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Mechanisms for inhibition of colon cancer cells by sulforaphane through epigenetic modulation and hTERT down-regulation

Samantha L. Martin¹, Rishabh Kala¹, and Trygve O. Tollefsbol^{1,2,3,4,5}

¹Department of Biology, University of Alabama at Birmingham, 1300 University Boulevard, Birmingham, AL 35294, USA

²Comprehensive Center for Healthy Aging, University of Alabama Birmingham, 1530 3rd Avenue South, Birmingham, AL 35294, USA

³Comprehensive Cancer Center, University of Alabama Birmingham, 1802 6th Avenue South, Birmingham, AL 35294, USA

⁴Nutrition Obesity Research Center, University of Alabama Birmingham, 1675 University Boulevard, Birmingham, AL 35294, USA

⁵Comprehensive Diabetes Center, University of Alabama Birmingham, 1825 University Boulevard, Birmingham, AL 35294, USA

Abstract

Epigenetic modulations such as DNA methylation and histone modifications are becoming increasingly important for their ability to upregulate, down-regulate or silence genes without altering the DNA sequence. Many bioactive compounds have been shown to modify genetic and epigenetic profiles in various forms of cancers. Of the many dietary phytochemicals, sulforaphane (SFN), found in cruciferous vegetables such as kale, cabbage and Brussels sprouts has been documented to have some of the most potent effects on cancer cells. Due to the importance of colon cancer as a major health problem, we evaluated the molecular mechanism of dietary SFN in colorectal cancer (CRC) cells and its impact on the regulatory gene telomerase, human telomerase reverse transcriptase (hTERT), which is elevated in about 90% of cancers and essential for their continued proliferation. At physiologically relevant concentrations of dietary SFN in both HCT 116 and RKO CRC cells. We show for the first time, that SFN treatment changed the morphology, significantly inhibited cell viability and induced apoptosis of the CRC cells. In addition, we also showed that SFN regulates HDAC1 and in turn affects hTERT mRNA levels. We also demonstrate that SFN down-regulated telomerase protein and enzymatic activity in both HCT 116 and RKO cell types. These findings suggest that HDAC1 is a promising target to delaying and/or preventing CRC and this may be accomplished via consumption of SFN in cruciferous vegetables through modulation of the regulatory subunit of telomerase.

Keywords

Sulforaphane; Epigenetics; Histone Deacetylase; Histone Deacetylase Inhibitor; Human Telomerase Reverse Transcriptase

Introduction

Colon cancer is the second leading cause of cancer-related deaths and the third most commonly diagnosed cancer in both men and women (Janout and Kollarova, 2001). Based on data reported by the American Cancer Society (ACS), the average overall risk of developing colon cancer is approximately 1 in 20 (5%) in the United States, with around 136,830 new cases reported yearly and an expected death rate of 50,310 in the year 2014 (2015). The mortality rate from colon cancer has dramatically decreased in the past 20 years due in part to early detection, identification of polyps, more effective treatments and diet. It is well known that a high consumption of phytochemicals prevalent in fruits and vegetables dramatically reduces the risk of various chronic diseases such as but not limited to coronary heart disease (Dauchet et al., 2006), diabetes (Jew et al., 2009) and cancer (Block et al., 1992). Accordingly, there has been considerable interest with respect to epigenetic regulation mediated by dietary phytochemicals.

Epigenetics, the study of heritable change in gene expression without altering the DNA sequence, is postulated to work through external factors such as dietary phytochemicals and is widely studied because of its reversibility (Delcuve et al., 2009) (Duthie, 2011). Epigenetic modifications include histone acetylation, deacetylation and DNA methylation and targeting epigenetic machinery may be the most effective strategy in the development of cancer (Montenegro et al., 2014). Sulforaphane (SFN), an isothiocyanate found in cruciferous vegetables such as, cabbage, broccoli, kale, Brussels sprouts and cauliflower, has been shown to contain many biological activities and functions to prevent or treat carcinogenesis (Martin et al., 2013; Park and Pezzuto, 2002).

Investigators in the past decade have revealed that dietary phytochemicals such as sulforaphane (SFN) influence the expression and activity of the aforementioned epigenetic modifications (Gerhauser, 2013). In cruciferous vegetables, SFN is found at elevated levels in the form of glucosinolate precursors. However, upon chewing, the compound is catalyzed by myrosinase, also found in the human gut, and it is converted into SFN (Gerhauser, 2013). In addition, copious studies have revealed that the dietary phytochemical SFN is a favorable mechanistic approach for the attenuation of epigenetic aberrations that are prevalent in cancer that can lead to the induction of apoptosis of cancer cells (Pledgie-Tracy et al., 2007). In fact, SFN has also been shown to increase global and local acetylation by inhibiting histone deacetylase (HDAC) activity, thereby epigenetically regulating various genes including tumor suppressors (Ho et al., 2009; Telang et al., 2009). HDAC inhibitors are known to reactivate epigenetically silenced genes in cancer cells, thus prompting surrogate markers to induce cell cycle arrest and apoptosis (Khan and La Thangue, 2012).

An overwhelming amount of pre-clinical and *in vitro* studies have demonstrated that SFN treatments are beneficial (Choi et al., 2008). Myzak et. al. performed a clinical study where

patients were placed on a diet consisting only of cruciferous vegetables which led to HDAC inhibition 3 h after the initial broccoli intake which led to a simultaneous induction of histone acetylation (Myzak et al., 2007). Many investigators have also found that the oral administration of cruciferous vegetables has an inverse correlation to a number of cancers such as liver, Hodgkin's lymphoma, cervical, prostate, lung, gastrointestinal tract and ovarian, lung, stomach, breast and colon cancer (Chung et al., 2000; Fahey et al., 2002; Hecht et al., 2002; Kassie et al., 2003; Lund, 2003; Murillo and Mehta, 2001; Nagle et al., 2003; Steinkellner et al., 2001; Zhang et al., 1994).

Understanding the etiology of colon cancer may aid us in preventing and treating this disease. Recently, studies have made major discoveries in scientific research using information obtained from non-coding RNA. MicroRNAs (mir) are a class of small non-coding RNAs that bind to the 3' untranslated region of target messenger RNAs, thereby causing posttranscriptional inhibition by degradation (Mirnezami et al., 2009). MicroRNAs play a vital role in cellular proliferation, differentiation and cell death and have been documented to act either as tumor suppressors or oncogenes in malignancy. Although microRNAs are relatively new to the field of research, there have been more than 900 identified in the human genome (Nugent et al., 2011). One of the major mir's implicated in human cancer is mir-21. Mir-21 has been documented to be up regulated in breast cancer, hepatocellular carcinomas, nasopharyngeal carcinoma, glioblastoma and colorectal cancer (Yang et al., 2013) (Nouraei et al., 2013). Studies have also shown that overexpression of mir-21 enhances cellular proliferation, migration, invasion and metastasis in copious cell lines (Kadera et al., 2013; Sanchez-Diaz et al., 2013). More importantly, investigators have implicated that aberrant expression of microRNAs may potentially be controlled by epigenetic processes such as methylation and deacetylation (Bandres et al., 2009; Li et al., 2009), thereby controlling vital epigenetic modifiers such as HDACs, DNMTs and hTERT.

The human telomerase reverse transcriptase (*hTERT*) gene is a promising marker in diagnosing malignancy (Ahmed and Tollefsbol, 2003). *hTERT*, a catalytic subunit of telomerase, is an epigenetically regulated gene that is expressed in more than 90% of human cancers, but not in detectable quantities in most somatic cells (Cong et al., 2002). HDAC inhibitors have been documented as vital suppressors of *hTERT* expression in breast cancer, leukemia and Hep2 cervical cancer cells (Meeran et al., 2010; Murakami et al., 2005; Woo et al., 2007). Due to the importance of SFN in regulating epigenetic processes and the role of hTERT in cancers such as colon cancer, we sought to explore the impact of SFN on epigenetically controlled *hTERT* in HCT 116 and RKO CRC cells. In this current study, we applied physiologically relevant concentrations of SFN to RKO and HCT 116 CRC cells to test their effects on *HDAC1* and *hTERT* gene relation as well as telomerase activity and apoptosis of the cells.

Materials and Methods

Cell culture and cell growth assay

Human RKO and HCT 116 colon cancer cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Epigenetically and genetically, RKO and HCT 116 colon cancer cell lines are similar with a wt-p53. Both cell types were maintained in

Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA) and 1% Penicillin and Streptomycin (Fischer Scientific) at 37° in a 5% CO₂ incubator. The cells were subcultured at 90% confluency and seeded in 6- and 96-well plates. Cells were treated at the indicated concentrations upon 24 h increments for 72 hours. Control cells were treated with DMSO (vehicle control) and medium was replaced every 24 h throughout the experiment.

Chemicals

R, S Sulforaphane (98.2% pure) was purchased from (LKT Laboratories, Minneapolis, MN). Sulforaphane was dissolved and diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 100 mmol/L and stored at -20°C.

MTT analysis

Cells were seeded at 1,000 cells per well in a 96-well plate and allowed to adhere for 24 h before daily treatments at the indicated concentrations. After the end of the 72 h treatment, 50 µL of MTT (5 mg/L) was added to each well and allowed to incubate for 4 h at 37°C. DMSO (150 °L) was added to each well, and an absorbance at 595 nm was read on a microplate reader (model 680, Bio-Rad).

Apoptosis Analysis

Cellular apoptosis was detected by Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit purchased by life technologies (Invitrogen Life Technologies, Grand Island NY) and analyzed using FACS.

Real-Time PCR

Cellular RNA was isolated from cultured cells using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA (5 µg) was reverse transcribed into cDNA using the Script cDNA synthesis kit (Bio-Rad Hercules, CA). GAPDH was used as internal control. Real-time quantitative PCR was carried out in a Bio-Rad MyiQ thermocycler (Bio-Rad, Hercules, CA) using Platinum SYBR Green detection system (Invitrogen, Carlsbad, CA). The primer sets were as follows: sense 5'-AGG GGC AAG TCC TAC GTC CAG T -3' and anti-sense 5'-CAC CAA CAA GAA ATC ATC CAC C-3' for hTERT, sense 5'-GAA GGT CGG AGT CAA CGG ATT T-3' and 5'-ATG GGT GGA ATC ATA TTG GAA C-3' antisense for GAPDH and sense 5'-ATGGACGATCTGTTTCCCCT-3' anti-sense 5'-CGGTTTACTCGGCAGATCTT-3' for HDAC1. GAPDH was the internal loading control. The Real-time quantitative PCR was conducted in a CFX96 Real-Time System (Bio-Rad) using the SYBR Green detection system (Bio-Rad, Hercules, CA, USA). The delta-delta CT method was used to determine the relative level for expression. The fold change in expression was determined by the formula $2^{-\Delta\Delta C_T}$ where $C_T = C_T(\text{Target})_{\text{normalized}} - C_T(\text{Control})_{\text{normalized}}$; $C_T(\text{target})_{\text{normalized}} = C_T(\text{gene of interest}) - C_T(\text{target GAPDH})$ and $C_T(\text{Control})_{\text{normalized}} = C_T(\text{gene of interest}) - C_T(\text{Control GAPDH})$.

Western Blotting

Protein was extracted from cultured cells using RIPA lysis buffer (Upstate Biotechnology, Lake Placid, NY) following the manufacturer's protocol. Equal amounts of protein were loaded into a 4–20% Tris-Bis gel and separated by electrophoresis at 165 V. Primary and secondary antibodies were probed using the SNAP i.d. (Millipore, Billerica, MA) for specific HDAC and hTERT (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized using the ECL detection system (Santa Cruz Biotechnology) and XRS chemi gel doc (Bio-Rad Hercules, CA) following the protocol of the manufacturer.

HDAC activity assay

Cell pellets were extracted using the Thermo Scientific NER-PER Nuclear and Cytoplasmic extraction kit (Waltham, MA USA 02451). HDAC activity was assayed using Epigentek HDAC Activity/Inhibition Assay (Colorimetric) (Farmingdale, NY 11735) according to the manufacturer's protocol.

Telomerase activity assay

Telomerase activity was performed using Telo *TAGGG* Telomerase PCR ELISA Kit (Roche applied science, Indianapolis, IN) according to the manufacturer's protocol.

MicroRNA analysis

Total RNA was extracted from cells using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. MicroRNA cDNA was reversed transcribed using specific primers obtained from Invitrogen (Invitrogen Life Technologies, Grand Island NY). MicroRNA real-time quantitative PCR was carried out in a Bio-Rad MyiQ thermocycler (Bio-Rad, Hercules, CA) using probes obtained from Invitrogen (Invitrogen, Carlsbad, CA) following the manufacturers instructions.

Statistical Analysis

The statistical significance of control groups and treated sulforaphane controls were determined using paired Student T test in Graph Pad Prism version 41 for Windows, Graph Pad Software, San Diego California USA (www.Graphpad.com), in each case, $P < 0.05$ was considered statistically significant.

Results

SFN inhibits proliferation and causes morphological changes in CRC cells

To ascertain an optimal dose of SFN and access the status of proliferation, we first performed cell growth and morphological analysis on RKO and HCT 116 CRC cell lines. As shown in Figures 1 and 2, RKO and HCT 116 cells, respectively, were treated with the indicated concentrations of SFN for 72 h. Following the 72 h treatment, we observed a dose-dependent cell growth inhibition with SFN treatment in both cell lines. In addition, there was a significant change in the morphology of both cell lines beginning at the concentrations of 10 μ M SFN (Figures 1C and 2C), thus solidifying it as an optimal concentration for future testing. In accordance with our previous findings (Figure 1 and 2), Figure 3 confirms that

cellular viability was significantly inhibited at the optimal concentration of 10 μ M, thus, strengthening the dose- and time-dependent (1 day data not shown) inhibition seen in the morphological assay previously performed. Collectively, cell growth was almost entirely inhibited at 20 μ M SFN at 72 h (Figures 1D, 2D and 3) indicating that 20 μ M SFN may be toxic in the aforementioned cell lines. Taken together, our results indicate that SFN at the optimal concentration of 10 μ M causes a significant reduction in proliferation in CRC cell lines RKO and HCT 116.

SFN induces apoptosis in RKO and HCT 116 CRC cells—It is well known that HDAC inhibitors promote cellular apoptosis in various forms of cancer cells (Liu et al., 2008; Singh et al., 2005). Given the aforementioned information, we assessed cell death by performing an apoptosis analysis via Annexin V flow cytometry. As shown in Figure 4 there is a significant increase in percent apoptosis for HCT 116 cells and there was a highly significant increase in percent apoptosis in RKO cells in response to SFN with a 72 h treatment.

SFN causes a significant down-regulation in oncogene microRNA-21

It has been documented that microRNAs participate in a wide variety of regulatory events and are exceptionally essential in gene expression. To further explore the mechanism of the mir-21 mediated down-regulation of hTERT, we assessed the expression of microRNA 21 by performing quantitative PCR. As shown in figure 6 our studies indicate that SFN significantly inhibited mir-21 in RKO CRC cells at our optimal concentration of 10 μ M SFN.

Effects of SFN on protein and gene expression

Studies have consistently shown that an imbalance of histone acetyl transferase (HATs) and HDACs generally lead to aberrant transcription of many epigenetic genes. HDACs are known to inhibit the accessibility of transcription machinery, thereby promoting a closed chromatin structure (Ropero and Esteller, 2007). It is also well known that telomerase is transcriptionally upregulated in numerous tumor cells (Janknecht, 2004). To further explore the molecular mechanism of SFN on HDAC and its effect on *hTERT* mRNA in CRC cells, we performed Real-time PCR. As shown in Figures 5A and 5B, there is a consistent upregulation at 5 and 10 μ M SFN in both *HDAC* and *hTERT* mRNA levels.

SFN causes a repression in HDAC and hTERT protein and enzymatic activity

It is well known that SFN serves as an HDAC inhibitor thus activating or repressing key genes responsible for carcinogenesis. Despite this, the potential of HDAC inhibitors repressing *hTERT* continues to remain controversial. To investigate this, we performed western blotting analyses on HDAC and hTERT as well as activity assays for these two enzymatic processes in RKO CRC cells. Our studies show although there was a significant down-regulation in hTERT mRNA in response to SFN treatments of RKO CRC cells (Figure 5B), there was no attenuation observed in hTERT protein expression (Figure 7), or telomerase activity at 72 h treatment (Fig. 8). In contrast, there was a clear trend between SFN and HDAC as it was down-regulated in its mRNA expression (Figure 5A), protein expression (Figure 7), and overall HDAC activity (Fig 7).

Discussion

Although many clinicians speculate that colon cancer is preventable, it still remains the second most frequent cause of cancer-related deaths in both men and women in the United States (Janout and Kollarova, 2001). Like most cancers, colon cancer is treated with surgery, radiation and/or chemotherapy depending on the stage of diagnosis and aggressiveness (2014). To avoid these extreme treatments, extensive efforts have been implemented to involve the use of plant-based derivatives to prevent the onset of carcinogenesis. Dietary compounds derived from an array of fruits and vegetables are known to contain phytochemicals that are documented to inhibit DNA damage, reactivate tumor suppressors and reverse the progression of premalignant cells. This process is termed chemoprevention. Chemopreventive agents are of increasing importance because of their ability to alter gene expression and delay oncogenesis by orally consuming dietary phytochemicals. Many studies suggest that these dietary phytochemicals exert protective mechanisms against carcinogenesis (Naithani et al., 2008).

Studies have reported that targeting HDAC inhibitors likely impacts cancer. Several HDAC inhibitors have been shown to exhibit exemplary anti-tumor activities, but still remain under phase I and II clinical investigations (Munster et al., 2009; Su et al., 2011). HDAC-inhibitor SFN has been shown to have some of the strongest effects on breast and ovarian cancer and is speculated to be among the most vital of HDAC inhibitors to date (Chen et al., 2013; Meeran et al., 2010). In addition, several *in vitro* studies have shown that SFN also has the ability to induce apoptosis and decrease cell viability in numerous cancer cell lines (Chu et al., 2009; Ho et al., 2009; Pledge-Tracy et al., 2007), but not yet reported in CRC cells RKO. Accordingly, in order to investigate the molecular effects of SFN, we treated the cells with physiologically relevant concentrations on two CRC cells, representative of a wide range of CRC phenotypes.

To determine toxicity levels and obtain optimum concentrations, we analyzed the effect of SFN for 24 (Data not shown) and 72 h (Figure 3) with concentrations ranging from 0–40 μ M and performed an MTT analysis. We found that cells exposed to 2.5 μ M SFN showed no change in cellular viability in HCT 116 and RKO CRC cells as compared to our vehicle control, DMSO. Our analysis also showed that cellular viability was almost completely inhibited at 20 μ M SFN, with a significant 82% and 85% decrease (** $P < .01$), in HCT 116 and RKO respectively, indicating that this higher concentration of SFN may be toxic to both types of CRC cell lines. Doses of 10 μ M SFN showed the most favorable results as both cell lines were selectively inhibited, independent of cytotoxicity. While HCT 116 cells rendered a 20 % decline (** $P < .01$) in cellular viability, RKO cells displayed an even greater reduction at 42% (* $P < .01$) after 72 h of treatment of SFN, thus solidifying it as the optimal concentration.

To further confirm what was seen in our cell growth and proliferation assay, we analyzed a range of SFN treatments from in MTT analysis and observed morphological changes and induction in apoptosis. As seen in Figures 1 and 2, for both RKO (Fig. 1A) and HCT 116 cells (Fig. 2A) the vehicle control showed no change in morphology after a 72 hour treatment. Consistent with our MTT analysis, we found that beginning with our optimal dose

of 10 μM SFN, there were considerable morphological changes (Figs. 1C and 2C). In addition, we observed a significant induction in percentage apoptosis with SFN treatment in both HCT 116 and RKO cells as shown in Figure 3, as compared to our vehicle control. Doses of 20 μM SFN showed a drastic change in morphology with an extremely significant induction of apoptosis in RKO. While the induction of apoptosis and changes in morphology was not as significant in HCT 116 cells as RKO cells, they were nevertheless significant, further solidifying physiologically relevant 10 μM SFN as our optimal dose. We propose that the difference in induction of apoptosis in the two types of colorectal cancer (CRC) cell may be due to the site of origin, stage of aggressiveness and genetic background of the two cell lines (Ahmed et al., 2013).

Chemoprevention and chemotherapeutic agents have been greatly warranted to counteract the toxicity levels seen in chemotherapy. Recently, SFN has made a major impact in the field of epigenetics for its role as an HDAC inhibitor (Ho et al., 2009; Myzak et al., 2007). It is well known that the constant equilibrium between histone acetyltransferases (HATs), which transfer acetyl groups, and HDACs, which remove acetyl groups, prevent aberrant patterns in histone modifications, thereby making it a crucial mechanism in regulating gene expression (Nian et al., 2009; Ropero and Esteller, 2007). To date, scientists have identified 18 mammalian HDACs and divided them into four classes based on their sequence similarities to yeast. Class I HDACs include HDAC1, HDAC2, HDAC3 and HDAC8, are Zn^{2+} -dependent and have a nuclear localization. Class II HDACs are divided into two subclasses: subclass IIa includes HDAC4, HDAC5, HDAC7 and HDAC9, whereas subclass IIb consists of HDAC6 and HDAC10. Both subclasses are Zn^{2+} dependent and are found in the nucleus and cytoplasm. Class III consists of 7 Zn^{2+} independent Sirtuins. While these groups of HDACs are NAD^+ , little is known about their exact subcellular location. Lastly, class IV consists of only HDAC11. This final class, which is homologous to class II and I have a nuclear localization (Gregoret et al., 2004).

Although some studies report that HDAC inhibitors have strong post-translational implications, others have found that in fact they have stronger effects post-transcriptionally (Hirsch and Bonham, 2004). Our findings are consistent with the latter. In the present study, we found that HDAC1 mRNA levels displayed a significant down-regulation beginning at our optimal concentration of 10 μM SFN. In addition, we demonstrated that there was an attenuation at the translational and enzymatic activity levels, as overall HDAC activity was deregulated starting at 5 μM and was significantly attenuated at our optimal dose of 10 μM SFN. Thus the attenuation of the HDAC1 gene, protein and deregulation of the enzyme could be due to the SFN-mediated binding to the catalytic site of HDAC1, thereby decreasing HDAC1 levels and overall HDAC activity.

Telomerase, a ribonucleoprotein that is responsible for maintaining genome stability by capping the ends of chromosomes, has recently become a promising target for therapeutics. hTERT, the catalytic subunit of telomerase, is widely expressed in over 90% of cancers, but remains at undetectable concentrations in somatic cells (Cong et al., 2002). In an attempt to identify changes in hTERT modulated by the HDAC inhibitor SFN, we performed transcriptional, translational and enzymatic activity assays. Our results showed that there was a significant transcriptional and translational down-regulation beginning at our optimal

concentration of 10 μ M SFN. Contradictory to our original hypothesis, our results also showed that the hTERT enzyme levels had no attenuation after 72 hours. However, for the first time, we showed that a 9-day treatment with our optimal concentration of 10 μ M SFN caused a significant down-regulation in the telomerase protein (Figure 7). This effect could be due to the extended half-life of the telomerase enzyme (Love et al., 2008). Furthermore, we also believe that the attenuation of the hTERT protein could be due to the recruitment of HDAC inhibitor SFN facilitating hyperacetylation and promoting an open chromatin formation, thereby allowing known repressor proteins to interact with the regulatory region to regulate the expression of hTERT.

Studies have documented that mir-21 is up regulated in various diseases including cardiovascular disease (CVD), inflammation and cancer (ref). Because of this, we attempted to down-regulate mir-21 by directly treating cells with SFN as our proposed dietary phytochemical. While it is well known that dietary phytochemicals play a crucial role in regulating epigenetic modifiers, there is little to no information on dietary phytochemicals and their effect on microRNAs. It was our hypothesis that there would be a significant down-regulation of mir-21 mediated by SFN, subsequently down-regulating hTERT transcription. Consistent with our hypothesis, for the first time, we showed that mir-21 was significantly down-regulated at our optimal concentration of 10 μ M SFN, identical to the trend seen in hTERT mRNA (Figure 5). We propose that the inhibition seen in hTERT mRNA at our optimal concentration of 10 μ M SFN could be due to an increase in well known tumor suppressor phosphatase and tensin homolog (PTEN), mediated by the down regulation of mir-21 caused by SFN. It has been implicated that mir-21 directly targets tumor suppressors such as but not limited to tropomyosin 1 (TMP1) and PTEN (Zhu et al., 2007). We suggest that the down-regulation of hTERT could be due to the inhibition of the AKT pathway by PTEN, thereby leading to decreased hTERT mRNA levels. Furthermore, it has also been documented that there is an inverse correlation between loss of PTEN and overexpression of telomerase, indicating that PTEN signaling may be vital in regulating telomerase and other epigenetic modifiers (Zhou et al., 2006), thereby strengthening our rationale.

In this present study, we demonstrated that SFN at the physiologically relevant concentration of 10 μ M SFN, changed cellular morphology, inhibited cellular proliferation, induced apoptosis, and down-regulated protein and enzyme activity levels of HDAC1 in both RKO and HCT 116 CRC cells. Furthermore, we also showed for the first time, with SFN treatment, that telomerase after a 9-day treatment was significantly inhibited at the optimal concentration of 10 μ M SFN when compared to our vehicle control. Although this work introduced novel *in vitro* findings, *in vivo* validation will be warranted. It is our hope that these present findings will invoke further testing in other cancers types, in addition to ultimately leading to a full mechanistic elucidations of the efficacy of SFN in colon cancer. Taken together, it is clear that there is interplay between mir-21 and hTERT, thereby making this a promising target for chemoprevention and chemotherapeutics in the very near future.

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Figure 1. Sulforaphane changes morphology in RKO colon cancer cell lines

A) DMSO treatment after 72 h. B) 5 μ M treatment after 72 h. C) 10 μ M treatment after 72 h. D) 20 μ M treatment after 72 h. The circular white floating cells are indicative of apoptosis.

Figure 2. Sulforaphane changes morphology in HCT 116 colon cancer cell lines

A) DMSO treatment after 72 h. B) 5 μ M treatment after 72 h. C) 10 μ M treatment after 72 h. D) 20 μ M treatment after 72 h. The circular white floating cells are indicative of apoptosis.

Figure 3. Sulforaphane inhibits cellular proliferation of HCT 116 and RKO colon cancer cells lines after 72 h treatment

Human RKO and HCT 116 colon cancer cells were treated with varying concentrations of SFN and cell viability was determined using MTT assay. Results were obtained from three independent experiments, mean $\pm$ SE. (Statistical significance; * $P < 0.05$, ** $P < 0.01$)

Figure 4. Sulforaphane causes a significant increase in apoptosis in RKO and HCT 116 CRC cell lines

A) RKO and HCT 116 CRC cells show a significant increase in percent apoptosis after 72 h treatments with increasing concentrations of SFN. The results were obtained using three independent experiments with mean $\pm$ S.E. Statistical significance; * $P < 0.05$), ** $P < 0.01$.

Figure 5. SFN regulates mRNA expression of several key epigenetic modifiers in RKO cancer cells

Human RKO colon cancer cells were treated with varying concentrations of 0, 5, and 10 μ M SFN. A.) Relative mRNA levels of *HDAC1* in control. B.) Relative mRNA levels of *hTERT* in control. Results were obtained from three independent experiments, mean $\pm$ SE. Statistical significance; * $P < 0.05$, ** $P < 0.01$.

A

Figure 6. Sulforaphane causes a down regulation in microRNA 21

Human RKO colon cancer cells were treated with concentrations of 0, 5 and 10 μ M SFN.

A.) Relative microRNA levels in RKO colon cancer cells. Results were obtained from three independent experiments, mean $\pm$ SE. Statistical significance; * $P < 0.05$, ** $P < 0.01$.

Figure 7. Sulforaphane causes attenuation in protein expression in several key epigenetic modifiers

SFN protein expression was assessed by western blot. A.) HDAC1 protein expression was obtained after 72 h treatment. B.) hTERT protein expression was obtained after a 72 h treatment. C.) β Actin was used as an internal loading control. Results were obtained from three independent experiments, mean $\pm$ SE. (Statistical significance * $P < 0.05$, ** $P < 0.01$).

Figure 8. SFN inhibits HDAC activity in RKO colon cancer cells

HDAC activity was assayed in control, as well as SFN-treated cells at increasing concentrations of SFN for 72 h. Results were obtained from three independent experiments, mean $\pm$ SE. Statistical significance * $P < 0.05$, ** $P < 0.01$.

Figure 9. SFN down-regulates hTERT protein expression in a dose-and time-dependent manner
SFN activity was assayed with control, in addition to SFN-treated cells for 72 h. Results were obtained from three independent experiments, mean $\pm$ SE. Statistical significance; * $P < 0.05$, ** $P < 0.01$.