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Disruption of Epigenetic Silencing in Human Colon Cancer Cells Lines Utilizing a Novel Supercritical CO_2 Extract of Neem Leaf (*Azadirachta indica*)

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Abstract. *Background/Aim: Aerial parts and seeds of the neem tree (Azadirachta indica) have long been used in traditional medicine such as Ayurveda for health-related purposes. Our interest in neem bioactives lies in their potential use as standalone anticancer agents, or as adjuvants to standard therapy. The aim of the present study was to explore a supercritical CO_2 extract (SCNE) of neem leaf and a prominent liminoid in neem leaf, nimbolide, for epigenetic activity. Materials and Methods: Human colorectal cancer cell lines (HCT116 and HT29) were cultured for 48 h in the presence of neem extract or nimbolide and evaluated for growth inhibition and evidence of suppression of histone deacetylation and DNA methylation. Results: Both SCNE and nimbolide suppressed the proliferation of colon cancer cells by inducing epigenetic modifications. Conclusion: Neem leaf contains bioactive constituents which modify epigenetic activity.*

Colorectal cancer is associated with high morbidity and mortality throughout the world and its incidence is increasing in the developing world accounting for over 9% of all cancer incidence (1-3). Early diagnosis has become possible due to improved detection techniques. Current treatments, including surgery, radiotherapy, and/or hormone therapies are useful for treating colon cancer, but there is still no effective cure for most patients suffering from advanced colon cancer and gains have been rather marginal (4, 5). Given that treatment options for patients with advanced colon cancer are limited, the use of natural products prior to, or along with conventional therapy may be a rational and an appealing strategy. The traditional use of medicinal plant extracts has stimulated research into their mechanisms of action to identify promising anti-cancer agents. Their appeal lies in their lack of comparative toxicity and the notion that their use may elicit a window of vulnerability in the cancer cell to which current therapies may be targeted.

DNA hypermethylation and histone deacetylation play key roles in activation/inactivation of many genes including tumor-suppressor genes such as p16 (also known as Ink4a, CDKN2A) (6, 7). Epigenetic change is a common mechanism for inactivation of p16 (Ink4a, CDKN2A), which plays a regulatory role in human carcinogenesis (8). Targeting DNA hypermethylation and histone deacetylation with pharmacological inhibitors is one way of altering genetic expression in disease (9, 10). Some pharmacological inhibitors such as 5-Aza-2'-deoxycytidine [5aza-dc, a DNA methyltransferase inhibitor (DNMT)] and suberoylanilide hydroxamic acid [SAHA, a histone deacetylase inhibitor (HDAC inhibitor)] have entered clinical testing, but are restricted in human patients due to their toxicity (11, 12).

Aerial parts and seeds of the neem tree (*Azadirachta indica*), have attracted worldwide prominence in recent years, owing to their wide range of traditional medicinal uses (13). Research studies have pointed out the neem tree as reservoirs of therapeutic compounds for several diseases, including cancer (14). Neem compounds present in bark, leaves, flowers, and seed oil have proven to have anticancer properties, including the capacity to suppress proliferation, enhance immunomodulatory effects, suppress inflammatory activity and induce p53-independent apoptosis (15). Not all neem extracts are alike and our previous studies employed a novel supercritical CO_2 neem extract (SCNE) of high purity (16). Specifically, this SCNE was shown to affect cell proliferation, inflammation, migration, and invasion in human colon cancer cells accompanied by growth inhibition in mice bearing HT29 and HCT116 xenografted tumors. Given the current interest in identifying natural products that influence the epigenetics of colon cancer, we evaluated, in the current study, the effects of the SCNE on the expression of HDACs and DNMTs, with the specific goal of de-

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silencing expression of the tumor-suppressor gene p16. Few cancer bioactives have been isolated from neem extracts to date. However, one potential candidate is nimbolide, a prominent liminoid found in neem leaf. In addition to the SCNE, we also evaluated nimbolide for epigenetic modifying capacity (17).

Materials and Methods

Cell lines and cell culture. Human colon cancer cell lines HCT116 and HT29 were obtained from American Type Culture Collection (ATCC) and used within 6 months. Both these cell lines were maintained in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA, M9309) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (Atlanta biologicals, S11150). The cultures were incubated at 37˚C in a humidifier incubator with 5% CO_2. To evaluate dose-dependent changes in protein and gene expression, cells were treated with different concentrations of SCNE (Nisarga Ltd., Satara, Maharashtra, India) (0-150 μg/ml) and nimbolide (Biovision, Milpitas, CA, USA) (1-100 μM) or an equal volume of dimethyl sulfoxide (DMSO) as a vehicle for different time periods as needed.

Cell viability assay. Human colorectal cancer lines, HCT116 and HT29 were plated in 96-well plates; the next day the cells were serum- starved for 24 h and then treated with SCNE (0-150 μg/ml) and nimbolide (1-100 μM) for 48 h. After treatment, cell viability was measured by MTT [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide] assay (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. Briefly, MTT (5 mg/ml) was added and plates were incubated at 37˚C for 4 h before dimethyl sulfoxide was added to each well. Finally, the absorbance of each well was read at a wavelength of 540 nm using a plate reader (Molecular Devices, Sunnyvale, CA, USA). The results were expressed as a percentage of surviving cells over non-treated cells.

Western blot analysis. HCT116 and HT29 cells were grown to confluency in 150 mm culture dishes. Cells were serum- starved for 24 h. The next day, cells were treated with different doses of SCNE and nimbolide versus the control (DMSO) for 48 h (HCT116) or 96 h (HT29) at 37˚C. For cytoplasm protein extraction cells were kept on ice for 10 min with low salt Lysis buffer (10 mM HEPES, 10 mM KCl, 1 mM EDTA), then scrapped and spun down. Cytoplasmic protein was collected from the supernatant. For nuclear protein extraction cell pellet was collected and 50-100 μl of high-salt lysis buffer (20 mM HEPES, 0.4 M NaCL, 1 mM EDTA) was added and incubated on ice for 30 min with regular vortexing. The tubes were spun down and nuclear protein was collected from the supernatant. Protein concentration was determined using NanoVue Plus (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Equal amounts of protein were separated on 4% or 10% SDS-PAGE. Then, proteins were transferred to Immun-Blot PVDF membranes for protein blotting (Bio-Rad, Hercules, CA, USA) and blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1 h at room temperature. Antibodies against DNMT1 (Cell Signaling Technology, Danvers, MA, USA (D63A6), DNMT3a (Cell Signaling Technology, D2361), Lamin B1 (Cell Signaling Technology, 13435S), HDAC1 (Abcam, ab53091), HDAC2 (Abcam, ab32117), HDAC3 (Abcam, ab32369), GAPDH (Abcam, ab181602) were diluted in 5% skim milk. Horseradish peroxidase-conjugated goat anti- rabbit (Cell Signaling Technology, 7074S) antibody was used as a secondary antibody. Blots were imaged on a Chemidoc™ Touch Imaging System (Bio-rad).

Determination of HDAC and DNMT activities. The Epigenase HDAC Activity/Inhibition Direct Assay kit and the EpiQuik DNMTs Activity/Inhibition Assay kit were purchased from Epigentek (Farmingdale, NY, USA) and were used for the determination of total HDAC and DNMT activity levels according to the manufacturer's instructions. In brief, for HDAC activity determination, nuclear cell lysates were prepared and 10 μg of extracts were incubated with an acetylated substrate for 90 min at 37˚C. Optical density values were obtained at $530_{ex}/590_{em}$ nm using a Tecan Spark 10M plate reader (Männedorf, Switzerland). For DNMT activity determination, nuclear proteins (10 μg) were spotted on the strip wells. The methylated histone was detected with a high-affinity antibody, and was quantified with a horseradish peroxidase-conjugated secondary antibody color development system. Optical density values were obtained at 450 nm with an optional reference wavelength of 655 nm using iMark plate reader (Biorad). Protein concentration was determined using NanoVue Plus (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed using the Piece™ Agarose Chip kit (Thermo Fisher, 26156) for cell line samples according to the manufacturer's protocols. Briefly, the crosslinked chromatin DNA was sonicated into fragments followed by fixation with 1% formaldehyde. The immunoprecipitation was done using the following antibodies: anti-Histone H3 (acetyl K9+K14+K18+K23+K27) (Abcam, 47915), anti-Histone H3 (tri methyl K27)(Abcam, ab192985), anti-Histone H3 (tri methyl K9) (Abcam, ab8898) and H3k4me3 antibodies (Abcam, ab8580) with a normal rabbit IgG as the negative control. Primers flanking the promoters of p16 were used for qRT-PCR.

Real-time PCR. Total RNA was isolated using Aurum total extraction kit (Bio Rad, 12183018A) and first- strand cDNA created using iScript cDNA synthesis kit (Bio Rad, 170-8841). The iQ SYBR supermix (100ng of cDNA) (Bio Rad, 172-5274) was utilized to measure p16 levels. Relative expression ratios (%) were calculated comparing ΔΔCT values using Gapdh as the normal expression target. Primers sequences were: P16 (F): 5'- GAGGGGAAGGAGAGAGCAGT-3', (R) 5'-GGGTGTTTGGTGTCATAGGG-3'; GAPDH (F) 5'-CT TTTGCGTCGCCAG-3', (R) 5'-TTGATGGCAACAATATCCAC-3'.

Statistical analysis. All the statistical analyses were performed using Graph Prism software (Version 6). To determine *p*-values, we used one-way analysis of variance (ANOVA) (and non-Parametric); and *p*-values<0.05 were considered significant. Results are depicted as the mean±SEM.

Results

SCNE and nimbolide decreased cell viability in human colon cancer cells. In order to understand the influence of SCNE and nimbolide exposure on the cell viability of human colon cancer cells, HCT116 and HT29 were treated with different concentrations of SCNE and nimbolide for 48

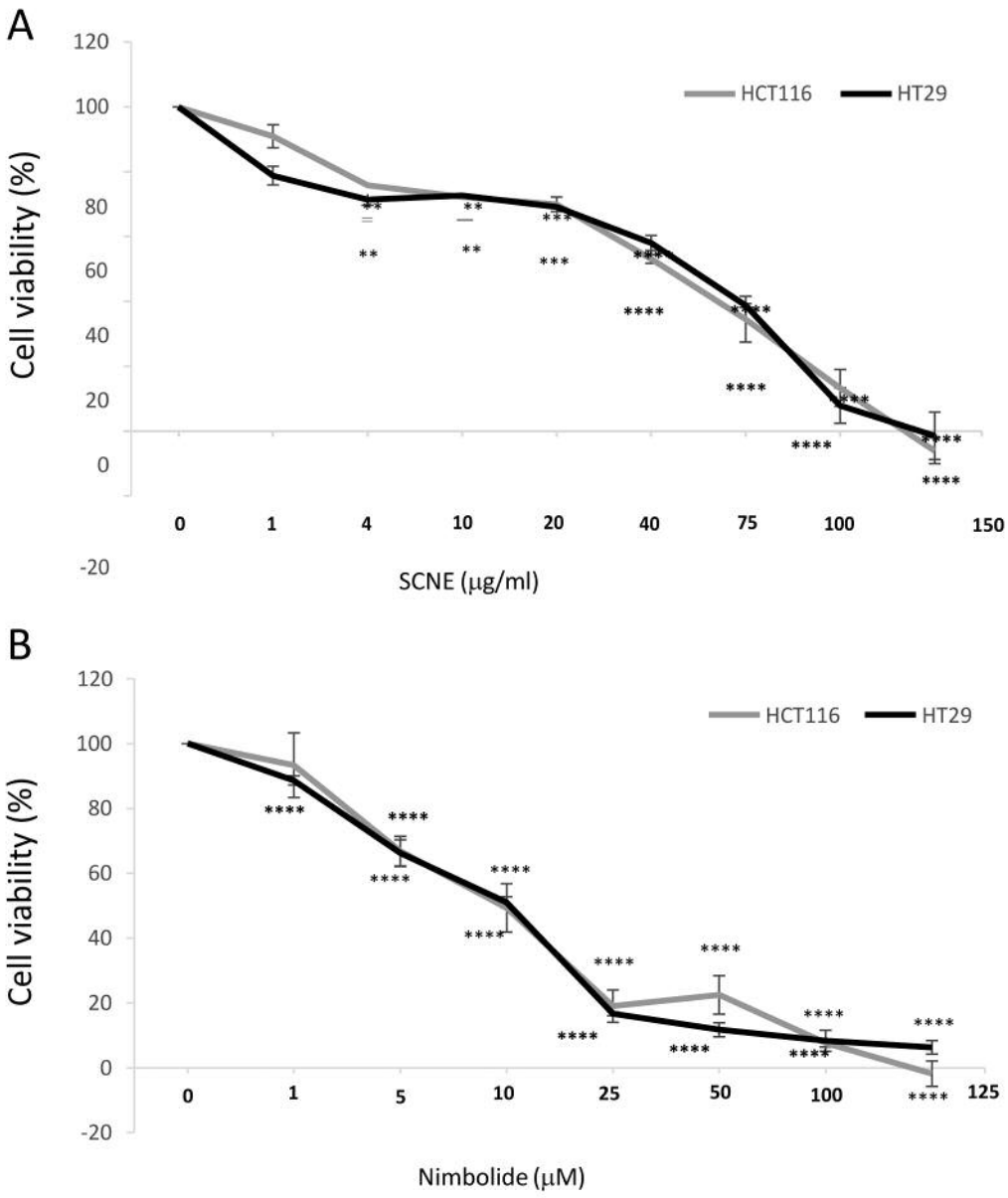

Figure 1. *SCNE and nimbolide reduced viability in CRC cells. HCT116 and HT29 cells were treated with different concentrations of SCNE (A) and nimbolide (B) for 48 h and the cell viability was measured by MTT assay. Data were expressed as mean±SEM of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 indicate significant difference versus vehicle control.*

h, respectively. The HCT116 cell line is reported to be epigenetically sensitive (*via* microsatellite instability) whereas the HT29 cell line is microsatellite stable (18). As shown in Figure 1, 48 h treatment with SCNE or nimbolide had similar trends on human colon cancer cell viability. These results suggest that SCNE and nimbolide inhibit cell viability in a dose-dependent manner. The IC_{50} for SCNE and nimbolide were determined to be <75 μg/ml and <10 μM, respectively, which is consistent with our previous finding (16). Data shown are the mean of three separate experiments±SEM and were statistically significant (p<0.05) by one-way analysis of variance. In subsequent experiments, cells were treated with SCNE at a dose of 40 and 75 μg/ml and nimbolide dose of 5 and 10 μM for 48 h and 96 h respectively.

SCNE and nimbolide inhibited the expression of HDACs and DNMTs in human colon cancer cell line HCT116 and HT29.

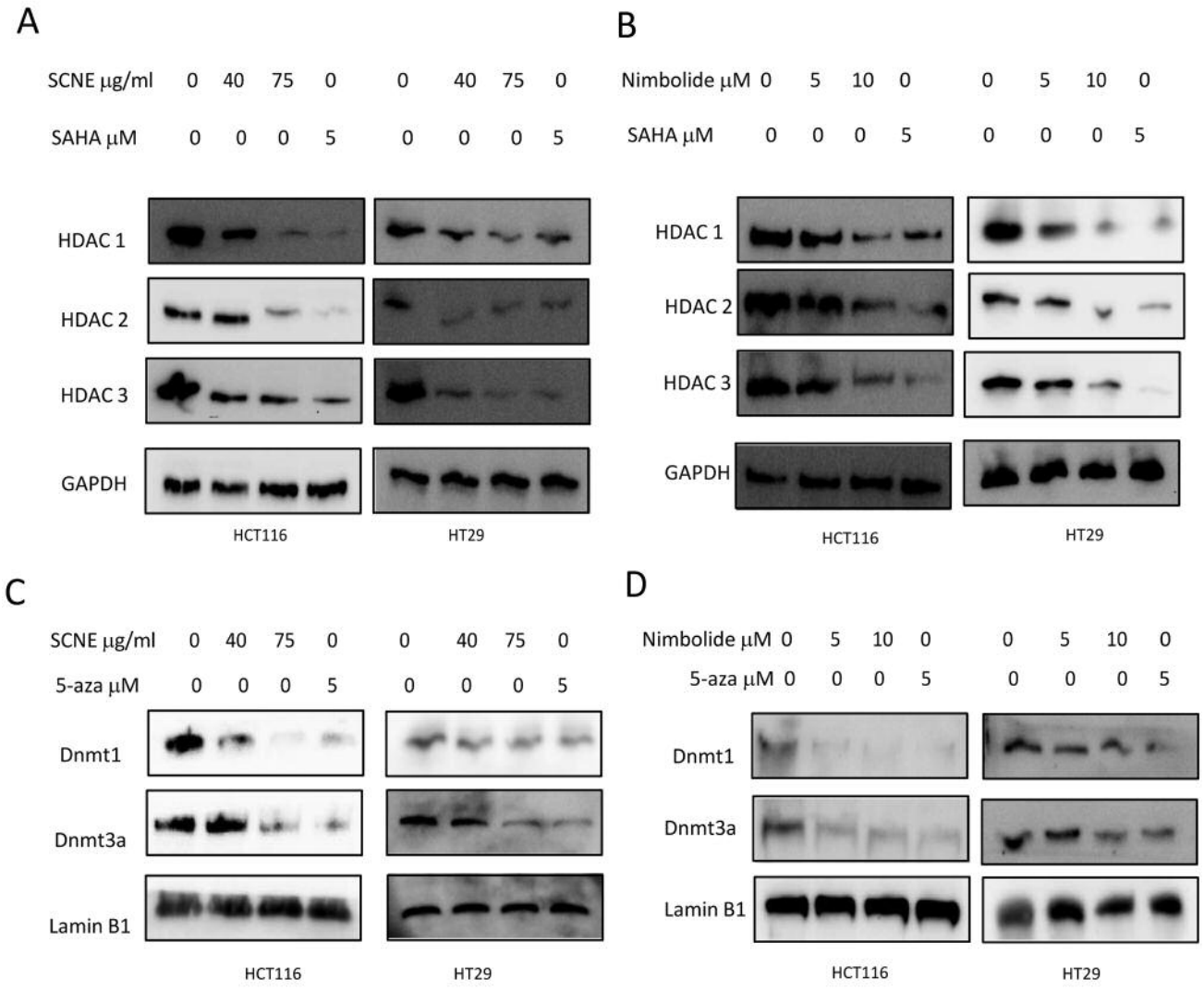

Figure 2. *SCNE and nimbolide decreased protein levels of HDACs and DNMTs in CRC cells. HCT116 and HT29 cells were treated with SCNE (40 and 75 μg/ml) and nimbolide (5 and 10 μM) for 48 h and 96 h, respectively. Expression of HDACs (A and B) and DNMTs (C and D) were determined by western blot. GAPDH and Lamin B1 were used as standards.*

After determining that cell viability was significantly repressed in both cell lines treated with SCNE and nimbolide, western blots were used to better interrogate the levels of HDACs and DNMTs following SCNE and nimbolide treatment. HDACs and DNMTs have been shown to play important roles in DNA modification (19). Therefore, we determined the expression of HDAC and DNMT isoforms in both human colon cancer cell lines treated with 40 and 75 μg/ml of SCNE and 5 and 10 μM of nimbolide for 48 h, as described previously (16). The western blots show a dose-dependent decrease in HDACs (Figure 2A and B) and DNMTs (Figure 2C and D) protein expression. Nimbolide seemed to be slightly more effective in inhibiting DNMTs than HDACs.

Treatment with SCNE, nimbolide and SAHA reduced HDAC and DNMT enzymatic activity in HCT116 and HT29 cells. The acetylation of histone tails influences the compression of the chromatin structure (19). Here, we show the effect of neem agents on the HDAC activity in nuclear extracts of HCT116 and HT29 cells. As shown in Figure 3A and B, the levels of HDAC activity were approximately decreased by SCNE dose of 40 μg/ml (26.98%) and 75 μg/ml (70.88%) in HCT116 for 48 h. The levels of HDAC activity were decreased by nimbolide dose of 5 μM (31.00%) and 75 μg/ml (53.80%) in HCT116 for 48 h. Moreover, the levels of HDAC activity were decreased by SCNE dose of 40 μg/ml (33.01) and 75 μg/ml (70.07%) in HT29 for 96 h. The levels of HDAC activity were also decreased by nimbolide dose of 5 μM (44.44%) and 10

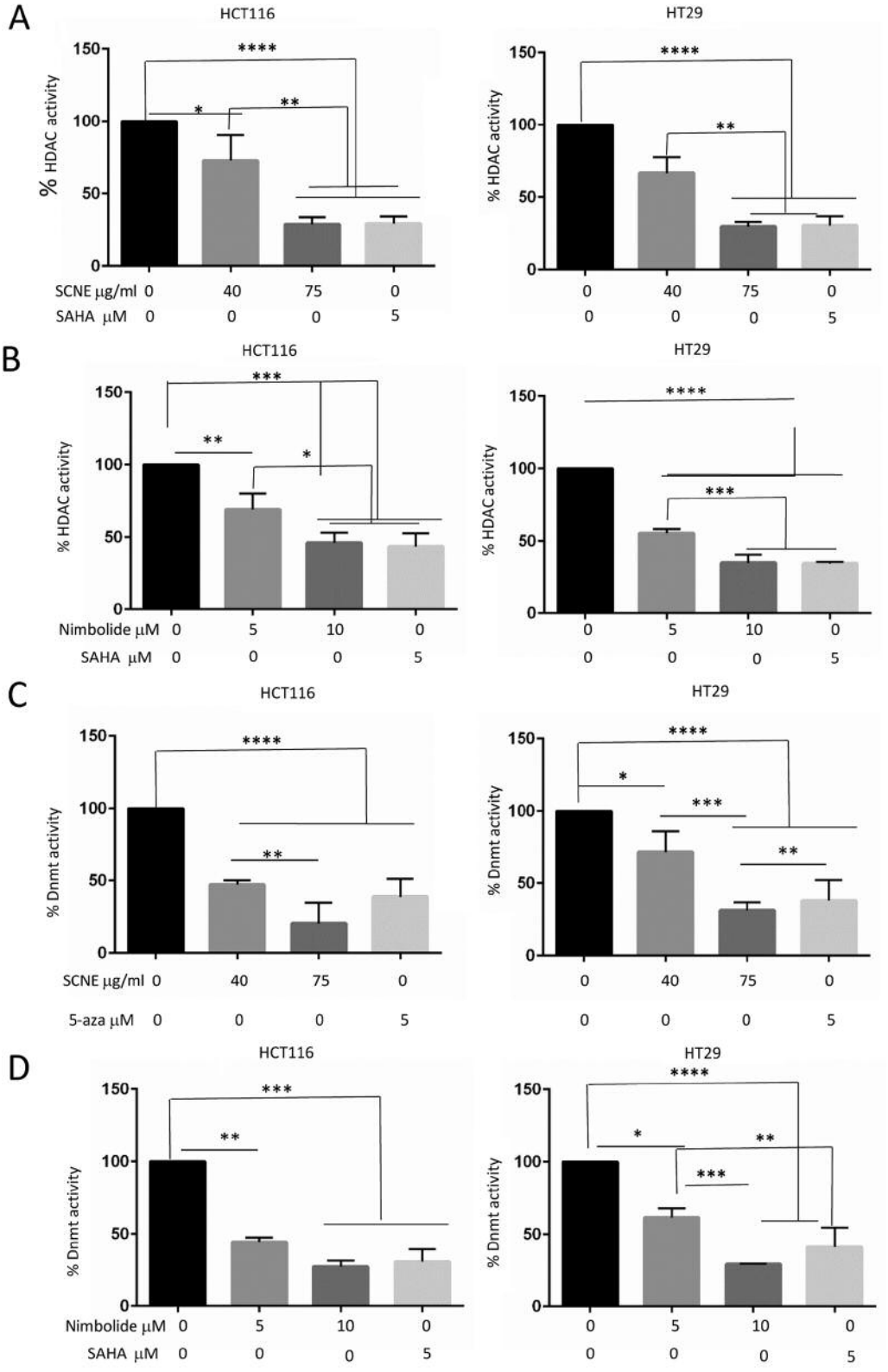

Figure 3. *SCNE and nimbolide reduced HDAC and DNMT activity in CRC cells. HCT116 and HT29 cells were treated with SCNE (40 and 75 µg/ml) and nimbolide (5 and 10 µM) for 48 h and 96 h, respectively. HDAC (A and B) and DNMT (C and D) activity was measured by ELISA. Data are expressed as mean±SEM. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 indicate significant difference versus vehicle control.*

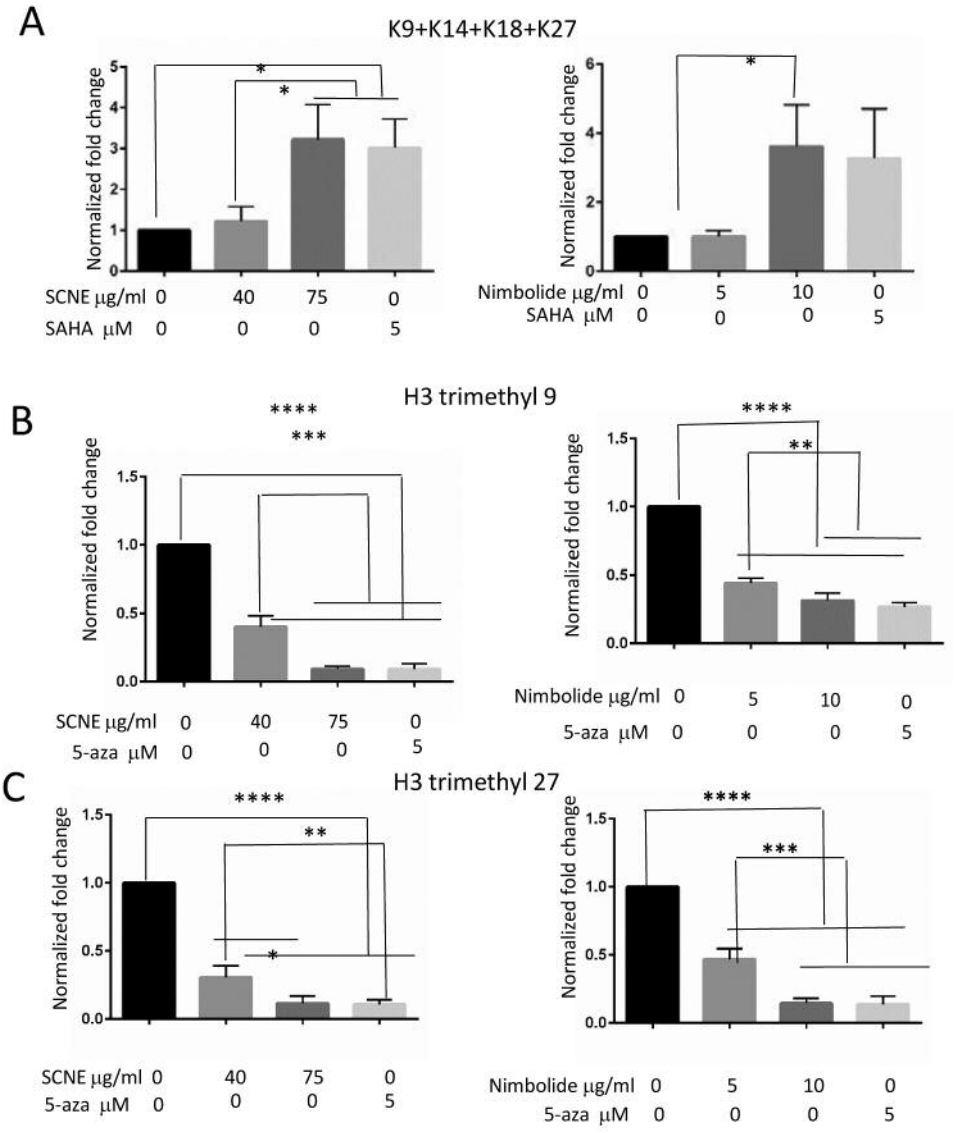

Figure 4. *SCNE and nimbolide treatment decreases promoter deacetylation and methylation of p16 in HCT116. Individual changes were examined using antibodies against specific marks: Histone H3 (acetyl K9+K14+K18+K23+K27) (A), Histone H3 (tri methyl K9) (B), Histone H3 (tri methyl K27) (C). *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 indicate significant difference versus vehicle control.*

µM (65.05%) in HT29 for 96 h. The data also indicates that SCNE and nimbolide have similar effects on HDAC activity in both cell lines, comparable to that of SAHA.

DNMTs modulate both maintenance and *de novo* methylation of DNA. However, repression of DNMT enzymatic activity most commonly suggests an increase in transcriptional activation (12). Here, a colorimetric DNMT activity assay was used to assess the overall enzymatic activity of DNMTs within HCT116 and HT29 cells treated with SCNE and nimbolide. As shown in Figure 3C and D, the levels of DNMT activity were decreased by treatment with 40 µg/ml (51.13) and 75 µg/ml (78.04%) of SCNE for 48 h in HCT116 cells. The levels of DNMT activity were decreased by 5 µM (28.27%) and 10 µM (68.52%) ofnimbolide in HCT116 cells for 48 h. Moreover, the levels of DNMTs activity were decreased by an SCNE dose of 40 µg/ml (24.19%) and 75 µg/ml (69.41%) in HT29 cells for 96 h. The activity of DNMTs was also decreased by nimbolide at doses of 5 µM (45.59%) and 10 µM (74.18%) in HT29 cells for 96 h. These data suggest that SCNE and nimbolide have similar effects on DNMT activity comparable to those of 5-azacytidine.

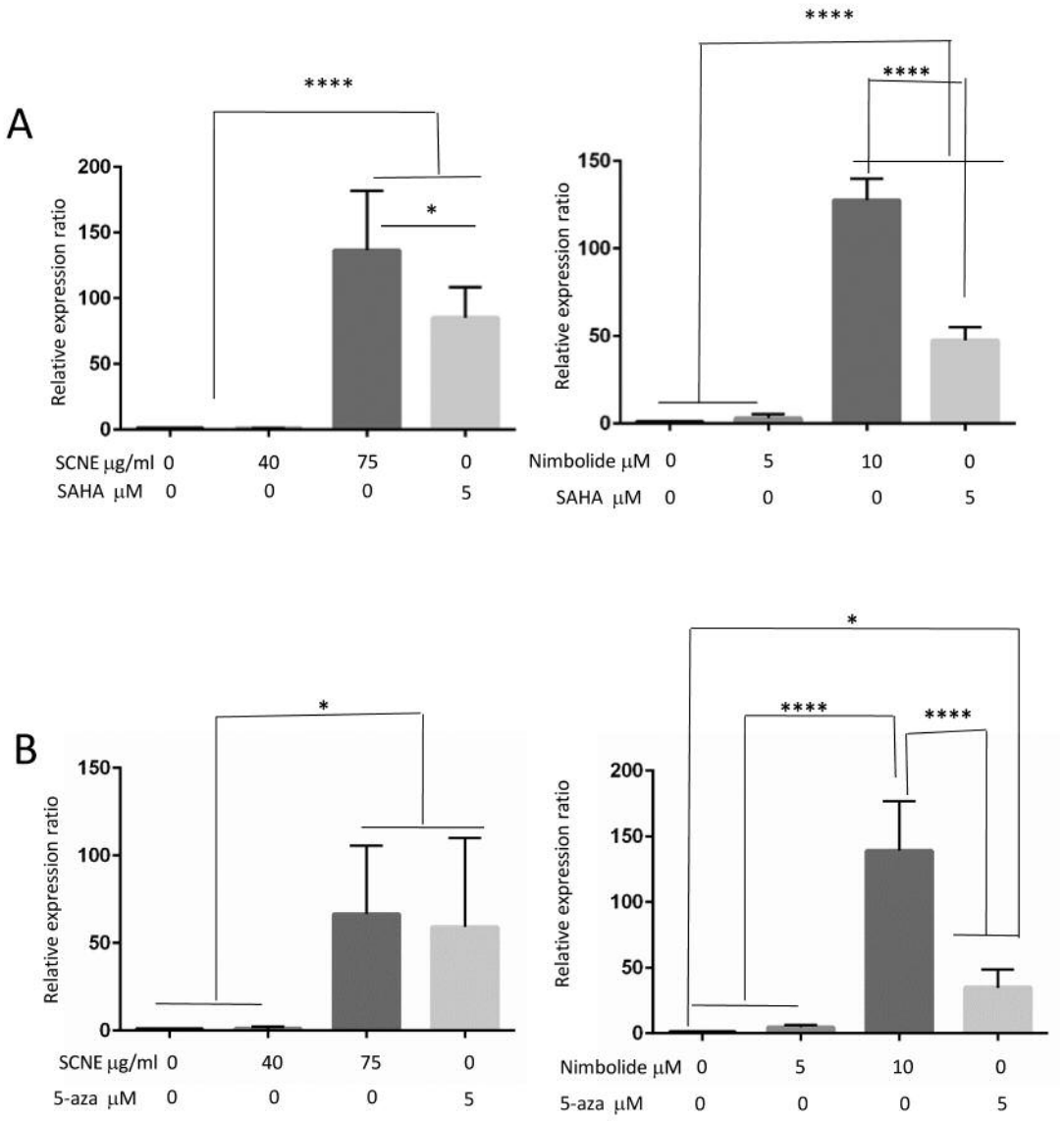

Figure 5. *SCNE and nimbolide increase expression of p16 transcripts in HCT116. Significant results are seen at the higher concentration of 75 mg/ml SCNE and 10 mM nimbolide compared to SAHA (A) and 5-aza (B). Data are expressed as the mean±SEM. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 indicate significant difference versus vehicle control.*

SCNE and nimbolide inhibited deacetylation of the p16 gene in the promoter region. To determine the relative effect on histone acetylation in the p16 promoter, human colon cancer cells were exposed to SCNE and nimbolide, and ChIP assays were used to assess the relative acetylation level of H3 Lys9 (H3K9), H3 Lys14 (H3K14), H4 Lys18 (H4K18), and H4 Lys27(H4K27). The results of the ChIP studies showed that, with increasing concentrations of SCNE and nimbolide, acetylation levels of H3K9, H3K14, H4K18, and H4K27 in the promoter region of p16 were significantly up-regulated by SCNE at a dose of 75 µg/ml and by 10 µM nimbolide (Figure 4A). Both the neem extract and nimbolide increased the expression of these activation marks.

Methylation analysis of p16 promoter in human colon cancer cells. To determine the relative contributions of histone methylation in the p16 promoter in human colon cancer cells exposed to SCNE and nimbolide, ChIP assays were used to assess the relative methylation level of H3K9me3 (Figure 4B), and H3K27me3 (Figure 4C). The results of the ChIP studies showed that, with increasing concentrations of SCNE and nimbolide, the expression of repression marks decreased.

Effects of SCNE and nimbolide on p16 mRNA expression in HCT 116 cells. To determine the effects of SCNE and nimbolide on the mRNA expression of p16 , HCT 116 cells were treated with SCNE or nimbolide for 48 h. Transcript expression was evaluated by quantitative real-time PCR (Figure 5). The resulting graphs show a statistically significant increase in p16 transcript in HCT116 cells after treatment with 75 μg/ml SCNE or 10 μM nimbolide.

Discussion

There is a wide interest in identifying new natural products that prevent or are therapeutic for colon cancer. One such class of natural products are those acting through epigenetic regulation. Safe and effective epigenetic modulators could reestablish the expression of critical tumor suppressor genes which are silenced in cancers. Using this strategy, it may be possible to re-enable regulatory control in some tumors making them more sensitive to therapy. In the search for such agents, previous work from our laboratory demonstrated that a highly purified extract of neem leaf has anticancer potential in colon and oral cancer models (16, 20). Neem contains a complex array of limonoids, which are a class of oxygenated triterpenes called tetranortriterpenoids (21). We continue to investigate this extract for other mechanisms relevant to the prevention or adjuvant therapy of cancer. Our previous studies confirmed a potential anti-cancer effect of purified SCNE by finding that SCNE treatment of human cancer cells resulted in reduced cell proliferation, inflammation, migration, and invasion (16). We also found that treatment of mice bearing HT29 and HCT116 xenografted colon tumors exhibited striking inhibition of colon tumor growth. However, the underlying mechanisms of this inhibition of colorectal cancer cell proliferation and metastasis remain to be fully elucidated. The intent of the current study was to gauge the effects of neem compounds on epigenetic pathways. The results suggest that alterations in the epigenetic machinery may be another mechanism by which neem and its constituents can contribute to cancer prevention and therapy.

Acetylation and methylation play important roles in reversible post-translational modifications affecting epigenetic modulation with critical roles in gene transcription, DNA replication, DNA repair, and cell-cycle progression. Overexpressed HDACs and DNMTs have been identified in many human cancers, resulting in repressed chromatin states that interfere with vital tumor suppressor functions. Inhibition of HDAC and DNMT activity has been pursued as a mechanism for re-activating repressed genes in cancers, with some HDAC and DNMT inhibitors showing promise in the clinical setting. In this study, we report that both SCNE of neem leaf and nimbolide demonstrated marked inhibition of epigenetic pathways. Both inhibited DNMT and HDAC expression and activity, promoted acetylation of the p16 gene promoter while inhibiting the expression of repressive marks in this tumor suppressor gene. These events associate with re-expression of the p16 gene.

The p16 protein is inactivated in a variety of human cancers (22). Thus, impairment of p16 by epigenetic silencing could impact the expression of genes associated with progression or inhibition of cancer that may represent important targets for chemoprevention or therapy. This is a different approach compared to using therapeutic drugs to enhance the re-expression of some silenced genes (23, 24). In this study we showed that SCNE enhances acetylation of the p16 promoter while reducing methylation of p16, both contributing to the restoration of p16 gene expression. Although the effective doses of 40-75 μg/ml SCNE, in short duration, used in this study, are attainable *in vitro*, they may not be biologically achievable in humans. Future experiments using lower, more biologically relevant doses over longer periods, as well as *in vivo* studies utilizing xenograft models will need to be conducted to validate these results. Bioavailability of SCNE and neem components (as well as other plant flavonoids) is a critical issue to be addressed in murine models of colorectal cancer, but our results indicate that certain constituents of the neem tree, such as nimbolide do have epigenetic modulatory promise. Whether other constituent liminoids from neem also demonstrate epigenetic modulatory activity awaits further research.

Conflicts of Interest

None of the Authors has any conflict of interest in regard to this study.

Authors' Contributions

ZQ carried out the experiments reported in this study with the assistance of KA and JM. JM prepared the figures for the study. All authors contributed to the preparation of the article. MJW contributed to the conception of the study. All authors read and approved the final article.

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