Chemopreventive Effects of an HDAC2-Selective Inhibitor on Rat Colon Carcinogenesis and APC^{min/+} Mouse Intestinal Tumorigenesis[§]

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ABSTRACT

Epigenetic modulators, particularly histone deacetylases (HDACs), are valid targets for cancer prevention and therapy. Recent studies report that HDAC2 overexpression is associated with colon tumor progression and is a potential target for colon cancer prevention. This study tested chemopreventive and dose-response effects of Ohio State University HDAC42 (OSU-HDAC42), a selective HDAC2 inhibitor, using a rat colon carcinogenesis model to assess aberrant crypt foci inhibition and a familial adenomatous polyposis model to assess intestinal tumor inhibition. Colonic aberrant crypt foci were induced by azoxymethane (AOM) (15 mg/kg body weight, once-weekly subcutaneous injections at 8 and 9 weeks age). One week after AOM treatment, groups of rats were fed an AIN-76A diet containing 0, 75, 150, and 300 ppm OSU-HDAC42 for 8 weeks, and colonic aberrant crypt foci were evaluated. To assess the inhibitory effect of OSU-HDAC42 on small-intestinal polyps and colon tumor growth, 6-week-old male C57Bl/6J-APC^{min/+} mice were fed an AIN-76A diet containing

150 ppm OSU-HDAC42 or 300 ppm pan-HDAC inhibitor suberoylanilide hydroxyamic acid (SAHA) for 80 days. Our results demonstrate that dietary OSU-HDAC42 produced dose-dependent inhibition of AOM-induced colonic aberrant crypt foci formation (13–50%; $P < 0.01$ to < 0.0001) and reduced multiple crypts with ≥ 4 crypts per focus (25–57%; $P < 0.01$ to < 0.0001) in F344 rats. Our findings show that 150 ppm OSU-HDAC42 significantly inhibited small-intestinal polyps ($>46\%$; $P < 0.001$), with polyp size measuring >1 mm ($P < 0.001$), and colon tumors ($>26\%$) in APC^{min/+} mice, whereas 300 ppm SAHA showed nonsignificant inhibition. Mice fed 150 ppm OSU-HDAC42 had significantly decreased HDAC2, proliferating cell nuclear antigen, B cell lymphoma 2, cyclin-dependent kinase 2, and cell division cycle homolog 25C expression levels and increased p53 expression levels. These observations demonstrate the chemopreventive efficacy of OSU-HDAC42 against chemically induced and polyposis models of intestinal tumorigenesis.

Introduction

Colorectal cancer is the third leading cause of cancer deaths in the United States, with an estimated 102,480 new cases of colon cancer and 40,340 new cases of rectal cancers and an estimated 50,830 deaths in 2013 (National Cancer Institute, 2013). Colorectal cancer has been studied extensively in the context of environmental as well as genetic alterations that consistently activate and inactivate tumor suppressor genes (Hanahan and Weinberg, 2000; Young et al., 2007; Markowitz and Bertagnolli, 2009). Recent studies suggest that epigenetic changes in DNA methylation and histone modifications are equally important during tumor progression (Esteller, 2008;

Sharma et al., 2010). Of these, DNA methylation is the most extensively studied in various cancer types (Paz et al., 2003).

Histone acetyltransferases add an acetyl group onto the ϵ -amino group of lysine, thereby reducing the positive charge on histones, which in turn reduces its ability to bind to negatively charged DNA and thus lead to relaxed chromatin structure and accessibility to various transcription factors and other transcription components (Shogren-Knaak et al., 2006). Conversely, histone deacetylases (HDACs) remove the acetyl group, thereby restoring the closed chromatin structure. Thus, HDACs have a dual role that includes chromatin structure regulation as well as post-translational modification of numerous proteins playing a critical role during normal cellular proliferation, differentiation, and carcinogenesis (Strahl and Allis, 2000). HDAC overexpression contributes to tumor progression by transcriptional repression of genes involved in growth arrest, differentiation, and apoptosis (Zhu

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ABBREVIATIONS: AOM, azoxymethane; APC, adenomatous polyposis coli; Bcl-2, B-cell lymphoma 2; CDC25C, cell division cycle homolog 25C; CDK2, cyclin-dependent kinase 2; HDAC, histone deacetylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OSU-HDAC42, Ohio State University histone deacetylase 42; OUHSC, University of Oklahoma Health Sciences Center; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; SAHA, suberoylanilide hydroxyamic acid (Vorinostat); TCF, T-cell factor.

et al., 2004). Thus, harnessing histone modifications particularly targeting HDAC led to the successful approval of suberoylanilide hydroxyamic acid (SAHA), a pan-HDAC inhibitor, for the treatment of advanced cancers (Kelly et al., 2003, 2005; Garcia-Manero et al., 2005; O'Connor et al., 2005). However, antitumor activities of SAHA against solid tumors are disappointing. Recent understanding of members of the HDAC family led to rationale design of isoform-selective HDAC inhibitors to target HDACs that are associated predominately with specific tumor types (Brehm et al., 1998; Jung et al., 2012; Harms and Chen, 2007).

Colon cancer is frequently associated with aberrant signaling through the Wnt pathway due to loss of both functional copies of the tumor suppressor adenomatous polyposis coli (APC) and/or mutations in the β -catenin gene, although additional mutations are required for cancer development (Kinzler and Vogelstein, 1996). Loss of functional APC leads to overexpression of HDAC2 by a Myc-dependent mechanism; the HDAC2 promoter has a putative E-box and may be a downstream target of c-Myc (Zeng et al., 1998; Huang et al., 2005). In addition, studies demonstrate that T-cell factor (TCF)-4/Lef1- and/or the TCF-4/Lef1-dependent Wnt pathway protein, including peroxisome proliferative activated receptor- δ , induces HDAC2 transcription (He et al., 1998). In addition, HDAC2, a class I HDAC, is significantly upregulated during early stages of tumor development and the increase becomes more profound as lesions progress from adenoma to adenocarcinoma in colorectal carcinogenesis (Huang et al., 2005; Noh et al., 2011). In vitro studies demonstrate that HDAC2 expression and activity are required to maintain a transformed phenotype with resistance to apoptosis in cultured HT-29 colonic cancer cells (Göttlicher et al., 2001; Krämer et al., 2001, 2003). This evidence clearly demonstrates that elevated HDAC2 expression contributes to

colon tumor progression in an APC/ β -catenin/TCF-dependent pathway mechanism. On the basis of its association with colon tumor progression, HDAC2 has been considered a potential target for colon cancer prevention and treatment.

We evaluated whether the HDAC2 selective inhibitor Ohio State University HDAC42 (OSU-HDAC42) would provide chemopreventive efficacy compared with the pan-HDAC inhibitor SAHA in preclinical models of colon cancer. In a model of azoxymethane (AOM)-induced colon carcinogenesis in F344 rats, we assessed the efficacy and dose-response effects of OSU-HDAC42 using aberrant crypt foci as an endpoint marker. In the APC^{min/+} mouse model, we assessed the relative efficacy of OSU-HDAC42 and SAHA using intestinal tumors as the endpoint. We tested whether OSU-HDAC42 modulates the expression of HDAC2 and assessed G₂/M phase cell cycle arrest by analyzing cell division cycle homolog 25C (CDC25C), cyclin-dependent kinase 2 (CDK2), B-cell lymphoma 2 (Bcl-2), and p53 expression levels, in addition to proliferating cell nuclear antigen (PCNA), a surrogate marker of tumor cell proliferation. Our studies show that OSU-HDAC42 produced a dose-dependent inhibition of AOM-induced colonic aberrant crypt foci and had better efficacy compared with the pan-HDAC inhibitor, SAHA.

Materials and Methods

Chemicals. OSU-HDAC42 (AR-42) (Fig. 1A) and SAHA (Vorinostat) (Fig. 1B) were procured from the Division of Cancer Prevention at the National Institutes of Health National Cancer Institute Chemoprevention Drug Repository (Rockville, MD). The primary antibody to HDAC2 was from OriGene Technologies (Rockville, MD). Anti-CDC25C, anti-CDK2, anti-PCNA, anti-Bcl-2, and anti-p53, as well as horseradish peroxidase-conjugated secondary antibodies,

Fig. 1. (A) Chemical structure of OSU-HDAC42 (AR-42). (B) Chemical structure of SAHA (Vorinostat). (C) Experimental design to determine the chemopreventive efficacy of OSU-HDAC42 (75, 150, and 300 ppm) administered in the diet to assess MTD and AOM-induced colonic aberrant crypt foci formation in male F344 rats. ACF, aberrant crypt foci; MTD, maximum tolerated dose.

were all from Santa Cruz Biotechnology (Santa Cruz, CA). All ingredients for the semi-purified diets were purchased from Bio-Serv (Frenchtown, NJ) and stored at 4°C before diet preparation.

Animals, Care, and Diet. All animal experiments were performed according to Institutional Animal Care and Use Committee guidelines at the University of Oklahoma Health Sciences Center (OUHSC). Weanling male F344 rats were received from pathogen-free breeding laboratories (Harlan, Indianapolis, IN) and were maintained at the OUHSC Rodent Barrier Facility. All rats were housed in ventilated cages under optimal conditions (21°C, 60% relative humidity, 12-hour light/dark cycle, 20 air changes per hour) and were allowed to feed on a modified AIN-76A diet and to access automated tap water purified by reverse osmosis until initiation of the experiment. A modified AIN-76A diet containing 5% corn oil by weight was prepared per American Institute of Nutrition instructions. OSU-HDAC42 (75, 150, and 300 ppm) and SAHA (300 ppm) were premixed with a small quantity of casein and then blended into the bulk diet using a Hobart mixer. Both control and experimental diets were prepared weekly and stored in a cold room. Uniform distribution of test agents in the experimental diets was ensured by periodic mixing of the diets before feeding the animals. Rats were allowed ad libitum access to their respective diets and automated tap water purified by reverse osmosis.

Colon Carcinogenesis Bioassay and Quantification of Aberrant Crypt Foci. At the beginning of age 8 weeks, rats were treated with subcutaneous injections of AOM (15 mg/kg body weight once a week for 2 weeks) (Fig. 1C). Rats intended for vehicle treatment were given normal saline. In groups of rats intended for the selective HDAC2 inhibitor, OSU-HDAC42 doses were administered in the experimental diet 1 week after AOM treatment. On the basis of the toxicity studies, we tested 75, 150, and 300 ppm OSU-HDAC42 in the diet to assess the chemopreventive efficacy and dose-response effects. Body weights were monitored at weekly intervals. All rats were killed by CO₂ asphyxiation 8 weeks after AOM treatment and were dissected and analyzed for colonic aberrant crypt foci. The colons were cut open longitudinally from the anus to the cecum and fixed flat in between two filter papers with mucosa on the upper side in 10% buffered formalin. The colons were cut into 2-cm segments to evaluate the number of aberrant crypt foci starting from the distal end to the proximal end and were stained with 0.2% methylene blue in Krebs-Ringer solution. Aberrant crypt foci were distinguished from normal crypts by their increased size, intense staining, and pericryptal zone. Aberrant crypt foci scoring was done by two readers blinded to the experimental groups, and their results were averaged to determine the occurrence and multiplicity of aberrant crypt foci. Crypt multiplicity was determined as the number of aberrant crypt foci containing 1, 2, 3, or ≥4 crypts.

Breeding and Genotyping of APC^{min/+} Mice. Male APC^{min/+} (C57Bl/6J) and female wild-type littermate mice were purchased initially from The Jackson Laboratory (Bar Harbor, ME) and maintained in our own breeding colony at the OUHSC Rodent Barrier Facility. Male APC^{min/+} mice were bred with wild-type female mice and the required number of APC^{min/+} mice were generated and genotyped by the polymerase chain reaction method using primers (IMR0033, 5'-GCC ATC CCT TCA CGT TAG-3'; IMR 0034, 5'-TTC CAC TTT GGC ATA AGG C-3'; and IMR0758, 5'-TTC TGA GAA AGA CAG AAG TTA-3') according to the vendor's instructions. All mice were housed three per cage in ventilated cages under optimal conditions as described above.

Intestinal Tumorigenesis Bioassay. Six-week-old APC^{min/+} mice ($n = 10$ per group) and wild-type mice ($n = 3$ per group) were fed AIN-76A diets containing 0 or 150 ppm OSU-HDAC42 and 300 ppm SAHA for 10 weeks, before analysis of intestinal tumors. Body weights, animal behavior, or any abnormalities were observed weekly for changes such as weight loss, lethargy, and feeding habits that might show toxicity of the test agents. After 10 weeks of experimental diet feeding, all mice were killed at 16 weeks of age by CO₂ asphyxiation, blood was drawn by cardiac puncture, and serum was separated by centrifugation and stored at -80°C for further analysis. After

dissection, the intestine was harvested, rinsed with 0.85% NaCl, and opened longitudinally from the stomach to the distal rectum. The tissue was flattened on a filter paper to expose the polyps and colon tumors for visual scoring. The number, size, and location (duodenal, jejunum, ileum, and colon) of the intestinal polyps were determined using a dissection microscope. Polyps were scored by two different observers. For further histopathological analysis, tissue samples were fixed in 10% formalin, embedded in paraffin blocks, and processed with routine hematoxylin and eosin staining or stored in liquid nitrogen for further molecular analysis.

Immunohistochemistry. Intestinal tumor and colonic tissues (containing aberrant crypt foci) were embedded in paraffin and sectioned for immunohistochemistry. The sections were deparaffinized in xylene, rehydrated through a graded series of ethanol solutions to distilled water, and washed in phosphate-buffered saline (PBS). Antigen retrieval was carried out by heating sections in 0.01 M citrate buffer (pH 6) for 30 minutes in a boiling water bath. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in PBS for 5–10 minutes. Nonspecific binding was blocked using protein block (normal serum) for 20 minutes. Sections were then incubated overnight at 4°C with 1:100 dilutions of HDAC2, CDC25C, CDK2, Bcl-2, PCNA, and p53 primary antibodies. After several washes with PBS, the slides were incubated with the appropriate secondary antibody for 1 hour. The color reaction was developed with 3,3'-diaminobenzidine, according to the manufacturer's instructions provided in the Histo-stain kit supplied by Invitrogen Corporation (Frederick, MD). Substituted nonimmune IgG was used as a negative control. All of the specimens were observed using an IX71 microscope (Olympus, Center Valley, PA), and the digital computer images were recorded with an Olympus DP70 camera. Scoring was done according to the intensity of nucleic or cytoplasmic cells (0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining) and the prevalence of stained cells (1, 1–40%; 2, 41–50%; 3, 50–60%; 4, 61–100%). The final immunoreactive score was determined by multiplying the intensity scores by the prevalence of the extent of cell staining, yielding a score ranging from 0 to 12.

Cell Culture. Human colon cancer HCT116 cells were purchased from American Type Culture Collection (Manassas, VA). The cells were maintained in McCoy's 5A medium containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ and were subcultured after trypsinization (0.5% trypsin/2.6 M EDTA). For the experiments, the cells were seeded at 1×10^6 in culture dishes (100 mm in diameter) and grown to 70–80% confluence.

Toxicity and Cell Viability Assay. A calorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) metabolic activity assay was used to determine cell viability and to assess the effect of OSU-HDAC42 on toxicity in human colon cancer HCT116 cells. For the assay, HCT116 cells (5×10^3) were seeded in 96-well culture plates and treated with 0–32 µM OSU-HDAC42 for 24 hours. MTT (20 µl; 5 mg/ml stock) solution then was added to the wells and incubated at 37°C for 4–5 hours. Thereafter, the medium was gently aspirated from the wells, and 200 µl of dimethylsulfoxide was added to each well to dissolve the purple formazan crystals. The absorbance at 570 nm was recorded in a microplate reader (FLUOstar OPTIMA; BMG Labtech, Cary, NC). Data were derived from at least three independent experiments, and the percentage of relative cell viability was calculated using the following formula: [(mean absorbance of treated cells)/(mean absorbance of control cells)] $\times 100$.

Detection of HDAC2 Activity. The Fluor de Lys-Green HDAC2 Assay Kit (catalog number BML-AK512; Enzo Life Sciences International, Plymouth Meeting, PA) was used to measure HDAC2 activity in human colon cancer HCT116 cells. The assay is based on the Fluor de Lys-Green substrate and Fluor de Lys developer combination. Reactions were carried out according to the manufacturer's instructions. The procedure involves two steps. In the first step, Fluor de Lys-Green substrate, which comprises an acetylated lysine side

chain, is incubated with HDAC2. Deacetylation of the substrate sensitizes the substrate so that treatment with the Fluor de Lys developer produces a fluorophore in the second step. The fluorophore was excited with 485 nm light (470–500 nm), and the emitted light (approximately 530 nm) was detected on a fluorometric plate reader. Fluor de Lys-Green is an especially sensitive substrate for HDAC2. Activity was readily measured with enzyme amounts ranging from 1 to 10 ng/well (0.36–3.6 nM in 50 μ l), thus enabling IC₅₀ determinations for high-affinity inhibitors.

Western Blot Analyses. Human colon cancer HCT116 cells were grown in McCoy's 5A medium containing L-glutamine and supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere with 5% CO₂ and subcultured after trypsinization (0.5% trypsin/2.6 M EDTA). For the experiments, the cells were seeded at 1×10^6 in culture dishes (100 mm in diameter), grown to 70–80% confluence, and lysed in ice-cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 $\times$ protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO)]. After brief vortexing, the lysates were separated by centrifugation at 12,000g for 15 minutes at 4°C, and protein concentrations were measured with the Bio-Rad protein assay reagent (Hercules, CA). Proteins (60 μ g/lane) from an aliquot were separated with electrophoresis through 10% SDS-PAGE and transferred to nitrocellulose membranes. After blocking with 5% milk powder, membranes were probed for expression of HDAC2, CDC25C, CDK2, and p53 with β -actin as the loading control in hybridizing solution (1:500 in Tris-buffered saline/Tween 20 solution) using the respective primary antibodies and then probed with their respective horseradish peroxidase-conjugated secondary antibodies. Detection was performed using the SuperSignal West Pico chemiluminescence procedure (Pierce, Rockford, IL). The bands were captured on Ewen-Parker (Elmsford, NY) blue sensitive X-ray films.

Statistical Analysis. The results are presented as the mean $\pm$ S.E.M. Statistical differences in body weights were analyzed using analysis of variance between the control and treated groups. Differences in aberrant crypt foci occurrence and multiplicity, tumor multiplicity, and differences in molecular markers were analyzed by the unpaired *t* test with Welch's correction. Differences between groups were considered significant at *P* < 0.05. All statistical analysis was performed with Prism software (version 5.0; GraphPad Software, Inc, San Diego, CA).

Results

General Observations from the Rat Colon Carcinogenesis Model. All F344 rats fed with the control diet had a steady body weight gain during and after administration of AOM. No significant overt toxicities were observed in major organs in rats administered 75, 150, or 300 ppm OSU-HDAC42 for 8 weeks. In addition, we observed no significant body weight loss or toxicity in rats administered 75 or 150 ppm OSU-HDAC42; however, significant body weight loss was observed in rats treated with 300 ppm OSU-HDAC42 (data not shown).

Dietary OSU-HDAC42 Inhibits Aberrant Crypt Foci Formation. Rats treated with vehicle and fed control or experimental diets did not show any signs of aberrant crypt foci formation in the colon. Inhibition of total aberrant crypt foci per colon and a reduction in the number of multicrypt (≥ 4) foci were considered as the efficacy end points. AOM-treated rats fed the control diet had a mean of 141 ± 8.2 total aberrant crypt foci per colon and a mean 17 ± 1.2 foci per colon containing multiple (≥ 4) aberrant crypts per focus. Rats fed

75, 150, and 300 ppm OSU-HDAC42 in the diet showed significantly reduced total aberrant crypt foci per colon, with mean values of 116 ± 4.7 (*P* < 0.01), 99 ± 4.7 (*P* < 0.0003), and 71.5 ± 5.4 (*P* < 0.0001), respectively (Fig. 2A). Aberrant crypts containing multiple foci (≥ 4) were reduced significantly, with mean values of 12.7 ± 1.3 (*P* < 0.013), 10.1 ± 1.2 (*P* < 0.001), and 7.4 ± 0.8 (*P* < 0.0001) in rats fed 75, 150, and 300, respectively (Fig. 2B). Our results demonstrate that OSU-HDAC42 produces a dose-dependent inhibition of AOM-induced total aberrant crypt foci per colon by 13–50% (*P* < 0.01 to *P* < 0.0001) and that it reduces the number of foci with four crypts per focus by 25–57% (*P* < 0.01 to *P* < 0.0001) in F344 rats.

Modulation of HDAC2, Cell Cycle, and Antiapoptotic Markers in Aberrant Crypt Foci. Although evidence shows that HDAC2 is overexpressed in colon cancer, the specific roles of HDAC2 in cell proliferation and apoptosis are unclear. Recent studies report that HDAC2 regulates cell-cycle components and that suppression of HDAC2 causes G₂/M phase arrest of cell cycle progression (Liu et al., 2013). We used immunohistochemistry to analyze expression of HDAC2 and other key modulators including CDC25C, CDK2, p53, and Bcl-2. Dietary administration of 75, 150, and 300 ppm OSU-HDAC42 caused a dose-dependent inhibition in the expression of HDAC2, CDC25C, CDK2, p53, and Bcl-2 (approximately 50–60%) in aberrant crypt foci from treated mice compared with control mice (Fig. 3). Immunohistochemistry was quantified as described in *Materials and Methods* to obtain the percent values. These results demonstrate that suppression of HDAC2 and G₂/M phase cell cycle progression markers occurs upon OSU-HDAC42 treatment. These results

Fig. 2. (A) Effect of dietary OSU-HDAC42 (75, 150, and 300 ppm) on AOM-induced colonic total ACF in F344 male rats. (B) Effect of dietary OSU-HDAC42 (75, 150, and 300 ppm) on AOM-induced colonic multicrypt ACF (number of crypts with ≥ 4 crypts per focus per colon) in F344 male rats. ACF, aberrant crypt foci.

Fig. 3. Effect of OSU-HDAC42 on HDAC2, CDC25C, CDK2, and p53 expression levels in AOM-induced colonic preneoplastic lesions (ACF). Immunohistochemical analysis was performed on paraffin-embedded and microsectioned colon tissues as described in *Materials and Methods*. Staining intensity was scored as described in *Materials and Methods*. Original magnification, $\times 60$. ACF, aberrant crypt foci.

also suggest that HDAC2 is upregulated in aberrant crypt foci but not in normal crypts; hence, targeting of HDAC2 may not alter normal cellular proliferation.

General Observations from the APC^{min/+} Intestinal Tumorigenesis Model. At the time of termination, no significant body weight change or noticeable toxicity was observed in the mice administered 150 ppm OS-HDAC42 or 300 ppm SAHA (Fig. 4, A and B). As expected, mice fed the control diet showed lower body weights. Body weight loss in mice fed the control diet might be due to the increased small-intestinal tumor burden with impairment of food absorption and anemia. Overall, the doses applied in the efficacy studies appear to be nontoxic.

Dietary OSU-HDAC42 Inhibits APC^{min/+} Mouse Intestinal Tumorigenesis. APC^{min/+} mice spontaneously develop small-intestinal polyps with fewer tumors in the colon; these are classified histopathologically as adenomas with no local invasion of the lamina propria. On the basis of these dose-tolerability results, we tested nontoxic doses of 150 ppm OSU-HDAC42 and 300 ppm SAHA to assess the intestinal tumor inhibition in APC^{min/+} mice. Figure 4 summarizes the chemopreventive effect of dietary OSU-HDAC42 (150 ppm) or SAHA (300 ppm) on tumor multiplicity in the small intestine.

Mice fed the control diet developed a mean 46.3 ± 5.28 intestinal polyps. In APC^{min/+} mice, OSU-HDAC42 administered at 150 ppm for 10 weeks significantly reduced total intestinal tumor multiplicity to 25.7 ± 1.37 ($P < 0.001$), which is a $>46\%$ inhibition (Fig. 4C). Mice fed 150 ppm OSU-HDAC42 showed a significant inhibition in polyps with sizes measuring >1 mm (mean 5.75 ± 1.09 per animal; $P < 0.001$) compared with controls (mean 19.40 ± 3.0 per animal), whereas mice fed 300 ppm SAHA showed a nonsignificant inhibition (mean 17.70 ± 2.79) (Fig. 4D). Mice fed the control diet had a mean 1.2 ± 0.37 colon tumors per animal, whereas mice fed 150 ppm OSU-HDAC42 showed a mean 0.8 ± 0.2 colon tumors per animal, which is an inhibition of $>26\%$ ($P \leq 0.05$) (Fig. 4E). However, mice treated with 300 ppm SAHA showed no significant changes in total small-intestinal polyps; furthermore, there was nonsignificant inhibition in colonic tumors compared with the group fed the control diet.

Modulation of HDAC2, Cell Cycle, and Antiapoptotic Markers in Tumors. From recent studies, it is understood that Wnt pathway components transcriptionally regulate HDAC2 and are identified as a target of the APC/ β -catenin pathway (Su et al., 1992; Fodde et al., 1994; Shoemaker et al., 1997). Further evidence suggests that HDAC2 is highly

Fig. 4. (A) Experimental design to determine the efficacy of dietary OSU-HDAC42 and SAHA on small-intestinal and colon tumorigenesis in the *APC^{min/+}* mice. (B) Effect of dietary 150 ppm OSU-HDAC42 and 300 ppm SAHA on body weight gain in *APC^{min/+}* mice. (C) Effect of dietary administration of OSU-HDAC42 or SAHA on small-intestinal polyp formation in *APC^{min/+}* mice. (D) Effect of dietary OSU-HDAC42 or SAHA on polyp size in *APC^{min/+}* mice. (E) Effect of dietary OSU-HDAC42 or SAHA on colon tumor formation in *APC^{min/+}* mice.

expressed in colon tumors and is a potential target of colon cancer. In the present study, we determined HDAC2 expression in intestinal tumors of *APC^{min/+}* mice and extended our study to test whether HDAC2 is a target of colon cancer chemoprevention. We performed immunohistochemistry to analyze protein expression levels of HDAC2 in the small intestine (Fig. 5), as well as in colon tumor tissues (Fig. 6), from control animals and animals treated with 150 ppm OSU-HDAC42. Dietary administration of 150 ppm OSU-HDAC42 caused a significant inhibition in the expression of HDAC2 (approximately 60–70%), CDC25C, CDK2, Bcl-2, and PCNA (approximately 50–60%) in treated tissues compared with control samples. These results suggest that suppression of HDAC2 upon treatment with OSU-HDAC42 might have an effect on the G₂/M cell cycle regulatory proteins and may possibly induce cell cycle arrest and prevent further cell cycle progression. However, further studies are warranted to understand the underlying molecular mechanism and the molecules involved that may lead to cell cycle arrest and prevent

further cell cycle progression. In addition, we observed decreased expression of Bcl-2, a key regulator of apoptosis. Furthermore, we observed increased p53 expression levels in tissues from the OSU-HDAC42-treated mice, suggesting a critical role of p53 in the signal transduction pathway leading to G₂/M arrest or apoptosis in response to DNA damage.

Effect of OSU-HDAC42 on HCT116 Cell Viability and HDAC2 Activity. To determine the effect of OSU-HDAC42 in human colon cancer HCT116 cells, we examined the cytotoxic effects of 0–32 μ M OSU-HDAC42 (24-hour treatment) on HCT116 cells using the MTT cytotoxicity assay. A dose-dependent MTT reduction (or color change from yellow to purple) was observed in OSU-HDAC42-treated cells (Supplemental Fig. 1A). On a 24-hour exposure to OSU-HDAC42, MTT activity was reduced to $\geq 50\%$ at a concentration of 3.2 μ M or higher, compared with that of untreated cells. We next examined the dose-dependent effects of OSU-HDAC42 on the HDAC2 activity in HCT116 cells using the Fluor de Lys-Green HDAC2 assay, as described in *Materials and Methods*. Our data

Fig. 5. Effect of OSU-HDAC42 on HDAC2, CDC25C, CDK2, p53, and Bcl-2 expression in small-intestinal polyps. Immunohistochemical analysis was performed on paraffin-embedded and microsectioned colon tissues as described in *Materials and Methods*. Staining intensity was scored as described in *Materials and Methods*. Original magnification, $\times 60$.

demonstrated that maximal HDAC2 inhibitory activity could be detected at concentrations $\geq 1.6 \mu\text{M}$ (Supplemental Fig. 1B).

Molecular Analysis of OSU-HDAC42 Treatment in HCT116 Cells. After analyzing the inhibition of HDAC2 activity by OSU-HDAC42 in HCT116 cells, we attempted to elucidate the expression of molecular markers including HDAC2, CDC25C, CDK2, and p53, which are involved widely in cell cycle arrest, differentiation, and apoptosis. Our data demonstrate that high concentrations of OSU-HDAC42 (3.2 and $16 \mu\text{M}$) induced a strong upregulation of p53 proteins and strong inhibition of HDAC2, CDC25C, and CDK2 was observed at concentrations $\geq 1.6 \mu\text{M}$ (Supplemental Fig. 1C).

Discussion

Previous reports demonstrated that histone modifications such as acetylation and deacetylation are involved in a number of biologic processes including cell growth and intracellular signaling (Grunstein, 1997; Kouzarides, 2000; Marks et al., 2001; Roth et al., 2001; Minucci and Pelicci, 2006). Recent evidence suggests that HDACs are important targets for treatment of various neoplastic diseases. Of the several isoforms of HDACs, HDAC2 is upregulated in colorectal polyps associated with a poor prognosis (Witt et al., 2009). Several molecular components of the Wnt pathway, including TCF and c-Myc (He et al., 1998, 1999), regulate HDAC2 at the

transcriptional level, causing increased expression. Upon knock-down of these prominent factors, there is reduced HDAC2 expression. Hence, it is understood that components of the Wnt pathway regulate HDAC2 gene expression and lead to tumor development in $\text{APC}^{\text{min/+}}$ mice. Therefore, HDAC2 is a good target for colon cancer chemoprevention. Consistent with this evidence, we have demonstrated that HDAC2, a class I HDAC, is highly expressed in preneoplastic lesions as well as in adenoma and adenocarcinomas in the colon (Figs. 3, 5, and 6).

In general, HDAC inhibitors are very potent antitumor agents and several pan-HDAC inhibitors have been entered into clinical trials. Previous studies have shown that OSU-HDAC42 and pan-HDAC inhibitors possess antitumor effect in various cancer types, including ovarian (Jin et al., 2008), hepatocellular carcinomas (Noh et al., 2006), prostate (Weichert et al., 2008), and lung (Jung et al., 2012) cancer. Due to the complex enzymatic nature and occurrence of several subtypes of HDACs, selective targeting of HDACs is not yet fully established. No systemic studies have been conducted to establish the potential effect of targeting HDAC2 for colorectal cancer prevention. Our present results clearly demonstrate that the novel and rationally designed HDAC2 inhibitor OSU-HDAC42 decreases total aberrant crypts per colon as well as formation of multiple crypts per foci in colons of $\text{APC}^{\text{min/+}}$ mice by decreasing the expression of HDAC2. However, rats fed 300 ppm OSU-HDAC42 showed significant

Fig. 6. Effect of OSU-HDAC42 on HDAC2, CDC25C, CDK2, p53, and Bcl-2 expression in colon tumors. Immunohistochemical analysis was performed on paraffin-embedded and microsectioned colon tissues as described in *Materials and Methods*. Staining intensity was scored as described in *Materials and Methods*. Original magnification, $\times 60$.

body weight loss. Thus, doses ≤ 150 ppm were considered to be optimal for tumor assay studies. Hence, in the tumor assay study in APC^{min/+} mice, we used 150 ppm OSU-HDAC42 and 300 ppm of the pan-HDAC inhibitor SAHA to assess the inhibition of intestinal tumor formation. A diet with 150 ppm OSU-HDAC42 caused $>46\%$ suppression in the development of small-intestinal polyps ($P < 0.001$), whereas 300 ppm SAHA showed a nonsignificant inhibition.

Determination of small-intestinal polyp size under a dissection microscope revealed that 150 ppm OSU-HDAC42 caused a significant suppression of polyps >1 mm, whereas a nonsignificant inhibition was observed with SAHA. In summary, we demonstrate that treatment with the selective HDAC2 inhibitor OSU-HDAC42 significantly suppressed both preneoplastic lesions such as colonic aberrant crypt foci in rats and intestinal tumor formation in APC^{min/+} mice. In addition, we demonstrate a significant downregulation of HDAC2 expression in tumors of the small intestine and colon. Overall, OSU-HDAC42, an HDAC2-selective inhibitor, provides better efficacy compared with SAHA, a pan-HDAC inhibitor. HDAC2 is a potential target for colon cancer chemoprevention and the HDAC2-selective inhibitor OSU-HDAC42 exhibits efficacy in the two colon cancer models examined here.

Although there is reduced adenoma formation due to inhibition of HDAC2 with OSU-HDAC42, it is not clear whether the reduced adenoma formation is due to apoptosis. It has been reported that HDAC2 inhibition increases the

apoptotic response by regulating p53 in H1299 lung carcinoma and Saos-2 human osteosarcoma cells (Juan et al., 2000). However, no studies have been carried out to explain the underlying mechanisms responsible for the oncogenic potential of HDAC2 in apoptosis and cell cycle regulation of colon cancer. Recent studies report that HDAC2 recruited to the E2F pocket proteins (Chan et al., 2001) repress E2F target genes including p53 (Hershko et al., 2005) and fail to effect cell cycle arrest, leading to increased cell proliferation, thus eliciting HDAC2 critical role in cell cycle progression. Cell cycle is a tightly controlled mechanism, and dysregulation of cell cycle and their components may lead to tumor formation. The sequence and timing of activation of the different Cdk isoforms determine the order of the major cell cycle transitions before progression to the next (Pagano et al., 1992, 1993; Rosenblatt et al., 1992). The complex association of cyclin A/cdk2 has mitotic functions and is active during early S phase and G₂ phase prior to the activation of G₂/M transition. CDC25C is a mitotic inducer and is catalyzed during early S phase activation. CDC25C activity is regulated by the repressor protein 14-3-3, by phosphorylating Ser216 on CDC25C and also by binding to CDC25C, thereby leading to sequestration of CDC25C activity. Binding of 14-3-3 is displaced when Thr130 of CDC25C is phosphorylated by CyclinA/Cdk2 complex and increases CDC25C activity during early S phase and is a direct substrate of CyclinA/Cdk2. Increased CDC25C activity is required for cell cycle progression. In this study, we demonstrated the aberrant expression of HDAC2 in colon

cancer and examined the effects of HDAC2 inhibition on the cell cycle, specifically in the G₂/M transition, by assessing CDC25C and CDK2 expression in intestinal tumors from in vivo model systems. Our findings demonstrated that in contrast to the pan-HDAC inhibitor SAHA, 150 ppm OSU-HDAC42 downregulated CDK2 protein levels in small-intestinal polyps and colon tumors concomitant with a decrease in the levels of G₂/M phase cell cycle regulatory proteins such as CDC25C. Although HDAC2 inhibition had an effect on CDK2 and CDC25C expression levels, more studies are warranted to understand the underlying molecular mechanisms and their role in the cell cycle progression during G₂/M phase.

Bcl-2 inhibits apoptosis by preventing cytochrome c release and causes inactivation of caspase-9 without disrupting mitochondrial integrity, whereas p53 is a critical component of cell cycle regulation as well as apoptosis when cells are under stress (Oren, 1999; Sionov and Haupt, 1999; Reed, 2002). A recent report indicated that a Bcl-2/p53 functional interaction represents a key regulator of apoptosis that can be activated by targeting Bcl-2 in colorectal cancer cells (Jiang and Milner, 2003). Additional evidence demonstrates that class I HDACs are capable of downregulating p53 function; the deacetylase activity of HDAC occurs on the region of p53 that is acetylated by p300/cAMP response element-binding protein, suggesting that interactions of p53 and HDACs result in p53 deacetylation, thereby reducing p53 transcriptional activity (Juan et al., 2000). In this study, we showed that HDAC2 inhibition upon treatment with OSU-HDAC42 was associated with decreased expression of Bcl-2 and increased expression of p53. Although the molecular mechanism is not clearly understood, our observations suggest that HDAC2 inhibition by OSU-HDAC42 regulates the G₂/M cell cycle phase in a p53-dependent manner and that Bcl-2/p53 interaction is a key regulator of apoptosis in colon cancer. However, further studies are warranted to test the inhibitory potential of OSU-HDAC42 in rat colon adenocarcinoma formation and in human phase 0 and phase I biomarker and toxicity studies.

In conclusion, our studies explore HDAC2 as an important target for colorectal cancer chemoprevention. The ability of OSU-HDAC42 to inhibit HDAC2, to decrease expression of G₂/M phase cell cycle regulatory proteins, and to decrease expression of Bcl-2 and upregulate p53 strongly suggests that OSU-HDAC42 is a promising chemopreventive agent for colorectal cancer.

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Authorship Contributions

Participated in research design: Ravillah, Mohammed, Qian, Brewer, Zhang, Biddick, Steele, Rao.

Conducted experiments: Ravillah.

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