shown to be highly expressed in the adenomas of ApcMin/+ mice (13). Additionally, we also compared the expression levels of phosphorylated c-Jun N-terminal kinase (p-JNK), phosphorylated extracellular signal-regulated kinases (p-ERKs) and...
Cancer chemoprevention of intestinal polyposis by sulforaphane

Table I. Tumor multiplicity in small intestine of ApcMin/+ mice after treatment with control diet or SFN containing diet

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Proximal (%)</th>
<th>Middle (%)</th>
<th>Distal (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11</td>
<td>6.6 ± 0.8 (100)</td>
<td>16.9 ± 1.6 (100)</td>
<td>20.4 ± 2.7 (100)</td>
<td>43.9 ± 4.4 (100)</td>
</tr>
<tr>
<td>SUL 300 p.p.m.</td>
<td>11</td>
<td>3.8 ± 0.5 (57.6)</td>
<td>13.2 ± 1.7 (78.1)</td>
<td>15.8 ± 3.0 (77.5)</td>
<td>32.8 ± 4.5 (74.7)</td>
</tr>
<tr>
<td>SUL 600 p.p.m.</td>
<td>11</td>
<td>2.0 ± 0.4 (30.3)</td>
<td>9.2 ± 1.4 (54.4)</td>
<td>12.0 ± 1.6 (58.8)</td>
<td>23.2 ± 2.9 (53)</td>
</tr>
</tbody>
</table>

*Values are presented as mean ± SE (% control).

Table II. Tumor size in small intestine of ApcMin/+ mice after treatment with control diet or SFN containing diet

<table>
<thead>
<tr>
<th>Tumor diameters (mm)</th>
<th>Treatment</th>
<th>300 p.p.m. (%)</th>
<th>600 p.p.m. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;1.0</td>
<td>Control</td>
<td>28 (5.8)</td>
<td>192 (53.2)</td>
</tr>
<tr>
<td>1.0–1.9</td>
<td>412 (85.9)</td>
<td>159 (44.0)</td>
<td>70 (27.5)</td>
</tr>
<tr>
<td>&gt;2.0</td>
<td>40 (8.3)</td>
<td>10 (2.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>480 (100)</td>
<td>361 (100)</td>
<td>255 (100)</td>
</tr>
</tbody>
</table>

Values are presented as total number of tumors in certain size range in each treatment group (% total number of tumor in each treatment group).

Materials and methods

Animal care and drug treatment

ApcMin/+ mice were obtained from Jackson Laboratories (Bangor, ME) at the age of 7 weeks. Drug treatment was started at the age of 8 weeks. They were divided into three groups of 11 ApcMin/+ mice receiving either (i) control AIN-76A diet; (ii) 300 p.p.m. SFN (LKT Laboratories) in AIN-76A diet; or (iii) 600 p.p.m. SFN in AIN-76A diet (Research Diets, New Brunswick, NJ), respectively. Animals were weighed weekly and checked daily for signs of ill health. Mice were killed by CO2 inhalation at the age of 11 weeks, before the tumor burden caused any obvious morbidity. All animal experiments were carried out under an Institutional Animal Care and Use Committee approved protocol.

Tissue processing

After killing the animals, the small intestines were stripped of associated mesenteric fat and blood vessels, then divided by length into three equal sections (proximal, middle and distal segments), opened longitudinally and rinsed thoroughly with saline for polyps enumeration. Next, one-fifth of each segment was snap-frozen in liquid nitrogen for drug concentrations assay. For histopathology examination and immunohistochemical (IHC) analysis, the intestines were stapled flat to a plastic sheet and placed in 10% phosphate-buffered formalin for 24 h. The intestinal samples were then washed twice in phosphate-buffered saline (PBS; pH 7.4) for 5 min each. Non-specific binding was blocked by incubating with 3% bovine serum albumin (BSA) for 10 min. Incubation with primary antibody took place at room temperature for 30 min for cyclin-D1 (1:200; Zymed), β-catenin antibodies (1:1000; Transduction Laboratories), p-JNK (1:200; Cell Signaling Technology), p-ERK (1:200; Cell Signaling Technology) and p-Akt (Ser473) antibodies (1:50; Cell Signaling Technology). For the subsequent reaction, a streptavidin–biotin complex peroxidase kit (LSAB+ kit, Dako) was used according to the manufacturer’s instructions. The peroxidase activity was developed with the substrate 3,3,5-tiamino benzidine tetrahydrochloride (DAB+, Dako) by incubating the sections in DAB for 2 min. The slides were then rinsed gently with distilled water and counterstained with hematoxylin for 30 s. The slides were dehydrated in alcohol prior to mounting. Negative controls were carried out by omitting the relevant primary antibodies from duplicate sections and substituted with PBS.

Apoptosis and proliferation

The apoptotic cells were detected using an ApopTag In Situ Apoptosis Detection Kit (Chemicon). The assay was performed according to the manufacturer’s manual. After deparaffinization, the tissues sections were incubated in proteinase K for 15 min at room temperature. The sections were then incubated with terminal deoxynucleotidyl transferase (TdT) enzyme at 37°C for 1 h, washed in three changes of PBS and incubated with anti-digoxigenin 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, Ki-67 antibody (1:50; Dako) was used. The assay was performed following the manufacturer’s protocols. The scoring of apoptotic and proliferative cells was done at ×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 Ki-67 staining, small intestinal crypt cells were used as an internal positive control.

Evaluation of staining and statistical analysis

For Ki-67 and cyclin-D1 staining, cells with blue nuclei were considered unlabeled/negative, while cells with brown nuclei were considered labeled/positive. The expression of cyclin-D1 as well as apoptotic and proliferative indices were calculated as number of positive cells in the crypts cells and the epithelial cells lining the villous surface of an adenomatous polyp divided by the total number of cells counted multiplied by 1000. For β-catenin, p-JNK, p-ERK and p-Akt (Ser473) staining, a modified semiquantitative scoring system (14,15) was used to evaluate the staining by the antibodies. The degree of positive staining for all antibodies was evaluated by scoring on a scale of 0–4 for percentage of positive cells and on a scale of 0–3 for strength of intensity of staining. The percentage of positive cells was evaluated using the following scale: 0, no staining of the mucosal epithelial cells in any field; 1+, <25% of the epithelium stain positive; 2+, 25–50% stain positive; 3+, 50–75% stain positive; and 4+, >75% stain positive. As for the evaluation of strength of intensity of staining, this was estimated using the following scale: 0, no staining of the mucosal epithelial cells in any field; 1+, <25% of the epithelium stain positive; 2+, 25–50% stain positive; 3+, 50–75% stain positive; and 4+, >75% stain positive. For Ki-67 staining, small intestinal crypt cells were used as an internal positive control.

Measurement of SFN and SFN–GSH (glutathione) levels in the plasma and small intestine

The drug levels in the blood plasma as well as in small intestines from the animals exposed to varying doses of SFN via the diet were examined. Drug concentrations were determined by using LC/MS (Finnigan, Spectra System, San Jose, CA). Plasma samples were passed through Centricron filters at 4°C (hydrophilic membrane, 3000 MW pore size; Amicon, Beverly, MA) to remove molecules with molecular weights >3000 Da. One volume of
acetonitrile was added to each filtrate. The intestinal samples were homogenized in 400 ml water followed by 30 s of sonication. The homogenates were centrifuged at 14 000 g for 10 min and the supernatant was obtained. One volume of acetonitrile was added to precipitate the protein and after centrifugation at 14 000 g for 10 min, the supernatant was passed through a 0.45 mm filter (Sartorius, Goettingen Germany). The LC/MS system is composed of a Finnigan series of binary pumps, a degasser, a cooled autosampler and a system controller (Spectra system, San Jose, CA). An analytical C18 column (Shimadzu) was equilibrated with solvent composed of 45:55 (v/v) A:B, where A is 0.1% (v/v) formic acid in water and B is 0.1% formic acid in acetonitrile, with a flow rate of 0.2 ml/min. After loading of 10 ml sample, the column was eluted with the equilibrium solvent isocratically for 5 min and then eluted in a linear gradient mode up to 100% solvent B over 15 min. The eluant was directed to a Finnigan mass spectrometer (LCQ DECA) equipped with its Turbo Ionspray heated at 45°C using the nebulizer gas and nitrogen as the auxiliary gas. The Ionspray needle was maintained at 5.5 kV under positive mode to generate the molecular ion (M+H)+. The m/z for SFN was 178 and for SFN–GSH was 485, respectively. The ion optics was adjusted to operate at unit mass resolution. Optimization and calibration were achieved with Xcalibur (Finnigan) software to obtain a state file for mass spectrometer.

**Results**

**Chemopreventive effect of SFN on intestinal polyps**

After 3 weeks of treatment, the average number of polyps in the small intestine in each mouse decreased 25.3 and 47% in the 300 and 600 p.p.m. treatment group as compared to the control animals, respectively (Table I). No polyps were observed in the colon. In addition, as shown in Table II, when the polyps were grouped based on their diameters, 85.9% of the polyps in control mice were in the 1.0–2.0 mm size group, and only 5.8% were in the <1.0 mm size group; while 53.2% of the polyps in the 300 p.p.m. SFN-treated group were in the <1.0 mm group; and 72.5% of the polyps in the 600 p.p.m. SFN-treated group were in the <1.0 mm group. These results showed that in ApcMin/+ mice treated for 3 weeks with SFN decreased both the polyps multiplicity and the size as compared to the control, in a dose-dependent manner. To investigate the efficacy of SFN in inducing apoptosis and suppressing cell proliferation, the ApopTag In Situ Apoptosis Detection Kit and Ki-67 monoclonal antibody were used. The results showed that SFN selectively induces apoptosis in the adenomas but not normal mucosa and the apoptotic indices were significantly higher in adenomas of SFN-treated ApcMin/+ mice (Figure 1A–D; P = 0.028 for 300 p.p.m. SFN versus control; P < 0.0001 for 600 p.p.m. SFN versus control; P = 0.048 for 300 p.p.m. SFN versus 600 p.p.m. SFN). In contrast, the proliferative indices were significantly lower in adenomas of SFN-treated ApcMin/+ mice in comparison to the control group (Figure 2A–D; P < 0.0001 for 300 or 600 p.p.m. SFN versus control). In normal mucosa, proliferative cells were predominantly found in the crypt cells of the intestinal tissues. However, the proliferative indices were not significantly different in the normal mucosa of ApcMin/+ mice from different groups (data not shown). To elucidate the underlying mechanisms in which SFN elicited its chemopreventive effect, the expression of cyclin-D1, β-catenin, p-Akt, p-JNK and p-ERK were also evaluated in the present study. Positive cyclin-D1 immunoreactivity was
predominantly found in the crypt and epithelial cells of the adenomatous polyps in comparison to the normal mucosa (Figure 3A). On the other hand, strong cytoplasmic and nuclear β-catenin immunoreactivity was observed in the epithelial cells of adenomatous polyps (Figure 3C). In normal mucosa, the expression of β-catenin was only observed in the cell-to-cell border. However, no significant difference in the expression of these two biomarkers was found between the control and SFN treatment groups (Figure 3B and D). While the expression of cyclin-D1 and β-catenin was unaffected by SFN, the expression of the p-JNK, p-ERK and p-Akt was significantly suppressed in the treatment group as compared to their control counterpart (Figures 4–6). Strong p-JNK, p-ERK and p-Akt immunoreactivity was observed in the nucleus and cytoplasm of adenomas. In normal mucosa, the immunoreactivity of these biomolecules was only found in the crypt cells (Figures 4–6). The mean of total score for p-JNK was 5.4, 3.8 and 2.5 for control, 300 and 600 p.p.m. groups, respectively \((P = 0.002\) and <0.001 for control versus 300 p.p.m. and control versus 600 p.p.m., respectively; Figure 4D). The mean of total score for p-ERK was 5.3, 3.8 and 2.5 for control, 300 and 600 p.p.m. groups, respectively \((P = 0.002\) and <0.001 for control versus 300 p.p.m. and control versus 600 p.p.m., respectively; Figure 5D). The mean of total score for p-Akt was 5.4, 4.6 and 4.3 for control, 300 and 600 p.p.m. groups, respectively \((P = 0.06\) and 0.01 for control versus 300 p.p.m. and control versus 600 p.p.m., respectively; Figure 6D).

**Steady-state levels of SFN in plasma and target tissue**

The relationship between the chemopreventive efficacy of SFN in ApcMin/+ mice and the concentration of the agent or its metabolites was investigated in the present study. To that end, steady-state levels were determined in the plasma and small intestine, the target tissue, which had received dietary SFN at either 300 or 600 p.p.m. for 3 weeks using LC/MS. SFN was present in the plasma at levels ranging from 124 to 254 nM and SFN–GSH was at the level from 579 to 770 nM. In the small intestine, SFN concentration varied between 3 and 13 nmol/g of tissue and SFN–GSH 14–32 nmol/g of tissue. SFN levels in the serum reflected differences in dose more consistently than the SFN–GSH level did, but in the small intestine the SFN–GSH level correlated better with the dose (Table III).

**Discussion**

It is evident that SFN inhibits carcinogenesis in carcinogen-induced animal models. SFN was found to be able to block 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumor formation in rats (8), reduced tumor formation in mouse mammary gland explants (16), suppressed azoxymethane (AOM)-dependent abnormal colon crypt formation in rats (9) and blocked benzo[a]pyrene-induced forestomach tumors in ICR mice (11). In our present study, we have demonstrated that ApcMin/+ mice fed with SFN-supplemented diet...
developed significantly less and smaller polyps in their small intestine than their control counterpart, in a dose-dependent manner. Our results showed that cancer chemopreventive effect of SFN on intestinal polyposis in ApcMin/+ mice were through the induction of apoptosis and inhibition of proliferation of the adenomas. Interestingly, the SFN-induced apoptosis and inhibition of proliferation were restricted to adenomas but not in normal mucosa. Previous studies have shown that SFN can inhibit the growth of human cancer cells in culture by inducing apoptosis and causing cell cycle arrest (17–20). However, the in vivo mechanism by which SFN induces apoptosis or suppresses tumor growth is not fully defined. As dysregulation of the Wnt signaling pathway is believed to play a major role in the carcinogenesis of ApcMin/+ model, we examined the expression of two important biomarkers from this signaling pathway, β-catenin and cyclin-D1 in the small intestine of ApcMin/+ mice. Accumulation of nuclear β-catenin is one of the hallmarks for the aberrant activation of the Wnt signaling pathway that is regulated by APC gene. One of the biological consequences of this activation is the transactivation of genes that control cell cycle or proliferation such as cyclin-D1. Increased β-catenin and cyclin-D1 expression have been previously reported in colorectal cancer (CRC) patients (21–23) and in ApcMin/+ mice (13). In agreement with these observations, we found that the expression of cyclin-D1 and β-catenin were significantly higher in the adenomas in comparison to the normal mucosa. However, no significant difference in the expression of these biomarkers was found between the control and SFN treatment groups. This observation is in contradiction with in vitro cell culture model in which cyclin-D1 is an inhibitory target of SFN (24,25). The reason for this discrepancy is not clear and may explain the importance of in vivo study before in vitro findings can be translated into human clinical trials. Another direct transcriptional target of TCF/β-catenin, EphB2 has been reported to be important in the carcinogenesis of diverse tumor types including CRC (26). It has been shown that reduction of EphB activity accelerates colon tumor progression in the ApcMin/+ mice (27). It will be interesting to know whether the inhibition of intestinal polyposis by SFN is mediated through modulation of EphB activity. However, our data shows that the expression of EphB2 was not affected by treatment of SFN (data not shown). Our results suggest that SFN may not directly modulate major biomolecules in the Wnt or TCF/β-catenin signaling pathway to exert its chemopreventive effects.

Cumulative evidence suggests that aberrant Wnt signaling pathway could trigger activation of pro-survival/anti-apoptotic signaling cascades such as the PI3K/Akt and/or MAPK signaling pathways to facilitate the clonal expansion of dysregulated crypt cells. It has been reported that overexpression of β-catenin stimulates the activation of ERK leading to proliferation of fibroblast cells (28). Furthermore, increased expression of Akt by β-catenin/Tcf-Lef was also reported in CRC cells (29). Therefore, we analyzed the expression levels of p-JNK, p-ERK and p-Akt in the intestinal tissues of...
ApcMin/+ mice. Our results showed that p-JNK, p-ERK and p-Akt were highly activated in the adenomas of ApcMin/+ mice. Consistent with our observations, increased activity of JNK, ERK and Akt was reported previously in patients with CRC (30,31). In addition, Moran et al. (32) has also reported the overexpression of p-Akt in ApcMin/+ mice. Importantly, treatment of ApcMin/+ mice with SFN significantly suppressed the levels of p-JNK, p-ERK and p-Akt in the adenomas. A large body of evidence indicates that the activation of mitogen-activated protein kinases (MAPKs), such as JNK, ERK and Akt signaling pathways, could be crucial for pathogenesis, progression and oncogenic behavior of human CRC (33). Mutations of KRAS, an upstream regulator of MAPK and PI3K/Akt signaling pathways were found in 36% of CRCs (34). Furthermore, 17% of colon cancers have PTEN mutations, with a higher frequency (60%) being observed in microsatellite unstable tumors (35). For this reason, a number of chemotherapeutic drugs targeting MAPK/Akt are currently undergoing preclinical testing for the treatment of CRC (36). The present study extends our understanding of APC-associated tumor formation by showing the involvement of MAPK (JNK and ERK) and PI3K/Akt (Akt) signaling in the tumorigenesis process. This observation also partly supported the hypothesis of possible crosstalk between Wnt, PI3K/Akt and MAPK signaling pathways. Therefore, we hypothesize that SFN inhibits intestinal polyposis of ApcMin/+ mice through concomitant suppression of p-JNK, p-ERK and p-Akt, leading to decrease proliferation and increase apoptosis of adenomas. This hypothesis is corroborated by a recent finding by Myzak et al. (37) who found that long-term treatment of SFN suppressed tumorigenesis in ApcMin/+ mice through inhibition of histone deacetylase. Histone deacetylase (HDAC) inhibitor has been shown to exert its antiproliferation and proapoptotic effects through modulation of various signaling pathway, including the PI3K/ and ERK1/2 pathways (38).

Emerging data suggests that chronic inflammation plays an important role in colorectal carcinogenesis. Increase oxidative stress induced by reactive nitrogen species has been previously reported to be correlated with development of intestinal polyposis in ApcMin/+ mice (39). Since SFN is also a potent antioxidant and anti-inflammatory agent, future experiments studying its effects on biomarkers such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) could provide additional insight into suppression of intestinal polyposis by SFN in vivo.

Intake of SFN at a dietary level of 300 p.p.m., which equates to ~40 mg/kg/day (or 5 μmol/day), prevented, or retarded, adenoma formation. A further increase in the dietary level by a factor of 2 to ~80 mg/kg/day (600 p.p.m.; 10 μmol/day) yielded additional efficacy, with an increase in the SFN and its metabolites’ concentrations in the plasma and the small intestine. SFN and its metabolite SFN–GSH, at 3–30 nmol/g of small intestine tissue, the range of levels obtained here, appears to be necessary to prevent or retard adenoma formation in the gastrointestinal tract of ApcMin/+ mice. These concentrations range are roughly equivalent to ~3–30 μM concentrations of total SFN. In the in vitro experimental

Fig. 4. Analysis of p-JNK expression in adenomas of ApcMin/+ mice. Representative slide of p-JNK immunoreactivity in the cytoplasm and nucleus of adenomas of ApcMin/+ mice fed with (A) AIN-76A, (B) AIN-76A + 300 p.p.m. SFN and (C) AIN-76A + 600 p.p.m. SFN for 3 weeks. Strong immunoreactivity of p-JNK was found in the cytoplasm and nucleus of adenomas. Weak to moderate immunoreactivity of these biomolecules was seen in the crypt cells of normal mucosa. Magnification x200. The immunoreactivity was evaluated as described in Materials and methods. (D) Bar represents the mean ± SD total score for the expression of p-JNK in the adenomas of ApcMin/+ mice from different groups. The results show that the total score of p-JNK was significantly lower in the 600 p.p.m. SFN treatment groups as compared to the control (P = 0.001 for 600 p.p.m. versus control and 0.24 for 300 p.p.m. versus control). (Double asterisks represent a statistically significant value with P < 0.01.)
Fig. 5. Analysis of p-ERK expression in adenomas of ApcMin/+ mice. Representative slide of p-ERK immunoreactivity in the cytoplasm and nucleus of adenomas of ApcMin/+ mice fed with (A) AIN-76A, (B) AIN-76A + 300 p.p.m. SFN and (C) AIN-76A + 600 p.p.m. SFN for 3 weeks. Strong immunoreactivity of p-ERK was found in the cytoplasm and nucleus of adenomas. Weak to moderate immunoreactivity of these biomolecules was seen in the crypt cells of normal mucosa. Magnification ×200. The immunoreactivity was evaluated as described in Materials and methods. (D) Bar represents the mean ± SD total score for the expression of p-ERK in the adenomas of ApcMin/+ mice from different groups. The total score of p-ERK was significantly lower in the treatment groups as compared to the control (P < 0.001 for 300, 600 p.p.m. versus control). (Double asterisks represent a statistically significant value with P < 0.01.)

Fig. 6. Analysis of p-Akt (Ser473) expression in adenomas of ApcMin/+ mice. Representative slide of p-Akt (Ser473) immunoreactivity in the cytoplasm and nucleus of adenomas of ApcMin/+ mice fed with (A) AIN-76A, (B) AIN-76A + 300 p.p.m. SFN and (C) AIN-76A + 600 p.p.m. SFN for 3 weeks. Strong immunoreactivity of p-Akt (Ser473) was found in the cytoplasm and nucleus of adenomas. Weak to moderate immunoreactivity of these biomolecules was seen in the crypt cells of normal mucosa. Magnification ×200. The immunoreactivity was evaluated as described in Materials and methods. (D) Bar represents the mean ± SD total score for the expression of p-Akt (Ser473) in the adenomas of ApcMin/+ mice from different groups. The total score of p-Akt was also found to be significantly lower in the 600 p.p.m. SFN treatment groups as compared to the control (P = 0.01 for 600 p.p.m. versus control and P = 0.06 for 300 p.p.m. versus control). Data represent mean ± SD. (Asterisks represent a statistically significant value with P < 0.05.)
systems using mammalian cell lines, SFN has been shown to inhibit the growth of human cancer cells in culture by inducing apoptosis and causing cell cycle arrest (19). All of these inhibitory actions of SFN require the \textit{in vitro} concentrations in the range of 1–50 μM, which is quite remarkably similar to the levels that were achieved in the target tissue of ApcMin/+ mice at efficacious dietary doses as described in our current study.

Naturally occurring components including ITCs have received considerable attention as potential agents of cancer chemoprevention. Although the etiology of colon cancer is considered to be multifactorial and complex, dietary factors such as a high animal fat intake is considered to be linked positively with an elevated incidence. We believe that naturally occurring food components are probably safer and more effective chemopreventive agents against colon cancer.

In conclusion, the study presented here corroborates the notion that SFN possesses chemopreventive activity in a model germane to human colorectal carcinogenesis involving \textit{Apc} mutations. This study defines for the first time the relationship between chemopreventive efficacy of SFN and its concentration in the ApcMin/+ mouse. These findings encourage, in principle, the potential evaluation of SFN for adenoma-retarding efficacy in familial adenomatous polyposis (FAP) patients.

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Conflict of Interest Statement: None declared.

References


Table III. Concentration of SFN and its metabolite SFN-GSH in small intestine of mice that received SFN at 300 and 600 p.p.m. in their diet for 3 weeks.

<table>
<thead>
<tr>
<th>SFN content in diet</th>
<th>Plasma levels (nM)</th>
<th>Small intestine levels (nmol/g)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SFN</td>
<td>SFN-GSH</td>
</tr>
<tr>
<td>300 p.p.m.</td>
<td>124 ± 44</td>
<td>579 ± 106</td>
</tr>
<tr>
<td>600 p.p.m.</td>
<td>254 ± 136</td>
<td>770 ± 103</td>
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Values are the mean ± SD of 11 animals.

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