RESEARCH ARTICLE

Isothiocyanate sulforaphane inhibits protooncogenic ornithine decarboxylase activity in colorectal cancer cells *via* induction of the TGF-β/Smad signaling pathway

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Scope: The objective of this study was to elucidate molecular mechanisms behind the anti-tumor activities of the isothiocyanate sulforaphane (SFN) in colorectal cancer cells.
Methods and results: Cell growth was determined by BrdU incorporation and crystal violet staining. Protein levels were examined by Western blot analysis. Ornithine decarboxylase (ODC) activity was assayed radiometrically. Reverse transcriptase-PCR was used for measuring mRNA expression. For reporter gene assays plasmids were transfected into cells via lipofection and luciferase activity was measured luminometrically. Acetyl-histone H3 and H4 chromatin immunoprecipitation (ChIP) assays were performed followed by PCR with TGF-β-receptor II promoter specific primers. We could show that SFN-mediated cell growth inhibition closely correlates with a dose-dependent reduction of protein expression and enzymatic activity of ODC. This effect seems to be due to reduced protein levels and trans-activation activity of transcription factor c-myc, a direct regulator of ODC expression, as a consequence of SFN-induced TGF-β/Smad signaling. The coherency of these results was further confirmed by using TGF-β receptor kinase inhibitor SB431542, which largely abolishes inhibitory effects of SFN on both, ODC activity and cell growth.
Conclusion: Since elevated ODC enzyme activity is associated with enhanced tumor development, SFN may be a dietary phytochemical with potential to prevent carcinogenesis.

Keywords:
c-myc / Colorectal cancer / Ornithine decarboxylase / Sulforaphane / Transforming growth factor-β signaling

1 Introduction

Colorectal cancer accounts for approximately 10% of all cancers and is the second leading cause of cancer-related death in Western countries with an estimated 146 970 new cases and 49 900 deaths in 2009 in the United States [1]. In addition to conventional cancer therapy, preventive measures that target the multi-step progress of carcinogenesis involving initiation, promotion and progression [2] are aimed at decreasing the incidence and mortality of cancer. In this context, increasing evidence suggests the importance of food and nutrition in the modification of the cancer development [3]. In particular, *in vitro* and *in vivo* data revealed that consumption of cruciferous vegetables is supposed to lower overall cancer risk, including colon cancer, especially during the early stages [4]. These anti-carcinogenic activities have been suggested to be partly due to the relatively high levels of sulforaphane (SFN), the major biologically active compound [5]. SFN, first isolated from broccoli in the early 1990s, was initially identified as a potent

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Abbreviations: BrdU, bromodeoxyuridine; **FCS,** fetal calf serum; **HDAC,** histone deacetylase; **HDACi,** histone deacetylase inhibitor; **ODC,** ornithine decarboxylase; **SBE,** Smad binding element; **SFN,** sulforaphane; **TGF-β,** transforming growth facor-β; **TGF-β RI,** transforming growth factor-β receptor I; **TGF-β RII,** transforming growth factor-β receptor II

phase 2 enzyme inducer, but in recent years numerous studies have implicated further chemopreventive effects, comprising cell growth inhibition, induction of apoptosis and reduction of angiogenesis [6–9], whereby the underlying molecular mechanisms remain largely unclear.

Recently, Myzak and Colleagues identified SFN as a novel histone deacetylase inhibitor (HDACi) in colon and prostate cancer cells [10]. HDACi, as a new class of chemotherapeutic agents, show significant promise against a variety of cancers in clinical trials [11]. Most available HDACi inhibit all class I and II histone deacetylases (HDACs), thereby increasing acetylation of histone and nonhistone protein targets [12]. *In vivo*, histone acetylation depends on the balance between histone acetyltransferases and HDACs, which has been proposed to play an important role in transcriptional regulation by altering chromatin structure [13]. Histone acetylation by histone acetyltransferases is associated with an open chromatin conformation, promoting gene transcription, whereas HDACs maintain the chromatin in the closed, transcriptional inactive state. HDAC inhibitors have been shown to induce the expression of several tumor suppressive genes, such as $p21^{WAF1}$ [14], growth-differentiation factor 11, a member of the transforming growth factor-β (TGF-β) superfamily [15] as well as TGF-β receptors I and II (TGF-βRI and TGF-βRII) [16, 17]. Thus, the pro-tumorigenic role of HDACs in colon cancer and the pre-clinical efficacy of these agents in colon cancer cells justify the use of HDACi in cancer prevention and therapy [18].

TGF-β has been implicated in various cellular processes, including regulation of cellular proliferation [19] and differentiation [20], immune modulation [21] and extracellular matrix remodeling [22]. It exhibits its antiproliferative functions by activating a signaling pathway that mediates cell cycle arrest and induction of apoptosis. TGF-β exerts its effects through heteromeric receptor complexes consisting of type I and type II serine/threonine kinase receptors. The signaling is initiated by ligand binding to the TGF-βRII cell surface receptor. This, in turn, recruits the TGF-βRI kinase, which then phosphorylates the R-Smad proteins, Smad2 and Smad3 [23]. Activated R-Smads form a complex with the Co-Smad, Smad4, which shuttles directly to the nucleus. Here, the complex can either act as a co-activator or DNA-binding transcription factor, participating in the regulation of target gene expression (*e.g.* p15, p21, c-myc) [23, 24]. TGF-β signaling is further controlled by a third class of Smads, the inhibitory Smad6 and Smad7 proteins, which negatively regulate R-Smad activation. Recently, Daniel *et al.* [25] could demonstrate that butyrate, another natural occurring HDACi, mediates anticarcinogenic effects in colorectal cancer cells, at least partly, through the activation of the TGF-β signaling pathway. Furthermore, Traka *et al.* [26] provide evidence that SFN enhances TGF-β signaling in cell cultures.

Previous studies could demonstrate that modulation of polyamine metabolism provides a chemopreventive strategy of different phytochemicals [27–31]. Although cellular polyamines spermidine and spermine, as well as their precursor putrescine, are essential for growth and DNA synthesis, increasing concentrations are associated with hyperproliferation and cell transformations [32]. Overall, polyamines are involved in almost all steps of colonic tumorigenesis. Regulation of polyamine levels is governed primarily by activity of ornithine decarboxylase (ODC), the key enzyme of polyamine biosynthesis. Hence, regarding the role of polyamines in colonic carcinogenesis, modulation of polyamine metabolism and, in particular, of ODC activity has been studied as a potent therapeutic strategy in cancer treatment and prevention [30, 33].

Thus, the aim of this project was to provide insight into the molecular mechanisms of SFN-mediated anti-tumor activities, in particular by investigating regulatory functions of SFN on ODC activity, also with regard to a possible involvement of the TGF-β signaling pathway.

2 Materials and methods

2.1 Cell culture and materials

Caco-2 cells were kept in DMEM, supplemented with 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids and were maintained at 37°C in an atmosphere of 95% air and 5% CO_2. The cells were passaged weekly using Dulbecco's PBS containing 0.1% trypsin and 1% EDTA. The cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24 h. For the ODC activity assay, the cells were synchronized in a medium containing 1% FCS 24 h before treatment. SFN (Calbiochem) was dissolved in DMSO at a concentration of 100 mM, SB431542 (Sigma-Aldrich) was dissolved in DMSO at a concentration of 10 mM and TGF-β-1 (PeproTech) was dissolved in BSA at a concentration of 10 μg/mL. DMEM, DMEM/Ham's F-12 medium, McCoy's 5A, FCS, DMSO, Sodium pyruvate solution, penicillin and streptomycin stock solutions were all obtained from PAA Laboratories GmbH.

2.2 Cell counts

Cells were suspended and cultured in 96-well dishes at a density of 10^4/well (0.28 cm^2). Twenty-four hours after plating, cells were incubated for 24–72 h with substances. At given time points, cell numbers were assessed by crystal violet staining. Medium was removed from the plates and cells were fixed with 5% formaldehyde for 5 min. After washing with PBS, cells were stained with 0.5% crystal violet for 10 min, washed again with PBS and destained with 33% acetic acid. Absorption, correlating linear with cell number, was measured at 620 nm.

2.3 Cell proliferation

The effects of SFN on DNA synthesis of cells was assessed using a cell proliferation ELISA kit (Roche Diagnostics). This assay is a colorimetric immunoassay for the quantification of cell proliferation based on the measurement of bromodeoxyuridine (BrdU) incorporation during DNA synthesis. Cells were grown in 96 well culture dishes (10^4 cells/well), incubated with SFN for different time intervals and then labeled with BrdU for a further 4 h. Incorporated BrdU was measured colorimetrically.

2.4 Plasmids

The human pGL3-c-myc promoter (−62–93) with the sequence TTCTCAGAGGCTTGGCGGGAAAAAGAACGG and the pGL3-c-myc-promotor (−62–93) TIEm with the sequence TTCTCAGA**TTAAA**GGCGGGAAAAAGAACGG (Smad binding site mutated) were kindly provided by Prof. Ellenrieder (Marburg, Germany).

The pCGN-Smad3 and pCGN-Smad4 expression plasmids were obtained from Dr. X. F. Wang (Durham, USA) and the 4 × SBE (Smad binding element)-luc promoter was generously provided by B. Vogelstein (Johns Hopkins, Baltimore, MD, USA) [34].

2.5 Reporter assays

Caco-2 cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Medium was changed after 4 h of transfection and the cells were incubated in a medium containing 10% serum for another 16–20 h. Later, the cells were treated with SFN (20 μM) or TGF-β (20 nM) and then subjected to luciferase assay. pCGN and pGL3, as empty vector plasmids, were used as a negative control for transfection and luciferase assay. Luciferase assays were carried out by using luciferase assay kit (Promega) and a luminometer (TECAN Spectra-Fluor^{Plus}). A cytomegalovirus-Renilla luciferase plasmid or a Simian Virus-40-Renilla luciferase plasmid (both from Promega) was used as a control, to normalize the transfection efficiency, and was assayed as described [35].

2.6 SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Caco-2 cells were seeded in 80-cm^2 flasks; 24 h after plating, cells were incubated with substances for different time intervals ranging from 1 to 48 h. After washing the cells with ice-cold PBS, followed by an incubation step with cell lysis buffer (Cell signalling) containing multiple protease inhibitors (Complete Mini®, Roche) for 20 min at 4°C, cells were harvested by scraping. Protein extracts were obtained after sonication of cell lysates (2 × 5 s) and centrifuged at 10 000 rpm for 10 min at 4°C. Samples were analyzed for their protein content using the BioRad® colorimetric assay according to the method of Bradford (BioRad Laboratories). After addition of sample buffer (Roti Load®, Roth) to the total cellular extract and boiling for 5 min at 95°C, 30–40 μg of total protein lysate was separated on a 10 or 12% SDS-polyacrylamide gel. Protein was transferred onto nitrocellulose membrane (Schleicher&Schuell) and the membrane was blocked for 1 h at room temperature with 5% skimmed milk in Tris-buffered saline containing 0.05% Tween-20. Next, blots were washed and incubated overnight at 4°C in Tris-buffered saline containing 0.05% Tween-20 containing either 5% BSA or 5% skimmed milk with a 1:1000 or 1:2000 dilution of primary antibodies for TGF-β1, TGF-βRI and TGF-βRII (all from Cell Signaling), c-myc and ODC (all from Santa Cruz Biotechnology). The secondary, horseradish peroxidase-conjugated, antibody (Santa Cruz Biotechnology) was diluted at 1:2000 or 1:4000 and incubated with the membrane for another 60 min in skimmed milk. Following chemoluminescence reaction (ECL, Amersham pharmacia biotech), bands were detected after exposure to Hyperfilm-MP (Amersham International plc). Blots were reprobed with β-actin antibody (Santa Cruz Biotechnology). For quantitative analysis, bands were evaluated densitometrically by ProViDoc system (Desaga), normalized for the density of β-actin.

2.7 Reverse transcriptase-PCR

Total cellular RNA was isolated by RNAzol BTM (Wak-Chemie), following manufacturer's instructions. Reverse transcription of total cellular RNA was carried out using Superscript II RNase H reverse transcriptase (Life Technologies) and random hexanucleotide primers (Promega). PCR was performed (on the cDNA) using the following sense and antisense primers, custom-synthesized by Biospring: *TGF-β1*: 5′-CAC GAT CAT GTT GGA CAA CTG CTC C-3′ and 5′-CTT CAG CTC CAC AGA GAA GAA CTG-3′; *GAPDH*: 5′-ATC TTC CAG GAG CGA GAT CC-3′ and 5′-ACC ACT GAC ACG TTG GCA GT-3′. Thermal cycling was performed as follows: denaturation for 30 s at 95°C, annealing for 30 s at 55°C and extension for 90 s at 72°C. Twenty-five to thirty-five cycles were performed. Primers were used at a final concentration of 10 μM each, dNTPs at 500 μM (Eurogentec) and $MgCl_2$ at 3 mM. Five units of Taq DNA Polymerase were used *per* 50 μL reaction. Ten microliter of PCR product were separated on a 1.5% agarose gel containing ethidium bromide and visualized by UV illumination.

2.8 ODC activity

The activity of ODC was assayed using a radiometric technique in which the amount of $^{14}CO_2$ liberated from

DL-[l-^{14}C]ornithine (207.2×10^4 MBq/mol, Amersham Pharmacia Biotech) was estimated, as described previously [36]. Briefly, after treatment, cell culture dishes were placed on ice and monolayers were washed three times with cold PBS. Cells were harvested by scraping in homogenizing buffer (50 mM Tris buffer, pH 7.2, 5 mM DTT, 100 μM EGTA), sonicated and centrifuged for 10 min at $15\,000 \times g$ at 4°C. Hundred microliter of the obtained supernatant was incubated in a stoppered tube with 74 μM DL-[l-^{14}C]ornithine in the presence of pyridoxal-5-phophate for 1 h at 37°C. $^{14}CO_2$, liberated by the decarboxylation of ornithine and trapped on filters impregnated with benzethonium hydroxide, was measured by liquid scintillation spectroscopy. Samples were analyzed for their protein content using the BioRad® colorimetric assay according to the method of Bradford (BioRad Laboratories). ODC is expressed in picomolar of released CO_2 *per* hour *per* milligram of protein. Controls always included samples for measurement of nonenzymatic release of $^{14}CO_2$.

2.9 Chromatin immunoprecipitation Assay

Chromatin immunoprecipitation assays were performed using Acetyl-Histone Immunoprecipitation Assay Kits (Upstate Biotechnology) following the manufacturer's instructions. Briefly, approximately 1×10^6 cells cultured in a 75 cm^2 dish were treated with different concentrations of SFN (10–20 μM). Histones were cross linked to DNA by adding 1% formaldehyde directly to culture medium for 10 min at 37°C. Each sample was lysed in SDS lysis buffer (kit component) containing protease inhibitors (1 mM PMSF, 1 μg/mL aprotinin and 1 μg/mL pepstatin A) followed by sonication on ice to shear DNA to lengths between 200 and 1000 bp and incubation with primary antibodies to acetylated histone H3 or acetylated histone H4 overnight at 4°C. Samples were incubated with Salmon Sperm DNA/Protein A Agarose-Slurry (kit component) for 1 h at 4°C with rotation. After washing several times with washing buffers (kit components), the pellet protein A agarose/antibody/histone complex was incubated with elution buffer (1% SDS and 0.1 M $NaHCO_3$) for 15 min at room temperature. Samples were incubated with 0.2 M NaCl for 4 h at 65°C. After DNA was recovered by phenol/chloroform extraction and ethanol precipitation, PCR was performed using the following primers for TGF-βRII promoter, sense: 5′-GAG AGA GCT AGG GGC TGG-3′; antisense: 5′-CTC AAC TTC AAC TCA GCG CTG C-3′; primer for β-actin gene promoter, sense: 5′-CCA ACG CCA AAA CTC TCC C-3′; antisense: 5′-AGC CAT AAA AGG CAA CTT TCG-3′ [17]. Results were finally normalized to β-actin.

2.10 Statistics

The data are expressed as mean ± SE of at least three independent experiments. Results were analyzed using GraphPad Prism software by a two-way ANOVA. A p-value < 0.05 was considered to be significant.

3 Results and discussion

3.1 Cell growth-inhibitory effects of SFN closely correlate with the reduction of ODC protein and activity

In colorectal cancer tissue, the activities of the polyamine-synthesizing enzymes ODC and *S*-adenosylmethionine decarboxylase as well as the content of polyamines are increased 3- to 4-fold over that found in the equivalent normal colonic tissue [37]. Increasing concentrations of polyamines are generally associated with cell proliferation and cell transformation induced by growth factors [38], carcinogens [39] or oncogenes [32]. Several studies suggest

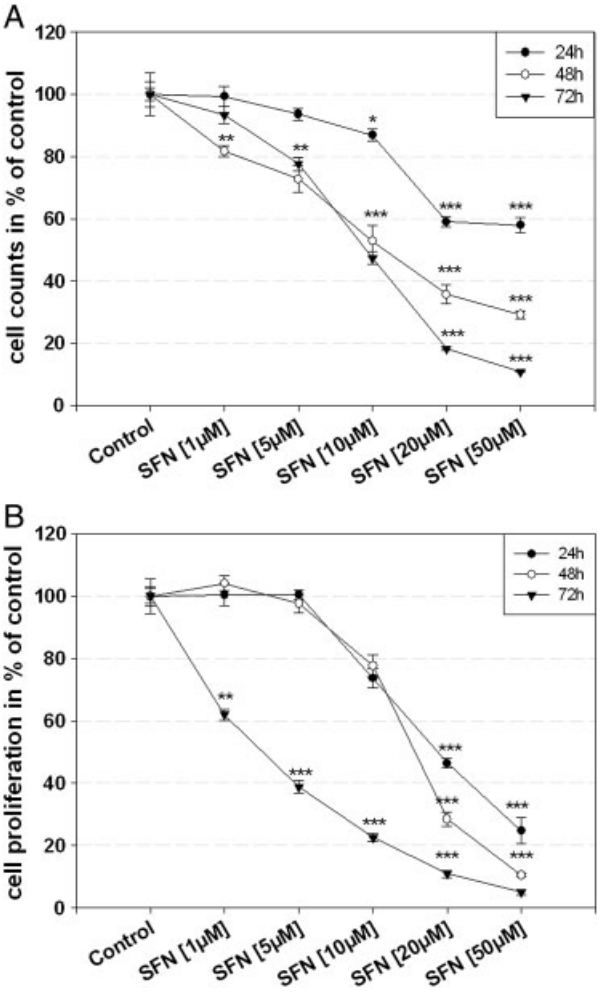

Figure 1. Cell counts and cell proliferation of Caco-2 cells (A, B) 24, 48, and 72 h after incubation without (control) or with SFN (1–50 μM). Values represent mean ± SE ($n = 3$); $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *versus* control.

that a number of potential chemopreventive agents down-regulate ODC expression/activity and subsequently deplete polyamine content in colon cancer cells [40]. Hence, inhibiting the polyamine metabolism, particularly the biosynthetic key enzyme ODC, is considered to represent an attractive target for both cancer chemotherapy and cancer chemoprevention. Interestingly, in their most recent publication, Furniss *et al.* reported modulator effects of broccoli extracts as well as SFN alone on polyamine metabolism in colorectal cancer cells. However, direct modes of action were not further specified [41]. In the present study, we could demonstrate that SFN (1–50 μM) inhibits cell counts and proliferation significantly in Caco-2 cells in a dose- and time-dependent manner ($^{***}p<0.001$) (Fig. 1A and B), which closely correlates with a dose-dependent reduction of ODC protein levels after 3 and 6 h (Fig. 2A) and activity after 24 h of incubation (Fig. 2B) ($^{***}p<0.001$). The crucial role of polyamine depletion was further suggested as addition of exogenous spermidine significantly ($^{***}p<0.001$) counteracted growth inhibitory effects of SFN after 24 h (Fig. 2C). Similar effects could be observed by the group of Lee *et al.* where SFN inhibited TPA-induced ODC activity in mouse epidermal ME308 cells [42].

ODC levels are tightly controlled either by transcriptional regulation or by rapid post-translational degradation. The proto-oncogene c-myc is a transcription factor that directly regulates the expression of ODC by binding to a specific CACGTG sequence in the gene promoter [43]. c-myc was subsequently found to be activated in various animal and human tumors and an amplification of the c-myc gene has been described in ~15% of all human tumors [44]. Since c-myc is a ubiquitous promoter of cell growth and proliferation, it functions as a transcriptional activator or inhibitor depending on the target gene [45, 46]. We could demonstrate that decreased ODC activity and protein levels in Caco-2 cells are accompanied by decreased protein levels of c-myc after 6 h of incubation (Fig. 2D), implicating a direct modulation of ODC gene expression by SFN.

3.2 Effects of SFN on TGF-β signaling

We previously reported that induction of the TGF-β signaling pathway in colon cancer cells is a crucial event in the anti-carcinogenic activities of butyrate, another natural occurring HDAC inhibitor [25]. This, together with the

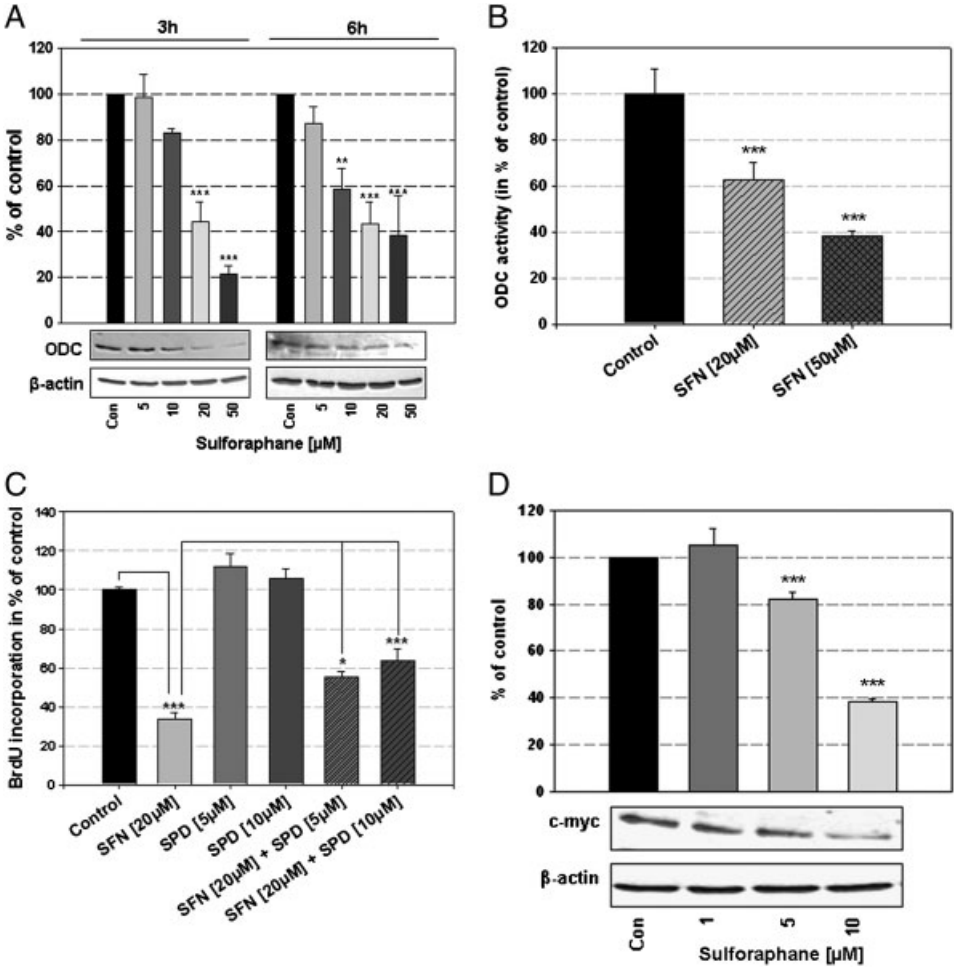

Figure 2. (A) Western blot of ODC (~53 kDa) in Caco-2 cells after incubation with SFN (5–50 μM) for 3 and 6 h. The graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE ($n=3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) Activity of ODC in Caco-2 cells after incubation with SFN (20–50 μM) for 24 h. Bar graphs represent mean ± SE ($n=4$); $^{***}p<0.001$ *versus* control. (C) Add-back experiment with spermidine. Cell proliferation was measured after simultaneous treatment of Caco-2 cells with SFN (20 μM) and spermidine (SPD) (5–10 μM) for 24 h. Bar graphs represent mean ± SE ($n=3$); $^{*}p<0.05$, $^{***}p<0.001$ *versus* control. (D) Western blot of c-myc protein expression (~67 kDa) in Caco-2 cells after incubation with SFN (1–10 μM) for 6 h. The bar graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE ($n=3$); $^{*}p<0.05$, $^{***}p<0.001$ *versus* control). Representative immunoblots of three independent experiments are shown.

findings of Traka *et al.*, who could demonstrate that TGF-β signaling also seems to play an important role in various SFN-mediated effects [26] lets us consider whether SFN possibly shows similar modes of action in our context. Thus, we measured protein as well as mRNA level of TGF-β in Caco-2 cells and could show an obvious increase of TGF-β precursor levels after 2 h (Fig. 3A) and an increase of mRNA after 1 and 3 h of incubation with SFN, indicating regulatory effects on the level of transcription (Fig. 3B). As already mentioned, not only TGF-β alone but also the expression status of TGF-βRI and TGF-βRII is essential for TGF-β-mediated actions. Therefore, protein levels of both receptors I and II were detected after incubation with SFN, and were found to be highly increased after 1 h (Fig. 3C) ($^{***}p<0.001$).

To further specify these regulatory mechanisms, we used chromatin immunoprecipitation analysis to investigate possible effects of SFN-mediated HDAC inhibition on the

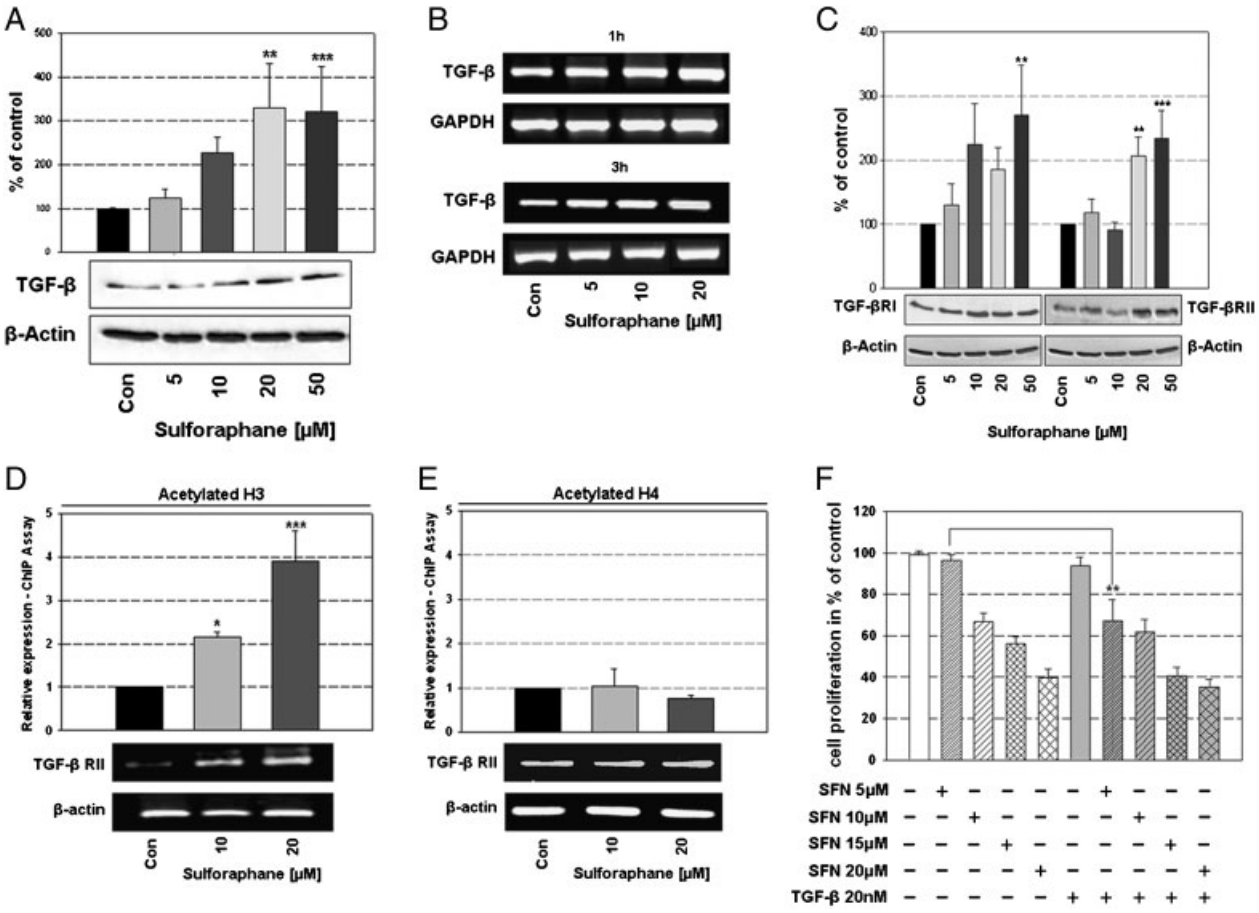

Figure 3. (A) Western blot of TGF-β1 precursor (~50 kDa) in Caco-2 cells after incubation with SFN (5–50 μM) for 2 h. A representative immunoblot of three independent experiments is shown. The bar graph presents densitometric analysis of the Western blot images normalized to β-actin (mean ± SE ($n=3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) RT-PCR of TGF-β mRNA (298 bp) in Caco-2 cells after incubation with SFN (5–20 μM) for 1 and 3 h. Representative agarose gels showing PCR products of three independent experiments for both time-points are shown. (C) SFN-induced expression of TGF-β receptors I and II protein level after 1 h of incubation. Caco-2 cells were treated without (control) and with SFN (5–50 μM). Whole-cell lysates were prepared and TGF-β receptors I and II protein expression were determined by Western blotting using either anti-TGF-β receptors I (~52 kDa) and II (~110 kDa) antibodies. Representative immunoblots of three independent experiments are shown. $^{**}p<0.01$, $^{***}p<0.001$ *versus* control. (D) Caco-2 cells were treated with SFN (10–20 μM) or vehicle for 1 h of incubation. DNA was cross-linked to proteins before harvesting. Chromatin immunoprecipitation (ChIP Assay) was performed against acetylated histone H3, and after DNA isolation and reversal of cross-linking, primers specific for TGF-β receptor II (101 bp) were used during PCR amplification. Results were normalized to β-actin (156 bp), and expressed relative to control, which was assigned an arbitrary value of 1.0. Graph presents the densitometric analysis after 1 h (mean ± SE ($n=3$); $^{*}p<0.05$, $^{***}p<0.001$ *versus* control). (E) Caco-2 cells were treated with SFN (10–20 μM) or vehicle for 1 h of incubation. DNA was cross-linked to proteins before harvesting. ChIP Assay was performed against acetylated histone H4, and primers specific for TGF-β receptor II (101 bp) were used during PCR amplification. Results were normalized to β-actin (156 bp), and expressed relative to control, which was assigned an arbitrary value of 1.0. Graph presents the densitometric analysis after 1 h. (F) Cell proliferation of Caco-2 cells after simultaneous treatment with SFN (5–20 μM) and TGF-β (20 nM) for 24 h. Values represent mean ± SE ($n=3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control.

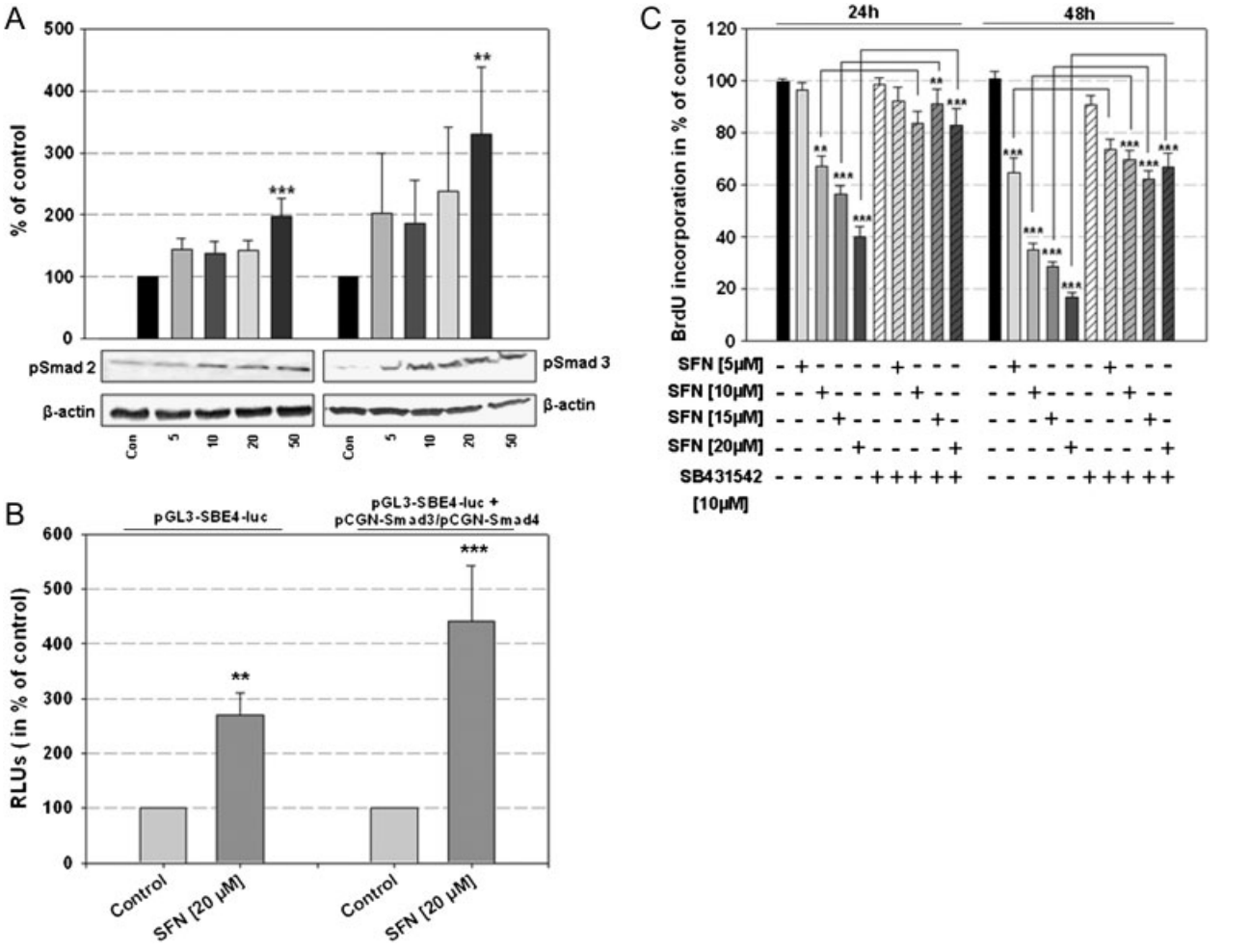

Figure 4. (A) Western blot of pSmad2 and pSmad3 in Caco-2 cells after incubation with SFN (5–50 μM) for 1 h. One representative immunoblot of three independent experiments for both proteins is shown. The bar graphs present densitometric analysis of the Western blot images normalized to β-actin (mean ± SE; $^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) The pGL3-SBE4-luc reporter gene construct (800 ng) alone and in combination with the expression vectors pCGN-Smad3/pCGN-Smad4 (100 ng each) and 20 ng SV-40-renilla were transiently transfected into Caco-2 cells *via* lipofection. Cells were treated without (control) or with SFN (20 μM) for 6 h before luciferase activity was determined. Results are presented as relative light *per* units (RLUs) in percentage of control, normalized to transfection efficiency (cotransfection of SV-40-renilla) and normalized to effects of the empty vectors pCGN and pGL3basic (mean ± SE ($n = 3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* untreated cells). (C) Cell proliferation of Caco-2-cells treated with SB431542 (10 μM) and SFN (5–20 μM). BrdU incorporation was measured after 24 and 48 h of incubation (mean ± SE ($n = 3$); $^{**}p<0.01$, $^{***}p<0.001$ *versus* control).

acetylation status of histone H3 as well as histone H4 associated with the TGF-βRII promoter (Fig. 3D and E). After 1 h, accumulation of RII with highly acetylated histone H3 was observed in SFN-treated Caco-2 cells in comparison to untreated control cells ($^{***}p<0.001$) (Fig. 3D). This SFN effect on RII is selective because the β-actin gene was not affected and obviously specific for acetylated H3 since acetylated histone H4 was not associated with the TGF-βRII gene after incubation with SFN for 1 h (Fig. 3E). The observed accumulation of acetylated histone H3 indicated histone acetylation to be involved in the transcriptional induction of RII and further suggests the importance of HDAC inhibitory properties of SFN in the regulation of TGF-β signaling. In earlier works, Osada *et al.* already demonstrated that HDAC inhibitors can increase the transcriptional activity of TGF-βRII *in vivo* and *in vitro* in human cancer cell lines [17]. In addition to these findings, Lee *et al.* reported [47] that another HDAC inhibitor MS-275 induces the accumulation of acetylated histones in the chromatin of the TGF-βRII gene, which is associated with an increase of TGF-βRII mRNA in human breast cancer cells, contributing to the restoration of TGF-β signaling. As both TGF-βRI and TGF-βII protein levels were regulated by SFN, we were interested to know whether co-incubation with exogenous

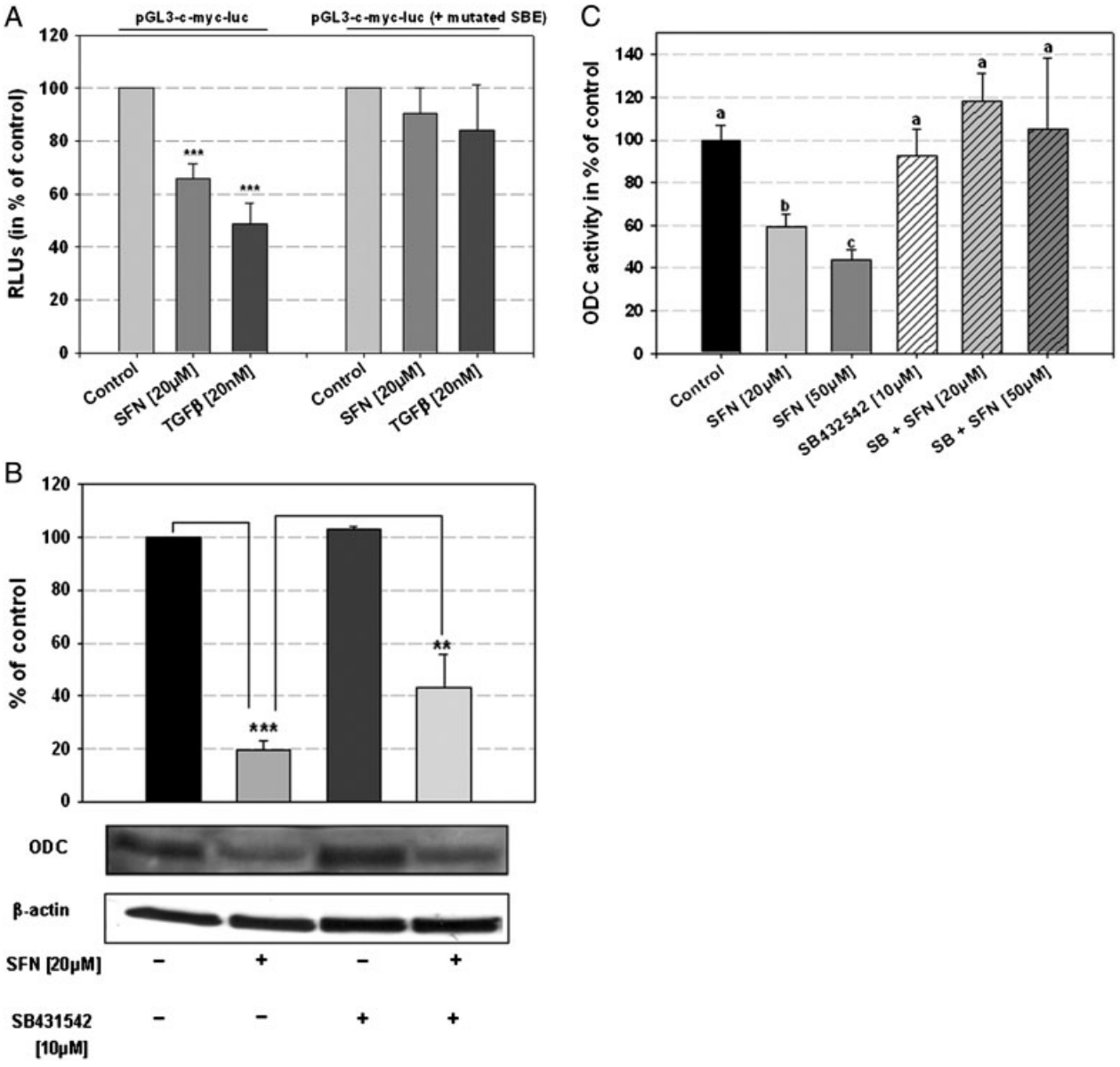

Figure 5. (A) Either pGL3-c-myc-luc or pGL3-c-myc-luc (ΔSBE) (400 ng) and 15 ng CMV-renilla were transiently transfected into Caco-2 cells *via* lipofection. Cells were treated with SFN (20 μM) or TGF-β (20 ng) for 6 h before luciferase activity was determined. Each experiment was performed in triplicates. Results are presented as mean ± SE of at least three independent experiments after normalization for transfection efficiency by cotransfection of CMV-renilla. Results are expressed with respect to pGL3-cotransfected cells ($^{**}p<0.01$, $^{***}p<0.001$ *versus* control). (B) Western blot of ODC protein in Caco-2 cells after 6 h of incubation with SFN (20 μM) or/and SB431542 (10 μM). One representative immunoblot of three independent experiments for both proteins is shown. The bar graphs present densitometric analysis of the Western blot images normalized to β-actin (mean ± SE; ($n=3$); $^{***}p<0.001$ *versus* control; $^{**}p<0.01$ *versus* SFN). (C) Activity of ODC after simultaneous treatment with SFN (20–50 μM) and/or SB431542 (10 μM) in Caco-2 after 24 h of incubation (mean ± SE; ($n=3$); values not sharing a letter differ significantly, $^{*}p<0.05$).

TGF-β (20 nM) might amplify SFN-mediated reduction of cell proliferation in Caco-2 cells. For this, we analyzed BrdU incorporation after 24 h of treatment, but could only detect significant additive effects at a concentration of 5 μM SFN ($^{**}p<0.01$) (Fig. 3F). That, in accordance with our above-mentioned findings on intracellular TGF-β levels, lets us hypothesize that higher doses of SFN might lead to intracellular TGF-β-saturation and thus resistance to exogenous TGF-β.

Upon ligand binding, receptors of the TGF-β family generally phosphorylate Smad proteins, which then move into the nucleus where they activate transcription of different target genes. For responding to the question whether SFN-mediated TGF-β-signaling also involves an activation of

Smads, we first analyzed the phosphorylation status of Smad2 and Smad3 in Caco-2 cells after 1 h of incubation with increasing concentrations of SFN, which was found to be significantly induced in a dose-dependent manner (Fig. 4A) ($^{***}p<0.001$). Then, we performed reporter gene assays with SBE_4luc, where four SBEs are cloned in the pGL3-vector. Reporter gene activity of SBE_4luc was significantly induced by SFN ($^{**}p<0.01$), whereas the induction could be further enhanced when expression vectors pCGN-Smad3/pCGN-Smad4 were cotransfected ($^{***}p<0.001$) (Fig. 4B). To give the direct evidence that SFN mediates growth inhibitory effects, at least partly *via* induction of the TGF-β-signaling pathway, we simultaneously treated the cells with SFN and the specific TGF superfamily type 1 activin receptor-like kinase inhibitor SB431542 alone and in combination and measured the BrdU incorporation after 24 and 48 h (Fig. 4C). In fact, cell growth inhibitory effects of SFN were largely abolished by SB431542 ($^{***}p<0.001$).

3.3 Involvement of the TGF-β signaling pathway in SFN-induced inhibitory effects on ODC expression and activity in Caco-2 cells

Since Smad proteins were able to suppress c-myc activity in human skin epithelial cells by directly binding to Smad-responsive elements in the *c-myc* promoter [48], we were interested whether the observed downregulation of c-myc protein by SFN might also be due to an activation of Smad signaling. Therefore, we have done reporter gene assays after 6 h of SFN-treatment on Caco-2 cells either transfected with wild-type c-myc-luc or with c-myc-luc bearing mutated SBEs. TGF-β was used as a positive control. While in Caco-2 transfected with the wild-type construct, both SFN and TGF-β significantly decreased reporter gene activity compared to untreated cells ($^{***}p<0.001$), no effects could be observed in cells transfected with a mutated c-myc-promoter (Fig. 5A). We conclude that due to the mutation in the SBEs, Smads could not efficiently bind to the promoter resulting in an abolishment of the SFN- and TGF-β-mediated inhibition of c-myc gene activity.

Controversial data exist about the effects of TGF-β on polyamine metabolism. On the one hand, TGF-β has been shown to induce ODC mRNA in H-ras-transformed fibrosarcoma cell lines on which TGF-β acts as a growth stimulator [49]. On the other hand, Motyl *et al.* [50] could demonstrate that TGF-β suppresses both cell growth and the activities of ODC and *S*-adenosyl-L-methionine decarboxylase in a human chronic myelogenous lymphoma cell line. Similar results were found in the group of Nishikawa *et al.* [51], showing inhibitory effects of TGF-β on polyamine metabolism. In accordance with these publications and since our results revealed an involvement of the TGF-β signaling pathway in SFN-mediated downregulation of the *c-myc* promoter, we suggested that modulation of TGF-β signaling might also affect downstream ODC expression and activity. Indeed we could observe that co-incubation of SFN with a specific TGF-β kinase inhibitor partly abolished SFN-induced reduction of ODC protein expression (Fig. 5B) and activity (Fig. 5C), which might be due to direct TGF-β/Smad signaling-mediated transcriptional repression of transcription factor c-myc, upstream of the ODC gene. This effect was investigated in detail by Chen *et al.*, who could demonstrate that repression of c-myc expression by TGF-β occurs by direct interaction of a repressor complex consisting of Smad3 the transcription factors E2F4/5 and DP1 and the retinoblastoma family member p107 with a regulatory Smad responsive element in the *c-myc* promoter [52].

4 Concluding remarks

In summary, the present study clearly points out that SFN mediates growth inhibitory effects in colorectal cancer cell lines, at least partly, *via* TGF-β-dependent inhibition of c-myc and thus reduced protein expression and activity of proto-oncogene ODC. Noting the fact that colorectal cancer is still one of the most commonly occurring malignancies worldwide, the use of nontoxic agents like SFN, which inhibit specific molecular steps in the carcinogenic pathway, might be a promising strategy for cancer chemoprevention.

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