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Targeting the epigenome, including the use of histone deacetylase (HDAC) inhibitors, is a novel strategy for cancer chemoprevention. 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/apoptosis in various cancer cells, through mechanisms that are poorly understood. Based on the structure of known histone deacetylase inhibitors, it was hypothesized that SFN may possess HDAC inhibitory properties. Initial studies confirmed that, indeed, at physiologically-relevant concentrations, SFN inhibited HDAC activity in human colorectal cancer cells, with a concomitant increase in acetylated

histones H3 and H4, induction of p21 expression, and increased acetylated histone H4 associated with the *P21* promoter. A metabolite of SFN, SFN-Cysteine, was found to be the active HDAC inhibitor. Furthermore, in BPH-1, LnCaP, and PC-3 human prostate epithelial cells, SFN inhibited HDAC activity and increased acetylation of histones. SFN also induced p21 expression, with an increase in acetylated histone H4 associated with the *P21* promoter in BPH-1 cells. The downstream effects of HDAC inhibition by SFN included induction of pro-apoptotic proteins and repression of anti-apoptotic proteins, and an increase in multi-caspase activity. Dietary SFN suppressed the growth of human prostate cancer PC-3 xenografts and inhibited HDAC activity in the xenografts, peripheral blood mononuclear cells (PBMC), and prostates. In time-course studies, a single oral dose of SFN induced histone acetylation at 6 and 24 h in mouse colonic mucosa, and long-term dietary SFN treatment increased histone acetylation in the ileum, colon, PBMC, and prostates. Moreover, dietary SFN suppressed intestinal tumorigenesis significantly in Apc^{min} mice, with an increase in acetylated histones detected in the normal-looking ileum and polyps and polyps from the colon. Overall, the data presented in this thesis support a novel mechanism for chemoprevention by SFN *in vivo*, through inhibition of histone deacetylase. The findings also imply that SFN will offer significant protection against at least two of the major cancer killers in the US, namely colon and prostate cancer.

A Novel Mechanism of Chemoprevention by Sulforaphane:

Inhibition of Histone Deacetylase

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CONTRIBUTION OF AUTHORS

CHAPTER 4: Dr. P. Andrew Karplus constructed the figure of the SFN-Cys-TSA overlay and was consulted on the structure of the metabolites of SFN. Dr. Fung-Lung Chung provided the SFN metabolites.

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CHAPTER 7: Gayle Orner was consulted for Apc^{min} mice animal studies.

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INTRODUCTION

Cancer was recently listed as the second-leading cause of death in the United States, after heart diseases in 2002. However, the most up to date current estimates, as of February 2005, rank cancer as the leading cause of death of Americans under age 85 (American Cancer Society).

Although progress has been made in cancer research over the past few decades, overall cancer mortality rates remain similar to those from the 1950s (American Cancer Society). Additional research to further understand the molecular basis of tumorigenesis is expected to identify novel targets for prevention and therapy, and hopefully lower these cancer statistics.

The etiological factors vary in each cancer type, but the general underlying scheme is that cancer results from a series of events that ultimately leads to selective growth advantage and survival of aberrant cells. Hallmarks of these aberrant or 'transformed' cells include loss of response to growth inhibitory factors, ability to produce growth promoting factors, inability to respond to apoptotic signals, infinite replicative potential, induction of angiogenesis, and tissue invasion and metastasis. These phenotypic characteristics are ultimately the result of multiple changes in gene expression. In general, activation of oncogenes and inactivation of tumor

suppressor genes are essential in the transformation from normal to malignant phenotype.

In the past few decades, the body of knowledge surrounding the molecular and cellular characteristics of cancer has grown exponentially. With this increased understanding of how cancer develops comes progress in elucidating mechanisms of cancer prevention. Chemoprevention is theoretically possible throughout numerous stages of tumorigenesis, and as stated by one leading scientist in this area, "Prevention is the best cure" (Dr. Bandaru Reddy, LPI seminar speaker, March 31, 2005). So-called 'primary' chemoprevention seeks to block initiating and promoting events early in tumorigenesis, whereas 'secondary' chemoprevention acts during later progression and metastasis stages. Finally, tertiary chemoprevention suppresses recurrence of primary cancers and/or development of secondary tumors which may arise from therapy.

Diet and nutrition have long been associated with tumorigenesis, with certain dietary factors identified as causative agents and others as preventive agents. Epidemiology studies suggest that, in general, diet plays a major role, and indeed might account for 1/3 to 2/3 of all *modifiable* risk factors in human cancer development (American Cancer Society). A plethora of dietary factors have been identified through experimental and epidemiological studies which possess chemopreventive activity, as reviewed in recent articles (1-3), and the search continues for novel compounds. Understanding the

mechanisms through which dietary factors modulate tumorigenesis will be critical in designing effective chemopreventive and therapeutic strategies.

This thesis examines the possible targeting of events at multiple-stages of tumorigenesis via a novel mechanism, namely alterations in histone acetylation, using a dietary chemopreventive agent from broccoli, sulforaphane. Early chapters which review the subject were recently accepted for publication.

Histone deacetylases as targets for dietary cancer preventive agents: Lessons learned with butyrate, diallyl disulfide, and sulforaphane

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Abstract

Cancer is a multi-factorial process involving genetic and epigenetic events which result in neoplastic transformation. Reversal of aberrant epigenetic events, including those that modulate the transcriptional activity of genes associated with various signaling pathways, holds the prospect of influencing multiple stages of tumorigenesis. Perturbation of normal histone acetylation status can result in undesirable phenotypic changes, including developmental disorders and cancer. Indeed, aberrant histone acetylation may be an etiological factor in several, if not all, types of cancer. In general, histone acetylation leads to chromatin remodeling and a de-repression of transcription. Histone deacetylase (HDAC) inhibitors may be useful for cancer prevention and therapy by virtue of their ability to 'reactivate' the expression of epigenetically silenced genes, including those involved in differentiation, cell cycle regulation, apoptosis, angiogenesis, invasion, and metastasis. Several natural and synthetic HDAC inhibitors have been shown to affect the growth and survival of tumor cells *in vitro* and *in vivo*. Interestingly, three dietary chemopreventive agents, butyrate, diallyl disulfide, and sulforaphane, also have HDAC inhibitory activity. This review discusses the role of aberrant histone acetylation in tumorigenesis and describes the potential for cancer chemoprevention and therapy with a particular emphasis on dietary HDAC inhibitors.

Introduction

In addition to the specific sequence of bases within DNA, the manner in which the genetic material is 'packaged' plays a pivotal role in determining the expression of a host of genes. In the nucleus, DNA is wound around a histone octomer core consisting of two sets each of histones H2A, H2B, H3, and H4 (4, 5). In recent years, it has become apparent that post-translational modifications of histones, such as acetylation, phosphorylation, ubiquitination, and methylation, are important in the regulation of gene expression (6-9). These modifications alter chromatin structure and influence binding of co-activators, co-repressors, and other proteins which read the so-called 'histone code' (6-9).

Histone acetylation, particularly of histones H3 and H4, is arguably the most extensively characterized chromatin modification studied thus far. Acetylation and deacetylation of histones exists in a dynamic equilibrium, mediated by the opposing activities of two classes of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Generally, acetylated histones are associated with transcriptionally active chromatin (10-12). By removal of acetyl groups from the ε-amino groups of lysine residues in the N-terminal tails of core histones, chromatin condensation leads to concomitant transcriptional repression; conversely, covalent addition of acetyl groups is associated with upregulation of gene expression (4, 10, 12). Addition of acetyl groups to histone tails results in an 'open' chromatin

conformation, facilitating transcription by allowing transcription factors access to DNA. In addition, acetylation can recruit chromatin remodeling factors, which may alter chromatin structure in such a way as to provide access to DNA by transcription factors (Figure 1). Several HATs and HDACs have been discovered, each of which seems to recognize specific substrates, although the substrates and pathways involved remain to be clarified in most (if not all) cases (reviewed in (13)). HDACs can be classified into two broad classes: Class I HDACs are thought to be expressed in all tissue types and are found almost exclusively in the nucleus, whereas Class II HDACs exhibit a more restricted expression pattern and can shuttle between the nucleus and the cytoplasm (reviewed in (13)). In recent years, it has become clear that inappropriate targeting or aberrant activity of these enzymes can be linked to tumorigenesis.

Epigenetics describes heritable changes in gene expression that do not arise directly from changes in DNA sequence. It has become increasingly clear that epigenetic events, including alterations in histone acetylation, are a driving force in tumorigenesis and may contribute to some hallmark phenotypic changes observed in neoplastic transformation, including loss of differentiation and loss of growth regulatory mechanisms affecting cell cycle arrest and apoptosis (6, 14-24). Epigenetic alterations have been observed in several cancer types, throughout the progression of tumorigenesis, from early initiation to subsequent promotion stages, and finally to metastasis.

Because epigenetic modifications are potentially reversible, unlike the heritable changes in DNA (i.e. mutations), they are a desirable target for possible chemoprevention strategies. In addition, epigenetic modifications can be early events in carcinogenesis; thus, prevention/reversal could be targeted to pre-neoplastic cells or early stages of tumorigenesis, before additional neoplastic changes take place.

Histone Acetylation Status and Cancer

Oncogenic mechanisms associated with aberrant histone acetylation and deacetylation have not been fully elucidated, but evidence from human cancers suggests interference with HAT activities through genetic alterations, loss of heterozygosity, chromosomal translocations, or viral inactivation. In addition, over-expression, aberrant cellular localization/compartmentalization, or mistargeting of HDACs through fusion proteins or improper co-repressor recruitment may be causative factors in the deregulation of histone acetylation (Figure 2). HATs, serving as transcriptional activators, play a key role in pathways leading to differentiation, cell cycle control, and apoptosis. By the same reasoning, HDACs which function as transcriptional repressors have an opposing role in the aforementioned pathways. In addition to histones, HATs and HDACs target transcription factors directly, and changes in the acetylation status of these proteins can result in an increase in DNA binding capability, thereby increasing transcriptional activation.

Aberrant histone acetylation has been linked with several human cancers (21, 23, 24). Inactivating mutations in *EP300*, a HAT, were identified in lung, breast, and colorectal cancer cell lines and primary tumors (25). Furthermore, in cases involving truncation mutations, the second allele was lost or inactivated, suggesting that *EP300* may act as a classical tumor suppressor gene through the Knudson two-hit hypothesis (25). Whereas genetic changes were rare in another mutational analysis of *EP300* in colon, breast, and ovarian carcinomas, loss of heterozygosity (LOH) was detected in 38% of colon, 36% of breast, and 49% of ovarian primary tumors, suggesting that bi-allelic inactivation of *EP300* is not necessary for tumorigenesis (26). In support of this notion, the developmental disorder Rubenstein-Taybi syndrome (RTS) results from haploinsufficiency of CREB binding protein (CBP), another HAT, through point mutations and chromosomal rearrangements (27, 28). Interestingly, RTS patients have an increased susceptibility to cancer (23), which links a specific type of regulation of gene expression to tumorigenesis. Certain viral oncogenes, such as E6 from human papillomavirus type 16, have been shown to interact with P300 and CBP, resulting in the loss of transcriptional activity by these HATs (29). Loss of function of HATs is observed throughout the progression of tumorigenesis.

Perhaps the best studied example of mistargeting by HDACs is acute promyelocytic leukemia (APL), which results from a transforming fusion protein of the promyelocytic leukemia zinc finger (PLZF) and the retinoic acid

receptor-alpha (RARα). In APL, HDACs are recruited to the PLZF domain and the RAR portion of the fusion protein directs it to retinoic acid target genes, ultimately resulting in prevention of differentiation due to repression of transcription by HDACs. Due to the presence of the fusion protein, treatment with retinoic acid fails to displace the HDACs and leukemias with this type of translocation are resistant to retinoic acid (30, 31).

Recent evidence suggests that Class II HDACs can shuttle between the nucleus and cytoplasm, and that this may be a mechanism to relay signal pathways to transcriptional control (22). For example, HDAC4 localizes to the nucleus following transfection with an oncogenic, constitutively active form of RAS (32), thus connecting 'classical' genetic alterations to epigenetics. While HDACs themselves may not be over-expressed *per se*, deregulation of upstream signaling events can prompt HDACs to enter the nucleus and interfere with pathways regulating differentiation.

Over-expression of HDACs has been observed in primary human prostate tumors and prostate cell lines. HDAC activity was increased in ND1, PC-3, DuPro, and TSUPr1 prostate cancer cells lines compared to cells derived from benign prostate hyperplasia (BPH-1) (33). Interestingly, HDAC1 mRNA expression was increased in all prostate cancer cell lines compared with BPH-1 cells (33). HDAC1 protein expression also was increased in TSUPr1 cells compared to BPH-1 cells, as determined by immunocytochemistry. Furthermore, HDAC1 expression was higher in

primary human prostate cancers compared to benign prostate hyperplasia (33). Increased expression of HDAC-1 also has been observed in human gastric cancer tissues, both at the level of mRNA and protein (20), suggesting that in at least some cancer types, over-expression of HDACs may be partially responsible for disrupting transcriptional regulation through alteration in histone acetylation.

In addition, the levels of acetylated histone H4 were reduced in gastric and colorectal carcinomas in comparison to non-neoplastic mucosa (34). The mechanism through which this loss of global histone acetylation occurred was not investigated, but may be due to either loss of activity of HATs or an increase in activity of HDACs. Strikingly, reduction in the amount of acetylated histone H4 is correlative to advanced tumor stage, depth of tumor invasion, and lymph node metastasis in gastric cancer (34). In colorectal cancer, reduction in global acetylation of histone H4 was observed in 80% of carcinomas and 39% of adenomas, and significantly correlated with advanced tumor stage and depth of tumor invasion, suggesting that loss of acetylation is progressive during colon tumorigenesis (34). Also, in primary human gastric carcinoma, a recent study demonstrated through chromatin immunoprecipitation that histone H3 was hypoacetylated in the *P21* promoter (35). Collectively, these various studies provide evidence that perturbation of the balance between acetylation and deacetylation is a factor in neoplastic transformation. Indirect evidence of the importance of acetylation status in

tumorigenesis also comes from the observation that HDAC inhibitors can halt and even reverse tumor cell growth, as discussed in the following section.

HDAC Inhibitors: Potential for Cancer Prevention

Although the precise molecular mechanisms remain to be determined, indirect evidence through the use of HDAC inhibitors strongly suggests that HDACs are intricately involved in several pathways, including those regulating differentiation, cell cycle arrest, and apoptosis, as well as angiogenesis, invasion, and metastasis. Mechanisms for transcriptional repression at specific promoters involve an increase in HDAC activity and/or a decrease in HAT activity, and HDAC inhibitors can effectively restore the intricate balance of acetylation and deacetylation. Interestingly, the downstream effectors of HDAC inhibition include the same types of effectors identified in many cancer prevention studies, such as p21, BAX, and caspases. Classical HDAC inhibitors have been extensively reviewed in terms of their activity in cell and animal models, as well as in clinical trials, and microarray technology has been applied to high-throughput screening of gene expression changes in response to HDAC inhibitors. This section will provide a few key examples of the more well established HDAC inhibitors, with particular emphasis given to those most likely to play a role in cancer chemoprevention or therapy.

HDAC inhibitors have been shown to increase global acetylation as well as acetylation associated with specific promoters of genes. Although the

equilibrium is shifted toward greater histone acetylation after treatment with HDAC inhibitors, the expression of only a relatively small number of genes is altered in an upward or downward direction (36-39). Importantly, only neoplastically transformed cells appear to respond to increased acetylation by undergoing differentiation, cell cycle arrest, or apoptosis; normal cells, despite the increased acetylation, do not respond in this manner to HDAC inhibitors (40). Several classes of HDAC inhibitors exist, including (1) small-chain fatty acids, (2) hydroxamic acids, (3) cyclic tetrapeptides, and (4) benzamides, as well as others that do not fit into these structural classifications (41-45). The original HDAC inhibitors were identified and isolated from natural sources, and based on their structure and the crystal structure of the active site pocket of a HDAC homologue from *Aquifex aeolicus*, various synthetic inhibitors have been developed (46-48). The general structure of HDAC inhibitors is comprised of a functional group at one end that interacts with a zinc atom and neighboring amino acids at the base of the HDAC active site, a spacer that reaches down into the channel region of the active site, and a cap group which is hypothesized to interact with external amino acid residues (Figure 3) (46, 49). Various HDAC inhibitors have been reviewed before (reviewed in (41-45)), and a brief discussion is presented here, followed by a more detailed focus on HDAC inhibitors specifically associated with the diet.

Prototype HDAC inhibitors

Before their mechanism of action was fully understood, small molecules containing hydroxamic acid moieties and cyclic tetrapeptides were found to influence cell differentiation. Trichostatin A (TSA) and trapoxin are examples of naturally-occurring compounds from two classes of HDAC inhibitors. TSA was shown to induce differentiation in murine erythroleukemia cells (50, 51), and later shown to be a potent inhibitor of HDACs (52). Subsequent studies showed that TSA can reverse morphological transformation of oncogenic *ras*-transformed NIH3T3 cells (53). In addition, TSA induced hepatocyte differentiation in human hepatoma cells, increasing hepatocyte-specific genes, ammonia removal, and albumin synthesis, with concomitant increases in acetylated histones H3 and H4 (54). Trapoxin was also shown to induce morphological reversion from transformed to normal in *sis*-transformed NIH3T3 fibroblasts (55, 56), and later studies demonstrated that trapoxin was an irreversible inhibitor of HDACs (57). Furthermore, trapoxin induced the production of plasminogen activator, a marker of differentiation, in F9 teratocarcinoma cells (57). The effectiveness of HDAC inhibitors as differentiating agents has been confirmed using several synthetic HDAC compounds, as reviewed elsewhere (41-44). Induction of differentiation is an early event in which HDAC inhibitors can intervene to influence tumorigenesis.

Given that HATs and HDACs are important in cell cycle regulation, it is not surprising that HDAC inhibitors have a profound effect on cell cycle arrest

in transformed cells, and both G1 and G2/M cell cycle arrest has been observed after treatment with HDAC inhibitors. Various mechanisms have been postulated to explain these effects on the cell cycle. For example, an HDAC inhibitor-sensitive checkpoint has been proposed, which appears to be defective in tumor cells, but intact in normal cells (58). It also has been suggested that HDAC inhibitors cause G2/M arrest by decreasing the amount of deacetylated histones required for chromatin formation with newly synthesized DNA (59). Certainly, the differences in genetic and epigenetic abnormalities in various cancers influence the effects of HDAC inhibitors. One of the most striking examples of HDAC inhibition is upregulation of the cyclin-dependent kinase inhibitor, p21$^{CIP1/WAF1}$ (P21). Interestingly, p21 induction by HDAC inhibitors is p53-independent, as demonstrated by cell lines expressing mutant forms of p53 (60). The effect of HDAC inhibitors on transcription of the *P21* gene is thought to be mediated through Sp1 sites in the promoter, which may recruit HDACs and co-repressor complexes (61-63). Several other genes involved in cell cycle regulation have been identified as targets of HDAC inhibitors, including *TOB-1* (64), *P27^{KIP1}* (65), *GADD45* (66), *CYCLIN B1* (67), *CYCLIN D1* (68), and *CYCLIN A* (69) (for review, see (13, 41, 43)). HDAC inhibitors also consistently downregulate certain genes, including *c-myc* (39, 70), which may inhibit several converging growth-promoting signaling pathways.

In addition to induction of cell cycle arrest, HDAC inhibitors can stimulate both intrinsic and receptor-mediated apoptosis in transformed cells and tumors. HDACs can interact with the tumor suppressor transcription factor, p53, and remove acetyl groups from the C-terminal tail, resulting in a decrease in transactivation (71-73). Conversely, HDAC inhibitors lead to an increase in acetylation of P53 and an increase in P53 transcriptional activity (74). Many downstream target genes of p53 are initiators of the intrinsic apoptotic pathway (75). Indeed, increased expression has been reported for BAX, PIG3, NOXA, and BAK after treatment with HDAC inhibitors (43, 76). Some HDAC inhibitors have been shown to increase CD95/CD95L (77) and FAS/FASL (78), which can induce receptor-mediated apoptosis. Markers of induction of apoptosis, such as caspase activation, have been observed upon administration of HDAC inhibitors (43). Thus, HDAC inhibitors can prevent growth of a tumor, or cause tumor regression, by induction of apoptosis.

Although HDAC inhibitors can act directly on tumor cells, modification of the surrounding tumor environment also may play a role in cancer prevention. Metastatic disease is often the cause of cancer morbidity, rather than the primary tumor itself. In order for a primary tumor to grow and ultimately metastasize, it requires vascularization to supply oxygen and nutrients. Hypoxia, a key factor in induction of angiogenesis, has been demonstrated to induce HDAC1 expression and activity (79). Since hypoxia induces HDAC1, it is logical to hypothesize that HDAC inhibitors inhibit

angiogenesis. Indeed, HDAC inhibitors have been shown to inhibit tumor angiogenesis *in vitro* and *in vivo* (20). TSA reduced both hypoxia-induced factor-1 (HIF-1) and vascular endothelial growth factor (VEGF), factors which are essential for angiogenesis (79). Inhibition of angiogenesis serves two purposes: it limits tumor size by restricting oxygen and nutrients, and it decreases the opportunities for metastatic cells to enter the circulatory system. In gastric carcinoma cells, TSA suppressed invasion, and induced the expression of genes thought to suppress invasion and metastasis, including *TIMP-1* (inhibition of extracellular matrix degradation), *nm23H1/H2* (suppression of cell motility and metastasis), and inhibitors of metalloproteinases (34). Finally, HDAC inhibitors augment immune-system activation and recognition of tumor cells. Stimulatory molecules, such as CD40, CD80, and CD86, as well as major histocompatibility (MHC) class I and class II proteins and interferons are upregulated by HDAC inhibitors (43). While these activities do not directly affect tumor growth/death, they are likely to play an important role in tumor survival.

Dietary HDAC Inhibitors

Because HDAC inhibitors have a broad spectrum of targets and act at several stages of tumorigenesis, they are ideal candidates for chemoprevention and therapy. One of the goals of prevention is to delay the onset of clinical, malignant cancers, and HDAC inhibitors possess the ability

to intervene at several steps in tumor progression. Interestingly, all of the dietary HDAC inhibitors discovered to date have been previously identified as cancer chemopreventive agents; elucidation of their activity as HDAC inhibitors has only been uncovered in the search for a mechanistic explanation of their chemopreventive actions. The following section will discuss chemoprevention by three HDAC inhibitors that are consumed in the human diet, namely butyrate, diallyl disulfide (DADS), and sulforaphane (SFN). These dietary HDAC inhibitors possess certain structural features that most likely facilitate their interactions with the HDAC active site (Figure 4), and they are believed to act on cell survival and turnover in an analogous manner to other known HDAC inhibitors.

BUTYRATE

Butyrate is formed in the digestive tract by fermentation of dietary fiber. Meta-analyses suggest a protective effect of consumption of fiber, although prospective studies have yet to confirm this possibility for specific types of fiber or their individual constituents. Butyrate itself shows inconsistent effects *in vivo*, possibly due to poor distribution/bioavailability at the target site when administered as the isolated parent compound, but it is an effective agent in various cancer cells *in vitro*. For example, butyrate has potent activity as a differentiating agent and inducer of cell cycle arrest and apoptosis in cell culture models.

Butyrate was one of the first modulators of histone acetylation to be identified. However, butyrate-induced differentiation was noted in mouse erythroleukemic cells (80), before its effects on histone acetylation were discovered. Since the first report of histone modifying effects of butyrate in HeLa and Friend leukemia cells (81), several hundred researchers have documented HDAC inhibition and increased acetylated histone expression in a myriad of cell lines treated with butyrate.

Several signaling pathways are affected by butyrate (82-84), suggesting multiple mechanisms, only some of which involve alteration of histone acetylation. However, it is clear that butyrate alters transcriptional regulation in a manner similar to other HDAC inhibitors, with similar consequences in terms of changes in cellular differentiation, cell cycle arrest,

apoptosis, and inhibition of invasion and metastasis. Rapid induction of *P21* mRNA expression in p53-null cells upon addition of butyrate was shown to be dependent on Sp1 response elements in the *P21* promoter (60), and subsequent studies reported an increase in acetylated histones associated with the *P21* promoter (63). A model has been proposed in which Sp1/Sp3 recruit HDACs to the *P21* promoter, resulting in deacetylation of histones associated with the *P21* promoter, whereas butyrate disrupts this process and 'derepresses' the chromatin (63). A similar model has been proposed for other HDAC inhibitors (62, 85). Butyrate exhibits additional modulatory activities on cell cycle regulation, as seen with the 'prototypical' HDAC inhibitors. For example, butyrate inhibits cyclin D1 (86) and cyclin B1 (67) expression, and induces cell cycle arrest in both G1 and G2/M (70, 86). As with TSA, butyrate causes down-regulation of *c-myc* at the mRNA level (70), which may also explain, in part, the effects of butyrate on cellular proliferation.

Similar to other HDAC inhibitors, apoptosis induction has been reported for butyrate, involving the activation of caspases and decreased levels of BCL-2 (67, 87-90). Activation of death receptor-mediated apoptosis also been proposed, in which butyrate increased death receptor 5 (DR5) expression, a receptor for TRAIL, leading to activation of caspase-8 and caspase-10 (91). Interestingly, whereas TSA induced only a transient effect on histone hyperacetylation in HT-29 cells, butyrate caused a sustained and prolonged change in histone hyperacetylation (87), suggesting that the latter

compound may be more effective for chemoprevention in some circumstances. Whether this is true *in situ* remains to be determined.

Evidence suggests that butyrate also may inhibit tumor invasion and metastasis. Butyrate induced expression of proteins known to inhibit invasion in a cell culture model (92), and also reduced liver metastasis of rat colon carcinoma cells *in vivo* (93). In addition, butyrate induced expression of proteins which may enhance immune system activation and recognition, such as CD86, MHC1, MHC2, and ICAM1 (reviewed in (43)).

Several clinical trials have been completed with butyrate or synthetic derivatives of butyrate. Despite initial success in inducing remission in a patient with leukemia (94), other therapeutic interventions have not been as promising. However, optimization of the route and length of administration of butyrate may increase its therapeutic effects. In addition, chronic dietary administration of butyrate through consumption of fiber may have significant chemopreventive effects through inhibition of HDACs.

DIALLYL DISULFIDE

DADS is an organosulfur compound found in garlic and other *Allium* vegetables. Inhibition of initiating events has been proposed, based on the ability of DADS to modulate Phase 1 and Phase 2 enzyme activities (reviewed in (95)). However, DADS also has been demonstrated to inhibit carcinogenesis *post*-initiation (96). Furthermore, differentiation, cell cycle

arrest and apoptosis have been reported in several cancer cell lines upon administration of DADS (reviewed in (97)).

The first report of alteration of histone acetylation by DADS was described in DS19 (mouse erythroleukemic) and K562 (human leukemic) cells, coupled with evidence for induction of differentiation (98). Histone acetylation also was induced in rat hepatoma and human breast cancer cells by DADS, and to a greater extent by a metabolite of DADS, allyl mercaptan (98). Rats administered DADS or allyl mercaptan had increased levels of acetylated histones in the liver (99), demonstrating that altered acetylated histone status is achievable *in vivo*. Recently, DADS was shown to induce acetylated histones H3 and H4 in two colon epithelial cell lines, CaCo-2 and HT-29; DADS also increased *P21* mRNA and protein levels in HT-29 cells through a P53-independent mechanism (100). Strikingly, 200 μM allyl mercaptan inhibited 91% of HDAC activity in nuclear extracts from CaCo-2 cells, reducing HDAC activity to the same level as nuclear extracts treated with TSA (200 nM), whereas 200 μM DADS parent compound inhibited to a lesser extent (29%) (100). Thus, one hypothesis is that *in vivo*, allyl mercaptan or one of its metabolites represents the active HDAC inhibitor(s) rather than the parent compound, DADS (Figure 4B).

As with other HDAC inhibitors, DADS induced a G2/M arrest in several cell types, including human colon and neuroblastoma cells (reviewed in (97)). One study reported p53-independent apoptosis in colon cells (101), and

several others demonstrated an increase in BAX expression in human lung and breast cancer cells (102, 103). Caspase-3 activation, indicative of cells undergoing apoptosis, also has been reported in human leukemia, lung cancer, breast cancer, and neuroblastoma cell lines (reviewed in (97)). Thus, several of the downstream signaling effects associated with changes in histone acetylation by DADS are similar to other well-known HDAC inhibitors.

Interestingly, another organosulfur compound, S-allylmercaptocysteine, also has been reported to affect histone acetylation status. In human colon and breast cancer cells, and in mouse erythroleukemic cells, S-allylmercaptocysteine induced growth arrest and increased the levels of acetylated histones H3 and H4 (104). Others have described G2/M arrest and apoptosis in human colon and mouse fibroblast cells (104, 105), with one mechanism hypothesized to be alteration of microtubule dynamics by S-allylmercaptocysteine (106). HDAC inhibitors can acetylate α-tubulin, resulting in altered microtubule dynamics (107) (108). Although S-allylmercaptocysteine has not been directly tested in terms of inhibition of HDAC activity, it is likely that it, too, acts through HDAC inhibition. HDAC inhibition may be a general mechanism for chemoprevention by organosulfur compounds found in garlic, depending on their structure and ability to fit within the enzyme active site.

SULFORAPHANE

SFN is an isothiocyanate found in cruciferous vegetables, such as broccoli. First discovered as a potent Phase 2 enzyme inducer (109), a plethora of blocking and suppressing chemopreventive activities have since been attributed to SFN (reviewed in Myzak and Dashwood 2005). Until recently, the mechanism(s) underlying the suppressing activities of SFN were unknown. We first reported on the inhibition of HDAC activity and concomitant increases in acetylated histones in human embryonic kidney 293 (HEK293) cells and HCT116 human colorectal cancer cells (85). SFN treatment increased acetylated histone H4 associated with the *P21* promoter, and increased p21 protein expression in HCT116 cells. Rather than the parent compound, a metabolite of SFN, SFN-cysteine, was the active form (Figure 4), inhibiting HDAC activity by approximately 45% at 15 μM (85). These effects also were observed in three human prostate epithelial cell lines, namely benign prostate hyperplasia (BPH-1) cells, androgen-dependent LnCaP cancer cells, and androgen-independent PC-3 cancer cells (Myzak et al, unpublished data). Furthermore, the increased histone acetylation induced by SFN caused prostate cells to undergo G2/M arrest and apoptosis, with an increase in p21 and BAX expression.

HDAC inhibitors have been hypothesized to disrupt checkpoints during G2/M. Several studies have demonstrated that SFN causes a G2/M arrest, and alters checkpoint 2 kinase activity (110). It has been suggested that HDAC inhibitors trigger a G2/M checkpoint control that is active in normal

cells but inactivated in tumor cells; reactivation in the cancer cell enables cell death to occur (58). Interestingly, HDAC inhibitors have been shown to cause an increase in acetylated tubulin (107, 108), which may alter the dynamics of tubulin polymerization. One of the proposed mechanisms of G2/M arrest by SFN is through tubulin depolymerization (111, 112), thus further connecting activities of HDAC inhibitors with those of SFN. SFN-treated cells exhibit characteristics of aberrant mitosis, as do other HDAC inhibitor-treated cells (58). A role for oxidative stress also has been proposed, with evidence for induction of thioredoxin by HDAC inhibitors in normal cells but not in cancer cells (113).

Another isothiocyanate, allyl isothiocyanate (allyl-ITC), found in mustard oil, was reported to induce histone acetylation (114). However, allyl-ITC itself did not exhibit HDAC inhibitory activity (114). Since allyl-ITC is metabolized in a similar fashion to SFN, the S-allylmercaptocysteine metabolite may be the active form, and indeed, S-allylmercaptocysteine itself caused an increase in histone acetylation (104). This suggests that other isothiocyanates, which have reported chemopreventive activities similar to SFN (reviewed in (115)), may act through inhibition of HDACs. Because the 'cap' group seems to determine specificity toward individual HDACs, different isothiocyanates may target individual HDACs to varying degrees.

Future Perspectives

As the quest for novel HDAC inhibitors continues, it is likely that other compounds will be identified from dietary sources. It will be important to focus on metabolites in addition to parent compounds, because these may possess more potent HDAC inhibitory activity, as is the case of metabolites of DADS and SFN. Given that the dietary compounds discussed in this review are established cancer chemopreventive agents *in vivo*, it is reasonable to assume that other HDAC inhibitors also may exert cancer chemopreventive activity. Importantly, although HDAC inhibitors increase global acetylation status in normal cells, only transformed cells respond to HDAC inhibitors in a manner that results in differentiation, cell cycle arrest, and apoptosis (40, 91). Due to their ability to act on tumor cells themselves via changes in differentiation, cell cycle arrest, and apoptosis, as well as modulating the tumor environment (immune system activation, angiogenesis, and metastasis), HDAC inhibitors warrant greater attention as cancer chemopreventive and therapeutic agents (Figure 5). An important distinction to be made from the potent agents currently under consideration for clinical trials, such as SAHA and related 'pharmacologic' compounds, is that dietary phytochemicals are likely to be weak agonists and modulate HDACs in more subtle ways. Thus, the potential exists for diet to play a role in the types and extent of HDC modulation and gene expression, on a more chronic basis throughout life. Understanding which diets or individual dietary compounds

might alter the balance in favor of 'chemoprevention,' especially in healthy populations, will be an important avenue for future research.

Figure Legends

Figure 1: Histone acetylation and deacetylation controls configuration of chromatin and regulates transcription. *A*. Repressor transcription factors bind to specific DNA sequences and recruit HDACs and other co-repressors. De-acetylated histone tails block access of transcription factors to DNA and repress transcription. *B*. Acetylation of histone tails allows these tails to move away from DNA, giving transcription factors access to DNA. Acetylation also recruits additional co-activators and results in loosening of chromatin.

Figure 2: Dysregulation of histone acetylation in tumorigenesis. Loss of HAT activity or increased HDAC activity plays a role in the progression from initiated cells to invasion and metastasis. See text for specific details of each step.

Figure 3: Structures of prototype HDAC inhibitors. Compounds such as trichostatin A and trapoxin have cap group attached to an 'arm', or spacer function, that reaches into the HDAC active site and positions the functional group adjacent to the zinc atom and neighboring amino acid residues.

Figure 4: Structures of dietary HDAC inhibitors (A) butyrate, (B) diallyl disulfide and (C) sulforaphane, and related metabolites. (D) Overlay of

trichostatin A (TSA) and sulforphane-cysteine (SFN-cys) in an HDAC active site, as reported previously (85).

Figure 5: HDAC inhibitors act through several mechanisms. Butyrate from dietary fiber breakdown, DADS from garlic, and SFN from broccoli inhibit HDAC and thereby cause de-repression of genes controlling differentiation, cell cycle progression, apoptosis, angiogenesis, invasion and metastasis. Each of the cancer protective mechanisms is described in further detail in the text.

Figure 1.

A.

Transcriptional Repression

B.

Transcriptional Activation
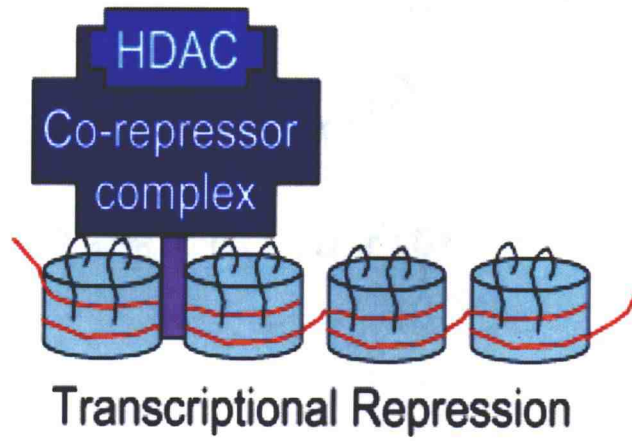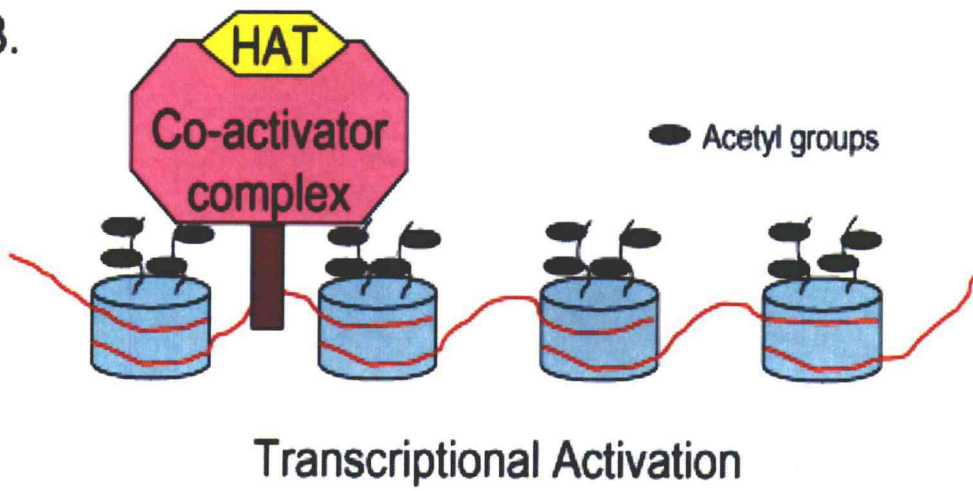
Figure 2.

32
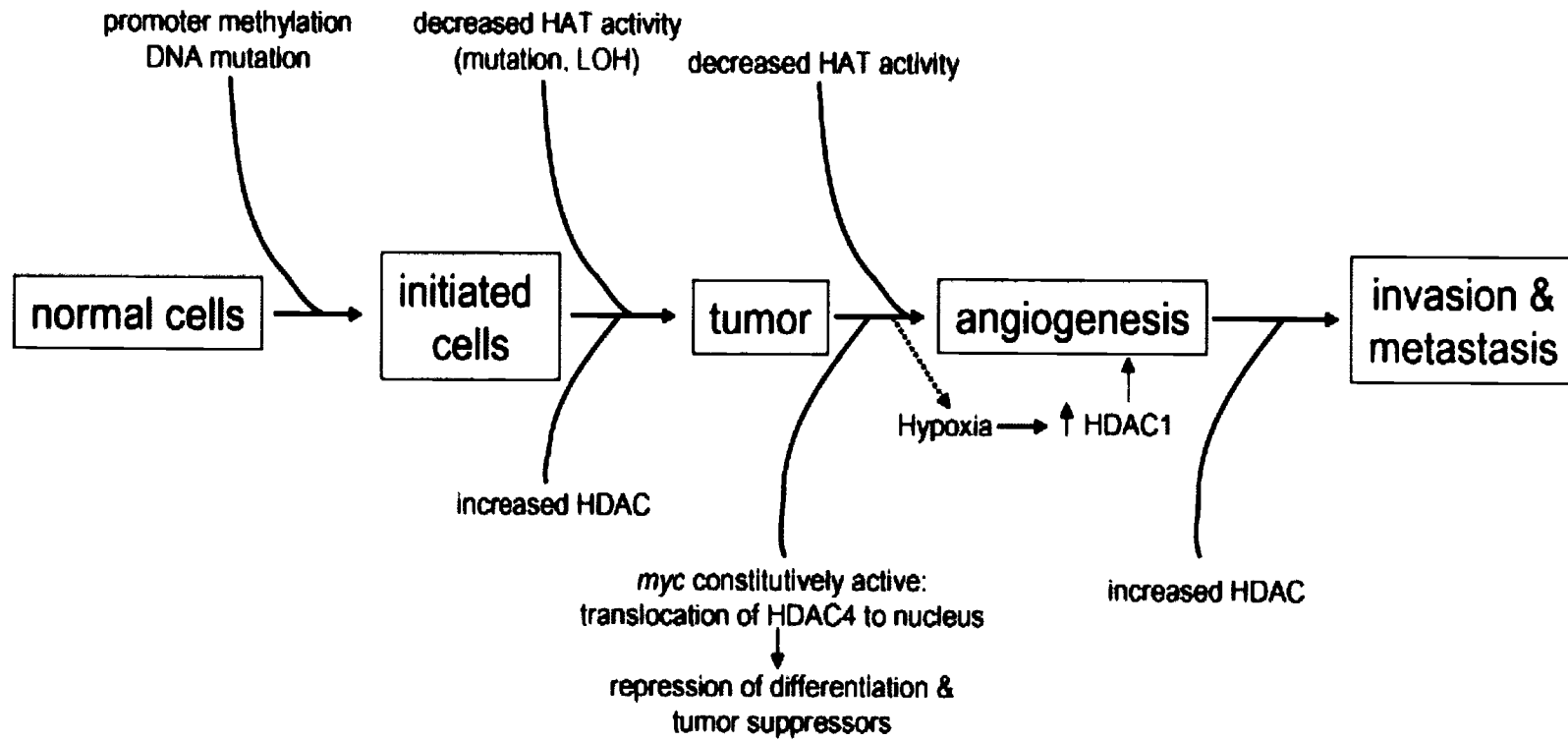
Figure 3.

A.

Cap | Spacer | Functional group
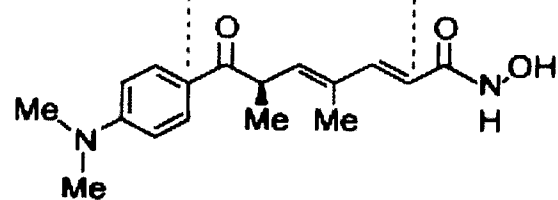
Trichostatin A

B.

Trapoxin

Figure 4.

A.

Me — OH
O

Butyrate

B.

S–S

Diallyl disulfide

S

Allyl mercaptan

NH₂

S–S

COOH

S-allylmercaptocysteine

C.

O
‖
S
Me — NCS

Sulforaphane

O
‖
S
Me — N
S
S
NH₂
O–C–O

Sulforaphane-cysteine

D.

SFN-Cys

TSA

Zn
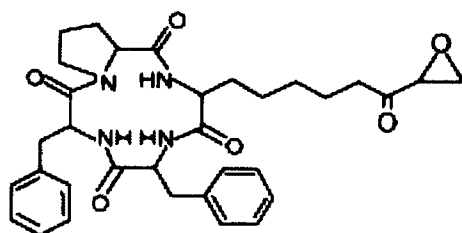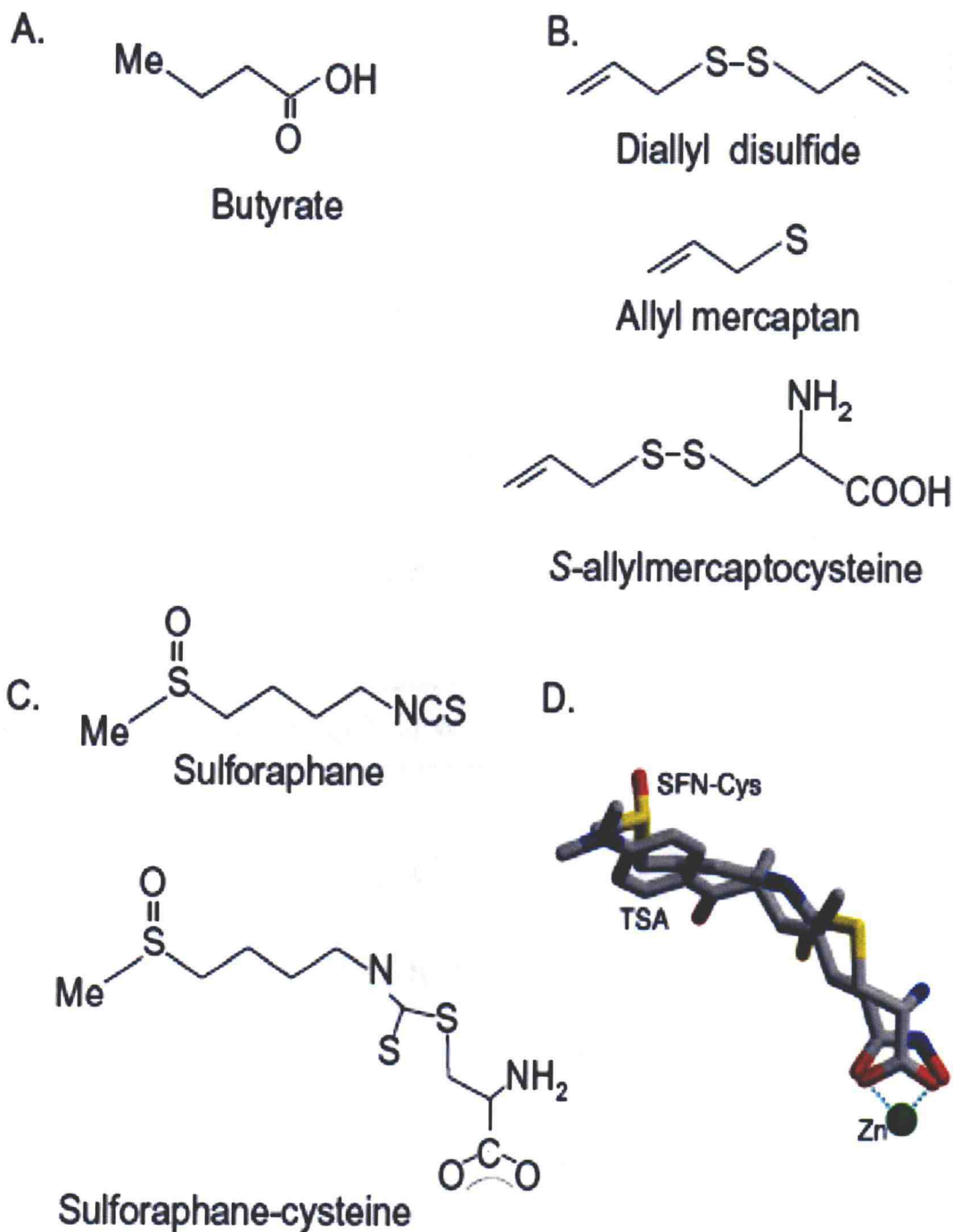
Figure 5.
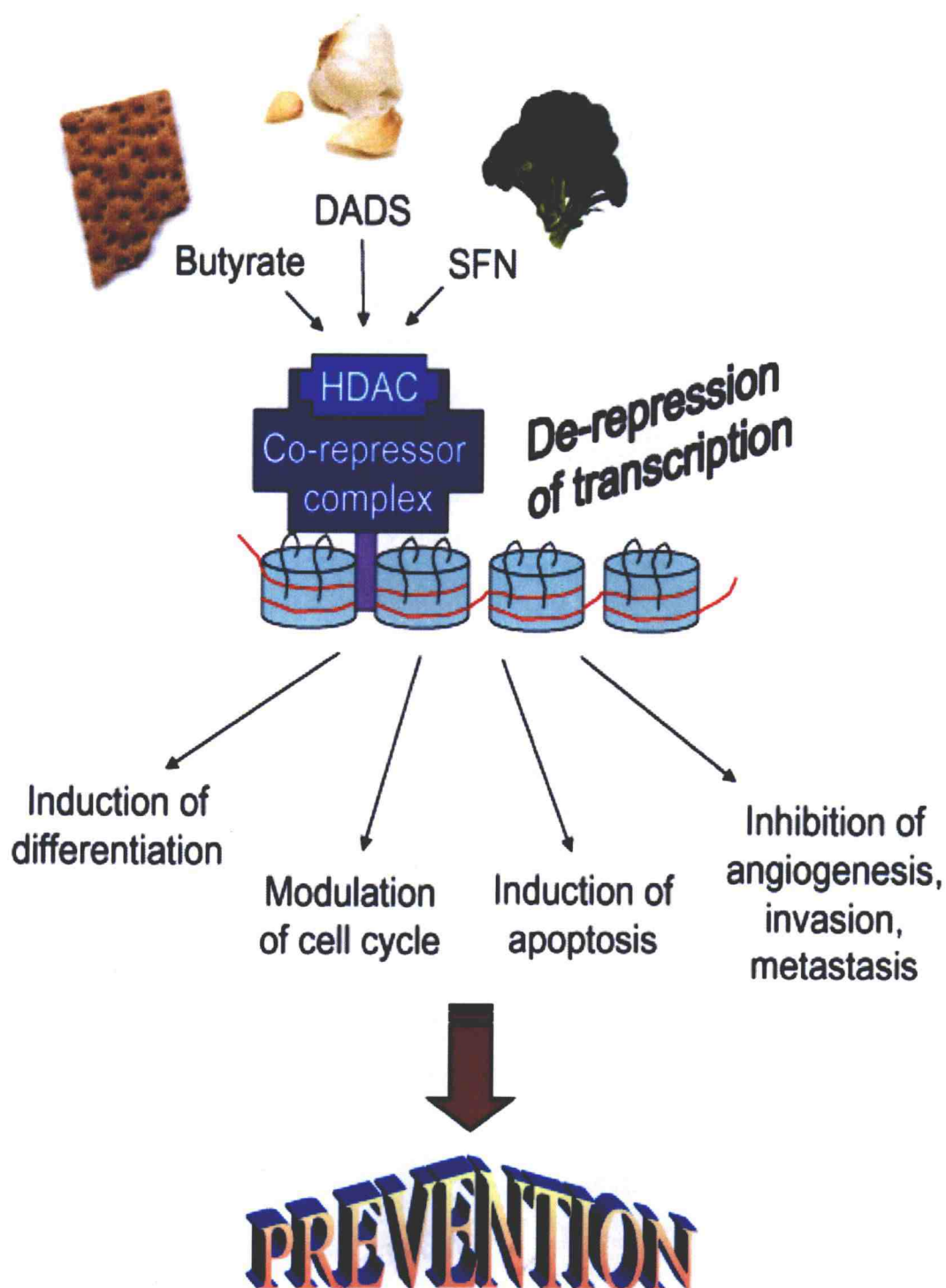
Chemoprotection by sulforaphane:
Keep one eye beyond Keap1.

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Abstract

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables, with particularly high levels detected in broccoli and broccoli sprouts. Over a decade ago, this phytochemical was identified as a likely chemopreventive agent based on its ability to induce Phase 2 detoxification enzymes, as well as to inhibit Phase 1 enzymes involved in carcinogen activation. Considerable attention has focused on SFN as a 'blocking' agent, with the ability to modulate the Nrf2/Keap1 pathway, but recent evidence suggests that SFN acts by numerous other mechanisms. SFN induces cell cycle arrest and apoptosis in cancer cells, inhibits tubulin polymerization, activates checkpoint 2 kinase, and inhibits histone deacetylase activity. The latter findings suggest that SFN may be effective during the post-initiation stages of carcinogenesis, as a 'suppressing' agent. Moreover, pharmacological administration of SFN may be a promising therapeutic approach to the treatment of cancers, including those characterized by increased inflammation and involving viral or bacterial-related pathologies. The present review discusses the more widely established chemoprotective mechanisms of SFN, but makes the case for additional work on mechanisms that might be of importance during later stages of carcinogenesis, beyond Keap1.

Introduction

An estimated 1.4 million Americans will be diagnosed with cancer in 2005, with over 570,000 mortalities (American Cancer Society). Although advances in treatment have been made, there continues to be a need for intervention strategies, including compounds that act as primary protective agents by preventing, delaying, or reversing pre-neoplastic lesions, as well as those that act on secondary or recurrent cancers as therapeutic agents. Compounds found in the diet are of particular interest because of their accessibility to the general population, and ongoing research continues to identify novel candidates for use in cancer chemoprevention clinical trials.

Isothiocyanates (ITCs) are found in cruciferous vegetables such as broccoli, Brussels sprouts, cauliflower, and cabbage. Sulforaphane (SFN) is an ITC found at high levels in broccoli and broccoli sprouts (109, 116). Since it was first identified in 1992 as a potential chemopreventive agent (109), there has been a sharp increase in PubMed citations mentioning SFN (Figure 6). Early research focused on Phase 2 enzyme induction by SFN, as well as inhibition of enzymes involved in carcinogen activation, but there has been growing interest in other mechanisms of chemoprotection by SFN.

Thus, in addition to its ability to act as a 'blocking' agent against early initiating events, recent evidence points to SFN as a 'suppressing' agent, helping to delay or reverse growth and/or survival of transformed cells (Figure 7). The precise mechanism(s) that operate during the post-initiation phase

are not well understood, and only a handful of suppressing agents have been studied in any detail. Nonetheless, this review considers the evidence that SFN might represent a multi-faceted chemopreventive agent, with the ability to act during blocking, suppressing and therapeutic stages. The various mechanisms will be discussed in turn, namely: (1) inhibition of Phase 1 enzymes, (2) modulation of Phase 2 enzymes, (3) eradication of infection, (4) inhibition of growth promoting signaling pathways, (5) alteration of signaling pathways, cell cycle arrest, and apoptosis, and (6) inhibition of recurrence (refer to Figure 7).

Prevention of chemical carcinogenesis: carcinogen detoxification

Pathways that augment carcinogen detoxification and excretion can reduce reactive intermediate(s) that might otherwise interact with DNA, thereby effectively blocking initiating events early in carcinogenesis. Evidence suggests that SFN accomplishes this through two mechanisms, namely inhibition of Phase 1 enzymes and induction of Phase 2 enzymes (Figure 7).

1. Inhibition of Phase 1 enzymes

Phase 1 enzymes can metabolically activate pro-carcinogens; thus, under certain circumstances, the inhibition of Phase 1 enzymes can be considered a preventive measure against chemically-induced carcinogenesis.

Activities of cytochromes P450 (CYPs) 1A1 and 2B1/2 were inhibited dose-dependently by SFN in rat hepatocytes, as determined by 7-ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-dealkylase (PROD), respectively (117). In human hepatocytes, SFN decreased mRNA levels of CYP3A4, and this translated to a decrease in CYP3A4 activity (117). Furthermore, studies of p-nitrophenol hydroxylase activity in acetone-induced rat liver microsomes established that SFN was a competitive inhibitor of CYP2E1 (118). Subsequent work in mouse hepatocytes showed dose-dependent inhibition of unscheduled DNA synthesis induced by N-nitrosodimethylamine (NDMA), a carcinogen known to be activated by CYP2E1, and SFN exhibited antimutagenic effects against NDMA at nanomolar concentrations in the Salmonella assay. SFN had no effects on the mutagenicity of sodium azide, a direct-acting mutagen (118), suggesting that SFN indeed inhibited one or more enzymes in the S9 fraction. Interestingly, SFN was reported to have no effect against CYP1A2 (118), an enzyme involved in the metabolic activation of heterocyclic amines (3). Nonetheless, SFN inhibited the S9-dependent mutagenic activities of several heterocyclic amines in the Salmonella assay, including 2-amino-3-methylimidazo[4,5-f]quinoline (IQ), 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), and 3-amino-1,4-dimethyl-5H-pyridol[4,3-b]indole (Trp-P-1) (119). Other mechanisms were not examined for SFN, such as complex formation with the carcinogen, degradation of the activated metabolites, or

scavenging of electrophiles (3), which can serve to limit the extent to which initiating agents interact with DNA and form covalent adducts. Interestingly, SFN decreased the number of PhIP-DNA adducts by approximately 40% in human HepG2 cells pre-treated with 1 μM SFN followed by 10 ng PhIP; however, there was no decrease in DNA adduct formation with post-treatment of SFN, suggesting that SFN had no effect on PhIP DNA-adduct repair, but rather prevented PhIP interactions with DNA (120). SFN was also shown to protect against benzo(*a*)pyrene (BaP)-induced single-strand DNA breaks in the comet assay, with concentrations as low as 5 μM SFN (121). Using THLE cells that express CYP2E1 or CYP1A2, SFN also inhibited double-strand breaks initiated by NDMA and IQ, respectively (122). In non-cancerous human mammary epithelial MCF-10F cells, SFN provided protection against DNA adduct formation by BaP and 1,6-dinitropyrene (123). In the latter studies, protection was observed by concentrations as low as 0.1 μM SFN, which inhibited 68% of BaP-DNA adducts, with a maximum of 80% inhibition being detected at 2 μM SFN. Interestingly, an increase in NADPH:quinone reductase (NQO1) and glutathione-*S*-transferase P1 (GSTP1) protein expression was also reported in these cells (123). Collectively, these reports provide evidence for protection by SFN against carcinogen-DNA damage *in vitro* and suggest a role for inhibition of certain Phase 1 enzymes, but they do not completely rule out the possibility that SFN operates via other blocking mechanisms.

2. Modulation of Phase 2 enzymes

An additional blocking mechanism, and indeed the first to be associated with SFN, is the potent induction of Phase 2 enzymes. SFN augments various pathways known to facilitate carcinogen detoxification and excretion. Using quinone reductase (QR) activity as a measure for inducer activity in Hepa 1c1c7 murine hepatoma cells, SFN was shown to double QR activity at concentrations on the order of 0.4-0.8 µM SFN (109). Furthermore, mice treated by gavage with 15 µmol SFN/mouse/day for five days had an increase in QR and glutathione-S-transferase (GST) in the liver, forestomach, glandular stomach, proximal small intestine, and lungs (109), establishing that SFN also induces Phase 2 enzymes *in vivo* following oral ingestion.

Although many cell lines respond to SFN, there is some variability in the extent and type of Phase 2 enzyme induction, depending on cell type. In human HepG2 cells, for example, SFN increases mRNA of UDP-glucuronosyltranferase (UGT) 1A1 and GSTA1 (120), increases QR activity (124), and increases UGT1A1 protein as well as bilirubin glucuronidation (125). An increase in GST and QR activities also was observed with SFN treatment in murine Hepa1c1c7 cells (124, 126). Whereas there was an apparent decrease in GST activity in HT29 cells, undifferentiated CaCo-2 cells demonstrated a dose- and time-dependent induction of GSTA1 and UGT1A1 upon SFN administration (127). Human prostate cells responded to

SFN with an increase in NQO1 and GSTA1 mRNA, as well as an increase in microsomal GST activity and QR activity (128). Furthermore, SFN increased γ-glutamylcysteine synthetase light chain mRNA, resulting in an increase in cellular glutathione (128). In primary rat hepatocytes, SFN has been reported to induce mRNA of GSTA1/2 and GSTP1 (117), whereas in human primary hepatocytes, SFN induced mRNA of GSTA1/2 and GSTM1 but decreased mRNA of CYP3A4 (117). The net result of the aforementioned responses to SFN is to increase cellular defenses, leading to enhanced carcinogen detoxification and protection against potential mutagenic events.

Importantly, these effects of SFN in cell culture can be extrapolated to *in vivo* situations. As previously mentioned, induction of GST and QR activities was observed in tissues of mice given SFN orally, demonstrating that systemic uptake and bioavailability of SFN and/or its metabolites was feasible (109). Confirming these observations, rats treated by gavage for five consecutive days demonstrated a dose-dependent (200-1000 μmol SFN/kg/day) increase in QR and GST activity in the liver, colon, and pancreas (126), and 40 μmol SFN/kg/day for five days by gavage increased QR and GST activities in the rat forestomach, duodenum, and bladder (129).

In recent years, two main mechanisms have been proposed to explain the induction of Phase 2 enzymes by SFN, namely disruption of Nrf2-Keap1 interactions and mitogen-activated protein kinase (MAPK) activation (Figure 8). These mechanisms may act synergistically to induce genes that contain

the so-called 'antioxidant response element' (ARE). In early experiments, SFN was shown to be the most potent inducer among several compounds tested in an ARE reporter assay (130). The disruption of Nrf2-Keap1 interactions, the translocation of Nrf2 to the nucleus, and the induction ARE-containing genes has been extensively reviewed for SFN (131-134). Recent evidence suggests that specific cysteine residues of Keap1, the anchor that prevents Nrf2 from entering the nucleus, act as 'sensors' that are modified by SFN and other Phase 2 enzyme-inducers (135). In mice treated by gavage with 9 μmol SFN/day for one week, several classes of genes were identified as targets of SFN in a transcriptional microarray, including cellular NADPH regenerating enzymes, xenobiotic metabolizing enzymes, antioxidant enzymes, and biosynthetic enzymes of glutathione and glucuronidation conjugation pathways (136). The importance of Nrf2 was illustrated through the use of Nrf2 knockout mice, in which the response to SFN in induction of the aforementioned classes of genes was abrogated. Further studies in which mice were fed broccoli seeds, which contain high levels of SFN, in the diet for seven days resulted in an increase in protein expression of NQO1, GSTA1/2, GSTA3, and GSTM1/2 in the stomach, small intestine, and liver of wild-type mice (137). Although the response in Nrf2 knockout mice was not as strong compared to wild-type mice, broccoli seeds nonetheless were able to increase GSTA1/2, GSTA3, and GSTP1 in the stomach and GSTA3 in the liver, hinting at other possible mechanisms.

An additional mechanism through which SFN might activate ARE-driven genes is via the mitogen activated protein kinase (MAPK) pathway (Figure 8). SFN was shown to increase ERK2 activity, which is downstream from Raf-1 and MEK (138). Use of a MAPK inhibitor attenuated activation of QR activity in HepG2 cells and ARE reporter activity, and SFN directly activated Raf-1 (138). Subsequently, MAPK activation was observed in rat livers two hours after rats were treated by gavage with 50 μmol SFN (139). This, along with other data (reviewed in (140, 141), suggests that MAPK activation may play a role in the activation of Nrf2, and that SFN may regulate ARE-mediated transcription both through its effects on Keap1-Nrf2 destabilization, as well as MAPK activation.

Blocking effects of SFN also have been reported in other studies, *in vivo*. Administration of 75 μmol or 150 μmol SFN three days prior and one day after a single dose of DMBA dose-dependently decreased mammary tumor incidence and multiplicity in Sprague-Dawley rats (142). Importantly, the latter study also showed a delay in onset of tumor formation. Hot water extracts from broccoli sprouts, containing high levels of the SFN precursor glucoraphanin, were inhibitory toward DMBA-induced mammary tumors (116). SFN blocked BaP-initiated forestomach tumors in mice (143). Interestingly, in a pre- versus post-initiation experiment, SFN and its metabolite SFN-*N*-acetylcysteine (SFN-NAC) demonstrated different outcomes in prevention of azoxymethane (AOM)-induced colonic aberrant

crypt foci (144). SFN administered both pre-and post-initiated effectively inhibited the total number of crypts and the number of rats with more than four crypts. However, SFN-NAC was active only *post*-initiation (144), suggesting that the parent compound, SFN, and the metabolite, SFN-NAC, may elicit different mechanisms of chemoprevention. The results of the latter study clearly suggest that SFN acts not only as a blocking agent, but also as a suppressing agent during later stages (Figure 7).

3. Eradication of infection

Although many studies with SFN have focused on detoxification of chemical carcinogens, evidence also has been reported for protective effects against viral or bacterial pathogens (Figure 3). For example, chronic infection with *Helicobacter pylori* (*H. pylori*) is associated with an increased risk of gastric cancer (145). SFN was recently shown to inhibit several strains of *H. pylori*, including antibiotic-resistant strains, with a minimal inhibitory concentration of 4 µg/ml (143). Moreover, SFN administered at 7.5 µmols per day for five days eradicated *H. pylori* from human gastric xenografts in nude mice (146). The precise mechanism(s) through which SFN protected in these studies remains unknown.

Chemoprevention: beyond Keap1

Only recently has SFN been described as a chemopreventive and therapeutic agent that might act through suppressing mechanisms, i.e. *post-initiation*. Some studies reported decreased viability of neoplastically transformed cells as compared with the corresponding non-transformed cells or pre-neoplastic cells, suggesting some selectivity of SFN action. The downstream effects of SFN appear to be dependent on cell type, as well as the concentration and length of SFN treatment. While several studies have described effects on signaling pathways, cell cycle arrest and/or apoptosis (Table 3.1), relatively few mechanisms have been explored to explain precisely how SFN mediates these changes.

4. Inhibition of growth-promoting signaling pathways

During the various steps of multi-stage carcinogenesis (Figure 7), tumor modulators can alter signaling pathways in a manner that promotes or inhibits cell growth. Whereas initiating events often constitutively activate survival and/or growth signaling pathways, chemopreventive agents can override the aberrant signaling events and thereby act as tumor suppressors. SFN interferes with two well-known signaling pathways, beyond Keap1. First, SFN acts as an anti-inflammatory agent through decreasing the DNA binding capability of nuclear factor κB (NFκB) (147). In murine RAW macrophages, SFN decreased lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E2, and tumor necrosis factor α (TNFα), as well as inducible

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nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) protein expression and mRNA levels (147). Second, SFN inhibited UVB-induced AP-1 activation in human keratinocytes at concentrations ranging from 1-10 μM (148). It was shown that SFN interfered with AP-1 binding to DNA, and cysteine residues in AP-1 may be important for the inhibitory effects (148).

5. Induction of cell cycle arrest and/or apoptosis

The effects of SFN on cell viability were described in HT-29 human colon cancer cells, where 100 μM SFN for 24 or 48 h resulted in a decrease in cell viability to 10% of controls (149). Further work established an IC$_{50}$ of 15 μM SFN in HT-29 cells and 50 μM in CaCo-2 cells, suggesting possible selective effects toward undifferentiated *versus* differentiated cells, respectively (149). Alterations in cell cycle progression also have been reported in HT-29 cells treated with SFN, with a decrease in the percentage of cells in G1 and an increase in G2/M, as well as increases in cyclin A and cyclin B1 protein levels (150), and hyperphosphorylation of the retinoblastoma tumor suppressor protein (Rb) (151). Furthermore, there was an increase in Bax protein expression, loss of cytochrome c from mitochondria, and concomitant elevation of cytosolic cytochrome c (150). Apoptotic markers also were observed upon treatment with SFN, such as phosphatidylserine translocation, nuclear condensation, membrane blebbing, and vesicle formation (150).

Similar responses have been observed in human prostate cancer cells after treatment with SFN. Androgen-dependent LnCaP cells exposed to 9 μM SFN underwent a G1 arrest, whereas increasing the dose to 50 μM caused the cells to become apoptotic, with activation of caspases and a dose-dependent decrease in DNA synthesis (152). SFN caused androgen-independent DU-145 cells to also undergo G1 cell cycle arrest, with inhibition of Cdk4 activity, an increase in p21$^{Cip1/Waf1}$ expression, and a decrease in cyclin D1 expression (153). Furthermore, apoptosis was induced in these cells, as evidence by increased terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL), increased caspase-3 (cleaved), and decreased Bcl-2 expression (153). While the latter studies with LnCaP and DU-145 cells reported a G1 arrest, SFN caused an apparent G2/M arrest in PC-3 cells (110). The latter cells also underwent apoptosis, as evidenced by an increase in DNA fragmentation, cleavage of poly-ADP ribose polymerase (PARP), and increased expression of Bax and cleaved caspase-3 and caspase-9 (154).

SFN-mediated cell cycle arrest and apoptosis has been reported in several other cell lines. For example, a G2/M arrest was described in murine sarcomatid mammary F311 cells (112) and human MCF-7 mammary cancer cells (111), with cytotoxic effects also reported in MCF-7 cells (155). Loss of cell viability was observed with concentrations as low as 1 μM SFN in lymphoblastoid cells (156), 4.3 μM SFN in HL60 cells (157), and 3 μM SFN in

human T-cell leukemia cells (158). Apoptosis induction in medulloblastoma cells occurred at 5 μM SFN, with an 8-fold increase in caspase activity at 10 μM SFN (159). SFN also elicited caspase-3 cleavage and G2/M arrest in human pancreatic cancer cells, and cellular toxicity due to SFN was positively correlated with a decrease in cellular GSH levels (160). Based on the numerous reports of cell cycle arrest and apoptotic activity induced by SFN in multiple cell types, SFN can potentially act against several types of cancer, and at early, intermediate and late stages (Figure 7).

Apoptotic markers have also been observed with SFN in xenograft models. For example, in BALB/c mice implanted with murine mammary carcinoma cells and subsequently given 15 nmol SFN for 13 days by *i.v.* injection there was a 60% decrease in tumor mass, a decrease in proliferating cell nuclear antigen (PCNA) levels, and an increase in cleaved PARP (112). PC-3 xenografts from mice treated by gavage with 5.6 μmol SFN three times per week for three weeks were approximately one-fourth the volume compared to controls, and the implants from SFN-treated mice exhibited an increase in TUNEL staining, and increases in the expression of pro-apoptotic proteins such as Bax and Bid (154).

Several mechanisms have been proposed to explain how SFN induces changes in cell cycle progression and apoptosis. First, two groups demonstrated that SFN maintains Cdc2 kinase in its active form, which was correlated with induction of apoptosis (112, 151). However, another study

reported reduction of Cdc25C, a phosphatase responsible for activation of Cdc2; this reduction of Cdc25C by SFN was mediated via activation of checkpoint 2 kinase (Chk2) (110). Reduction of Chk2 by siRNA slightly decreased SFN-mediated G2/M arrest, suggesting that Chk2-independent mechanisms also contribute to SFN-induced cell cycle arrest, but Chk2 protein levels may influence the sensitivity of cells to SFN (110). Based on observations that SFN treatment resulted in condensed, unaligned chromatin at the metaphase plate in MCF-7 cells (111), and aberrant and mildly depolymerized spindles in F311 cells (112), it was hypothesized that SFN disrupts tubulin polymerization, resulting in cell cycle arrest during mitosis. SFN did decrease tubulin polymerization *in vitro*, albeit at non-physiological concentrations (100-300 µM) (111, 112).

Finally, SFN was recently identified as a novel histone deacetylase (HDAC) inhibitor in human embryonic kidney 293 (HEK293) and in HCT116 human colon cancer cells (85). HDAC inhibitors have been shown to induce cell cycle arrest and apoptosis (41), and they appear to have less effect on normal cells, perhaps due to induction of thioredoxin (113). At physiologically relevant concentrations, SFN increased acetylated histones H3 and H4, and expression of p21[Cip1/Waf1]. Moreover, in a chromatin immunoprecipitation (ChIP) assay, SFN dose-dependently increased the amount of acetylated histone H4 associated with the *P21* promoter. A metabolite of SFN, SFN-cysteine, was shown to be the likely inhibitor of HDAC activity (85). These

findings were recapitulated in benign prostate hyperplasia (BPH-1), LnCaP, and PC-3 human prostate cells, together with marked changes in cell cycle progression and apoptosis (M.C. Myzak et al, unpublished data). Importantly, the promoter region of the *P21* gene appears to lack an ARE, supporting a Nrf2-independent mechanism by SFN. HDAC inhibition provides an important new avenue for future research on SFN, both as a tumor suppressing agent and as a chemotherapeutic agent during late stages of carcinogenesis.

Conclusions

Accumulating evidence suggests that SFN is a highly promising dietary preventive agent, due to its ability to confer chemoprotection through several distinct pathways and via multiple mechanisms of action. Early studies focused on the potent Phase 2 enzyme inducing activities of SFN, and alterations in Nrf2/Keap1 signaling. However, recent reports have identified SFN effects on cell cycle checkpoint controls, apoptosis mediators, and HDAC activity, suggesting the need to keep one eye beyond Keap1. Due to its ability to induce cell cycle arrest and apoptosis in various tumor cells, SFN has the potential to act against secondary and possibly recurrent cancers (Figure 7), and the diversity of mechanisms makes SFN a strong candidate for possible therapeutic application, either alone or in combination with other agents that provide synergistic or additive protection. Epidemiological

evidence suggests a reduced risk for cancers of the prostate (161-163), lung

(164-166), breast (167), and colon (168, 169) in people who consume

cruciferous vegetables. Further studies will be required to determine to what

extent these protective effects of whole vegetables can be attributed to SFN

and/or its metabolites, and the various mechanisms discussed in the present

review and outlined in Figure 9.

Table 3.1. Responses to SFN treatment in various cell lines

Cells	Dose (μM)	Treatment time (h)	Changes detected	References
HT-29	15	48	Decreased cell viability; increased Bax, cyclin A, cyclin B1, p21, Rb phosphorylation, cdc2 kinase activities; cytochrome c release from mitochondria; G2/M arrest; roscovitine decreased SFN-induced apoptosis	(149-151)
HCT116	15	48	Increased p21, acetylated histones H3 and H4	(85)
CaCo-2	50	48	Decreased cell viability	(149)
LnCaP	9-50	30	G1 arrest; sub-G1 peak; decreased DNA synthesis	(152)
DU-145	3-90	24-48	G1 arrest; increased p21, TUNEL, caspase activity; decreased cyclin D1	(153)
PC-3	20-40	4-72	G2/M arrest; increased sub-G1, increased Chk2 kinase activity, caspase activity, Bax; decreased cell viability, cyclin B1, Cdc25B, Cdc25C	(110, 154)
F3II	5 100-300 15	24 1 36	IC$_{50}$=5 μM; G2/M arrest; interference with tubulin polymerization in vitro; increased cdc2 kinase activity; decreased Bcl-2	(112)
MCF-7	15 13.7	24 48	G2/M arrest; increased cyclin B1; disruption of microtubule polymerization IC$_{50}$=13.7 μM by sulforhodamine B assay	(111, 155)
MCF-12A	40.5	48	IC$_{50}$=40.5 μM by sulforhodamine B assay	(155)
Lympho-blastoid	3.9 1	48 48	IC$_{50}$=3.9 μM for loss of cell viability; increased apoptosis	(156)
HL-60	3.4	72	IC$_{50}$=3.4 μM for loss of cell viability	(157)
Jurkat	30	48	G2/M arrest; increased Bax, p53, apoptosis; decreased Bcl-2	(158)
DAOY	2 10 10	24 18 24	Increased caspase activity; increased TUNEL, cleaved PARP; loss of cell viability (40% of controls)	(159)
MIA PaCa-2	10-40	24	G2/M arrest; caspase-3 cleavage; decreased cell viability, GSH levels; no Chk1 activation	(160)
PANC-1	5	24	G2/M arrest; decreased cell viability, GSH levels	(160)

Figure Legends

Figure 6. Increasing interest in SFN, based on annual citations in PubMed.

Figure 7. SFN acts at various steps during multi-stage carcinogenesis. This figure illustrates the various 'blocking' and 'suppressing' mechanisms by which SFN inhibits multi-stage carcinogenesis. Each of the protective mechanisms (*1-6*) is described in further detail in the text. Adapted from a previous review (3).

Figure 8. SFN induces ARE expression through disruption of the Keap1-Nrf2 complex. SFN can interact directly with sulfhydryl residues on Keap1, causing Nrf2 to be released. Alternatively, SFN can activate the MAPK pathway, causing phosphorylation of Keap1 and release of Nrf2. Once released, Nrf2 enters the nucleus, where it transactivates ARE-responsive genes.

Figure 9. Multiple mechanisms of chemoprotection by SFN. This figure combines empirically tested as well as postulated activities of SFN, based largely on *in vitro* studies, plus *in vivo* findings where available. Each of the protective mechanisms is described in further detail in the text.

Figure 6.

Figure 7.

STAGE OF PROTECTION   PROTECTIVE 'MECHANISM'

Procarcinogen

CYP3A4
CYP1A1
CYP2B1/2
CYP2E1
CYP1A2

1. Inhibition of Phase 1 enzymes
(block metabolic activation)

Carcinogen
(Activated Metabolites)

GST
NQO1
UGT1A1

2. Modulation of Phase 2 enzymes
(induce detoxifying pathways)

DNA Adducts

Phase 2 Conjugations
(Excreted)

Bacterial/viral infection

3. Eradication of infection

Mutation
(Initiated Cell)

Fewer adducts

Inherited mutation

4. Inhibition of growth-promoting
signaling pathways

Pre-neoplastic lesion

NEOPLASIA

5. Alteration of signaling pathways
(cell cycle arrest and apoptosis)

Fewer tumors

Metastasis

Fewer secondary tumors

6. Inhibition of recurrence?

BLOCKING

SUPPRESSION/THERAPY

Figure 8.

Figure 9.

Inhibition of Phase 1 enzymes

Inhibition of HDAC activity

Induction of Phase 2 Enzymes (Keap1-Nrf2 disruption, MAPK activation)

Inhibition of tubulin polymerization

Sulforaphane

Inhibition of inflammation (interference with NFκB)

Activation of Chk2 kinase

Apoptosis (induction of Bax, caspase activity)

Cell cycle arrest (induction of p21)

Inhibition of signal pathways (interference with AP-1)

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

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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/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 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 (41, 170). 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 (41, 171). Natural inhibitors of HDAC, such as trichostatin A (TSA), have received considerable interest as anti-cancer agents due to their ability to induce proteins such as p21$^{Cip1/Waf1}$, leading to cell cycle arrest, differentiation, or apoptosis in neoplastically transformed cells (41, 47, 48, 171, 172). 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 (41).

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 (64, 67, 172). The findings with butyrate have raised an awareness about other HDAC inhibitors which, 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 (173).

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 (116, 142, 144). 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 (174-177). A recent study in the rat reported plasma concentrations of SFN on the order of 20 µM (178). Based on 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 ATCC (Manassas, VA) and cultured in minimum essential media (MEM) (GIBCO-BRL; Grand Island, NY) with 10% horse serum, 1 mM sodium bicarbonate, and 1 mM sodium pyruvate at 37^0C under 5% CO_2. 1×10^6 HEK293 cells were seeded 36-48 h prior to transfection into 60 mm culture dishes coated with 0.2% gelatin. Human HCT116 colorectal cancer cells, also obtained from ATCC, were cultured in McCoy's 5A medium (GIBCO-BRL) with 10% fetal bovine serum and penicillin/streptomycin. HCT116 cells (1.2×10^6) were seeded 24 h prior to transfection into 60 mm culture dishes. Transfections were done at approximately 70% cell confluency.

Transient Transfections. Reporter assays contained pTOPflash (TOPflash), a luciferase construct containing multiple TCF/LEF binding sites (179, 180). TOPflash (0.5 μg/plate) and β-galactosidase (0.1 μg/plate) were co-transfected with human β-catenin (0.5 μg/plate), TCF4 (0.5 μg/plate), and/or HDAC1 (1-3 μg/plate) using 6 or 12 μl TransFast (Promega; Madison, WI) transfection reagent per μg DNA for HEK293 cells and HCT116 cells, respectively. Cells were harvested 48 h post-transfection. In some experiments, the glutathione S-transferase (GST) inhibitor ethacrynic acid (EA, 100 μM) (Sigma; St. Louis, MO) was added, 1 h after transfection. SFN (LKT Laboratories; St. Paul, MI) was added 1 h after transfection, or 2 h after addition of EA. TSA (100 ng/mL) (Biomol; Plymouth Meeting, PA) was added

8 h prior to harvesting. SFN, EA 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 transfections were done in triplicate, and each experiment was repeated on three or more separate occasions.

Reporter Assays. As reported previously (181, 182), the Bright-Glo Luciferase assay (Promega) was used to determine TOPflash activity in cytoplasmic extracts, and standardized to β-galactosidase levels, which were determined using Galacto-Star (Tropix; Applied Biosystems; Foster City, CA). Detection was by an Orion Microplate Luminometer (Berthold). Results were first calculated as relative light units (RLU), and then converted to relative TOPflash activity by assigning an arbitrary value of 1.0 to transfections with TOPflash alone.

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 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% BSA, followed by overnight incubation with primary antibody at 4^0C, and 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; Santa Cruz, CA); acetylated Histone H3, 1:100 (Upstate; Charlottesville, VA); acetylated Histone H4, 1:100 (Upstate), p21, 1:400 (Biosource; Camarillo, CA); PARP, 1:500 (Biosource); 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 Fluor-de-Lys HDAC activity assay kit (Biomol). Incubations were performed at 37^{0}C for 10 min with HeLa nuclear cell extracts (supplied with the kit), and the HDAC reaction was initiated by the addition of Fluor-de-Lys substrate. After 10 min, Fluor-de-Lys Developer was added and incubated for another 10 min at room temperature. In some experiments, cytoplasmic and nuclear extracts (25 μg 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 (ChIP).** HEK293 cells were transfected with 2 μg TOPflash and 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 manufacturers instructions, with anti-acetylated Histone H4 or anti-acetylated Histone H3 antibody and primers to

TOPflash (Forward primer: 5'-CATGTCTGGATCCTCTAGAGTCG-3'; Reverse primer: 5'-AGTCGCGGTTGGAGTAGTAG-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: $94^{\circ}C$ for 30 s, $50^{\circ}C$ for 30 s, and $72^{\circ}C$ for 1 min. HCT116 cells were treated with 9 or 15 μM SFN, 100 ng/ml TSA, or 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'-GGTGTCTAGGTGCTCCAGGT-3'; Reverse primer: 5'-GCACTCTCCAGGAGGACACA-3'). PCR products were detected after 30 cycles with the cycling conditions as follows: $94^{\circ}C$ for 30 s, $56^{\circ}C$ for 30 s, and $72^{\circ}C$ for 30 s, with an additional 10 cycles of $95^{\circ}C$ for 1 min, $56^{\circ}C$ for 1 min, and $72^{\circ}C$ for 1 min.

SFN Metabolites. The metabolites SFN-glutathione (SFN-GSH), SFN-cysteine (SFN-Cys), and SFN-*N*-acetylcysteine (SFN-NAC) were kindly provided by Dr. Clifford Conaway. Each compound was dissolved in DMSO:20 mM potassium phosphate, pH 5.0 (1:1 vol/vol), and 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 SFN-Cys and SFN-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 homolog from *Aquiflex aeolicus* (46). 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: (i) carboxylate binding in a bidentate fashion to the active site zinc, (ii) minimizing steric conflict between substrate and enzyme, based on a fixed protein, (iii) maintaining favorable torsion angles, (iv) having a hydrogen-bond partner for buried polar atoms, and (v) following the favored position of the bound TSA molecule. A solution satisfying these requirements was found for SFN-Cys, but the bulky acetyl group in SFN-NAC did not fit without moving one or more protein atoms within the active site.

Statistical analyses. Pairwise comparisons were made between SFN- or TSA-treated cells and the corresponding vehicle-treated controls, using Students' *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 TOPflash (183). We first verified that HDAC1 acts in a similar manner towards TCF4; in HEK293 cells co-transfected with TCF4, β-catenin and TOPflash, there was a dose-dependent decrease in reporter activity with increasing amount of exogenous HDAC1 in the assay (Fig. 10A). In these experiments, the reporter activity was suppressed to basal levels, equivalent to TOPflash alone, after transient transfection with 3 μg HDAC1 construct. The suppression by 3 μg HDAC1 was reversed in the presence of TSA or SFN, and the combination of SFN plus TSA produced a 5-fold increase in reporter activity compared with controls given no HDAC inhibitor (Fig. 10B). Thus, the order of activity in the reporter assays was SFN+TSA > SFN > 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 TOPflash reporter activities (Fig. 10A,B) and the corresponding HDAC activities; thus, nuclear HDAC activity was increased dose-dependently with HDAC1 in the assay (Figure 10C), and the test inhibitors suppressed HDAC activity in the order SFN+TSA > SFN > TSA (Fig. 10D).

Subsequently, HEK293 cells were treated with different doses of SFN in the absence of exogenous β-catenin and TCF4. There was a concentration-dependent increase in reporter activity with each addition of SFN (3, 9, 15 μM), in the presence and absence of TSA, and the combination of 100 ng/ml TSA plus 15 μM SFN enhanced reporter activity >8-fold compared with TOPflash alone (Fig. 11A). This increase in reporter activity was not due to induction of cytoplasmic or nuclear β-catenin, or changes in nuclear HDAC protein levels, which remained essentially unaffected by SFN and/or TSA under these experimental conditions (Fig. 11B-D). The lack of any change in endogenous protein expression for β-catenin and HDAC1 supports a mechanism involving de-repression of the TOPflash promoter by TSA and SFN.

In the cytoplasmic and nuclear extracts of HEK293 cells treated with the test compounds (Fig. 12A and 3B, respectively), 100 ng/ml TSA and 15 μM SFN inhibited HDAC activity significantly. However, when HeLa nuclear extracts supplied with the HDAC assay kit were incubated with SFN parent compound, no significant inhibitory activity was detected (Fig. 12C). In marked contrast, when HEK293 cells were treated with the corresponding doses of SFN and the cell-free media was added to HeLa nuclear extracts, there was a concentration-dependent decrease in HDAC activity (Fig. 12D). These findings suggested that one or more SFN metabolite(s) might be responsible for the inhibition of HDAC activity in HEK293 cells.

To examine this possibility further, the GST inhibitor EA (184) was used to block the conversion of SFN to its major metabolites. Thus, HEK293 cells were incubated with 100 µM EA shortly after transfection with TOPflash, and prior to the addition of SFN or TSA. No effect was seen with EA alone, but EA blocked the ability of SFN to enhance TOPflash reporter activity (Fig. 13A). In contrast, the TSA-mediated increase in reporter activity was further augmented by EA (Fig. 13A). These results suggested that SFN metabolites, rather than 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 EA augmented the inhibition produced by TSA (Fig. 13B), and the former was confirmed using specific metabolites of SFN (Fig. 13C); whereas the parent compound and SFN-GSH had little or no inhibitory activity, SFN-Cys and SFN-NAC metabolites inhibited HDAC activity in a concentration-dependent manner. At the highest concentration tested (15 µM), 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. 14A and 14B); 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

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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, ChIP 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.145C). 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. 15). As in HEK293 cells, SFN activated TOPflash reporter activity in a concentration-dependent manner (Fig. 15A), this was paralleled by a decrease in nuclear HDAC activity (Fig. 15B), and immunoblots revealed an increase in acetylated histone H3, acetylated histone H4, and p21$^{Cip1/Waf1}$ (Fig. 15C). Finally, TSA and 15 μM SFN each produced an ~4-fold increase in the ChIP assay, using antibody to acetylated histone H4 followed by primers to the promoter of *P21* (Fig. 15D).

DISCUSSION

Billin et al. (183) 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 (116, 142, 144), and is thought to induce Phase 2 detoxification enzymes through the interaction of Nrf-2 with the antioxidant response element (ARE) (135, 136, 185). However, SFN also has a marked effect on cell cycle checkpoint controls and cell survival/apoptosis in various cancer cell lines, through molecular mechanisms that remain poorly understood (121, 149, 150, 153, 154, 158, 159, 186, 187). 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 without 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 ChIP 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 media 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 media 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 (188). In vivo, SFN acts both as a blocking agent and suppressing agent of colonic aberrant crypts in azoxymethane-treated F344 rats, whereas SFN-NAC only was effective *post*-initiation (144). Other studies have shown that the NAC conjugate of isothiocyanates such as phenethyl isothiocyanate, as well as SFN-NAC itself, can modulate growth and

apoptosis of human prostate cancer cells (152, 189). In HepG2-C8 cells (175), SFN and SFN-NAC differed in their ability to induce ARE-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 EA, 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, EA 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 (190). Based on the results with EA, 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. 13C). Molecular modeling studies revealed a plausible interaction for SFN-Cys within the active site of HDAC-like protein (HDLP), with the carboxylate group of SFN-Cys positioned as a bidentate Zn ligand (Fig. 16), similar to that of the hydroxamic acid group of TSA bound to HDLP (46). From the results obtained to date, we hypothesize that HDAC recognizes 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 (116, 121, 142, 144, 149, 150, 152-154, 158, 159, 186, 187, 189).

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 (191, 192). 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. 15). As with other HDAC inhibitors (47, 48, 64, 67, 173, 193-197), such as TSA, suberoylanilide hydroxamic acid (SAHA) and sodium butyrate, SFN markedly increased p21$^{Cip1/Waf1}$ protein expression, and ChIP assays revealed a corresponding increase in acetylated histone H4 bound to the promoter region of *P21*. A search of the *P21* promoter revealed no ARE sites, supporting an Nrf-2-independent mechanism. The physiologically-relevant concentrations of SFN used here had no effect on apoptosis in the attached cell population at 48 h, based on studies of Caspase-3 activation, PARP cleavage, and cell morphology (data not presented). However, there was evidence for a decrease in the overall rate of cell growth, and further studies are in progress on the cell cycle regulation and differentiation of colon cancer

cells treated with SFN and its major metabolites. Collectively, our results suggest that, in addition to its early "blocking" activity, SFN may be effective during later stages, both as a tumor-suppressing agent and as a chemotherapeutic agent, alone or in combination with other HDAC inhibitors currently undergoing clinical trials (19, 41, 171).

ACKNOWLEDMENTS

FIGURE LEGENDS

Fig. 10. Activation of the TOPflash reporter is indicative of HDAC inhibition. A, HEK293 cells were transiently transfected with β-catenin and TCF4, or the corresponding empty vector, together with the TCF/LEF-responsive luciferase reporter construct 'TOPflash,' in the presence of exogenous HDAC1 (the wedge symbol for 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-catenin, TCF4, and 3 µg HDAC1 construct. Cells were treated with TSA in DMSO (100 ng/mL) or DMSO alone, 8 h prior to harvest. Sulforaphane (SFN; 15 µM) was added 47 h prior to 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.

Fig. 11. Sulforaphane increases TOPflash reporter activity without inducing β-catenin or HDAC1 protein levels. A, HEK293 cells were transiently

transfected with TOPflash (no exogenous β-catenin or TCF/LEF) and treated with increasing concentrations of SFN, alone or in combination with TSA (100 ng/mL). TSA and SFN were added 8 and 47 h prior to 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 (B) cytoplasmic or (C) nuclear extracts, as well as (D) HDAC1 in the nuclear extracts. The wedge symbol indicates 3, 9, 15 μM SFN. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by Student's t-test, compared with TOPflash alone.

Fig. 12. Cytoplasmic and nuclear HDAC activity, and the lack of inhibition by SFN parent compound. HEK293 cells were treated with increasing concentrations of SFN, or with TSA (100 ng/mL) as a positive control, and (A) cytoplasmic or (B) nuclear extracts were used in the HDAC activity assay. The wedge symbol indicates 3, 9, 15 μM SFN. C, SFN parent compound was incubated with HeLa nuclear extracts, provided with the HDAC activity assay kit. The wedge symbol indicates 0, 3, 9, 15 μM SFN. D, Media from HEK293 cells treated with 0, 3, 9 or 15 μM SFN was incubated with HeLa nuclear extracts in the HDAC activity assay. Control, no media added (open bar). Results indicate mean±SD, $n=3$, and are representative of the findings from

three or more separate experiments. * p<0.05 by Student's *t*-test, compared with the vehicle control.

Fig. 13. Ethacrynic acid attenuates the effects of SFN in TOPflash reporter and HDAC activity assays; metabolites of sulforaphane directly inhibit HDAC activity. *A*, HEK293 cells were transfected with TOPflash and then treated with 100 µM ethacrynic acid (EA) prior to SFN (15 µM) or TSA (100 ng/mL) exposure. *B*, Media from HEK293 cells in (*A*) was incubated with HeLa nuclear extracts in the HDAC activity assay. Control, no media added (open bar). *C*, SFN metabolites were incubated with HeLa nuclear extracts and assayed for HDAC activity. The wedge symbol indicates 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. SFN-GSH, glutathione conjugate; SFN-Cys, cysteine conjugate; SFN-NAC, *N*-acetylcysteine conjugate. * p<0.05, ** p<0.01, by Student's *t*-test.

Fig. 14. Sulforaphane increases global and localized histone acetylation. HEK293 cells were treated with SFN and/or TSA, or with sodium butyrate as a control, and (*A*) acetylated histone H3 or (*B*) acetylated histone H4 was assessed by immunoblotting. Relative densitometry values for each lane are shown above the corresponding blots. *C*, HEK293 cells were transiently

transfected with 2 µg TOPflash and treated with 9 µM SFN or vehicle (–SFN). DNA was cross-linked to proteins prior to harvesting, chromatin immunoprecipitation (ChIP) was performed against acetylated histone H3 or acetylated histone H4, and following DNA isolation and reversal of cross-linking, primers specific for TOPflash were used during PCR amplification. Results were first normalized against the corresponding input controls, and then expressed relative to TOPflash alone (–SFN), which was assigned an arbitrary value of 1.0.

Fig. 15. Sulforaphane 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 (*A*) TOPflash reporter activity and (*B*) nuclear HDAC activity, as described for HEK293 cells (Fig. 2*A* and Fig. 3*B*, respectively). Results (mean±SD, *n*=3) are representative of the findings from two separate experiments. The wedge symbol indicates 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*, 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 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.

Fig. 16. Modeling of SFN-Cys in an HDAC active site. The TSA molecule and Zn atom are shown as they are observed to interact in the crystal structure of the HDAC homolog from *Aquiflex aeolicus* (Protein Data Bank entry 1C3R; (46)). Overlaid is SFN-Cys, showing an assumed bidentate interaction of the carboxylate group with the Zn-atom; the rest of SFN-Cys can be modeled to fill roughly the same space as does bound TSA, with no steric clashes with the surrounding protein. As modeled, the buried Cys α-amino group is positioned to make a single hydrogen bond with His-132. Carbon atoms are shown in gray, oxygen in red, nitrogen in blue, sulfur in yellow, and Zn in green. Dotted lines indicate the modeled carboxylate-Zn bonds. Figure created using Molscript and Raster3D.

Figure 10.

Figure 11.

85

Figure 12.

Figure 13.

A

B

C

Figure 14.

88

Figure 15.

A HCT116 cells

B

SFN

C
Acetylated H3
Acetylated H4
p21^Cip1/Waf1

0 μM   15 μM SFN

D
P21 promoter
Input controls

H4

SFN   TSA
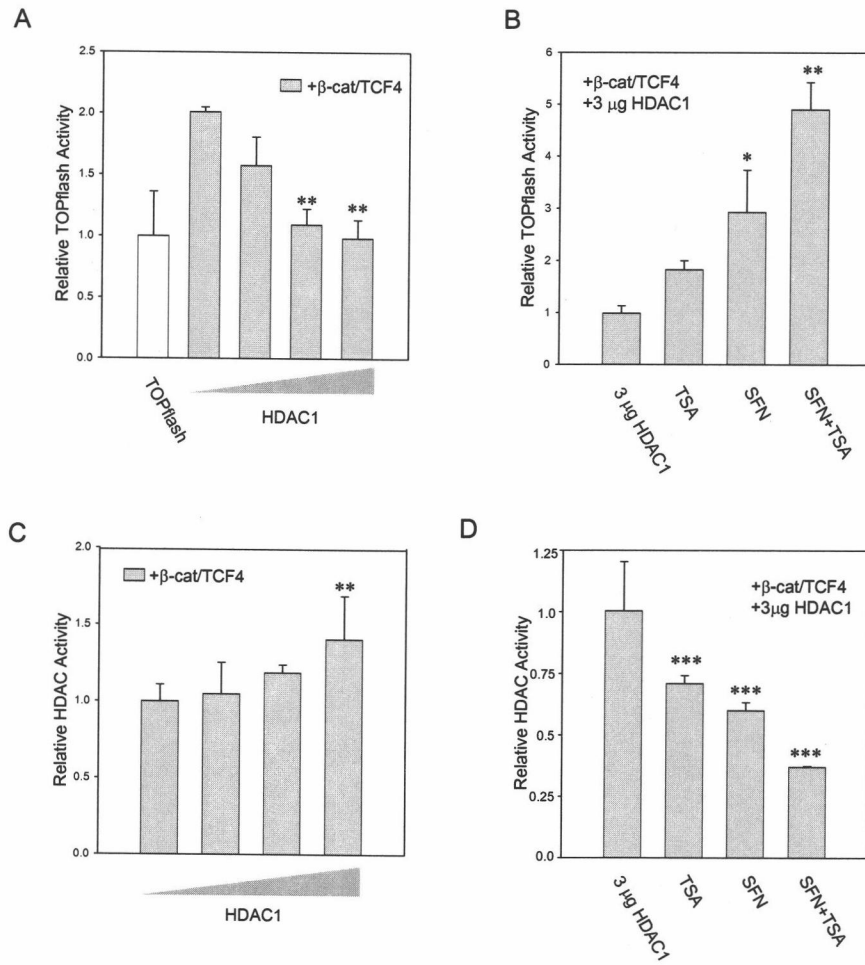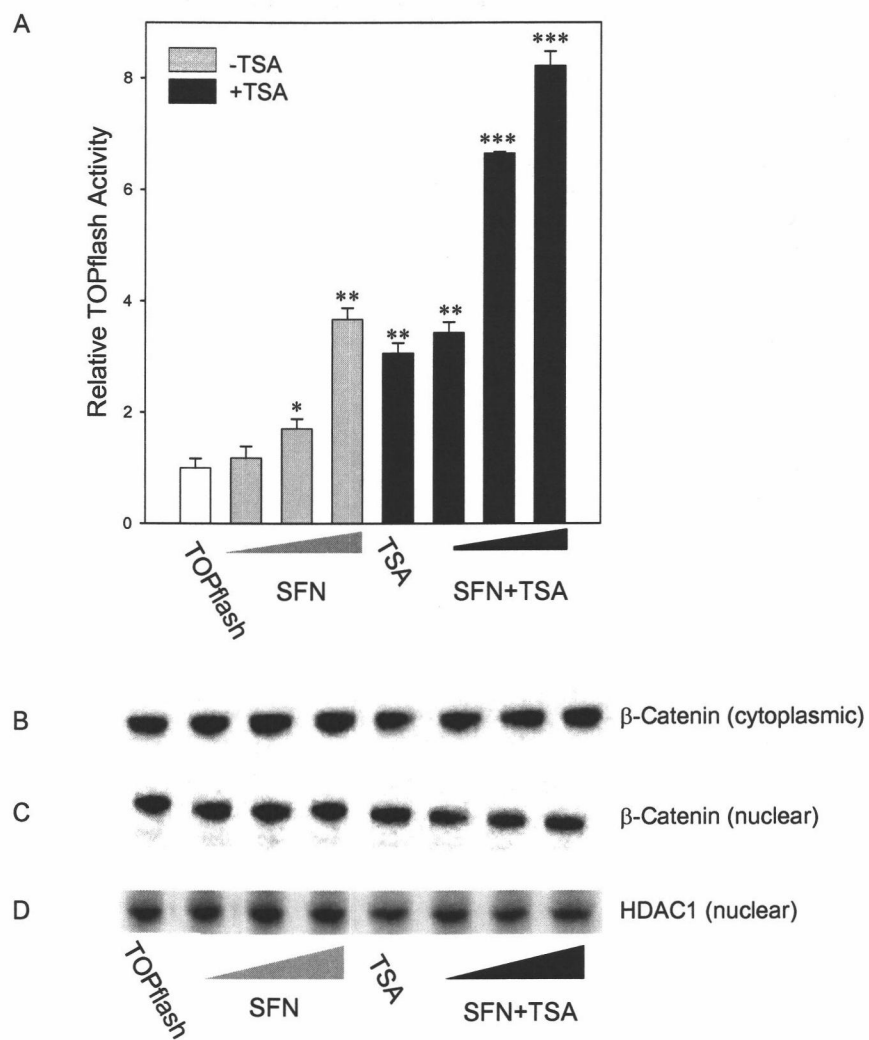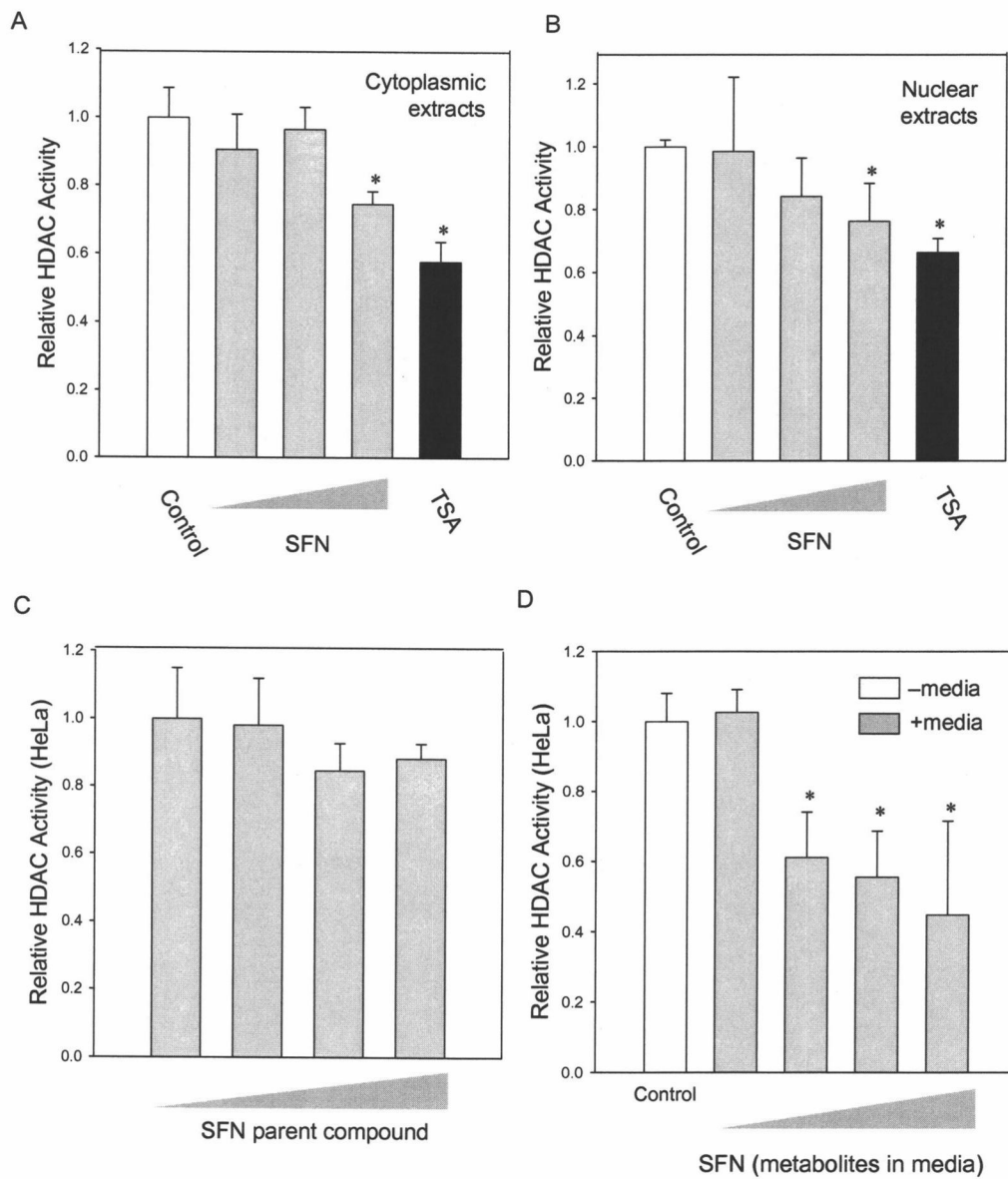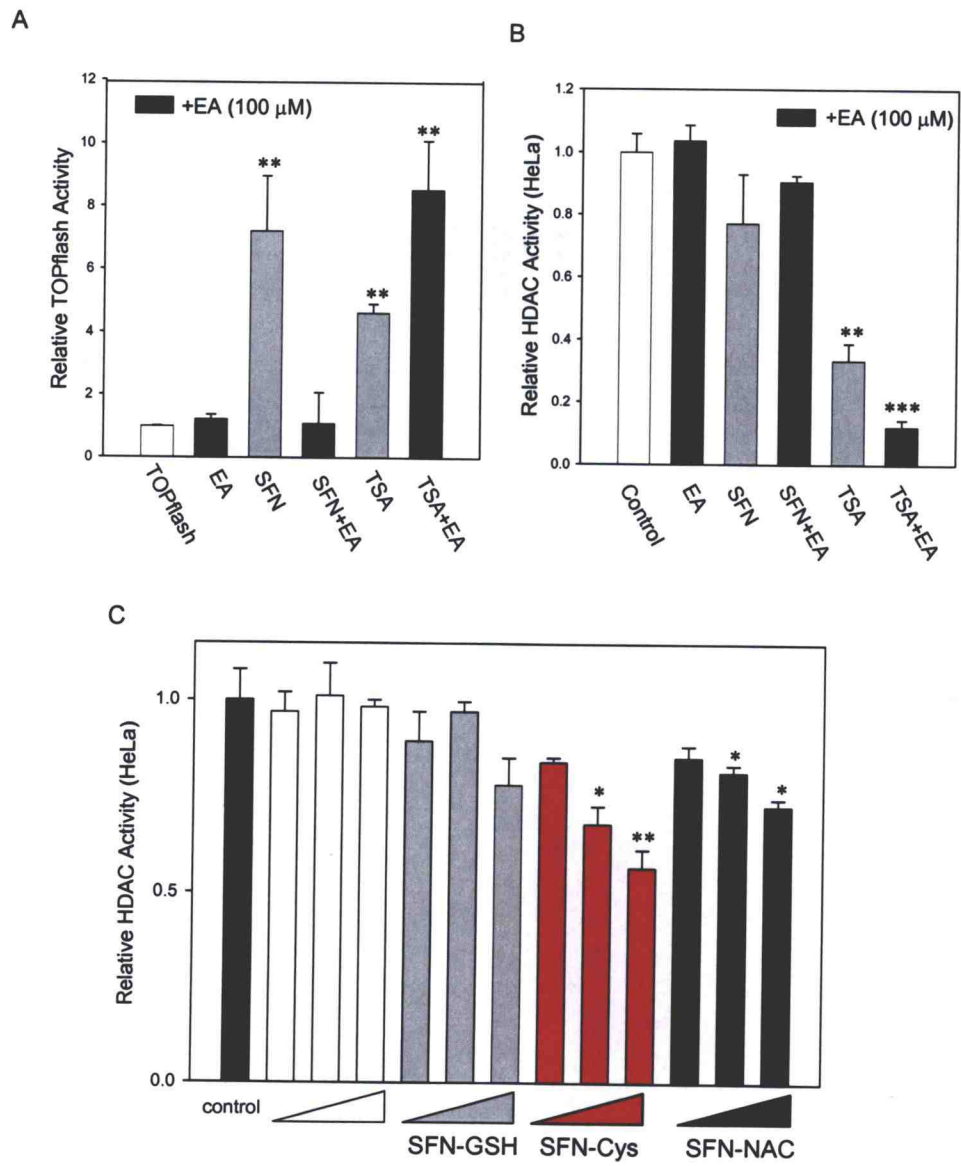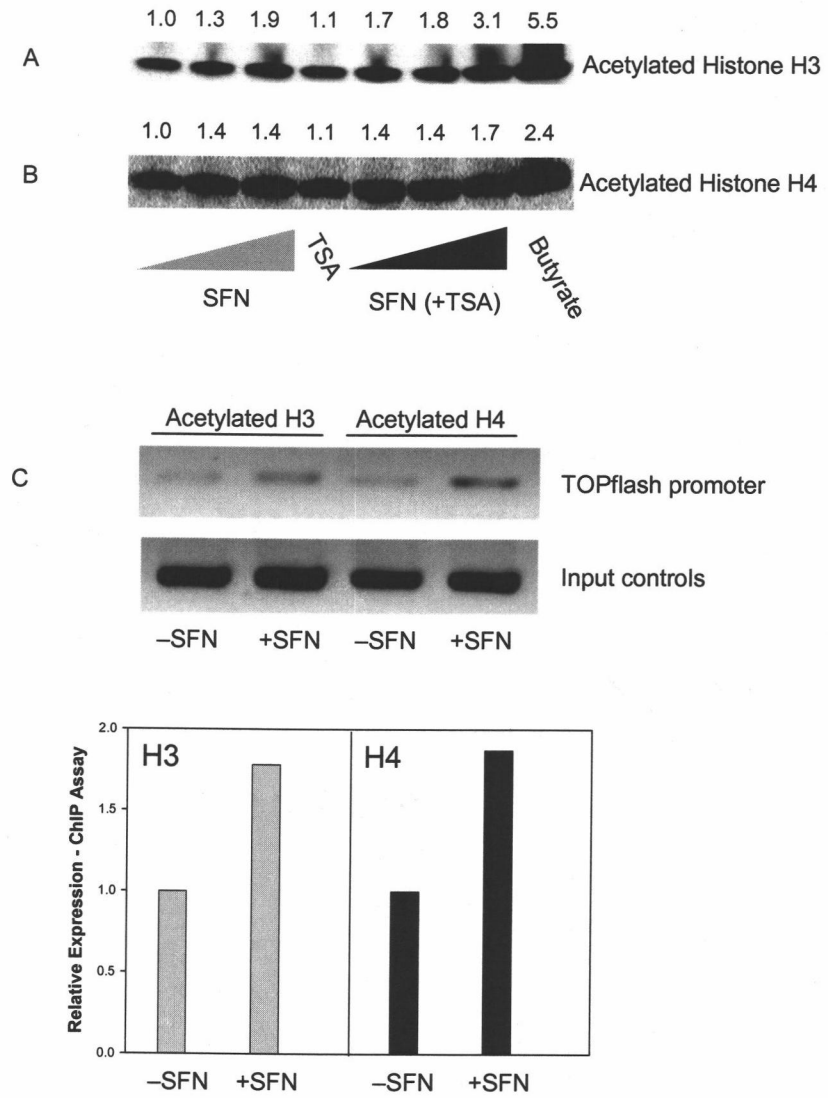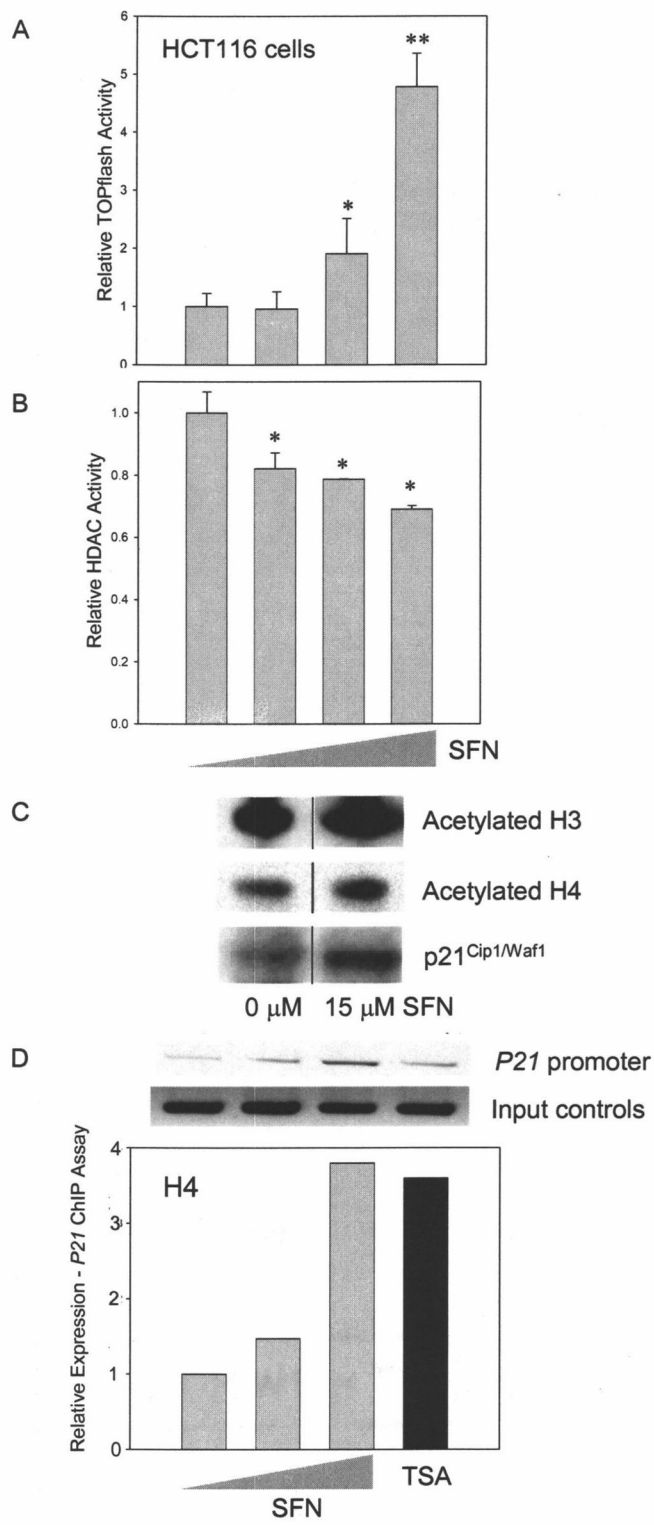
Figure 16.

Sulforaphane Inhibits Histone Deacetylase Activity in BPH-1, LnCaP, and PC-3 Prostate Epithelial Cells

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Abstract

Sulforaphane (SFN), an isothiocyanate first isolated from broccoli, exhibits chemopreventive properties in prostate cancer cells through mechanisms that are poorly understood. We recently reported on a novel mechanism of chemoprotection by SFN in human colon cancer cells, namely the inhibition of histone deacetylase (HDAC) (198). Here, we report that physiologically relevant concentrations of SFN (3-15 μM) also significantly inhibited HDAC activity in BPH-1, LnCaP, and PC-3 prostate epithelial cells. The inhibition of HDAC was accompanied by a global increase in acetylated histones in all three prostate cell lines, and in BPH-1 cells treated with SFN there was enhanced interaction of acetylated histone H4 with the promoter region of the *P21* gene and the *bax* gene. A corresponding increase was seen for p21[Cip1/Waf1] and Bax protein expression, consistent with previous studies using HDAC inhibitors such as trichostatin A. The downstream events included cell cycle arrest and activation of apoptosis, as evidenced by changes in cell cycle kinetics and induction of multi-caspase activity. These findings provide new insight into the mechanisms of SFN action in benign prostate hyperplasia, androgen-dependent prostate cancer, and androgen-independent prostate cancer cells, and they suggest a novel approach to chemoprotection and chemotherapy of prostate cancer through the inhibition of HDAC.

INTRODUCTION

Prostate cancer is the second leading cause of cancer-related death in men. With over 230,000 men estimated to be diagnosed with prostate cancer in 2004, preventive measures that target the various steps involved in cancer initiation and progression could significantly decrease the incidence and mortality of prostate cancer. Epidemiological studies suggest that cruciferous vegetable intake may lower overall risk of prostate cancer, particularly during the early stages (161-163, 199), and there is growing interest in identifying the specific chemoprotective constituents and their mechanisms of action.

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables and is especially high in broccoli and broccoli sprouts (109). SFN is an effective chemoprotective agent in carcinogen-induced animal models (116, 142, 144), as well as in xenograft models of prostate cancer (154). Recent work has clearly implicated multiple mechanisms of SFN action, although the majority of studies have focused on SFN as a potent Phase-2 enzyme inducer and effects on cell cycle arrest and apoptosis (121, 149, 150, 152-154, 158, 159, 186, 187).

We reported on a novel mechanism of chemoprotection by SFN in human colon cancer cells, namely the inhibition of histone deacetylase (HDAC) (198). SFN was shown to (i) inhibit nuclear HDAC activity, (ii) augment the levels of acetylated histones, (iii) increase acetylated histone H4

interactions with the *P21* promoter, and (iv) elevate the expression of p21$^{Cip1/Waf1}$ in HCT116 cells.

Several clinical trials are currently ongoing aimed at establishing the chemotherapeutic efficacy of HDAC inhibitors, based on evidence that cancer cells undergo cell cycle arrest, differentiation and apoptosis *in vitro*, and that tumor volume and/or tumor number may be reduced in animal models (19, 41, 171).

Inhibitors of HDAC have generated considerable recent interest as novel chemoprotective agents because they target epigenetic events that can occur at various stages of cancer development (14, 45). Moreover, since epigenetic mechanisms exist in a wide array of target tissues, HDAC inhibitors have the potential for broad applicability. In the case of prostate cancer, for example, it has been shown that HDAC activity increases in metastatic cells compared with prostate hyperplasia (33) and over-expression of HDAC1 in PC-3 cells results in an increase in cell proliferation and an overall decrease in cell differentiation (200). This may be of particular importance in the progression to androgen independence. Importantly, inhibitors of HDAC, including suberoylanilide hydroxamic acid (SAHA), valproic acid, depsipeptide, and sodium butyrate have been demonstrated to be effective against prostate cell lines and xenograft models (201-203).

Based on the studies with HDAC inhibitors in prostate cancer cell lines and our recent work in human colon cancer cells, we sought to test the broad

applicability of HDAC inhibition by SFN using various prostate cell lines, including consideration of the downstream consequences of HDAC inhibition on apoptosis and cell cycle arrest. We report here, for the first time, that physiologically relevant concentrations of SFN inhibit HDAC activity in BPH-1, LnCaP, and PC-3 cells, and provide support for SFN as an effective chemopreventive agent for prostate hyperplasia and/or cancer.

MATERIALS AND METHODS

Cell Culture: Human benign prostate hyperplasia epithelial cells (BPH-1), androgen-independent prostate cancer epithelial cells (PC-3), and androgen-dependent prostate cancer epithelial cells (LNCaP) were obtained from American Type Tissue Collection (Manassas, VA). Cells were grown and maintained in RPMI 1640 with glutamine plus 10% FBS, and treated with test agents at approximately 50-70% confluency. Unless stated otherwise, cells were harvested 48 h after treatment with 3–15 μM D,L-SFN (LKT Labs, St. Louis, MN), or 8 h after treatment with 100 ng/ml trichostatin A (TSA, Biomol, Plymouth Meeting, PA). The final concentration of vehicle, DMSO, did not exceed 0.1%. Dose and treatment times for TSA and SFN were identical to our recent study (1), and based on a prior investigation by Billin *et al.* using TSA (183), and another by Hu *et al.* (139) which reported plasma concentrations for SFN in the rat on the order of 20 μM. Pilot experiments

identified the peak of TSA inhibitory activity to occur 8 h after treatment (Myzak and Dashwood, unpublished results).

Western Blotting: Protein concentrations were determined by Lowry assay from attached cell populations. Proteins (10-20 mg) 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 with Amido Black staining and β-actin levels. The membrane was blocked for 1 h with 2% bovine serum albumin, followed by either overnight incubation with primary antibody at 4°C or 1 h incubation with primary antibody at room temperature, and was finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Antibody dilutions were as follows: acetylated histone H3, 1:100 (Upstate, Charlottesville, VA); acetylated histone H4, 1:100 (Upstate); p21, 1.5 mg/ml (Neomarkers, Fremont, CA); HDAC1, 1:100 (Santa Cruz Biotechnologies, Santa Cruz, CA); poly(ADP-ribose) polymerase, 1:500 (Biosource, Camarillo, CA); Bax, 1:500 (BD Pharmingen, San Diego, CA), Bcl-2 (Santa Cruz Biotechnologies, Santa Cruz, CA), p53 (Santa Cruz Biotechnologies) and, β-actin, 1:5000 (Sigma, St. Louis, MO). Detection was by Western Lightning Chemiluminescence Reagent Plus (PE Life Sciences, Boston, MA) with image analysis on an AlphaInnotech photodocumentation system.

HDAC Activity Assay: The Fluor-de-Lys HDAC activity assay kit (Biomol) was used, as reported previously (198). Attached cell lysates (15 mg total protein) from cells treated with SFN or TSA were incubated with Fluor-de-Lys substrate for 10 min at 37°C to initiate the HDAC reaction. Fluor-de-Lys Developer was then added, and the mixture was incubated for another 10 min at room temperature. Fluorescence was measured using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices), with excitation 360 nm and emission 460 nm.

Chromatin Immunoprecipitation (ChIP): BPH-1 cells were treated with SFN as above. Formaldehyde (Sigma) was added directly to media to a final concentration of 1% and incubated for 10 min at 37°C. Attached cell lysates were sonicated eight times for 15 s using a Heat Systems-Ultrasonics Sonicator (Model W-225R) on setting 5. A Chromatin Immunoprecipitation kit (Upstate) was used according to the manufacturer's instructions, with anti-acetylated histone H4 antibody. Primers and PCR conditions used for amplification of the *P21* promoter were exactly as reported previously (198). Primers and PCR conditions used for amplification of the *bax* promoter were as follows: F: 5'-TAATCTCAGCACTTTGGGAGG; R: 5'-GACAGGGTCTCACTGTGTTGC. PCR products were detected after 30

cycles of the following cycling conditions: 94^0 C for 30 s, 52^0 C for 30 s, and 72^0 C for 30 s.

Real time PCR: Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA) from BPH-1 cells 12 h after treatment with 0 or 15 µM SFN, and first-strand cDNA was prepared from 4 µg total RNA using an Omniscript Reverse Transcriptase Kit (Qiagen). Levels of *bax* mRNA were quantified by real time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), using the following primers: *bax* forward primer 5' TGC TTC AGG GTT TCA TCC AG 3' and reverse primer 5' GGC GGC AAT CAT CCT CTG 3'; *GAPDH* forward primer 5' GAA GGT GAA GGT CGG AGT C 3' and reverse primer 5'GAA GAT GGT GAT GGG ATT TC 3'. PCR was conducted over 40 cycles (95°C/10 s, 56°C/20 s, 72°C/20 s) using an Opticon Monitor 2 system (Finnzymes, Finland), in 50 µl containing cDNA, SYBR Green I dye (DyNAmo master solution, Finnzymes, Finland), and the corresponding primers.

Multi-caspase Activity: Multicaspase activity was assessed using a flow-cytometry based Multicaspase assay kit (Guava Technologies, Hayward, CA,). Cells were trypsinized, washed in D-PBS and stained with a fluorochrome-conjugated caspase inhibitor, sulforhodamine-valyl-alanyl-aspartyl-fluoromethylketone (SR-VAD-FMK) according to the manufacturer's

instructions. This inhibitor readily crosses cell membranes and covalently binds to the active forms of multiple caspases, which are cleaved from inactive precursors (procaspases) during apoptosis induction. Subsequently, cells were washed 3 times and stained with 7-AAD (7-amino-actinomycin-D) for 15 min prior to analysis on a Guava Personal Cell Analyzer (Guava Technologies).

Cell Cycle Analysis: A flow cytometric assay also was performed to assess effects of SFN on cell cycle. BPH-1 and PC-3 cells were treated with SFN and cells (attached and floating) were harvested by trypsinization at 24 or 48 h post-treatment. One million cells were fixed by slow, dropwise addition of 2 ml cold 70% ethanol, followed by storage at $4^{\circ}C$ for 24 hours. After fixation, cells were washed, pelleted, and resuspended in 0.04 mg/ml propidium iodide and 100 mg/ml RNase in PBS. The sample was incubated at room temperature for 30 min and analyzed on the Guava PCA. Multi-Cycle analysis software (Phoenix Flow Systems, San Diego, CA) was used to generate histograms and determine number of cells in each phase of the cell cycle.

Statistics: One-way analysis of variance (ANOVA) was performed to assess the differences between groups. Differences in means among treatments

were tested by Dunnett's test, and the level of significance was designated as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

RESULTS

We recently identified SFN as an HDAC inhibitor in human colorectal cancer cells (198), and the present study sought to verify that SFN also acts as an HDAC inhibitor in benign prostate hyperplasia and prostate cancer cells. HDAC activity was inhibited by 40%, 30% and 50% in BPH-1, LnCaP and PC-3 cell lysates compared with the corresponding controls (Fig. 17) at the physiologically relevant concentration of 15 μM SFN. Under the present assay conditions, the positive control TSA lowered HDAC activity by 30% in BPH-1 cells, but had no significant effect in LnCaP or PC-3 cells.

Immunoblotting confirmed that SFN increased the expression of acetylated histone H3 and acetylated H4 in all three prostate cell lines, compared with untreated controls (Fig. 18). An increase in acetylated histones also was detected in cells treated with TSA, most notably in PC-3 cells (Fig. 18C), despite the apparent lack of HDAC inhibition by TSA in these cells (Fig. 17).

Because the SFN-mediated increase in acetylated histones H3 and H4 was most striking in BPH-1 cells (Fig. 18), these cells were examined for changes in the acetylation status of histone H4 associated with the promoter region of the *P21* gene. Using anti-acetylated histone H4 antibody followed by PCR with primers specific for the *P21* promoter, ChIP assays showed a concentration-dependent increase in the amount of acetylated histone H4 associated with the *P21* promoter (Fig. 19*A*). Expression of the p21 protein, a

well known target of HDAC inhibitors (47, 48, 64, 67, 173, 193-197), was increased by SFN in all three prostate cell lines, compared with untreated controls (Fig. 19*B*).

To determine the downstream effects of HDAC inhibition by SFN, pro- and anti-apoptotic protein expression was examined by immunoblotting. An increase in the pro-apoptotic protein Bax was detected in all three prostate cell lines (Fig. 20*A-C*), and SFN also caused an increase in p53 protein levels in BPH-1 and LnCaP cells, compared with controls (Fig. 20*A,B*). As expected, p53 was not detected in p53-null PC-3 cells (Fig. 20*C*). The increased expression of pro-apoptotic proteins was coupled with a decrease in the anti-apoptotic protein Bcl-2 in SFN-treated BPH-1 and PC-3 cells (Fig. 20*A,C*), but not in LnCaP cells (Fig. 20*B*), which express low levels of Bcl-2 protein. Changes in Bcl-2 family proteins have been described with other HDAC inhibitors (reviewed in (13)), but a direct association between the histone acetylation status of a specific apoptosis-related gene and its expression has not been reported. Thus, we performed ChIP as described above for *P21*, but used primers specific for the *bax* promoter; there was a marked increase in acetylated histone H4 associated with the *bax* promoter (Fig. 20*D*), and a significant increase in *bax* mRNA expression, 12 h after SFN treatment (Fig. 20*E*).

Changes induced by SFN in Bcl-2 and Bax expression were associated with an increase in multi-caspase activity (Fig. 21). Cells stained

positive in this assay contain one or more active (cleaved) caspases, which is indicative of apoptosis. Treatment of BPH-1 cells with 15 μM SFN resulted in a significant increase in multi-caspase activity at 24 h, and a concentration dependent increase was seen for SFN in these cells at 48 h (Fig. 21A,B). In PC-3 cells, an increase in multi-caspase activity also was observed 24 and 48 h after treatment with SFN (Fig. 21 C,D). Interestingly, 15 μM SFN induced caspase activity at 48 h but not 24 h, whereas the two lower concentrations of 3 and 9 μM SFN were effective at both time points. One interpretation is that 15 μM SFN activated additional mechanisms that delayed the onset of apoptosis in PC-3 cells.

The increase in multi-caspase activity induced by SFN in BPH-1 cells (Fig. 21A,B) was paralleled by an increase in the number of floating cells, and a concomitant decrease in the attached cell population (Fig. 22A,B). In PC-3 cells, a concentration dependent increase in floating cells was observed (Fig. 22C), coupled with a concentration dependent decrease in the attached cell population (Fig. 22D). Under the microscope, the floating cells had morphological hallmarks clearly distinct from the attached cells, consistent with the cells detaching and undergoing apoptosis.

The induction of apoptosis, and the detachment of cells, is frequently preceded by changes in cell cycle kinetics (204). In BPH-1 cells there was a significant loss of cells in the G1 phase of the cell cycle and an increase in G2/M cells, 24 h after SFN treatment (Table 1). In the comparison of 0

versus 15 μM SFN, the proportion of BPH-1 cells in G2/M increased from 15.31% to 65.34%, implying the presence of a G2/M arrest. Interestingly, no such changes were detected in PC-3 cells at 24 h (data not shown), but at 48 h there was a concentration-dependent decrease in G1 and an increase in G2/M indicating a G2/M arrest (Table 5.1).

DISCUSSION

We recently provided the first evidence for SFN as an HDAC inhibitor in human embryonic kidney 293 cells and HCT116 human colorectal cancer cells (198). The present investigation has confirmed that SFN acts in a similar manner in human prostate cell lines, and we extend these findings by showing that the possible downstream consequences of HDAC inhibition in BPH-1, LnCaP, and PC-3 cells include cell cycle arrest and apoptosis. Previous reports described cell cycle arrest in LnCaP, PC-3, and other prostate cell lines treated with SFN (152-154), but not in the context of HDAC inhibition, and to our knowledge this is the first study with SFN in BPH-1 cells.

In vivo studies have established that SFN is an effective cancer chemopreventive agent in several animal models (116, 142, 144), and it is thought to induce Phase 2 detoxification enzymes through interaction of Nrf-2 with the antioxidant response element (ARE) (135, 136, 185). However, SFN also has a marked effect on cell cycle checkpoint controls and cell survival/apoptosis in various cancer cell lines, through molecular mechanisms that remain poorly understood (121, 149, 150, 152-154, 158, 159, 186, 187). The present investigation provides possible insight into this question by showing, for the first time, that SFN is an inhibitor of HDAC activity in prostate epithelial cells.

In all three prostate cell lines, HDAC inhibition by SFN occurred at physiologically relevant concentrations, which are on the order of ~20 μM

(178). Importantly, immunoblotting for HDAC1 revealed no loss in HDAC protein expression (data not presented), which recapitulated previous findings with SFN in colon cancer cells (198). It is noteworthy that androgen-independent PC-3 cells responded particularly well to SFN, with significant HDAC inhibition seen at concentrations as low as 3 µM SFN (data not presented). This may be attributed to the fact that these cells generally have higher HDAC activity compared with normal prostate epithelial cells (33).

As with other HDAC inhibitors (47, 48, 64, 67, 173, 193-197), such as TSA, suberoylanilide hydroxamic acid (SAHA) and sodium butyrate, SFN increased p21 protein expression in prostate cells, and ChIP assays confirmed an increase in the expression of acetylated histone H4 associated with the *P21* promoter. The *P21* promoter lacks an ARE, supporting an Nrf-2-independent mechanism for the induction of p21 by SFN. Importantly, 15 µM SFN also increased acetylated histone H4 association with the *bax* promoter and augmented *bax* mRNA expression (Fig. 20*D,E*), indicating that SFN might directly mediate Bax induction through chromatin remodeling. Further studies are in progress to determine whether SFN-induced changes in Bcl-2, Bax and multi-caspase activity necessarily result from HDAC inhibition rather than other mechanisms, such as modulation of cell cycle control proteins, tubulin polymerization, and MAPK (reviewed in (205)). It is noteworthy that PC-3 cells, which lack p53 (see Fig. 20*C*), had strong induction of p21 (Fig. 19*B*), supporting a p53-independent mechanism for SFN in these cells.

Apoptosis induction and cell cycle alterations have been reported for HDAC inhibitors (19, 41, 171), including dietary agents (206). In previous studies with SFN by others, a G2/M arrest was noted in PC-3 cells (110), whereas a G1 arrest was observed in DU-145 and LnCaP cells (152, 153). Similarly, we demonstrated that SFN treatment in BPH-1 and PC-3 cells induced pro-apoptotic markers (Figure 4-6) and a G2/M cell cycle arrest (Table 5.1). Further mechanistic studies are required, especially in the context of other known targets of HDAC inhibitors, such as the cyclins (13). Nonetheless, one important implication of these findings is that both benign prostate hyperplasia and androgen-independent prostate cancer might respond favorably to SFN treatment, via the induction of cell cycle arrest and apoptosis.

Previously, we identified SFN-Cys, a metabolite of SFN, as the likely HDAC inhibitor in colon cancer cells (198). SFN is metabolized via the mercapturic acid pathway, with the initial step involving glutathione (GSH) conjugation catalyzed by glutathione-S-transferases (GST) (115, 188, 207, 208). In prostate cancer, GSTP1 is frequently turned off by epigenetic modifications through methylation of its promoter (209, 210). According to a recent study, CpG island hypermethylation in the promoter region of GSTP1 was observed in LnCaP and PC-3 cell lines (209). Although GSTP1 is epigenetically silenced in LnCaP and PC-3 cell lines (the status is unknown in BPH-1), SFN nonetheless exhibited significant HDAC inhibitory activity (Fig.

17). It has been demonstrated that conjugation of SFN to GSH can occur as a facile reaction, without catalysis by GST (188), and it may be that slower conjugation enhances HDAC inhibition by acting as a "time-release" mechanism, such that the SFN-Cys metabolite is available for a more sustained period. To explore this hypothesis, future studies should examine the kinetics of SFN-Cys generation and turnover in cell lines with altered GST expression.

In summary, the results presented here suggest that SFN may be effective against benign prostate hyperplasia, androgen-dependent prostate cancer, and androgen-independent prostate cancer through the inhibition of HDAC activity. The present work focused on p21 and Bax, which are well established targets of HDAC inhibitors and key regulators of cell cycle kinetics and apoptosis, but additional targets of HDAC inhibition warrant further investigation. Collectively, the results suggest that, through modulation of HDAC-regulated gene expression, SFN might be useful both as a chemopreventive agent as well as a chemotherapeutic agent in the prostate.

Acknowledgements

Table 5.1.

Cell Line	[SFN] (μM)	% cells in phase		
		G1	S	G2/M
BPH-1	0	57.82 ± 3.94	26.86 ± 1.10	15.31 ± 0.98
	3	57.82 ± 0.94	28.11 ± 1.11	14.06 ± 1.70
	9	65.55 ± 2.95**	19.63 ± 1.72	14.97 ± 1.52
	15	19.02 ± 4.72**	15.75 ± 6.08**	65.24 ± 6.47**
PC-3	0	54.37 ± 2.25	25.85 ± 1.93	28.27 ± 1.60
	3	51.69 ± 2.16	22.82 ± 2.26	24.79 ± 2.37
	9	44.69 ± 1.72*	29.28 ± 0.65	26.02 ± 2.37
	15	25.46 ± 1.04**	26.16 ± 3.36	48.37 ± 2.33**

LEGENDS

Fig. 17. SFN inhibits HDAC activity in prostate epithelial cells. BPH-1 (*A*), LnCaP (*B*) and PC-3 cells (*C*) were harvested 48 h after treatment with SFN (15 μM), or 8 h after TSA exposure (100 ng/ml), and cell lysates were analyzed for HDAC activity, as reported (85). Results = mean±SD, *n*=3. *$P<0.05$, **$P<0.01$.

Fig. 18. SFN increases acetylated histone levels in prostate cell lines. Cells were treated with SFN or TSA as described in the legend to Fig.1. and acetylated histone H3 or acetylated histone H4 levels were assessed by immunoblotting. Equal protein loading was confirmed using β-actin. Results are representative of two or more separate experiments.

Fig. 19. SFN increases p21 expression in prostate cells. *A*, BPH-1 cells were treated with SFN and 48 h later DNA was cross-linked to proteins, chromatin immunoprecipitation (ChIP) was performed using acetylated histone H4, and following reversal of cross-linking and isolation of DNA, PCR was performed with primers to the *P21* promoter. *Wedge symbol*, 3, 9, 15 μM SFN; *control*, 0 μM SFN (vehicle alone). *B*, Cells were treated with 0 or 15 μM SFN and 48 h later the attached cells were isolated and cell lysates were

immunoblotted for p21 protein. Equal protein loading was confirmed using β-actin. Results are representative of two or more separate experiments.

Fig. 20.   SFN alters the expression of pro- and anti-apoptotic proteins. Attached cells were harvested 48 h after treatment of BPH-1 (*A*), LnCaP (*B*), and PC-3 cells (*C*) with 0 or 15 μM SFN, and cell lysates were immunoblotted for Bcl-2, Bax and p53, as indicated.   Equal protein loading was confirmed using β-actin.   Results are representative of two or more separate experiments.   *D*, BPH-1 cells were treated with 0 or 15 μM SFN and ChIP was performed as described in the legend to Fig. 3, except that PCR was performed with primers to the *bax* promoter. *E*, real time PCR results for *bax* mRNA expression in BPH-1 cells 12 h after treatment with 0 or 15 μM SFN; mean±SD, *n*=3; *P<0.05.

Fig. 21.   SFN increases multi-caspase activity in prostate epithelial cells. BPH-1 cells (*A,B*) and PC-3 cells (*C,D*) were harvested 24 h or 48 h after treatment with 0, 3, 9, or 15 μM SFN, as indicated, and the attached cells were examined for multi-caspase activity using a Guava Personal Cell Analyzer (PCA).   Results represent mean±SD, *n*=3, from experiments conducted on two or more separate occasions.   *P<0.05, ** P<0.01, ***P<0.001.

Fig. 22. SFN increases the proportion of prostate cells in the floating population. BPH-1 (*A,B*) and PC-3 cells (*C,D*) were treated with 0, 3, 9, or 15 μM SFN and 48 h later the floating and attached cells were counted. Results are given as mean±SD, *n*=3. * *P*<0.05, ** *P*<0.01, ***P*<0.001.

Table 5.1. SFN alters cell cycle distribution in BPH-1 and PC-3 cells.
Cells were treated with 0, 3, 9, or 15 µM SFN, as indicated, and harvested
after 24 h (BPH-1) or 48 h (PC-3). Attached and floating cells were fixed in
70% ethanol and stained with propidium iodide, and cell cycle kinetics were
examined using the Guava PCA, followed by data analysis with Multi-Cycle
software. Results indicate mean±SD, n=3. * P<0.05, ** P<0.01.
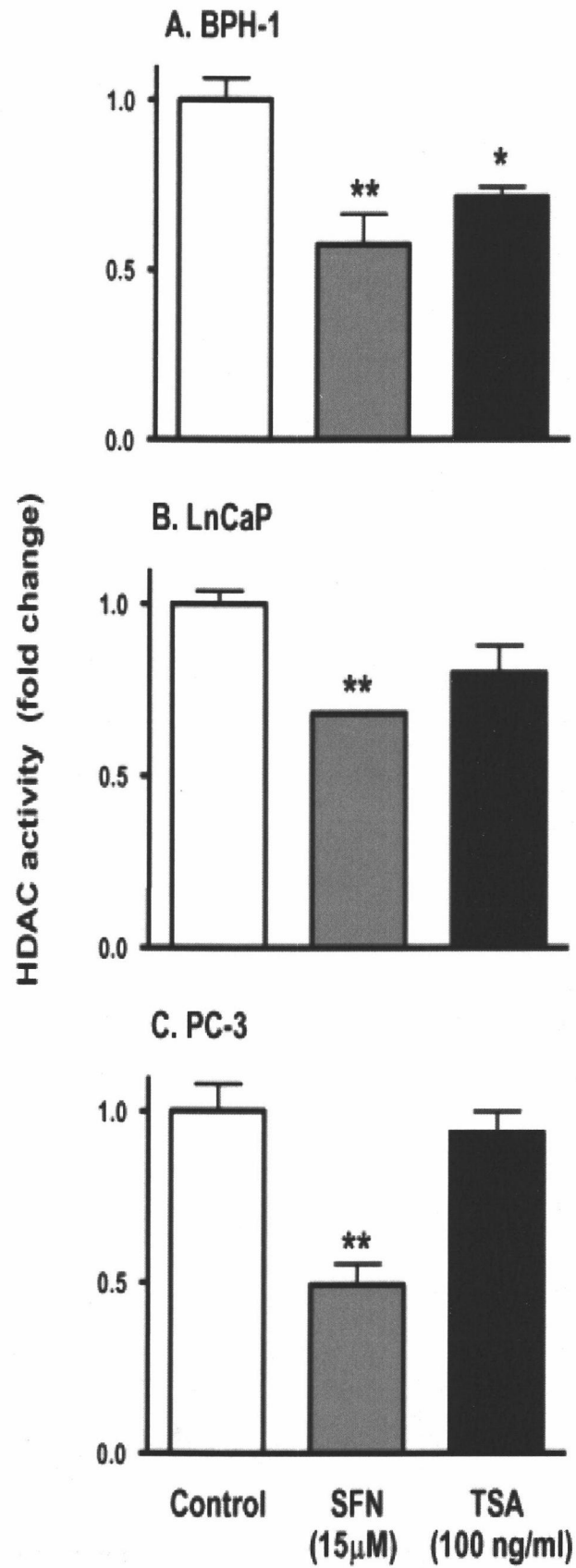
114

Figure 17.

Figure 18.
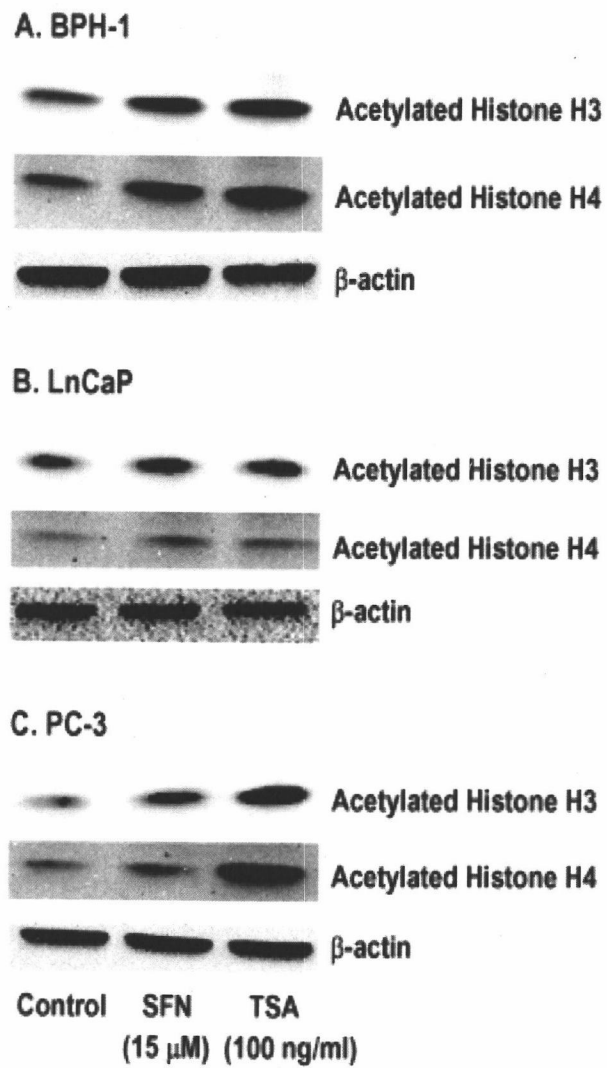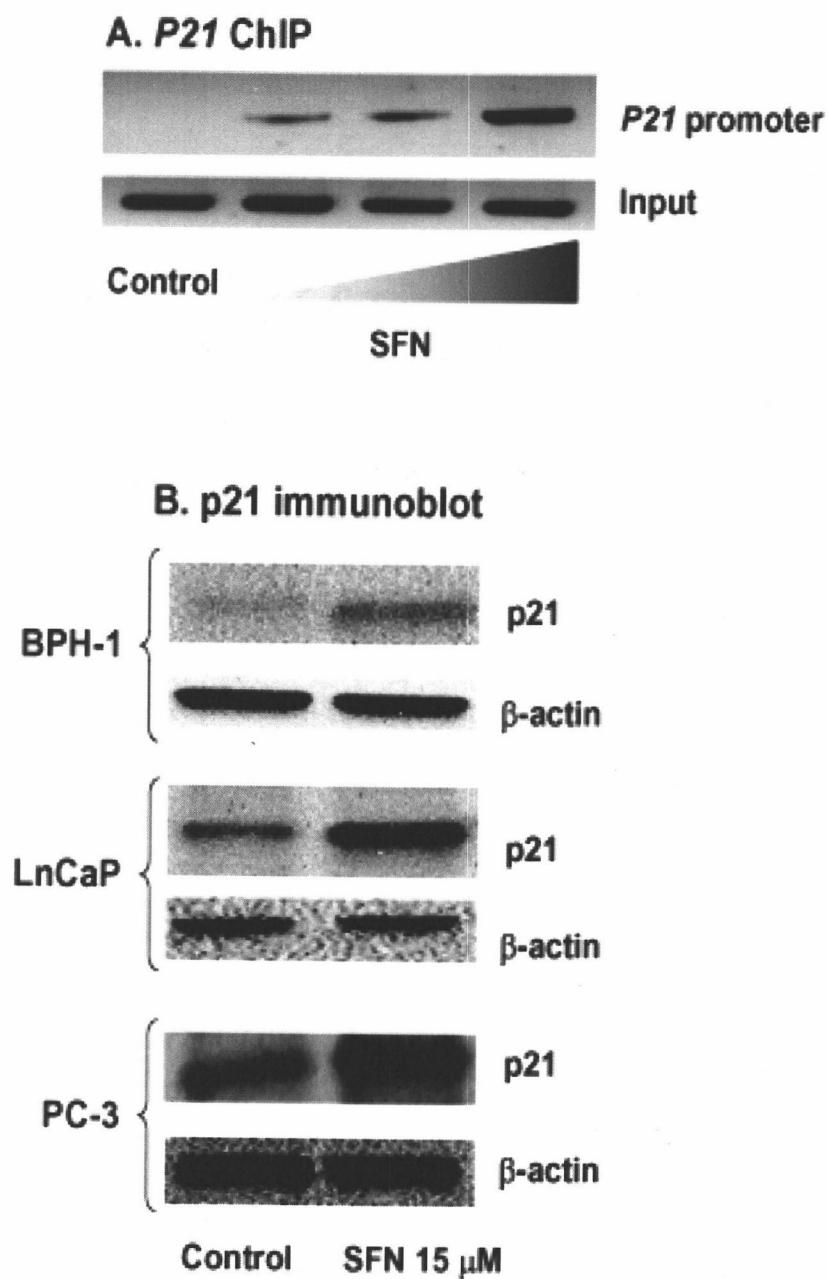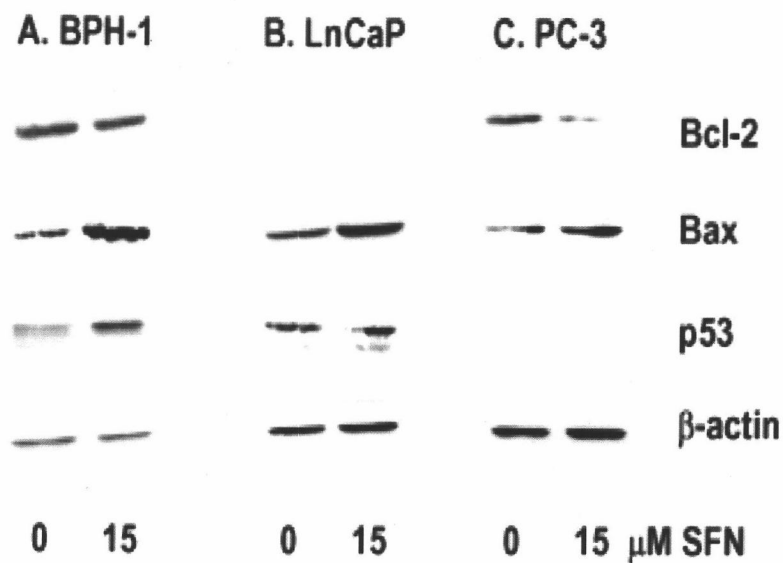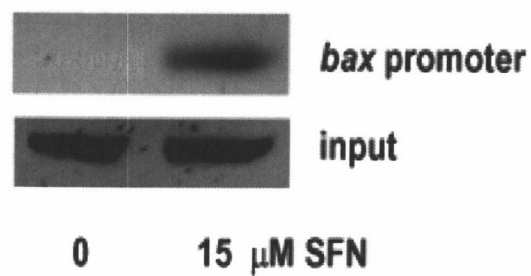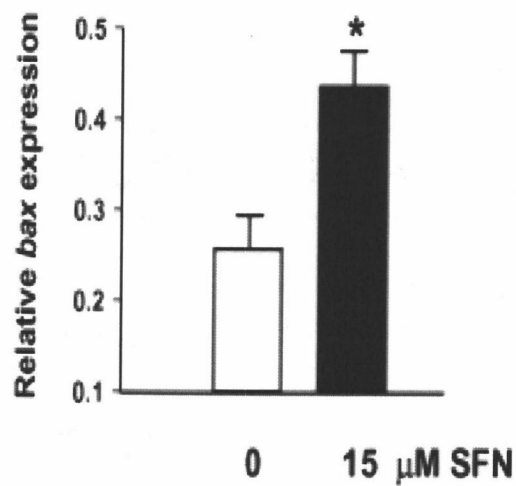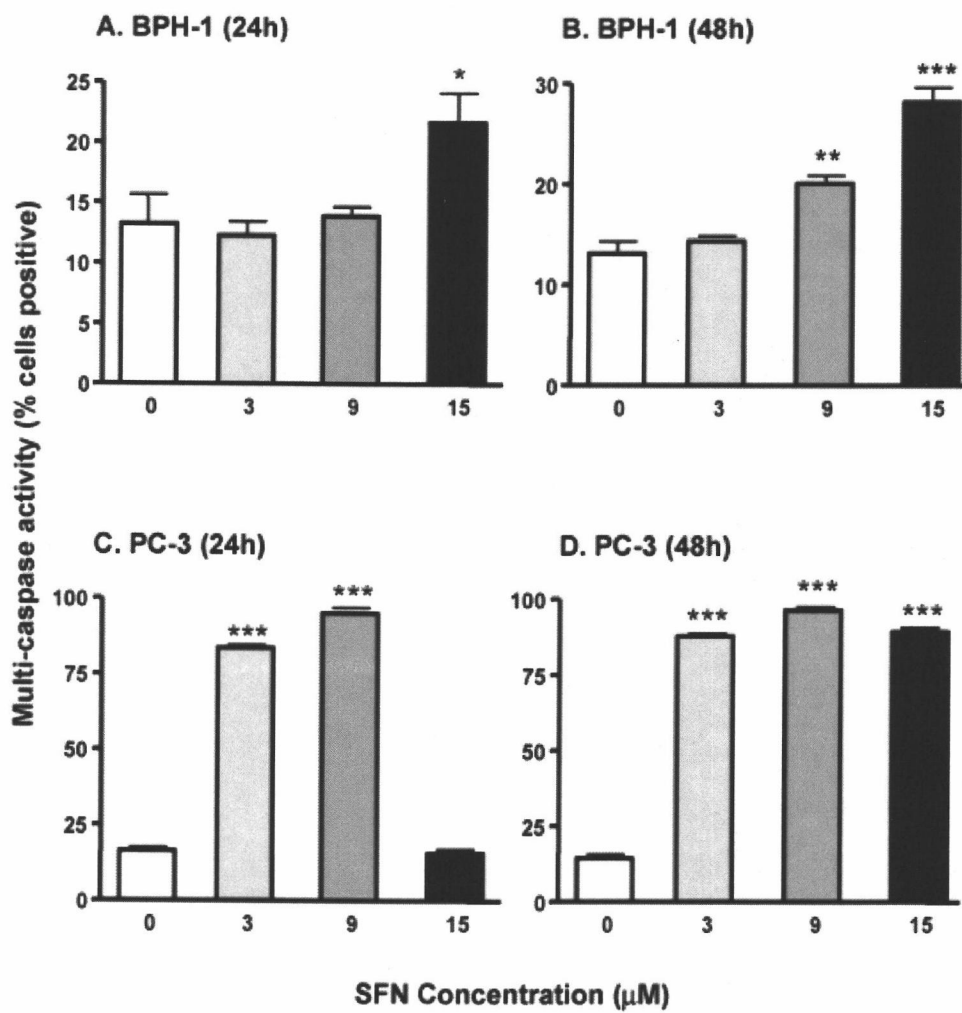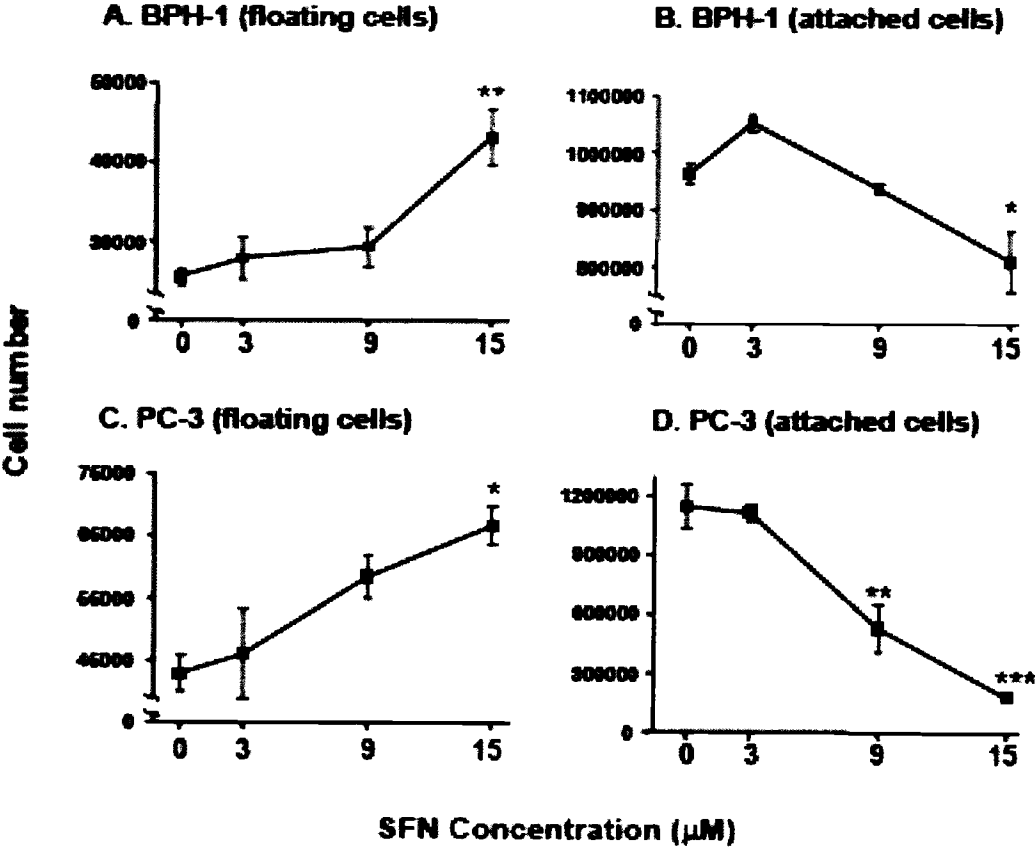
A. BPH-1

Acetylated Histone H3

Acetylated Histone H4

β-actin

B. LnCaP

Acetylated Histone H3

Acetylated Histone H4

β-actin

C. PC-3

Acetylated Histone H3

Acetylated Histone H4

β-actin

Control   SFN   TSA
          (15 μM)   (100 ng/ml)

Figure 19.

A. *P21* ChIP

P21 promoter

Input

Control

SFN

B. p21 immunoblot

BPH-1

p21

β-actin

LnCaP

p21

β-actin

PC-3

p21

β-actin

Control   SFN 15 μM

Figure 20.

A. BPH-1　　B. LnCaP　　C. PC-3

Bcl-2

Bax

p53

β-actin

0　　15　　　0　　15　　　0　　15　μM SFN

D. *bax* ChIP

bax promoter

input

0　　　15　μM SFN

E. *bax* real time–PCR

0　　　15　μM SFN

Figure 21.

A. BPH-1 (24h)

B. BPH-1 (48h)

C. PC-3 (24h)

D. PC-3 (48h)

SFN Concentration (μM)

Figure 22.

SFN Concentration (μM)

Sulforaphane retards the growth of human PC-3 prostate cancer xenografts and inhibits HDAC activity *in vivo*.

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Abstract

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables such as broccoli. This anticarcinogen was first identified as a potent inducer of Phase 2 enzymes, but evidence is mounting that SFN acts through other cancer chemopreventive mechanisms. We recently reported on a novel mechanism of chemoprotection by SFN in human colon cancer cells and prostate epithelial cells, namely the inhibition of histone deacetylase (HDAC). In the present investigation, we sought to test, for the first time, whether SFN also might inhibit HDAC activity *in vivo*. When consumed in the diet at an average daily dose of 7.5 μmol per animal, SFN suppressed the growth of human PC-3 prostate cancer cells in male nude mice. There was a significant decrease in HDAC activity in the xenografts, as well as in the prostates and peripheral blood mononuclear cells (PBMC) of mice treated with SFN, compared to controls. There also was evidence for an increase in global histone acetylation in the xenografts, prostates and PBMC, although this did not reach statistical significance in mice treated with SFN alone. Interestingly, however, there was a significant increase in acetylated histone H3 in the prostates of mice treated with SFN in combination with a second HDAC inhibitor, trichostatin A (TSA; 500 μg/kg, by daily *s.q.* injection). TSA alone retarded the growth of PC-3 xenografts, albeit less effectively than SFN, and the combination of SFN plus TSA did not result in greater inhibition of tumor growth under the present conditions. These findings provide

evidence that one mechanism through which SFN acts as a cancer chemopreventive agent is through the inhibition of HDAC activity.

INTRODUCTION

Histone deacetylase (HDAC) inhibitors are receiving increasing interest as potential anti-cancer agents (19, 41, 43, 205, 206). Most studies on HDAC inhibitors have focused on therapeutic intervention after the formation of a clinical malignancy, but there is increasing interest in their chemopreventive potential. HDAC inhibitors are interesting candidates for prevention because they enable re-expression of epigenetically-silenced genes involved differentiation, cell cycle regulation, apoptosis, angiogenesis, invasion, and metastasis.

HDAC changes have been linked to the progression of several cancers (13, 19, 43, 206). For example, *HDAC1* mRNA levels are higher in several prostate cancer cell lines compared to benign prostate hyperplasia, with a concomitant increase in HDAC activity (33). Moreover, HDAC1 expression was increased in primary human prostate cancer tissue compared to prostate hyperplasia (33). Over-expression of HDAC1 in PC-3 cells resulted in an increase in cell proliferation and an overall decrease in cell differentiation (200). HDAC1 expression was highest in hormone refractory prostate cancer (200), suggesting that alterations in HDAC may be of particular importance in the progression to androgen independence. Importantly, inhibitors of HDAC, including suberoylanilide hydroxamic acid (SAHA), valproic acid, depsipeptide, and sodium butyrate have been demonstrated to be effective against prostate cell lines and xenograft models

(201-203). Prostate cancer is the second leading cause of cancer-related death in men. With over 230,000 men in the US estimated to be diagnosed with prostate cancer in 2005, preventive measures that target the various steps involved in cancer initiation and progression could significantly decrease the incidence and mortality of this disease.

Epidemiological evidence suggests that consumption of cruciferous vegetables decreases overall risk for prostate cancer, particularly during the early stages (161-163, 199). SFN is a constituent of cruciferous vegetables, found at high levels in broccoli and broccoli sprouts (109, 116). SFN has been identified as an effective cancer chemoprotective agent in animal models (116, 142, 144), as well as in xenograft models of prostate cancer (154). Although the majority of early studies focused on SFN as a potent Phase-2 enzyme inducer, recent work has implicated other mechanisms of SFN action (reviewed in (205)). For example, studies using various cancer cell lines have shown either cell cycle arrest or apoptosis upon treatment with SFN (121, 149, 150, 152-154, 158, 159, 186, 187).

We recently identified SFN as a novel HDAC inhibitor in colon and prostate cancer cells (198, 211). HDAC inhibition was associated with global increases in histone acetylation, enhanced interactions of acetylated histones with the promoter regions of the *P21* and *BAX* genes, and elevated expression of p21$^{Cip1/Waf1}$ and BAX proteins. In addition to a G2/M cell cycle

arrest, there was evidence for loss of BCL-2 expression and increased muticaspase activity, resulting in apoptosis.

Based on these findings (198, 211), we sought to determine whether SFN might act as an HDAC inhibitor *in vivo*, using a prostate xenograft model. We also examined the combination of SFN and a second HDAC inhibitor, namely trichostatin A (TSA), since previous studies suggested synergistic effects of SFN plus TSA in human cancer cells (198). Here, we report on growth suppression of human PC-3 prostate cancer cells by dietary administration of SFN, and provide the first evidence that SFN may exert its chemopreventive effects, at least in part, through inhibition of HDAC activity *in vivo*.

MATERIALS AND METHODS

Xenografts: Male athymic nude BALB/c (nu/nu) mice (5-week old) were purchased from Charles River (Wilmington, MA) and maintained in accordance with Institutional Animal Care and Use Committee guidelines. PC-3 cells were mixed in a 1:1 ratio of complete media (RPMI 1640 + 10% FBS) and High Concentration Growth Factors Matrigel Matrix (Becton Dickinson; Bedford, MA). A suspension of 10^6 cells (50 µl) was injected subcutaneously into the right flank of each mouse. Tumor volume was calculated using the following formula: length x width^2 x 0.5236.

Treatments: Mice were randomized into four groups of ten animals. The treatment groups were as follows: control, trichostatin A (TSA), sulforaphane (SFN), and combination of TSA and SFN. TSA (Biomol) was prepared in 10% sterile DMSO and an average of 0.5 mg/kg was administered daily by subcutaneous injection for the duration of the study, starting on the day of implantation. SFN (LKT, St. Paul, MN) was mixed into pelleted AIN93G diet without *t*-butylhydroquinone at a concentration of 443 mg/kg diet (Research Diets). Diets were γ-irradiated at 2.5 MRads and then fed to mice *ad libitum*. Food intake and body weights were monitored throughout the study and the animals were killed 21 days after xenograft implantation.

PBMC isolation: Spleens were homogenized with a Dounce homogenizer and a single cell suspension was made by passing the homogenate through a 21 gauge needle followed by a 23 gauge needle. The cell suspension was placed on top of Fico/Lite LE (Atlanta Biologicals, Lawrenceville, GA) and centrifuged for 25 minutes at 2000 x g. The PBMC layer was removed and placed into a clean tube and centrifuged for 10 minutes at 900 x g. The PBMC pellet was placed in 90% FBS/10% DMSO and slow-frozen in isopropyl alcohol at -80 C overnight.

Western Blotting: Frozen portions of tumor tissue were placed in lysis buffer and ground using a Potter-Elvehjem homogenizer (teflon drill). Whole prostates were homogenized and centrifuged at 15,000 rpm for 5 min, and the supernatant was collected. Protein concentrations were determined by Lowry assay. Proteins (10-20 mg) 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 with Amido Black staining and β-actin levels. The membrane was blocked for 1 h with 2% bovine serum albumin, followed by either overnight incubation with primary antibody at 4°C or 1 h incubation with primary antibody at room temperature, and was finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Antibody dilutions were as follows: acetylated histone H3,

1:100 (Upstate, Charlottesville, VA); acetylated histone H4, 1:100 (Upstate); Bax, 1:100 (Santa Cruz Biotechnologies, Santa Cruz, CA); and β-actin, 1:5000 (Sigma, St. Louis, MO). Detection was by Western Lightning Chemiluminescence Reagent Plus (PE Life Sciences, Boston, MA) with image analysis on an AlphaInnotech photodocumentation system. Image quantification was determined by NIH ImageJ.

HDAC Activity Assay: HDAC activity was determined using the Fluor-de-Lys HDAC activity assay kit (Biomol), as reported previously (198). Homogenates (25 mg of total protein) from tumor tissue, prostates, or PBMC were incubated with Fluor-de-Lys substrate in triplicates for 12 min at 37°C to initiate the HDAC reaction. Fluor-de-Lys Developer was then added, and the mixture was incubated for another 10 min at room temperature. Fluorescence was measured using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices), with excitation 360 nm and emission 460 nm.

Statistics: One-way analysis of variance (ANOVA) or Student's *t*-test were used to assess the differences between groups. Differences among treatments were tested by Dunnett's test.

RESULTS

RESULTS

We previously identified SFN as an HDAC inhibitor in human prostate epithelial cells, with HDAC inhibition resulting in cell cycle arrest and apoptosis (211). In the present study, we sought to verify that SFN was an HDAC inhibitor *in vivo* leading to retardation of PC-3 xenograft growth, and to determine if the combination of two HDAC inhibitors (SFN and TSA) would result in greater growth inhibition than either agent alone. SFN administered in the diet inhibited xenograft growth significantly compared to controls as early as three days after implantation, and continued to protect through the duration of the study (Fig 23A). In addition, TSA and the combination of SFN+TSA repressed xenograft growth up to 12 days *post*-implantation, but the protective effects diminished somewhat at later times (Fig 23A). Interestingly, the combination of SFN+TSA was not significantly different from SFN or TSA alone, suggesting no additive effects on xenograft growth under the present conditions (Fig 23A).

None of the treatments had any adverse effects on animal health, food intake, or body weight. The average daily food intake did not differ between controls and treatment groups (Table 6.1). Moreover, body weight was not significantly different throughout the duration of the study, suggesting that SFN, TSA, and the combination of SFN+TSA was essentially non-toxic (Fig 23B).

HDAC activity assays were performed on xenografts to establish whether the prevention of xenograft growth was correlated with changes in HDAC activity. SFN and the combination of SFN+TSA significantly inhibited HDAC activity in xenografts (***P<0.001; Fig 24A). Interestingly, immunoblotting of xenografts showed a trend towards increased acetylation of histones H3 and H4 among the treatment groups, compared with controls (Fig 24B,C). These changes, however, were not statistically significant. In xenografts, induction of BAX expression was observed, and this was significant in animals fed SFN (Fig 24B,C). Because PC-3 cells lack P53, the increase in BAX might be attributed to increased acetylation at the *BAX* promoter, as observed recently in PC-3 cells treated with SFN *in vitro* (211).

Although HDAC inhibition was detected in the xenografts, it was of interest to determine the effects of the test agents in mouse tissues, such as the prostate. HDAC activity was significantly inhibited in the prostates from mice treated with SFN or SFN+TSA (Fig 25A). Paralleling the inhibition of HDAC activity, prostates from both the SFN and SFN+TSA treatment groups showed increased acetylated histones H3 and H4, whereas there was no change in the prostates from the TSA-treated mice (Fig 25B,C). A concomitant increase in BAX expression was observed in SFN- and SFN+TSA-treated mice (Fig 25B,C).

Several HDAC inhibitors are currently undergoing clinical trials; an important biomarker of HDAC inhibition used in these trials is acetylation of

histones from peripheral blood mononuclear cells (PBMC). Thus, HDAC activity and histone acetylation status were determined in PBMC isolated from the mice in this study. HDAC activity in PBMC was inhibited significantly in SFN-fed mice, compared with controls (**$P<0.01$, Fig 26A). Surprisingly, in groups given TSA or TSA+SFN there was an apparent increase rather than a decrease in mean HDAC activities, and scatter plots suggested the existence of two populations of PBMC, one with low HDAC activity and another with high HDAC activity (Fig. 26A). In all three test groups, the sub-population of 'responders' had significantly lower HDAC activity than the controls (***$P<0.0001$, Fig. 26A inset). Acetylated histones were essentially undetectable in PBMC from control mice, but were detected in PBMC of mice treated with SFN, TSA, or SFN+TSA; however, there was considerable variation within and between treatment groups (Fig 26B,C). Compared with controls, BAX expression was elevated significantly in PBMC of mice treated with one or both of the HDAC inhibitors (Fig 26B,C).

DISCUSSION

We recently showed that SFN inhibited HDAC activity in BPH-1, LnCaP, and PC-3 prostate epithelial cells, resulting in cell cycle arrest and apoptosis (211). Other laboratory groups have previously shown that SFN given at a dose of 5.6 μmol three times per week by gavage slowed the growth of PC-3 xenografts, and caused induction of apoptosis (154). In the present study, we confirmed that dietary administration of SFN also retarded the growth of PC-3 xenografts. Moreover, similar to our *in vitro* studies, we demonstrate for the first time that dietary administration of SNF effectively inhibits HDAC activity *in vivo*.

Several reports have described SFN as an effective chemopreventive agent *in vivo*, and most studies have focused on Phase 2 enzyme induction as the 'central' mechanism (116, 142, 144, 154). However, SFN acts as a suppressing as well as a blocking agent against colonic aberrant crypt foci formation (144), suggesting a role for mechanisms *post*-initiation. SFN induced apoptosis in, and slowed growth of, PC-3 xenografts *in vivo* (154), and cell culture models have demonstrated cell cycle arrest and apoptosis using SFN as a test agent (110-112, 149-155, 158-160, 198, 212). There is at present little information on the mechanisms by which SFN modulates cell cycle arrest and apoptosis in cancer cells (reviewed in (205)). Based on the present findings, and our previous work in colon and prostate cancer cells

(198, 211), we propose that SFN operates as an HDAC inhibitor, similar to compounds such as TSA.

TSA was not as effective as SFN at inhibiting xenograft growth in the present study (Fig 23A). TSA is a potent HDAC inhibitor *in vitro*, but has contradictory activities *in vivo*. Whereas TSA showed no anti-tumor effects in a melanoma xenograft model (213), post-initiation administration of 500 µg/kg TSA daily for four weeks decreased *N*-methyl-*N*-nitrosourea-induced mammary tumor volume by 75% compared to controls (214). Pharmacokinetic studies in mice revealed that TSA appears in the plasma as early as two minutes after *i.p.* administration and undergoes rapid Phase 1 biotransformation, such that it is undetectable in the plasma 24 hours after administration (215). TSA and the first metabolite, *N*-demethyl-TSA, possess HDAC inhibitory activity; however, all other metabolites are inactive as HDAC inhibitors (215). In the present investigation, rapid bio-transformation might explain why TSA was not as effective as SFN at inhibiting PC-3 xenograft growth. Indeed, PBMC from TSA-treated mice showed no increase in acetylated histone levels compared to controls (Fig 26), possibly because TSA was administered 24 h prior to collection of PBMC and therefore was already inactive through metabolism. Future studies are needed to resolve this question.

Based on the aforementioned findings with TSA, it was perhaps not surprising that the combination of TSA and SFN had little or no additive effect

on xenograft growth. This was in contrast to previous studies in cell culture, in which SFN+TSA combined resulted in a larger increase in acetylated histones and a greater inhibition of HDAC activity compared with either compound alone (198). Phase 1 and Phase 2 enzymes may have played a role in the bioavailability of active metabolites *in vivo*, affecting their interactions with HDAC. We previously demonstrated that inhibition of glutathione-S-transferases (GSTs) resulted in greater HDAC inhibitory activity by TSA, suggesting that TSA may be inactivated by conjugation to glutathione (198). SFN is a potent inducer of Phase 2 enzymes, including GSTs and other glutathione-generating enzymes (reviewed in (115, 205)). The SFN-cysteine metabolite appears to be the active form for HDAC inhibition (198), thus any alteration in the metabolism of SFN may result in a reduction of HDAC inhibitory activity. It would be of interest to determine whether co-administration of SFN+TSA alters the spectrum of metabolites of one or both compounds in mice. Further studies will be required to address this issue. Results for SFN+TSA illustrate the importance of examining combinatorial treatments not only in cell culture but also *in vivo*.

Several clinical trials are currently ongoing with HDAC inhibitors, including SAHA and depsipeptide (see www.clinicaltrials.gov). Phase 1 clinical trials have been completed for both of these HDAC inhibitors, and the levels of acetylated histone H3 from PBMC were used as an indicator of activity *in vivo* (216, 217). In the present investigation, SFN increased

acetylated histone levels in several (but not all) mice and inhibited HDAC activity in PBMC (Fig. 26). It is noteworthy that HDAC inhibitors in clinical trials were administered by continuous *i.v.* infusion for 2 h (216, 217), whereas in this study SFN was given in the diet for 3 weeks. Thus, dietary SFN might provide for a more sustained level of histone acetylation.

Mice in the TSA and SFN+TSA groups received the same dose of TSA by s.c. injection; however, there was an apparent sub-grouping of animals into 'responders' and 'non-responders' based on HDAC activities in PBMC (Fig. 26A), and acetylated histone status in PBMC (Fig. 26B,C). It will be necessary to confirm these findings in follow-up studies, but the results raise an important question for clinical trials with HDAC inhibitors, in which PBMC data might be used to stratify those individuals most likely to respond favorably from those that might be more resistant to HDAC inhibitors.

Although the prostate cancer xenograft model is useful for testing potential chemopreventive and chemotherapeutic agents, it is important to verify that the agents under investigation actually reach the intended target, which in this case was the prostate. The accumulation of acetylated histones detected in the prostate of SFN-treated mice, and the activation of BAX, suggests that SFN indeed entered the prostate in a bioactive form and attained concentrations necessary for HDAC inhibition.

Interestingly, the HDAC activity levels varied between tissue types. For example, average HDAC activity in the xenografts (human PC-3 prostate

cancer) from control mice were approximately 8-fold higher than in the prostates and PBMC from these mice (compare controls in Fig. 24A versus Figs. 25A and 26A). PC-3 cells are derived from an androgen-independent, late-stage human prostate carcinoma; the high HDAC activity in these cells compared to normal murine prostate tissue supports a role for HDAC in the progression to prostate cancer. In the present investigation, inhibition of xenograft development was most obvious early after implantation (Fig 23A); it is unclear whether acquired resistance might have occurred with further treatment, but this possibility warrants further investigation due to its important implication for the clinical efficacy of HDAC inhibitors.

In summary, results from the present investigation showed that dietary administration of SFN retarded the growth of PC-3 prostate cancer cells in nude mice. SFN inhibited HDAC activity in the xenografts, prostates, and PBMC, and there was a trend towards increased histone acetylation by SFN. Under the present conditions, TSA was not as effective as SFN, possibly due to its rapid turnover to inactive metabolites, and as a consequence there was no additive effect of SFN+TSA on xenograft growth. Overall, this investigation provides evidence that SFN acts as an HDAC inhibitor *in vivo*, and suggests that further studies are warranted on this novel mechanism of cancer chemoprevention and chemotherapy.

ACKNOWLEDGEMENTS

Table 6.1. No Effect of SFN and TSA on food intake and body weight gain.

	Average daily food intake (g)	Average body weight at end of study (g)
Controls	3.11 ± 0.99	20.48 ± 1.22
SFN	3.26 ± 0.98	20.58 ± 1.23
TSA	3.22 ± 0.95	20.60 ± 1.02
SFN+TSA	3.33 ± 1.01	21.19 ± 1.69

FIGURE LEGENDS

Figure 23. SFN, TSA, and SFN+TSA retard the growth of PC-3 xenografts. (*A*) PC-3 cells were implanted into forty male nude mice, which were randomized into four groups: control, SFN, TSA, or SFN+TSA. SFN was administered in the diet (2.5 μmol/g) and TSA was given by *s.c.* injection at an average of 500 μg/kg daily, beginning on the day of implantation. Tumor volume was determined as described in the Materials and Methods. (*B*) Average mouse body weight for each treatment group. Data = mean±SE; *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 24. HDAC inhibition by SFN and SFN plus TSA in xenografts. (*A*) HDAC activity was determined in xenografts as described in Materials and Methods. Data = mean±SE, n=10; ***$P<0.001$; (*B*) Xenografts were immunoblotted for acetylated H3, acetylated H4, and BAX; β-actin was the loading control. Each lane represents a xenograft from an individual animal, and the blot is a representative sample from each group of ten animals. (*C*) Quantification of immunoblots using NIH Image J, normalized for β–actin. Data = mean±SE; *$P<0.05$.

Figure 25. HDAC inhibitors increase acetylated histones and induce BAX expression in mouse prostates. (*A*) HDAC activity was determined in prostates as described in Materials and Methods. Data = mean±SE, *n*=10; **P<0.05; **P<0.01*; (*B*) Prostates were immunoblotted for acetylated H3, acetylated H4, and BAX; β-actin was included as a loading control. Each lane represents a prostate from an individual animal, and the blot is a representative sample from each group of ten animals. (*C*) Quantification of immunoblots using NIH Image J, normalized for β–actin. Data = mean±SE; ***P<0.01*.

Figure 26. SFN inhibits HDAC activity and induces BAX expression in mouse peripheral blood mononuclear cells (PBMC). (*A*) HDAC activity was determined as described in Materials and Methods; PBMC data for each mouse are shown in triplicate, for ten mice in each group; ***P<0.01* for SFN *versus* controls. Inset: data for 'responders', showing significant inhibition in each group *versus* controls. (*B*) PBMC were immunoblotted for acetylated H3, acetylated H4, and BAX; β-actin was the loading control. Each lane represents PBMC from an individual animal, and the blot is a representative sample from each group of ten animals. (*C*) Densitometry data determined by NIH Image J, and normalized for β–actin. Data = mean±SE; **P<0.05, **P<0.01*.

Figure 23.

Figure 24.

A.

B.

C.

143

Figure 25.

A.

B.

C.
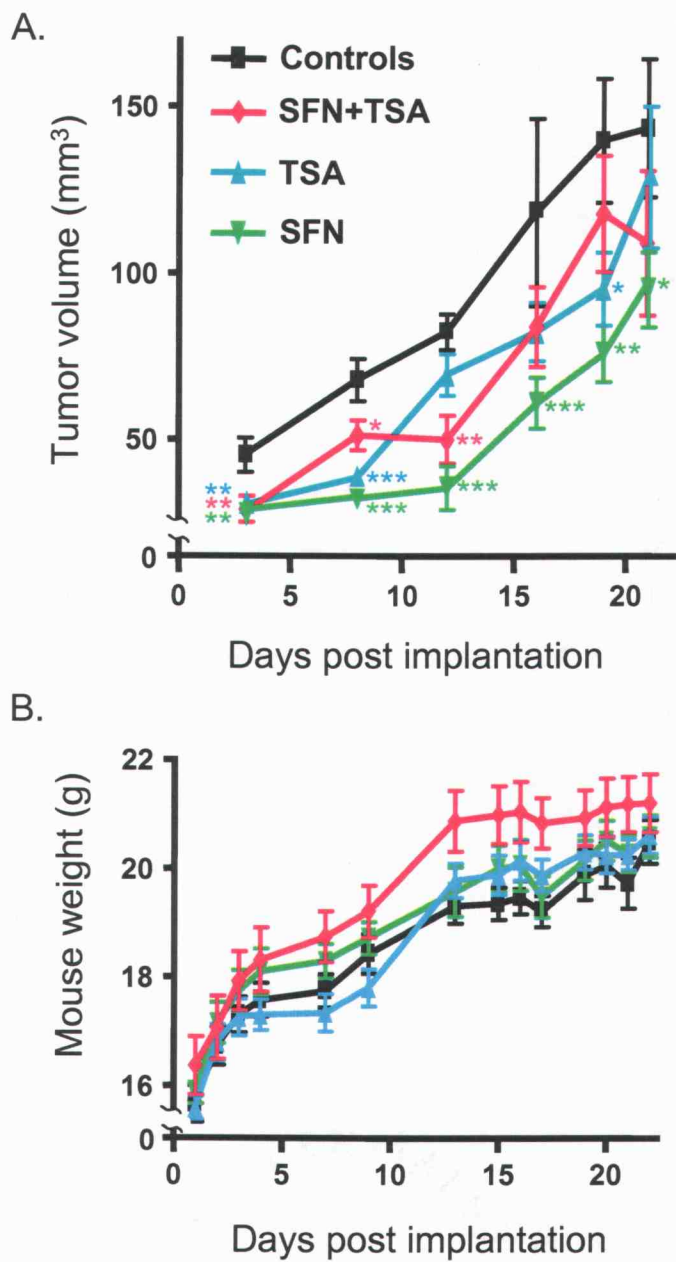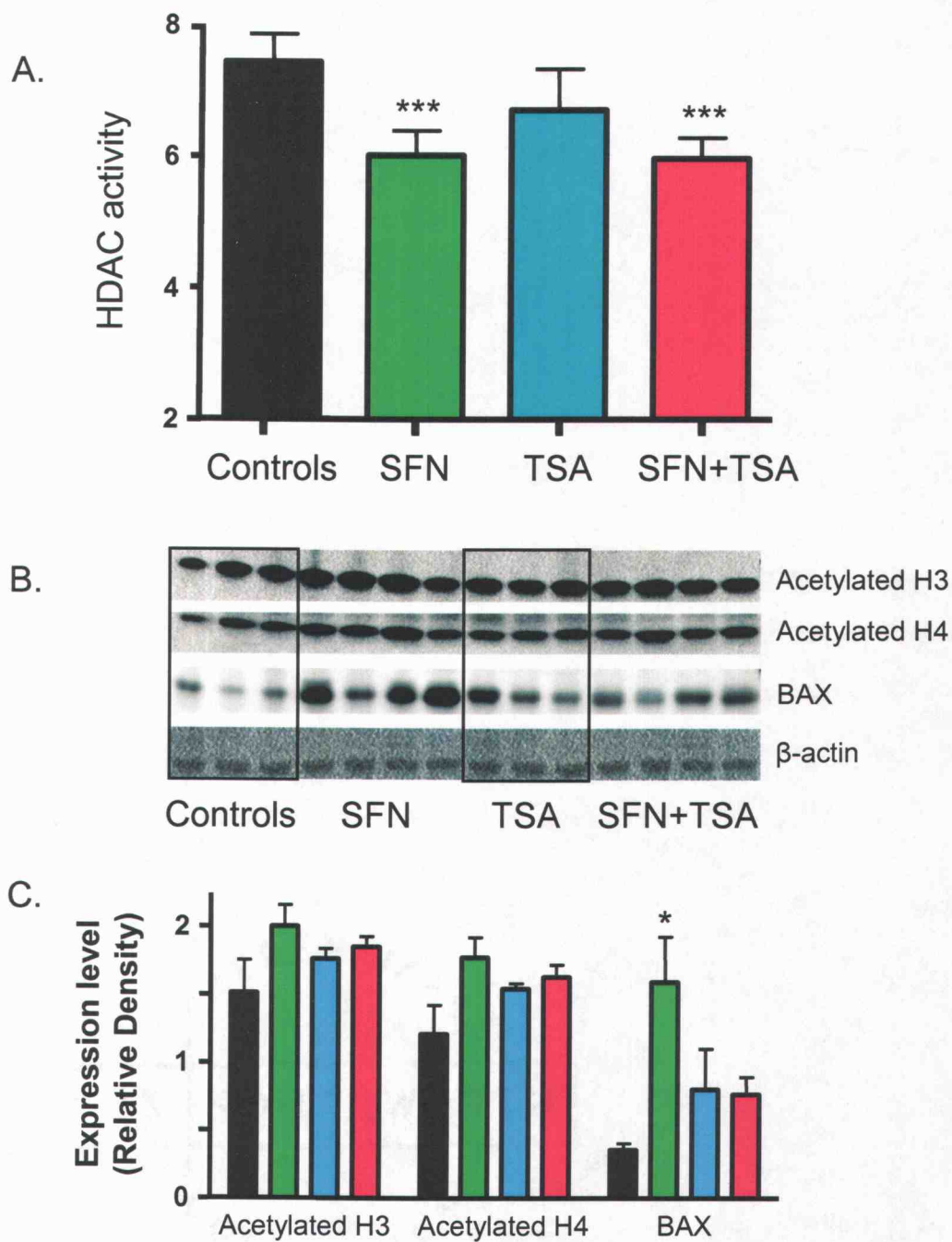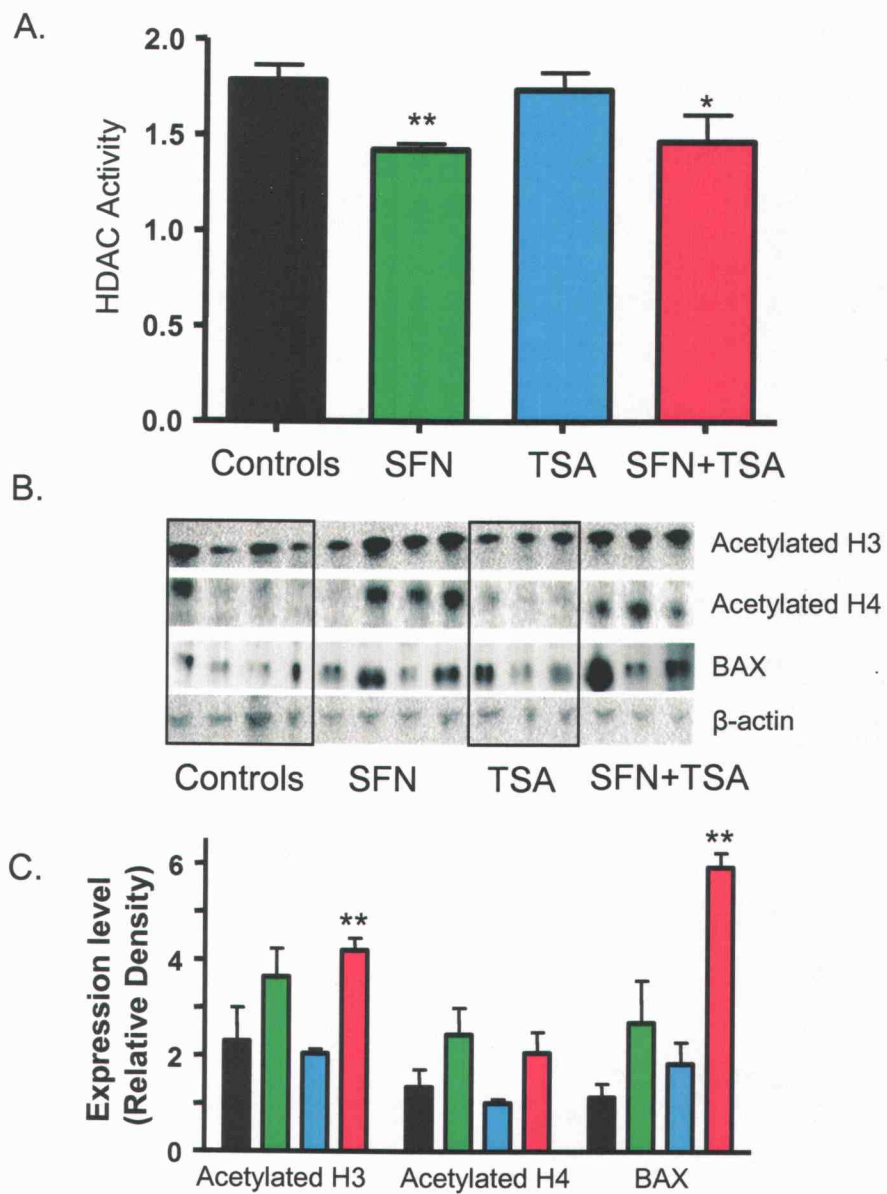
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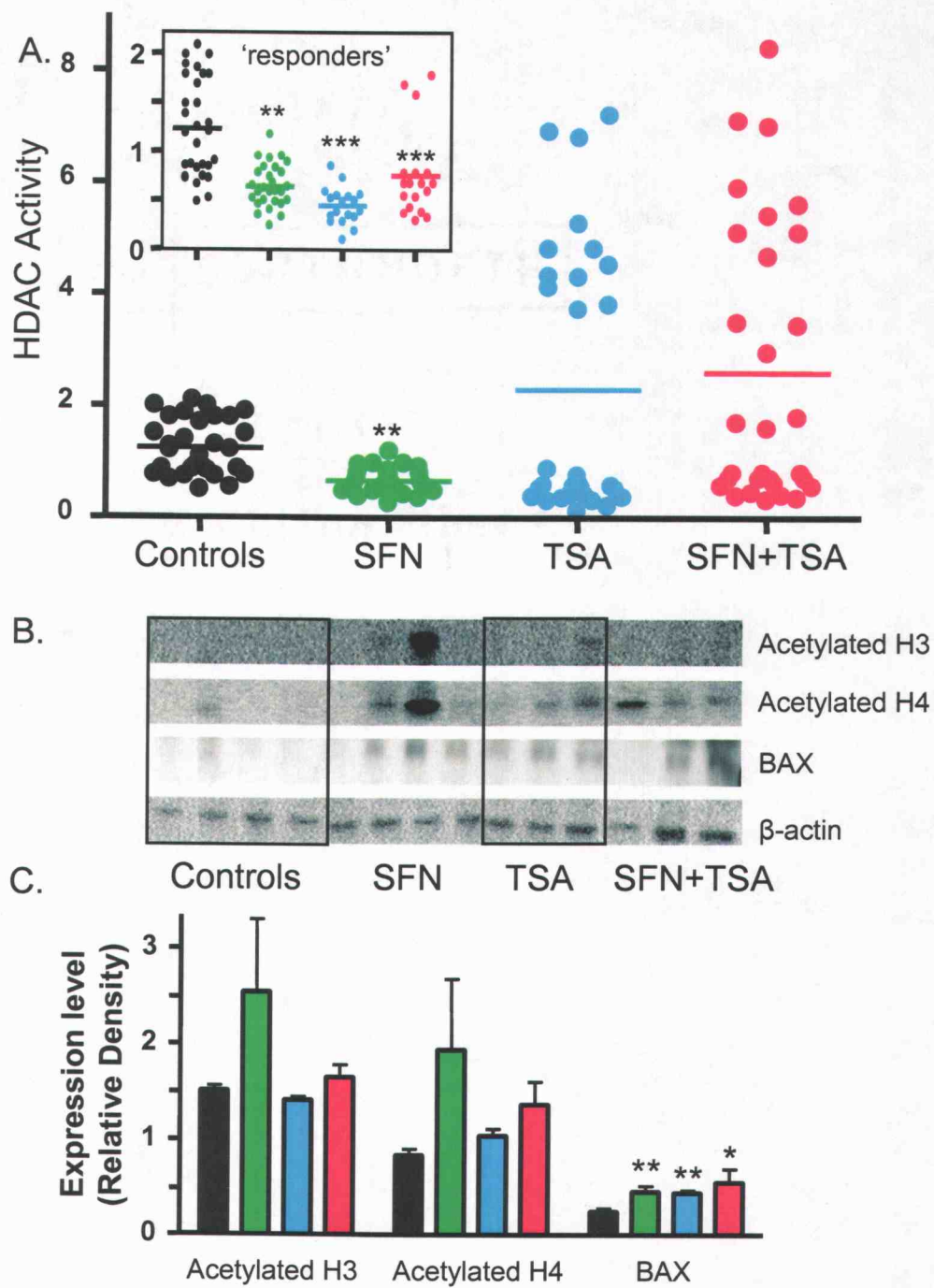
Sulforaphane prevents tumorigenesis in *Apc*[min] mice through inhibition of histone deacetylase activity

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ABSTRACT

Sulforaphane (SFN) is an isothiocyanate found in cruciferous vegetables such as broccoli. Early studies with SFN demonstrated potent induction of Phase 2 detoxification enzymes, and more recent work revealed cell cycle changes and induction of apoptosis in cancer cells. We recently provided evidence that SFN acts as a histone deacetylase (HDAC) inhibitor in human colon cancer cells and prostate epithelial and cancer cells *in vitro*. Here, we show that SFN also acts as an HDAC inhibitor *in vivo*. Mice were treated by gavage with a single oral dose of 10 µmol D,L-SFN, and a significant increase was seen in acetylated histones H3 and H4 at 6 h in colonic mucosa, persisting up to 24 h post-administration. HDAC activity was attenuated significantly in the colonic mucosa, and an increase in p21 was detected up to 48 h post-administration. Longer term treatment with SFN in the diet (~6 µmol per animal per day) resulted in accumulation of acetylated histones in the ileum, colon, peripheral blood mononuclear cells (PBMC), and prostates of wild-type mice. Moreover, dietary SFN suppressed tumor multiplicity in Apc^{min} mice significantly compared to controls, and this was correlated with an increase in acetylated histones and induction of Bax. The results provide further evidence for HDAC inhibition by SFN *in vivo*, and more broadly imply that HDAC inhibition may be an important mechanism for the chemoprevention and therapy of cancers.

INTRODUCTION

Colon cancer is the third-leading cause of cancer-related deaths in the United States, with over 145,000 new cases and 56,000 deaths estimated in 2005 (American Cancer Society). Studies have suggested that dietary factors play an important role in colon tumorigenesis, both in terms of causative and preventive agents (3, 218, 219). Indeed, epidemiological evidence suggests that consumption of cruciferous vegetables reduces the incidence of colon cancer (168, 169). Cruciferous vegetables are rich in isothiocyanates, several of which have been identified as potential chemopreventive agents. In particular, one isothiocyanate found at high levels in broccoli and broccoli sprouts (109, 116), sulforaphane (SFN), has been shown to possess chemoprotective activity against colon cancer in several *in vivo* studies (116, 142, 144).

Most *in vivo* studies to date have focused on the effects of SFN as a so-called 'blocking' agent (3), through induction of Phase 2 enzymes. However, experimental evidence in various cancer cells lines suggests that SFN also modulates carcinogenesis as a 'suppressing' agent *post*-initiation (reviewed in (205)). Recently, we provided evidence that SFN acts as a histone deacetylase (HDAC) inhibitor in human colorectal cancer cells and prostate epithelial and cancer cells in culture (198, 211). HDAC inhibition by SFN resulted in cell cycle arrest and apoptosis (211). In addition, dietary SFN suppressed the growth of human PC-3 prostate cancer xenografts *in vivo*,

and there was inhibition of HDAC activity in xenografts, prostates, and peripheral blood mononuclear cells (PBMC) in nude mice, as well as evidence for accumulation of acetylated histones (220).

HDAC inhibitors are receiving increasing interest as chemopreventive and chemotherapeutic agents against various cancers (reviewed in (206)). Evidence suggests that alterations in acetylated histone levels may be important in the progression of colorectal cancer. For example, reduction in global acetylation of histone H4 was observed in 80% of colon carcinomas and 39% of adenomas, and significantly correlated with advanced tumor stage and depth of tumor invasion, suggesting a progressive loss of histone acetylation during colon tumor development (34). Moreover, HDAC2 was elevated after loss of APC function, and inhibition of HDAC2 by siRNA in cells expressing a mutant form of APC resulted in a decrease in cell viability and induction of apoptosis (221). Importantly, HDAC2 protein is elevated in human primary colon carcinomas compared to matched controls (221), suggesting that HDAC2 may be a target for prevention and therapy. Indeed, success has been achieved *in vitro* with trichostatin A, butyrate, and other inhibitors of HDAC2 and related HDACs (37, 64, 66, 67, 88, 222).

Based on the epidemiological evidence for protection against colorectal cancer by cruciferous vegetable consumption and the ability of HDAC inhibitors to induce apoptosis in colon cancer cells lines, we sought to test the hypothesis that SFN can act as an HDAC inhibitor *in vivo* in the

mouse gastrointestinal (GI) tract and suppress tumorigenesis in the Apc^{min} mouse. Here, we show for the first time, that a single oral dose of SFN inhibited HDAC activity and induced histone acetylation in the mouse colon. Moreover, long-term dietary administration of SFN resulted in accumulation of acetylated histones in the ileum, colon, prostate, and PBMC of wild-type mice and inhibited tumorigenesis in the Apc^{min} mouse through induction of histone acetylation. This is the first report of chemoprevention by SFN in a non-carcinogen-induced model, providing evidence for prevention beyond the blocking phase, and supporting HDAC inhibition as a viable mechanism through which SFN exerts its protective effects *in vivo*.

MATERIALS AND METHODS

Animals:

Pilot study: Twelve control (i.e. non-*Apc*^min) mice were obtained from a breeding colony at Oregon State University (223). Three mice each were treated by gavage with 10 µmol L-SFN or D,L-SFN (LKT Labs, St. Paul, MN), or with SFN-NAC (gift of F.L. Chung and C. Conaway), in 200 µl DMSO, or DMSO alone. Mice were euthanized 6 h later and the colons were removed and cleaned with PBS. Colons were opened longitudinally and the colonic mucosa was scraped, placed in lysis buffer, and snap-frozen in liquid nitrogen.

Short-term study: Thirty-six C57BL/6J mice at 5-weeks of age, obtained from Jackson Laboratory (Bar Harbor, ME), were housed 3 per cage and fed pelleted AIN93G diet without *t*-butylhydroquinone (Research Diets) for one week. Mice were treated by gavage with 10 µmol D,L-SFN (LKT Labs) in buffered water. Six, 24, and 48 h after administration, mice were euthanized by CO_2. Spleens were taken in order to isolate peripheral blood mononuclear cells (PBMC), and colons were removed and processed as in the pilot study.

Tumor study: Fifteen male *Apc*^min and six C57BL/6J^{+/+} mice (wild-type), obtained from Jackson Laboratory at 6-weeks of age, were housed 3 per cage and given water and pelleted AIN93G diet (Research Diets) without *t*-butylhydroquinone. Diets were formulated with or without 443 mg/kg SFN (LKT) and fed *ad libitum*. The study was terminated when mice were

approximately 16 weeks of age. Mice were euthanized after fasting for 12 h, blood was collected by cardiac puncture, livers and spleens were removed and weighed, and PBMC were isolated from the spleens. Prostates were removed and snap-frozen in liquid nitrogen. The GI tract was excised, divided into sections (duodenum, jejunum, ileum, and colon), and cleaned with ice-cold PBS. The sections were opened longitudinally and the number, size, and location of polyps along the GI tract were recorded by individuals blinded to genotype and treatment. Polyps and adjacent normal-looking tissue were removed and snap-frozen in liquid nitrogen for Western blot analysis. Normal tissue was also collected from wild-type mice.

PBMC Isolation: Spleens were homogenized with a Potter-Elvehjem homogenizer (teflon drill) and a single cell suspension was made by passing the homogenate through a 21 gauge needle followed by a 23 gauge needle. The cell suspension was placed on top of Fico/Lite-LE (Atlanta Biologicals, Lawrenceville, GA) and centrifuged for 25 min at 2000 x g. The PBMC layer was removed and placed in a clean tube and centrifuged for 10 min at 900 x g. The PBMC pellet was placed in 90% FBS/10% DMSO and slow frozen in isopropyl alcohol at $-80^{\circ}C$ overnight.

Western Blotting: Frozen portions of tissue or mucosa were placed in lysis buffer and ground using a Potter-Elvehjem homogenizer (teflon drill). Whole prostates were homogenized and centrifuged at 15,000 rpm for 5 min, and the supernatant was collected. Protein concentrations were determined by

Lowry assay. Proteins (10-20 mg) 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 with Amido Black staining and β-actin levels. The membrane was blocked for 1 h with 2% bovine serum albumin, followed by either overnight incubation with primary antibody at 4°C or 1 h incubation with primary antibody at room temperature, and was finally incubated for 1 h with secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Antibody dilutions were as follows: acetylated histone H3, 1:100 (Upstate, Charlottesville, VA); acetylated histone H4, 1:100 (Upstate); Bax, 1:100 (Santa Cruz Biotechnologies, Santa Cruz, CA); and, β-actin, 1:5000 (Sigma, St. Louis, MO). Detection was by Western Lightning Chemiluminescence Reagent Plus (PE Life Sciences, Boston, MA) with image analysis on an AlphaInnotech photodocumentation system. Image quantification was determined by NIH ImageJ.

HDAC Activity Assay: HDAC activity was determined using the Fluor-de-Lys HDAC activity assay kit (Biomol), as reported previously (198). Homogenates (25 mg of total protein) from colonic mucosa, prostates, or PBMC were incubated with Fluor-de-Lys substrate for 12 min at 37°C to initiate the HDAC reaction. Fluor-de-Lys Developer was then added, and the mixture was incubated for another 10 min at room temperature. Fluorescence was measured using a Spectra Max Gemini XS fluorescent

plate reader (Molecular Devices), with excitation 360 nm and emission 460 nm.

***In vivo* Chromatin Immunoprecipitation (ChIP):** After removal of polyps and 'normal-looking' tissue for Western blot analysis, the ileum and colonic mucosa were scraped. The mucosa was placed in 10% formalin for 15 min and then centrifuged at 3000 rpm for 5 min. The pellet was washed twice with ice-cold PBS containing protease inhibitors and then re-suspended in SDS-lysis buffer and frozen at -80 ^{0}C. Samples were thawed and sonicated on ice for 5 times for 20 s each. A Chromatin Immunoprecipitation kit (Upstate) was used, following the instructions provided with the kit, using anti-acetylated histone H3 (Upstate). DNA was isolated by phenol-chloroform extraction. PCR was done with the following cycling conditions: 95 ^{0}C 1 min, 52 ^{0}C for 1 min, 72 ^{0}C for 1 min, for 45 cycles. F: CATAGATGTATGTGGCTCTGC; R: GCTGCCTCCTTATAGCGTCGG.

Statistics: One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, Mann-Whitney, or Student's *t*-test was performed to assess the differences between groups; the level of significance was designated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

RESULTS

We first undertook a pilot study to determine the best form of SFN to use in subsequent experiments. HDAC activity was inhibited significantly in mouse colonic mucosa 6 h after treatment with SFN-NAC, L-SFN, or DL-SFN, with the latter compound producing the greatest degree of inhibition among the three agents tested (Fig *27A*). Acetylated histones corresponded well with HDAC inhibition, and acetylated histones H3 and H4 were increased significantly by D,L-SFN (Fig *27B,C*). Based on these results, D,L-SFN was used in subsequent experiments.

The time-course for HDAC inhibition in colonic mucosa was examined 6, 24, and 48 h after a single dose of D,L-SFN. D,L-SFN increased histone acetylation significantly at 6 h (Fig *28A*), and this still evident at 24 h (Fig *28B*) but returned to control levels by 48 h (Fig *28C*). Expression of p21, a target of HDAC inhibitors *in vitro* (47, 48, 64, 67, 173, 193-197), was increased by D,L-SFN only at 48 h (Fig *28C*).

We next examined the effects of chronic dietary administration of D,L-SFN on Apc^{min} mice and the corresponding wild type controls. Food intake was slightly higher in wild type mice and lower in Apc^{min} mice given D,L-SFN *versus* control diet, but the average consumption of ~6 μmol D,L-SFN/day for 10 weeks did not adversely affect body weight (Table 7.1). Packed cell volume (hematocrit) was increased and the spleen somatic index (SSI) was decreased in SFN-treated animals compared to the controls (Table 7.1).

In the ileum and colon of wild type mice (Fig 29 *A,B*), there was a trend towards increased acetylated histones H3 and H4 with D,L-SFN treatment, but this did not reach statistical significance. There was strong induction of acetylated histones in the PMBC and prostates for two out of three wild type mice given D,L-SFN, with a concomitant increase in p21 (Fig 30*A,B*). There also was significant inhibition of HDAC activity in the prostates of wild type mice given D,L-SFN (**$P<0.01$, Fig 30*C*).

In Apc^{min} mice, D,L-SFN suppressed tumor multiplicity significantly (**$P<0.01$, Fig *31A*). There was consistent protection in all sections of the GI tract, but the greatest inhibition occurred in the ileum and the jejunum (Fig *31B*).

Global acetylated histone status was examined in the ileum and colon from the Apc^{min} mice (Fig 32 and Fig 33, respectively). There was some inter-individual variability within the D,L-SFN group; nonetheless, compared to Apc^{min} mice given control diet, acetylated histones were strongly increased in four of six normal-looking ileum (Fig 32*A*) and three of six ileum polyps (Fig 32*B*), as well as two of three colon polyps (Fig 33*B*). An increase in Bax protein expression also was observed in the ileum polyps (Fig 32*C*) and colon polyps of D,L-SFN-treated mice (Fig 33*C*). Induction of Bax expression has been reported for other HDAC inhibitors (reviewed in (13, 43)), and we recently demonstrated a SFN-mediated increase in acetylated histone H4 associated with the promoter region of the *BAX* gene in prostate epithelial

cells *in vitro* (211). An increase in acetylated histone H3 associated with the promoter region of the *p21* gene was observed in the 'normal-looking' and polyps of the ileum and colon (Fig 32*D*, 33*D*).

When the data for all SFN-fed groups were normalized to controls, the latter being assigned an arbitrary value of 1.0, there was a consistent trend of increased acetylated histones H3 and H4 in all tissues, except in the normal-looking colon of *Apc*^min mice (Fig. 34). For all data combined (Fig. 34), there was an average increase of 2.4-fold for acetylated H3 and 2.2-fold for acetylated H4 in rats given SFN diet *versus* control diet

DISCUSSION

We recently identified SFN as an HDAC inhibitor in human colorectal (198) and prostate cancer cells *in vitro* (211). We also showed that dietary SFN slowed the growth of human PC-3 prostate cancer xenografts in mice, and inhibited HDAC activity in the xenografts, PBMC and prostates (220). In the present study, we confirmed that SFN also acted as an HDAC inhibitor in colonic mucosa of mice given a single bolus dose of 10 μmol SFN (Fig 27). There also was increased histone acetylation in various tissues of mice given D,L-SFN in a short-term dosing study as well as following chronic dietary administration. In each tissue examined from mice consuming SFN long-term in the diet, there was an increase in accumulation of acetylated histones, but due to high inter-animal variation, the differences were not significant (Fig 34).

SFN was first identified as a potent Phase 2 enzyme inducer (109). Subsequent work confirmed that SFN protected during the blocking phase of chemically-induced carcinogenesis (116, 142-144), and one study provided evidence for suppression of carcinogen-induced colonic aberrant crypts *post-initiation* (144). However, to our knowledge, there have been no reports with SFN using an *in vivo* cancer model that did not involve carcinogen treatment. Thus, we present the first evidence that SFN acts as a chemopreventive agent in *Apc*^{min} mice (Fig 31), an animal model that is genetically pre-disposed to the development of intestinal polyps (224). In this model, there is

no possibility that the effects of SFN are due to alteration of carcinogen metabolism since no carcinogen is administered.

In the pilot study, all three forms of SFN inhibited HDAC activity and increased accumulation of acetylated histones (Fig 27). Interestingly, SFN-NAC had comparable activity to D,L-SFN in terms of HDAC inhibition and induction of acetylated histones. The latter findings *in vivo* confirm and extend the evidence from *in vitro* studies, showing that SFN-NAC and SFN-CYS were effective HDAC inhibitors, and that the latter compound fits within the HDAC active site (198). The *in vivo* activity of SFN-NAC may be attributed to one or both of the following mechanisms: i) SFN-NAC is recognized by HDACs, resulting in the removal of the *N*-acetyl group and subsequent inhibition by SFN-CYS; ii) SFN-NAC is deconjugated *in vivo* to the parent compound, SFN, which is then metabolized through the mercapturic acid pathway, with the SFN-CYS metabolite being the active form in HDAC inhibition.

Accumulation of acetylated histones after a single bolus dose of D,L-SFN was detected at 6 and 24 h, but this had diminished by 48 h in colonic mucosa. This is in contrast to colorectal cancer cells in culture, in which accumulation of acetylated histones remained for up to 48 h (198). *In vivo*, SFN is probably metabolized to the active form(s) within 6 h, but has been largely eliminated by 48 h, and thus is unable to inhibit HDAC at the latter time-point. Interestingly, p21 was elevated at 48 h in colonic mucosa,

suggesting sustained increase in this known target of HDAC inhibitors for some time after global histone acetylation status returns to control levels. Further studies are needed to investigate the possibility that acetylated histones associated specifically with the *p21* promoter remain elevated, despite the lack of an apparent increase in global histone acetylation at 48 h.

Importantly, there was a trend towards increased histone acetylation in the ileum, colon, PBMC and prostates of wild type mice given D,L-SFN in the diet for several weeks (Figs 29 and 30), as well as in the ileum, ileum polyps and colon polyps of *Apc*^{min} mice (Figs 32 and 33), albeit somewhat variable for mice with each group. These changes were seen despite a 12 h fast prior to necropsy, and the large increase in acetylated histones and p21 expression in PBMC for two of three mice given D,L-SFN is noteworthy (Fig *30A,B*), because these end-points were used in clinical trials with HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) and depsipeptide (216, 217).

The inhibition of ileum polyps by SFN was highly significant and accompanied by an accumulation of acetylated histones in 4 of 6 *Apc*^{min} mice (Fig 32). In the *Apc*^{min} mice, low hematocrits and high SSI are highly correlative to tumor multiplicity, and efficacious chemopreventive agents used in this model somewhat reverse the this phenotype to that of a wild-type mouse (225); the hematocrits were increased and the SSI was decreased in SFN-treated mice over controls (Table 7.1). Expression of Bax, a reported

target of HDAC inhibitors *in vitro*, was generally higher in polyps and normal-looking tissues of D,L-SFN-treated mice *versus* the corresponding tissues of mice fed control diet, but there was considerable variability between animals within each group, suggesting the possibility of two populations: responders and non-responders, which may reflect polymorphisms in the populations (Fig 32C). Previous reports found that normal-looking intestinal tissue from Apc^{min} mice contains micro-adenomas (226); therefore, a small increase in Bax expression observed in the normal-looking' ileum and a larger increase in Bax expression in ileum polyps might reflect some degree of selectivity of SFN (as an HDAC inhibitor) for transformed cells.

The Apc^{min} mouse has certain advantages as a tumor model, but one limitation is the low prevalence of colon polyps as compared with ileum polyps. It is noteworthy that D,L-SFN was less effective, statistically, at suppressing polyps in the colon compared with the ileum, and had less effect on acetylated histones and Bax. To date, it is unknown whether wild-type mice and Apc^{min} mice differ in terms of HDAC levels and/or activities in the colonic mucosa. Wild-type mice were generally more responsive to D,L-SFN than Apc^{min} mice in terms of histone acetylation and p21 induction (Fig 30, and data not presented), especially in experiments using short duration exposure, and further studies are warranted on the possibility of acquired resistance following prolonged exposure to HDAC inhibitors, as described for other drugs such as NSAIDs (see below). Interestingly, a recent study

demonstrated a direct link between levels of HDAC2 expression and loss of Apc, such that the ileum of wild-type mice had no detectable HDAC2 protein, but normal-looking ileum and ileum polyps of mice with a truncated form of Apc (Apc[1638N]) had a marked increase in HDAC2 (221). Moreover, *i.p.* injection of *Apc*[min] mice with another HDAC inhibitor, valproic acid, diminished tumor multiplicity, with an increase in acetylated histone H3 in normal-looking tissue and a decrease in HDAC2 expression in polyps from treated mice (221). Valproic acid is somewhat of a unique HDAC inhibitor, in that it shows specificity for Class I HDACs, and targets HDAC2 for proteasomal degradation (227, 228), resulting in both inhibition of existing HDACs and reduction in HDAC protein levels. Unlike valproic acid, SFN does not seem to show specificity for a specific HDAC and it does not alter HDAC1 protein levels, although its effects on other HDACs have not been investigated (198).

The present investigation suggests that chemoprevention by SFN involves inhibition of HDAC activity *in vivo*, but this does not rule out additional mechanisms. For example, sulindac and other NSAIDS suppress tumorigenesis in the *Apc*[min] mouse model, and one of the main mechanisms is thought to involve decreased inflammation (223, 225, 229, 230). SFN reportedly acts as an anti-inflammatory agent by attenuating the DNA binding capability of nuclear factor κB (NFκB) (147). In murine RAW macrophages, SFN decreased lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E2, and tumor necrosis factor α (TNFα), as well as inducible

nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) protein expression and mRNA levels (147). Other mechanisms of SFN action have been proposed based on studies in cell culture (for review, see (205)), such as activation of checkpoint 2 kinase and inhibition of tubulin polymerization. One of the defining characteristics of cancer cells is the multitude of pathways that have been altered; the ability to target several signaling pathways, as is the case with SFN, may be an important trait of a successful chemopreventive agent.

In summary, results from the present investigation showed a consistent increase in accumulation of acetylated histones H3 and H4 with a single, bolus dose of D,L-SFN by gavage in short-term studies (6-48 h), as well as in long-term dietary administration of SFN. Increased histone acetylation histones was accompanied by induction of downstream 'effector' proteins, such as p21 and Bax. Most importantly, dietary SFN suppressed intestinal tumorigenesis in the Apc^{min} mouse, with approximately 50% reduction seen in all four sections of the GI tract (duodenum, jejunum, ileum and colon). Overall, this investigation provides evidence that SFN acts as an HDAC inhibitor *in vivo* in the intestinal tract, prostate, and PBMC, and that HDAC inhibition contributes in an important way to the chemopreventive and therapeutic activities of SFN *in vivo*.

ACKNOWLEDGEMENTS

TABLE 7.1.

Effects of dietary SFN on food consumption, body weight, hematocrit, and spleen somatic index (SSI).

	Average daily food consumption (g)	Mouse weight at end of study (g)	Hematocrit % PCV	SSI
Wild-type controls	5.0±0.53	23.7±0.59	49.2±2.57	0.35±0.06
Wild-type D,L-SFN	5.4±0.67****	24.8±1.37	51.2±2.84	0.32±0.08
Apc^{min} controls	4.9±0.63	21.3±2.65	20.4±2.57	1.34±0.20
Apc^{min} D,L-SFN	4.7±0.61**	22.7±2.34	26.9±8.66	1.20±0.41

FIGURE LEGENDS

Figure 27. L-SFN, D,L-SFN, and SFN-NAC inhibit HDAC activity and induce accumulation of acetylated histones in mouse colonic mucosa. Mice (n=3 per group) were treated by gavage with 10 μmol L-SFN, D,L-SFN, SFN-NAC, or vehicle alone, and colonic mucosa was isolated after 6 h. (*A*) HDAC activity was determined as described in Materials and Methods. (*B*) Colonic mucosa from each mouse was immunoblotted for acetylated H3 and acetylated H4; β–actin was the loading control. (*C*) Densitometry data were quantified by NIH Image J and normalized for β–actin; mean±SE. *P<0.05, ***P<0.001.

Figure 28. Time-course of D,L-SFN-induced changes in acetylated histones and p21 in mouse colonic mucosa. Mice were treated by gavage with 10 μmol D,L-SFN (n=3 per group) and colonic mucosa was isolated after (*A*) 6 h, (*B*) 24 h, or (*C*) 48 h, and immunoblotted for acetylated H3, acetylated H4, and p21. β–Actin was the loading control. Densitometry data were quantified by NIH Image J and normalized for β–actin; mean±SE. **P<0.01.

Figure 29. **Effects of long-term dietary treatment with D,L-SFN on histone acetylation in wild-type mouse ileum and colon.** Wild-type mice consumed approximately 6 μmol SFN per day for 10 weeks. Tissues from the ileum (*A*) or the colon (*B*) were immunoblotted for acetylated H3 and acetylated H4; β–actin was the loading control. Densitometry data were quantified by NIH Image J and normalized for β–actin; Data = mean±SE.

Figure 30. **Effects of long-term dietary treatment with D,L-SFN on histone acetylation and p21 in wild-type mouse PMBC and prostates.** Wild-type mice consumed approximately 6 μmol SFN per day for 10 weeks. PBMC (*A*) or prostates (*B*) were immunoblotted for acetylated H3, acetylated H4, and p21. β–actin was the loading control. Densitometry data were quantified by NIH Image J and normalized for β–actin; Data = mean±SE. (*C*) HDAC activity was determined in prostates as described in Materials and Methods. Data = mean±SE; **$P<0.01$, ***$P<0.001$.

Figure 31. **Inhibition of intestinal polyps by dietary D,L-SFN in *Apc*^{min} mice.** *Apc*^{min} mice consumed approximately 6 μmol SFN per day for 10 weeks. (*A*) Suppression of tumor multiplicity; (*B*) Suppression according to tumor distribution in the GI tract. Data = mean±SE; *$P<0.05$; **$P<0.01$.

Figure 32. **Histone acetylation status in normal-looking tissue and polyps from the ileum of _Apc_^{min} mice after dietary D,L-SFN treatment.** _Apc_^{min} mice consumed approximately 6 μmol SFN per day for 10 weeks. Immunoblots were performed for each control and D,L-SFN-treated mice, on two or more separate occasions; data shown are representative findings for each animal (numbered above the corresponding lane). (_A_) Normal-looking tissue or (_B_) polyps from the ileum were immunoblotted for acetylated H3, acetylated H4, and Bax; β–actin was included as a loading control. (_C_) Densitometry data were quantified by NIH Image J and normalized for β–actin; Data = mean±SE. (_D_) Ileum mucosa was scraped and DNA was cross-linked to proteins, chromatin immunoprecipitation (ChIP) was performed using acetylated histone H3, and following reversal of cross-linking and isolation of DNA, PCR was performed with primers to the _p21_ promoter.

Figure 33. **Histone acetylation status in normal-looking tissue and polyps from the colon of _Apc_^{min} mice after dietary D,L-SFN treatment.** _Apc_^{min} mice consumed approximately 6 μmol SFN per day for 10 weeks. Colon tissues were analyzed exactly as described for ileum, see legend to Fig. 32 for details.

Figure 34. **Histone acetylation status among all groups given SFN _versus_ control diet.** Histone acetylation data from Figs 3-7 for mice given

SFN in the diet were normalized to the respective controls given control diet (the latter being assigned an arbitrary value of 1.0). All data were combined for groups with respect to increased acetylated histone H3 and H4; a ~2-fold increase in acetylated histones was seen in mice given SFN *versus* control diet (p=0.058).

169

Figure 27.

A.

B.

C.
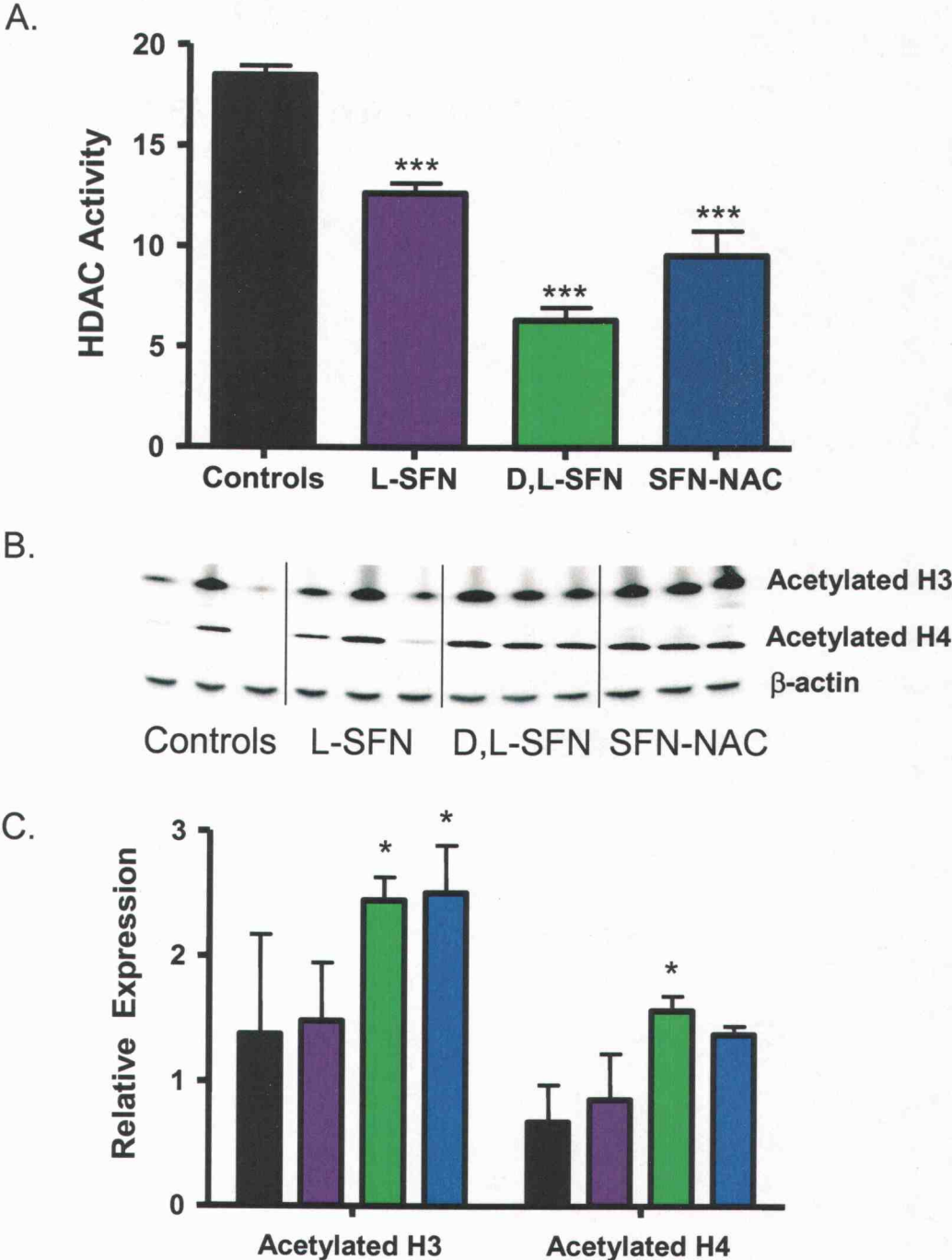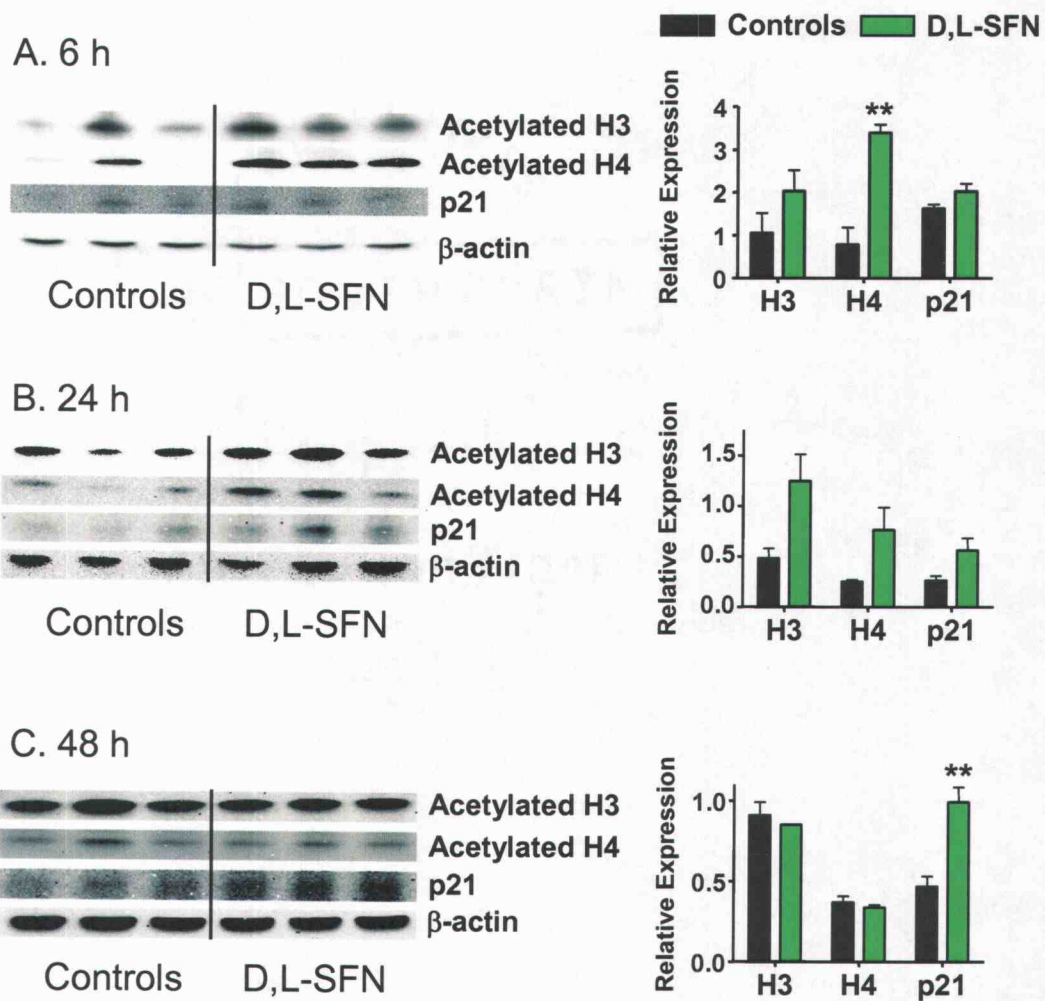
170

Figure 28.

A. 6 h

Acetylated H3
Acetylated H4
p21
β-actin

Controls   D,L-SFN

B. 24 h

Acetylated H3
Acetylated H4
p21
β-actin

Controls   D,L-SFN

C. 48 h

Acetylated H3
Acetylated H4
p21
β-actin

Controls   D,L-SFN

Figure 29.

A. Ileum

Acetylated H3

Acetylated H4

β-actin

Controls   D,L-SFN

Relative Expression

0.6
0.4
0.2
0.0

H3   H4

■ Controls
■ D,L-SFN

B. Colon

Acetylated H3

Acetylated H4

β-actin

Controls   D,L-SFN

Relative Expression

0.6
0.4
0.2
0.0
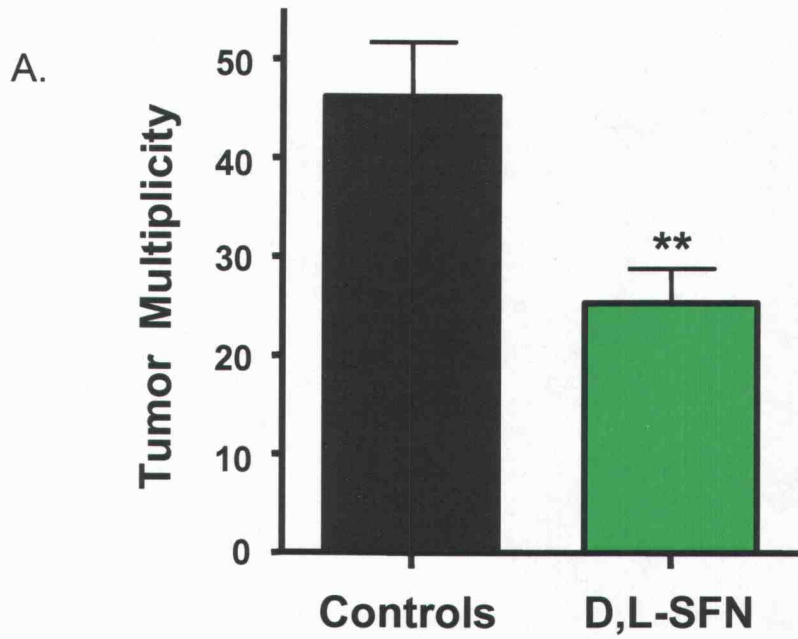
H3   H4

Figure 30.
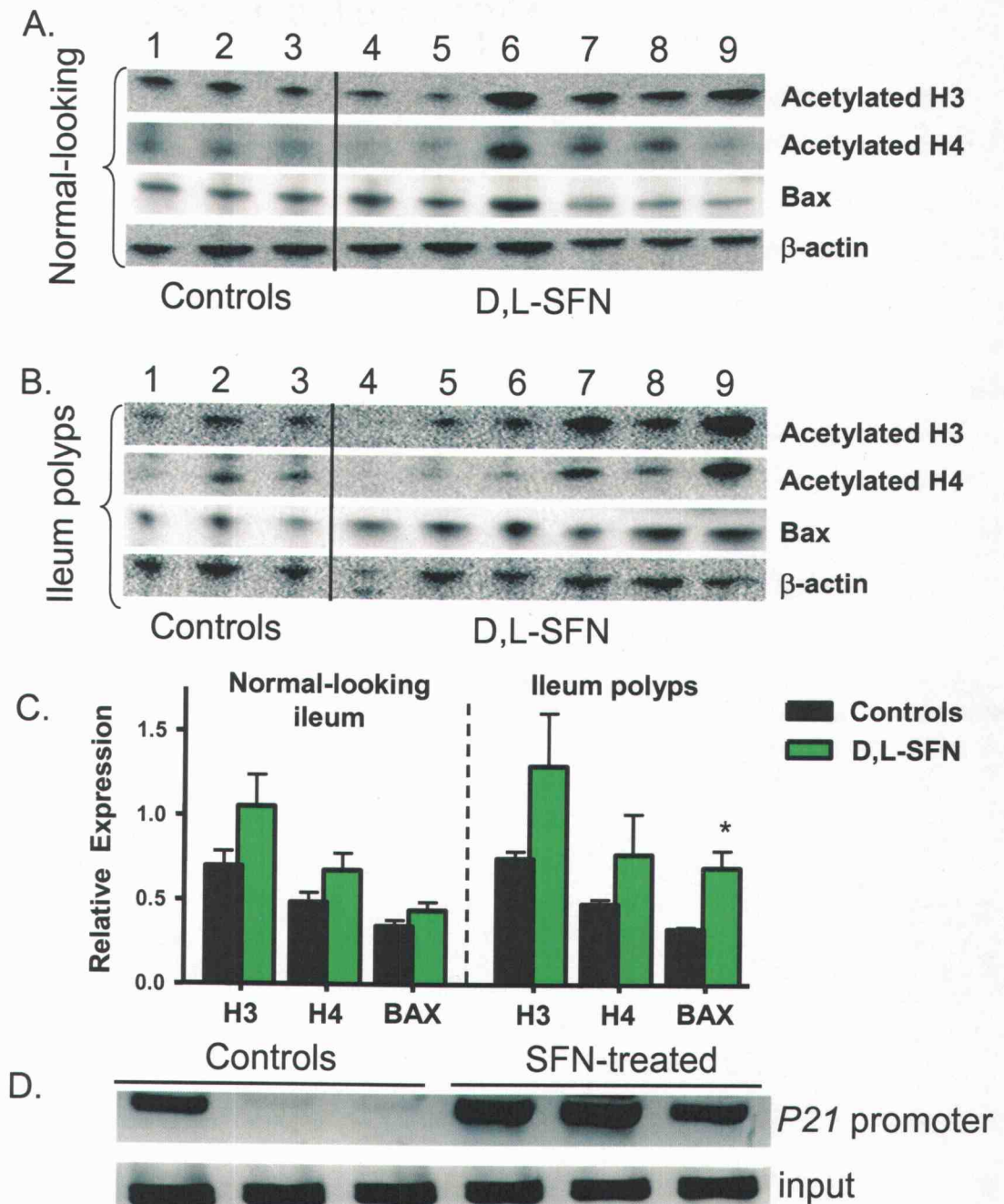
Figure 31.

A.

B.

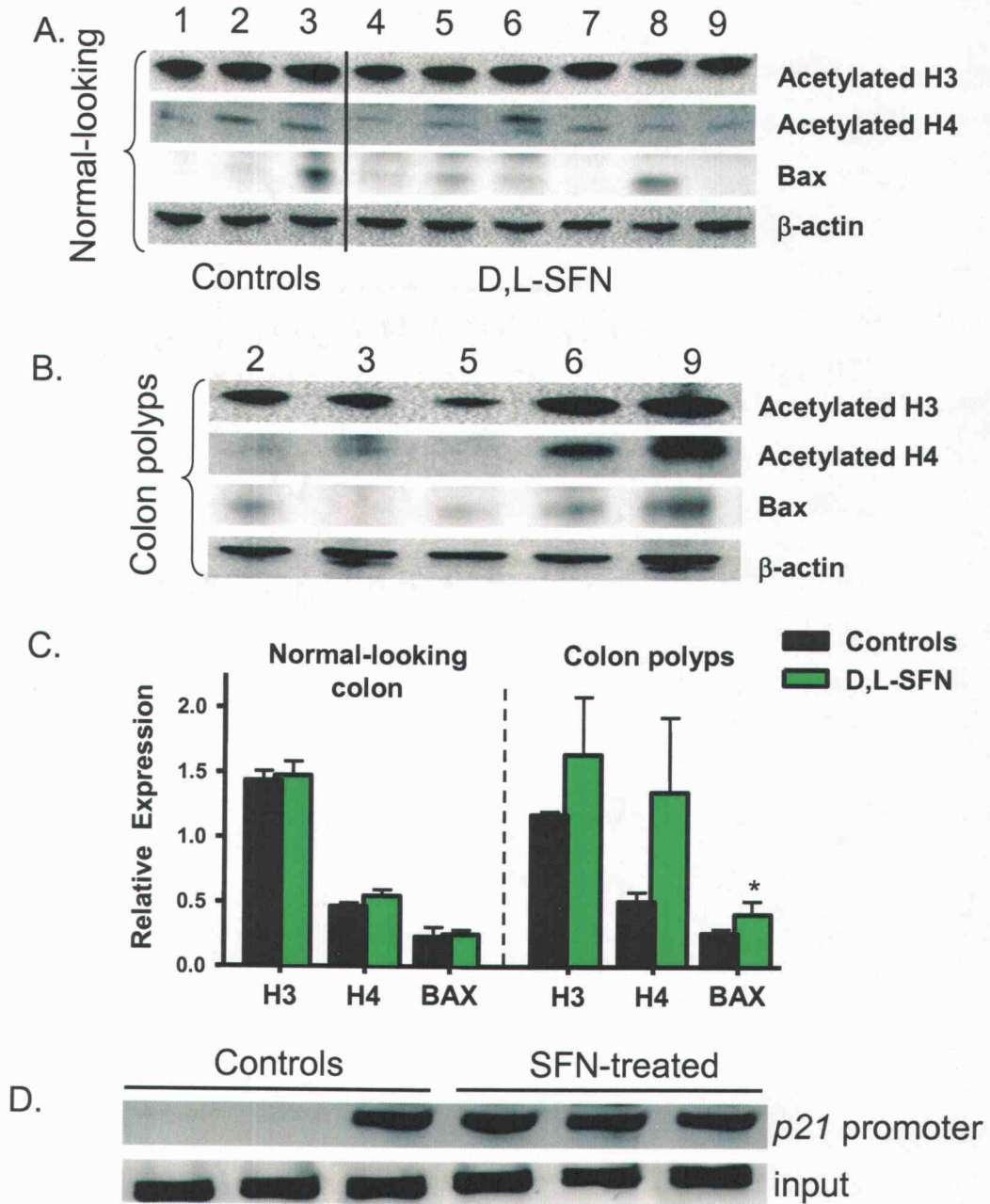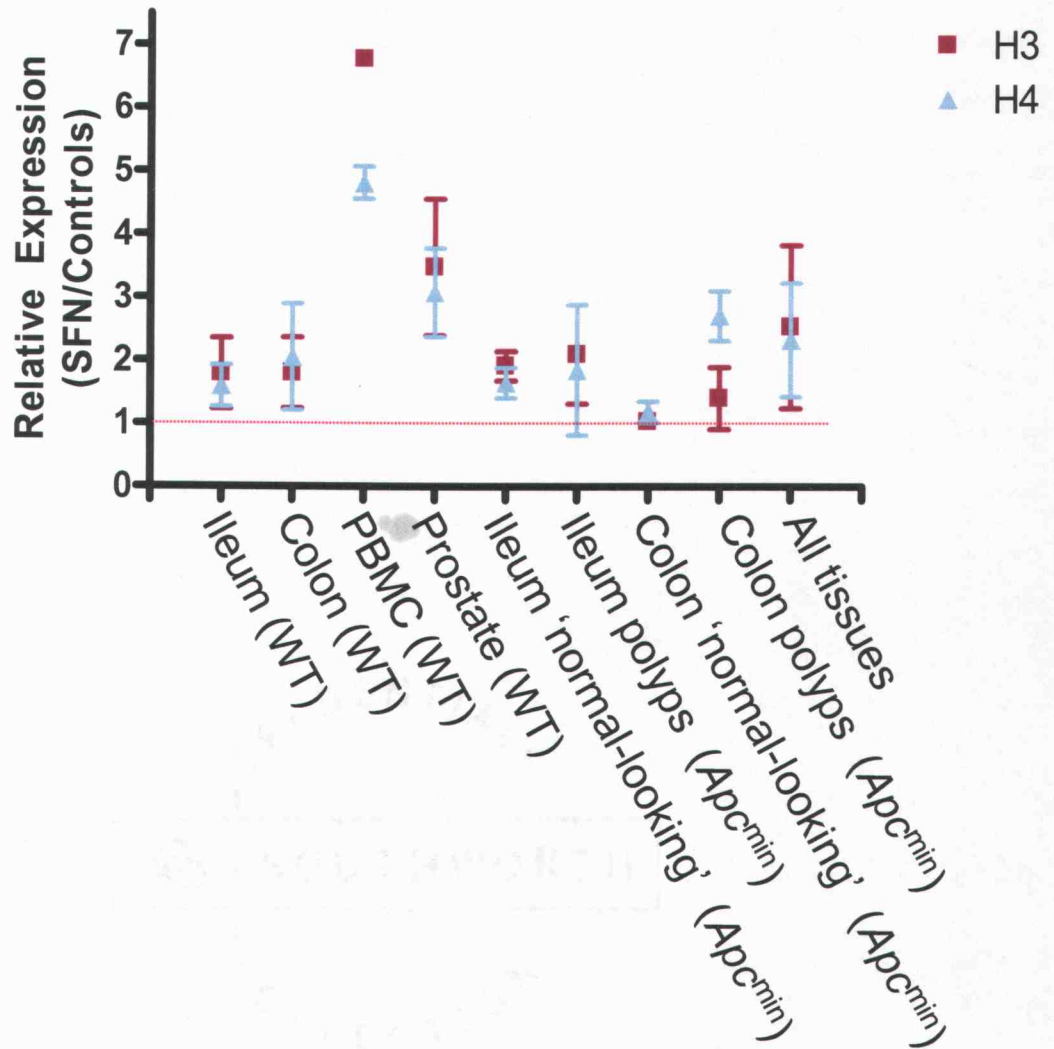Figure 32.

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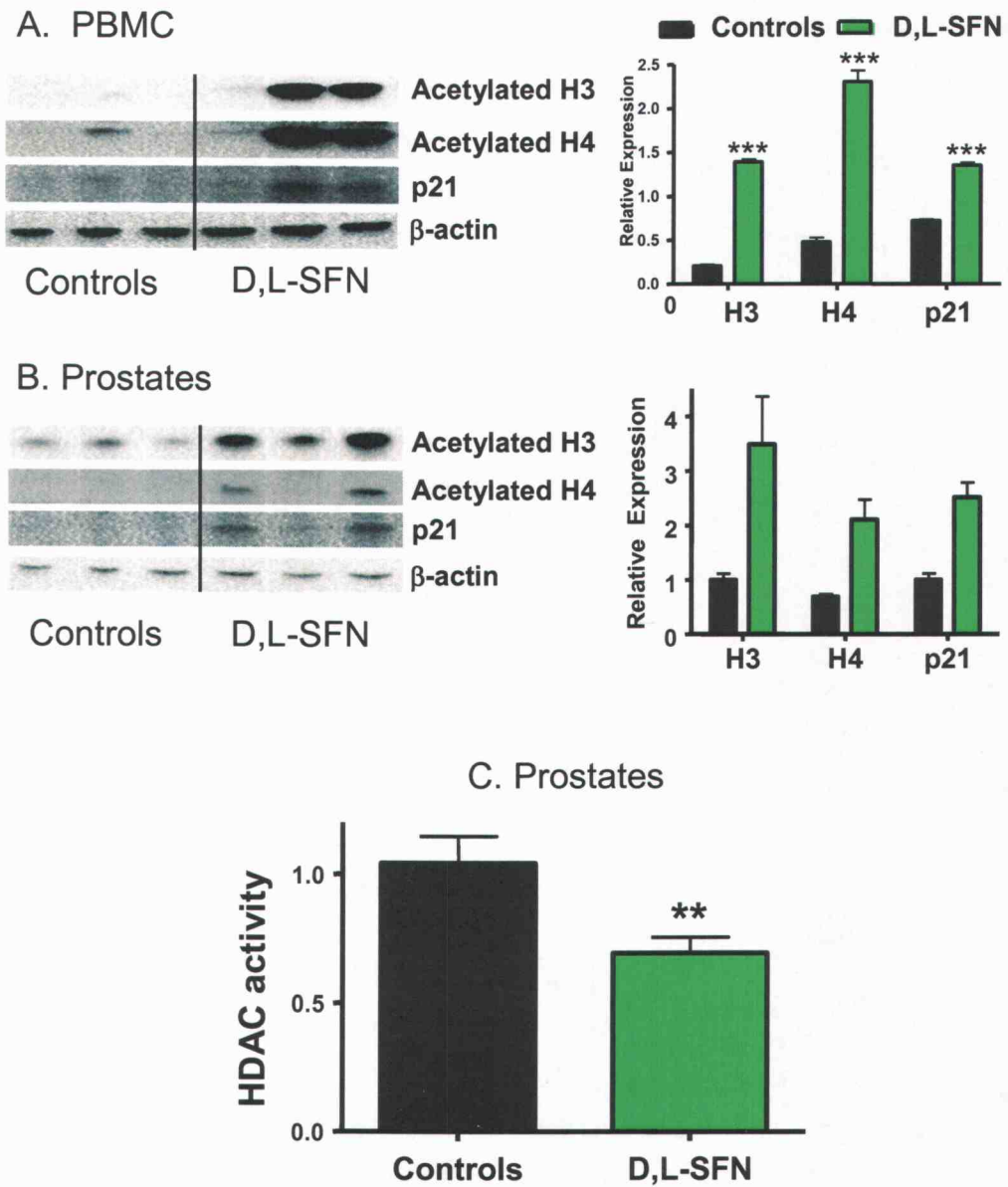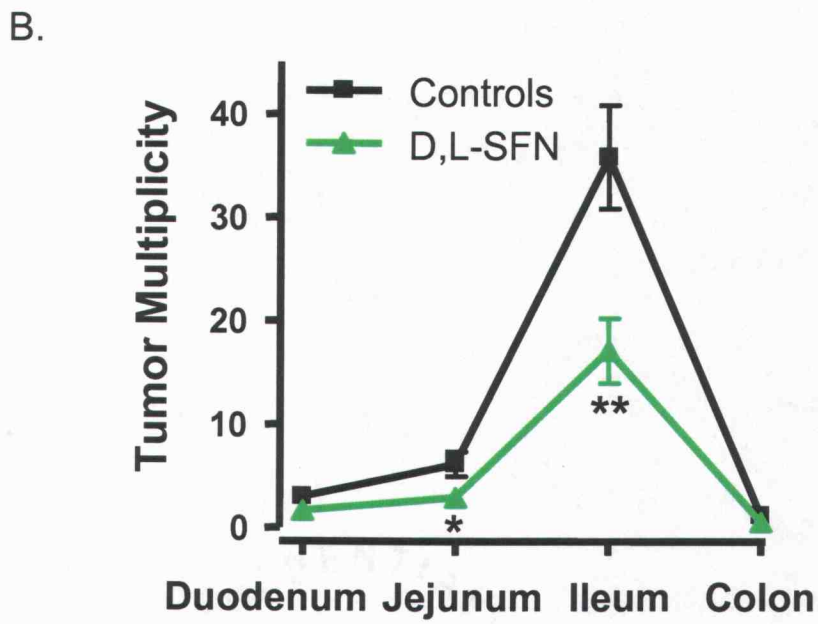
Figure 33.

Figure 34.

DISCUSSION

OVERVIEW and SUMMARY

The impetus for the studies described in this thesis came from an MCB lecture given by Dr. Mark Leid, in which HDAC inhibitors were mentioned as possible cancer preventive agents. A Medline/Pubmed search showed few if any studies published on HDAC inhibition by _dietary_ factors, although butyrate was mentioned as one such possibility (63, 64, 67). Structural considerations suggested SFN as a candidate HDAC inhibitor; as it turned out, this hypothesis was correct, but for the 'wrong' reasons. Specifically, it was the metabolite SFN-Cy that was identified as the likely HDAC inhibitor, rather than parent SFN, thanks in large part to discussions with Dr. Andrew Karplus (Department of Biochemistry and Biophysics).

Cell culture models initially were used in this thesis work; SFN inhibited nuclear and cytoplasmic HDAC activity in HEK293 cells, and at this point the thesis committee recommended experiments with SFN in cancer cells. In human colon cancer cells, SFN caused inhibition of nuclear HDAC activity, increased acetylation of histone H3 and H4, induced expression of p21$^{Cip1/Waf1}$, and there was accumulation of acetylated histone H4 associated with the _P21_ promoter.

These findings were extended into another type of cancer, namely prostate cancer, confirming that HDAC inhibition by SFN was not specific to one cell type or cancer type. In cells derived from benign prostate

contrast to results in HEK293 cells, where the combination of SFN+TSA resulted in greater inhibition of HDAC activity and greater accumulation of acetylated histones. *In vivo*, bioinactivation of TSA may have prevented combined (i.e. additive or synergistic) effects from being seen, although further studies are needed in this area. Importantly, however, the xenograft study provided proof-of-concept that inhibition of HDAC activity by SFN was feasible *in vivo*.

To confirm that SFN can inhibit HDAC activity in other tissues, and to examine the effects of short- and long-term administration of SFN, time-course and tumor prevention studies were performed. In the initial 'pilot' investigation, D,L-SFN and SFN-NAC increased acetylated histones H3 and H4, and inhibited HDAC activity 6 h post-gavage in colonic mucosa. Time-course studies revealed that 10 μmol SFN induced accumulation of acetylated histones as early as 6 h post-gavage, which were sustained for at least 24 h in colonic mucosa. Interestingly, SFN induced p21 in the colonic mucosa, beginning at 24 h post-gavage, and levels were maintained for at least 48 h. Long-term (10 week) dietary administration of SFN (~6 μmol/mouse/day) increased acetylated histones in the ileum, colon, PBMC, and prostates of wild-type mice. Moreover, p21 expression was induced in PBMC and prostates, and HDAC activity was inhibited in prostates. Importantly, dietary SFN suppressed tumor multiplicity in Apc^{min} mice significantly, and in the 'normal-looking' tissue and polyps from the ileum SFN

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induced histone acetylation and Bax expression (in polyps more so than 'normal-looking' tissue). Increased histone acetylation and Bax expression also were observed in the colon polyps.

Overall, the results from this thesis suggest that inhibition of HDAC activity by SFN is a viable mechanism, both *in vitro* and *in vivo*. HDAC inhibition by SFN results in apoptosis and cell cycle arrest in cultured human cancer cells, retards human prostate cancer xenograft growth, and suppresses intestinal tumorigenesis in the Apc^{min} mouse. It is worth noting, as a point of reference, the inhibition by other suppressing agents in the Apc^{min} mouse; potent NSAIDs such as sulindac (80 ppm in diet) (223, 225, 229) and Celecoxib (1500 ppm in diet) (231), as well as green tea and white tea (1.5% in drinking water) (223, 225), all inhibit to approximately the same degree as SFN (see figure below). The possible synergistic effects of combined agents warrants future attention, such as SFN plus tea or SFN plus NSAIDs.
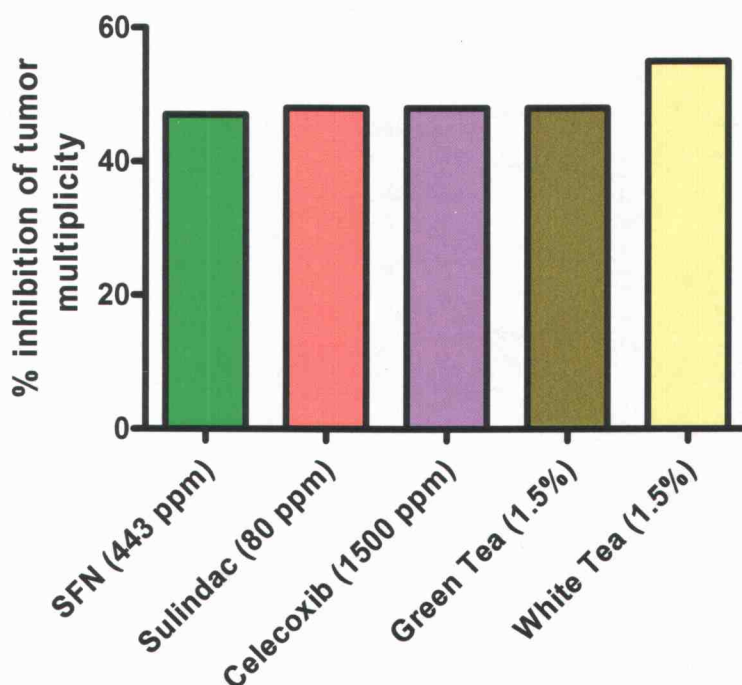
OTHER MECHANISMS

Although SFN inhibited HDAC activity, and increased global as well as localized histone acetylation on the promoters of genes such as *P21* and *BAX*, the possibility exists for other mechanisms to be involved *in vitro* and *in vivo*. For example, it is well established that SFN disrupts Keap1-Nrf2 interactions, allowing Nrf2 to enter the nucleus and induce transcription of ARE-containing gene promoters (185, 232). Nrf2 interacts with small Maf transcription factors at the ARE (131, 232); however, the presence of additional co-factors has not been elucidated. Moreover, no potential repressor complexes interacting with the ARE have been described.

Translocation of Nrf2 to the nucleus may disrupt co-repressor complexes or help to assemble co-activator complexes at AREs. Although a computer-based search suggested no ARE in the *P21* promoter, at least 10 kb upstream of the transcription start site, long-range changes cannot be ruled out. These may still induce *P21* expression and affect regulatory elements located kilobases away. One way to examine the role of Nrf2 in SFN-induction of *P21* or other apparent non-ARE-containing gene promoters is through the use of Nrf2 knockout cells/mice. A "chip on ChIP" experiment, that is, a microarray using DNA from a chromatin immunoprecipitation for acetylated histones, from Nrf2 knockout cells and normal cells treated with SFN may reveal specific target genes and may help elucidate the relative role each mechanism plays in regulation of transcription. Previous reports demonstrated that induction of Phase 2 enzymes in Nrf2 knockout mice by SFN was somewhat, but not completely, abrogated (137), suggesting that other factors may be involved in ARE-mediated gene expression. The latter studies examined transcripts, but not protein expression levels, of genes lacking ARE in their promoters.

Moreover, effects of HDAC inhibitors on promoters *containing* an ARE have not been investigated in any detail. A global increase in acetylated histones may increase expression of genes containing an ARE in their promoters. Thus, the potent induction of Phase 2 enzymes reported by SFN may be due to two mechanisms: disruption of the Keap1-Nrf2 complex with

translocation of Nrf2 to the nucleus and inhibition of HDACs present in co-repressor complexes interacting with the ARE.

FUTURE STUDIES

Based on the studies present in this thesis, it will be important to test the chemopreventive efficacy of SFN in an animal model of prostate cancer. Evidence from the investigations presented here suggests that SFN is bioavailable *in vivo* and can modulate HDAC activity in the prostate, but the effects of HDAC inhibition are unknown in terms of prostate cancer chemoprevention. The hormone-induced rat prostate cancer model and transgenic prostate cancer models, such as TRAMP mice, are potential systems in which to test the hypothesis that SFN prevents prostate tumorigenesis, at least in part, through HDAC inhibition, and to examine the relative contribution of other mechanisms, such as Nrf2 induction.

Also of significant interest is the extension of these findings into humans. Initially, HDAC activity and histone acetylation status in PBMC of human volunteers might be compared after ingestion of purified SFN, or broccoli sprouts, which are rich in the precursor to SFN called glucoraphanin. Based on the inter-individual variability in terms of HDAC inhibition and accumulation of acetylated histones in the *in vivo* studies from this thesis, PBMC probably should be compared before and after intake of SFN for each individual in human studies. Thus, each person would serve as their own

control, in a 'cross-over' design. In the future, clinical trials may be carried out to determine the preventive and therapeutic effects of SFN in humans in various cancer types.

Effects of long-term HDAC inhibition in humans have not been explored. It is possible that exposure to an HDAC inhibitor over a long period of time will result in a compensatory, recuperative response, whereby HDACs no longer respond to inhibitors or global histone acetylation is not altered by inhibitors. A 'cocktail' of HDAC inhibitors may be useful in that situation, to prevent development of resistance.

IMPLICATIONS OF DIETARY HDAC INHIBITORS

This work should give impetus to the search for other novel test compounds from the diet, and almost certainly some of these will be discovered to act as dietary HDAC inhibitors. The human diet is likely to contain many compounds with HDAC inhibitory activity; compounds containing a 'cap' group, 'spacer,' and cysteine conjugate at the other end may be a general structure for dietary HDAC inhibitors, and may include compounds metabolized through the mercapturic acid pathway. Some other possible dietary HDAC inhibitors, based on the somewhat conserved structure of HDAC inhibitors, include known anticarcinogenic isothiocyanates, as well as lipoic acid, biotin, and vitamin E metabolites. As the cellular and molecular mechanisms for individual HDACs are elucidated, the signaling

events that control HDAC activity will become more defined, and the role of aberrant histone acetylation in tumorigenesis further appreciated, including the opportunity to better target specific agents to specific HDACs. The discovery of novel dietary HDAC inhibitors that can be directed to specific HDACs will streamline approaches to chemoprevention and 'rational' chemoprevention strategies may be discovered.

Finally, dietary HDAC inhibitors may subtly 'tweak' HDACs throughout an individuals' lifetime. Since the human diet is variable, constant modulation of HDACs by dietary HDAC inhibitors may help to circumvent resistance that can arise with chronic administration of a single potent test compound. The diet contains numerous compounds that affect a myriad of pathways; as novel targets for chemoprevention are discovered and the mechanisms of specific dietary components are elucidated, a prescription for 'personalized chemoprevention' might be made, on a case-by-case basis, in the not too distant future.

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