Research Strategy (major revisions from the last submission are shown in Calibri font)

### Background and Significance

According to the American Cancer Society, about 142,820 people will be diagnosed with colorectal cancer and about 50,830 people will die of the disease in 2013, making it the third most commonly diagnosed cancer and the third leading cause of cancer death. A major contributor to colorectal cancer risk is diet. Beneficial effects of cruciferous vegetables such as broccoli, cauliflower, Brussels sprouts, watercress wasabi and cabbage have been attributed to their content of isothiocyanates (ITCs), compounds characterized by an N=C=S functional group. Our recent studies revealed that ITCs target the DNA damage response pathway through epigenetic mechanisms, which provides the basis for the current application and its central hypothesis.

Sulforaphane (SFN) and related ITCs inhibit histone deacetylase (HDAC) activity [2] and increase HDAC protein turnover [6], under conditions in which DNA damage signaling may be triggered. This leads to increased DNA double-strand breaks and checkpoint activation, culminating in cell growth arrest. HDAC inhibition by ITCs can enhance the acetylation of histone proteins as well as non-histone proteins, including those involved in DNA repair, such as CtIP, leading to their degradation and turnover. Notably, ITC-treated cancer cells are more susceptible than normal cells because the latter exhibit more efficient double-strand-break processing and repair. We propose to examine dietary HDAC inhibitors and the acetylation status of histone and non-histone proteins with critical roles in DNA damage and repair. As a corollary, the turnover/degradation of key players involved in DNA damage signaling will be assessed, along with phenotypic changes (cell cycle arrest and cell death pathways). The working model and its implications for enhancing the sensitivity of cancer cells to DNA damaging agents, is shown in Fig 1.

#### Need for improvement in colon cancer therapy

Colorectal cancer (CRC) is one of the leading causes of cancer-related death in the US. Beyond early detection and surgical removal of polyps, therapeutic approaches to CRC are currently inadequate. Improved treatments for CRC are needed for patients who are not detected early and/or respond poorly to standard therapies. The current treatment options for such patients include combining surgery with radiation and/or chemotherapy. Most chemotherapy protocols are based on the use of 5-fluorouracil (5-FU), which may be combined with other agents, such as Oxaliplatin [7]. A key mechanism of action of such drugs is through DNA damage pathways, but cancer cells can counteract these events by activating DNA repair [8]. This typically results in drug resistance and poor clinical responses. In the absence of alternative approaches, especially for high-risk patients with metastasis, there is an urgent need to define adjunct therapies that interfere with the DNA repair machinery and increase the efficacy of current treatments for CRC. In fact, DNA repair inhibitors, including those targeting poly(ADP-ribose) polymerase [9], have entered clinical trials for breast cancer. CAM natural products provide a new and potentially more effective avenue to address these concerns, in a mechanistically-defined fashion.

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Fig 1 Role of CAM natural products with HDAC inhibitory activity in altering protein acetylation and DNA damage signaling. Adapted from [5].
Modulation of the DNA damage response by reversible epigenetic mechanisms

This application addresses DNA damage and genomic instability in the context of chromatin remodeling. Histone deacetylases (HDACs) are chromatin modifiers that alter gene expression, but also exert a broader range of functions by deacetylating non-histone proteins. Recently, HDACs normally found to be overexpressed in cancer cells have been implicated in protecting such cells from genotoxic insults [5]. Recruitment and de-recruitment of HDACs, histone acetyltransferases (HATs), and binding partners at sites of DNA damage produces localized areas of open chromatin, increasing the potential for damage induced by UV, IR, and chemotherapeutic agents. HDAC inhibitors appear to facilitate cancer cell death by removing the protective effects of HDACs on DNA, creating an open chromatin state, enabling access to genotoxins, and inhibiting DNA repair mechanisms though modifying the acetylation status of key repair proteins (Fig 2).

CAM natural products with HDAC inhibitory activity modulate the DNA damage response

ITCs inhibit HDAC activity and cause histone hyperacetylation in cancer cells [1,11-14]. Recently, BITC [12] and SFN [6] were shown to decrease HDAC protein expression. HDAC3 was identified as the early 'sentinel' HDAC in SFN-treated colon cancer cells, resulting in turnover of the HDAC3/SMRT corepressor complex [6]. Other ITCs inhibit HDAC activity (Fig 3), and preliminary data support the loss of HDAC activity coinciding with HDAC protein turnover and histone hyperacetylation, increasing in potency with alkyl chain length (Fig 4). In some circumstances HDAC inhibition can trigger the DNA damage response [10]. ITCs also can exert genotoxic effects in cancer cells [16-18], as illustrated by comet formation and double-strand breaks (DSBs) in HCT116 cells (Fig 5). Because HDAC inhibition and turnover occurred under the exact same conditions (Fig 4), we examined CtIP expression in co-IP assays with anti-acetyl-lysine antibody; ITC-triggered loss of CtIP protein coincided with increased CtIP acetylation (Fig 6). The rationale for studying CtIP was as follows. HDAC inhibitors such as trichostatin A (TSA), vorinostat, and...
valproic acid (VPA) affect DNA repair proteins such as Ku70, involved in non-homologous end joining (NHEJ) [19,20] and CtIP, involved in homologous recombination (HR) [3]. HR is instigated by DSB-end resection [21,22], generating ssDNA through the combined actions of proteins that include CtIP [23,24] and BRCA1 [25]. The ssDNA is bound by Replication Protein A (RPA), leading to the formation of a ssDNA-RAD51 nucleoprotein filament that mediates HR [26]. CtIP plays a role in modifying cellular tolerance to anti-cancer drugs [27].

and was recently reported to be deacetylated by SIRT6 [4]. HDAC inhibition increased CtIP acetylation, enhancing its turnover [3]. Because ITCs also triggered CtIP acetylation and turnover (Fig 6), we knocked down HDAC3 (an early target of ITCs), and this recapitulated the increase in DNA damage (pH2AX) and CtIP hyperacetylation (Fig 7). Aim1 follows up on these findings with additional HDAC knockdown/rescue experiments. Importantly, we found that colon cancer cells were markedly more sensitive than normal colonic epithelial cells to SFN-induced DSBs, which went unrepaired in cancer cells, whereas normal colon cells repaired the damage more effectively and had reduced pH2AX levels (Fig 8). In addition, normal cells accumulated RPA phosphorylation on Ser4/8, a marker for resected DSBs (Fig 9). Thus, we hypothesize that SFN and other ITCs target cancer cells over normal cells via their differential susceptibilities to DNA damage, coupled with inadequate repair (Fig 10). This work provides a foundation for the stated central hypothesis and experiments in Aims 1-3. Chronic inflammation and inflammatory mediators either directly or indirectly affect DSB processing [53]. Thus, we will also test the central hypothesis in cells undergoing inflammatory/oxidative stress.

b Innovation

The innovative aspects of the application emerge from the fact that epigenetic modifications, which are potentially more druggable than genetic changes, are targeted through HDAC inhibition/turnover, protein hyperacetylation, and dysregulated DNA damage/repair mechanisms in cancer cells as compared to normal cells (Fig 10). Proteomic analyses of the acetylome will define novel targets of CAM natural products for further evaluation, including the first comparative studies in a newly established and clinically-relevant Pirc and DSS/Pirc animal model. In addition to the tumor suppression outcomes expected for ITCs alone and in combination with anticancer agents, normal versus inflammatory/oxidative stress versus cancer theme integrates all three aims. This will be facilitated by the use of tissue microarrays and laser-capture microdissection to examine mechanistically-defined molecular targets in Aims 1-3.

c Approach

Aim 1: Test the hypothesis that specific HDACs and/or sirtuins are targeted for turnover by dietary ITCs and/or their metabolites, resulting in dysregulated DNA damage/repair in colon cancer cells, but not in normal cells. In addition to known epigenetic targets, such as CtIP, novel candidates will be identified via LC-MS/MS analyses of the cellular acetylome.
Methods -

i. **Cell culture and test agents** Human colon cancer (HCT116, HT29, SW48 and SW480) and normal colonic epithelial cells (CCD841, FHC) will be obtained from the American Type Culture Collection (Manasas, VA, USA). Cancer cells in exponential growth will be seeded at 0.1x10^6 cells and cultured in a 60-mm dish in McCoy’s 5A medium (GIBCO-BRL). Normal cells will be seeded at 1x10^5 cells in Modified Eagle’s medium (GIBCO-BRL). PMA (10 ng/ml), LPS (5 µg/ml) and H2O2 (100 µM) obtained from Sigma will be incubated with cells to induce cellular stress. Growth medium will be supplemented with 10% FBS/1% penicillin-streptomycin. SFN and 6-SFN purchased from Toronto Research Chemicals, Ontario will be prepared as 50 mM stock solutions in sterile DMSO, diluted in sterile PBS and tested in a range of doses starting from 100 nm to 30 µM. Aliquots stored at −20 °C will be thawed for single use. Metabolite studies will initially focus on SFN, since the corresponding mercapturic acid standards and LC/MS methods are established and available under a separate grant (P01 CA090890, R. Dashwood, PD). Metabolites of 6-SFN will be identified as proposed in Aim 1B.

ii. **Overexpression and knockdown assays** HDAC3, HDAC6 and/or SIRT6 as transfection-ready DNA in vector, empty vector, siRNA (Trilencer-27) and control siRNA will be procured from Origene (Rockville, MD). Cells will be transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a ratio of 1:3-1:4 in reduced serum medium (OPTI-MEM, Invitrogen), using the manufacturer’s protocol.

iii. **Immunoblotting** Cyttoplasmic and nuclear extracts prepared using the NE-PER kit (Pierce, #78833) will be immunoblotted as described previously [6]. Briefly, proteins (10–20 µg) will be separated by SDS-PAGE on NuPAGE 4–12% Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membrane (Invitrogen). Equal protein loading will be confirmed by Amido Black staining and β-actin. The membrane will be blocked for 1 h with 2% bovine serum albumin, followed by incubation with primary antibody overnight at 4°C, and then probed with secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) for 1 h. Primary antibodies will be against HDAC1, HDAC2, HDAC3, HDAC4, HDAC6, HDAC9, HDAC10, SIRT1, SIRT3, SIRT6, pH2AX Ser139, CtIP, Ku70, pATR Ser428, pATM Ser1981, pBRCA1 Ser1524, pCHK1 Ser296, pCHK2 Thr68, H2AX, ATR, ATM, BRCA1, CHK1, CHK2, pKAP1 S824, KAP1, pRPA32 S4/S8, RPA32 and RAD51. Immunoreactive bands will be visualized via Western Lightning Plus-ECL Enhanced Chemiluminescence Substrate (Perkin Elmer, Inc, Waltham, MA) and detected via FluorChem-8800 chemiluminescence (Alpha Innotech imager).

iv. **Flow cytometry** Cell cycle analysis will be carried out as reported [6]. Briefly, cells in the exponential growth phase will be seeded at a cell density of 0.1 × 10^6 cells in 60-mm culture dishes and treated with vehicle or ITC test compounds. Adherent and non-adherent cells will be collected at selected time points (3, 6, 9, 24, 48, and 72 h) in cold PBS, fixed in 70% ethanol, and stored at 4°C for at least 48 h. Fixed cells will be washed with PBS and resuspended in propidium iodide (PI)/Triton X-100 staining
solution containing RNaseA. Samples will be incubated in the dark for 30 min before cell cycle analysis. DNA content will be detected using a capillary cytometer (Guava Technologies, Hayward, CA).

v. Comet assays Single-cell gel electrophoresis will be performed as reported [29]. In brief, ~10^6 cells treated with or without ITCs will be mixed with low melting agarose to form a cell suspension. After slide preparation, immersion in cold lysis solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10.0, 1% sodium sarcosinate, 1% Triton X-100, 10% DMSO) will be performed overnight at 4 °C. Slides will be placed in a horizontal tank filled with fresh solution (1 mM Na₂EDTA, 300 mM NaOH, pH 13.0) for 30 min, followed by electrophoresis at 0.8 V/cm for 30 min. After rinsing at 4 °C to neutralize excess alkali, slides will be stained with ethidium bromide, cover-slipped, and 25 randomly chosen nuclei per duplicate slide will be analyzed using a Nikon E400 fluorescence microscope linked to Comet Assay III software (Perspective Instruments, Suffolk, UK). Percent of comet tail area and length will be reported.

vi. ROS Generation Intracellular ROS generation in DMSO-treated control and ITC-treated cells at different doses (100nm – 30 μM) and time points (6-24 h) will be measured using the H2DCFDA assay [50]. Upon cleavage of the acetate groups by intracellular esterases and oxidation, nonfluorescent H2DCFDA is converted to the highly fluorescent 2',7'-dichlorofluorescein (DCF), measured using the Guava EasyCyte flow cytometer.

vii. Statistics Each experiment will be repeated at least three times. For SFN, 6-SFN, and selected key metabolites (see below), comparisons will be made between treated and non-treated cancer cells versus normal cells. Analysis of groups will be by an overall test (3 d.f.) followed by the three a priori contrasts. The specific method will depend on the nature of the response. For example, the response for the comet assay (number of damaged cells out of 50) could conditionally have the mean-variance relationship of an over-dispersed binomial distribution. The variation between replicates could be used to estimate the over dispersion in a quasi-likelihood analysis within a generalized linear model framework.

b) Metabolites of SFN (Fig 11) and 6-SFN may be more effective than parent compound in terms of HDAC turnover, CtIP acetylation, and DNA repair defects in cancer cells. We will use inhibitors of the mercapturic acid pathway, as reported (e.g., ethacrynic acid [1]), coupled with LC-MS/MS methodologies [52]. 6-SFN-Cys would be of interest based on predicted fit of the –Cys group in the HDAC pocket [1]. However, the new HDAC/corepressor turnover model [6] implies that other metabolites might be involved in HDAC turnover, CtIP acetylation/turnover and pH2AX induction, and distinguishable mechanistically from parent compound in terms of ROS generation.

Methods -

i. Identification of ITC metabolites ITCs and their major metabolites will be assessed in cells using the LC-MS/MS methodology reported [42-44]. Briefly, colon cancer and normal cell lines will be treated with SFN or 6-SFN for 6 h or 24 h and cell lysates and used cell media will be acidified with formic acid (0.5% v/v) and loaded on pre-conditioned Bond-Elut C2 cartridges (500 mg, 3 ml, Varian Inc., Walnut Creek, CA) followed by washing with 2 ml MeOH:water (5:95). Parent and metabolites will be eluted from the cartridges with MeOH and acetonitrile. Eluants will be dried under N₂ and reconstituted in 0.1% formic acid in acetonitrile for LC-MS/MS analysis. Preliminary data of SFN metabolites in HCT116 cells (Fig 12) predict -GSH, -CG, and –Cys as key intermediates for study in SFN and 6-SFN experiments. The major metabolites will be isolated and tested individually for effects on CtIP turnover, pH2AX levels, and ROS generation.
c) Lysine acetylation is a reversible posttranslational modification that plays a role in regulating gene expression and protein turnover/stability. There are over 1750 proteins with Lys acetylation sites so far identified [30]. Using LC-MS/MS (Fig 13) we will examine the acetylome of normal cells and colon cancer cells after incubating with SFN and 6-SFN, or following knockdown of selected HDACs. Identified target proteins will be further interrogated in normal and colon cancer cells via expression and functional analyses.

Methods -

i. **Cell treatments** with SFN and 6-SFN will be based on results from Aim 1A, optimized in terms of time and concentration. In a different set of experiments, cancer cells with siRNA knock-down of HDAC3, HDAC6, or both will be generated as described in Aim 1A.

ii. **Gel Electrophoresis** Cytoplasmic and nuclear extracts (NE-PER kit, Pierce, #78833) from cells will be subjected to gel electrophoresis as described in Aim 1A with each experiment repeated a minimum of 3 times. In addition, 2-D electrophoresis followed by immunoblotting will be performed, if required, to aid in more accurate identification of acetylated proteins, using methodologies described earlier [31,32]. To obtain acetylated proteins for LC-MS/MS, an identical duplicate SDS gel will be stained with Coomassie blue (Pierce, Rockford, IL) for 2 h at room temperature. Bands immunoreactive with anti-acetylated lysine antibody that are found to correspond to those in the immunoblot will be excised followed by in-gel digestion using Promega protease enhancer+trypsin (modified, gold) following the manufacturer’s protocol (Promega, Madison, WI). Peptides will be passed through Amicon Microcon centrifugal filters (10 kDa MWCO) to remove trypsin and membrane fragments before subjecting to LC-MS/MS analysis.

iii. **Identification of acetylated proteins** Proteomic analyses will be performed by OSU’s EHSC mass spectrometry facility and core. Protein digests from excised SDS-PAGE bands will be assessed based on published methods [31]. Tryptic peptides will be trapped on a Michrom Peptide CapTrap column and a C18 column (Agilent Zorbax 300SB-C18, 250 x 0.3 mm, 5 μm). A binary solvent system consisting of solvent A, 2% aqueous acetonitrile with 0.1% formic acid, and solvent B, acetonitrile with 0.1 % formic acid will be used. Peptides will be trapped and washed with 1% solvent B for 3min. Peptide separation will be achieved using a linear gradient from 3% B to 30% B at a flow rate of 4 μL/min over 35 mins. LC-MS/MS analysis will be conducted on a LTQ-FT MS instrument coupled to a Waters nanoAcquity UPLC system. The LTQ-FT mass spectrometer will be operated using data-dependent MS/MS acquisition with a MS precursor ion scan, performed in the ICR cell, from 350-2000 m/z with the resolving power set to 100,000 at m/z 400, and MS/MS scans performed by the linear ion trap on the five most abundant doubly or triply charged precursor ions detected in the MS scan. Thermo RAW data files will be processed with Proteome Discoverer v1.3.0. For database searching Mascot (v2.3) will be used to search the SwissProt database using the following parameters: the digestion enzyme will be set to Trypsin/P and two missed cleavage sites will be allowed. The precursor ion mass tolerance will be set to 10 ppm, while fragment ion tolerance of 0.8 Da will be used. Dynamic modifications that will be considered: carbamidomethyl (+57.0215 Da) for Cys, oxidation (+15.9994 Da) for Met and acetylation (+42.0106 Da) for Lys. Scaffold_3.3.1 (Proteome Software, Portland, OR) will be used for proteomic data compilation and data evaluation. Co-I Dr. Claudia Meier provided a support letter.

iv. **Immunoprecipitation (IP) of acetylated proteins** Results of the proteomics screen will be verified via IP assays, using the basic methodology reported previously [6]. In brief, nuclear or cytoplasmic extract (500 μg) will be precleared with 100 μl Protein A Sepharose CL-4B (Amersham Biosciences) on a rotator at 4°C for 1.5 h. Pre-cleared supernatant will be collected following overnight IP with anti-acetyl lysine antibody (10 μg/mg protein, Millipore, # AB3879). 100 μL of Protein A Sepharose beads will be added and samples allowed to rotate at 4°C for 1.5 h. Supernatants will be removed and the beads washed 3 times with PBST. Beads will be denatured in standard loading buffer and the proteins examined by immunoblotting, using commercially available antibodies for proteins identified in the...
proteomics screen. The stability and turnover of acetylated proteins, post-ITC treatment or following HDAC knockdown, will be monitored by immunoblotting as described in Aim 1A.

**Anticipated results, potential pitfalls, and alternative strategies** We expect to verify that specific HDACs (HDAC3, HDAC6, SIRT6) and their substrates (e.g., CtIP) will be targeted for efficient turnover in colon cancer cells but not in normal cells incubated with SFN, 6-SFN, and their metabolites. If differential uptake in cancer vs. normal cells is observed, mechanistic insights will be drawn based on intracellular (rather than applied) concentrations of SFN, 6-SFN, or the metabolite. Under severe oxidative stress conditions, even ‘normal’ cells might experience prolonged ROS-induced DNA damage which, in the presence of ITC-induced HDAC inhibition and CtIP turnover, goes unrepaired. This is a key question in the context of IBD, and preclinical studies under new Aim 2C. In this scenario, the hypothesis is that even oxidative stress-induced precancerous cells in the population will be removed by apoptosis, leaving truly undamaged normal cells unaffected. Protein targets with critical roles in DNA damage/repair, such as CtIP, plus others involved in inflammation and oxidative stress will be identified in proteomics studies based on hyperacetylated states and altered stability/turnover. This work will help to prioritize which specific DNA damage/repair pathways become most dysregulated in colon cancer cells vs. normal cells, combined with studies of pH2AX, ATM/ATR signaling, or mismatch repair players. Although HDAC3 was the early sentinel HDAC for SFN [6], it is possible that other HDACs/sirtuins will be important for 6-SFN, and this would be verified by time-course experiments and corresponding HDAC overexpression (‘rescue’) and knockdown studies, together with evaluation of phenotypic outcomes (cell viability/cell cycle/cell death assays, as reported earlier [6]). A potential pitfall is that the acetyome could reveal numerous target proteins of interest. We will focus initially on the targets with known (or suspected) relevance to the central hypothesis, and for which antibodies are commercially available. If one or more novel players arise in the acetyome as a ‘dominant signal’, knockdown and overexpression experiments will be used to probe their roles. HATs that regulate the acetylation of histone and non-histone proteins might themselves be regulated by reversible acetylation. HAT versus HDAC regulatory mechanisms of CAM natural products and their metabolites have received scarce attention, especially in the context of DNA damage/repair defects in cancer cells.

**Aim 2:** Using the polyposis in rat colon (Pirc) and Pirc/DSS models, define the ITC metabolite levels achieved in colonic mucosa and the relationship to HDAC turnover, CtIP acetylation, and antitumor activity. Sub-aims will use laser-capture microdissection, tissue microarrays, and LC-MS/MS to discern epigenetic changes in normal versus cancer cells.

**Rationale.** The adenomatous polyposis coli (APC) tumor suppressor is the ‘gatekeeper’ of CRC, and mutational or epigenetic silencing of the APC gene activates Wnt/β-catenin signaling [33]. In the Apcmin mouse model, we provided the first evidence that SFN and SFN-N-acetylcysteine (SFN-NAC) reduced HDAC activity and increased histone acetylation coincident with p21 induction and tumor suppression [2]. A major disadvantage of the Apcmin mouse is that the majority of polyps arise spontaneously in the ileum, with few in the colon. The Pirc model is the rat counterpart of the Apcmin mouse, but with the distinct advantage of developing tumors mainly in the colon. Specifically, the Pirc model develops colon tumors with 100% incidence by 4 months, and by 6 months of age 20% of tumors become invasive [34]. Importantly, loss of Apc function in this model involves haploinsufficiency and/or monoallelic epigenetic silencing [35]. Treatment with dextran sulfate sodium (DSS) further augments colon tumor formation [45], providing a new colitis model that we believe will better advance studies of inflammatory bowel disease (IBD) and the potential benefits of CAM agents acting via epigenetic mechanisms. Thus, integrating with Aim 1, Aim 2A will first determine the minimum effective single oral gavage dose of SFN and 6-SFN that inhibits HDAC expression leading to CtIP loss and induction of DNA damage responses, followed in Aim 2B by chronic administration of SFN and/or 6-SFN to demonstrate tumor suppression in the colon.
Previous rat studies with SFN showed that single oral gavage doses (200-800 μmol/kg; 35-140 mg/kg), or repeated doses (17-280 μmol/kg; 3-50 mg/kg), were safe [36-40]. There also was no toxicity in rats given broccoli sprout extract (BSE) at 40-160 μmol ITC/kg body weight daily for 14 days, when ~80% of the ITC dose was eliminated in the urine within 24 h [41]. Based on this information, we will start with a single oral dose of ~40 mg SFN/kg in the Pirc model and escalate the dose to attain significant HDAC inhibition and protein acetylation in the colon. Male and female rats will be examined for potential gender-related differences, including tissue metabolites such as -GSH, -CG, -NAC and -Cys, and molecular analyses will be performed on normal versus cancer cells in the colon.

a) ITCs will be dosed by oral gavage to identify the maximum tolerated dose (MTD), following OECD’s up-and-down procedure (Fig 14). At least 4 tumor bearing rats (> 4 months age) will be dosed at the MTD to determine parent and metabolite levels in target tissue (colon), using our standard LC-MS/MS methodology [42-44]. Single bolus administration at MTD, 0.5xMTD, and 0.1xMTD will be used to examine colon (and other) tissues 3, 6 and 24 h after SFN or 6-SFN treatment. The lowest effective dose based on HDAC activity, DNA damage and target acetylation status (histones, CtIP) will be selected for colon tumor suppression studies. In a pilot experiment, colon tumors and adjacent normal tissues were harvested from F344/NTac ApcPirc/+ males with an average of 12.8±5 tumors/animal (n=5) at 8-12 months of age. Tumors showed higher expression of HDAC3 and pH2AX compared with adjacent normal tissue (Fig 15 A,B). Six hours after a single ITC dose, there was reduced HDAC3 and HDAC6 expression in the tumors and increased pH2AX (Fig 15C). Densitometry data did not reveal a marked difference in potency among the ITCs, although the doses were probably not fully optimized.

b) For long-term studies in the Pirc model (Fig 16), animals of either sex will be fed AIN93 control diet or diet supplemented with SFN or 6-SFN at a dose predetermined from the pilot experiments. QA/QC studies on the diet will be performed on a monthly basis to confirm the correct concentration and stability of the test agent. We do not anticipate food aversion with SFN or 6-SFN, as sometimes seen with BITC, but if necessary pair-feeding will be performed for the corresponding controls. Littermates will be randomized to the dietary treatments. Body weight and food consumption will be monitored regularly. At sacrifice, blood will be obtained by cardiac puncture. Colon will be opened longitudinally, washed with PBS, and the tumors...
c) Treatment of Pirc rats with DSS increases the multiplicity of colon tumors (Fig 17). This study parallels Aim 2B exactly, except that rats at 1 month of age will be given 4% (w/v) DSS in the drinking water. There will be two rounds of DSS, each lasting 7 days and separated by a 7-day period without DSS, based on published studies in the Pirc model [45]. At the end of the DSS cycle, and an additional recovery of 1 week, 2-month old rats will be randomized to AIN93 or ITC diet. We will double the number of animals per group compared to Aim 2B, since about half of the DSS-treated rats experience weight loss caused by the high tumor burden [45]. Terminal studies will be carried out as in Aim 2B (Fig 16). Additionally, histological scores of epithelial damage and inflammation, nitrotyrosine and cytokine (TNF-α, IL-6) levels, will be monitored.

d) LC-MS/MS studies in Aim 2D will define for the first time the differential acetylomes in Pirc and Pirc/DSS models. As outlined in Fig 18, tumor and adjacent normal cells from frozen tissues will be separated by laser capture microdissection (LCM), solubilized, and run on a 2D gel. Protein spots will be analyzed by LC-MS proteomics to identify novel acetylated proteins, as in Aim 1C mechanism studies.

Methods -

i. Animal studies Rats are housed in standard caging with free access to water and AIN93 diet, minus t-butylhydroquinone (Research Diets, New Brunswick). F1 hybrid rats will be generated as published [45] by breeding ACI Apc+/+ females (Harlan, Indianapolis) to F344/NTac ApcPirc/+ males (Taconic, Hudson, NY). DSS will be purchased from Fisher Scientific (Pittsburgh, PA). SFN and 6-SFN from Toronto Research Chemicals, Ontario will be formulated in AIN93 diet at concentrations that provide daily doses of the range determined by the dose escalation study (Aim 2A).

ii. Immunoblotting Frozen samples of tumor and adjacent normal-looking colonic mucosa will be thawed, homogenized in lysis buffer (20 mM (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin), centrifuged at 15,000 rpm for 5 min, and the supernatant collected. Immunoblotting will be carried out as described in Aim 1. Initial work will probe HDAC3 (1:200, Santa Cruz, #11417) and pH2AX Ser139 (1:200, Santa Cruz, #101696). Anti-nitrotyrosine (06-284, Millipore), other HDACs/sirtuins, DNA damage/repair, and oxidative stress proteins will be prioritized from Aim 1, using the antibodies listed. Serum IL-6 and TNFα levels will be detected using Rat ELISA kits (Invitrogen).

iii. Colon biopsies and tissue microarrays (TMAs) Tumors and adjacent normal tissue will be harvested, fixed in formalin, and processed by standard techniques in the Epigenetic/Translational Biomarkers core (under P01 CA090890, R Dashwood, PD). Slides will be as reported [46,47], allowing for serial sections of each TMA block. One slide will be examined by routine H&E staining, whereas others will be processed for laser capture microdissection (LCM) of stroma and adenocarcinoma cells using a Zeiss PALM MicroBeam IV Laser Capture System (Carl Zeiss, Thorn-wood, NY) as shown in Fig 19. In addition, some slides will be incubated with primary antibody, starting with HDAC3 (1:200, Santa
Cruz, #11417) and pH2AX Ser139 (1:200, Santa Cruz, #101696). As samples permit, other HDACs, DNA damage/repair proteins, and oxidative stress mediators will be assessed using antibodies listed in Aim 1. Immunostaining will be performed in a Dako Autostainer Universal Staining System, with detection via Nova Red/hematoxylin. As necessary, high temperature antigen retrieval with BDTM Retrieval A solution (Dako, Carpantaria, CA) may be employed. Control slides will be stained with Dako Universal Negative Mouse or Rabbit. Percent cells and total area staining positive will be recorded in a given field (reader blinded to treatment group), and data then entered into a spreadsheet. Sections of snap-frozen tissue also will be processed for TMA slides, LCM, and qPCR, as reported [46].

iv. Tissue metabolites of SFN and 6-SFN in colon tissues will be assessed using LC-MS/MS [42-44]. Please refer to the text adjacent to Fig 12.

v. Identification of acetylated proteins LCM of frozen sections of colon tissue will separate adenocarcinoma regions from stroma and normal-looking mucosa. Tissue lysates will be trypsin digested and subjected to 2D gel electrophoresis/proteomic analyses, as in Aim 1C.

vi. Statistics Comparisons between control and ITC-diet will be made for each response of interest with the analysis method depending on the nature of the response. Tumor incidence of 100% is expected in both groups based on previous reports [34,45]. Colon tumor multiplicity (number of tumors/tumor bearing animal) will be compared using methods appropriate for counts of this nature. Our previous study in the Apc^min mice has shown that SFN inhibited tumor multiplicity by ~50% [2]. In a recent Pirc study using the same strain of rats [45], males and females in control groups had means of ~ 30 and 6 tumors, respectively. Further, variance was roughly proportional to the mean (ratio 1.1 - 1.3). Assuming similar multiplicity (30 ± 1.3), 15 male rats/group will give high (>95%) power to detect even a 30% reduction in multiplicity. Because females are expected to have lower multiplicity (6 ± 1.3), ~35 female rats/group will have >80% power to detect a 30% reduction. For detecting a larger reduction (e.g., 50%), fewer female rats (e.g., <15) would be required to have the same power. Tumor volume initially will be analyzed using linear models, possibly on the log transformed scale. Non-parametric models will be used when necessary. Models will include litters as blocks and, if effective, the power could be increased. The final data will be analyzed in collaboration with statistician Dr. Pereira (see letter of support). Additional rats were included in the Pirc/DSS studies in anticipation of likely weight loss, and to provide enough tissue for the proposed molecular analyses.

Anticipated results, potential pitfalls, and alternative strategies We expect (i) to define a dose of SFN and 6-SFN that following single oral gavage in the Pirc model inhibits HDAC activity, lowers HDAC3 and/or HDAC6 expression, and induces DNA damage and acetylation of histone and non-histone proteins (e.g., CtIP) in the colon; (ii) to provide the first evidence in the Pirc and Pirc/DSS models for tumor suppression by two different dietary ITCs, together with predicted molecular changes according to the central hypothesis; (iii) to further delineate the differential effects of SFN and 6-SFN on normal versus cancer cells, in Pirc colon biopsies; (iv) to relate epigenetic molecular changes to key tissue metabolites, such as -NAC and -Cys (integrating with Aim 1); and (v) to define novel inflammation-associated biomarkers in Pirc versus Pirc/DSS colon tissues, based on differential protein acetylation status. Liver tissue and metastases also will be examined, since the Pirc model provides the added advantage of testing the central hypothesis (cancer versus normal cells) in a separate tissue from the primary site.
Because the samples available from LCM will be limited, we will prioritize molecular targets based on Aim 1 findings. Most likely, HDAC3 and/or HDAC6, plus pH2AX would be first priorities. It is possible that HDACs other than HDAC3 and HDAC6 will be implicated in the inhibition/turnover studies, or that targets other than histones and CtIP will be involved. We would integrate with Aim 1, in particular the acetylome data, to investigate targets implicated in chronic inflammation, oxidative stress, and colon cancer in vivo. It is conceivable, though unlikely, that SFN and 6-SFN will exhibit poor efficacy in the Pirc model, in which case we would use 9-SFN (Fig 15). Alternatively, dosing by gavage, longer ITC treatment, and/or switching to Pirc on other genetic backgrounds could also be considered, and then carried through Aim 3.

Aim 3: Test the hypothesis that ITCs chemo-sensitize human colon cancer cells to conventional anticancer agents, due to intrinsic DNA damage defects. Integrating mechanistic and preclinical findings from Aims 1 and 2, SFN and 6-SFN will be tested in combination with standard DNA damaging anticancer drugs, to define conditions for optimal HDAC turnover, CtIP acetylation, and increased pH2AX. The best ITC+drug combination will be tested in Pirc or Pirc/DSS models.

**Rationale.** The rationale for combination therapies was provided under Background & Significance. Aim 3 provides a formal test of the central hypothesis using standard chemotherapy drugs+ITCs.

a) ITC+drug combinations will be evaluated *in vitro*, including the timing of ITC treatment (pre-, co- or post-incubation) relative to drug exposure. Cell viability (CCK-8 assay) and growth will be used to determine combination index (CI) values [48], based on the isobologram, and synergistic, additive, or antagonistic outcomes. The most promising combinations will be further evaluated in terms of HDAC turnover and DNA damage markers. A pilot study with SFN+Mitomycin C showed enhanced pH2AX, cleaved PARP, and cleaved caspase-3 in HCT116 cells, although HDAC3 turnover was equally effective with SFN alone (Fig 20). ITC+drug combinations will be tested in cancer, normal, and normal + oxidative stress conditions, as in Aim 1A.

**Methods**

i. **Chemicals** Drugs used clinically to treat colon cancer and known to work through induction of DNA damage, *i.e.*, 5-fluorouracil, Oxaliplatin, Irinotecan and Cisplatin, will be purchased from Sigma (St. Louis, MO). Drugs will be diluted and stored as stipulated in the Material Data Safety Sheet. SFN and 6-SFN will be sourced as described in Aim 1.

ii. **Chemosensitivity** Cell lines (see Aim 1) will be exposed to serial dilutions of SFN or 6-SFN and drugs spanning the IC<sub>50</sub> values, nominally Cisplatin (2 μM), 5-FU (6.16 μM), Oxaliplatin (1.24 μM), or Irinotecan (2.57 mM). Cells also will be pretreated with either PMA (10 ng/ml), LPS (5 μg/ml) or H<sub>2</sub>O<sub>2</sub> (100 μM) for 24 h followed by ITC+drug treatment. Co-incubation and sequential treatment regimens will further define the most effective dosing schedule for chemosensitization. After 48 h, cell viability will be determined (CCK-8 assay, Dojindo, Rockville, MD). Additional assays will test reversibility after drug removal.

iii. **Data Analysis** Cytotoxic IC<sub>50</sub> values will be determined from log concentration-effect curves in GraphPad Prism, using nonlinear regression analysis on three independent experiments. To test combination effects, data will be analyzed using the median-effect method [48], utilizing CalcuSyn version 2 software (Biosoft, Cambridge, UK). Each concentration-response curve (individual agents as well as combinations) will be fitted onto a linear model using the median effect equation, allowing calculation of a median effect value. The formula will be applied CI = d1/D1 + d2/D2, where D1 and D2 represent the concentration of drug 1 and 2 alone, required to produce a certain effect, and d1 and d2 are the concentration of drugs 1 and 2 in combination required to produce the same effect.

iv. **DNA damage assessment** ITC+drug combinations will be tested in HCT116 cells grown in 60 mm tissue culture dishes, followed by immunoblotting for pH2AX and comet assays, as in Aim 1.
b) From Aim 3A, combinations that best enhanced DNA damage in cultured colon cancer cells and/or cells exposed to oxidative stress will be tested in the *Pirc* or *Pirc/DSS* model, respectively. The study design (Fig 21) nominally uses SFN in combination with 5-FU. Actual test combinations and inclusion of metastasis arm of the study will be guided by Aim 3A. Tissues from the combination treatment will be examined for molecular changes in HDACs, protein acetylation, inflammation, and DNA damage biomarkers in Aim 3C.

Methods (as in Aim 2B) -

i. **Animal treatment** Rats will be given saline or with 5-FU i.v. at a dose of 50 mg/kg once a week for 4 weeks, a clinically relevant schedule that was efficacious in a rat colon cancer model [49]. ITC dose will be based on data from Aim 2.

ii. **Chemosensitization** Treatment will be stopped after 4 months followed by terminal studies as described in Aim 2. Possible toxicity will be monitored by body weights, and by histopathological analyses of blood, kidney, liver, colon and other organs at sacrifice.

vii. **Statistics** Results from Aim 2B or 2C will inform on the planning and number of rats assigned. For multiplicity, using the same assumptions as in Aim 2B regarding control mean and variance, a sample size of 15 rats/group will give >90% power to detect an additional 30% reduction due to a combination of agents relative to an initial 30% reduction caused by a single agent. Nominally, 20 rats will be used in the combination arm(s) to provide sufficient tumor tissues for molecular analyses, anticipating a greater degree of tumor suppression than for either agent alone. The final data will be analyzed in collaboration with statistician Dr. Pereira (see letter of support).

**Anticipated results, potential pitfalls, and alternative strategies** According to the central hypothesis (i) ITCs will synergize with drugs to enhance DNA damage in colon cancer cells, and in colonic epithelial cells under oxidative stress, but not in normal cells; (ii) this will be correlated with HDAC inhibition/turnover and increased acetylation of histone and non-histone proteins, such as CtIP; (iii) in the *Pirc* or *Pirc/DSS* model, ITC+drug combination will be more effective for tumor suppression than either agent alone; and (iv) the DNA damage response from the *in vitro* studies will be recapitulated *in vivo* in target tissue (colon), and will be greater in adenocarcinoma cells and cells under oxidative stress than surrounding stroma and normal colonic mucosa. Descriptive correlations from *in vivo* experiments with ITC+drug combination treatments will lead to further mechanistic work *in vitro*, including the use of knockdown and/or overexpression strategies as outlined in Aim 1. At this point, there is no specific plan to move into a knockout model (e.g., HDAC3 loss targeted to colon), but this could be pursued later. It is possible that the proposed combination of SFN+5-FU (Fig 21) will be substituted with a different ITC+drug combination for use in the *Pirc/DSS* model, especially if prior aims support a novel epigenetic molecular target that is a better candidate for clinical translation than HDAC3, HDAC6, or CtIP.

**Tentative schedule for completing the Specific Aims**

<table>
<thead>
<tr>
<th>Aim</th>
<th>Year 1</th>
<th>Year 2</th>
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<th>Year 4</th>
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<tr>
<td>Aim 1A</td>
<td>HDAC turnover and DNA damage, CtIP acetylation</td>
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<td>Aim 1B</td>
<td>ITC metabolite profiling</td>
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<td>Aim 1C</td>
<td>LC-MS/MS studies to probe acetylation</td>
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<td>Aim 2A</td>
<td>Dose-escalation</td>
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<td>Aim 2B</td>
<td>Anti-tumor activity in Pirc model</td>
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<td>Aim 3A</td>
<td>Anti-tumor activity in DSS/Pirc model</td>
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Fig 21 ITC+drug combination in *Pirc* rats, with or without DSS treatment starting at 1 month.