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Dietary HDAC inhibitors: time to rethink weak ligands in cancer chemoprevention?

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Abstract

There is growing interest in the various mechanisms that regulate chromatin remodeling, including modulation of histone deacetylase (HDAC) activities. Competitive HDAC inhibitors disrupt the cell cycle and/or induce apoptosis via de-repression of genes such as *P21* and *BAX*, and cancer cells appear to be more sensitive than non-transformed cells to trichostatin A and related HDAC inhibitory compounds. This apparent selectivity of action in cancer cells makes HDAC inhibitors an attractive avenue for drug development. However, in the search for potent HDAC inhibitors with cancer therapeutic potential there has been a tendency to overlook or dismiss weak ligands that could prove effective in cancer prevention, including agents present in the human diet. Recent reports have described butyrate, diallyl disulfide and sulforaphane as HDAC inhibitors, and many other dietary agents will be probably discovered to attenuate HDAC activity. Here we discuss ‘pharmacologic’ agents that potently de-repress gene expression (e.g. during therapeutic intervention) versus dietary HDAC inhibitors that, as weak ligands, might subtly regulate the expression of genes involved in cell growth and apoptosis. An important question is the extent to which dietary HDAC inhibitors, and other dietary agents that affect gene expression via chromatin remodeling, modulate the expression of genes such as *P21* and *BAX* so that cells can respond most effectively to external stimuli and toxic insults.

Introduction

Chromatin remodeling—direct versus indirect HDAC inhibition

Considerable attention has focused on the silencing and unsilencing of genes through changes in DNA methylation (1), but such epigenetic modifications in DNA often require prior alterations at the level of the histones. The ‘histone code’ refers to an ever increasing complexity of histone modifications, including acetylation, methylation, phosphorylation, ubiquitination and biotinylation (2). There is growing interest in these post-translational changes and their implications for cancer development. Global loss of monoacetylation and tri-methylation of histone H4 is a common hallmark of human tumor cells (3). A recent commentary (4) also discussed novel protective functions of p53 associated with chromatin remodeling on a global scale, including transcriptional mechanisms that recruit or displace histone deacetylase (HDAC). Direct HDAC inhibitors also can affect changes in gene expression and impact on key regulators of apoptosis and the cell cycle (5–10), such as

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p21^{Cip1/Waf1}, cyclins (A, E, B1, D1 and D3), apoptosis mediators (CD95, Bax and Bcl-2), transcription factors (GATA-2, c-Myc) and retinoic acid receptors (RAR).

RARs are targets of retinoids, which have been reviewed extensively in terms of their promise and pitfalls for cancer prevention (11,12). One area of particular interest has been that of acute promyelocytic leukemia (APL), because APL patients respond to pharmacologic doses of retinoic acid with disease remission. Molecular studies have focused on the PML/RAR oncogenic transcription factor, and models have been developed to explain the therapeutic mechanisms of retinoids in APL (Figure 1)

(http://www.nature.com/nrc/journal/v1/n3/animation/nrc1201-181a_swf_MEDIA1.html, http://www.broad.mit.edu/chemobio/lab_schreiber/anims/animations/trich_retin.html). In brief, RAR/PML binding to the retinoic acid response element (RARE) recruits the CoR/SIN3/HDAC complex and represses transcription (Figure 1A), but agonists such as retinoic acid interact with the RAR and displace CoR/SIN3/HDAC (13), thereby activating gene expression in association with CoA/HAT complexes (Figure 1B). Resistance to retinoic acid treatment can occur in APL, due to the presence of a RAR–PLZF fusion protein and the inability to effectively displace HDAC (14,15). However, therapeutic efficacy in such cases can be improved when retinoids are combined with agents such as trichostatin A or suberoylanilide hydroxamic acid (SAHA), which interact directly with HDAC as competitive inhibitors (16–19) (Figure 1C). The latter compounds have provided valuable insights into the role of specific residues in the HDAC catalytic mechanism and the geometry of the substrate-binding pocket (6). Important structural features for HDAC inhibition appear to be a functional group that interacts with the buried zinc atom, a spacer or linker ‘arm’ that fits within the HDAC pocket, and in many (but not all) inhibitors a ‘cap’ group that sits just outside the active site (6–10).

HDAC inhibitors have been reported to disrupt the cell cycle in G₂, allowing cells to prematurely enter the M phase, as well as interfering directly with the mitotic spindle checkpoint (20). Cell cycle arrest and/or apoptosis is mediated through the de-repression of genes such as *P21* and *BAX*, and cancer cells appear to be more sensitive than non-transformed cells to the actions of HDAC inhibitors. The mechanistic basis for this apparent selectivity of action against cancer cells is far from clear, although recent studies have implicated thioredoxin and intracellular thiol status, the accumulation of reactive oxygen species, and induction of TRAIL (Apo2L, TNFSF10), DR4 and DR5 (21,22).

For the reasons alluded to above, HDAC inhibitors provide an attractive avenue for drug development, and considerable attention has focused on potent, high-affinity agents related to trichostatin A and SAHA (7–10). However, in the search for HDAC inhibitors with cancer therapeutic potential, we believe that there has been a tendency to overlook or dismiss weak ligands that could prove effective in cancer prevention, including agents present in the human diet.

Dietary HDAC inhibitors—weak ligands in cancer prevention

A recent review discussed the cancer chemopreventive properties of three reported dietary HDAC inhibitors (23), namely butyrate, diallyl disulfide (DADS) and sulforaphane (SFN). In general, these dietary agents are weak ligands and inhibit HDAC activity at higher concentrations than trichostatin A or SAHA, which are effective in the nanomolar to low micromolar range. A pertinent question, then, concerns the concentrations needed for inhibition of HDAC activity by dietary compounds, and the likelihood that these levels might be achieved under normal physiological conditions.

Butyrate is the smallest known HDAC inhibitor and contains a simple three carbon ‘spacer’ attached to a carboxylic acid group (Figure 2A). Based on the known structural features of the HDAC pocket (6), it is assumed that butyrate fully enters into the active site of the enzyme,

and that the carboxylate group of the short-chain fatty acid forms a bidentate ligand with the buried zinc atom. HDAC activity is inhibited at high micromolar to low millimolar concentrations *in vitro*, levels nonetheless considered to be achievable in the GI tract, where butyrate serves as the principal oxidative fuel for the colonocytes (24–27).

A second dietary agent reported to inhibit HDAC activity is the garlic compound DADS (28), which after metabolic conversion to *S*-allylmercaptocysteine (29) resembles butyrate in having a ‘spacer’ ending with a carboxylic acid functional group (Figure 2B). A similar spacer and carboxylic acid functional group also is present in SFN–cysteine (SFN–Cys) (Figure 2C), a metabolite of the compound SFN, which is found in broccoli and broccoli sprouts. Molecular modeling studies with SFN–Cys provided good support for complex formation with HDAC (Figure 3). Thus, the SFN–Cys *α*-amino group was positioned to H-bond with buried His residues in the enzyme pocket, and the SFN–Cys carboxylate group formed a bidentate ligand with the active site zinc atom. *In vitro*, SFN–Cys inhibited HDAC activity significantly in a cell-free system when tested at concentrations in the range 3–15 μM , whereas SFN parent compound had no effect on HDAC unless it was incubated with cells to allow for metabolism (30). Inhibition of the first step in the mercapturic acid pathway (see below) blocked the HDAC inhibition associated with SFN in human colon cancer cells (30), thus establishing the importance of metabolism for HDAC inhibition by SFN. As with most dietary agents, little is known about the concentrations of parent SFN or its active form(s) in different tissues (e.g. colon, prostate), or the temporal changes that occur following normal human dietary intake. In a study with four human volunteers who consumed broccoli sprouts (31), peak plasma concentrations were reported to be in the range 0.94–2.27 μM , based on an assay that detected total isothiocyanate and dithiocarbamate levels, rather than specific SFN metabolites.

The ‘spacer’ theory—predicting dietary HDAC inhibitors

Based on the SFN studies conducted to date, we propose that the ‘ultimate’ HDAC inhibitor is SFN–Cys, generated from the parent compound via the mercapturic acid pathway (Figure 4, lower left). In addition to SFN–Cys formed from SFN–glutathione (SFN–GSH), there may be removal of the acetyl group from SFN–*N*-acetylcysteine (SFN–NAC) by HDAC, leading to regeneration of SFN–Cys. The latter possibility is interesting, because it represents a mechanism-based (deacetylation) pathway, with the product reversibly inhibiting the enzyme. As mentioned above, direct addition of SFN parent compound to nuclear extracts *in vitro* had no effect on HDAC activity, and SFN–GSH also had minimal inhibitory activity, whereas SFN–NAC and SFN–Cys attenuated HDAC activity significantly (Figure 4A).

According to the working hypothesis, other reported ‘anti-carcinogenic’ isothiocyanates and allyl compounds from cruciferous vegetables and garlic (32–34) may be potential HDAC inhibitors, if they are metabolized via the mercapturic acid pathway and have the correct geometry to fit the HDAC pocket. We examined this possibility for several isothiocyanates, using a screening methodology in which HCT116 human colon cancer cells were treated with test compound and the corresponding cell lysates were added to an *in vitro* HDAC activity assay (Figure 4B). HDAC inhibition was seen, reproducibly, in cells treated with SFN, in contrast to the lack of inhibition obtained upon addition of parent compound to isolated nuclear extracts (compare SFN data in Figure 4A and B). Isothiocyanates with a similar ‘spacer’ length as SFN, such as sulforaphene, erucin and phenylbutyl isothiocyanate exhibited comparable HDAC inhibitory activities, as did compounds with a longer or shorter spacer, such as 6-SFN, 9-SFN, 6-erucin and phenethyl isothiocyanate. This is consistent with molecular modeling studies showing the HDAC active site occupied with the Cys moiety, and the isothiocyanate ‘cap’ group influencing accessibility to the binding pocket (Figure 3). Similar findings have been reported for structural analogs of trichostatin A, in which the spacer and hydroxamic acid group were retained while substituting the cap group (7–10). Interestingly, NAC and cysteine

alone had no effect when incubated with HCT116 colon cancer cells at the same concentration of 15 μM , and the cell lysates were used in the HDAC assay (Figure 4B).

We make a distinction here between HDAC inhibition at lower doses of SFN (<20 μM) and the effects reported more typically at higher concentrations. For example, we would expect little if any meaningful contribution of the HDAC mechanism in colon or prostate cancer cells treated with 40–100 μM SFN; SFN in this dose-range has been shown to generate reactive oxygen species, and activates a mitochondrial/Bcl-2-associated apoptotic pathway that was blocked by 1–10 mM GSH or NAC (35,36). The latter compounds were included ostensibly for their ‘antioxidant’ activities, but other mechanisms might be involved when cells are treated with a ~ 10–100-fold molar excess of GSH or NAC, compared with SFN. In human hepatoma cells, 1 mM NAC protected against Ni-induced histone hypoacetylation (37), but had no apparent effect on histone acetylation in HL-60 human leukemia cells (38).

Based on the model proposed, it is interesting to speculate about other putative HDAC inhibitors in the human diet. Retinoic acid (Figure 1B) has a cap group, spacer, and carboxylic acid functional group, and might be considered a candidate HDAC inhibitor. However, drug-resistant cases of APL involving the PLZF fusion protein respond to trichostatin A but not retinoic acid (Figure 1C). This might be due, in part, to poor fit between the retinoic acid and the specific HDAC that associates with PLZF, rather than all HDACs *per se*. Derivatives of retinoic acid are available (e.g. 9-*cis*, 11-*cis*, 13-*cis* and all-*trans*) with a different degree of flexibility and orientation in the spacer. As far as we are aware, retinoids have not been examined formally for fit in the HDAC pocket and for inhibition of enzyme activity.

As mentioned above, the HDAC inhibitor butyrate is a short-chain fatty acid, but there are numerous other dietary fatty acids, which in theory might gain access to the HDAC active site, with their terminal carboxylic acid group positioned adjacent to the zinc atom. One particularly interesting example is conjugated linoleic acid (CLA), which has anticarcinogenic and antiatherogenic properties, and enhances growth and feed efficiency, through mechanisms that are poorly understood (39). CLA in fact comprises several structurally-related isomers, and the *cis*-9, *trans*-11 and *cis*-10, *trans*-12 isomers have distinct biological activities, quite different from each other and from the *cis*-9, *cis*-12 isomer, linoleic acid (39). Interestingly, *c*10, *t*12 CLA decreases Bcl-2 and induces p21, a known HDAC target (39). The shape of the fatty acid tail is strongly influenced by the position and number of double bonds, which might influence access to the HDAC pocket. Other bioactive lipids also have a spacer and $-\text{COO}^-$ arrangement, such as the prostanoids; although not strictly ‘dietary’, they warrant consideration in terms of possible effects on HDACs.

Molecular modeling studies revealed that the Cys moiety fits well in the HDAC active site when attached to SFN, but no effect was seen on enzyme activity with free Cys (Figure 4B). Free amino acids from dietary sources are unlikely to substitute for Cys, owing to poor fit with the HDAC pocket, and because of the rapid turnover by aminotransferases during normal intermediary metabolism.

Other dietary compounds with the spacer and carboxylic acid functional group include biotin, which is noteworthy since it is used to modify histone tails as part of the histone code (see above), as well as the ‘antioxidant’ lipoic acid (40). Fat-soluble antioxidants such as α -tocopherol, γ -tocopherol and tocotrienols undergo ω -hydroxylation of the side chain and sequential rounds of β -oxidation, leading to carboxyhydrochroman derivatives with appropriate spacer lengths attached to a carboxylic acid group (41,42). Whether such metabolites would ever reach sufficiently high levels to affect HDAC activity in the nucleus is an open question, but ‘class II’ HDACs also reside in the cytoplasm and might be targets for inhibition (see next section).

Dietary HDAC inhibitors—a double-edged sword?

‘Classical’ HDACs include both class I and class II HDACs that are inhibited by trichostatin A (5–10). Class I HDACs are found exclusively in the nucleus and are expressed in most cell types, whereas class II HDACs shuttle between the nucleus and cytoplasm and appear to have a more restricted tissue distribution. Studies with potent agents related to trichostatin A and tapoxin have clearly established the concept of iso-form-specific HDAC inhibitors (10); however, there is much work to be done to clarify downstream signaling pathways for each HDAC family member and the response to dietary factors, alone as well as in combination, where synergistic or antagonistic effects might be anticipated. Tissue-specific metabolism and the levels of ‘ultimate HDAC inhibitor’, such as SFN–Cys, that might be achieved at the target site, further complicate this issue. In addition, certain dietary agents may increase rather than attenuate HDAC activity, as reported for theophylline in alveolar macrophages from patients with chronic obstructive pulmonary disease (43), and for resveratrol in the activation of human SIRT1 (44). The latter enzyme belongs to the recently discovered NAD⁺-dependent SIR2 family, designated as class III HDACs, which do not typically respond to trichostatin A. HDAC inhibitors thus have the potential to increase or decrease gene expression, and therapy or prevention based on such mechanisms is still largely a ‘black box’ awaiting further investigation.

Pauling often used the term ‘orthomolecular’ medicine (45,46), which in the context of the present discussion can be viewed as the regulation of genes by the right concentration of a dietary factor in order to maintain optimal cell growth, differentiation, and apoptosis, within a homeostatic norm. By this definition, we distinguish between ‘pharmacologic’ agents that potently de-repress gene expression (e.g. during therapeutic intervention) and dietary HDAC inhibitors that, as weak ligands, more subtly regulate the expression of genes involved in cell growth and apoptosis. An important question is the extent to which dietary HDAC inhibitors, and other dietary agents that affect gene expression in the context of chromatin remodeling [e.g. reversal of DNA hypermethylation by soy isoflavones (47)], modulate genes such as *P21* and *BAX* so that cells can respond most effectively to external stimuli and toxic insults. Studies with dietary HDAC inhibitors are in their infancy, but we believe it is important not to dismiss these agents simply because they are weak ligands; their ability to modulate gene expression in subtle ways and, through a lifetime of exposure, to impact cancer chemoprevention warrants further investigation.

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Abbreviations

APL	acute promyelocytic leukemia
CLA	conjugated linoleic acid
HDAC	histone deacetylase
RAR	retinoic acid receptors

RARE	retinoic acid response element
SAHA	suberoylanilide hydroxamic acid
SFN	sulforaphane
SFN–Cys	SFN–cysteine
SFN–GSH	SFN–glutathione
SFN–NAC	SFN– <i>N</i> -acetylcysteine

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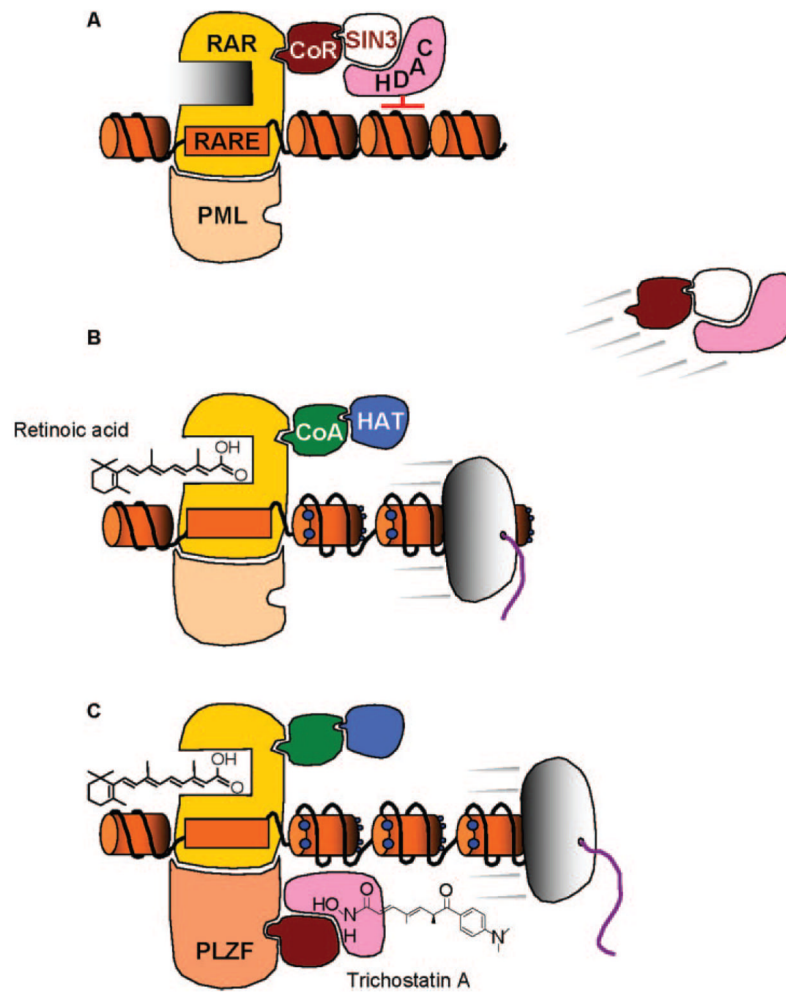


Fig. 1. Indirect and direct mechanisms of HDAC modulation, leading to de-repression of silenced genes. **(A)** Binding of the retinoic acid receptor (RAR) to the retinoic acid response element (RARE), in conjunction with the promyelocytic leukemia (PML) protein, recruits corepressor-Sin3-HDAC (CoR-SIN3-HDAC) complexes. HDAC removes acetyl groups from histones and causes chromatin condensation, leading to gene silencing. **(B)** Retinoic acid binds to RAR and induces a conformational change in the protein, leading to the release of CoR-SIN3-HDAC and recruitment of co-activator-histone acetyltransferase (CoA-HAT) complexes. HATs, such as p300 and CREB-binding protein, transfer acetyl groups (blue dots) to the histone amino-terminal tails, leading to nucleosomal repulsion, chromatin relaxation and gene transcription. **(C)** Resistance to retinoic acid treatment, as in the case of the RAR-PLZF (promyelocytic leukemia zinc finger) fusion protein and its associated CoR/HDAC complexes, can be circumvented through the use of competitive inhibitors of HDAC, such as trichostatin A.

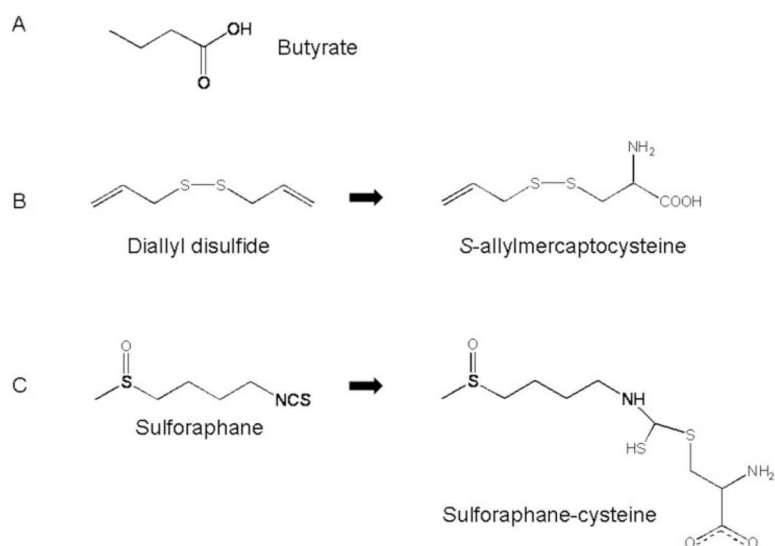


Fig. 2. Structures of dietary HDAC inhibitors. **(A)** The smallest known HDAC inhibitor, butyrate, contains a short three carbon ‘spacer’ attached to a carboxylic acid functional group. **(B)** The dietary HDAC inhibitor diallyl disulfide, which is present in garlic, can be converted to the metabolite *S*-allylmercaptocysteine, containing a spacer and a carboxylic acid functional group. **(C)** Sulforaphane, an isothiocyanate found in broccoli and broccoli sprouts, can be converted to the metabolite SFN–Cys, which has spacer and carboxylic acid functional that fits within the HDAC active site (Figure 3).

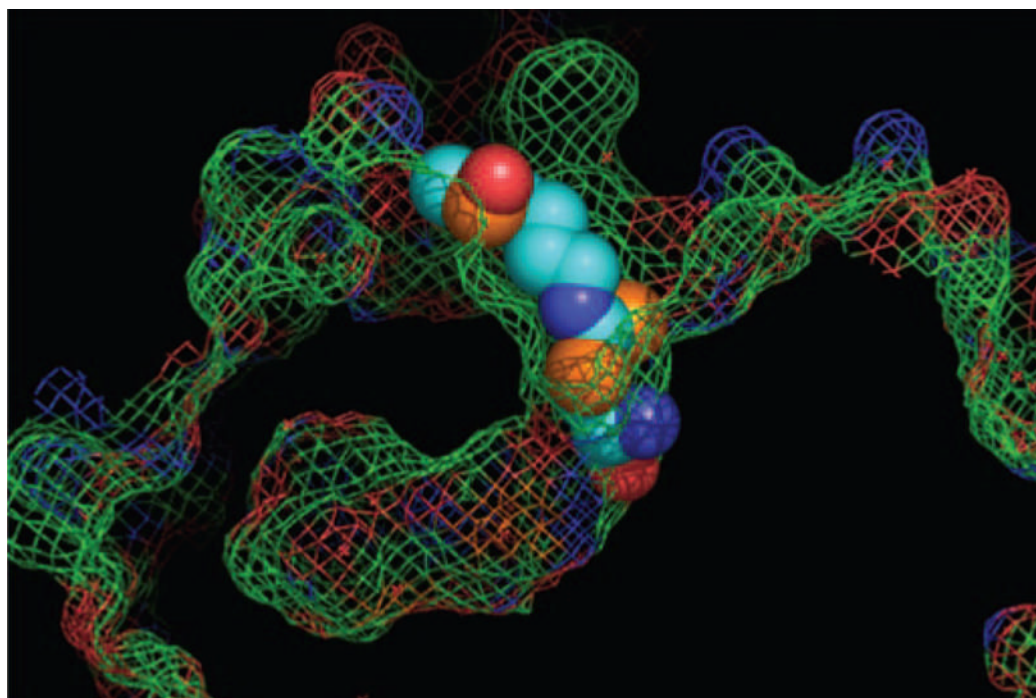


Fig. 3. SFN-Cys/HDAC interaction. Molecular modeling studies revealed a plausible interaction for SFN-Cys within the active site of an HDAC, with the carboxylate group of SFN-Cys positioned as a bidentate ligand with the buried zinc atom. See (30) for further details.

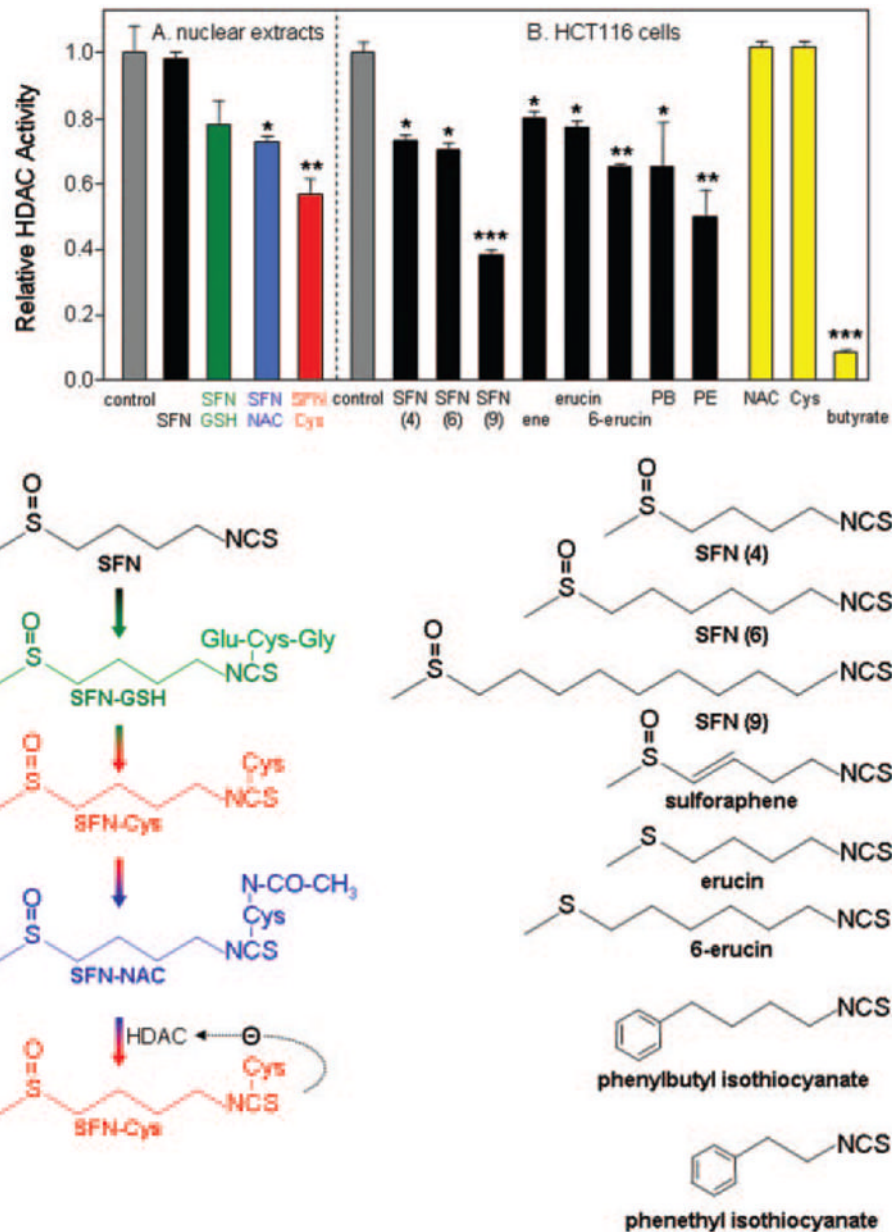


Fig. 4. HDAC inhibitory activity of sulforaphane (SFN) and structurally related isothiocyanates. **(A)** The mercapturic acid pathway converts SFN sequentially to SFN-GSH (SFN-glutathione), SFN-GSH to SFN-Cys (SFN-cysteine) and SFN-Cys to SFN-NAC (SFN-N-acetylcysteine). SFN-Cys formed from SFN-GSH, or after deacetylation of SFN-NAC by HDAC, leads to competitive enzyme inhibition, according to the working hypothesis (see text). Direct addition of SFN or SFN-GSH to isolated nuclear extracts *in vitro* had no significant effect on HDAC activity, whereas SFN-NAC (blue bar) and SFN-Cys (red bar) attenuated HDAC activity significantly. **(B)** Incubation of SFN with HCT116 human colon cancer cells followed by testing of the cell lysates revealed significant HDAC inhibition, as seen with several other structurally related isothiocyanates. All concentrations were 15 μ M, except butyrate which was 1 mM. Data = mean \pm SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, using Student's *t*-test versus the

corresponding control (gray bar). For details of the HDAC assay and other conditions, see (30).