

Synergistic Mediation of Sulforaphane and Vitamin D on the *Wnt*-signaling Pathway

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Colorectal cancer is a leading cause of death in The United States. Research suggests that colorectal cancer is strongly associated with aberrant expression of the *Wnt*-signaling pathway. Sulforaphane (SFN) is a bioactive component found in broccoli and is a histone deacetylase inhibitor that may play a role in regulation of the *Wnt*-signaling pathway. Binding of vitamin D to its receptor activates the vitamin D receptor element, which in turn enhances recruitment of histone acetylation factors. The present study investigated the synergistic effect of SFN and vitamin D on the *Wnt*-signaling pathway. We hypothesized that synergistic treatment using both compounds would show greater down-regulation of the *Wnt*-signaling pathway than treatment with either compound on its own. Caco-2 cells were harvested using treatments of various concentrations of SFN supplemented with and without vitamin D. Histone deacetylase inhibitor TSA was used as a positive control. RNA was isolated from cells and used to prepare cDNA. Quantitative real-time PCR was performed to assess expression of *Wnt*-pathway targeting genes *Axin2*, *Cyclin D1*, and *C-Myc* relative to the expression of housekeeping gene *GAPDH*. Our results reveal a trend indicating that as vitamin D treatment is supplemented with increasing concentrations of SFN, expression of *Wnt*-pathway-related genes is increasingly repressed. These findings represent preliminary data that warrant further investigation of SFN and vitamin D epigenetic regulation of the *Wnt*-signaling pathway. *Journal of Nature and Science*, 1(2):e36, 2015.

Sulforaphane | Vitamin D | *Wnt* Pathway

The *Wnt*-signal pathway is a highly conserved mechanism of proteins that mediate cell-to-cell interactions by transmitting received surface signals through the cytoplasm and into the nucleus (1). This complex provides essential control of cell growth, differentiation, and survival (2), but aberrant gene expression of components related to the *Wnt*-signal cascade is known to result in uncontrolled stem cell renewal, effectively leading to various human pathologies, including tumorigenesis and cancer (3). *Wnt*-signaling is particularly associated with colorectal cancer. In fact, the vast majority (>90%) of colorectal tumors in humans possess significant alterations to the *Wnt*-pathway (4). Colorectal cancer is the second leading cause of cancer-related death in the United States (5); therefore, it is of high priority to seek a clear understanding of *Wnt*-pathway regulation.

The activated *Wnt*-signaling pathway is known to cause both cancer and tumor progression and it has been hypothesized that this is due to inappropriate gene activation by β -catenin (6), an intracellular *Wnt*-signal transducer. *Axin2* is a tumor suppressor protein that is responsible for maintaining the destruction complex that oversees the degradation of β -catenin (3). In the active state of the *Wnt*-signal pathway, β -catenin accumulates in the cytoplasm and interacts with transcription factors TCF and LEF in the nucleus causing transcription of *Wnt*-target genes (6). Activation of the *Wnt*-signaling pathway (Figure 1) occurs in the absence of *Wnt* antagonists, some of which include SFRPs and WIFs, which bind *Wnt* proteins (10), and DKFs and the WISE/SOST families, which bind the LRP5/6 membrane receptor (11). Disrupted interaction of the *Axin*-APC β -catenin destruction complex can also promote *Wnt*-signaling (12).

Sulforaphane (SFN) is a bioactive food component found naturally in broccoli and broccoli sprouts and is known for its

anti-inflammatory and chemoprotective properties (13). Previous research has demonstrated that SFN acts as a histone deacetylase (HDAC) inhibitor (14) and in this way plays an important role in regulating the critical balance of histone acetyl transferase and histone deacetylase activity. Disruption to this correspondence of gene regulation can result in the progression of cancer (15). In a study using breast cancer cell lines, SFN was shown to be a suppressor of the *Wnt*-pathway (16). However, the molecular mechanism by which SFN alters *Wnt* activity remains poorly understood.

The present study investigated the influence of SFN and vitamin D on the *Wnt*-pathway by assessing the relative expression of *Wnt*-signaling reporter genes *Axin2*, *Cyclin D1*, and *C-Myc*. *Axin2* is a direct target of the *Wnt*-pathway (17) whereas *Cyclin D1* and *C-Myc* are indirect targets regulated by the β -catenin/TCF-4 complex (15,16). Decreased relative expression of these genes would therefore indicate inhibition of the *Wnt*-pathway. We are interested in using a synergistic approach with vitamin D based on previous research that suggests vitamin D also participates in chromatin remodeling related to histone acetylation. Upon vitamin D-induced activation, the vitamin D receptor (VDR) binds to the vitamin D response element (VDRE) within promoter regions of genes and in turn enhances the recruitment of histone acetylation related cofactors (18). We will test the hypothesis that SFN and vitamin D synergistically mediate the *Wnt*-signaling pathway. We predict that the combined treatment of SFN and vitamin D will be more effective in repressing the *Wnt*-signaling pathway than treatment with either SFN or vitamin D alone. Trichostatin A (TSA) is a known natural HDAC inhibitor (14) and will be used as a positive control for the study.

Methods and Materials

Sampling and Culture Conditions

The present study was conducted using a previously treated harvest of Caco-2 cells obtained from Dr. Richard Wood's lab (University of Massachusetts, Amherst). The cell harvest consisted of two groups of four samples. Each group contained a control sample, a sample treated with a dose of 1.0 μ M SFN, a sample treated with a dose of 2.5 μ M SFN, and a sample treated with a dose of 1.0 μ L TSA, the positive control. Additionally, all samples in one group (the vitamin D group) received a 100 μ M dose of 1,25-dihydroxyvitamin D; the other group (the non-vitamin D group) represented the samples without the combined vitamin D treatment. All cells were harvested using 1 ml of TRIzol Reagent per well. The cell harvest was stored in Eppendorf tubes and frozen at -80 degrees Celsius.

RNA isolation

Phase separation was accomplished by adding 200 μ L chloroform reagent to each homogenate. Isopropanol (0.5 mL) was used to precipitate the RNA from each sample. Following precipitation,

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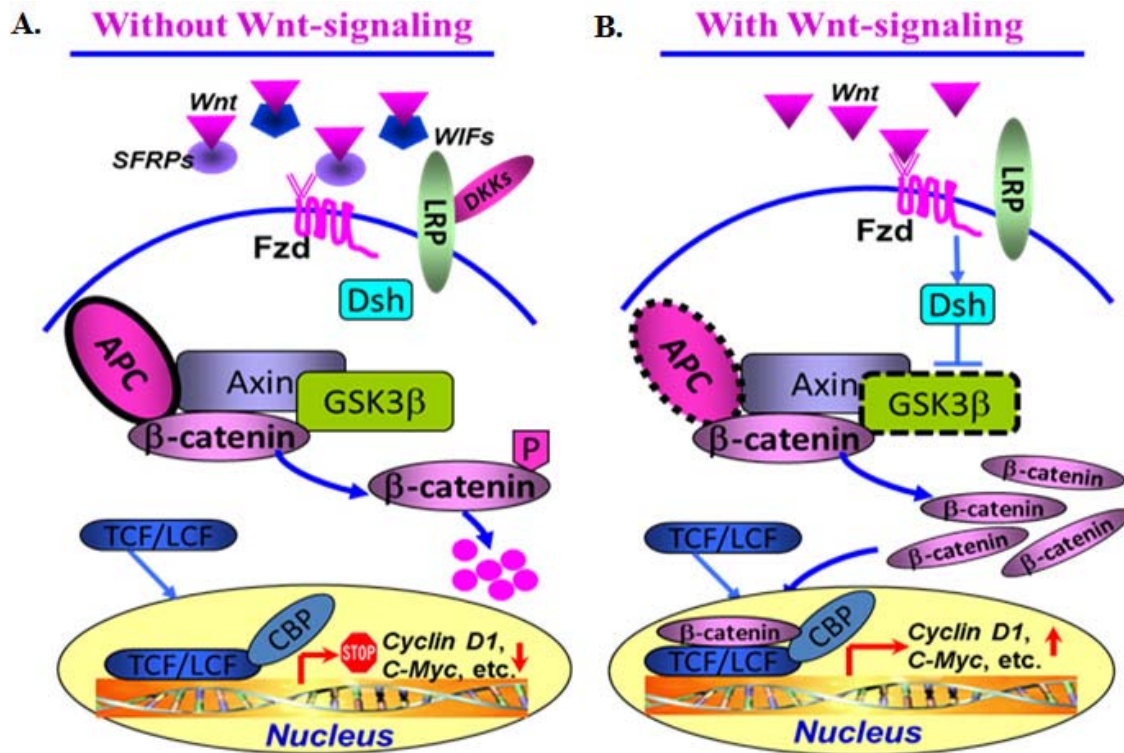


Figure 1. Wnt-signaling activation. a) The Wnt-signaling pathway is kept in its inactive form by antagonists and the intact APC-Axin complex. Antagonists including sFRPs and WIFs inhibit Wnt from binding to its receptor and DKKs prevent the formation of the LRP-Wnt-Frizzled (Fzd) complex by interacting with LRP (7). APC enables Axin to promote the phosphorylation and subsequent ubiquitination of β -catenin (8). b) Activation of Wnt-signaling occurs when Wnt associates with membrane receptors Fzd or LRP (9) or when the APC-Axin complex becomes unstable. Consequently, β -catenin is not phosphorylated and accumulates in the cytoplasm, eventually associates with TCF in the nucleus, thereby promoting the transcription of Wnt target genes (3).

three washes using 1 ml 75% ethanol were performed to wash the RNA. 40 μ L DEP-C water were added to each sample, followed by a ten minute incubation period at 56 degrees Celsius in order to solubilize the RNA. The RNA samples were quantified using a spectrophotometer using their absorbance at $\lambda=260$ (2 μ L RNA sample in 498 μ L DEPC water).

cDNA synthesis

cDNA was prepared using the SuperScript III First-Strand Synthesis System for RT-PCR and oligodT primer (Life Technologies, Grand Island, NY). The manufacturer's instructions were followed. cDNA was finally quantified using $\lambda=260$ (1 μ L cDNA sample in 499 μ L MiliEq water).

Real-time PCR

Quantitative real-time PCR was performed in order to measure relative expression levels of Wnt readout genes Axin2, C-Myc, and Cyclin-D1. We additionally analyzed expression of housekeeping gene GAPDH in order to provide an internal control. Primer sets were designed for Axin2, C-Myc, Cyclin-D1, and GAPDH. For each sample, 4 μ L cDNA were added to preparations containing 4 μ L appropriate primer set, 7 μ L ddH₂O, and 15 μ L SYBR® Green dye, yielding a total reaction volume of 30 μ L. The reactions, in triplicates, were loaded into 96-well microplates with a transparent lid and the reaction was performed in the Step One Plus system (Applied Biosystems). A heating lid was used and the plate was held at 95 degrees Celsius for 20 seconds before proceeding into 40 cycles of 95 degrees Celsius for 1 second followed by 60 degrees Celsius for 20 seconds.

Statistical analysis

Expression signals of the real-time PCR were evaluated using Step One V 2.0 Software and the relative expression of each gene was assessed using the $\Delta\Delta$ Ct method. Average Ct values were calculated for all sample replicates. Δ Ct for each sample average

was calculated by normalizing each experiment to the expression level of the constitutive gene, GAPDH. Average Δ Ct values for each set of replicates were determined. The experimental mean Δ Ct value obtained was compared to Δ Ct of the control (without SFN or vitamin D) and the control in the vitamin D group (without SFN), yielding $\Delta\Delta$ Ct, which was used to calculate overall expression fold change. Statistical significance was considered as $P<0.05$.

Results

As stated in the introduction, SFN has been demonstrated as an HDAC inhibitor (14) and also plays a role in the downregulation of the Wnt-signal pathway (16). However, data investigating if SFN's effect on the Wnt-pathway is related to its epigenetic function is elusive. Using real-time PCR, we assessed the relative expression of Axin2, Cyclin D1, and C-Myc, three Wnt-pathway reporter genes, after treatment using different concentrations of SFN and synergistic treatment using vitamin D, which is involved in the recruitment of histone acetylase factors (18). Expression of HDAC inhibitor TSA was used as a positive control. All results were normalized to expression of housekeeping gene GAPDH. Statistical analysis using the $\Delta\Delta$ Ct method to evaluate fold-change was performed and the results were assessed.

Effect of SFN and vitamin D on Axin2 expression

Data analysis of our real-time PCR reports revealed increased SFN concentration negatively correlated with Axin2 expression, both in the non-vitamin D group and the vitamin D group. Compared to the non-vitamin D control (Figure 2a), the 1.0 μ M SFN treatment was lower (-0.20), but not significant. The 2.5 μ M SFN treatment showed even lower expression (-0.71) and was of significant value ($P<0.05$). When comparing the vitamin D group to the vitamin D control (Figure 2b), we observed a similar trend. 1.0 μ M SFN showed decreased (-0.04), but not significant expression, but the decrease using 2.5 μ M SFN did show a very significant ($P<0.01$) further decrease (0.45). The TSA in the non-vitamin D group did

not show a significant fold decrease (-0.26), but when treated with vitamin D, it displayed a very significant ($P < 0.01$) decrease (-0.30) from the vitamin D control. When examining the experimental conditions compared to the control (Figure 2c), as vitamin D treatments are combined with increasing doses of SFN, we can see a general downward trend of Axin2 expression. Additionally, it is clear that TSA when combined with vitamin D results in a larger fold decrease than TSA alone.

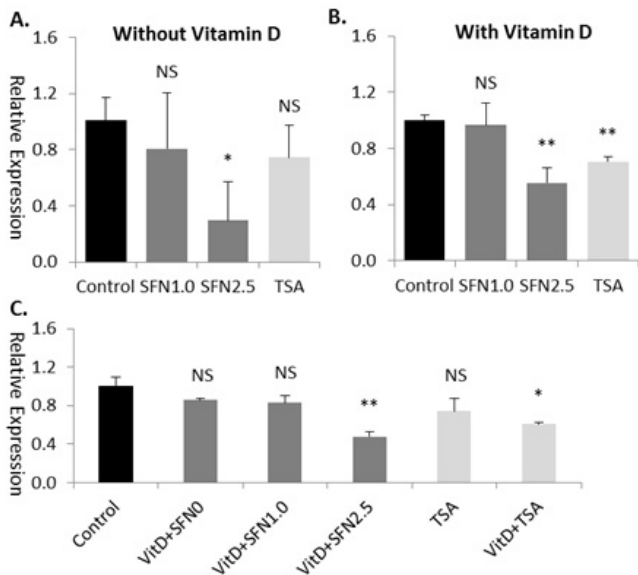


Figure 2. Axin2 mRNA Relative Expression. a) Effect of SFN on Axin-2 expression with TSA positive control. Experimental caco-2 cells were treated with SFN (1.0 μ M or 2.5 μ M) or TSA (1 μ L). Real-time PCR was performed using SYBR Green. All values for mRNA expression were normalized to corresponding expression of internal control, GAPDH. Axin-2 expression is presented relative to expression of no-treatment control. ** $P < 0.05$, NS, not significant. b) Synergistic effect of SFN and vitamin D on Axin-2 expression, with TSA + Vitamin D positive control. All experimental caco-2 cells were treated with 1,25-dihydroxyvitamin D (vitamin D) (100 μ M) and SFN (1.0 μ M or 2.5 μ M) or TSA (1 μ L). Real-time PCR was performed and Axin-2 mRNA expression was normalized using GAPDH expression. Axin-2 expression is presented relative to expression of vitamin D control. ** $P < 0.01$, NS, not significant. c) Summary of SNF and vitamin D effects on Axin-2 expression. - Experimental caco-2 cells were treated with vitamin D (100 μ M); vitamin D + SNF (1.0 or 2.5 μ M); TSA (1.0 μ L); or vitamin D μ M + TSA (1.0 μ L). Real-time PCR was performed and Axin-2 mRNA expression was normalized using GAPDH expression. Axin-2 expression is presented relative to expression of no treatment control. ** $P < 0.01$, * $P < 0.05$, NS, not significant.

Effect of SFN and vitamin D on Cyclin D1

Next, we evaluated relative expression of Cyclin D1. Treatment of the non-vitamin D group (Figure 3a) with 1.0 μ M SFN and 2.5 μ M SFN both decreased expression of cyclin D1 (-0.61 and -0.64, respectively). The fold change using the higher concentration was just slightly larger, but neither value was statistically significant. TSA treatment decreased expression by a 0.74-fold and this value was very significant ($P < 0.01$). In the vitamin D group (Figure 3b), 1.0 μ M SFN resulted in a non-significant expression decrease (-0.06) compared to the vitamin D control and 2.5 μ M SFN yielded a further decrease (-0.64) that was significant ($P < 0.05$). TSA + vitamin D treatment resulted in a significant ($P < 0.05$) 0.42-fold decrease. When comparing the non-vitamin D control to the experimental conditions involving vitamin D combined with increasing concentrations (Figure 3c), another consistent downward trend in fold change is observable and all measurements are significant ($P < 0.05$ or $P < 0.001$). However, unlike the Axin2 results, expression fold decrease of Cyclin D1 was not higher when TSA was combined with vitamin D than treatment with TSA alone.

Effect of SFN and vitamin D on C-Myc

Finally, we evaluated the effect of our experimental conditions on the relative expression of C-Myc. As shown in Figure 4a, 1.0 μ M SFN treatment without vitamin D resulted in a 0.45 fold decrease that was not significant. Unlike previous findings, 2.5 μ M SFN did not further repress the expression of C-Myc; rather, the fold-change decreased to 0.35, although this was also not a significant value. TSA treatment also resulted in non-significant, decreased C-Myc expression (-0.28). In the vitamin D group (Figure 4b), treatment using 1.0 μ M SFN did not decrease expression when compared to the vitamin D control; in fact, expression increased by a 0.12 fold.

Similarly, relative expression increased with 2.5 μ M SFN by a 0.01 fold. Additionally, combined TSA and vitamin D also increased (+0.23) expression of C-Myc. None of the values were significant. When we compare the synergistic treatment to the non-vitamin D control (Figure 4c), results resemble trends found earlier, however, treatment using vitamin D and no SFN achieved a larger fold decrease (-0.46) than either vitamin D and 1.0 μ M SFN (-0.39) or vitamin D and 2.5 μ M SFN (-0.45); all of these values were significant ($P < 0.05$). In this comparison, we can see that vitamin D combined with TSA results in a larger fold decrease (-0.33) than TSA alone (-0.28).

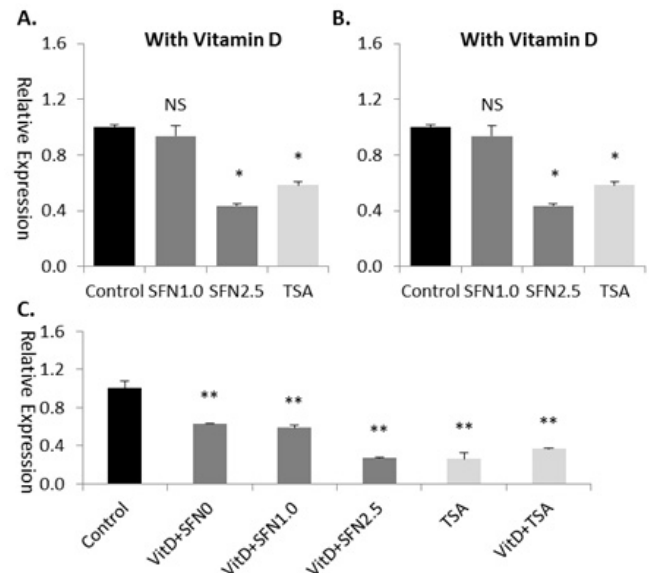


Figure 3. Cyclin D1 Relative Expression. a) Effect of SFN on Cyclin D1 expression with TSA positive control. Experimental caco-2 cells were treated with SFN (1.0 μ M or 2.5 μ M) or TSA (1 μ L). Real-time PCR was performed using SYBR Green. All values for mRNA expression were normalized to corresponding expression of internal control, GAPDH. Cyclin D1 expression is presented relative to expression of no-treatment control. ** $P < 0.05$, NS, not significant. b) Synergistic effect of SFN and vitamin D on Cyclin D1 expression, with TSA + Vitamin D positive control. All experimental caco-2 cells were treated with 1,25-dihydroxyvitamin D (vitamin D) (100 μ M) and SFN (1.0 μ M or 2.5 μ M) or TSA (1 μ L). Real-time PCR was performed and Cyclin D1 mRNA expression was normalized using GAPDH expression. Cyclin D1 2 expression is presented relative to expression of vitamin D control. * $P < 0.05$, NS, not significant. c) Summary of SNF and vitamin D effects on Cyclin D1 expression. - Experimental caco-2 cells were treated with vitamin D (100 μ M); vitamin D + SNF (1.0 or 2.5 μ M); TSA (1.0 μ L); or vitamin D μ M + TSA (1.0 μ L). Real-time PCR was performed and Cyclin D1 mRNA expression was normalized using GAPDH expression. Cyclin D1 expression is presented relative to expression of no treatment control. ** $P < 0.01$, * $P < 0.05$, NS, not significant.

Discussion

Colorectal cancer is the third most commonly diagnosed cancer in men and women in the US and represents the second most common cause of death by cancer (19). The *Wnt*-signaling pathway is known to be over-active in many types of cancers and is especially associated with colorectal cancer (3,4). SFN is a bioactive food

component that has been proven to down-regulate the *Wnt*-pathway (16) and might do so by exerting its property as an HDAC inhibitor (14). However, the causative mechanism by which SFN has an effect on *Wnt*-signaling is elusive. Upon activation by vitamin D, VDR binds to VDRE and enhances the recruitment of histone acetylation factors (18). The present study investigated the hypothesis that SFN and vitamin D synergistically mediate the *Wnt*-signaling pathway by performing real-time PCR to measure changes that occurred in the relative mRNA expression of *Wnt*-pathway readout genes Axin-2, Cyclin D1, and C-Myc.

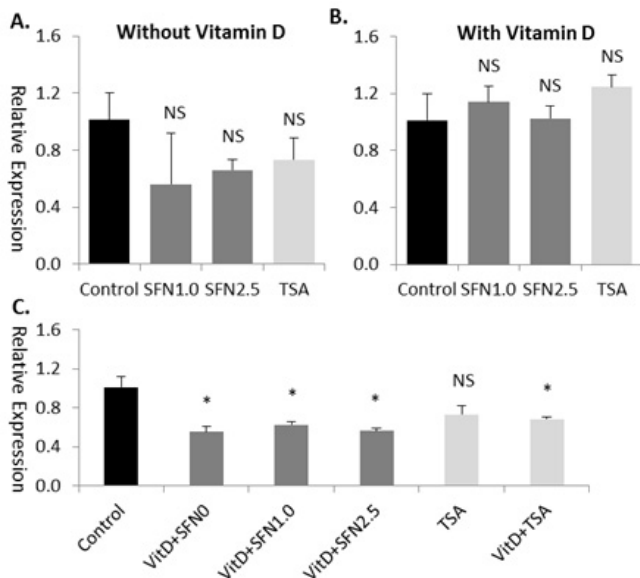


Figure 4. C-Myc Relative Expression. a) Effect of SFN on C-Myc expression with TSA positive control. Experimental caco-2 cells were treated with SFN (1.0 μ M or 2.5 μ M) or TSA (1 μ L). Real-time PCR was performed using SYBR Green. All values for C-Myc mRNA expression were normalized to corresponding expression of internal control, GAPDH. C-Myc expression is presented relative to expression of no-treatment control. NS, not significant. b) Synergistic effect of SFN and vitamin D on C-Myc expression, with TSA + Vitamin D positive control. All experimental caco-2 cells were treated with 1,25-dihydroxyvitamin D (vitamin D) (100 μ M) and SFN (1.0 μ M or 2.5 μ M) or TSA (1 μ L). Real-time PCR was performed and C-Myc mRNA expression was normalized using GAPDH expression. C-Myc expression is presented relative to expression of vitamin D control. NS, not significant. c) Summary of SFN and vitamin D effects on C-Myc expression. - Experimental caco-2 cells were treated with vitamin D (100 μ M); vitamin D + SFN (1.0 or 2.5 μ M); TSA (1.0 μ L); or vitamin D μ M + TSA (1.0 μ L). Real-time PCR was performed and C-Myc mRNA expression was normalized using GAPDH expression. C-Myc expression is presented relative to expression of no treatment control. * P <0.05, NS, not significant.

Our results, shown above, reveal a conspicuous trend in gene expression after treatment with different concentrations of SFN and vitamin D. Most notably, when comparing the synergistic treatment to the non-vitamin D control (Figures 2c, 3c and 4c), a consistent

decreasing trend in gene expression is observable for all genes. This suggests that as vitamin D treatment is supplemented with increasing concentration of SFN, the *Wnt*-signaling pathway expression negatively correlates. This in part supports our original hypothesis that vitamin D and SFN synergistically mediate the *Wnt*-pathway.

However, our results also indicate that in many cases, treatment using SFN *without* the 100 μ M vitamin D was more effective at inhibiting expression of the gene of interest than the treatment using SFN synergistically with vitamin D. This can be observed when comparing Axin2 expression (Figures 2a and 2b) using both 1.0 μ M and 2.5 μ M SFN; Cyclin D1 expression (Figures 3a and 3b) using 1.0 μ M SFN; and C-Myc expression (Figures 4a and 4b) using both 1.0 μ M and 2.5 μ M SFN. This suggests that vitamin D may somehow inhibit SFN from exerting its entire potential as an HDAC inhibitor on the *Wnt*-pathway. Additionally, it is also important to consider that TSA was more effective at repressing the expression of Axin 2 and C-Myc when it was in combination with vitamin D (Figures 2c and 4c). However, this was not true of Cyclin D1 expression, which was more effectively inhibited by TSA treatment alone (Figure 3c). We predict that these inconsistencies are the result of using a concentration of vitamin D that was too high (100 μ M). We expect that using vitamin D at the lower concentration of 10 μ M would more accurately showcase a synergistically mediated repression of *Wnt*-pathway gene expression.

Although our results somewhat support that SFN and vitamin D synergistically mediate the *Wnt*-signaling pathway, many of the determined fold-change values were not statistically significant; thus, it is difficult to draw conclusions based on the results from this study alone. A key explanation for the insignificant results lies in the fact that the experiment was conducted using only one cell passage. In a future modified version of this experiment, we would aim to use at least three cell passages. While the results from this experiment do present a notable trend that somewhat supports our original hypothesis, it is needless to say that further experimentation is warranted in order to draw finite conclusions. If reliable results are produced, this information could be used to fuel an in-depth study involving immunoprecipitation and a luciferase reporter assay to monitor histone acetylation and the dynamics of *Wnt*-pathway activation in response to SFN and vitamin D treatment.

In conclusion, we tested SFN and vitamin D synergistic mediation of the *Wnt*-signaling pathway by assessing their effect on expression of *Wnt*-pathway readout genes. We found that as increasing concentration of SFN was supplemented to vitamin D treatment, the expression of *Wnt*-signaling targeting gene negatively correlated. These findings represent preliminary data that warrant further investigation of SFN and vitamin D epigenetic regulation of the *Wnt*-signaling pathway.

Acknowledgments

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