ABSTRACT

Sulforaphane (SFN), a compound found at high levels in broccoli and broccoli sprouts, is a potent inducer of phase 2 detoxification enzymes and inhibits tumorigenesis in animal models. SFN also has a marked effect on cell cycle checkpoint controls and cell survival and/or apoptosis in various cancer cells, through mechanisms that are poorly understood. We tested the hypothesis that SFN acts as an inhibitor of histone deacetylase (HDAC). In human embryonic kidney 293 cells, SFN dose-dependently increased the activity of a β-catenin-responsive reporter (TOPFlash), without altering β-catenin or HDAC protein levels. Cytoplasmic and nuclear extracts from these cells had diminished HDAC activity, and both global and localized histone acetylation was increased, compared with untreated controls. Studies with SFN and with media from SFN-treated cells indicated that the parent compound was not responsible for the inhibition of HDAC, and this was confirmed using an inhibitor of glutathione S-transferase, which blocked the first step in the metabolism of SFN, via the mercapturic acid pathway. Whereas SFN and its glutathione conjugate (SFN-GSH) had little or no effect, the two major metabolites SFN-cysteine and SFN-N-acetylcysteine were effective HDAC inhibitors in vitro. Finally, several of these findings were recapitulated in HCT116 human colorectal cancer cells: SFN dose-dependently increased TOPFlash reporter activity and inhibited HDAC activity, there was an increase in acetylated histones and in p21(Cip1/Waf1), and chromatin immunoprecipitation assays revealed an increase in acetylated histones bound to the p21 promoter. Collectively, these findings suggest that SFN may be effective as a tumor-suppressing agent and as a chemotherapeutic agent, alone or in combination with other HDAC inhibitors currently undergoing clinical trials.

INTRODUCTION

The reversible acetylation of nuclear histones is an important mechanism of gene regulation (1, 2). A balance exists in normal cells between histone acetyl transferase and histone deacetylase (HDAC) activities, and when this balance goes awry, cancer development can ensue (3, 4). Natural inhibitors of HDAC, such as trichostatin A (TSA), have received considerable interest as anticancer agents because of their ability to induce proteins such as p21(Cip1/Waf1), leading to cell cycle arrest, differentiation, or apoptosis in neoplastically transformed cells (2–6). Some of the most potent HDAC inhibitors have TSA-like chemical structures, with a hydrophobic group attached to a “spacer” and a distal hydroxamic acid moiety that fits into the HDAC catalytic active site (2).

The simplest HDAC inhibitor identified to date is butyric acid, a short-chain fatty acid produced in the gut in millimolar concentrations, and that causes cell cycle blockade, differentiation or apoptosis in a number of transformed cell lines (4, 7, 8). The findings with butyrate have raised an awareness about other HDAC inhibitors that, although less potent than TSA, might nonetheless contribute to cancer chemoprevention as a result of their chronic, daily ingestion in the diet. One recent study showed that diallyl disulfide, a chemopreventive organosulfur compound from garlic, increased p21(Cip1/Waf1) expression in human colon cancer cells via the inhibition of HDAC (9).

It is likely that the human diet contains many other HDAC inhibitors, and, in this context, we became interested in the dietary chemopreventive agent sulforaphane (SFN). This isothiocyanate was first isolated from broccoli as a potent inducer of phase 2 detoxification enzymes, as well as being an inhibitor of phase 1 enzymes that activate chemical carcinogens, and SFN has been shown to prevent cancer in laboratory animals (10–12). High cellular accumulation of SFN has been observed in mammalian cells, up to millimolar concentrations, and appreciable levels of SFN and its metabolites are excreted in the urine of humans consuming broccoli (13–16). A recent study in the rat reported plasma concentrations of SFN on the order of 20 μM (17). On the basis of considerations of the HDAC active site and the chemical features of known HDAC inhibitors, we sought to test the hypothesis that SFN would inhibit HDAC activity. The present paper reports, for the first time, that two of the major metabolites of SFN indeed act as HDAC inhibitors, with evidence for altered histone acetylation status and increased p21(Cip1/Waf1) expression in human embryonic kidney 293 cells and HCT116 colon cancer cells.

MATERIALS AND METHODS

Cell Culture. Human embryonic kidney 293 (HEK293) cells were obtained from American Type Culture Collection (Manassas, VA) and were cultured in minimum essential media (Life Technologies, Inc., Grand Island, NY) with 10% horse serum, 1 mM sodium bicarbonate, and 1 mM sodium pyruvate at 37°C under 5% CO2. HEK293 cells (1 × 106) were seeded 36–48 h before transfection into 60-mm culture dishes coated with 0.2% gelatin. HEK293 cells and HCT116 cells, respectively. Cells were harvested 48 h posttransfection. In some experiments, the glutathione S-transferase (GST) inhibitor ethacrynic acid (100 μM; Sigma, St. Louis, MO) was added 1 h after transfection. SFN (LKT Laboratories; St. Paul, MN) was added 1 h after transfection or 2 h after the addition of ethacrynic acid. TSA (100 ng/ml; Biomol, Plymouth Meeting, PA) was added 8 h before harvesting. SFN, ethacrynic acid, and TSA were dissolved in DMSO; DMSO (Sigma) alone was used as vehicle control. Cytoplasmic and nuclear extracts were obtained using the Ne-Per kit (Pierce; Rockford, IL). All of the transfections were done in triplicate, and each experiment was repeated on three or more separate occasions.

Reporter Assays. As reported previously (20, 21), the Bright-Glo Luciferase assay (Promega) was used to determine TOPFlash activity in cytoplasmic extracts and was standardized to β-galactosidase levels, which were determined with pGL3-Basic Vector (Promega) transfections in HEK293 cells and HCT116 cells, respectively. Cells were cultured in McCoy’s 5A medium (Life Technologies, Inc., Grand Island, NY) with 10% fetal bovine serum and penicillin/streptomycin. HCT116 cells (1.2 × 106) were seeded 24 h before transfection into 60-mm culture dishes. Transfections were done at ~70% cell confluency.

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Western Blotting. Protein concentrations were determined using the Lowry assay with bovine serum albumin as a standard. Proteins (15–30 μg) were separated by SDS-PAGE on a 4–12% bis-Tris gel (Novex, San Diego, CA) and were transferred to nitrocellulose membrane (Invitrogen, Carlsbad, CA). Equal protein loading was confirmed by Amido Black staining. The membrane was blocked for 1 h with 2% bovine serum albumin, followed by overnight incubation with primary antibody at 4°C, and was finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Antibody dilutions were as follows: β-catenin, 1:500 (Transduction Labs, Lexington, KY); HDAC1, 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA); acetylated histone H3, 1:100 (Upstate, Charlottesville, VA); acetylated Histone H4, 1:100 (Upstate); p21, 1:400 (Biosource, Camarillo, CA); poly(ADP-ribose) polymerase, 1:500 (Biosource); and caspase-3, 1:2000 (Calbiochem, San Diego, CA). Detection was by Western Lightning Chemiluminescence Reagent Plus (PE Life Sciences, Boston, MA) with image analysis on an AlphaInnotech photodocumentation system.

HDAC Activity Assay. HDAC activity was determined using the Fluorode-Lys HDAC activity kit (Biomol). Incubations were performed at 37°C for 10 min with HeLa nuclear cell extracts (supplied with the kit), and the HDAC reaction was initiated by the addition of Fluorode-Lys substrate. After 10 min, Fluor-de-Lys Developer was added, and the mixture was incubated for another 10 min at room temperature. In some experiments, cytoplasmic and nuclear extracts (25 μg of total protein) from cells treated with SFN and/or TSA were substituted for HeLa cell extracts and assayed for HDAC activity, as above. Fluorescence was measured using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices), with excitation 360 nm and emission 460 nm.

Chromatin Immunoprecipitation. HEK293 cells were transfected with 2 μg of TOPflash and were treated with SFN or TSA as above. Cell lysates were sonicated eight times for 15 s using a Heat Systems-Ultrasonics Sonicator (Model W-225R) on setting 5. The Chromatin Immunoprecipitation kit (Upstate) was used according to the manufacturer’s instructions, with anti-acetylated histone H4 or anti-acetylated histone H3 antibody and primers to TOPflash (forward primer, 5’-CATGCTGATCCTCTTAGAGTCG-3’; reverse primer, 5’-AGTCGCGTTGGATGTTAGT-3’). The DNA was purified using Wizard PCR Preps DNA purification system (Promega). PCR products were detected after 28 cycles with the cycling conditions as follows: 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. HCT116 cells were treated with 9 or 15 μM SFN or 100 ng/ml TSA, or with DMSO as control, and cell lysates were prepared as above. Anti-acetylated Histone H4 antibody was used with primers to the P21 promoter (forward primer, 5’-GGTGCTTAGGGCTCCAGGT-3’; reverse primer, 5’-GACCTGCTCCAGGACACA-3’). PCR products were detected after 30 cycles with the cycling conditions as follows: 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, with an additional 10 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min.

Fig. 1. Activation of the TOPflash reporter is indicative of HDAC inhibition. A, HEK293 cells were transiently transfected with β-catenin (β-cat) and TCF4, or the corresponding empty vector, together with the TCF/LEF-responsive luciferase reporter construct “TOPflash,” in the presence of exogenous HDAC1 (wedge symbol; HDAC1, indicates 0, 1, 2, 3 μg transfected DNA). The cell lysates were assayed for reporter activity. After normalizing for transfection efficiency using β-galactosidase, relative light units were converted to “Relative TOPflash Activity” by assigning an arbitrary value of 1.0 to assays containing TOPflash alone. B, HEK293 cells were transiently transfected with TOPflash, β-cat, TCF4, and 3 μg of HDAC1 construct. Cells were treated with TSA in DMSO (100 ng/ml) or with DMSO alone, 8 h before harvest; SFN (15 μM) was added 47 h before harvesting. C, nuclear extracts from HEK293 cells treated as in A were used in HDAC activity assays. D, nuclear extracts from HEK293 cells treated as in B were used in HDAC activity assays. Results indicate mean ± SD (n = 3) and are representative of the findings from three or more separate experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001, by Student’s t test, compared with vehicle controls.
**SDN Metabolites.** The metabolites SDN-glutathione (SDN-GSH), SDN-cysteine (SDN-Cys), and SDN-N-acetylcysteine (SDN-NAC) were kindly provided by Dr. Clifford Conaway. Each compound was dissolved in DMSO and 20 mM potassium phosphate (pH 5.0; 1:1 v/v) and was tested for inhibitory activity in the HDAC assay as described above, using HeLa nuclear extracts as the enzyme source.

**Molecular Modeling.** The covalent geometries of SDN-Cys and SDN-NAC were generated using the Insight II modeling package (Accelrys, San Diego, CA). Each structure was then manually docked into the active site of an HDAC homologue from *Aquilax acelicus* (22). The structure with TSA bound (Protein Data Bank entry 1C3R) was chosen as the best representative of a ligand-bound form of the enzyme. Modeling was guided by the following constraints: (a) carboxylate binding in a bidentate fashion to the active site zinc; (b) minimizing steric conflict between substrate and enzyme, based on a fixed protein; (c) maintaining favorable torsion angles; (d) having a hydrogen-bond partner for buried polar atoms; and (e) following the favored position of the bound TSA molecule. A solution satisfying these requirements was found for SDN-Cys, but the bulky acetyl group in SDN-NAC did not fit without moving one or more protein atoms within the active site.

**Statistical Analyses.** Pairwise comparisons were made between SDN- or TSA-treated cells and the corresponding vehicle-treated controls, using Student’s *t* test, and the levels of significance shown in the figures were as follows: *P* < 0.05; **P** < 0.01; ***P*** < 0.001.

**RESULTS**

Previous studies with TSA demonstrated that β-catenin-HDAC1 interactions regulate the transition of LEF1 from a transcriptional repressor to an activator in reporter assays using TOPlflash (23). We first verified that HDAC1 acts in a similar manner toward TCF4; in HEK293 cells cotransfected with TCF4, β-catenin, and TOPlflash, there was a dose-dependent decrease in reporter activity with increasing amount of exogenous HDAC1 in the assay (Fig. 1A). In these experiments, the reporter activity was suppressed to basal levels, equivalent to TOPlflash alone, after transient transfection with 3 µg of HDAC1 construct. The suppression by 3 µg of HDAC1 was reversed in the presence of TSA or SDN, and the combination of SDN plus TSA produced a 5-fold increase in reporter activity, compared with controls given no HDAC inhibitor (Fig. 1B). Thus, the order of activity in the reporter assays was SDN + TSA > SDN > TSA.

Nuclear extracts from HEK293 cells transfected with β-catenin, TCF4, and HDAC1 were used in the HDAC activity assay. There was an inverse relationship between the TOPlflash reporter activities (Fig. 1A) and the corresponding HDAC activities; thus, nuclear HDAC activity was increased dose dependently with HDAC1 in the assay (Fig. 1C), and the test inhibitors suppressed HDAC activity in the order SDN + TSA > SDN > TSA (Fig. 1D).

Subsequently, HEK293 cells were treated with different doses of SDN in the absence of exogenous β-catenin and TCF4. There was a concentration-dependent increase in reporter activity with each addition of SDN (3, 9, 15 µM), in the presence and absence of TSA, and the combination of 100 ng/ml TSA plus 15 µM SDN enhanced reporter activity >5-fold compared with TOPlflash alone (Fig. 2A). This increase in reporter activity was not due to the induction of cytoplasmic or nuclear β-catenin, or to changes in nuclear HDAC protein levels, which remained essentially unaffected by SDN and/or TSA under these experimental conditions (Fig. 2B–D). The lack of any change in endogenous protein expression for β-catenin and HDAC1 supports a mechanism involving de-repression of the TOPlflash promoter by TSA and SDN.

In the cytoplasmic and nuclear extracts of HEK293 cells treated with the test compounds (Fig. 3, A and B, respectively), 100 ng/ml TSA and 15 µM SDN inhibited HDAC activity significantly. However, when HeLa nuclear extracts supplied with the HDAC assay kit were incubated with SDN parent compound, no significant inhibitory activity was detected (Fig. 3C). In marked contrast, when HEK293 cells were treated with the corresponding doses of SDN and the cell-free medium was added to HeLa nuclear extracts, there was a concentration-dependent decrease in HDAC activity (Fig. 3D). These findings suggested that one or more SDN metabolite(s) might be responsible for the inhibition of HDAC activity in HEK293 cells.

To examine this possibility further, we used the GST inhibitor ethacrynic acid (24) to block the conversion of SDN to its major metabolites. Thus, HEK293 cells were incubated with 100 µM ethacrynic acid shortly after transfection with TOPlflash, and before the addition of SDN or TSA. No effect was seen with ethacrynic acid alone, but ethacrynic acid blocked the activity of SDN to enhance TOPlflash reporter activity (Fig. 4A). In contrast, the TSA-mediated increase in reporter activity was further augmented by ethacrynic acid (Fig. 4A). These results suggested that SDN metabolites, rather than the parent compound, inhibit HDAC activity in HEK293 cells, whereas TSA parent compound is a more potent HDAC inhibitor than its metabolite(s). The latter was confirmed by the addition of cell-free media to HDAC assays, in which ethacrynic acid augmented the inhibition produced by TSA (Fig. 4B), and the former was confirmed using specific metabolites of SDN (Fig. 4C); whereas the parent compound and SDN-GSH had little or no inhibitory activity, SDN-Cys and SDN-NAC metabolites inhibited HDAC activity in a concentration-dependent manner. At the highest concentration tested (15 µM),

![Fig. 2. SDN increases TOPlflash reporter activity without inducing β-catenin or HDAC1 protein levels. A, HEK293 cells were transiently transfected with TOPlflash (no exogenous β-catenin or TCF/LEF) and were treated with increasing concentrations of SDN, alone (–TSA) or in combination with TSA (+TSA; 100 ng/ml). TSA and SDN were added 8 and 47 h before harvesting, respectively, and the cell lysates were assayed for reporter activity. Results indicate mean ± SD (n = 3) and are representative of the findings from three separate experiments. HEK293 cells treated as in A were subjected to immunoblotting for β-catenin in cytoplasmic (B) or nuclear (C) extracts, as well as for HDAC1 (D) in the nuclear extracts. The wedge symbol indicates 3, 9, 15 µM SDN. *P* < 0.05; **P** < 0.01; ***P*** < 0.001 by Student’s *t* test, compared with TOPlflash alone.](Image)
the relative order of inhibitory activity was as follows: SFN-Cys > SFN-NAC > SFN-GSH = SFN.

In HEK293 cells treated with SFN or SFN + TSA, immunoblotting revealed an increase in global acetylation of histones H3 and H4 (Fig. 5A); TSA plus 15 μM SFN caused a 3.1- and a 1.7-fold increase, respectively, in acetylated histones H3 and H4, compared with 5.5- and 2.4-fold for the control, sodium butyrate. The SFN-mediated increase in global histone acetylation was reproducible in HEK293 cells as well as in human colon cancer cells (see below). To examine histone acetylation associated specifically with the promoter of TOPflash, chromatin immunoprecipitation was performed with antibodies to acetylated histones H3 or H4, followed by PCR using primers specific for TOPflash; SFN produced an ~2-fold increase compared with cells given no SFN (Fig. 5B and C). These results provide evidence for global and localized changes in histone acetylation status after exposure of HEK293 cells to SFN.

To extend these findings, we treated HCT116 human colorectal cancer cells with SFN (Fig. 6). As in HEK293 cells, SFN activated TOPflash reporter activity in a concentration-dependent manner (Fig. 6A), this was paralleled by a decrease in nuclear HDAC activity (Fig. 6B), and immunoblots revealed an increase in acetylated histone H3, acetylated histone H4, and p21Cip1/Waf1 (Fig. 6C). Finally, TSA and 15 μM SFN each produced an ~4-fold increase in the chromatin immunoprecipitation assay, using antibody to acetylated histone H4 followed by primers to the promoter of P21 (Fig. 6D).

DISCUSSION

Billin et al. (23) observed that TSA activated TOPflash reporter activity in HEK293 cells in the presence and absence of exogenous β-catenin and LEF1, and provided evidence that HDAC1 switches LEF1 from a repressor to a transcriptional activator. Our results confirm that TSA increases TOPflash reporter activity, and demonstrate that the effect of HDAC1 on LEF1 can be extended to TCF4, the major form of TCF/LEF in colonic mucosa. Thus, under certain circumstances, HDAC1 might influence whether TCF4 acts as a transcriptional activator or repressor in the Wnt signaling pathway. The present results also show that TOPflash reporter activity can be used as an indirect measure of HDAC activity, with an increase in reporter activity corresponding to a decrease in HDAC activity in cells treated with TSA or SFN.

SFN is an effective cancer chemopreventive agent in several animal models (10–12) and is thought to induce phase 2 detoxification enzymes through the interaction of Nrf-2 with the antioxidant response element (25–27). However, SFN also has a marked effect on cell cycle checkpoint controls and cell survival and/or apoptosis in various cancer cell lines, through molecular mechanisms that remain poorly understood (28–36). The present investigation provides possible insight into this question by showing, for the first time, that SFN is an inhibitor of HDAC activity.

SFN dose-dependently increased TOPflash reporter activity with-
out altering protein levels of β-catenin or HDAC1, indicating that the activity of HDAC itself was altered by SFN. In parallel experiments, cytoplasmic and nuclear extracts from HEK293 cells treated with SFN had diminished HDAC activity compared with untreated cells, global histone acetylation was increased by SFN, and chromatin immunoprecipitation assays revealed a greater amount of TOPflash template bound to acetylated histones H3 and H4. Collectively, these findings provide direct evidence that SFN activates the TOPflash reporter by increasing its association with acetylated histones.

Interestingly, when SFN parent compound was incubated with HeLa nuclear extracts, there was no effect on HDAC activity. However, when medium from HEK293 cells treated with SFN was incubated with HeLa nuclear extracts, a dose-dependent decrease in HDAC activity was observed. This suggested that one or more SFN metabolite(s) present in the medium from cells treated with SFN might be the active HDAC inhibitor(s). SFN is metabolized through the mercapturic acid pathway, starting with the formation of SFN-GSH by GST, and further metabolism yields the SFN-Cys and SFN-NAC metabolites (37). In vivo, SFN acts as both a blocking agent and a suppressing agent of colonic aberrant crypts in azoxymethane-treated F344 rats, whereas SFN-NAC was effective only after initiation (10). Other studies have shown that the NAC conjugate of isothiocyanates such as phenethyl isothiocyanate, as well as SFN-NAC itself, can modulate the growth and apoptosis of human prostate cancer cells (38, 39). In HepG2-C8 cells (14), SFN and SFN-NAC differed in their ability to induce antioxidant response element-related gene expression and apoptosis, and these biological effects were blocked by exogenous GSH, possibly acting through redox changes in Keap-1 rather than via the modulation of GST.

Using an inhibitor of GST, namely ethacrynic acid, we observed an increase in the HDAC inhibitory activity of TSA (Fig. 4B), indicating that the parent compound was more effective than its metabolites. In contrast, ethacrynic acid blocked the effects of SFN in the TOPflash reporter assay, supporting the view that SFN metabolites are important for HDAC inhibition. Metabolism of other HDAC inhibitors, such as FK228 (FR901228), has been demonstrated to be necessary for HDAC inhibitory activity (40). On the basis of the results with ethacrylic acid, we tested purified SFN metabolites and observed that SFN-NAC and SFN-Cys each produced a concentration-dependent inhibition of HDAC activity, whereas SFN and SFN-GSH had little inhibitory activity in vitro (Fig. 4C). Molecular modeling studies revealed a plausible interaction for SFN-Cys within the active site of the HDAC-like protein, with the carboxylate group of SFN-Cys positioned as a bidentate Zn ligand (Fig. 7), similar to that of the hydroxamic acid group of TSA bound to HDAC-like protein (22). From the results obtained to date, we hypothesize that HDAC recog-

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**Fig. 4.** Ethacrynic acid attenuates the effects of SFN in TOPflash reporter and HDAC activity assays; metabolites of SFN directly inhibit HDAC activity. A, HEK293 cells were transfected with TOPflash and then were treated with 100 μM ethacrynic acid (+EA) before SFN (15 μM) or TSA (100 ng/ml) exposure. B, medium from HEK293 cells in A was incubated with HeLa nuclear extracts in the HDAC activity assay. Control, no medium added (open bar). C, SFN metabolites were incubated with HeLa nuclear extracts and were assayed for HDAC activity. The wedge symbol, 3, 9, 15 μM SFN or SFN metabolite. Results (mean ± SD; n = 3) are representative of the findings from three separate experiments. Control, vehicle alone. *, P < 0.05; **, P < 0.01, by Student’s t test.
nizes the acetylated metabolite SFN-NAC, and that deacetylation generates SFN-Cys as a direct inhibitor of HDAC. This novel mechanism-based inhibition opens an avenue for new drug design centered on compounds that become effective HDAC inhibitors only after metabolism, possibly including other dietary isothiocyanates with demonstrated cancer chemoprotective activities (10–12, 28–36, 38, 39).

Finally, the activation of TOPflash reporter activity by TSA or SFN might be viewed, in a general sense, as evidence for enhanced β-catenin/TCF/LEF signaling, which is a known oncogenic mechanism in colon cancer (41, 42). We performed studies with SFN in the human colorectal cancer cell line HCT116, which contains high endogenous levels of mutant β-catenin, and showed that SFN enhanced TOPflash reporter activity, inhibited nuclear HDAC activity, and increased the levels of acetylated histones H3 and H4 (Fig. 6). As with other HDAC inhibitors (5–9, 43–47), such as TSA, suberoylanilide hydroxamic acid (5772 HDAC INHIBITION BY SULFORAPHANE)

![Figure 5](image-url)  
**Fig. 5.** SFN increases global and localized histone acetylation. **A**, HEK293 cells were treated with SFN and/or TSA, or with sodium butyrate as a control, and acetylated histone H3 or acetylated histone H4 was assessed by immunoblotting. Numbers above the lanes, relative densitometry values for the corresponding blots. **B**, HEK293 cells were transiently transfected with 2 μg of TOPflash and were treated with 9 μM SFN (+SFN) or vehicle (−SFN). DNA was cross-linked to proteins before harvesting, chromatin immunoprecipitation (ChIP Assay) was performed against acetylated histone H3 or acetylated histone H4, and after DNA isolation and reversal of cross-linking, primers specific for TOPflash were used during PCR amplification. **C**, ChIP results were first normalized against the corresponding input controls, and then were expressed relative to TOPflash alone (−SFN), which was assigned an arbitrary value of 1.0.

![Figure 6](image-url)  
**Fig. 6.** SFN inhibits HDAC activity in HCT116 cells. Human HCT116 colorectal cancer cells were transiently transfected with TOPflash and treated with increasing concentrations of SFN. Assays were conducted for TOPflash reporter activity (**A**) and nuclear HDAC activity (**B**), as described for HEK293 cells (Fig. 2A and Fig. 3B, respectively). Results (mean ± SD; n = 3) are representative of the findings from two separate experiments. The wedge symbol, 0, 3, 9, 15 μM SFN. C, immunodetection of acetylated histone H3, acetylated histone H4, and p21 Cip1/Waf1 in HCT116 cells treated with 0 or 15 μM SFN. D, chromatin immunoprecipitation assay (P21 ChIP Assay) with antibody to acetylated histone H4 and primers to the P21 promoter. For quantification, results were first normalized to the corresponding input control, and then were expressed relative to −SFN control, which was assigned an arbitrary value of 1.0. Wedge symbol, 0, 9, 15 μM SFN; TSA, 100 ng/ml. *, P < 0.05; **, P < 0.01 by Student’s t test, compared with the vehicle controls.
acid, and sodium butyrate, SFN markedly increased p21 Cip1/Waf1 protein expression, and chromatin immunoprecipitation assays revealed a corresponding increase in acetylated histone H4 bound to the promoter region of P21. A search of the P21 promoter revealed no antioxidant response element sites, supporting an Nrf-2-independent mechanism. The physiologically relevant concentrations of SFN used alone or in combination with other HDAC inhibitors currently under-


A Novel Mechanism of Chemoprotection by Sulforaphane: Inhibition of Histone Deacetylase
