ABSTRACT

Title of Document: SYNERGISTIC ANTI-CANCER EFFECTS OF CAPSAICIN AND 3,3'-DIINDOLYLMETHANE IN HUMAN COLORECTAL CANCER, INVOLVEMENT OF P53 AND NF-κB

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Cancer is the second leading cause of death in the United States and a leading cause of morbidity and mortality worldwide. A promising area of cancer research is focused on chemoprevention by nutritional compounds. Epidemiological studies have shown a strong negative correlation between fruit, vegetable and spice intake and rates of cancer. Although individual active compounds have demonstrated significant anti-cancer activity, an emerging area of research is focusing on the combination of multiple dietary compounds on cancer that act synergistically to exert greater effects. In the current study, we evaluated potential synergistic effects of capsaicin, an active compound from red chili peppers, in combination with 3,3'-Diindolylmethane (DIM), from cruciferous vegetables. A synergistic induction of apoptosis and inhibition of cell proliferation was observed in multiple cancer cell lines treated with combination of capsaicin and DIM. We also observed that these two compounds activated transcriptional activity of p53 and NF-κB synergistically. Combination treatment stabilized nuclear p53 and up- or down-regulated expression of several target genes that are downstream of NF-κB and p53. The present study suggests capsaicin and DIM work synergistically to inhibit cell proliferation and induce apoptosis in colorectal cancer through modulating transcriptional activity of NF-κB, p53 and expression of their target genes associated with apoptosis and cell cycle.

SYNERGISTIC ANTI-CANCER EFFECTS OF CAPSAICIN AND 3,3'-DIINDOLYLMETHANE IN HUMAN COLORECTAL CANCER, INVOLVEMENT OF P53 AND NF-κB

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Masters of Science
2014

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1.1 Introduction

Cancer is a major public health problem worldwide. In the United States, cancer remains the second leading cause of death [1]. The National Cancer Institute (NCI) estimates that 1,660,290 men and women will be diagnosed with and 580,350 men and women will die of cancer of all sites in 2013 [2]. In addition, current cancer treatments remain expensive, invasive and ineffective, particularly in the advanced stages. Consequently there has been much research focused on the prevention of cancer across the population. The epidemiologic review of almost 200 studies that examined the relationship between fruit and vegetable intake and cancer incidence clearly demonstrated that individuals with the highest intakes of fruits and vegetables have the lowest occurrence of cancers of the lung, colon, breast, cervix, esophagus, oral cavity, stomach, bladder pancreas and ovary. The risk of cancer for most sites was twice as high in persons whose intake of fruit and vegetables was low compared with those with high intake [3]. This strong association has been attributed to these foods being rich sources of numerous bioactive compounds and has led researchers to focus attention on the active ingredients found in fruits and vegetables and the molecular mechanisms of action as potential novel targets for cancer prevention and therapy [4-8].

The active ingredients found in plants have been shown to act on multiple molecular targets to suppress carcinogenesis and cancer progression [9-11]. While individual active compounds have demonstrated significant anti-cancer activity, an emerging area of research is focusing on the combination of multiple dietary compounds on cancer that

work synergistically through multiple cell-signaling pathways to exert greater effects [12-15].

When discussing therapeutic targets of cancer it is essential to discuss the hallmarks of cancer, first identified in a seminal paper by Hanahan and Weinberg in 2000 [16]. They identified six biological capabilities acquired during tumorigenesis that offer insight to the complexities of cancer as well as targets of therapeutic action of anti-cancer compounds. The acquired hallmarks of cancer are sustaining proliferative signaling, evading growth suppressors, activating invasion and metastasis, enabling replicative immortality, inducing angiogenesis and resisting cell death. Identifying and focusing on these particular properties enables concentration on treatments that offer broader efficacy specific to tumor cells.

1.2 Dietary Phytochemicals have chemopreventive properties

The term "chemoprevention" was first used by Dr. Michael B. Sporn in 1976 to describe the use of naturally occurring or synthetic chemical agents to inhibit, delay or reverse the disease process of carcinogenesis [17]. Thirty years following that landmark paper there have been many naturally-occurring dietary compounds that have been found to possess chemopreventive properties [7, 18, 19]. Chemopreventive strategies using phytochemicals found in fruits, vegetables, teas and spices are a promising area of cancer research because they are inexpensive, safe and readily available while possessing diverse inhibitory effects against cancer initiation, promotion, progression and metastasis. Much research has been done to elucidate the underlying mechanisms of action of these phytochemicals and numerous cellular mechanisms have been implicated in the cancer preventative effects of dietary phytochemicals that include antioxidant enzymes,

inhibiting cell cycle progression and cell proliferation, inducing apoptosis, suppressing oncogenes and activating tumor-suppressor genes by modulating cellular signaling pathways [8, 10, 20].

1.3 Capsaicin induces cell cycle arrest and apoptosis in cancer cells

Capsaicin is a homovanillic acid derivative (trans-8-methyl-*N*-vanillyl-6-nonenamide) and the spicy component of hot chili peppers that has been studied for its anti-cancer [21-23], anti-oxidant [24], anti-inflammatory, anti-obesity [25] and analgesic properties[26, 27]. Capsaicin has been studied in a variety of cancers but the effects on carcinogenesis are still controversial due to conflicting results in epidemiological and basic research studies [28, 29]. There is also still incomplete data regarding the exact cellular mechanisms of how capsaicin induces cell cycle arrest and apoptosis.

A thorough review of capsaicin by Bley et. al noted that capsaicin appears to inhibit the growth of or induce apoptosis in over forty distinct cancer cell lines [30]. The loss of a fully functional apoptotic program is considered one of the hallmarks of most, if not all types of malignant cells [31]. Apoptosis is an essential barrier to cancer development that cancer cells can avoid through multiple strategies, by suppressing pro-apoptotic regulators and increasing the function of anti-apoptotic ones. One reason the growth rate of cancer cells is much more rapid than that of normal cells is because of deregulation or malfunctioning of their cell-growth and cell-death systems. Therefore, induction of apoptosis or cell-cycle arrest by dietary compounds such as capsaicin is a promising approach to inhibit the promotion and progression of carcinogenesis. In recent years cancer research has focused on targeting specific signaling pathways that drive inappropriate cell growth and survival.

Capsaicin induces apoptosis in various cancer models including colon [32], prostate [33, 34], liver [35], esophagus [36], skin [37], leukemia [38], bladder [39], lung [40], pancreas [41] and endothelial cells [21], leaving normal cells unharmed [38, 41, 42]. Various mechanisms of capsaicin-induced apoptosis have been proposed from different cancer cell models. For example, capsaicin-induced apoptosis is in a p53-dependent mechanism in leukemia [38], but in prostate cancer cells, capsaicin's effect is p53 independent [34]. Capsaicin down-regulates the prostate-specific antigen and androgen receptor by inhibition of TNFα-stimulated degradation of NF-κB in prostate cancer [34] and inhibits VEGF-induced proliferation, DNA synthesis, chemotactic motility, and capillary-like tube formation of primary cultured human endothelial cells [21]. Capsaicin-induced apoptosis is elevated by co-treatment of the AMPK activator in HT-29 colon cancer cells [32], and TRPV6 mediates capsaicin-induced apoptosis in gastric cells through a p53 and JNK-dependent pathway [43]. Lu et. al. reported that capsaicin increased ROS and Ca^{2+} and decreased mitochondrial membrane potential which led to decreased anti-apoptotic protein Bcl-2, increased pro-apoptotic Bax and activated caspase-8, -9 and -3 [22]. Capsaicin has been shown to possess potent anti-cancer and chemopreventive mechanisms through diverse molecular mechanisms but there is still much to be discovered regarding the exact methods of actions due to different variations according to cancer type and the effect is dependent on the cell or tissue context.

1.4 3,3'-Diindolylmethane exerts anti-cancer activities through diverse mechanisms

3,3'-diindolylmethane (DIM) is a major acid condensation product of Indole-3-carbinol (I3C) and generated in the acidic environment of the stomach following dimerization of I3C, which is an autolysis product of glucosinolate, a naturally occurring component of

Brassica species of cruciferous vegetables (cabbage, broccoli, cauliflower, and brussels sprouts) [44]. Both I3C and DIM have been established as chemopreventive agents, however recent evidence suggests that DIM has greater bioavailability in vivo and more effective results in vitro at exerting anti-cancer activity due to its stability [45]. In fact DIM, not I3C was detected in serum of women following ingestion of a single dose of oral I3C [46]. DIM was also detected in the livers and feces of rodents fed I3C, whereas the parent I3C compound could not be detected in tissues of these rodents [47, 48]. Thus, the biological effects of I3C may be attributable to DIM, but not I3C *in vivo*.

DIM and its derivatives have demonstrated the ability to suppress cell proliferation and induce apoptosis in colon cancer cells [49, 50] as well as other types of cancer cells including prostate [51], breast [52], bladder [53], pancreas [54], hepatoma [55], and umbilical vascular endothelium [56]. Lerner et. al. found that DIM induced apoptosis in two colon cancer cell lines, HCT 116 and Colo-320 [57]. DIM induced apoptosis in a dose dependent manner in both cell lines as evidenced by DNA fragmentation and apoptotic morphology, condensation and fragmentation of the nucleus. They also discovered that DIM increased expression of N-myc downstream regulated gene-1 in the poorly differentiated Colo-320 cells. Kim et al. found that treatment of two colon cancer cell lines, HCT116 and HT-29 with DIM resulted in a substantial decrease in the number of viable cells and induced apoptosis in a dose-dependent manner [58]. DIM increased the activation of caspase-3, -7, -8, and -9 and enhanced poly (ADP-ribose) polymerase cleavage and increased the translocation of cytochrome c and Smac/Diablo from the mitochondria to the cytoplasm.

Treatment of cancer cells with DIM has been shown to inhibit carcinogenesis, decrease cell proliferation, increase apoptosis and induce G1 cell cycle arrest by targeting genes, transcription factors and cell signaling cascades that regulate the cell cycle. DIM induces apoptosis by inactivating the Akt pathway in breast cancer cells [52] and activating TRAIL expression and Nur77 in colon cancer cells [59]. DIM induces anti-tumorigenic genes, IFNγ [60] and caveolin expression via a PPARγ-dependent pathway [50, 53] and cell cycle arrest by Sp1-mediated activation of $p21^{WAF1/CIP1}$ expression [61]. DIM also suppressed metastasis by inhibiting Ras signaling and VEGF-induced neovascularization in endothelial cells [56]. DIM suppresses cell proliferation and induces tumor-suppressing gene NAG-1 expression at the transcription level in HCT-116 cells [62]. DIM has been studied for years in many cancer lines, most notably in prostate and breast cancer but remains very relevant for continuing studies in other cancer cell lines. According to the NCI Clinicaltrials.gov homepage there is currently one Phase 2 trial to investigate how well DIM works in treating patients with stage I or stage II prostate cancer undergoing radical prostatectomy with the rationale that the compound helps to slow the growth of tumors. Further research of DIM in a variety of cancers is needed to continue to elucidate the anti-cancer potential of this compound.

1.5 Physiological concentrations and bioavailability of Capsaicin and DIM

While there are many published articles regarding *in vitro* effects of capsaicin and DIM, the data on *in vivo* physiological activity is very limited, particularly in humans, which makes it difficult to assess the physiological concentrations and bioavailability of the compounds in regards to their physiological potential in humans.

In a study of capsaicin distribution in rats following an oral gavage of 30mg/kg body weight dose, the researchers observed that nearly 94% of the dose was absorbed and maximum concentration of 1.9 μg/ml was observed in the blood 1 hour after administration. This concentration decreased to less than half (0.83 μg/ml) in 6 hours and was not detectable after 48 hours [63]. During a study on capsaicin and insulin control, blood concentrations of the compound was estimated at 2.47 ng/ml (equivalent to 8.1 nM) in adult males following intake of 0.4 mg/kg body weight [64]

There have been a few studies in which oral administration of I3C or DIM resulted in measureable amounts in serum and tissues, indicating that absorption does occur [47, 65-67]. During a phase I trial in women, participants were administered 400, 600, 800, 1000 or 1200 mg of Indole-3-carbinol and then provided serum samples to analyze blood concentrations of the metabolites. It was the major product of acid-catalyzed condensation, DIM, and not indole-3-carbinol that was detected in serum. While there was significant inter-individual variation in the serum concentration, the average max concentration at 2 hours post ingestion was 61 ng/mL in the 400 mg dose group and 607 ng/mL following the 1,000 mg dose. No further increase was observed following a 1,200 mg dose [46].

In a study in rats, DIM was present in circulation after 15 minutes following administration of 200 mg/kg dose. The maximum plasma DIM concentration achieved at 1 hour after administration was 0.15 μg/mL [68].

I3C and DIM are currently sold as nutritional supplements available to consumers in health food stores. The recommended maximal human dose of DIM supplement is 2 mg/kg per day or about 100-200 mg for women and 200-400mg for men.

1.6 Toxicity of Capsaicin and DIM

Published studies on the toxicity of capsaicin have been inconsistent with both positive and negative effects being reported in some of the typical genetic toxicology assays.[69-73] Inconsistencies between the tests may be due to methodology as well as the source of capsaicin, whether it is pure synthetic capsaicin or extracted from chili peppers. Toxicology studies that analyzed pure capsaicin may differ in results attained with extracts because of inconstant contents and potentially toxic impurities such as aflatoxins B1 and G1 in the extracts [30].

Chili extracts were found to be mutagenic in five strains of Salmonella typhimurium as well as in the mammalian micronucleus test [74]. However, an Ames test with highly pure synthetic capsaicin and found no mutagenic activity in five different bacteria strains. The same group looked at in vitro chromosomal aberrations assays using human peripheral blood lymphocytes and found no significant increases in chromosomal aberrations, polyploidy, or endoreduplication [70].

Another paper using high-purity capsaicin also found no genotoxic activity in the bacterial mutation and chromosome aberration tests. Also, no evidence of cytotoxicity or genotoxicity was observed in rat bone marrow micronucleus test. The researcher's conclusion at the end of the paper was "that pure capsaicin is not active in the standard battery of genotoxicity assays recommended by the International Conference on Harmonisation for evaluation of new medicines. Earlier reported in vitro genotoxic activity is probably associated with mutagenic impurities in commercial grades of the material" [75].

There have also been inconsistent reports on the effects of high chili consumption and stomach cancer in epidemiological studies. In Mexico and India, human dietary exposure to capsaicin from high intakes of chili peppers has been linked with gastric cancer [76, 77] but a study done in Italy showed a protective effect [78].

Indole-3-carbinol and DIM have also had inconsistent toxicology reports. A study found that DIM induced immunotoxic effects including decreases in various immune cells and increase in apoptosis in the spleen of neonatal mice [79]. However a long-term toxicity study analyzed both Indole-3-Carbinol (I3C) and DIM intake in Sprague-Dawley (SD) rats and found no toxic effects. The rats were fed a control diet, 1 or 10x the current human dose found in nutritional supplements of 2 mg/kg of absorption-enhanced DIM or 5-7x the maximal recommended dose of I3C. No significant differences were observed between the groups and there was no observed toxicity from either I3C or DIM, even after 12 months of treatments[67].

1.7 NF-κB targeting in cancer

The transcription factor NF-κB is a regulator of genes encoding cytokines, cytokine receptors, and cell adhesions molecules that control immune and inflammatory response. NF-κB plays a major role in development and progression of cancer because it regulates more than 500 genes and aberrant NF-κB regulation has been observed in many cancers [80]. NF-κB can affect all six hallmarks of cancers through the transcriptional activation of genes associated with cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation and apoptosis. The NF-κB family is composed of five members: Rel A (p65), RelB, REL (cRel), NF-κB1 (p50 and its precursor p105) and NF-κB2 (p52 and its precursor p100).

Under normal conditions, NF-κB is sequestered in the cytoplasm, bound to a member of the IkB family of inhibitory proteins. Stimulation by one of a range of signals results in phosphorylation and ubiquitination of IkB followed by proteosome-mediated degradation. Dissociation from IkB results in NF-κB nuclear translocation and transcriptional regulation of numerous target gene that have both pro and anti-apoptotic effects [52, 81-83].

The roles of NF-κB in cancer progression and anti-cancer therapeutics are complex. In fact, different activation pathways of NF-κB may cause the expression of proteins that promote apoptosis like Fas-L, c-myc, p53, IκBα, or inhibit apoptosis through Traf2, IAP proteins, Bcl-2 like proteins [84]. Also, NF-κB activation has been shown to control the regulation of cell cycle proteins such as cyclin D1 and CDK2 kinase under certain stimulus.

Typically, NF-κB activation is associated with the action of inflammatory cytokines such as TNF and bacterial or viral infection. The activity of the NF-κB family of transcription factors, and RelA (p65) in particular, is closely linked to p53 and Mdm2 function [85, 86]. NF-κB is also activated by many stimuli that induce p53 activity, such as DNA-damaging agents and other forms of cellular stress. In addition to targeting genes, NF-kB can interact indirectly with p53 to regulate many genes related to tumorigenesis through transcriptional cross-talk [87]. The NF-κB subunit RelA (p65) has been shown to inhibit p53 dependent transactivation, while p53 can also suppress NF-κB transcriptional activity. One example of indirect p53-dependent repression is the regulation of the Cyclin D1 gene by crosstalk with the NF-κB pathway [88].

1.8 p53 targeting in cancer

p53 is a well-known tumor-suppressor that is mutated in about half of all cancers [89]. Therefore, reactivating its function to suppress cancer progression has been the focus of much research. Of the roughly 150 genes targeted by p53, most are associated with regulation of genomic integrity, the cell cycle, DNA repair mechanisms and cell death in response to a variety of stress signals or damage to the cell. These processes function to prevent inappropriate proliferation of damaged cells. When a cell is confronted by stress, p53 is stabilized in the nucleus, where it initiates cellular responses through transcriptional activation or repression of target genes resulting in cell cycle inhibition, apoptosis, genetic stability and inhabitation of angiogenesis [90, 91]. Phosphorylation at the Ser-15 residue of p53 is critical for p53-dependent transactivation. Accumulation of p53 by inhibiting the interaction between p53 and MDM2 also stimulates p53-dependent transactivation. While much of the activity of p53 is in the nucleus, it can also act outside of the nucleus to induce apoptosis by binding with anti-apoptotic proteins such as Bcl-2[92].

Capsaicin has demonstrated its ability to induce phosphorylation of p53 at the Ser-15 residue in myeloid leukemia cells (NB4) which also resulted in apoptosis [93]. Interestingly, they found the results were not as significant in p53 defective cells. In contrast, Mori, et al. found that capsaicin inhibited the growth and induced apoptosis in a p53-independent manner in prostate cancer cells in vitro and xenografts in vivo [94]. Treatment with Capsaicin in urothelial cancer cells resulted in increased expression of p53, and phosphorylation at Ser 15, 20 and 392 as well as significant cell cycle arrest and apoptosis [95]. Taken together, these results suggest that p53 and NF-κB are targets of

capsaicin's anticancer activity, but that it holds antiproliferative activity independent of p53 as well.

1.9 Synergistic effect of multiple phytochemicals

Studying the synergism of multiple phytochemicals holds promise for enhanced anti-cancer capabilities because the ability of chemopreventive phytochemicals to prevent tumor development is likely the outcome of the combination of several distinct sets of intracellular effects, rather than any one single biological response. Cancer is often the result of the accumulation of multiple mutations in genes that result in disruption of normal cell signaling and maintenance so it is probable that targeting multiple actions would result in greater results [14]. Many studies have suggested that phytochemicals in fruits, vegetables and spices can have complementary and overlapping mechanisms of action, including modulation of detoxification enzymes, scavenging of oxidative agents, stimulation of the immune system, regulation of gene expression in cell proliferation and apoptosis, hormone metabolism, and antibacterial and antiviral effects [5, 15, 96, 97]. Combination treatment may also result in therapeutic synergy between individual compounds at physiologically relevant doses, allowing for a reduction in their individual concentrations and toxicities [98]. The therapeutic doses for individual anti-carcinogens may far exceed what an individual could naturally ingest from plant sources however the combination of several antitumorigens at subtherapeutic levels have been shown to result in significant antitumor effects [15, 99, 100]. Thousands of phytochemicals are present in fruits and vegetables and differ in molecular size, polarity, and solubility, which may affect the bioavailability and distribution of each phytochemical. Therefore, use of these

dietary compounds with distinct molecular mechanisms is beginning to receive attention and is considered more promising for higher efficacy in cancer research.

It is currently common practice in clinical settings to use combination drug therapies in the treatment of cancers to overcome the common problem of drug resistance and improve outcomes [101, 102]. A promising area of research is identifying dietary compounds that can exert chemotherapeutic actions alone or to sensitize cancer cells to traditional chemotherapy drugs [7, 20, 103]. A major concern regarding the prevention and treatment of cancer is the relative toxicity of chemopreventive and/or therapeutic agents, and many of these agents are associated with undesirable dose-related toxicity. Dietary compounds are therefore especially appealing for prevention and treatment of cancer due to their low toxicity.

Modern cancer research is seeking out ways to improve the efficacy of chemotherapy through the development and/or identification of novel chemotherapeutic agents and through identification of novel molecular targets. Combining dietary compounds to exert greater anti-cancer properties and identifying their molecular targets will have obvious benefits in regards to treatment options available for patients.

The high number of publications suggests that novel combination treatment with common cancer therapies and chemopreventive agents may enhance anti-tumor activity through synergic action [14, 104, 105]. Combining cherry extract with suboptimal levels of sulindac reduced total tumors in the small intestine of Apc^{Min+} mice compared to mice fed sulindac alone [106]. In humans, a combination of supplements curcumin and quercetin regressed adenomas in patients with FAP [107]. DIM synergistically suppressed the proliferation of human colorectal cancer cells when combined with

sulforaphane [108]. DIM in combination with genistein resulted in decreased proliferation by working on Estrogen and Androgen receptors in vitro LNCaP PCa cells [109]. Most notably, they found that the combination of the two enabled them to use low concentrations of the compounds while still seeing significant results. Hwang et al. found that genistein with capsaicin resulted in decreased breast cell cancer proliferation and increased apoptosis in vitro and in vivo in a breast cancer rat model. [13] They discovered that in TPA-treated conditions both in vivo and in vitro downregulated MAP kinase and COX 2 and activated AMPK to exert anti-cancer activity. Manikandan et al. studied the synergistic anticancer activity of curcumin and catechin in vitro in human colon cancer cells. They found that individually the compounds inhibited growth but in combination had the highest level of growth inhibition [12]. They found the dietary compounds together induced cytotoxicity, nuclear fragmentation and condensation and DNA fragmentation associated with apoptosis but did not analyze the molecular actions that resulted in the apoptosis. Furthermore, in previous data, mixture of I3C and resveratrol was much more effective in NAG-1 induction compared to resveratrol alone [62]. Therefore, use of multiple dietary compounds with distinct and complementary molecular mechanisms and gene targets is beginning to receive much attention and is considered promising for cancer research.

CHAPTER 2: Materials and Methods

2.1. Cell culture and reagents

Human colorectal adenocarcinoma cells HCT116, LoVo, CaCo2, SW480, HT-29, human lung cancer A549 and human prostate cancer PC3 cells were purchased from American Type Culture Collection (ATCC; Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM/F12) supplemented with 10% fetal bovine serum (FBS). Normal human colon cells, CCD112CoN was purchased from ATCC and grown in Eagle's Minimal Essential Medium (EMEM) supplemented with 10% FBS. The cells were maintained at 37 ºC under a humidified atmosphere of 5% CO_2.

2.2 Cell proliferation and Apoptosis

Human colorectal adenocarcinoma cells HCT116, LoVo, SW480, CaCo-2, HT-29, human lung cancer cells A549, human prostate cancer cells PC3 and normal human colon cells, CCD112CoN were treated with different concentrations of capsaicin (0-100 µM), DIM (0-25 µM) or combination (50 µM capsaicin and 12 µM DIM). Capsaicin was dissolved in ethanol and DIM was dissolved in dimethyl sulfoxide (DMSO). Ethanol and DMSO was used as a vehicle in the control groups and the final concentrations of ethanol and DMSO did not exceed 0.1% (v/v).

Cell proliferation was assessed by the MTT [(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (Sigma, St. Louis, MO) method. Cells were plated at 4000 cells/well onto a 96-well plate in three replicates and grown overnight. The cells were treated with different concentrations of capsaicin (0-100 µM), DIM (0-25 µM) or combination (50 µM capsaicin and 12 µM DIM) in media supplemented with 1% Fetal Bovine Serum (FBS) for 24 and 48 hours at 37ºC under 5% CO_2. Then, cells were

incubated with 20 μL of MTT solution for 3 hours at 37ºC. The optic density was recorded at 490 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc., Winooski, VT).

Apoptosis for HCT-116 was performed using FACS analysis. Briefly, both attached and floating cells were collected and resuspended in 80% ice-cold ethanol. After overnight at −20 °C, the cells were rehydrated with PBS and stained with 70 μM propidium iodide solution including 1 mg/mL RNase A. Apoptosis and cell cycle distribution were analyzed using Beckman Coulter Epixs XL flow cytometer equipped with EXPO32 ADC and ModiFit LT software. And apoptosis for other human colorectal cancer cells (LoVo, CaCo2, HT-29 and SW480) was tested with Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instruction.

2.3 Transient transfection and luciferase assay

Transient transfection for *NF-κB Luc* and *p53 Luc* plasmid was performed using a Polyjet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD) according to the manufacturer's instructions. HCT116 cells ($2x10^5$ cells/well) are plated in 12-well plates in three replicates and incubated overnight. The next day, plasmid mixtures containing 1 μg of *NF-κB Luc* or *p53 Luc* and 0.1 μg of *pRL-null* were transfected into the cells for 24 hours. The cells were washed and harvested in 1x luciferase lysis buffer. The luciferase activity was measured and normalized to the *pRL-null* luciferase activity using a dual-luciferase assay kit (Promega, Madison, WI).

2.4 Isolation of cytosol and nucleus fraction

Cytosol and nuclear fraction of cells were prepared according to the manufacturer's protocols of a nuclear extraction kit (Active Motif, Carlsbad, CA). Briefly, after HCT116

cells were treated in the compounds for 24 hours, the cells were washed twice with ice-cold PBS containing phosphatase inhibitors (Sigma Aldrich, St. Louis, MO). Cells were harvested with hypotonic buffer containing detergent and incubated at 4ºC for 15 min. The supernatants (cytoplasmic fraction) were collected after centrifugation at 14,000 g for 1 minute at 4ºC, and stored at -80ºC. For nuclear fractions, cell pellets were resuspended with lysis buffer and incubated at 4ºC for 30 minutes under shaking. After 30 min, nuclear suspensions was centrifuged at 14,000 g for 10 minutes at 4ºC, and the supernatants (nuclear fraction) were stored at -80ºC for further analysis

2.5 SDS-PAGE and Western Blot

The cells were washed with ice-cold 1x phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bioproduct Inc, Ashland, MA) supplemented with protease and phosphatase inhibitor cocktail (Sigma Aldrich) and centrifuged at 12,000 g for 10 minutes at 4º. After protein concentration is determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL), equal amounts of proteins were subjected to 10% or 12% SDS-PAGE, and the separated protein was transferred onto nitrocellulose membranes (Osmonics, Minnetonka, MN). The membranes were blocked for non-specific binding with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 hour at room temperature and then probed with primary antibodies overnight at 4ºC, followed by incubation with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 hour at room temperature. Chemiluminescence was detected with Pierce ECL Western Blotting substrate (Thermo Scientific) and visualized by Chemidoc MP Imaging system (Bio-Rad, Hercules, CA).

2.6 RT-PCR

Total RNA was isolated from cells using the RNeasy Micro Kit (QIAGEN, Valencia, CA) and cDNA synthesized using cDNA synthesis kit. Quantitative real-time PCR was performed and targeted primers for Bak, p21 and GAPDH were used. Amplification was performed under conditions of 95 °C for 10 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s. Primers sequences are following: Bak: forward 5'-TCTGGCCCTACACGTCTACC-3', and reverse 5'ACAAACTGGCCCAACAGAAC-3', p21: forward 5'GAGCGATGGAACTTCGACTT-3', and reverse 5'-CAGGTCCACATGGTCTTCCT-3', GAPDH: forward 5'ACCCAGAAGACTGTGGATGG-3', and reverse 5'TTCTAGACGGCAGGTCAGGT-3'. Reaction products were analyzed on 1% agarose gel.

2.7 Statistics

Statistical analysis was performed with IBM SPSS and the data was analyzed by 1-way ANOVA with Tukey's post hoc comparisons. Data is expressed as means ± SD and differences were considered significant at $P < 0.05$.

Chapter 3: Synergistic anti-cancer properties of capsaicin and DIM

The initiation, promotion and progression of cancer are typically a long-term process that proceeds through many steps. There is increasing evidence from cancer epidemiological and pathological studies suggesting that many human cancers could be prevented or their progression slowed down or even halted with chemoprevention [3, 4]. Chemopreventive agents can act as blocking agents, which impede the initiation stage, or suppressing agents, which arrest or reverse the promotion and progression of cancer by targeting factors that control cell proliferation, differentiation, senescence or apoptosis. Many active compounds found in fruits, vegetables and spices have been shown to possess the ability to both block and suppress carcinogenesis. The anti-carcinogenic function of these compounds might be attributed to a combination of these effects and their ability to target multiple pathways [20].

Typically the growth rate of cancer cells is much more rapid than that of normal cells because of malfunctioning or dysregulation of their cell-growth and cell-death mechanisms. Therefore, induction of apoptosis or cell-cycle arrest by dietary chemopreventive compounds presents a promising approach to inhibit the promotion and progression of carcinogenesis and to remove genetically damaged, precancerous or cancerous cells from the body. We and others reported that several phytochemicals including capsaicin, 6-gingerol, epicatechin, indole-3 carbinol and DIM possess potent anti-cancer effects. In previous study, we screened the combinational effects of capsaicin with other dietary compounds based on expression of tumor suppressor genes such as NAG-1 and ATF3. As a result, we found that combination of capsaicin and DIM showed synergistically induced expression of these genes and programmed cell death. The

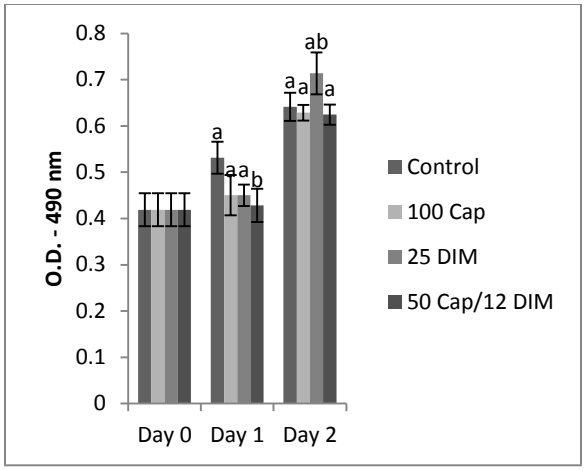
hypothesis is that the combination of capsaicin and DIM can have significant synergistic anti-cancer effects at relatively lower concentrations than individual compounds. To test this hypothesis, multiple cancer cell lines were treated with individual concentrations (100 μM Capsaicin and 25 μM DIM) and then combination of the two at half the concentration of the individual ones (50 μM Capsaicin and 12 μM DIM). The results suggest that the combination of Capsaicin and DIM can have significant synergistic anti-cancer effects as compared to higher concentrations of the individual compounds and these are due to mechanisms involving p53 and NF-κB.

3.1 Combination treatment of Capsaicin and DIM suppresses cell proliferation in multiple cancer cell lines

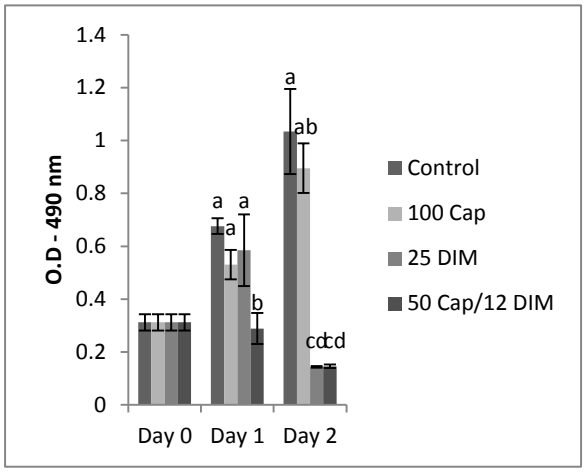
To explore the effect on cell proliferation of individual and combination treatment with capsaicin and DIM in multiple cell lines, normal human colon CCD-112CoN **(Fig 3.1A)**, human colon adenocarcinoma HCT116 **(Fig 3.1B)**, LoVo **(Fig 3.1C),** CaCo2 **(Fig 3.1D)**, HT-29 **(Fig 3.1E)**, SW480 **(Fig 3.1F),** human lung carcinoma A549 **(Fig 3.1G)** and human prostate carcinoma PC3 **(Fig 3.1H)** were treated with the stated concentrations of the compounds. As shown in Fig 3.1A, treatment of single compounds did not change cell proliferation in normal human colon CCD-112Con cells. Co-treatment of capsaicin and DIM slightly but significantly decreased cell proliferation on day 1 but not on day 2**.** Overall, this result is in agreement with the literature that suggests that the individual compounds of capsaicin and DIM are nontoxic to normal cells [30, 67, 95]. Although the pattern that human colorectal cancer cells to respond to capsaicin are different according to types of cells, combination of low dose capsaicin and DIM showed similar activity with high dose of single compounds in terms of suppressing cell proliferation **(Fig 3.1.B-**

F). In addition, synergistic suppression of cell growth was observed in non-colorectal cancer cells **(Fig. 3.1.G,H)**. Taken together, these results indicate that the combination of the two compounds restricts cell proliferation in multiple cancer cell lines while being nontoxic to normal cells.

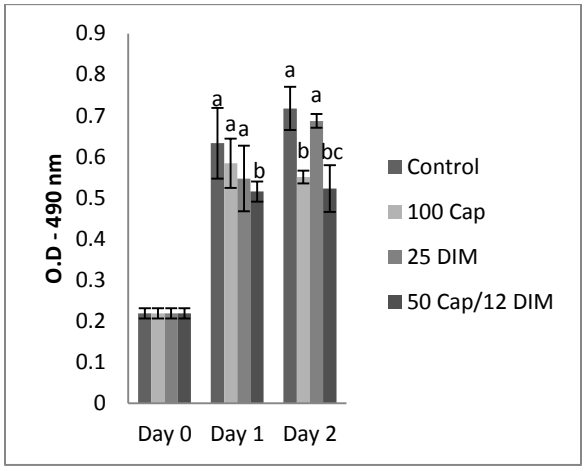
(A) CCD112CoN



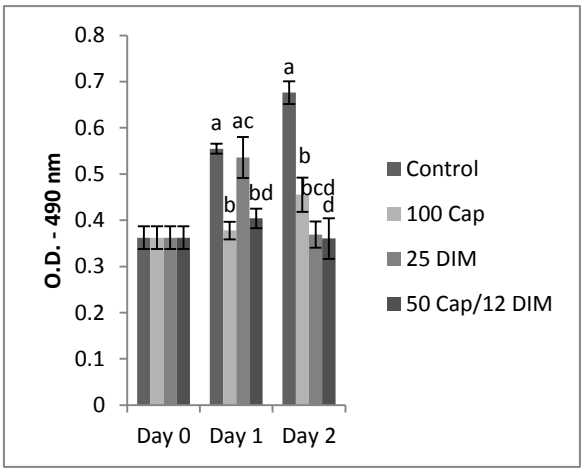
(B) HCT-116



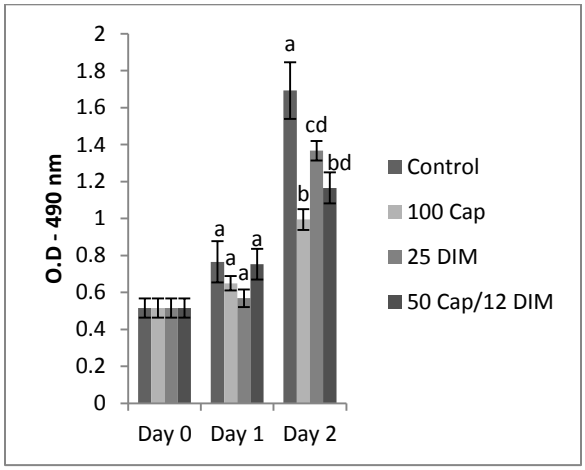
(C) LoVo



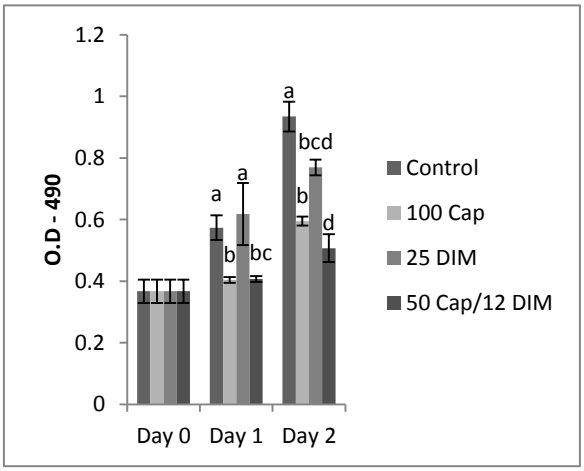
(D) CaCo2



(E) HT-29



(F) SW480



(G) A549 **(H) PC3**

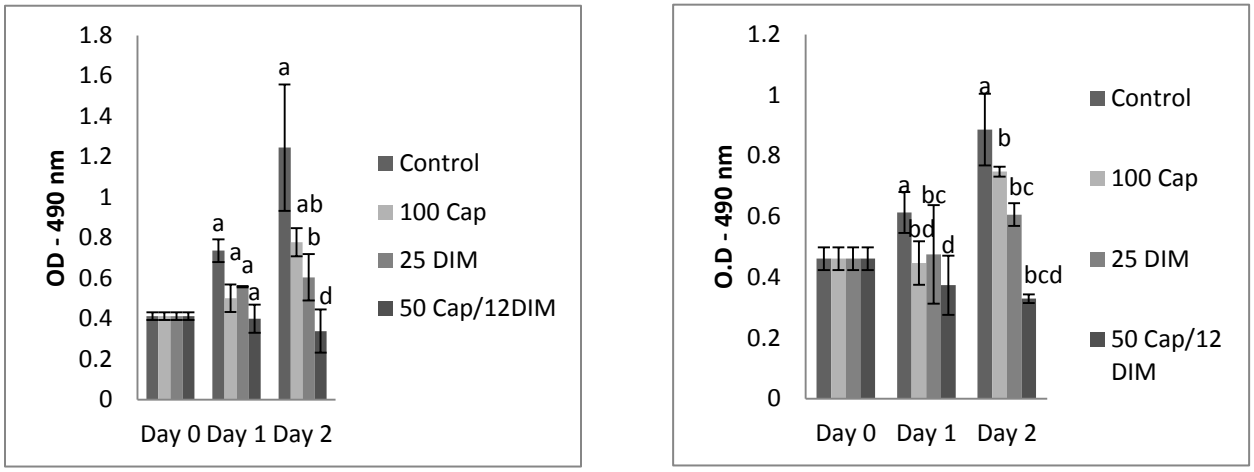


Figure 3.1 Combination treatment inhibits cell proliferation in multiple cancer cell lines, but not normal human colon cells. Normal human colon CCD112CoN (A) HCT116 (B), LoVo (C), CaCo-2 (D), HT-29 (E), SW480 (F), A549 (G), and PC3 (H) cells were plated onto a 96-well plate and grown overnight. The cells were treated with different concentrations of capsaicin (0-100 µM), DIM (0-25 µM) or combination (50 µM capsaicin and 12 µM DIM) in media supplemented with 1% FBS for 24 and 48 hours at 37ºC under 5% CO_2. Cell proliferation was assessed by the MTT [(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] (Sigma, St. Lous, MO) method. The optic density was recorded at 490 nm using an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc., Winooski, VT). Values are means ± SD, n = 3. Means without a common letter differ, $P < 0.05$.

3.2 Synergistic induction of apoptosis in multiple cancer cells lines.

Apoptosis is an essential way for the body to eliminate abnormal cells that pose a threat to the organism's life and is an essential barrier against tumorigenesis. We depend on a functional apoptotic program for proper tissue development and maintenance to eliminate cells that are irreparably damaged. However, if a cell is able to evade apoptosis with certain damages or mutations that inhibit apoptosis and that cell continues to multiply, this can lead to tumor initiation, progression and/or metastasis [31]. Also, there is strong evidence that defective apoptosis is a cause of drug resistance and cancer treatment failure. Since it is the alteration of apoptosis that contributes both to tumor development and drug resistance, compounds that can trigger apoptosis in previously resistant cells has been the focus of much research [110, 111].

To determine whether capsaicin and DIM had a synergistic effect on apoptosis, the cells were treated with various concentrations of capsaicin individually (100 μM) and DIM individually (25 μM) as well as in combination (50 μM capsaicin and 12 μM DIM) and compared with a control group. While the individual compounds resulted in apoptosis, interestingly the combination treatment of capsaicin and DIM induced a marked reduction in the number of viable cells in all cell lines, which suggest these compounds individually induce apoptosis, but together have an even greater effect **(Fig 3.2).**

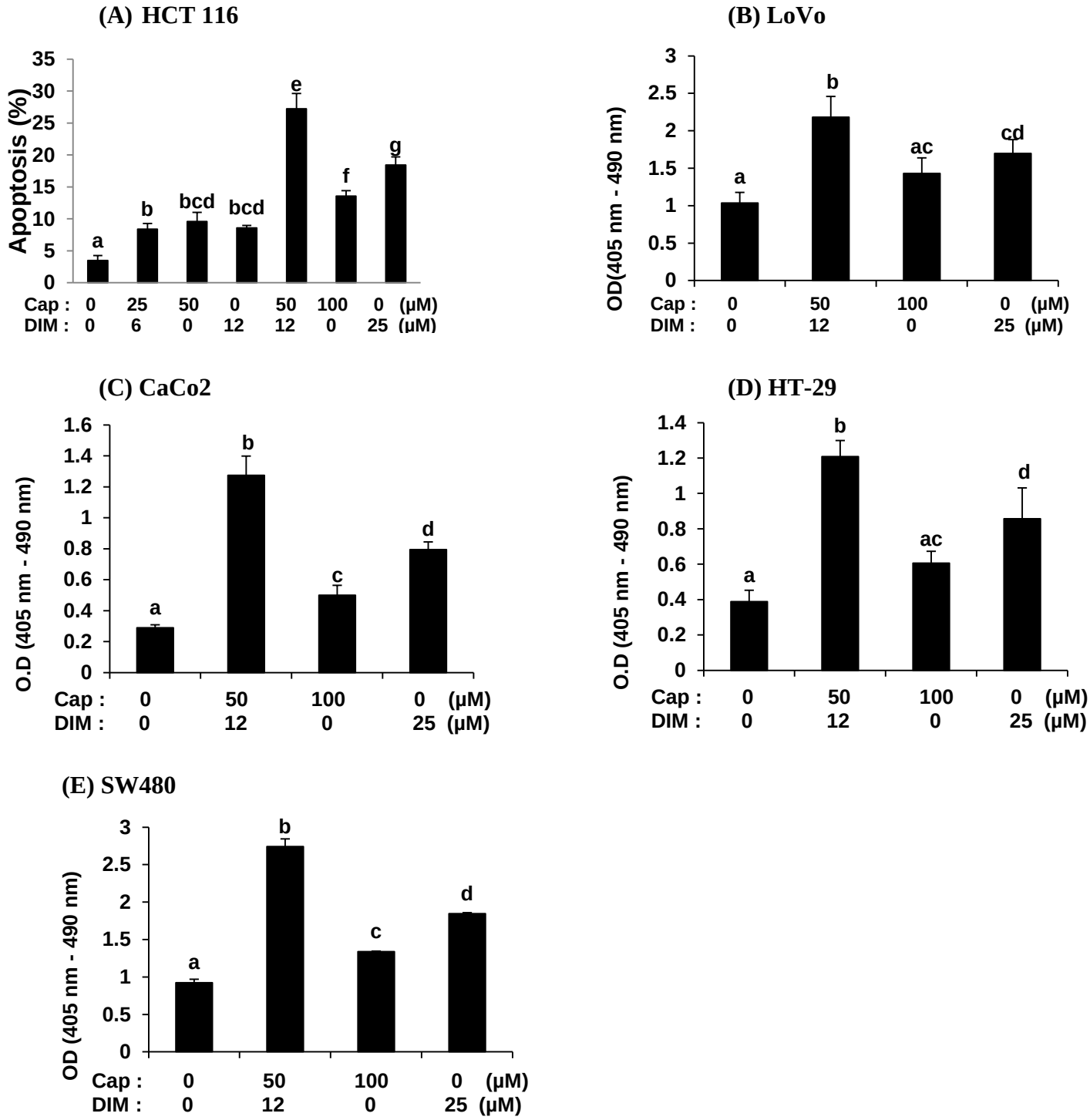


Figure 3.2 Synergistic effects of capsaicin and DIM in apoptosis of multiple cell lines. Apoptosis of different human colorectal cancer cells, HCT116 (A), LoVo (B), CaCo2 (C), HT-29 (D) and SW480 (E), was tested with FACS (HCT-116 cells) and Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics, Indianapolis, IN) for LoVo, CaCo2, HT-29 and SW480 cells according to the manufacturer's instruction. Values are means ± SD, n = 3. Means without a common letter differ, $P < 0.05$.

3.3 Combination treatment increases transcriptional activity of p53 and increases expression in the nucleus.

A major reason that cancer cells are able to evade apoptosis and grow in a rapid and uncontrolled manner is through mutations in the p53 tumor suppressor gene [112]. The p53 gene is the most frequently mutated gene in human cancers and the cells with p53 mutation will lack the DNA-damage-sensing capability that would normally induce cell cycle arrest, DNA repair and the apoptotic cascade. This is seen in p53 null (-/-) and p53 mutant cells that are resistant to drug induced apoptosis [89]. One reason for this is that the majority of the genes and proteins that are transcriptional targets of p53 regulate the cell cycle, senescence, DNA repair and apoptosis; all essential barriers to tumor initiation and progression. Therefore, targeting p53 and the downstream transcriptional targets in cancer cells offer promising therapeutic interventions for cancer prevention and treatment [91].

p53 has been shown to have two mechanisms for regulating cell cycle and initiating apoptosis [112]. Certain stimuli such as oxidative stress and genotoxic injury can cause p53 nuclear accumulation and activation of downstream proapoptotic gene expression such as PUMA, Bak or Bax to induce cell death. However, p53 has also been shown to have transcription-independent proapoptotic effects in the cytoplasm through direct interactions with the Bcl-2 family proteins [113]. Since it was observed that capsaicin and DIM can initiate apoptosis and cell cycle arrest, the transcriptional activity and expression of p53 was analyzed.

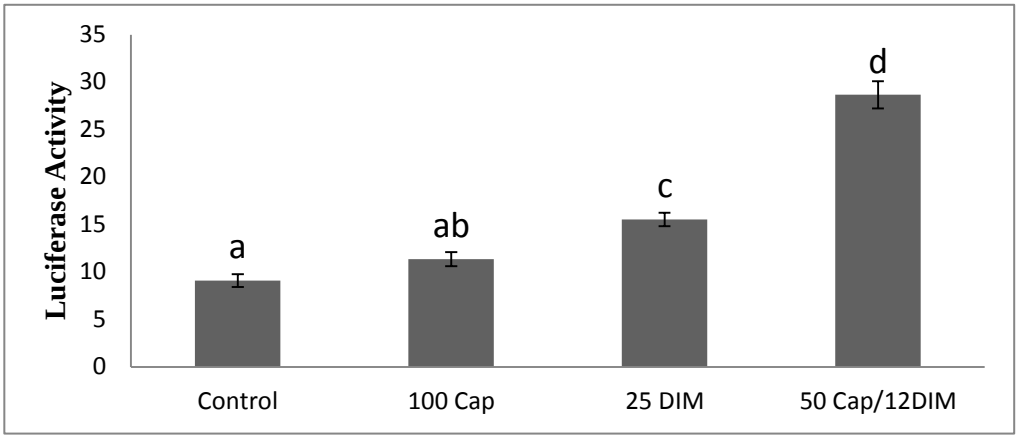
HCT116 cells were seeded on 12 well plates in three replicates. After growth overnight, the cells were transfected with plasmid containing 1 μg of p53 luciferase plasmid and 0.1

μg pf pRL-null vector for 24 hours at 37ºC. The transfected cells were then treated with vehicles as the control, capsaicin and DIM individually or in combination for 24 hours. The cells were harvested in 1x luciferase lysis buffer, and luciferase activity was measured. The transcriptional activity of p53 significantly increased with combination treatment as compared with the individual compounds **(Fig 3.3A).**

To assess the level of p53 proteins in the cells, HCT116 cells were treated with the compounds for 24 hours, harvested and the cytoplasm and nucleus were separated. The cell lysates were analyzed by Western Blotting for p53 and TATA-binding protein (TBP) was used as nuclear fraction marker and loading control in nuclear fraction. As a result, there was increase in the level of p53 in the nucleus of the cells incubated with the combination of both capsaicin and DIM **(Fig 3.3B)**. p53 protein levels in the nucleus are usually kept low by rapid degradation through ubiquitin-dependent proteolysis [114]. The levels of p53 within a cell are not as dependent on the creation of new p53, but on the rate of degradation by the MDM2 protein. Activation of p53 depends on the inhibition of this degradation and subsequent stabilization of the p53 protein which is allowed to rapidly accumulate. It is believed that accumulation of p53 in nucleus after co-treatment of capsaicin and DIM is caused by enhanced p53 stability. Mechanisms such as phosphorylation at serines 15, 37 or 20 have been shown to reduce the interaction between p53 and MDM2 in vitro and result in nuclear stabilization and activation [115]. p53 has demonstrated transcription-independent proapoptotic effects as well, as it has been shown that particular mutations that prevent the transactivating functions of p53 and/or the deletion of p53 nuclear localization signal which causes its retention in the

cytoplasm, do not completely eliminate p53 apoptosis [113, 116]. The cytoplasmic targets of p53 appear to be the Bcl-2 family of proteins, most notably Bak and Bax [117].

(A)



(B)

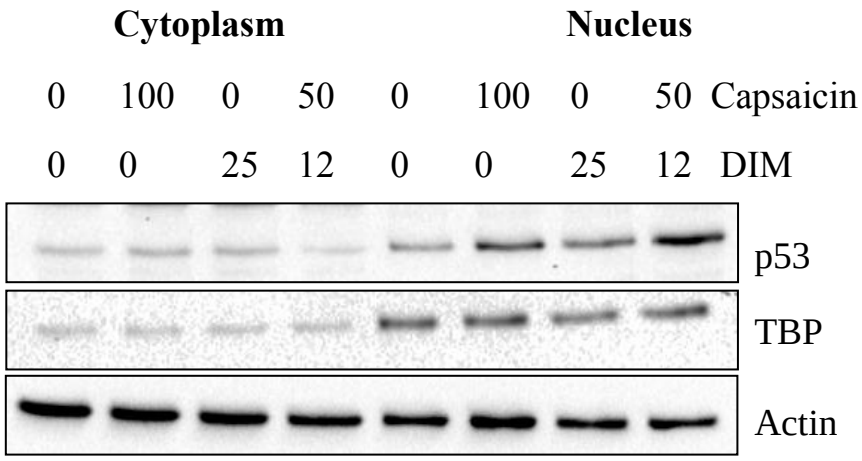


Figure 3.3 Combination treatment synergistically increases transcriptional activity of p53 and increases expression of p53 in nucleus. HCT116 cells were transfected with plasmid containing 1μg of p53 luciferase plasmid and 0.1 μg pf pRL-null vector for 24 hours at 37ºC. The transfected cells were then treated with the vehicle as the control, 100 μM capsaicin, 25 μM DIM individually or combination for 24 hours. The cells were harvested in 1x luciferase lysis buffer, and luciferase activity was measured. Values are means ± SD, n = 3. Means without a common letter differ, $P < 0.05$. **(A)** HCT116 cells were treated with 100 μM capsaicin, 25 μM DIM individually or combination for 24 hours. Cells were washed with ice cold PBS and harvested. The cytoplasm was separated from the nucleus and cell lysates were analyzed by Western Blot for p53, TATA-binding protein (TBP) and actin. TBP was used as a nuclear loading control. **(B)**

3.4 Effect of combination treatment on expression of target proteins that regulate apoptosis and cell cycle.

After observation of the effect of combination treatment on cell proliferation, apoptosis and p53 transcription and expression it was hypothesized that genes and proteins known to be regulated by p53 could be modulated through combination treatment. Western blot analysis was used to analyze target proteins that are involved in these processes and RT-PCR was used to confirm mRNA levels of the target genes.

Cell cycle progression is a sequential process that directs dividing cells through G1, S, G2 and M phases. Transitions between G1-S or G2-M phases function as checkpoints to halt cell division if necessary. The balance between interactions among cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (CDIs) regulates the progression of the cell cycle and disruptions of any of these proteins can effect and block the continuous proliferation of tumorigenic cells [118, 119].

Two well-known regulators of the cell cycle are the tumor suppressor proteins, p21 and p27. Increases of these two proteins result in suppression of the actions of CDK4 and CDK6, therefore preventing a cell from continuing through the cell cycle and proliferating [120].

Combination treatment resulted in increased expression of both p21 and 27 with subsequent inhibition of CDK4 and CDK 6 **(Fig 3.4A).**

There are several pathways which regulate apoptosis within a cell. The extrinsic pathway is triggered by the binding of a death receptor, such as Fas or tumor necrosis receptor 1, with this extracellular ligand, Fas-L. When this binding occurs, Fas-L combines with Fas to form a death complex. The Fas/Fas-L complex recruits death domain-containing

protein (FADD) and pro-caspase-8 to become the death-inducing signaling complex which results in a cascade of reactions which result in the execution of the apoptotic program [110].

Another very important pathway is the intrinsic pathway which is regulated by the Bcl-2 family of cytoplasmic proteins. It directly interacts many pro- and anti-apoptotic members that determine whether the cell survives or begins down the apoptotic pathway. Treatment of HCT-116 cells with the combination of capsaicin and DIM resulted in increased expression of multiple pro-apoptotic proteins **(Fig 3.4B).** Bak, Bax, BID, PUMA (p53-upregulated modulator of apoptosis) and Fas-L are all pro-apoptotic proteins that are downstream of p53.

Research into mechanisms that promote cell survival and resistance to apoptosis have identified a number of genes that are up-regulated by NF-κB. Expression of several genes including Bcl-2, Bcl-Xl, cIAP, survivin, cyclin D1, TRAF1, TRAF2 have been reported to be targets of NF-κB that promote tumorigenesis through resistance to apoptosis and cell cycle arrest [82]. Combination treatment resulted in downregulation of pro-survival protein, Bcl-2 **(Fig 3.4C)**. Bcl-2 inhibits apoptosis and has been shown to be over-expressed in many types of cancer cells [121]. Reduced Bcl-2 expression promotes apoptotic responses to anticancer drugs, while increased expression leads to resistance to chemotherapeutic drugs and radiation therapy and is a target to prevent drug resistance and increased apoptosis. Tumor necrosis factor receptor-associated factor 2 and -3 (TRAF2 and TRAF3) have also been shown to be oncogenes that regulate the noncanonical signaling of NF-κB. Previous groups have shown that suppression of

TRAF2 and TRAF3 results in decreased proliferation and tumorigenesis [122]. Combination treatment resulted in downregulation of these two oncogenes **(Fig 3.4C).** Following treatment with capsaicin and DIM, cells were harvested and reverse transcription-polymerase chain reaction was performed to analyze levels of mRNA for p21 and Bak **(Fig 3.4D).** Increased levels of p21 and Bak mRNA were identified following treatment of both capsaicin and DIM.

Nonsteroidal anti-inflammatory drug (NSAID) activated gene-1 (NAG-1) is a divergent member of the transforming growth factor-beta (TGF-β) superfamily that has been shown to possess various tumor suppression activities both *in vitro* as well as in mouse models [62, 123, 124]. The expression of NAG-1 has been shown to induce apoptosis in colorectal cancer cells[62], pancreatic cancer [125] and prostate cancer [126]. Given the potential of this gene, researchers have focused on compounds that can increase the expression of this gene as a target for anti-cancer drugs and phytochemicals. When HCT116 cells were treated with capsaicin and DIM, the combination resulted in a significant increase in expression of NAG-1 **(Fig 3.4E).**

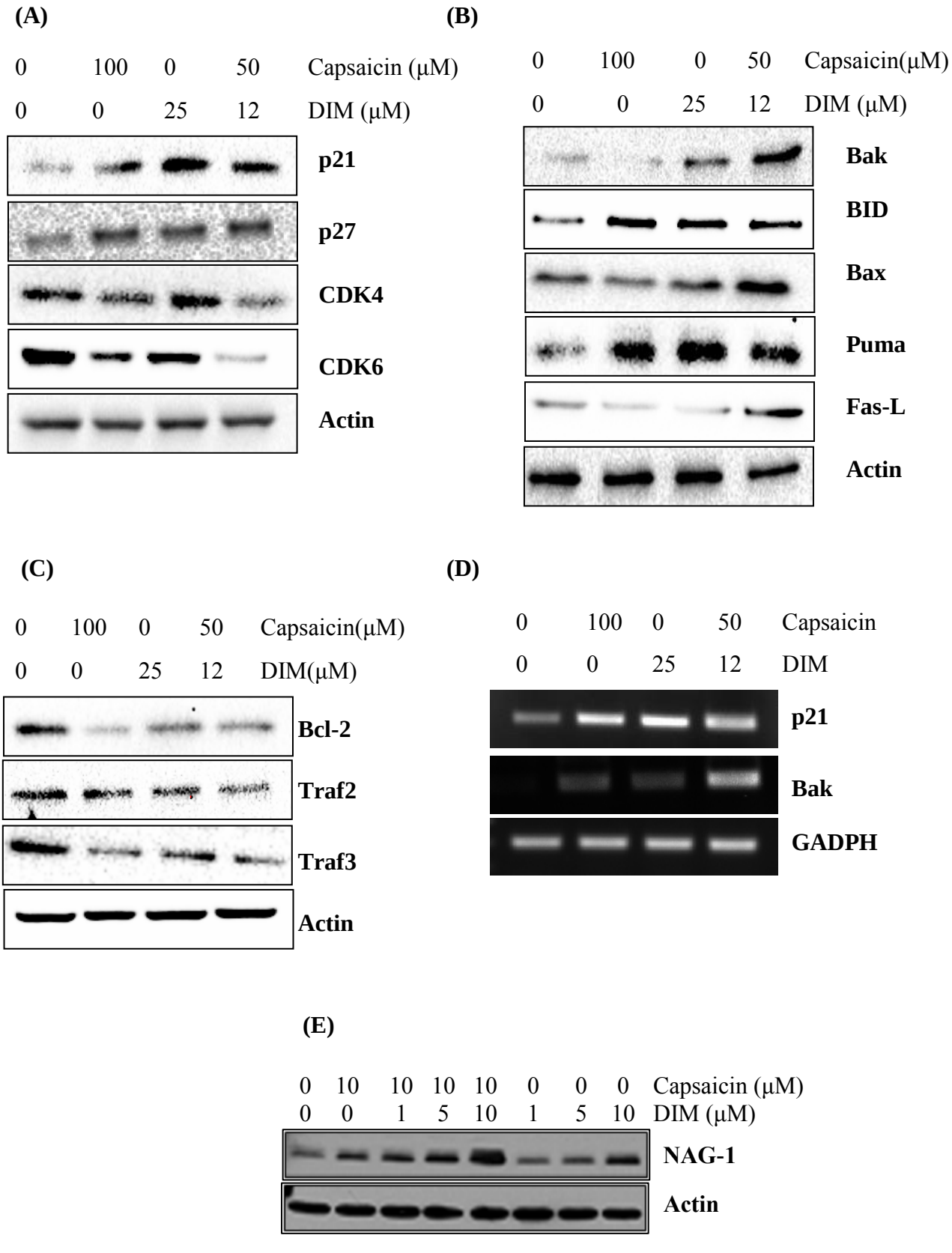

(A)
0 100 0 50 Capsaicin (μM)
0 0 25 12 DIM (μM)
p21
p27
CDK4
CDK6
Actin
(B)
0 100 0 50 Capsaicin(μM)
0 0 25 12 DIM (μM)
Bak
BID
Bax
Puma
Fas-L
Actin
(C)
0 100 0 50 Capsaicin(μM)
0 0 25 12 DIM(μM)
Bcl-2
Traf2
Traf3
Actin
(D)
0 100 0 50 Capsaicin
0 0 25 12 DIM
p21
Bak
GADPH
(E)
0 10 10 10 10 0 0 0 Capsaicin (μM)
0 0 1 5 10 1 5 10 DIM (μM)
NAG-1
Actin

Figure 3.4 Analysis of protein and mRNA expressions in HCT116 cells treated with Capsaicin and DIM. HCT116 cells were treated with different concentrations of capsaicin (0-100 μM), DIM (0-25 μM) or combination (50 μM capsaicin and 12 μM DIM) and incubated for 24 hours. The cells were harvested and cell lysates were analyzed with Western Blot for target proteins. Proteins involved in regulating the cell cycle,p21, p27, CDK4 and CDK6 were analyzed **(A).** Proteins with pro-apoptosis functions, Bak, BID, Bax, Puma and Fas-L were analyzed **(B)**. Anti-apoptotic proteins Bcl-2, Traf2, Traf3 were analyzed **(C)**. HCT116 cells were treated with capsaicin (100μM), DIM (25μM) or combination (50 μM capsaicin and 12 μM DIM) and then total mRNA was harvested. RT-PCR was performed **(D).** HCT116 cells were treated with various concentrations of capsaicin and DIM and harvested. Western Blot for NAG-1 was performed **(E).**

3.5 Modulation of target genes is p53 dependent

The p53 tumor suppressor protein performs a critical role in inducing apoptosis. Many of the proapoptotic Bcl-2 family members including PUMA, Bax and Bak have been reported to be transcriptional targets of p53 [117]. Also the cell cycle regulating proteins, p27 and p21 are also transcriptional targets of p53 [91]. To test if the target genes modulated by combination treatment identified previously were dependent on p53, HCT116 p53 null cells were plated and treated with various concentrations of capsaicin individually (100 μM) and DIM individually (25 μM) as well as in combination (50 μM capsaicin and 12 μM DIM). The cells were harvested and the lysates were subjected to analysis by Western Blot. Interestingly, p27, p21, Fas-L and Bak were all upregulated in a p53-dependent manner **(Fig 3.5)**.

(A)

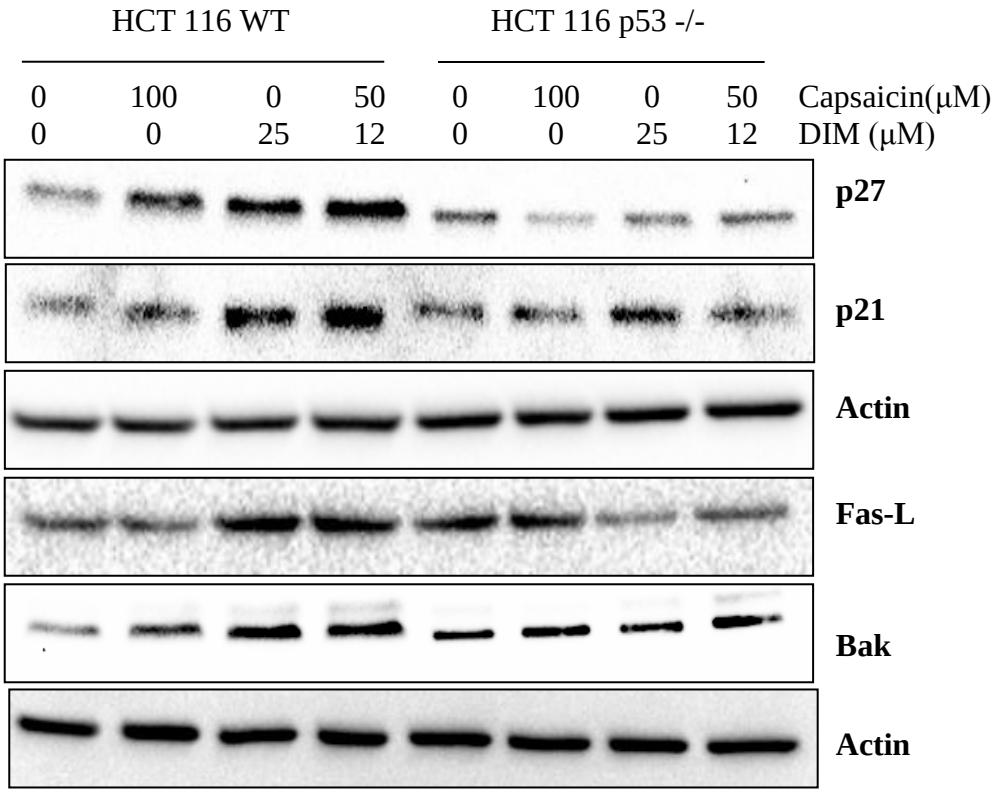


Figure 3.5 Modulation of target proteins in a p53-dependent manner. HCT116 Wild Type (WT) and HCT116 p53 null (-/-) cells were treated with different concentrations of capsaicin (0-100 μM), DIM (0-25 μM) or combination (50 μM capsaicin and 12 μM DIM) and incubated for 24 hours. The cells were harvested and cell lysates were analyzed with Western Blot for target proteins.

3.6 Combination treatment increases transcriptional activity of NF-κB

NF-κB family of transcription factors have been shown to play an important role in regulating apoptosis, both in inducing apoptosis or blocking it. It has also been shown to act on cell cycle regulation through sensitizing or desensitizing a cell to apoptotic signals. The dual roles of NF-κB transcription factors have led to much debate regarding its role in cancer initiation and progression [80, 83]. Given that both p53 and NF-κB are transcription factors stimulated with cellular stress, and the compounds already have demonstrated the ability to increase p53 transcriptional activity, the transcriptional activity of NF-κB was measured.

HCT116 cells were seeded on 12 well plates in three replicates. After growth overnight, the cells were transfected with plasmid containing 1 μg of NF-κB luciferase plasmid and 0.1 μg pf pRL-null vector for 24 hours at 37ºC. The transfected cells were then treated with the vehicle as the control, capsaicin and DIM individually or in combination for 24 hours. The cells were harvested in 1x luciferase lysis buffer, and luciferase activity was measured **(Fig 3.6A).** Interestingly, the transcriptional activity of NF-κB significantly increased with combination treatment as compared with the individual compounds.

p53 and NF-κB have been shown to be induced by similar stimuli including DNA-damaging agents, hypoxia, oxygen free radicals and ionizing radiation and there is evidence to suggest p53 itself induces NF-κB activation [127, 128]. NF-κB activity in cancer is complex as there is evidence to show that it both inhibits and contributes to apoptosis depending on the stimulus, cell line and other factors. The result of this project supports others that have shown that NF-κB and p53 play important roles in cell cycle arrest and apoptosis in *in vitro* cancer cells.

(A)

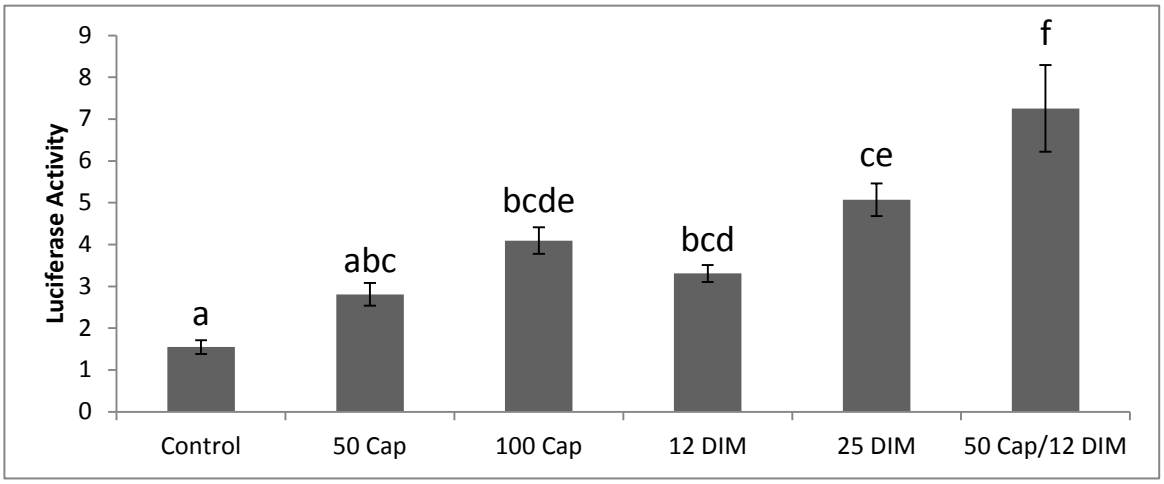


Figure 3.6 Synergistic increase in transcriptional activity of NF-κB.

HCT116 cells were transfected with plasmid containing 1 μg of NF-κB luciferase plasmid and 0.1 μg pf pRL-null vector for 24 hours at 37ºC. The transfected cells were then treated with the vehicle as the control, 100 μM capsaicin, 25 μM DIM individually or combination for 24 hours. The cells were harvested in 1x luciferase lysis buffer, and luciferase activity was measured. Values are means ± SD, n = 3. Means without a common letter differ, $P < 0.05$.

Chapter 4. Discussion and Conclusions

4.1 Discussion

Cancer remains a major health concern worldwide and current treatment options often have debilitating side effects in addition to being expensive and invasive. Therefore there is a growing interest in the prevention of cancer by plant-derived, natural compounds due to their ability to prevent or halt the initiation and progression of cancer. Many individual plant-derived compounds have demonstrated significant effectiveness with minimized toxicity in many different types of cancer [6]. It is not just researchers that are interested in identifying active compounds in fruits, vegetables and spices to prevent chronic disease, but consumers as well. Sales of dietary supplements in the United States totaled an estimated $30 billion in 2011[129]. While there is great interest in preventing cancer and other chronic diseases through a healthier diet and supplements, more research is still needed regarding the efficacy of supplement use across the population. Decades of research on individual phytonutrient and vitamin supplements have resulted in mixed outcomes and more questions than answers [4, 5, 96].

A promising area of research is the identification of novel combinations of phytonutrients that have the ability to target multiple pathways involved in the hallmarks of cancer while limiting toxic side effects to healthy tissues. With this study, I have analyzed the anti-cancer properties of the combination of two phytochemicals and compared it with higher concentrations of the individual compounds. The interesting finding is that the combination of these two compounds exerted significant and sometimes greater anti-cancer activities than the individual compounds at higher concentrations. It is significant that combination treatment exerted synergistic upregulation of tumor suppressor genes,

suppressed oncogenes, inhibited cell proliferation and initiated apoptosis. The hallmarks of cancer offer targets of cancer therapy to prevent the initiation or halt the progression of cancer given that the dysregulation of multiple pathways involved in the cell cycle and apoptotic program is critical for cancer development and progression.

Both p53 and NF-κB pathways play crucial roles in human cancer; roles that are still being investigated due to their complexity [80, 130]. These two pathways have been the focus of much research due to the hope that they will offer further insight on the specific mechanisms of cancer progression and reveal potential therapeutic targets. The ability of the combination of capsaicin and DIM to increase the transcriptional activity of both p53 and NF-κB is noteworthy. Together these two transcriptional factors regulate hundreds of genes that play integral roles in all the hallmarks of cancer and represent promising targets for cancer chemoprevention.

Many of the target genes for p53 are critical to the establishment of the early and late stages of cancer, particularly genes involved in apoptosis and cell cycle. Cell cycle progression is regulated by coordinated activation of cyclin-dependent kinase (cdk)/cyclin complexes. Cyclins bind and activate specific cdk4/6, in association with cyclin D1, and cdk2 to phosphorylate proteins of the retinoblastoma tumor suppressor (Rb) family. Phosphorylation of Rb determines whether a cell will enter S phase. CDK inhibitors, such as p21 and p27, negatively regulate cell cycle progression by inhibiting the activity of the cyclin D1/cdk4/6 complexes, thereby decreasing the level of hyper-phosphorylated Rb [131]. In our study, treatment of capsaicin and DIM induced CDK inhibitors (p21 and p27) and proapoptotic proteins (Bak and Fas L) via p53 dependent mechanisms (**Figure 3.5**).

Here, we propose two mechanisms by which combination of capsaicin and DIM increase apoptosis and changes expression of cell growth and apoptosis-related genes. Firstly, we observed that the compounds increased p53 nuclear accumulation (**Figure 3.3A**) and transcriptional activity (**Figure 3.3B**). p53 accumulation in the nucleus seems to be associated with its protein stability. Ser15 phosphorylation of p53 suppresses ubiquitination of p53 [132] and subsequent 26S proteasome-mediated degradation by reducing the interaction of p53 with E3 ligase, MDM2 and eventually promotes the accumulation and transactivation [133]. Most studies support that p53 degradation occurs exclusively on cytoplasmic proteasome with focusing on nuclear export of p53 via the CRM-1 pathway. However, proteasomes are abundant in both cytosol and nucleus and many studies support proteosomal degradation of transcription factors in the nucleus. In addition, inhibition of proteasomal degradation using MG-132 leads to localization of p65 and p53 in nucleolus (fibrillar centers) compartments [134].

Secondly, in the present study, we observed that combination of capsaicin and DIM synergistically increased transcriptional activity of NF-κB. The transcription factor NF-κB is newly recognized as a key mediator of the cellular stress response upon anticancer therapy, and the activation of NF-κB can lead to a pro-death response. It is believed that increased NF-κB mediates apoptosis induced by anti-cancer agents [135-138]. Thus, further study is required to investigate how compounds activate transcriptional activity of NF-kB.

Although the main control of NF-κB activation is cytoplasmic IκB, recently many studies propose additional mechanisms that are required to activate or prevent activity of NF-κB protein. In fact, NF-κB recruitment is dynamically regulated by multiple signaling inputs

and NF-κB depends entirely on cooperative interactions with partner transcription factors for recruitment to some genes. NF-κB also can be regulated in the nucleus by complex mechanisms that are still obscure. They include post-translational modification and nuclear distribution of p65 which could determine cell fate, pro-apoptotic or anti-apoptotic, and influence its transcriptional activity. For example, an increased accumulation of p65 in nucleoplasm results in apoptosis whereas accumulation of p65 in nucleolus is a mechanism of apoptosis activation in response to apoptotic stimuli [139]. Another interesting point is that p53 and NF-κB have shown an ability to interact with each other and coordinate induction of apoptosis. For example, many proapoptotic stimuli such as DNA-damaging agents lead to transcriptional co-activation of p53 and NF-κB. Ryan et al. suggested that p53 directly activates transcriptional activity of NF-κB which is required for p53-induced apoptosis [127]. Other studies demonstrate that p53-dependent NF-κB activation could contribute to the apoptotic response [128], and the loss of p53 could abrogate NF-κB-mediated apoptotic response [140]. p65 has been reported to promote apoptosis by actively repressing transcription of antiapoptotic genes through association of p65 with histone deacetylase-containing complexes acting as corepressor [141]. Overall, even though we did not study direct interaction between p53 and NF-κB, it is possible that both NF-kB and p53 may be essential for apoptosis induced by combination treatment. Fas ligand (FasL) is believed to be common target for p53 and NF-κB and induction of FasL is required for p53- and NF-κB-dependent apoptosis [142-144]. So FasL could be a good target protein studying an apoptosis associated with p53 and p65.

Although we claim that p53 mediates capsaicin and DIM's effect, we do not exclude the possibility that this combo suppressed cell proliferation and induces apoptosis through p53-independent mechanism because some cell lines such as SW480 and Caco-2 are p53 mutant.

TRAF2 and TRAF3 are adapter proteins and signal transducers that regulates NF-κB and play a role in apoptosis. TRAF3 recruits TRAF2, which forms a heterodimeric complex with TRAF1 which then recruits the inhibitor-of-apoptosis proteins (IAPs) and other apoptotic suppressors for the inhibition of caspase activation [122]. Therefore, TRAF2 and TRAF3 are seen as mediating the anti-apoptotic signals from the TNF receptors [145, 146]. While we did not test to see the effects of the downstream regulation on IAPs as a result of treatment, it is noteworthy that two known inhibitors of apoptosis were downregulated as a result of combination treatment.

Another interesting target of apoptosis is NAG-1. NSAID-activated gene-1 (NAG-1) is a novel member of the transforming growth factor-beta (TGF-β) superfamily and show pro-apoptotic and anti-tumorigenic gene in human cancer models including colon, breast, and prostate [147]. Transgenic mice overexpressing NAG-1 are resistant to azoxymethane (AOM)-induced aberrant crypt foci (ACF), and NAG-1-Tg-*Min* mice showed less tumor load in the small intestine compared with littermate *Min* control mice [148]. NAG-1 expression is induced not only by NSAIDs, but also by several anti-tumorigenic compounds. Our group already reported that both capsaicin and DIM increase expression of NAG-1 [62, 123]. In this study, these compounds synergistically increased expression of this tumor suppressor genes and NAG-1 expression is also

mediated by status of p53 [149, 150]. These results indicate that NAG-1 acts as a molecular target for these two dietary compounds.

A strength of this study is the number of different cancer lines studied to analyze the effects of the phytochemicals. Each cancer cell line has a unique genetic background and therefore it is substantial that the compounds exerted significant anti-cancer effects in multiple cell lines.

A limitation of this study is that it is an *in vitro* design. Therefore, one must use caution in extrapolating the data to make application and recommendations in humans. Caution is always required because it is not known whether exactly the same signaling mechanisms would be operational *in vivo* and there is a possibility of differences in the therapeutic doses in vivo as compared to the concentrations and conditions *in vitro.* However, studies in cell cultures can provide valuable insights into the detailed cellular mechanisms that would otherwise be difficult if not impossible to observe in human subjects however, more studies are needed, particularly in humans, regarding how combinations of phytonutrients such as capsaicin and DIM could help prevent or suppress the initiation and progression of cancer.

4.2 Conclusion

In conclusion, I believe this study can be used to further confirm the findings of other researchers that the combination of multiple phytochemicals can be used to exert greater chemopreventative effects rather than a single compound. This is in support of the recommendations set by top nutrition authorities to eat at least 5 servings a day of a variety of fruits and vegetables.

The further study of novel combinations of phytochemicals that have chemopreventive or therapeutic activities holds a lot of promise. However, which foods and/or components to combine for maximum cancer prevention remain to be determined. Identifying the many active components in foods and their mechanisms of action towards cancer prevention is still needed. This is very complicated because the effects of dietary components can be cell and dose dependent in addition each one can possess multiple mechanisms of action. In time, with increased knowledge regarding novel combinations and improved research technologies we may be able to identify foods to eat to help prevent specific cancers and perhaps begin to see declines in the occurrences and mortality of cancers.

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