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Combination Regimens Using Dietary Components For The Chemoprevention Of Colorectal Cancer and Inflammation

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**COMBINATION REGIMENS USING DIETARY COMPONENTS FOR THE
CHEMOPREVENTION OF COLORECTAL CANCER
AND INFLAMMATION**

A Dissertation Presented

By

CHRISTINA DIMARCO-CROOK

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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The Department of Food Science

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DEDICATION

To God from whom all good things come

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ABSTRACT

COMBINATION REGIMENS USING DIETARY COMPONENTS FOR THE
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AND INFLAMMATION
SEPTEMBER 2018

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Dietary components have been found to effectively modulate multiple deregulated signaling pathways associated with the initiation and progression of carcinogenesis and inflammation in cellular and animal models. However, clinical studies have shown mixed results when examining the efficacy of individual dietary components, perhaps suggestive of the synergism that exists between multiple components within a particular food and the diet as a whole. Additional research is needed to identify and characterize the unknown interactions and potential chemopreventive and anti-inflammatory properties within combination regimens using dietary components.

Nobiletin a polymethoxyflavone (PMF) found primarily in the peel of sweet (*C. sinensis*) and bitter (*C. aurantium*) orange has demonstrated significant anti-cancer and anti-inflammatory effects in both cellular and animal models of colon cancer; therefore it is important to investigate the biological activities and interactions of its metabolites with other dietary components in order to better understand the possible mechanisms of nobiletin *in vivo*. One of the primary metabolites of nobiletin in the mouse 3',4'-didemethylnobiletin (DDMN), has been identified as the metabolite with the strongest anti-proliferative effects in HCT116 wild-type p53 colon cancer cells. Colonic concentration of nobiletin in the mouse is also much lower than its primary metabolites,

of which DDMN is reported to exhibit stronger anti-cancer and anti-inflammatory effects than its parent compound nobiletin. In addition, curcumin, apigenin and luteolin have each been shown individually to exhibit significant anti-carcinogenic and anti-inflammatory effects in various colon cancer model systems; however the interaction of these dietary components in combination with DDMN has yet to be explored. Our results find for the first time apigenin or luteolin, two flavones though similar in structure, to have strikingly different responses when combined with DDMN in HCT116 wild-type p53 colon cancer cells. Apigenin and DDMN are additive in combination with no apparent interaction whereas luteolin when combined with DDMN exhibits an antagonistic response with diminished anti-proliferative effects. Remarkably, in sharp contrast to these findings the combination of curcumin and DDMN in HCT116 wild-type p53 colon cancer cells demonstrates strong synergism with enhanced anti-proliferative effects which greatly exceed the effects of individual treatments.

Additional examination of the synergistic combination of curcumin and DDMN reveals significant cell cycle arrest and extensive apoptosis induced by the combination, which were much stronger than the effects induced by the treatments with curcumin or DDMN alone. Proteins associated with cell cycle arrest and apoptosis were analyzed by Western Blot to confirm the change in expression of these proteins were much greater in response to the combination treatment of curcumin and DDMN than each compound alone. The synergy between curcumin and DDMN offers a possible novel mechanism for nobiletin in combination with curcumin and warrants further investigation on their combination to determine its chemopreventive and anti-inflammatory potential for colon cancer *in vivo*.

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CHAPTER 1
REVIEW OF COMBINATION REGIMENS USING DIETARY COMPONENTS
FOR CANCER CHEMOPREVENTION

1.1 Abstract

Chemopreventive agents that the general population can consume for prolonged periods of time with minimal risk of any side effects are of great interest to all in search of a solution to the pervasive incidence of cancer. Dietary bioactive components have been found to modulate many deregulated molecular pathways associated with the initiation and progression of different types of cancer. Combination regimens with dietary bioactive components are a promising strategy for cancer chemoprevention because they may offer enhanced protective effects against cancer development but cause little or no adverse effects. This article provides an overview of studies examining the combination of dietary bioactive components for the chemoprevention of major types of cancer. A better understanding of existing research on the combination of dietary bioactive components will provide an important basis for the rational design of future combination studies and the successful development of cancer chemoprevention strategies.

1.2 Keywords

Dietary bioactive components, cancer chemoprevention, combination, synergy, whole foods, colorectal cancer, prostate cancer, breast cancer, lung cancer, dietary supplements

1.3 Introduction

Cancer is currently estimated to be responsible for one out of every four deaths in the United States, a staggering number that reflects its far-reaching impact on society (Jemal, Siegal et al. 2010). Although many advances have been made in the early diagnosis and treatment of cancer, significant challenges to lowering cancer mortality rates still remain. Accumulating evidence suggests that a promising strategy for controlling cancer mortality rates is to prevent cancer from progressing to the advanced malignant stages during which available treatment options are very limited. Cancer chemoprevention is the use of natural or synthetic chemicals to reverse, suppress, or prevent cancer development, thereby reducing an individual's risk of cancer (Greenwald 2002). Dietary bioactive components have been investigated intensely for their potential chemopreventive effects in different cancers. One major advantage of dietary components as chemopreventive agents is that they generally have none or few of the adverse effects frequently associated with pharmaceutical drugs after long-term administration (Chen and Kong 2005; Ramos 2008; Davis, Emenaker et al. 2010; Neergheen, Bahorun et al. 2010).

There is a growing body of evidence suggesting that the combination of cancer chemopreventive agents with distinct mechanisms of action may produce a synergistic type of interaction (Xiao and Yang 2008). The synergy among these agents can result in considerably stronger cancer chemopreventive effects than those produced by each agent individually. The enhanced efficacy of the combination regimen can also lower the dose required for each agent in the combination which lowers the cost of the regimen and offers a positive economic impact on society. Moreover, the lowered doses of

chemopreventive agents can potentially reduce or eliminate unwanted side effects frequently associated with long-term administration of high-dose chemopreventive agents. Numerous studies have explored the benefits of combining multiple pharmaceutical drugs and combining dietary bioactive components with pharmaceutical drugs to fight cancer (Schwartz, Birk et al. 2004; Tsuda, Ohshima et al. 2004; Constantinou, White et al. 2005; Fresco, Borges et al. 2006; Martin 2006; Xiao and Yang 2008). However, less is understood about the combination of dietary bioactive components for cancer chemoprevention. Herein, the combination studies on dietary bioactive components are reviewed to investigate their ability to alter the pathology of cancer. The principle and methods of determining the modes of interaction (i.e., synergistic, additive, and antagonistic) among multiple agents have been discussed elsewhere (Greco, Bravo et al. 1995; Chou 2006; Lee, Kong et al. 2007). In this review, the interaction among dietary bioactive components is examined on the basis of the combination index (CI) or relevant statistical analyses (Xiao and Yang 2008). This review summarizes existing research for the potential use of dietary bioactive components in combination for cancer chemoprevention.

1.4 Colorectal Cancer

Accumulating research in colorectal cancer models suggests dietary bioactive components in combination may enhance the anticarcinogenic effects attributed to each individual component (**Table 1.1**). The estimated transition from precancerous cells to a malignant colorectal tumor takes, on average, 10-15 years (Boursi and Arber 2007).

Aberrant crypt foci (ACF) are believed to be the first identifiable precursor lesions of colorectal cancer (Bird 1995).

1.4.1 Aberrant Crypt Foci (ACF) and Familial Adenomatous Polyposis (FAP)

The chemopreventive efficacy of curcumin (a major bioactive component found in turmeric), green tea catechins [mainly (-)-epigallocatechin-3-gallate, EGCG], and their combination was determined in male Wistar rats in which colon carcinogenesis was induced by 1,2-dimethylhydrazine (Xu, Ren et al. 2010). After 12 weeks of dietary treatments, the formation of colonic ACF was significantly decreased by the combination treatment of curcumin and tea catechins. This inhibitory effect was stronger than that of the treatments with individual components. Furthermore, following 32 weeks of dietary treatments, the colonic tumor incidence of the combination treatment group was significantly lower than that of the curcumin or catechin treatment groups. These results suggest that the combination treatment with curcumin and green tea catechins produces enhanced inhibitory effects on both the initiation and progression stages of colon carcinogenesis. Studies using whole foods in combination have also demonstrated strong anticarcinogenic effects on ACF formation (Challa, Rao et al. 1997; Sengupta, Ghosh et al. 2004). A study using the azoxymethane-induced rat model showed that the garlic and tomato combination dietary treatment produced a stronger effect on the inhibition of abnormal cell proliferation, the induction of apoptosis, and greater decreases in ACF formation than garlic or tomato alone (Sengupta, Ghosh et al. 2004). The pronounced inhibitory effect on ACF formation by the combination of garlic and tomato has been attributed in part to the reduction of cyclo-oxygenase-2 (COX-2) and induction of

Table 1.1 Studies examining the combination of dietary bioactive components on colorectal cancer

Combination Treatment	Model System Mechanism/Effect	References
Indol-3-carbinol Genistein*	HT29 colon cancer cells, ↓autophagy, ↓Akt phosphorylation, ↑caspase-dependent apoptosis	Nakamura <i>et al</i>
Sulforaphane, 3,3'-diindolymethane*	HCT116 Clone colon cancer cells, ↑G ₂ /M cell-cycle arrest, ↓cell proliferation	Pappa <i>et al</i>
Indole-3-carbinol, 4-methoxyindole-3-carbinol	DLD-1 and HCT116 colon cancer cells, ↓cell proliferation	Kronbak <i>et al</i>
Isothiocyanates Indoles	LS-174 colon cancer cells, ↓single-strand DNA breaks	Bonnesen <i>et al</i>
Resveratrol, Chrysin, Curcumin	Caco-2 colon cancer cells, ↑UGT1A1 ↓cell proliferation	Iwuchukwu <i>et al</i>
Butyrate Carnitine	Caco-2 colon cancer cells, ↓Cox-2, ↑apoptosis, ↓cell proliferation	Roy <i>et al</i>
Resveratrol Grapeseed extract*	HCT116 colon cancer cells, ↑, ↑Bax, ↓Bcl-2, ↑apoptosis, ↓cell proliferation	Radhakrishnan <i>et al</i>
Resveratrol Curcumin*	HCT116 colon cancer cells, ↓pEGFR, ↓pHER-2, ↓pHER-3, ↓pIGF-IR, ↑S phase cell-cycle arrest	Majumdar <i>et al</i>
Total Cranberry Extract Polyphenols	HCT116 and HT29 colon cancer cells, ↓cell proliferation	Seeram <i>et al</i>
Pomegranate Juice Polyphenols	HCT116 and HT29 colon cancer cells, ↓cell proliferation, ↑apoptosis, ↓lipid peroxidation	Seeram <i>et al</i>
Sulforaphane Apigenin	CaCo-2 colon cancer cells, ↑UGT1A1	Svehlikova <i>et al</i>
Docosahexaenoic acid (DHA), Butyrate	HCT116 colon cancer cells, ↑apoptosis ↑ mitochondrial Ca ²⁺	Kolar <i>et al</i>

Table 1.1 Continued

Combination Treatment	Model System Mechanism/Effect	References
Sulforaphane, Iberin Selenium	Caco-2 and HCT116 colon cancer cells, No effect on abnormal methylation	Barrera <i>et al</i>
Green Tea Selenium	Azoxymethane treated Sprague-Dawley rats, ↓DNMT1, ↑histone H3 acetylation, ↓ACF, ↓cell proliferation, ↓CyclinD1, ↑SFRP5	Hu <i>et al</i>
β-ionone geraniol	Dimethylhydrazine induced Wistar rats, ↓bioavailability	Viera <i>et al</i>
Sulforaphane Dibenzoylmethane	Apc ^{Min/+} mice, No significant effects as compared to individual components	Shen <i>et al</i>
(-)-Epigallocatechin-3-gallate, Fish oil	Apc ^{Min/+} mice, ↓tumor occurrence, ↓large tumors, ↓PGE2, ↓nuclear β-catenin, ↓p-Akt	Bose <i>et al</i>
Diosmin Hesperidin	Azoxymethane treated F344 rats, No significant effects as compared to individual components	Tanaka <i>et al</i>
Curcumin Green Tea Catechins	Dimethylhydrazine induced Wistar rats, ↓ACF, ↓tumor incidence, ↓cell proliferation	Xu <i>et al</i>
Green Tea Phytic acid	Azoxymethane treated Fisher 344 rats, ↓ACF incidence	Challa <i>et al</i>
Tomato Garlic	Azoxymethane treated Sprague Dawley rats, ↓cell proliferation, ↓apoptosis, ↓ACF	Sengupta <i>et al</i>
Fish oil, Corn oil, Butyrate	Azoxymethane induced Sprague Dawley rats, ↑↓ACF, ↑p21, ↑↓apoptosis	Crim <i>et al</i>
Peppermint, Sage Rosemary*	SW-480 colon cancer cells, ↓cell proliferation	Yi <i>et al</i>
Eicosapentaenoic acid (EPA), Lycopene	HT29 colon cancer cells, ↓cell proliferation, ↓p-Akt, ↓p-mTOR, ↑Bax, ↑Fas	Tang <i>et al</i>

*This study used the Combination Index (CI) to determine synergism

glutathione-S-transferase (Sengupta, Ghosh et al. 2003). Examination of the flavonoids diosmin and hesperidin in combination revealed a significant decrease in ACF formation in azoxymethane-treated rats (Tanaka, Makita et al. 1997). However, the combination effect on ACF was not statistically stronger as compared to treatments with individual agents.

The $Apc^{Min/+}$ mouse model has been used frequently to determine chemopreventive effects of dietary components against intestinal tumorigenesis. The $Apc^{Min/+}$ mouse model is a model for human familial adenomatous polyposis (FAP), which is a hereditary disease characterized by the inactivation of one allele of the adenomatous polyposis coli (APC) gene. Individuals diagnosed with FAP experience multiple colonic polyps that without removal may eventually progress to colon cancer (Rao and Reddy 2004). Although FAP only accounts for a small portion of diagnosed cases of colorectal cancer, APC gene mutations are a common early occurrence in the carcinogenesis process of the majority of colorectal tumors; therefore, use of the $Apc^{Min/+}$ mouse model can offer insight into these early genetic events and aid in the development of future chemopreventive agents (Powell, Zilz et al. 1992). The combination of sulforaphane and dibenzoylmethane (a compound found in licorice) was investigated in the $Apc^{Min/+}$ mouse model (Shen, Khor et al. 2007). The combination treatment caused a significant inhibition of tumor development as compared to the control. However, when the chemopreventive effects of half-dose combinations of sulforaphane and dibenzoylmethane were compared with those produced by full-dose combinations of sulforaphane and dibenzoylmethane alone, no statistical difference was found; therefore, further studies are required to examine a broad range of doses to determine the mode of

interaction (additive or synergistic). A combined treatment of EGCG (a major green tea polyphenol) and fish oil was used in the high fat diet of female $Apc^{Min/+}$ mice to determine if the combination decreased tumors more effectively than either agent alone (Bose, Hao et al. 2007). The combination treatment for nine weeks resulted in a significant decrease in the number of large tumors as compared to the control, a greater effect than individual treatments. A combination treatment of EGCG and fish oil resulted in tumors with decreased levels of prostaglandin E_2 (PGE_2), nuclear B-catechin, and phosphorylated Akt, three potential procarcinogenic factors for colorectal cancer. The combination of dietary components has also been found effective in inhibiting human FAP. For example, in a case study of five FAP patients, all were found to have a reduction in the number and size of adenomas following a six-month dietary treatment of 480 mg of curcumin and 20 mg of quercetin (a flavonol found in many fruits, vegetables, and grains) in combination three times a day (Cruz-Correa, Shoskes et al. 2006), highlighting the promising potential of dietary combination regimens for cancer chemoprevention.

1.4.2 Fish Oil –Docosahexaenoic and Eiosapentaenoic Acid

Besides the aforementioned study on the combination of fish oil and EGCG in $Apc^{Min/+}$ mice, several other studies have focused on the combination of fish oil with other dietary factors. In azoxymethane-treated rats, the dietary combination treatment of fish oil plus butyrate for 11 weeks resulted in inhibition on colonic ACF formation accompanied by increased apoptosis. In contrast, the combination of corn oil plus butyrate led to increased ACF formation and had no effect on apoptosis (Crim, Sanders et

al. 2008). Although both combinations increased the expression of p21, the fish oil plus butyrate combination was chemopreventive, whereas the corn oil plus butyrate combination was promotive of colon carcinogenesis. These findings highlight the important role of interactions between dietary components and their effects on biological outcomes. Studies evaluating fish oil components such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) offer clues to possible mechanisms involved in combinations with fish oil. In human colon cancer cells and primary cultures of rat colonic crypts, enhanced induction of apoptosis observed with the combination of butyrate and DHA appeared to occur through a p53-independent mechanism that relied on the upregulation of the mitochondrial Ca^{2+} -dependent pathway (Kolar, Barhoumi et al. 2007). Lycopene and EPA in combination demonstrated a significant reduction of cell proliferation in HT29 colon cancer cells ($p < 0.05$), which was associated with inhibition of the phosphatidylinositol 3-kinase/Akt signaling pathway (Tang, Cho et al. 2009).

1.4.3 Glucosinolates

One group of compounds under frequent investigation for their chemopreventive effects against colon cancer are glucosinolates, in particular the glucosinolate breakdown products such as sulforaphane, indole-3-carbinol, and their related derivatives. The main source of glucosinolates in the human diet is cruciferous vegetables, some of which include broccoli, cauliflower, Brussels sprouts, kale, and cabbage (Lund 2003; McGrath and Spigelman 2008). Indole-3-carbinol and genistein (a soy isoflavone) in combination were found to synergistically inhibit cell viability of HT29 human colon cancer cells ($CI < 1$) (Nakamura, Yogosawa et al. 2009). This inhibition was associated with

inactivation of Akt, an important protein involved in the key signaling pathways known to be overactive in colorectal cancer (Osaki, Oshimura et al. 2004). Synergistic inhibitory effects on cell proliferation were also found in a combination study examining the interaction between two glucosinolate products, 3,3'-diindolylmethane (DIM) and sulforaphane, in a colorectal cancer cell line (Pappa, Strathmann et al. 2007). DIM, a condensation product and dimer of indole-3-carbinol, is formed in the acidic environment of the stomach and is the primary form of indole-3-carbinol absorbed in humans. In the cell proliferation assay, a dose response effect was observed as lower concentrations of the sulforaphane plus DIM combination became synergistic in its effects on decreasing cell proliferation. The authors postulated the antagonistic interaction was due to the induction of phase II enzyme expression by sulforaphane at low concentrations, which increased metabolism of DIM and decreased its activities. Others have found less pronounced effects on cell proliferation by the combination of glucosinolate products such as indole-3-carbinol and 4-methoxyindole-3-carbinol in HCT116 colon cancer cells (Kronbak, Duus et al. 2010). Glucosinolate products can be protective in the early stages of carcinogenesis by diminishing the effects of genotoxic compounds (Bonnesen, Eggleston et al. 2001). Sulforaphane and indolo[3,2b]carbazole (ICZ, a condensation product of indole-3-carbinol) were studied in LS-174 colon cancer cells to determine their effects on carcinogen/genotoxin exposure of benzo(a)pyrene or H₂O₂. In each case, the combination treatment with sulforaphane and ICZ prior to carcinogen/genotoxin exposure achieved greater DNA protection than either agent alone. It is notable that the protection against DNA damage did not occur if the combination treatment followed benzo(a)pyrene or H₂O₂ exposure. Multiple studies also suggest glucosinolate

derivative combinations and other polyphenol combinations are more effective than their individual components in halting initiation of the carcinogenesis process through the induction of phase II enzymes (Nho and Jeffery 2004; Svehlikova, Wang et al. 2004; Iwuchukwu, Tallarida et al. 2011; Saw, Cintron et al. 2011).

1.4.4 Multiple Component Interaction based on Food Source

One area of interest is the interaction of components within a specific food. Butyrate and carnitine, both present in milk, have been found in combination to induce apoptosis in colon cancer cells by increasing the expression of proapoptotic proteins Bax and Bak and decreasing the expression of the COX-2 protein (Roy, Dionne et al. 2009). Although the proapoptotic effects of butyrate and carnitine in combination were significant as compared to the control, these enhanced effects were not significant when compared to those exhibited by butyrate alone. Resveratrol and grape seed extract, both found in grapes, have demonstrated synergistic effects in combination on the inhibition of cancer cell proliferation and induction of apoptosis through a p53-dependent mechanism in insulin-like growth factor-1 (IGF-1) stimulated HCT116 colon cancer cells (Radhakrishnan, Reddivari et al. 2011). These findings may have important implications for future chemopreventive strategies, as IGF-1 appears to play a pivotal role in the development of obesity-driven colorectal cancer and p53 is characteristically intact in colonic polyps, thereby offering a measureable outcome of treatment efficacy. The combination of one agent with different agents may elicit distinct mechanisms of actions. For example, when resveratrol was combined with a compound not found within grapes, such as curcumin, the combination elicited a synergistic response that was p53-

independent (Majumdar, Banerjee et al. 2009). This was different from the combination of resveratrol and grape seed extract.

1.4.5 Whole Foods – Fractions and Extracts

The combination effects of dietary components on colon cancer have been demonstrated in studies where the whole food and its fractions were compared. Total cranberry extract was examined and separated into fractions of sugars, organic acids, total polyphenols, proanthocyanidins, and anthocyanins to compare the efficacy of different fractions in the inhibition of colon cancer cell growth (Seeram, Adams et al. 2004). The greatest antiproliferative effect occurred with the total polyphenol fraction in HCT116 colon cancer cells, thereby suggesting a possible synergistic interaction among several polyphenol components. In a similar study, pomegranate juice demonstrated greater inhibition on HT-29 and HCT116 colon cancer cells than isolated individual polyphenol components of pomegranate juice (Seeram, Adams et al. 2005). Upon further examination, the pomegranate juice was found to have a significant effect on the induction of apoptosis in HT-29 cells but no significant effect on apoptosis in the HCT116 cell line. However, the individual polyphenol components of pomegranate juice significantly increased apoptosis in HCT116 cells, suggesting different mechanisms are responsible for the inhibitory effects of pomegranate juice in HT-29 colon cancer cells. Whole food extracts have also been investigated for their combination effects. A peppermint extract plus sage extract combination treatment synergistically inhibited the growth of SW-480 colon cancer cells. However, rosemary extract and sage extract alone was much stronger than peppermint extract in its inhibitory effects (Yi and Wetzstein

2011). Notably, studies using crude food extracts in cell culture models have very limited physiological relevance in terms of bioavailability and biotransformation of different dietary components in humans. Nevertheless, these types of studies may provide potential leads for further investigations using experimental models of better physiological relevance.

1.4.6 Epigenetic Effects

Epigenetic events have been shown to play critical roles in regulating carcinogenesis. Recent studies have examined epigenetic biomarkers to determine if the combination of bioactive dietary components elicits enhanced epigenetic responses in colorectal cancer models. Aberrant methylation frequently reported in colorectal cancer was unaffected by the combination of isothiocyanates and selenium in Caco-2 and HCT116 colon cancer cells (Barrera, Johnson et al. 2013). In contrast, a marked reduction of abnormal methylation was indicated with decreased expression of DNA methyltransferase-1 (DNMT1) following green-tea-plus-selenium combination treatment in a rat colorectal cancer model (Hu, McIntosh et al. 2013). Interestingly, the same combination also exhibited a greater induction of histone H3 acetylation than green tea or selenium alone, an important finding as deacetylated histone H3 has been positively associated with colorectal cancer development. Given the importance of epigenetic events, the role of epigenetic mechanisms in the combination of dietary components in colorectal cancer warrants further investigation.

1.5 Prostate Cancer

As a cancer of high frequency, prostate cancer has been frequently investigated as a target of combination treatments (**Table 1.2**). Effective modulation of the pathways mediated by androgens (such as testosterone) and their products is an important strategy for the inhibition of prostate carcinogenesis.

1.5.1 Soy Isoflavones and Soy Phytochemical Concentrate (SPC)

Both soy and selenium have shown the ability to individually work within these androgen mediated pathways in animal models, thereby altering the course of disease. Efficacy of soy isoflavones and selenium in combination was investigated in a Noble rat model of hormone-induced prostate cancer (Legg, Tolman et al. 2008). The two oral dosages of soy isoflavones were based on either the high consumption of soy among the Asian population (often associated in epidemiological studies with a decreased risk of prostate cancer) or the lower consumption of soy in the Western diet. The high concentration combination treatment of selenium plus isoflavones was found to significantly lower 5 α -reductase activity as compared to the low concentration combination treatment in the male Noble rats. The enzyme 5 α -reductase is involved in the reduction of testosterone to dihydrotestosterone (DHT), often elevated in prostate cancer. Moreover, results from a complementary study in male Noble rats demonstrated that combined dietary treatment with selenium and isoflavones produced stronger effects in reducing prostate cancer risk factors than either agent individually, and timing of dietary supplementation can greatly impact treatment efficacy (Tolman, Lephart et al. 2008). The combination of soy isoflavone genistein and resveratrol was investigated in

SV-40 tag rats, a transgenic model of spontaneously developing prostate cancer (Harper, Cook et al. 2009). The low-dose dietary combination regimen of genistein plus resveratrol (both at 83 mg/kg diet) did not affect cancer outcome, whereas the high-dose combination (both at 250 mg/kg diet) was effective in reducing the overall occurrence of prostate cancer. However, there was no evidence of a synergistic interaction between genistein and resveratrol that may increase chemopreventive effects of the individual agents. The expression of the androgen receptor (AR) protein was significantly increased by the single treatment of resveratrol, but the high-dose combination of genistein and resveratrol exhibited no increase in the expression of the AR protein. These findings suggest a potential opposing mechanism exists between genistein and resveratrol in regulating the expression of the AR protein. Cancer chemopreventive effects on androgens and their products have also been observed in the combination of soy and tea. A soy phytochemical concentrate (SPC) was used in combination with tea infusions of black or green tea as the sole source of drinking water in severe combined immunodeficiency (SCID) male mice inoculated with LNCaP human prostate cancer cells (Zhou, Yu et al. 2003). The SPC plus tea combinations were more effective in the reduction of tumor weight and metastasis to lymph nodes than individual treatments. Of particular interest, green tea alone elevated serum levels of DHT, whereas the SPC plus green tea combination significantly reduced serum levels of DHT, thereby suggesting a modulatory role of the androgen system as one of the mechanisms responsible for the combinatorial anticarcinogenic effects between SPC and green tea.

Table 1.2 Studies examining the effects of the combination of dietary bioactive components in prostate cancer

Combination Treatment	Model System Mechanism/Effect	References
Genistein, Quercetin, Biochanin A	PC-3, LNCaP, DU-145 prostate cancer cells, ↑bax, ↑caspase-3, ↓bcl-2, ↑ER-β, ↑p-JNK, ↓p-ERK, ↓PCNA, ↓cell proliferation ↑apoptosis	Kumar <i>et al</i>
Genistein Selenium	PC-3 and LNCaP prostate cancer cells, ↓MMP-2, ↑apoptosis, ↓cell growth	Kumi-Diaka <i>et al</i>
Resveratrol β-sitosterol	PC-3 prostate cancer cells, ↓ROS, ↓prostaglandin, ↓cell growth	Awad <i>et al</i>
Epigallocatechin gallate, Genistein, Quercetin	CWR22Rv1 prostate cancer cells, ↑p53, ↓cell proliferation, ↑NQO2, ↑AR	Hsieh <i>et al</i>
Quercetin Epigallocatechin gallate	CD44+ and CD133+ cancer stem cells isolated from PC-3, LNCaP and prostate cancer tumors ↑apoptosis, ↓cell proliferation, ↓cell viability, ↓colony formation, ↓migration, ↓invasion	Tang <i>et al</i>
Thearubigin Genistein	PC-3 prostate cancer cells, ↓cell growth, ↑G2/M phase cell cycle arrest	Sakamoto <i>et al</i>
Pomegranate juice polyphenols, seed oil, peel polyphenols*	DU 145 and PC-3 prostate cancer cells, ↓cell proliferation, ↓PLA2, ↓invasion	Lansky <i>et al</i>
Ellagic acid Urolithin A*	PC-3 prostate cancer cells, ↓cell proliferation	Vicinanza <i>et al</i>
Ginger Biophenolics*	PC-3 prostate cancer cells, ↓cell proliferation	Brahmbhatt <i>et al</i>
Daidzein Genistein	LNCaP and C4-2B prostate cancer cells, ↓cell proliferation, ↑apoptosis	Dong <i>et al</i>
Whole soy extract Daidzein, Genistein	PC-3 and LnCap prostate cancer cells, ↑apoptosis, ↑ sub-G1 cell population	Hsu <i>et al</i>

Table 1.2 Continued

Combination Treatment	Model System Mechanism/Effect	References
Isoflavones Selenium	Noble rats hormone induced prostate Cancer, 5alpha-reductase activity	Legg <i>et al</i>
Selenium Isoflavones	Noble rats hormone induced prostate cancer, ↓IGF-1, ↑leptin, ↓weight	Tolman <i>et al</i>
Soy phytochemical extract(SPC), green and black tea	SCID mice, ↓dihydrotestosterone, ↓tumor weight, ↓metastasis to lymph nodes	Zhou <i>et al</i>
Green Tea Soy	Noble rats implanted with estradiol and testosterone, ↓p50, ↓IL-6, ↓IL-1β, ↓TNF-alpha, ↓infiltrating inflammatory cells	Hsu <i>et al</i>
Tomato Broccoli	Copenhagen rats Dunning R3327-H, ↓tumor weight, ↓tumor growth	Canene-Adams <i>et al</i>
Tomato Powder Soy Germ	Mouse prostate TRAMP model, No significant effects as compared to individual components	Zuniga <i>et al</i>
Lycopene, Vitamin E, Selenium	Copenhagen rats Dunning R3327-H, No significant effects as compared to individual components	Lindshield <i>et al</i>
Lycopene, Vitamin E Selenium	Lady transgenic mice model, ↓PCNA, ↑apoptosis, ↓prostate cancer incidence	Venkateswaran <i>et al</i>
Lycopene Soy Isoflavones	Phase II clinical trial men w/prostate cancer, Opposing effects on PSA stabilization	Vaishampayan <i>et al</i>
Lycopene Lutein	AT3 rat prostate cancer cells, No significant effects as compared to individual components	Gunasekera <i>et al</i>
Genistein Resveratrol	SV-40 Tag rats, opposing effects on AR receptor ↓cell proliferation	Harper <i>et al</i>

*This study used the Combination Index (CI) to determine synergism

A substantial amount of research suggests soy isoflavones exhibit the ability to modulate multiple pathways involved in the pathology of prostate cancer. Genistein was shown to demonstrate strong combinatorial effects with different agents in the downregulation of cell proliferation and induction of apoptosis in both hormone-dependent and –independent prostate cancer cell lines. For example, genistein and selenium in combination significantly decreased cell proliferation and increased apoptosis and caspase-3 activity as compared to individual treatments in PC-3 (hormone-independent) and LNCaP (hormone-dependent) prostate cancer cells, thus implying the effects of the genistein plus selenium combination are not hormone dependent within these cell lines (Kumi-Diaka, Merchant et al. 2010). The interaction of genistein plus quercetin plus biochanin A was studied in LNCaP, DU-145, and PC-3 prostate cancer cells (Kumar, Verma et al. 2011). The triple combination was found to be more efficacious in the inhibition of cell proliferation in both estrogen receptor (ER)-dependent and –independent pathways than each component alone. Further mechanistic investigation in PC-3 cells demonstrated that the combination treatment of genistein plus quercetin plus biochanin A induced apoptosis, increased the activation of caspase-3, and up-regulated the expression of the ER-B gene (a gene important for the regulation of tumor repressor activity in prostate cancer). Indeed, the triple combination was more effective than individual or double combinations in modulating some of the key regulatory proteins, including BAX, bcl-2, pJNK, pERK-1/2, and PCNA in PC-3 prostate cancer cells.

1.5.2 Tea Components

Other studies point to the increased anticarcinogenic effects that occur when quercetin or genistein are combined with tea components. The combination of quercetin and EGCG was examined in prostate cancer stem cells and was found to produce pronounced inhibitory effects on cell viability, colony formation, metastasis, and cell invasion (Tang, Singh et al. 2010). Likewise, combined treatment of thearubigin (a tea component) plus genistein in PC-3 prostate cancer cells resulted in a substantial decrease in cell growth, and this inhibitory effect was stronger than that produced by thearubigin or genistein alone (Sakamoto 2000). Different mechanisms of action by thearubigin and genistein in the regulation of the G2/M cell cycle phase were proposed to be responsible for the observed suppression of cell proliferation. A triple combination of genistein plus EGCG plus quercetin was found to produce an enhanced inhibition of cell proliferation in CWR22Rv1 prostate cancer cells, a model reflective of the transition from androgen dependence to hormone refractory prostate cancer (Hsieh and Wu 2009). Although the known mechanisms of the individual components within a combination can complement each other and result in a potentiated effect, a new mechanism that is not evident to the individual components within the combination can also be revealed from the combination treatment. For example, in the Noble rat model of prostate carcinogenesis, the combination of soy and green tea produced an anti-inflammatory interaction that is not apparent for soy or green tea individually (Hsu, Bruno et al. 2011).

1.5.3 Multiple Component Interaction within the Same Food

The results from studies where components from the same food are subjected to investigation must be interpreted carefully. For example, fermented pomegranate juice polyphenols, pomegranate peel polyphenols, and the pomegranate seed oil were analyzed to determine their effects individually and in combination on the proliferation of DU-145 prostate cancer cells (Lansky, Jiang et al. 2005). It was found that both the oil plus juice and peel plus juice combinations exhibited synergistic effects on diminishing proliferation of the cancer cells. However, the pomegranate juice and peel contained many of the same compounds. Therefore, the combination effects produced by the peel plus juice combination could be merely a dose response rather than a meaningful interaction. However, research findings continue to show that multiple dietary components from the same food in combination cause greater anticarcinogenic effects than individual components. For example, the antiproliferative effects of the pomegranate metabolites ellagic acid and urolithin A in combination were determined in PC-3 prostate cancer cells, and the combined treatment was found to result in a marked synergistic decrease in cell proliferation (Vicinanza, Zhang et al. 2013). Similarly, the combination of ginger biphenolic compounds exhibits a strong synergism of antiproliferative effects in PC-3 prostate cancer cells (Brahmbhatt, Gundala et al. 2013). Furthermore, combined treatment of soy isoflavones (genistein plus daidzein) was found to be more effective in the induction of apoptosis and suppression of proliferation than individual isoflavones in a prostate cancer progression model (Dong, Xu et al. 2013).

1.5.4 Whole Foods

Recent studies examining whole foods have revealed enhanced anticarcinogenic effects that are attributed to the combination of multiple components present in the whole food or several foods combined (Basu and Imrhan 2007). Some studies have reported inhibition of prostate carcinogenesis by lycopene, the bioactive compound found in tomatoes (Siler, Barella et al. 2004). However, other studies have found that the whole tomato was more efficacious. Indeed, whole tomato powder at 10% of the diet significantly decreased tumor weight in a Dunning R3327-H prostate adenocarcinoma model, whereas lycopene as part of the diet at a very high dose did not exhibit any significant effect in decreasing tumor weight. Whole broccoli powder at 10% of the diet significantly decreased tumor weight. Most interestingly, the combination of whole tomato powder plus broccoli powder had an even greater effect on diminished tumor weight (Canene-Adams, Lindshield et al. 2007). In another study, the combination of whole tomato powder and soy germ was effective in diminishing prostate cancer incidence in the mouse prostate TRAMP model, but the inhibitory effects were not significantly different from those produced by each individual treatment (Zuniga, Clinton et al. 2013). Notably, whole soy extract significantly increased apoptosis in LnCap and PC-3 prostate cancer cells as compared to single treatment of individual soy isoflavones, genistein and daidzein.

1.5.5 Clinical Outcome vs Preclinical Findings

Recent prostate cancer clinical trials utilizing combinations warrant caution when extrapolating preclinical findings to a human population. A Phase II clinical trial

investigated the consumption of lycopene alone and in combination with soy isoflavones to determine their efficacy in the modulation of rising serum prostate-specific antigen (PSA) levels of 71 men with prostate cancer (Vaishampayan, Hussain et al. 2007). Treatment groups were randomized with 38 subjects receiving 15 mg of lycopene alone and 33 subjects receiving 15 mg of lycopene plus 40 mg of a soy isoflavone mixture twice daily for six months. Surprisingly, PSA stabilization for a minimum of three months occurred for 95% of the lycopene group but occurred for only 67% of the combination group. These results may suggest an antagonistic effect between lycopene and soy isoflavone mixtures in stabilizing PSA. The Selenium and Vitamin E Cancer and Prevention Trial (SELECT) examined the efficacy of the combination of selenium and vitamin E for prostate cancer prevention (Klein, Thompson et al. 2011). Although preclinical studies of prostate cancer have reported anticarcinogenic effects of vitamin E and selenium alone and in combination, the clinical outcome has proven to be quite different as vitamin E was found to increase the occurrence of prostate cancer when used alone and to a lesser degree in combination with selenium (Venkateswaran, Fleshner et al. 2004; Reagan-Shaw, Nihal et al. 2008). In agreement with these clinical findings, an animal study using the Lady transgenic model indicated that the combination of vitamin E and selenium was not chemopreventive but rather causative of an increased occurrence of prostate cancer (Venkateswaran, Klotz et al. 2009). Interestingly, when lycopene was added to the combination of vitamin E and selenium, the results were quite different such that the triple combination treatment significantly decreased the occurrence of prostate cancer in mice. However, the same triple combination of vitamin E plus selenium plus lycopene showed no inhibitory effect in male Copenhagen rats transplanted with

androgen-dependent Dunning R3327-H rat prostate adenocarcinomas (Lindshield, Ford et al. 2010). These conflicting results exemplify how an accumulation of cellular and animal findings is not always indicative of clinical outcome.

1.6 Breast Cancer

Table 1.3 summarizes the major studies on breast cancer and the chemopreventive effects of combination treatments with dietary components.

Epidemiological studies suggest certain foods commonly consumed in the Asian diet may be protective against the development of breast cancer (Butler, Wu et al. 2010).

1.6.1 Soy, Tea and their Components

Two foods that encompass a prominent position within the Asian diet that have been explored for their inhibitory effects on breast cancer are soy and tea. In one such study, a soy phytochemical concentrate (SPC) was used in combination with either green tea or black tea infusions as part of the diets of female SCID mice bearing implanted tumors of MCF-7 breast cancer cells (Zhou, Yu et al. 2004). The soy concentrate plus green tea combination was found to be more effective in tumor weight reduction as compared to the control. Potential mechanisms underlying the actions of the combination treatment were proposed to include downregulation of ER- α , reduction of serum IGF-1 levels, and inhibition of angiogenesis. Another study reinforced the effects of the combination on IGF-1 and ER- α by showing a substantial reduction in serum IGF-1 and estrogen concentration following a soy concentrate plus green tea combination treatment in FVB/N mice (Zhou, Li et al. 2007). When EGCG was added to the resveratrol plus y-

tocotrienol combination, it did not result in any increase in the inhibition of proliferation of MCF-7 breast cancer cells (Hsieh and Wu 2008). However, a pronounced increase was noted in the induction of quinone reductase NQO1 (a phase II enzyme) following exposure to the triple combination treatment of EGCG plus resveratrol plus γ -tocotrienol. Evaluation of the combination effects on the Rb/E2F protein complex, an important regulatory complex of the cell cycle progression, revealed greater inhibition of the Rb protein by each of the two component combinations tested than by individual treatment alone. Surprisingly, a diminished inhibitory effect on the Rb protein occurred following the exposure to the triple combination of EGCG plus resveratrol plus γ -tocotrienol in the breast cancer cells. These findings highlighted the complexity that is involved when combining multiple components for cancer inhibition and underscored the need for more combination studies to evaluate the variable interactions that can exist between different dietary bioactive components.

Soy isoflavones have been investigated for their use in combination treatments to determine their ability as phytoestrogens to suppress breast cancer through the regulation of the ERs and estrogen-responsive genes (Willard and Frawley 1998). The effects of genistein and indole-3-carbinol in combination were studied on the expression of BRCA1 and BRCA2 in estrogen-responsive MCF-7 breast cancer cells (Fan, Meng et al. 2006). Both BRCA1 and BRCA2 have been identified as tumor suppressor genes for hormone-responsive breast cancer, and the absence or decrease in the expression of BRCA1 has been observed in a significant proportion of sporadic breast cancers, suggesting its protective role against breast carcinogenesis. Combination treatment of genistein and

Table 1.3 Studies examining the effects of the combination of dietary bioactive components on breast cancer

Combination Treatment	Model System Mechanism/Effect	References
Eicosapentaenoic acid Genistein*	MDA-MB-231 and MCF-7 breast cancer cells, ↓cell proliferation	Nakagawa <i>et al</i>
Epigallocatechin, Resveratrol, γ-tocotrienol	MCF-7 breast cancer cells, ↑NQO1, opposing effects on Rb/E2F protein	Hsieh <i>et al</i>
Indole-3-carbinol genistein	MCF-7 breast cancer cells, ↑BRCA1, ↑BRCA2	Fan <i>et al</i>
Genistein,3,3'-diindolylmethane (DIM)	MDA-MB-231 breast cancer cells, ↓CXCR4, ↓chemoinvasion	Hsu <i>et al</i>
Pomegranate extract Genistein	MCF-7 breast cancer cells, ↓cell growth↑apoptosis	Jeune <i>et al</i>
Curcumin Genistein	MCF-7 breast cancer cells, ↓cell proliferation	Verma <i>et al</i>
Quercetin 3-β-D-glucoside (Q3G), Apple extract*	MCF-7 breast cancer cells, ↑Q3G stabilization, ↑bioavailability, ↓cell proliferation	Yang <i>et al</i>
Docosahexaenoic acid Curcumin*	SK-BR-3 breast cancer cells, ↑bioavailability, ↓cell proliferation, ↑p-53, ↑PPARγ	Altenburg <i>et al</i>
Soy phytochemical concentrate (SPC), Black tea, Green tea	SCID mice model of MCF-7 tumor growth, ↓tumor weight, ↓estrogen receptor-α, ↓serum insulin-like growth factor (IGF)-I	Zhou <i>et al</i>
Soy, Green Tea, Black Tea components	FVB/N mice, ↓serum estrogen, ↓serum insulin-like growth factor (IGF-I)	Zhou <i>et al</i>
Diallyl sulfide, ellagic acid, S-methylselenocysteine, Selenomethionine, Quercetin	Dimethylbenz[a]anthracene (DMBA) treated Sprague-Dawley, ↓tumor incidence	Ip <i>et al</i>

Table 1.3 Continued

Combination Treatment	Model System Mechanism/Effect	References
Flaxseed Soy protein	Ovariectomized athymic nude mice with MCF-7 tumor, ↓tumor growth, ↓pMAPK, ↓IGF-1Rβ	Power <i>et al</i>
Soy protein Fish oil	Sprague-Dawley rats, ↑apoptosis	Kramer <i>et al</i>
Genistein Daidzein	Nude mice model of green fluorescent protein tagged (GFP) MDA-MB-435 tumors, ↑metastasis to lung, liver, heart, kidney, Bones	Martinez-Montemayor <i>et al</i>
Flaxseed Soy protein	Ovariectomized athymic nude mice with MCF-7 tumor, ↓cell proliferation, ↓tumor growth, ↑apoptosis	Saarinen <i>et al</i>

*This study used the Combination Index (CI) to determine synergism

indole-3-carbinol in MCF-7 breast cancer cells resulted in a significant increase of the levels of BRCA1 and BRCA2 protein, and these effects were greater than those produced by genistein or indole-3-carbinol treatment alone. These pronounced chemopreventive effects were speculated to be initiated by the activation of the endoplasmic reticulum stress response signaling pathway, thereby leading to the expression of downstream target genes by genistein and DIM, a metabolite of indole-3-carbinol. Interestingly, the combination treatment of genistein and DIM also demonstrated anticarcinogenic effects in estrogen-negative MDA-MB-231 breast cancer cells (Hsu, Chen et al. 2009). Genistein and DIM in combination were efficacious in inhibiting the chemoinvasion of cells toward CXCL12, a chemokine receptor and ligand necessary for the metastasis of breast cancer. ER-positive MCF-7 breast cancer cells were found to be highly responsive to the

combination treatment of genistein plus pomegranate extract that led to stronger inhibition of cell growth and induction of apoptosis as compared to individual components (Jeune, Kumi-Diaka et al. 2005). Genistein and curcumin in combination exhibited a strong inhibitory effect on cell proliferation in MCF-7 breast cancer cells following 17-B estradiol and estrogenic pesticide exposure (Verma, Salamone et al. 1997). The combination treatment of genistein plus EPA has been reported to synergistically inhibit the growth of both MCF-7 and MDA-MB-231 breast cancer cells (Nakagawa, Yamamoto et al. 2000). The MCF-7 cells showed a greater sensitivity to the combination of genistein and EPA than the MDA-MB-231 cells. This variation in response appeared to be independent of ER status, thereby suggesting different mechanisms at work in ER-positive and ER-negative breast cancer cells.

1.6.2 Increased Bioavailability and Cellular Uptake

Many of the synergistic effects observed in the combination treatments have been attributed to complementary mechanisms, some of which suggest increased bioavailability as a possible mechanism for enhanced anticarcinogenic effects. Apple extract and quercetin 3-B-glucoside (Q3G) in combination synergistically decreased cell proliferation of MCF-7 breast cancer cells (Yang and Liu 2009). The synergistic antiproliferative effects of the combination were speculated to entail mechanisms associated with stabilization and increased bioavailability of Q3G. These findings are of particular interest in the use of compounds with poor bioavailability such as curcumin, as only a very small fraction of ingested curcumin are absorbed in humans; indeed, only a low micromolar concentration is present in the bloodstream from the ingestion of several

grams of curcumin (Garcea, Berry et al. 2005). For example, curcumin in combination with DHA was found to synergistically inhibit cell proliferation of ER-negative HER-2 positive SK-BR-3 breast cancer cells (Altenburg, Bieberich et al. 2011). A combination treatment of curcumin plus DHA in SK-BR-3 cells increased phosphorylation of p53 and expression of PPAR γ more than each compound alone. In the presence of DHA, the cellular uptake of curcumin in SK-BR-3 cells was greatly increased, perhaps through changes in the lipid membrane by DHA. This increased cellular uptake of curcumin within the SK-BR-3 cells could be a key mechanism for the enhanced anticarcinogenic effects of the curcumin plus DHA combination. Indeed, this combination could have important clinical implications. It utilized a targeted treatment strategy to offer enhanced bioavailability of anticancer agents within a particular phenotype of cancer cells, which in turn led to a greater anticancer efficacy.

1.6.3 Combinatorial Mechanisms Offset Negative Effects

Dietary bioactive components may work as chemopreventive agents at different stages of carcinogenesis, and the combination of these components may produce stronger inhibition on carcinogenesis. Three different combination treatments were studied in a dimethylbenz[a]anthracene (DMBA)-induced mammary tumor model in Sprague-Dawley rats, including diallyl sulfide plus Se-methylselenocysteine, ellagic acid plus selenomethionine, and diallyl sulfide plus quercetin (Ip and Ganther 1991). Selection criteria for the combination were based on the inclusion of one compound effective prior to carcinogen exposure and another compound effective post initiation; the individual compound was given either before or after DMBA exposure, based on the mode of

action. Each of the three combinations achieved a greater degree of tumor suppression than individual compounds. Indeed, only the combination treatment significantly decreased both tumor incidence and number as compared to the control. The combination of flaxseed and soy, which was based on the premise that flaxseed may modulate the effects of soy to offset the negative effects attributed to the ingestion of soy by at-risk populations, provides a recent example of a complementary combinatorial mechanism of dietary components (Power and Thompson 2007). Although soy isoflavones have been touted for their many health benefits, accumulating data point to the potential adverse effects of these compounds for postmenopausal women and those with existing breast cancer (Martinez-Montemayor, Otero-Franqui et al. 2010). Flaxseed in combination with soy protein has been shown to reduce the tumor-stimulating effects of soy in ovariectomized athymic mice (Saarinen, Power et al. 2006; Power, Chen et al. 2008). Therefore, this combinatorial interaction could be beneficial in allowing women at increased risk of breast cancer to ingest soy with flaxseed, thereby obtaining the health benefits of soy without the added risk. Along this line of findings, a complementary role of fish oil was identified in another study when it was combined with soy protein and found to offset the overexpression of ER- α , thus diminishing the potential negative tumorigenic effects of soy protein (Kramer, Johnson et al. 2009).

1.7 Lung Cancer

Lung cancer is the most commonly occurring cancer; however, few combination studies have been conducted to investigate multiple dietary bioactive components and their influence on lung carcinogenesis. Tea components in combination with other

compounds have been studied and have exhibited enhanced chemopreventive effects. Lung cancer cell lines expressing wild-type p53 were found to be more sensitive than cell lines not expressing p53 to the combination treatment of EGCG plus luteolin (Amin, Wang et al. 2010). Notably, the combination of EGCG plus luteolin induced extensive apoptosis in lung cancer cells expressing wild-type p53, and these effects were stronger than those produced by EGCG or luteolin alone. Indeed, knockdown of p53 expression in lung cancer cells resulted in a great inhibition of apoptosis induced by the combination treatment, thereby suggesting a p53-dependent mechanism for inducing apoptosis by the combination. Importantly, the efficacy of the EGCG plus luteolin combination was further confirmed in vivo in a xenograft tumor model in nude mice that showed a significant inhibition of tumor growth by the combination treatment. Another tea polyphenol, (-)-epicatechin (EC), was combined with curcumin in the treatment of PC-9 and A549 lung cancer cells (Saha, Kuzuhara et al. 2010). EC alone had no suppressive effect on either cell line in the test concentrations. However, EC in combination with curcumin exhibited approximately four times greater suppression of the growth of PC-9 cancer cells than curcumin alone. Similar results were observed in A549 cells. The enhanced anticancer effects were ascribed to the modulation of MAPK signaling by the combination treatment. Theaflavin-3-3'-digallate plus ascorbic acid and EGCG plus ascorbic acid, both combination treatments using tea polyphenols, were found to exhibit synergism in the inhibition of cell proliferation of SPC-A-1 lung cancer cells (Li, Wu et al. 2010). Cell cycle arrest at G0/G1 phase is responsible for the synergistic effects. Chemopreventive effects of green tea polyphenols have been demonstrated in many studies, but these beneficial effects were limited due to poor bioavailability of tea

polyphenols. One factor contributing to the poor bioavailability of tea polyphenols is that they undergo extensive biotransformation such as methylation, which may decrease their bioactivities. Interestingly, in the presence of quercetin, methylation of EGCG was significantly reduced in cancer cells. Consequently, the combination of quercetin and EGCG produced enhanced inhibitory effects on proliferation of lung cancer cells (Wang, Heber et al. 2012). To confirm these findings in vivo, SCID mice were given brewed green tea and quercetin alone or in combination for two weeks. In agreement with the in vitro results, the quercetin treatment greatly reduced the amount of methylated EGCG in the lung and kidney of mice. Indole-3-carbinol and silibinin, a flavonoid of milk thistle, have been used in combination to inhibit lung cancer, both in vitro and in vivo (Dagne, Melkamu et al. 2011). Low concentrations of indole-3-carbinol and silibinin in combination exhibited much stronger inhibitory effects on lung cancer cells than those produced by the individual compounds at higher concentrations. The inhibitory effects by the combination were associated with inactivation of Akt and ERK. These findings were confirmed in A/J mice where combination treatment of indole-3-carbinol and silibinin significantly inhibited lung carcinogenesis and downregulated Akt and ERK pathways. Moreover, the effects produced by the combination treatment were stronger than those of individual compounds.

1.8 Other Cancers

As discussed above, combination treatments with dietary bioactive components have demonstrated enhanced anticarcinogenic effects in colon, prostate, breast, and lung cancer models, but few studies have examined the efficacy of these combinations in less

frequently occurring cancers. Some of the key dietary components used in aforementioned combination studies appear to exhibit similar chemopreventive actions in other less common cancers.

1.8.1 Curcumin, Soy Isoflavone and Tea Components

Curcumin in combination with carnosic acid displayed a strong synergism in inhibiting acute myeloid leukemia cells accompanied by enhanced activation of apoptotic pathways (Pesakhov, Khanin et al. 2010). Combination treatment of curcumin plus isoflavone synergistically inhibited cell growth of BxPC-3 pancreatic cancer cells (Wang, Desmoulin et al. 2008). Furthermore, the combination treatment produced stronger inhibition on Notch 1 and NF-kB-mediated pathways than that attained by either compound alone.

EGCG in combination with curcumin showed pronounced synergism in an oral cancer cell model and exhibited underlying mechanisms involving G1 and G2 cell cycle arrest. The combination treatment of tea catechins EC plus EGCG induced apoptosis more than twofold that of the individual compounds in gastric carcinoma cells, suggesting a synergistic interaction as EC alone had almost no effect on apoptosis (Horie, Hirabayashi et al. 2005). Additionally, the combination of black tea polyphenol and resveratrol caused an enhanced inhibition of tumor incidence, tumor size, and tumor number in a two-stage mouse skin carcinogenesis model.

1.8.2 Resveratrol and Glucosinolates

Resveratrol was studied in combination with polyphenolic compounds ellagic acid and quercetin in MOLT-4 leukemia cells (Mertens-Talcott and Percival 2005). The resveratrol plus quercetin combination resulted in a greater-than-additive effect in inducing apoptosis and less-than-additive effect in decreasing cell proliferation; the resveratrol plus ellagic acid combination, however, produced a greater-than-additive effect in both inducing apoptosis and decreasing cell proliferation. Examination of resveratrol in combination with indole-3 carbinol in SK-OV-3 ovarian cancer cells revealed a significant increase of G1 and G2 arrest by the combination treatment (Raj, Abd Elmageed et al. 2008). Enhanced proapoptotic effects were produced by the combination of resveratrol with sulforaphane in human U251 glioma brain cancer cells, which was accompanied by a pronounced decrease of Akt activation and an increase of caspase-3 activation.

In PANC-1 pancreatic cancer cells, sulforaphane plus benzyl isothiocyanate combination treatments produced a potent inhibition of cell viability that was associated with inactivation of STAT3 and increase of apoptosis (Hutzen, Willis et al. 2009). Substantial reductions in the growth of mouse melanoma (B16F10) cells in vitro and in vivo with diminished MMP-9 expression were observed following a combination treatment of sulforaphane plus quercetin. The combination treatment showed strong inhibitory effects on melanoma both in vitro and in vivo, and these effects were stronger than those of each individual treatment (Pradhan, Mishra et al. 2010).

1.8.3 Garlic Components

Combinations of S-allylcysteine (an organosulfur component in garlic) and lycopene in vivo produced a greater induction of apoptosis in gastric cancer of Wistar rats in comparison to individual treatments (Velmurugan, Mani et al. 2005). Another garlic compound, diallyl sulfide, in combination with pomegranate fruit extract demonstrated a marked inhibition of a two-stage skin carcinogenesis in a mouse model (George, Singh et al. 2011). Combined treatment led to a significant regression in tumor volume; however, no effect was found on tumor number.

1.9 Concluding Remarks and Future Directions

Accumulating studies have demonstrated the potential of combination regimens with dietary bioactive components as a promising strategy for cancer chemoprevention. However, much remains to be explored for establishing a better understanding of the mechanisms of action of different dietary components in combination. Important information on the efficacy of combination regimens in humans is currently lacking. Additionally, more retrospective and prospective epidemiological studies should be conducted to determine the combinatorial effects of dietary bioactive components in inhibiting carcinogenesis of different organs. The promising leads identified in the epidemiological studies can be further validated in animal carcinogenesis models, especially for whole foods and their crude fractions; in vitro models such as cell culture assays offer very limited ability to account for the bioavailability and biotransformation of dietary components in humans. For example, results obtained with dietary compounds at concentrations too high to be achievable in human blood or specific tissues have a low

probability of being realized in humans. Ultimately, randomized, double-blind, placebo-controlled trials are needed to clearly demonstrate the efficacy of the combinations of dietary bioactive components.

Currently, only a small fraction of existing studies employ combination index-based statistical methods to design combination studies and to determine the modes of interaction among different dietary bioactive components (**Tables 1.1-1.3**). This is far from adequate to provide important information that can be conveniently compared and analyzed to establish efficacy and mechanisms of action of specific combination regimens. Therefore, standardization of protocols for combination studies is essential. Use of the same statistical method such as the combination index-based method provides a consistent interpretation of interactions allowing meaningful cross-examination of results from different laboratories.

Although numerous studies show that complementary mechanisms of individual dietary components can result in enhanced anticarcinogenic effects when used in combination, caution is warranted as certain dietary component combinations can be antagonistic with diminished anti-cancer effects and in some instances can even act as a promoter of carcinogenesis. In these cases, it is critical to understand how these components interact within the particular combinations so that recommendations can be made to avoid potential adverse effects. These types of recommendations are of particular importance to consumers taking dietary supplements, as many bioactive components are already combined in dietary supplement products, which is further complicated by consumers' tendency to consume multiple supplements at the same time.

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CHAPTER 2

NON-SYNERGISTIC ANTI-PROLIFERATIVE EFFECTS OF SELECTED FLAVONOIDS AND FLAVONOID METABOLITE IN COMBINATION FOR CHEMOPREVENTION OF COLORECTAL CANCER

2.1 Abstract

Flavonoids and their metabolites have been highlighted for their anti-carcinogenic and anti-inflammatory properties in colorectal cancer animal and cellular models, however many of their effects in combination have yet to be explored. Herein, we examine the anti-proliferative effects of two structurally similar flavones, luteolin or apigenin (differing only in hydroxylation at 3' of the B-ring) in combination with 3',4'-didemethylnobiletin (DDMN), a primary metabolite of nobiletin in the mouse. The individual anti-proliferative order of potency in HCT116 colon cancer cells was found to be apigenin > luteolin > DDMN. Our findings suggest the methoxylated A-ring on the DDMN structure is associated with a lower anti-proliferative effect than observed in luteolin which differs structurally only in the A-ring containing a hydroxyl group at the 5- and 7-position with no methoxyl group. Moreover, results indicate that apigenin and DDMN are additive when combined in a 1:1 ratio in HCT116 colon cancer cells at 1.5, 3, 4.5, 6 and 7.5 μM with no apparent interaction; whereas luteolin in combination with DDMN in a 1:1 ratio at 1.5, 3, 4.5, 6 and 7.5 μM exhibits an antagonistic response with diminished anti-proliferative effects in a dose dependent manner. Therefore, these findings suggest the presence of the 3' hydroxyl group on the B-ring of luteolin is associated with decreased inhibition of cellular growth, as compared to apigenin, following combination treatment with DDMN in HCT116 colon cancer cells. Taken

together, our research demonstrates for the first time the antagonistic anti-proliferative effects of luteolin and DDMN in combination in HCT116 colon cancer cells.

2.2 Keywords

Flavonoids, diet-based strategies, combination, cancer chemoprevention, colorectal cancer, flavonoid metabolites, anti-proliferative, antagonistic, additive, non-synergistic, flavones, secondary plant metabolites, interaction, flavonoid structure

2.3 Abbreviations

DDMN: 3',4'-didemethylnobiletin; ACF: aberrant crypt foci; PMF: polymethoxyflavone; AOM: azoxymethane; DMH: dimethylhydrazine

2.4 Introduction

Colorectal cancer is the third most commonly occurring cancer in the United States currently diagnosed in both men and women (Siegel, Miller et al. 2017). Rates of incidence and mortality have continued to decline in the 50-65+ age group; some of these changes have been attributed to decreased consumption of red meat, increased use of daily aspirin and the avoidance of smoking. Advances in colon cancer prognosis have also been made in recent years through early diagnosis. However, troubling statistics indicate that incidence and mortality rates have been increasing in younger adults age <50 by 22% (2000 to 2013) and 13% (2000-2014) respectively; more research is needed to better understand the mechanisms involved and to identify efficacious treatments that

can be used for extended periods of time with no toxicity, to prevent or halt the carcinogenic process.

Diet-based strategies for the chemoprevention of colorectal cancer are of particular interest due to their low toxicity and anti-cancer effects. In addition, dietary components come into direct contact with the colon, avoiding many of the bioavailability challenges often encountered in other cancers with diminished delivery of the active components into the bloodstream and to the target organ of interest. Therefore, the dietary components direct contact with the colon is likely to result in more of the parent compound and its active metabolites successfully reaching the colon for their therapeutic effects (Sak 2014; Ullah, Bhat et al. 2016).

Flavonoids are secondary plant metabolites, a group of polyphenolic compounds with anti-carcinogenic, anti-inflammatory and antioxidant properties (Pan, Lai et al. 2010; Gonzalez, Ballester et al. 2011). An abundance of flavonoids can be found in many fruits, vegetables, herbs and tea (Hoensch and Oertel 2011). Many anti-cancer effects of flavonoids have been identified in cellular colorectal cancer model systems (Kuo 1996). Further investigation in animal models suggest flavonoids may play a role in the inhibition of aberrant crypt foci (ACF) and colonic polyps, both of which can be precursors of colorectal cancer (Miyamoto, Yasui et al. 2010). Clinical and epidemiological evidence has been mixed, with a number of studies suggesting a decrease in colorectal cancer risk is associated with flavonoid consumption, whereas some studies find no significant correlation exists (Theodoratou, Kyle et al. 2007; Bobe, Sansbury et al. 2008; Nimptsch, Zhang et al. 2016). Remarkably, other clinical findings

have shown flavonoids can decrease the recurrence of colon cancer tumors after surgical removal (Hoensch, Groh et al. 2008).

More than 4000 flavonoids have been identified and are categorized based on their structure. The flavonoid basic structure consists of two benzene rings (A and B) connected with three carbons in an oxygenated heterocycle ring (C) (Liu 2004). It is variations on this C ring that determines the organization of the flavonoids into six different subgroups most commonly found in the diet as flavones, flavonols, flavanols, flavanones, anthocyanidins and isoflavones (Beecher 2003). Notably, of these flavonoid subgroups the flavones (luteolin, apigenin and nobiletin) have shown significant anti-cancer effects in animal and cellular models of colorectal cancer (Figure 2.1).

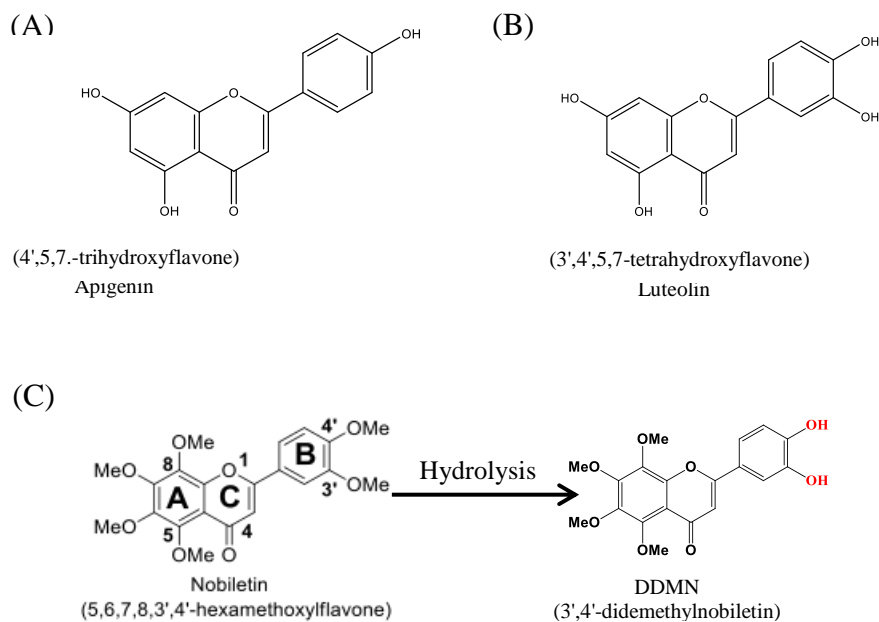


Figure 2.1. Chemical structures of (A) apigenin, (B) luteolin and (C) DDMN (3',4'-didemethylnobiletin)

Luteolin is found in celery, parsley, apple skin, cabbage, carrots, broccoli and green peppers. *In vitro* studies suggest luteolin can operate its effects in multiple cell signaling pathways associated with colorectal cancer. Researchers using a cellular model of intestinal inflammation found luteolin treatment to exhibit anti-inflammatory effects through the inhibition of the JAK/STAT pathway (Nunes, Almeida et al. 2017). Luteolin treatment in HT29 colon cancer cells initiates the induction of apoptosis and cell cycle arrest accompanied by a decrease in insulin-like growth factor-1 receptor (IGF-1R) signaling that leads to a reduction in activation of the PI3K/Akt and ERK1/2 pathways (Lim, Cho et al. 2012). Chemotherapeutic resistant colon cancer cell lines treated with luteolin were found to have increased sensitivity to oxaliplatin's anticancer effects; these results appear to be correlated with inhibition of the Nrf2 pathway and HO-1 expression (Chian, Li et al. 2014). Aberrant crypt foci (ACF) are one of the first signs of carcinogenic change that occurs in the colonic mucosa followed by the formation of colonic polyps which potentially can lead to the development of colon cancer. Luteolin appears to operate in both the initiation and promotion stage of the colorectal carcinogenic process. Treatment with luteolin significantly decreased the occurrence of aberrant crypt foci (ACF) in azoxymethane (AOM) induced colon carcinogenesis in mice (Ashokkumar and Sudhandiran 2008). In studies utilizing the dimethylhydrazine (DMH)-induced animal model of colon carcinogenesis in rats, luteolin treatment significantly decreased colon cancer incidence, number of colonic polyps and tumors, both in number and size (Manju and Nalini 2007; Osman, Said et al. 2015).

Apigenin, a flavone found in chamomile, grapefruit, onions, celery and parsley has been credited for its' powerful anti-carcinogenic and anti-tumor effects (Shukla and

Gupta 2010). Moreover, apigenin has been shown to favorably modulate cell cycle-related signaling proteins such as p53 and p21; both important for the regulation of cell cycle progression in colorectal carcinogenesis. Apigenin appears to stabilize p53 in normal cells but also displays anti-cancer effects in p53 mutant cells. A p53 independent mechanism is responsible for apigenin induced G2/M cell cycle arrest and upregulation of p21 expression in the p53 mutant colon cancer cell line HT29 (Takagaki, Sowa et al. 2005). Examination of HCT116 p53 wild-type colon cancer cells following apigenin treatment revealed a dose dependent suppression of cell proliferation with G2/M cell cycle arrest accompanied by increased expression of p53 and p21 (Lee, Sung et al. 2014). Additional investigation by Zhong *et al* in Apcmin+ mice, a colorectal cancer animal model, discovered apigenin treatment resulted in a decrease in polyp number and a concurrent increase in p53 activation (Zhong, Krisanapun et al. 2010). Other animal models of colon carcinogenesis have displayed similar positive effects with an inhibition of ACF in the distal region of the mouse colon and a decreased number of high multiplicity ACF observed following apigenin treatment in azoxymethane (AOM) induced CF-1 mice and AOM treated rats respectively (Au, Li et al. 2006; Leonardi, Vanamala et al. 2010).

Nobiletin a polymethoxyflavone (PMF), is found primarily in the peel of sweet (*C. sinensis*) and bitter (*C. aurantium*) orange. Accumulating evidence reveals nobiletin can operate in multiple signaling cascades associated with various disease processes to bring about positive effects (Luo, Guan et al. 2008; Lee, Cha et al. 2010; Huang, Li et al. 2016). In addition, studies indicate nobiletin is effective in the inhibition of colorectal cancer cell growth with G1 cell cycle arrest and upregulation of p21 (Morley, Ferguson et

al. 2007; Wu, Song et al. 2015). Results from preclinical studies using animal models confirm these cellular findings. In AOM /DSS treated mice expression of p21 and p53 were upregulated in colonic mucosa following nobiletin dietary treatment; these findings were associated with a significant reduction in incidence and multiplicity of colonic tumors (Wu, Song et al. 2015). Nobiletin dietary treatment resulted in the inhibition of ACF, decreased cell proliferation and expression of prostaglandin E₂ in the colonic mucosa of azoxymethane (AOM)-induced rat colon carcinogenesis (Kohno, Yoshitani et al. 2001; Suzuki, Kohno et al. 2004). Similar findings in F344 rats revealed nobiletin significantly reduced the number of colonic aberrant crypt foci (Tang, Ogawa et al. 2011).

Flavonoid metabolites are of great interest to researchers as they are present at higher concentrations than their parent compounds in both blood and colonic tissue; therefore it is likely the metabolites are responsible for many of the positive effects. Accumulating evidence appears to suggest flavonoid metabolites have different biological and antioxidant properties than their associated parent compound, often more potent in their response (Williamson and Clifford 2010; Lotito, Zhang et al. 2011). Nobiletin and its major metabolites as a mixture, in the same proportion as that found in the colon of mice fed nobiletin, upregulated the Nrf-2 pathway in RAW 264.7 cells and increased expression of p53 in HCT116 colon cancer cells (Wu, Song et al. 2017). Colonic concentration of nobiletin in the mouse is much lower than its three primary metabolites of which 3',4'-didemethylnobiletin (DDMN) has been identified as the metabolite with the strongest anti-proliferative effects in HCT116 colon cancer cells (Wu, Song et al. 2015). DDMN appears to inhibit multiple signaling pathways associated with

inflammation and carcinogenesis (Lai, Li et al. 2008; Su, Yen et al. 2012). Recent findings have shown 3',4'-didemethylnobiletin (DDMN), a primary metabolite of nobiletin in the mouse to have even stronger anti-inflammatory and anti-cancer effects than its parent compound nobiletin (Li, Sang et al. 2007; Lo, Pan et al. 2010; Su, Yen et al. 2012; Wu, Song et al. 2015). Indeed, anti-inflammatory properties of DDMN have been identified in RAW264.7 cells with DDMN exhibiting potent inhibition in the induction of iNOS, such that it greatly exceeds nobiletin's anti-inflammatory response (Li, Sang et al. 2007). DDMN initiates G2/M cell cycle arrest with an upregulation of p21 in HCT116 colon cancer cells; demonstrating stronger growth inhibition and induction of apoptosis than nobiletin in both HCT116 and HT29 cell lines (Wu, Song et al. 2015).

Combination of dietary components has shown many enhanced effects in various cancers (DiMarco-Crook and Xiao 2015). Multiple pathways dysregulated in the carcinogenic process warrants the use of several components with complementary mechanisms. Although many studies have examined the positive effects of individual flavonoids, few animal or clinical studies have investigated flavonoid to flavonoid combinations in colorectal cancer; however some positive findings have been reported (Tanaka, Makita et al. 1997; Hoensch, Groh et al. 2008). In a small clinical study of 29 patients with resected colon cancer and polyp removal half were given a combination of 20mg apigenin and 20mg epigallocatechin-gallate, (found in green tea) and matched to untreated controls. Interestingly, the untreated group had a 47% recurrence rate of colon cancer whereas the flavonoid treatment group had only a 7% recurrence. Synergistic anti-inflammatory and anti-oxidant effects have also been reported in RAW 264.7 cells

with a number of flavonoid to flavonoid combinations (Harris, Qian et al. 2006; Funaro, Wu et al. 2016; Phan 2017). Moreover, multiple colorectal cancer cell lines including HCT116, HT29, SW480 and Caco-2 cells have been used with flavonoid to flavonoid combination treatments and have demonstrated significant synergistic inhibition of cell proliferation (Wang, VanAlstyne et al. 2004; Lambert, Kwon et al. 2008; Gomez-Alonso 2012; Jaramillo-Carmona 2014). Remarkably, no studies have been done thus far to our knowledge using a combination treatment of a flavonoid to flavonoid metabolite to examine the inhibition of cell growth in colorectal cancer.

Based on these findings, the aim of this study is to examine the anti-proliferative effects in HCT116 colon cancer cells of two structurally related flavones, apigenin or luteolin in combination with 3'4'- didemethylnobiletin (DDMN), a primary metabolite of nobiletin in the mouse. Although apigenin, luteolin and DDMN are each effective in the inhibition of HCT116 colon cancer cells individually no information currently exists examining the potential synergy, additive or antagonistic effects of either flavone in combination with the metabolite DDMN. Therefore, for the first time this study distinguishes between the structurally related flavones apigenin or luteolin and their interaction with the flavone metabolite DDMN to determine their combinatory anti-proliferative effects in HCT116 colon cancer cells.

2.5 Materials and Methods

2.5.1 Cell Culture

HCT-116 human colon cancer cells were purchased from American Type Cell Collection (ATCC, Manassas, VA, USA). RPMI 1640 media (Mediatech, Herndon, VA,

USA) comprised of 5% fetal bovine serum (FBS), 100U/ml of penicillin and 0.1mg/ml of streptomycin was used as the growth medium for the cells and changed every several days. The HCT116 colon cancer cells were held in an incubator at 37 °C with 5% CO₂ and the cells selected for the experiments were between 3 to 25 passages.

2.5.2 Cell Treatments

Luteolin (>98%) and apigenin (>98%) were obtained from Quality Phytochemicals Inc. (Edison, New Jersey). The 3', 4'-didemethylnobiletin (DDMN, 98%) was produced after a multi-step process with its chemical structure verified using MS and NMR spectra methods as described (Li, Pan et al. 2007; Li, Sang et al. 2007). DMSO was used for the delivery of DDMN and curcumin to the cells in each experiment, resulting in a final concentration of 0.1% DMSO.

2.5.3 Cell Viability Assay

HCT-116 cells were seeded at 2,000 cells per well in 96-well plates and treatments of DDMN, apigenin, luteolin or their combination were administered in serial concentrations after 24 hour incubation in 200 µl of serum complete media. Once the associated treatment times were met, the replacement of media with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) containing media (Sigma-Aldrich) was performed to quantify cell viability as outlined in prior experiments (Qiu, Dong et al. 2010).

2.5.4 Analyses of Synergy

The analyses of synergy utilized a linear regression model based on the Chou Talalay method with some alterations as previously described (Chou and Talalay 1984; Xiao, Zhang et al. 2008; Guo, Qiu et al. 2012). Results were examined using the R program and synergy was categorized utilizing the interaction index parameters, with the combination interaction (CI) characterized as exhibiting additivity (=1), synergy (<1), or antagonism (>1).

2.6 Results and Discussion

2.6.1 Apigenin and DDMN Additively Inhibit Cell Viability in HCT116 Colon Cancer Cells

HCT116 human colon cancer cells were treated with serial doses of DDMN (3 μ M-15 μ M) and apigenin (3 μ M-15 μ M) individually and at half-dose serial concentrations in combination for 48 hours to determine growth inhibitory effects using the MTT assay. Cell viability as measured by the MTT assay revealed that combined treatment with DDMN and apigenin exhibited growth inhibitory effects after 48 hours of compound exposure (Figure 2.2). Individual treatment with DDMN alone provided only minimal inhibition of growth (13%-15%) at low concentrations (3 μ M-6 μ M) but was substantially more effective at inhibition of growth (30%) with the highest concentration (15 μ M). Apigenin individual treatment exhibited a dose dependent response with a much stronger inhibition (18%-91%) of growth at all concentrations (3 μ M-15 μ M) than DDMN. Combination of DDMN and apigenin at half-dose serial concentrations inhibited cell viability in a dose dependent manner with an additive effect that appeared

to not greatly differ from the individual treatment effects therefore no apparent interaction occurred as determined by the combination index previously described.

Additive was defined as combination interaction index $CI = 1$.

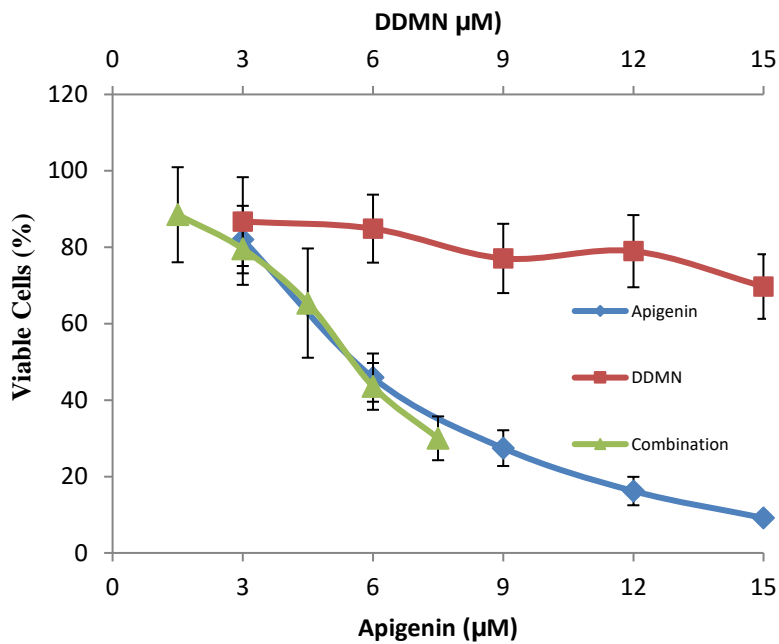


Figure 2.2 Growth inhibitory effects of apigenin and DDMN individually and in combination following serial concentrations of compound treatments in HCT116 human colon cancer cells. Cell viability was measured by MTT assay as previously described in Section 2.2.

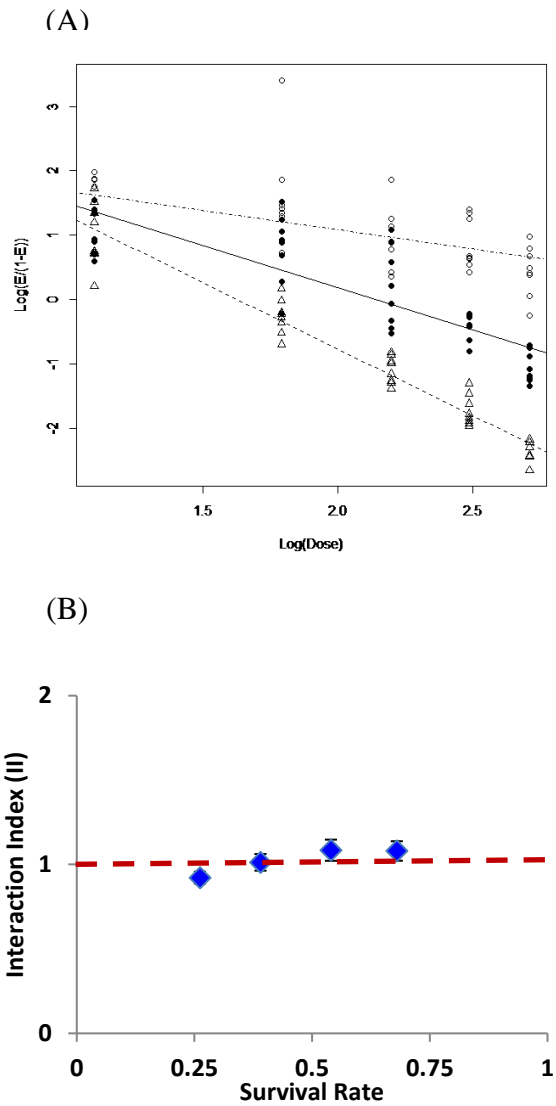


Figure 2.3 Combined treatment with apigenin and DDMN exhibited additive growth inhibitory effects after 48 hr of compound exposure. (A) Median effect plot of apigenin, DDMN and their combination on growth inhibition of HCT116 cells. (B) Interaction index plot for the combination effects of apigenin and DDMN on growth inhibitory effects of HCT116 colon cancer cells. Additive was defined as CI interaction index = 1. Median effect plot and interaction index plot constructed as described in Materials and Methods.

2.6.2 Luteolin and DDMN Demonstrate Antagonism in the Inhibition of Cell Viability in HCT116 Colon Cancer Cells

HCT116 human colon cancer cells were treated with serial doses of DDMN (3 μ M-15 μ M) and luteolin (3 μ M-15 μ M) individually and at half-dose serial concentrations in combination for 48 hours to determine growth inhibitory effects using the MTT assay. Cell viability as measured by the MTT assay revealed that combined treatment with DDMN and luteolin displayed growth inhibitory effects after 48 hours of compound exposure (Figure 2.3). Individual treatment with DDMN alone provided inhibition of growth (25%-32%) at low concentrations (3 μ M-6 μ M) but was substantially more effective at inhibition of growth (56%) with the highest concentration (15 μ M). Luteolin individual treatment exhibited a dose dependent response with a similar inhibition (23%-56%) of growth at all concentrations (3 μ M-15 μ M) as DDMN. The combination of DDMN and luteolin at half-dose serial concentrations inhibited cell viability in a dose dependent manner exhibiting an antagonistic effect of less inhibition than the individual treatments separately, as determined by the combination index previously described. Antagonism was defined as combination interaction index $CI > 1$.

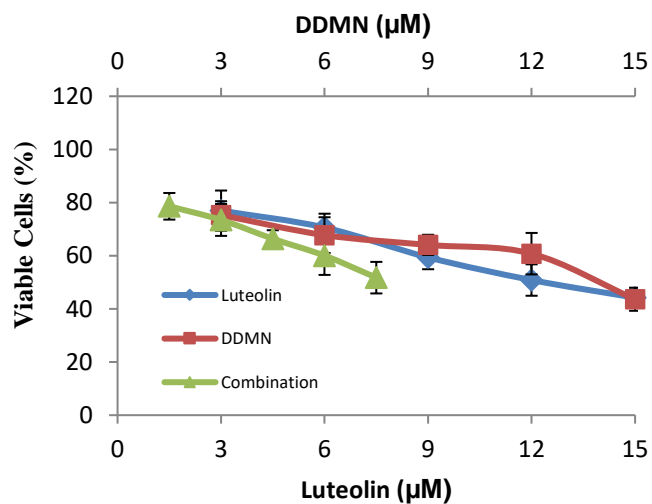


Figure 2.4 Growth inhibitory effects of luteolin and DDMN individually and in combination following serial concentrations of compound treatments in HCT116 human colon cancer cells. Cell viability was measured by MTT assay as previously described in Section 2.2.

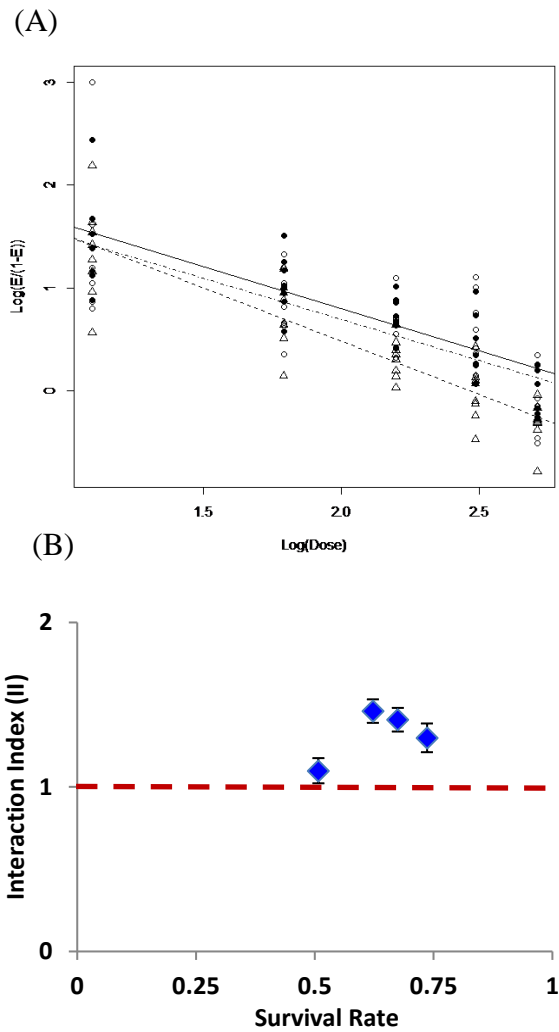


Figure 2.5 Combined treatment with luteolin and DDMN exhibited antagonistic growth inhibitory effects after 48 hr of compound exposure. (A) Median effect plot of luteolin, DDMN and their combination on growth inhibition of HCT116 cells. (B) Interaction index plot for the combination effects of luteolin and DDMN on growth inhibitory effects of HCT116 colon cancer cells. Antagonism was defined as CI interaction index > 1 . Median effect plot and interaction index plot constructed as described in Materials and Methods.

2.6.3 Discussion

Studies identifying specific vegetables and fruits exhibiting anti-proliferative activity conclude the inhibitory effects on cellular growth are likely due to individual components or a class of phenolic components present in the vegetable or fruit (Chu, Sun et al. 2002; Sun, Chu et al. 2002). Flavonoids are one such group of polyphenolic compounds found abundantly in vegetables and fruits (Liu 2004). A number of flavonoid interactions have been described as synergistic with enhanced anti-proliferative, anti-oxidant and anti-inflammatory effects (Freeman, Eggett et al. 2010; Hidalgo 2010; Gomez-Alonso 2012; Jaramillo-Carmona 2014). Although studies suggest flavonoids in combination can contribute to greater health promoting effects; other findings are mixed, pointing to the need to investigate flavonoids in combination to determine if their positive attributes are unchanged (additive) or negatively impacted resulting in antagonistic effects and diminished benefit (Freeman, Eggett et al. 2010; Colon and Nerin 2016). In addition, few studies have examined the combination of a flavonoid with a flavonoid metabolite offering the potential for greater anti-cancer effects using a colon cancer cellular model. Therefore, we report for the first time on the anti-proliferative effects of luteolin or apigenin both flavones, in combination with 3',4'- didemethylnobiletin (DDMN), a primary metabolite of the flavone nobiletin in the mouse in HCT116 colon cancer cells; findings indicate no interaction (additive) between apigenin and the metabolite occurred whereas an antagonistic combination response with luteolin and DDMN appears to exist. These results suggest the flavones although similar in structure can display divergently different interactions with the metabolite of a

polymethoxyflavone, exhibiting a range of response from no additional benefit to a reduction in anti-proliferative effectiveness.

Multiple studies have explored the relationship between the flavonoid structure and its anti-proliferative, anti-oxidant and/or anti-inflammatory effects (Kuo 1996; Chidambara Murthy, Kim et al. 2012). Results indicate flavonoid inhibition of cell growth is associated with the presence of a double bond at C2-C3 of which luteolin, apigenin and DDMN all possess; additional variations of the C ring appear to be accompanied by a reduction or loss of inhibitory activity (Agullo, Gamet-Payraastre et al. 1996; Depeint, Gee et al. 2002; Chang, Mi et al. 2008). Some researchers report heightened or diminished inhibition of cell growth can occur by flavonoids due to the location and/or number of hydroxyl groups on the flavonoid structure; whereas others find anti-proliferative effects do not appear to be impacted by hydroxyl group variations (Agullo, Gamet-Payraastre et al. 1996; Kuntz, Wenzel et al. 1999; Chang, Mi et al. 2008; Chidambara Murthy, Kim et al. 2012). The structure of apigenin (4',5,7-trihydroxyflavone) and luteolin (3',4',5,7-tetrahydroxyflavone) differ only in one hydroxyl group located on the B ring at the 3' position that luteolin contains but apigenin does not (Figure 2.1). Free radical scavenging activity has been associated with a greater number of hydroxyl groups on the B ring particularly the di-hydroxy at 3' and 4' is very important for strong radical absorbing activity; therefore luteolin exhibits a higher antioxidant activity with its di-hydroxy groups at 3' and 4' position as compared to apigenin that lacks a hydroxyl group at the 3' position (Rice-Evans, Miller et al. 1996; Kumar and Pandey 2013). Additional anti-oxidant and anti-inflammatory effects appear

to be correlated with the presence of the di-hydroxy groups at 3' and 4' (Cao, Sofic et al. 1997; Ueda 2002).

Polymethoxylated flavonoids (PMFs) such as nobiletin, have a benzo- γ -pyrone structure with a minimum of four methoxyl groups on the A, B and/or C ring (Li 2008; Ho 2012). High anti-proliferative activity has been associated with a C-8 methoxyl group as part of the polymethoxyflavone structure in a number of cancer cell lines (Kawaii, Tomono et al. 1999). Biotransformation of nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) occurs on the B ring at the 3' and 4' positions; such that 3',4'-didemethylnobiletin (Figure 1), a primary metabolite of nobiletin in CD-1 mice has recently been identified and found to have greater anti-proliferative, anti-oxidant and anti-cancer effects than the parent compound nobiletin (Li 2009). In contrast, other flavonoid metabolites are reported to have diminished anti-proliferative effects as compared to their parent compound (Delgado, Fernandes et al. 2014). Indeed, accumulating research of flavonoids and their metabolites suggests the number of methoxyl and hydroxyl groups and their position on the flavonoid structure can greatly influence their biological effects and antioxidant capacity (Rice-Evans, Miller et al. 1996; Tripoli 2007; Li 2008; Hidalgo 2010; Lotito, Zhang et al. 2011; Delgado, Fernandes et al. 2014; Kongpichitchoke, Hsu et al. 2015; Chen, Teng et al. 2016).

The concentrations used in this study for apigenin, luteolin and DDMN suppressed cell growth at all concentrations in a dose dependent manner (Figures 2.2 & 2.3). Individual treatment with apigenin was substantially more potent in its inhibitory effects than individual treatment with luteolin or DDMN. The absence of a hydroxyl group at the 3' position of apigenin does not have a diminished effect on apigenin's

individual anti-proliferative inhibition in this study using HCT116 colon cancer cells; rather apigenin exceeds luteolin in its inhibition of cell growth demonstrating the lack of a 3' hydroxyl group is associated with a greater reduction of cell proliferation in this cell line. The anti-proliferative effects of the individual compounds on HCT116 colon cancer cells were greatest in apigenin > luteolin > DDMN based on their inhibitory potency in this study. These findings are in agreement with the results of a comparison study of different flavones to determine their inhibition of HCT116 colon cancer cells; researchers found apigenin to exhibit the strongest anti-cancer inhibitory effects of decreased cell proliferation of all the flavones tested, including luteolin (Zhong, Krisanapun et al. 2010). In contrast, Manthey *et al* conducted a comparison of the anti-proliferative actions of flavones in HT29 colon cancer cells and found luteolin to have greater inhibitory effects than apigenin (Manthey and Guthrie 2002). Similarly, using the same HT29 cell line other researchers find flavonoids that lack the 3' hydroxyl (such as apigenin) are not as effective in the inhibition of cell growth as those with the 3' hydroxyl as a part of their flavonoid structure (Agullo, Gamet-Payrastre et al. 1996). Based on these findings and the results of this study, cell specificity appears to be involved in the measured anti-proliferative response of apigenin or luteolin in different colon cancer cell lines.

DDMN, a metabolite of nobiletin in the mouse was not examined in either of the aforementioned comparison studies however DDMN's dose dependent suppression of cell growth in this study is consistent with the anti-proliferative effects of DDMN previously described by Wu *et al* in HCT116 colon cancer cells (Wu, Song et al. 2015). Both luteolin and DDMN contain a di-hydroxy at the 3'- and 4'-position of the B-ring and

differ structurally only in the A-ring of which luteolin has hydroxyl groups at the 5- and 7-position whereas DDMN contains methoxyl groups at the 5-, 6-, 7- and 8-position. As such, DDMN structurally distinct from luteolin, with methoxyl groups on the A-ring is found in this study to be associated with a smaller anti-proliferative effect than that observed in luteolin in HCT116 colon cancer cells.

Luteolin and apigenin both flavones, have displayed synergism in the enhancement of anti-proliferative and anti-carcinogenic properties when combined with flavonoids in several cellular models of cancer (Amin, Wang et al. 2010; Shih, Liu et al. 2010; Huang, Wei et al. 2016). A synergistic combinatorial effect is likely due to complimentary mechanisms of food components used in combination; as such an antagonistic interaction could occur in a combination of food components with opposing mechanisms, however more research is needed as much remains to be determined (Pappa, Strathmann et al. 2007; Funaro, Wu et al. 2016). Our findings suggest the combinations of DDMN with apigenin or luteolin are not synergistic in the inhibition of cell proliferation; such that no interaction is apparent between DDMN and apigenin whereas an antagonistic interaction appears to occur in the combination of DDMN with luteolin (Figures 2.4 & 2.5).

Although the results of this study can indicate the nature of the interaction and its effects on anti-proliferation in combination these findings cannot determine causative mechanisms; they do however warrant further investigation through examination of the literature regarding luteolin's antagonistic interactions with other food components to offer insight into our finding of an antagonistic combination of luteolin and DDMN.

Luteolin has been reported to exhibit an antagonistic interaction with another flavone in several research studies. Notably, luteolin appears to have an antagonistic effect on the polymethoxyflavone nobiletin, the parent compound of DDMN in Caco-2 colon cancer cells, an intestinal absorption model; luteolin inhibited nobiletin uptake by 26%, researchers speculate this finding suggests the two flavones share the same transporter perhaps due to the similarity of their structures (Kimura, Ohta et al. 2014). In this study, the metabolite DDMN structurally distinct from its parent compound nobiletin containing a di-hydroxy at 3' and 4' of the B ring exhibits an antagonistic interaction with luteolin upon examination of anti-proliferative effects in HCT116 colon cancer cells. Indeed, the difference in structure of apigenin and luteolin suggests the absence of the 3' hydroxyl is associated with no interaction or an additive response when apigenin is combined with DDMN whereas the presence of the 3' hydroxyl on the luteolin structure is associated with an antagonistic response with diminished anti-proliferative effects in combination with DDMN. The ratio or proportion of luteolin that is used in combination has also been shown to be influential in determining an antagonistic or synergistic interaction with apigenin in the examination of cell cycle arrest. In SW480 p53 mutant colon cancer cells, the combination of luteolin at low concentrations (5 μ M-30 μ M) with apigenin at 20 μ M, significantly increased G2/M cell cycle arrest whereas luteolin at high concentrations (>30 μ M) combined with apigenin at 20 μ M resulted in antagonism with a significant decrease in the level of G2/M cell cycle arrest (Wang, VanAlstyne et al. 2004). The low concentrations of luteolin (3 μ M-15 μ M) used in this study in combination with the low concentrations of DDMN (3 μ M-15 μ M) were not synergistic in the suppression of cell growth rather luteolin in low concentrations exhibited antagonism

in a dose dependent manner when combined with DDMN resulting in a decreased anti-proliferative effect in HCT116 p53 wild-type colon cancer cells.

Interestingly, luteolin has recently been identified as a negative regulator of p53 with the significant down-regulation of p21, a downstream target of p53 that is involved in mediating G2 cell cycle arrest in HCT116 p53 wild type colon cancer cells (Sakai 2012). In contrast, both DDMN and apigenin individually have been found to significantly increase the induction of p21 in HCT116 p53 wild type cells (Zhong, Krisanapun et al. 2010; Wu, Song et al. 2015). As such, further research is warranted to explore whether an antagonistic interaction between luteolin and DDMN in HCT116 p53 wild type colon cancer cells could potentially occur in part due to luteolin possessing an opposing mechanism to DDMN in the regulation of p53 and subsequent downregulation of p21.

2.7 Conclusion

In summary, we report for the first time to our knowledge luteolin in combination with 3',4'-didemethylnobiletin (DDMN), the primary metabolite of nobiletin in the mouse, to exhibit antagonism in the inhibition of anti-proliferative effects in HCT116 colon cancer cells. Furthermore, these antagonistic effects appear to be associated with the presence of the 3' hydroxyl group on the B-ring of luteolin. Taken together, more research is needed to fully characterize the mechanism responsible for this antagonistic combinatory interaction of luteolin with DDMN and to determine if diminished anti-proliferative effects occur in other flavonoid metabolite combinations.

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CHAPTER 3

SYNERGISTIC ANTI-CANCER EFFECTS OF CURCUMIN AND FLAVONOID METABOLITE IN COMBINATION FOR CHEMOPREVENTION OF COLORECTAL CANCER

3.1 Abstract

Chemoprevention strategies employing the use of multiple dietary bioactive components in combination offer advantages due to their relative low toxicity and potential synergistic interactions, however research is needed to determine their overall combination effects. Herein, we evaluate the combination of curcumin and 3', 4'-didemethylnobiletin (DDMN) a primary metabolite of nobiletin in the mouse, to determine their anti-carcinogenic combinatory effects in HCT116 colon cancer cells. Isobologram analysis reveals a synergistic interaction exists between curcumin and DDMN in the inhibition of colon cancer cell growth in HCT116 colon cancer cells. Additionally, combination treatment induced significant G₂/M cell-cycle arrest and extensive apoptosis, which greatly exceeded the effects of individual treatments with curcumin or DDMN. Proteins associated with these heightened anti-carcinogenic effects were p53, p21, HO-1, c-PARP, cdc2 and cdc25c; each of the proteins was confirmed to be substantially impacted by the combination treatment, more than by individual treatments alone. This newly identified synergy between curcumin and DDMN should be explored further to determine its chemopreventive value and potential contribution to colorectal cancer *in vivo*.

3.2 Keywords:

Dietary bioactive components, combination, cancer chemoprevention, colorectal cancer, flavonoid metabolite, curcumin, turmeric, nobiletin, polymethoxyflavone, synergistic

3.3 Abbreviations

DDMN: 3',4'-didemethylnobiletin; ACF: aberrant crypt foci; PMF: polymethoxyflavone; AOM: azoxymethane; DMH: dimethylhydrazine

3.4 Introduction

Dietary bioactive components offer a viable option to current therapies for many diseases as they appear to be beneficial for their prevention and early treatment through the modulation of deregulated signaling pathways (Garcia-Lafuente, Guillamon et al. 2009; Pan, Lai et al. 2009; Tunon, Garcia-Mediavilla et al. 2009). Colorectal cancer is a complex disease characterized by multiple deregulated signaling pathways that are responsible for the promotion and progression of the carcinogenic process (Bird 1995). Multi-drug therapy is often used in the treatment of cancer as an alternative to targeting one gene or one signaling cascade; however toxicity and dosage tolerance remains a concern (Goldin and Mantel 1957; Humphrey, Brockway-Lunardi et al. 2011). Similarly, the use of drugs for the chemoprevention of colorectal cancer are limited due to undesirable side effects associated with their long-term use (Psaty and Potter 2006; Das, Arber et al. 2007; DuPont, Arguedas et al. 2007). Numerous *in vitro* and *in vivo* studies have highlighted the anti-carcinogenic effects of natural compounds found in food (Tsuda, Ohshima et al. 2004; Chen and Kong 2005; Fresco, Borges et al. 2006). Accumulating evidence suggests dietary bioactive components in combination may play

an important role in the chemoprevention of many cancers (DiMarco-Crook and Xiao 2015). Indeed, a multi-agent strategy of combination treatment utilizing several bioactive components is desirable as the dietary components are likely to have complementary effects thereby eliciting a more powerful anti-inflammatory and anti-cancer response with low toxicity (Liu 2003; Murakami, Takahashi et al. 2003; Rather, Bhat et al. 2013; DiMarco-Crook and Xiao 2015).

Polymethoxyflavones (PMFs) are a group of dietary bioactive components being investigated for their beneficial effects; a class of citrus flavonoids found primarily in citrus peel that have demonstrated anti-carcinogenic properties in a variety of cancer model systems (Sergeev, Ho et al. 2007; Xiao, Yang et al. 2009; Qiu, Dong et al. 2010; Lai, Tsai et al. 2011). PMFs also appear to offer an increased potential for absorption and bioavailability as compared to their polyhydroxylated flavonoid counterparts; due to the methoxyl groups on the PMF structure PMFs are more lipophilic and thus may result in higher permeability of the small intestine and subsequent higher absorption into the bloodstream (Li, Lo et al. 2006). Nobiletin, a polymethoxyflavone of particular interest has been reported to inhibit carcinogenesis in colon, prostate and breast cancer models (Tang, Ogawa et al. 2011; Chen, Ono et al. 2014). Notably, 3',4'-didemethylnobiletin (DDMN), the second most abundant metabolite of nobiletin in the mouse and the most potent of the metabolites, is reported to be present at a much higher concentration in mouse colonic mucosa than nobiletin; therefore, these findings suggest many of the observed positive effects of nobiletin could be attributed to its' metabolite DDMN (Zheng, Bi et al. 2015). Indeed, numerous research studies demonstrate 3',4'-didemethylnobiletin (DDMN) a primary metabolite of nobiletin in the mouse (Figure

1), exhibits a much stronger anti-oxidant, anti-inflammatory and anti-carcinogenic response than the parent compound nobiletin (Li, Sang et al. 2007; Lai, Li et al. 2008; Lo, Pan et al. 2010; Su, Yen et al. 2012).

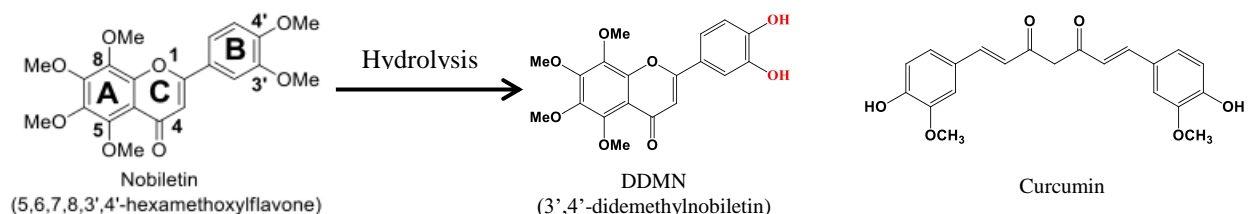


Figure 3.1 Chemical Structures of DDMN (3',4'-didemethylnobiletin) and curcumin

Curcumin, a bioactive component found in turmeric, (Figure 3.1) has been investigated as an alternative strategy for the chemoprevention of colorectal cancer and is widely credited for its anti-carcinogenic effects that include the downregulation of pro-inflammatory signaling cascades, initiation of cell cycle arrest and induction of pro-apoptotic pathways (Teiten, Eifes et al. 2010; Chen, Wang et al. 2014). The frequent use of turmeric in the diet among residents of India and the low occurrence of colorectal cancer reported within this population has led researchers to explore the role of curcumin in the chemoprevention of colorectal cancer (Ferrucci, Daniel et al. 2010; Park and Contreas 2010). Indeed, an accumulating body of research demonstrates curcumin operates within the same deregulated signaling pathways associated with the development of colorectal cancer, yet without the reported negative side effects of chemopreventive drugs. Although curcumin has displayed limited bioavailability *in vivo*, clinical studies examining curcumin intervention in patients for the prevention and treatment of colorectal cancer have generated positive preliminary findings of anti-cancer

effects (Carroll, Benya et al. 2011; Gupta, Patchva et al. 2013). Direct contact of curcumin with the colon overcomes some of the bioavailability challenge; however an effective combination treatment utilizing curcumin can also be beneficial in lowering the dosage required to obtain the maximum treatment effect of curcumin *in vivo*.

Curcumin and polymethoxyflavones (PMFs) have interacted synergistically with a number of other dietary bioactive components in combination, however no information currently exists that examines the anti-cancer effects of the combination of curcumin with a polymethoxyflavone metabolite in colon cancer. (Majumdar, Banerjee et al. 2009; Pesakhov, Khanin et al. 2010; Guo, Qiu et al. 2012; Funaro, Wu et al. 2016). Based on this information we will evaluate for the first time the anti-cancer inhibitory effects of the combination of curcumin and 3',4'-didemethylnobiletin (DDMN), a primary metabolite of nobiletin in the mouse, in the HCT116 human colon cancer cell line.

3.5 Materials and Methods

3.5.1 Cell Culture

HCT-116 human colon cancer cells were purchased from American Type Cell Collection (ATCC, Manassas, VA, USA). RPMI 1640 media (Mediatech, Herndon, VA, USA) comprised of 5% fetal bovine serum (FBS), 100U/ml of penicillin and 0.1mg/ml of streptomycin was used as the growth medium for the cells and changed every several days. The HCT116 colon cancer cells were held in an incubator at 37 °C with 5% CO₂ and the cells selected for the experiments were between 3 to 25 passages.

3.5.2 Cell Treatments

Curcumin (96%) was obtained from Quality Phytochemicals, LLC (New Jersey, USA). The 3', 4'-didemethylnobiletin (DDMN, 98%) was produced after a multi-step process with its chemical structure verified using MS and NMR spectra methods as described (Li, Pan et al. 2007; Li, Sang et al. 2007). DMSO was used for the delivery of DDMN and curcumin to the cells in each experiment, resulting in a final concentration of 0.1% DMSO.

3.5.3 Cell Viability Assay

HCT-116 human colon cancer cells were seeded in 96-well plates and treatments of DDMN, curcumin or their combination were administered in serial concentrations after 24 hour incubation. Once the associated treatment times were met, the replacement of media with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) containing media (Sigma-Aldrich) was performed to quantify cell viability. Further detail as outlined above and in prior experiments (Qiu, Dong et al. 2010).

3.5.4 Analyses of Synergy

The analyses of synergy utilized a linear regression model based on the Chou Talalay method with some alterations as previously described (Chou and Talalay 1984; Xiao, Zhang et al. 2008; Guo, Qiu et al. 2012). Results were examined using the R program and synergy was categorized utilizing the interaction index parameters, with the combination interaction (CI) characterized as exhibiting additivity (=1), synergy (<1), or antagonism (>1).

3.5.5 Cell Cycle Analyses

HCT-116 cells were seeded in 6-well plates at a rate of 8×10^4 cells per well. Once cells were attached following 24h incubation, treatments of DDMN, curcumin or their combination were added to cells for another 24h incubation period. In preparation for analysis following incubation, previously adhered cells were removed using trypsin ((0.25% trypsin-EDTA; Mediatech) and were combined with the floating cells. Further steps necessary for analysis were undertaken as previously described (Qiu, Dong et al. 2010). BD LSRII flow cytometry was used to examine cell cycle distribution and the subsequent data produced was analyzed with Modifit LT software.

3.5.6 Apoptosis Detection

HCT-116 cells were seeded in 6-well plates (8×10^4 cells per well), incubated for 24 hours and prepared for analysis as outlined above. Apoptotic cells were calculated following 48h treatments using BD LSRII flow cytometry and the Annexin V/PI double staining assay to distinguish between early apoptotic cells as Annexin V-positive/PI-negative cells and late apoptotic cells as Annexin V-positive/PI-positive cells.

3.5.7 Immunoblotting

HCT-116 cells were seeded in 15cm petri dishes, incubated for 24h and then treated with DDMN, curcumin or their combination for another 24h or 48h incubation. Whole cell lysate was isolated and retrieved for each treatment after cells were washed with iced PBS. Preparation for Western Blot and the subsequent analysis was performed as previously described (Xiao, Zhang et al. 2008; Qiu, Dong et al. 2010; Guo, Qiu et al.

2012). Antibodies for p53, p21^{Cip1/Waf1}, poly ADP ribose polymerase (PARP), cdc-2 (cdk-1), and cdc25c were each acquired from Cell Signaling Technology (Beverly, MA, USA); antibody for β -actin was obtained from Sigma Aldrich.

3.5.8 Statistical Analysis

Results are conveyed as the mean \pm standard deviation (SD). To compare the differences between more than 2 groups the analysis of variance model (ANOVA) was used. In addition, for multiple comparisons the Post-hoc test Tukey Range Honesty Significant Difference was employed. A 1% level of significance was established for all tests.

3.6 Results and Discussion

3.6.1 Curcumin and DDMN Synergistically Inhibit Cell Viability in HCT116

Colon Cancer Cells

HCT116 human colon cancer cells were treated with serial dosages of DDMN (3 μ M-15 μ M) and curcumin (2 μ M-10 μ M) individually and at half-dose serial concentrations in combination for 48 hours to determine growth inhibitory effects using the MTT assay (Figure 3.2). Cell viability as measured by the MTT assay revealed that combined treatment with DDMN and curcumin potentiated the growth inhibitory effects after 48 hours of compound exposure. Individual treatment with DDMN alone provided only minimal inhibition of growth (10%-12%) at low concentrations (3 μ M-9 μ M) but was substantially more effective at inhibition of growth (27%) with the highest concentration (15 μ M). Curcumin individual treatment exhibited a dose dependent response with a much stronger inhibition (20%-79%) of growth at all concentrations (2 μ M-10 μ M) than

DDMN. Remarkably, the combination of DDMN and curcumin at half-dose serial concentrations synergistically inhibited cell viability in a dose dependent manner with a more pronounced effect than individual treatments as determined by the combination index previously described in Section 2.3. Synergy was defined as combination interaction index $CI < 1$ (Figure 3.3).

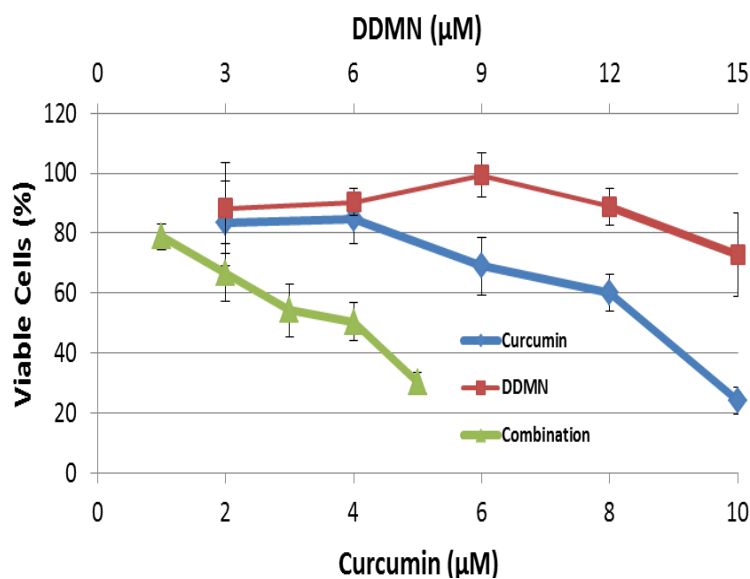


Figure 3.2 Growth inhibitory effects of curcumin and DDMN individually and in combination following serial concentrations of compound treatments for 72 hours in HCT116 human colon cancer cells. Cell viability was measured by MTT assay as previously described in Section 2.2.

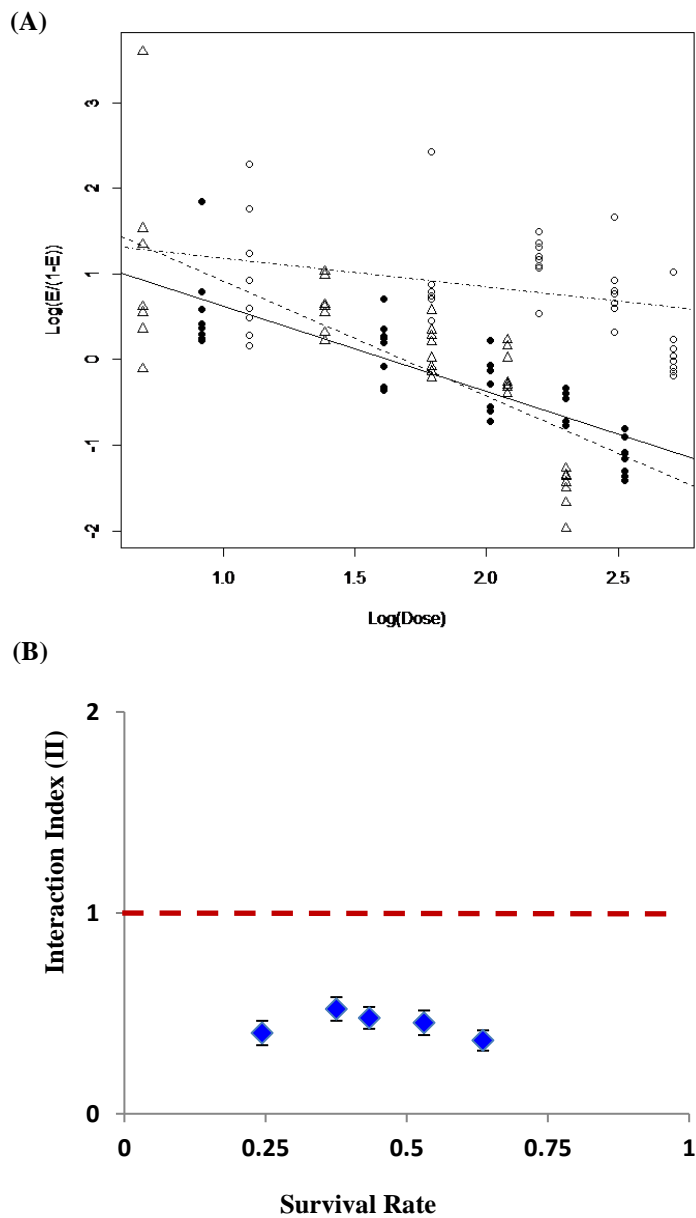


Figure 3.3 (A) Median effect plot of DDMN, curcumin and their combination on growth inhibition of HCT116 cells. (B) Interaction index plot for the combination effects of DDMN and curcumin on growth inhibitory effects of HCT116 colon cancer cells. Synergy was defined as CI interaction index < 1 . Median effect plot and interaction index plot constructed as described in Materials and Methods.

3.6.2 Combination of Curcumin and DDMN induce extensive apoptosis as compared to individual compounds in HCT116 Colon Cancer Cells

To investigate the role of apoptosis on the observed inhibition of cell growth, HCT116 colon cancer cells were subjected to 48 hour treatment of 5 μ M curcumin and 7.5 μ M DDMN alone and in combination then analyzed to determine the level of early and late apoptotic cells attributed to each treatment (Figure 3.4). Individual treatment concentrations of 5 μ M curcumin, 7.5 μ M DDMN, half dose combination treatment with 2.5 μ M curcumin+3.75 μ M DDMN and full dose combination treatment with 5 μ M curcumin +7.5 μ M DDMN were found to increase early apoptotic cells by 5.3-fold, 1.7-fold, 5.4-fold and 28.2-fold respectively whereas, the late apoptotic cells increased 3.3-fold, 1.7-fold, 3-fold and 7.9-fold respectively. Surprisingly, the full dose combination treatment resulted in a 28-fold increase in early apoptotic cells, a finding that greatly exceeded the 5.4-fold increase observed following half dose combination treatment, similar to the 5.3-fold increase exhibited by curcumin alone. The full dose combination treatment also exhibited a greater increase in late apoptotic cells of 7.9-fold, more than double the 3-fold increase produced in response to the half dose combination treatment, once again similar to the 3.3-fold increase of curcumin alone. These results demonstrated significant induction of apoptosis by the full dose combination as compared to control (Figure 3.3), with a substantial increase in early and late apoptotic cells, based on ANOVA followed by Tukey's HSD post-hoc test ($p < 0.01$, $n=3$).

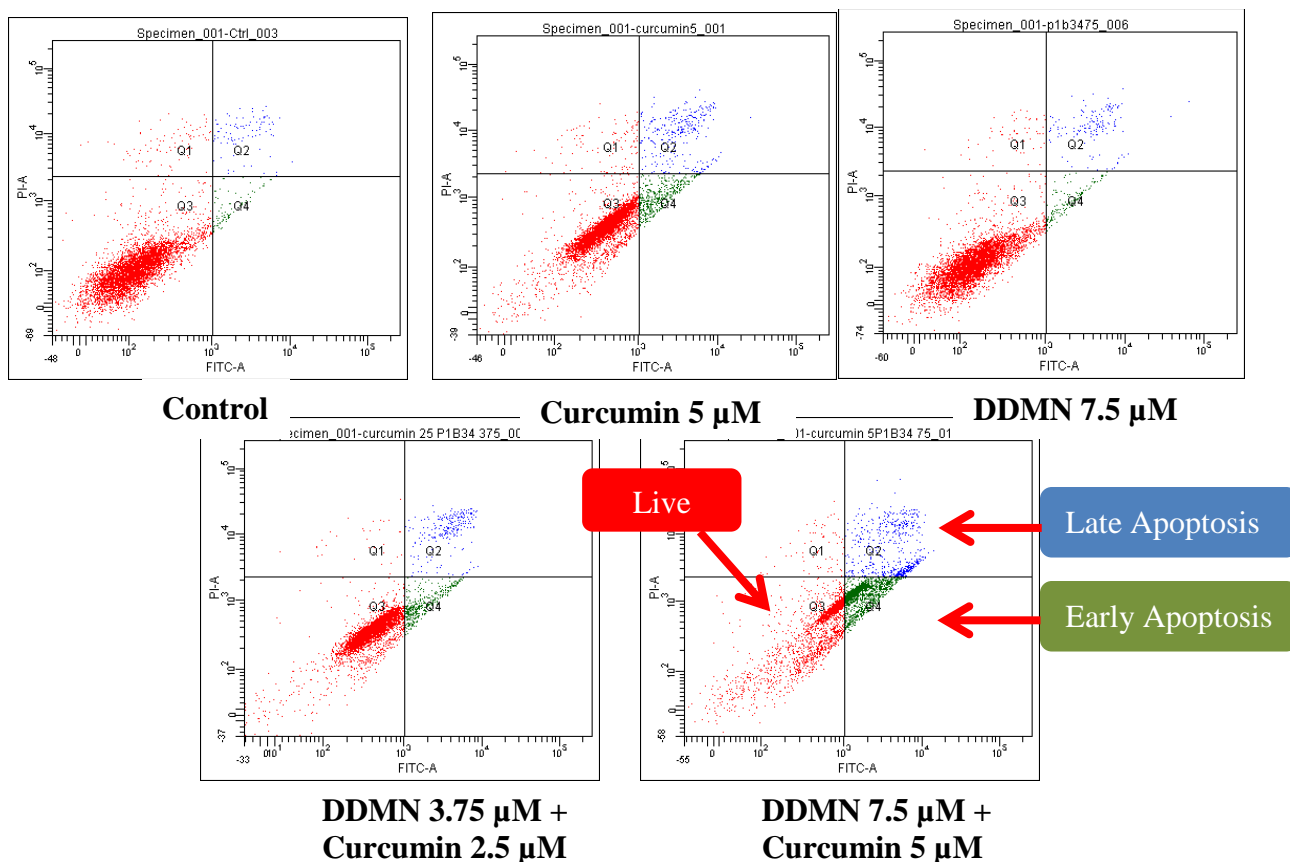


Figure 3.4 Effects of DDMN, curcumin, and DDMN/curcumin full dose combination on apoptosis in HCT116 colon cancer cells. HCT116 cells were seeded in 6-well plates then treated with concentrations as noted above at 24 hours. After another 48 hours cells were collected and apoptosis analyses were performed as previously described. Annexin V/PI dot plots indicate a much greater intensity in early apoptosis induction by the full-dose combination treatment as compared to the control or individual treatments.

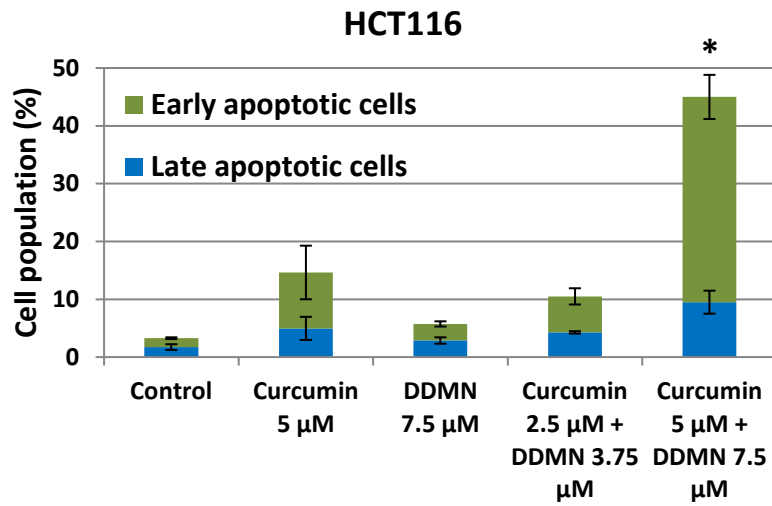


Figure 3.5 Quantification of effects by curcumin, DDMN and full-dose combination on apoptosis in HCT116 colon cancer cells. All data represents the mean \pm SD. The asterisk in the bar charts represents statistical significance of DDMN/curcumin full-dose combination treatment group as compared to the control, DDMN or curcumin treatment groups at full concentrations based on ANOVA followed by Tukey's HSD post-hoc test ($p < 0.01$, $n=3$).

3.6.3 Combination of Curcumin and DDMN exhibits greater G₂/M cell-cycle arrest than individual compounds in HCT116 Colon Cancer Cells

To further investigate the mechanism responsible for the anti-proliferative effects of the combination treatment, cell-cycle analysis was conducted. Figure 3.4 shows the results of curcumin, DDMN and the combination of curcumin + DDMN on cell-cycle arrest in HCT116 colon cancer cells. Treatment concentrations of 7.5 μ M DDMN and 5 μ M curcumin were evaluated individually, in half dose combination treatment and in full dose combination treatment in HCT116 cells after 24 hour incubation. Increased G₂/M phase cell-cycle arrest was observed for the individual curcumin treatment (5 μ M) although not significant and was much stronger than DDMN individual treatment (7.5 μ M) which increased G₂/M arrest only nominally. Interestingly, when combined at half-dose the effect was similar to curcumin alone but a significant accumulation of cells in the G₂/M phase occurred when the full dose combination treatment was examined as compared to control, based on ANOVA followed by Tukey's HSD post-hoc test ($p < 0.01$, $n=3$).

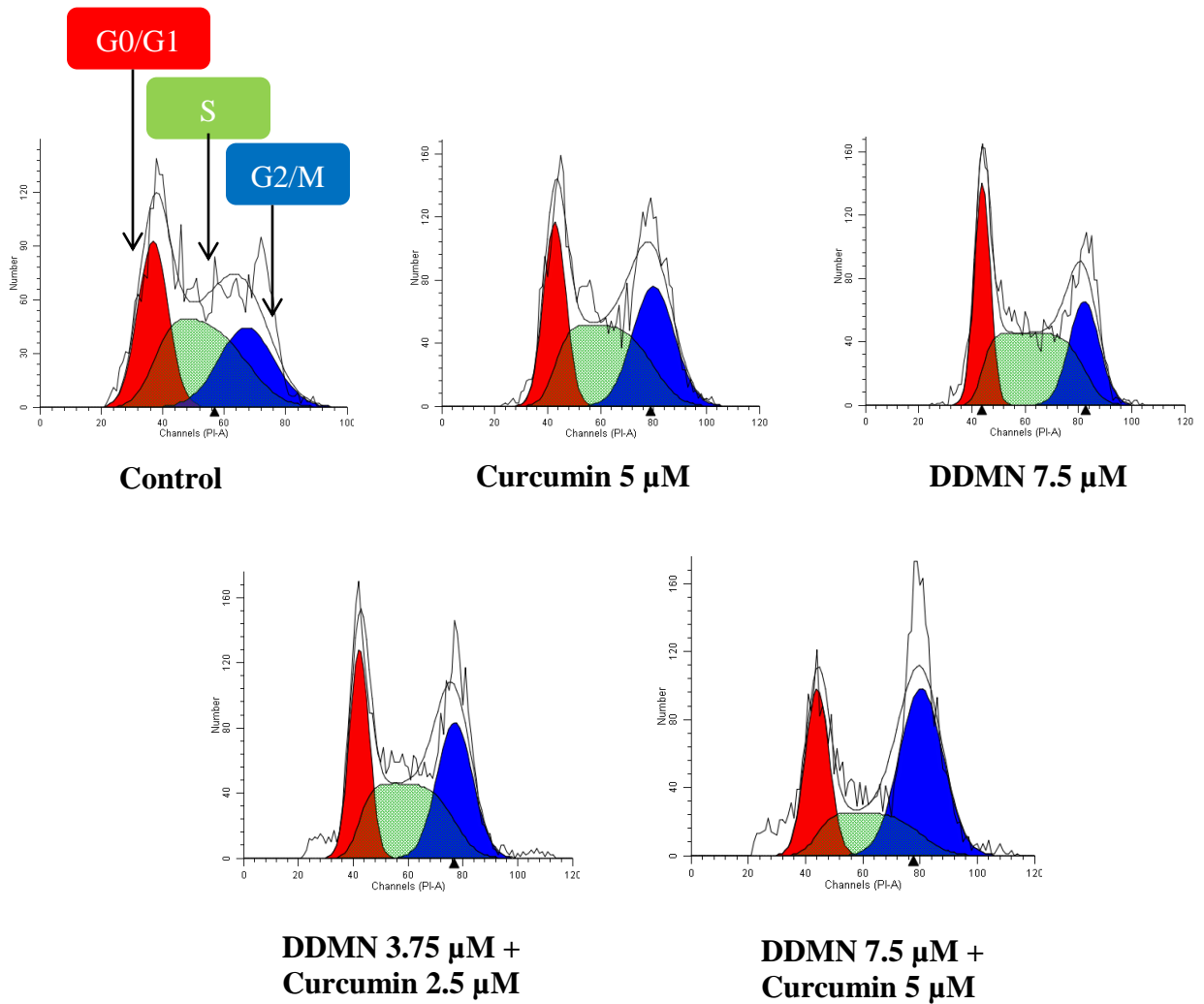


Figure 3.6 Effects of DDMN, curcumin, and DDMN/curcumin combination on cell cycle arrest in HCT116 colon cancer cells. HCT116 cells were seeded in 6-well plates then treated with concentrations as noted above at 24 hours. After another 24 hours cells were collected and cell cycle analyses were performed as previously described. Flow cytometer analyses revealed full-dose combination treatment significantly increased cell cycle arrest in G₂M phase as compared to control or individual treatments.

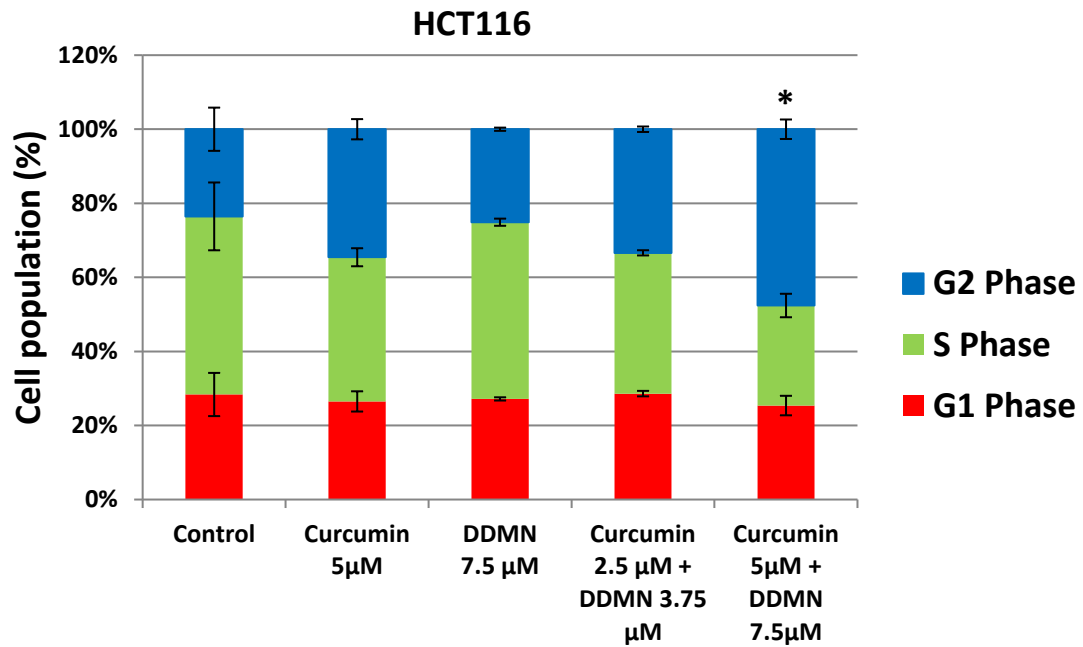


Figure 3.7 Quantification of effect by curcumin, DDMN and full-dose combination treatment on cell cycle in HCT116 colon cancer cells. All data represents the mean \pm SD. The asterisk in the bar chart represents the statistical significance of DDMN/curcumin full-dose combination treatment to increase G₂/M phase cell cycle arrest as compared to the control, DDMN and curcumin treatment groups at full concentrations based on ANOVA followed by Tukey's HSD post-hoc test ($p < 0.01$, $n=3$).

3.6.4 Combination treatment exhibited a pronounced effect on the modulation of proteins involved in cell-cycle and apoptosis signaling

Additional analysis was conducted to explore the mechanisms responsible for the heightened cell-cycle G₂/M arrest and induction of apoptosis observed following the combined full dose treatment of curcumin 5 μ M and DDMN 7.5 μ M in HCT116 colon cancer cells. Immunoblot examination of proteins that have been identified to be involved in these processes revealed the proteins of interest were greatly impacted by the combination treatment. Indeed, full dose concentrations of curcumin 5 μ M combined with DDMN 7.5 μ M in HCT116 colon cancer cells significantly increased expression of p21, p53, HO-1 and cleaved PARP whereas the combination significantly decreased expression of cdc2 and cdc25c (Figure 3.5).

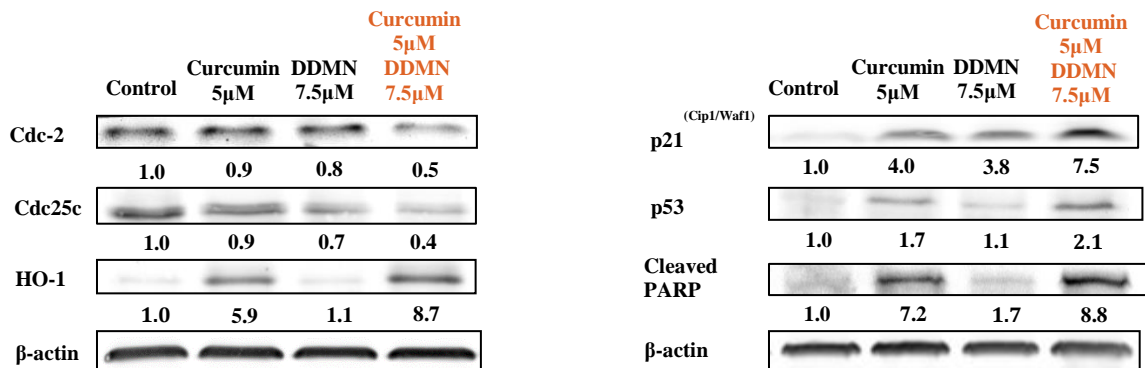


Figure 3.8 Effects of curcumin, DDMN and their combination on proteins associated with cell cycle and apoptosis. Full-dose combination treatment was found to potently alter key oncogenic markers in HCT 116 colon cancer cells. The number below each protein band represents the relative intensity of each band as measured by Image J software.

3.6.5 Discussion

Combination regimens are increasingly being explored for the chemoprevention of colorectal cancer (Zhou, Cheng et al. 2012; Mohammed, Janakiram et al. 2013). Multiple *in vitro* studies reveal pharmacological agents in combination with natural dietary bioactive components or their metabolites can result in heightened chemopreventive effects (Lev-Ari, Strier et al. 2005; Meyskens, McLaren et al. 2008; Kanthamneni, Chaudhary et al. 2010; Chaudhary, Sutaria et al. 2011; Wu, Song et al. 2017). Interestingly, a growing number of studies suggest several dietary bioactive components in combination can have similar anti-inflammatory and anti-cancer properties as observed in pharmacological agent combinations but without the side effects often associated with their use (Liu 2003; Murakami, Takahashi et al. 2003; Rather, Bhat et al. 2013; DiMarco-Crook and Xiao 2015). Moreover, the use of two or more dietary components in combination has been found to exhibit enhanced anti-carcinogenic effects in colorectal cancer both *in vitro* and *in vivo* (Bose, Hao et al. 2007; Nakamura, Yogosawa et al. 2009).

The combination of curcumin with pharmacological agents or other dietary components has been examined for anti-cancer effects in a number of colon cancer model systems (Lev-Ari, Strier et al. 2005; Cruz-Correa, Shoskes et al. 2006; Howells, Mitra et al. 2007; Majumdar, Banerjee et al. 2009). This study is the first to examine the combination of curcumin with 3',4'-didemethylnobiletin, a primary metabolite of nobiletin in the mouse. Our findings of decreased cellular growth, increased apoptosis and G₂/M cell-cycle arrest in response to individual DDMN or curcumin treatment are consistent with other researcher's findings however combination effects have not yet

been explored (Moragoda, Jaszewski et al. 2001; Wu, Song et al. 2015). This study for the first time has shown the combination of DDMN (7.5 μ M) + curcumin (5 μ M) results in synergistic inhibition of cell growth, extensive induction of apoptosis and G₂/M cell-cycle arrest in HCT116 colon cancer cells that is greater than individual treatment effects. Surprisingly, a significant increase in apoptosis or cell cycle arrest was not observed in the half dose combination of curcumin (2.5 μ M) + DDMN (3.75 μ M) although decreased cell viability was found to be synergistic at these combination levels. Others have reported differing mechanisms involved in curcumin's anti-cancer effects that are concentration based, with low concentrations of curcumin exhibiting an antioxidant effect and higher concentrations of curcumin demonstrating a pro-apoptotic effect (Park and Contreas 2010). A key step that indicates apoptosis has occurred is the cleavage of PARP; as Figure 3.5 demonstrates after 48 hour treatment the cleavage of PARP was substantially increased with curcumin (5 μ M) individual treatment (620%) and DDMN (7.5 μ M) individual treatment (70%), however a combined full dose treatment resulted in a much higher increase (780%) of cleaved PARP. Our findings of increased levels of cleaved PARP following individual treatment of HCT116 colon cancer cells with DDMN or curcumin are in agreement with other researcher's findings (Moragoda, Jaszewski et al. 2001; Wu, Song et al. 2015). Remarkably, we now find in this study that the combination of these two compounds results in a more pronounced cleavage of PARP than by DDMN or curcumin alone.

Proteins p21 and p53, reported to be important for the apoptotic process of many cancers, were shown to have a heightened increase in expression following DDMN (7.5 μ M) + curcumin (5 μ M) combination treatment as compared to control. After 24 hour

treatments with DDMN (7.5 μ M), curcumin (5 μ M), or full dose (5 μ M+7.5 μ M) combination, expression of p21 was elevated by 280%, 300% and 650% respectively, with the greatest effect observed as a result of the combination treatment. Similarly, following 24 hour exposure the full dose combination treatment exhibited a substantial 110% increase in p53 expression as compared to control, resulting in a much stronger response than individual curcumin (70%) or DDMN (10%) treatments. Research examining the role of p53 and p21 in apoptosis following curcumin treatment has had mixed findings (Jee, Shen et al. 1998; Choudhuri, Pal et al. 2002; Radhakrishna Pillai, Srivastava et al. 2004; Watson, Hill et al. 2008; Watson, Hill et al. 2010). Watson *et al* suggests curcumin utilizes a p-53 and p-21 independent mechanism to promote apoptosis in HCT116 colon cancer cells although evidence does exist to suggest that p21 may play a role in decreasing cell proliferation via cell-cycle arrest. Other researchers compared curcumin sensitivity in p53 wild type HCT116 colon cancer cells with a p53 knockout HCT116 cell line to conclude that both wild type p53 and p21 are required for curcumin sensitivity (Howells, Mitra et al. 2007). Interestingly, clinical findings following curcumin treatment in colorectal cancer patients suggest a correlation exists between an increased expression of p53 in tumor tissue with an increase in apoptotic tumor cells and increased body weight (He, Shi et al. 2011). To our knowledge this is the first time DDMN has been investigated individually to determine its effect on the expression of p53 and in our study individual treatment of DDMN at a concentration of 7.5 μ M increased p53 only nominally by 10% as compared to control. Again, we find for the first time the combination of DDMN and curcumin is more effective in the induction of p21 and p53 than by individual treatments in HCT116 colon cancer cells.

Two of the key proteins involved in cell cycle control, *cdc2* and *cdc25c* were markedly reduced in response to DDMN (7.5 μ M) + curcumin (5 μ M) combination treatment whereas levels of *cdc2* and *cdc25c* showed only a small reduction in expression following 24 hour exposure to individual compounds of DDMN or curcumin. *Cdc2*, a part of the *cdc2/cyclinB1* complex that is required to enter mitosis from the G₂ phase, was decreased only nominally by individual treatments of curcumin (5 μ M) 10% and DDMN (7.5 μ M) 20% as compared to control however full dose combination treatment exhibited a pronounced 50% decrease in expression. Likewise *cdc25c*, a protein necessary for the activation of *cdc2* was also less impacted by individual treatments with curcumin or DDMN resulting in a decrease in expression of only 10% and 30% respectively. In contrast, the combined full dose treatment of curcumin + DDMN resulted in a dramatic 60% reduction in *cdc25c* expression. Some research has suggested the binding of p21 to the *cdc2/cyclin B* complex can keep *cdc2* inactive in the nucleus preventing *cdc25c* activation (Collett and Campbell 2004). Interestingly, other researchers have demonstrated that the addition of p53 to p53-null HCT116 cells down regulated both *cdc2* and *cdc25c* promoters thereby implicating the involvement of p53 in the decreased expression of *cdc2* and *cdc25c* (Le Gac, Esteve et al. 2006). Our research reveals for the first time the decreased expression of *cdc2* and *cdc25c* by DDMN both alone and in combination with curcumin in HCT116 colon cancer cells. Moreover, we discovered the combination treatment of DDMN (7.5 μ M) + curcumin (5 μ M) markedly downregulated *cdc2* and *cdc25c* as compared to control and individual treatments.

Heme oxygenase-1 (HO-1), a phase 2 enzyme induced in response to oxidative stress was found to dramatically increase following our treatment of HCT116 colon

cancer cells with DDMN (7.5 μ M), curcumin (5 μ M) or full dose combination treatment by 490%, 10% and 770% respectively. Murphy *et al* reports curcumin treatment of HCT116 cells resulted in expression of HO-1 accompanied by cell shrinkage, membrane blebbing, apoptotic bodies and chromatin condensation (Murphy, Testa et al. 2014). To our knowledge, DDMN's effect on HO-1 has not yet been explored in a colon cancer model but DDMN has been found to increase induction of HO-1 in PC12 cells, a cell line used to study neuronal differentiation and cell death (Su, Yen et al. 2012). Controversy currently exists regarding the role of HO-1 in carcinogenesis as many reports of HO-1 expression have been associated with a pro-tumor effect that has been implicated in a variety of different cancers (Jozkowicz, Was et al. 2007). Other pre-clinical findings suggest an anti-carcinogenic role for HO-1 in prostate and colon cancer (Gueron, De Siervi et al. 2009; Andres, Fermento et al. 2014). Investigation using a chemical induced animal model of colon cancer found the degree of tumor progression was directly correlated with increasing amounts of HO-1 protein. This finding is in agreement with a study of Korean colon cancer patients that found a higher level of HO-1 expression was present in tumor tissues than in adjacent normal tissues (Kang, Maeng et al. 2012). Clinical findings from several other studies appear to support the positive role of HO-1 in colon cancer; demonstrating colonic HO-1 expression is associated with a greater long term survival rate in colorectal cancer patients (Becker, Fukui et al. 2007; Andres, Fermento et al. 2014). Researchers have conducted further analysis to explore the mechanism involved in the anti-cancer effects of HO-1 in HCT116 p53wt and p53-/- colon cancer cells. Results indicate the induction of cell-cycle arrest and apoptosis is dependent upon a fully functioning p53 protein that also is associated with a requirement

for increased p21 expression in order for the full expression of HO-1 and its' apoptotic effects to occur. Other research appears to support the integral role of a functioning p53; Kalo *et al* have found that HO-1 displays an anti-apoptotic effect in Caco-2 colon cancer cells with a mutant p53 and in other p53 mutant variants (Busserolles, Megias et al. 2006; Kalo, Kogan-Sakin et al. 2012). Taken together, our research for the first time indicates the combination of curcumin (5 μ M) and DDMN (7.5 μ M) results in a pronounced increase in HO-1 expression that is also associated with the increased induction of p53 and p21 accompanied by increased apoptosis in HCT116 colon cancer cells.

3.7 Conclusion

In this study, curcumin and DDMN demonstrate synergism in combination as compared to individual treatments for the decreased proliferation of HCT116 colon cancer cells. These findings appear to be attributed to the G₂/M cell-cycle arrest exhibited after 24 hour treatment and dramatic induction of apoptosis following prolonged 48 hour exposure of curcumin and DDMN in combination. Therefore, this article reports for the first time that the combination of curcumin and DDMN, a primary metabolite of nobiletin in the mouse, is synergistic in decreasing cell proliferation, potentiating G₂/M cell-cycle arrest and induction of apoptosis in HCT116 colon cancer cells. In addition, our research suggests the involvement of p53, p21, HO-1, c-PARP, cdc2 and cdc25c proteins in these enhanced anti-cancer effects of the combination treatment. These significant findings warrant further investigation into the synergistic properties and chemopreventive potential of curcumin and DDMN in combination for colorectal cancer.

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CHAPTER 4

SYNERGISTIC ANTI-INFLAMMATORY EFFECTS OF CURCUMIN AND FLAVONOID METABOLITE IN COMBINATION *IN VITRO*

4.1 Abstract

Chronic inflammation of the colon or inflammatory bowel disease (IBD) is associated with an increased risk of developing colon cancer. Clinical studies suggest the longer the duration of IBD and the higher the degree of inflammation the greater the colon cancer risk. Effective long-term anti-inflammatory dietary strategies free of undesirable side effects are urgently needed for the prevention of IBD-related colon cancer. Previously we identified the synergistic interaction between curcumin and 3', 4' didemethylnobiletin (DDMN) a primary metabolite of nobiletin in the mouse, to exhibit enhanced anti-carcinogenic combinatory effects in HCT116 p53 wild-type colon cancer cells. A p53 mutation is an early event in inflammatory colon cancer and a defining event in the adenoma to carcinoma transition of sporadic colon cancer; therefore in this study we explored the anti-proliferative effects of the combination in HT29 colon cancer cells, a p53 mutated cell line. To further characterize the synergism of this combination we also examined the anti-inflammatory effects of DDMN (5 μ M-25 μ M), curcumin (3 μ M-15 μ M) and their half- dose serial concentrations in combination at a 1.7:1 ratio in RAW 264.7 cells. Non-synergistic and diminished anti-proliferative effects were observed in the p53 mutated colon cancer cell line HT29 following combination treatment; whereas isobologram analysis reveals a synergistic interaction exists between curcumin and DDMN in the inhibition of LPS induced inflammation as evidenced by decreased NO production in RAW 264.7 cells. The anti-inflammatory synergy between curcumin and

DDMN offers a potential novel mechanism for the interaction of curcumin and nobiletin *in vitro* and warrants further investigation.

4.2 Keywords:

Dietary bioactive components, combination, anti-inflammatory, colon cancer, flavonoid metabolite, curcumin, nobiletin, synergistic, antagonistic, chronic intestinal inflammation

4.3 Abbreviations

DDMN: 3',4'-didemethylnobiletin; iNOS: inducible nitric oxide synthase; NO: nitric oxide; IBD: inflammatory bowel disease; UC: ulcerative colitis; ROS: reactive oxygen species; PMF: polymethoxyflavone; RNS: reactive nitrogen species; RONS: reactive oxygen nitrogen species

4.4 Introduction

Inflammation has been reported to play a key role in the establishment or progression of many diseases including atherosclerosis, obesity, arthritis, diabetes and cancer. The prevalence of inflammation in such a broad array of chronic disease states emphasizes the urgent need to develop anti-inflammatory diet-based strategies to alter the course of disease. Acute inflammation can be protective of healthy cells, transitory and self-limiting whereas chronic or long-term inflammation has been correlated with an increased incidence of tumor development in epidemiological and clinical studies (Pal, Bhattacharjee et al. 2014). Indeed, evidence suggests inflammation plays a prominent role in the establishment and promotion of many cases of colon cancer (Lakatos and

Lakatos 2008; Terzic, Grivennikov et al. 2010; Zhu and Li 2012). Inflammatory bowel disease (IBD) or chronic intestinal inflammation has afflicted an estimated 3.1 million Americans and appears to be occurring more frequently in the U.S. in recent years; however the reason for this increase is not fully understood (Siegel, Miller et al. 2017). Ulcerative colitis (UC) and Crohn's disease are the two most frequently reported types of IBD. Findings indicate the more colonic surface that is involved in the IBD-related inflammation and the longer the duration of IBD the greater the colon cancer risk (Itzkowitz and Yio 2004).

One of the leading theories of inflammation has involved the integral role of oxidative stress in the development of cancerous growth in chronic inflammation. It is this oxidative stress that is also associated with cellular damage and the pathogenesis of IBD. Oxidative stress refers to the harmful effects of uncontrolled reactive oxygen species (ROS) and reactive nitrogen species (RNS) accumulation to cells, also referred to as reactive oxygen nitrogen species collectively (RONS). Normal cell processes involve reactive oxygen species (ROS) at low levels (Kalo, Kogan-Sakin et al. 2012). Chronic inflammation generates an accumulation of RONS or free radicals that can operate as mutagenic agents in combination with increased cell proliferation and potentially bring about oncogenic transformation in epithelial cells; DNA damaged cells may be allowed to proceed through the cell cycle without being repaired to reproduce leading to tumorigenesis (Mladenova and Kohonen-Corish 2012).

A mutation in the tumor suppressor gene p53 is found to occur frequently as an early initiating event in inflammatory colon cancer and is also considered one of the defining events in the promotion of an adenoma to a carcinoma in sporadic colon cancer,

occurring in approximately 40-50% of diagnosed cases (Itzkowitz and Yio 2004; Li, Zhou et al. 2015). A fully functioning p53 is important in apoptosis for the destruction of cancer cells and suppression of the carcinogenic process; for this reason p53 is often referred to as the “guardian of the genome”(Cooks, Harris et al. 2014). Accumulating research suggests p53 is also important for the inhibition of inflammation (Gudkov, Gurova et al. 2011). A high occurrence of p53 mutations have been reported in the inflamed mucosa of ulcerative colitis (UC) patients that do not have cancer suggesting a p53 mutation is associated with chronic inflammation of the colon (Hussain, Amstad et al. 2000). Notably, in IBD when cancerous lesions are found they are typically located in the colonic mucosa involved with inflammation (Rubin, Haggitt et al. 1992).

Current treatment modalities for chronic colon inflammation are limited due to undesirable side effects associated with their long-term use. Research has shown diet to be a powerful manipulator in the prevention and promotion of inflammation. Many studies suggest individual dietary food components exhibit anti-inflammatory properties, yet little research exists examining these dietary components in combination (Benavente-Garcia and Castillo 2008; Pan, Lai et al. 2009; Tunon 2009). Preliminary pre-clinical studies appear to demonstrate synergistic anti-inflammatory effects in the combination of dietary components (Hemalswarya 2006; Cheung, Khor et al. 2009).

Polymethoxyflavones (PMFs) are one such group of dietary bioactive components being investigated; a class of citrus flavonoids found primarily in peels that have been credited with operating in a variety of capacities including exhibiting potent anti-inflammatory effects (Li, Lo et al. 2006; Walle 2007).

Nobiletin, a polymethoxyflavone (PMF) of particular interest for its anti-inflammatory effects, has shown significant inhibition of key proteins necessary for the promotion of the inflammatory process (Murakami, Nakamura et al. 2000; Tanaka, Sato et al. 2004; Harada, Tominari et al. 2011). Guo *et al* reports the combination of nobiletin, (the parent compound of DDMN) and sulforaphane results in potent synergistic anti-inflammatory effects in RAW 264.7 macrophage cells that include enhanced inhibition of nitric oxide (NO) and expression of heme oxygenase-1 (HO-1) that is greater than individual treatments (Guo, Qiu et al. 2012). Curcumin, the dietary component found in turmeric, is well established for its anti-inflammatory properties and has shown strong activity in the induction of HO-1 along with the inhibition of NO and iNOS in RAW 264.7 macrophages (Kim, Pae et al. 2008). Synergistic anti-inflammatory effects were observed in the combination of curcumin and sulforaphane in RAW 264.7 macrophages; the combination was found to synergistically down-regulate NO and upregulate the induction of heme-oxygenase-1 (HO-1) (Cheung, Khor et al. 2009). Although both nobiletin and curcumin have demonstrated synergism with sulforaphane in RAW 264.7 cells, their anti-inflammatory effects in combination with each other have yet to be explored.

Some researchers find the more potent anti-inflammatory effects of flavonoids are associated with the metabolites rather than their parent compounds (Warner, Zhang et al. 2016). Interestingly, research examining 3',4'-didemethylnobiletin (DDMN), a major metabolite of nobiletin in the mouse demonstrates anti-inflammatory effects with decreased transcription of nitric oxide synthase (iNOS) when topically applied to a TPA-induced mouse skin inflammation model (Lai, Li et al. 2008). Indeed, DDMN was found

to have stronger anti-inflammatory effects than nobiletin its parent compound *in vitro* with suppression of LPS induced nitric oxide (NO) production and downregulation of inducible nitric oxide synthase (iNOS).

Herein, to our knowledge this will be the first time DDMN and curcumin are investigated in combination in HT29 p53 mutant colon cancer cells and RAW 264.7 macrophage cells, to further characterize the synergistic interaction observed between DDMN and curcumin in HCT116 p53 wild-type colon cancer cells. This research will aid in the determination of whether the synergistic anti-proliferative effects of DDMN and curcumin in combination in HCT116 cells appear to utilize a p53-dependent or p53-independent mechanism for the inhibition of colon cancer *in vitro*. In addition, the investigation of an anti-inflammatory mechanism will be beneficial in establishing a basis for nobiletin and curcumin in combination for future application with inflammatory colon cancer and other inflammatory related diseases.

4.5 Materials and Methods

4.5.1 Cell Culture

HT29 cells were received from the American Type Culture Collection (ATCC, Manassas, VA) and prepared as previously described; maintained in RPMI-1640 media with added 5% heat-inactivated fetal bovine serum (FBS). RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI-1640 media with added 10% heat-inactivated FBS (Mediatech, Herndon, VA) along with 100 units/ml of penicillin, and 0.1 mg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO) at 37°C with 5% CO₂ and 95% air.

4.5.2 Cell Treatments

Curcumin (96%) was obtained from Quality Phytochemicals, LLC (New Jersey, USA). The 3', 4'-didemethylnobiletin (DDMN, 98%) was produced after a multi-step process with its chemical structure verified using MS and NMR spectra methods as described (Li, Pan et al. 2007; Li, Sang et al. 2007). DMSO was used for the delivery of DDMN and curcumin to the cells in each experiment, resulting in a final concentration of 0.1% DMSO.

4.5.3 Cell Viability Assay

HT-29 human colon cancer cells were seeded in 96-well plates (2000 cells per well) and treatments of DDMN, curcumin or their combination were administered in serial concentrations after 24 hour incubation. Once the associated treatment times were met, the replacement of media with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) containing media (Sigma-Aldrich) was performed to quantify cell viability. Further detail as outlined above and in prior experiments (Qiu, Dong et al. 2010).

4.5.4 Nitrite Assay

NO production by the Griess reaction will be determined by measuring the nitrite concentration. The culture media will be mixed with an equal amount of Griess reagent A at 1% sulfanilamide in 5% phosphoric acid and reagent B at 0.1% naphthylethylenediamine dihydrochloride in water. The absorbance at 540 nm is read using a plate reader (Elx800TM absorbance microplate reader, BioTek Instrument,

Winooski, VT). The concentrations of nitrite will be calculated utilizing a standard curve with sodium nitrite as a standard.

4.5.5 Analyses of Synergy

The analyses of synergy will be determined utilizing a linear regression model based on the Chou Talalay method with some alterations as previously described (Chou and Talalay 1984; Xiao, Zhang et al. 2008; Guo, Qiu et al. 2012). Results were examined using the R program and synergy was categorized utilizing the interaction index parameters with the combination interaction (CI) characterized as exhibiting additivity (=1), synergy (<1), or antagonism (>1).

4.6 Results and Discussion

4.6.1 Curcumin and DDMN Demonstrate Synergism in the Inhibition of NO in RAW 264.7 macrophage cells

To establish nontoxic dose ranges for DDMN and curcumin in RAW 264.7 cells; the effects of these compounds were determined on cell viability utilizing the MTT assay as previously described. We found that DDMN and curcumin concentrations up to 25 μ M and 15 μ M respectively as well as their combined treatment did not cause a significant decrease on the viability of RAW 264.7 cells. These nontoxic dose ranges were then used to determine the inhibition of NO production by DDMN, curcumin and their combination in LPS-stimulated RAW 264.7 macrophage cells. The cells were treated with serial concentrations of DDMN (5 μ M-25 μ M) and curcumin (3 μ M-15 μ M) individually and at half-dose serial concentrations in combination at a 1.7:1 ratio to

determine nitric oxide (NO) inhibitory effects using the nitrite assay (Figure 4.1). NO inhibition as measured by the nitrite assay revealed that combined treatment with DDMN and curcumin, each at half-dose synergistically potentiated the NO inhibitory effects. Individual treatment with DDMN alone exhibited a dose dependent response and provided inhibition of NO (5%-45%) at all concentrations (5 μ M-25 μ M). Curcumin individual treatment at concentrations (3 μ M-15 μ M) exhibited a dose dependent response with a slightly stronger inhibition (5%-56%) of NO production than DDMN at similar concentrations. Remarkably, the combination of DDMN and curcumin at half-dose serial concentrations synergistically inhibited NO with a more pronounced effect (21%-56%) than individual treatments as determined by the combination index previously described in Section 2.3. Notably, combined treatments at the lower half-dose concentrations of DDMN (2.5 μ M-5 μ M) and curcumin (1.5 μ M-2.5 μ M) exhibited the strongest synergy with inhibition of NO production by the combination (21%-33%) more than doubling the NO inhibition of each individual (5%-16%) treatment (Figure 4.2). Synergy was defined as combination interaction index $CI < 1$.

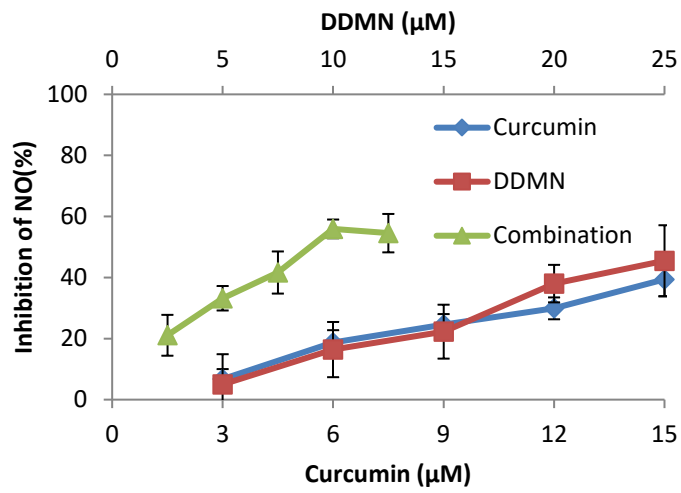


Figure 4.1 Percentage of inhibition on NO production by curcumin and DDMN individually and in combination in LPS-stimulated RAW 264.7 macrophage cells. Cells were treated with LPS as a positive control or LPS with serial concentrations of curcumin, DDMN or their combination.

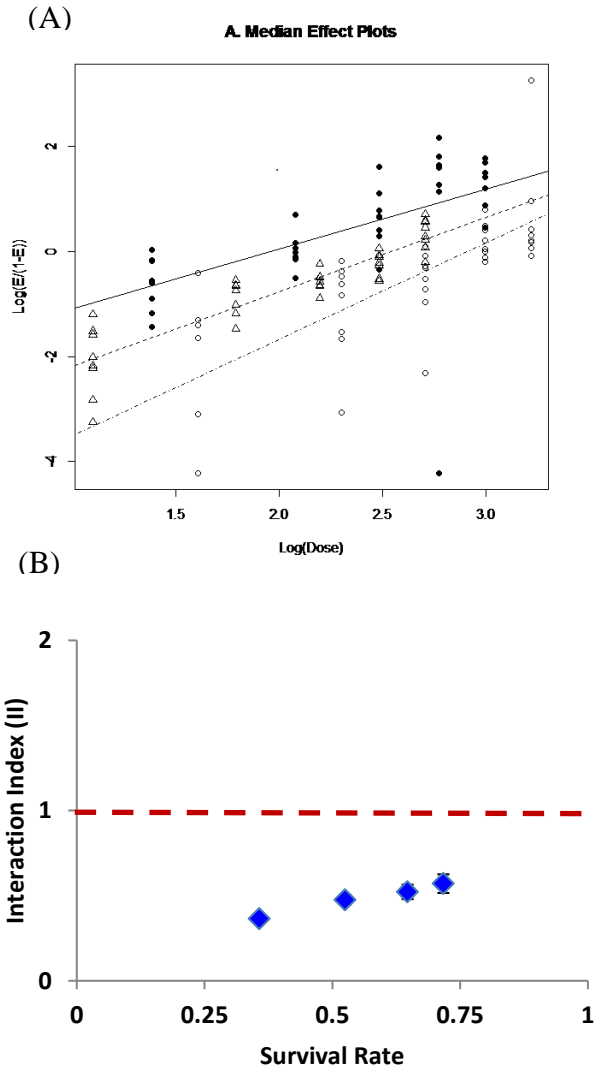


Figure 4.2 (A) Median effect plot of DDMN, curcumin and their combination on inhibition of NO production in LPS-stimulated RAW 264.7 macrophage cells. (B) Interaction index plot for the combination effects of DDMN and curcumin on NO production in LPS-stimulated RAW 264.7 macrophage cells. Synergy was defined as CI interaction index < 1 . Median effect plot and interaction index plot constructed as described in Materials and Methods.

4.6.2 Curcumin and DDMN Demonstrate Antagonism in the Inhibition of Cell Viability in HT29 p53 Mutant Human Colon Cancer Cells

HT29 p53 mutant human colon cancer cells were treated with serial doses of DDMN (3 μ M-15 μ M) and curcumin (2 μ M-10 μ M) individually and at half-dose serial concentrations in combination to determine growth inhibitory effects using the MTT assay. Cell viability as measured by the MTT assay revealed that combined treatment with DDMN and curcumin displayed growth inhibitory effects after 48 hours of compound exposure (Figure 4.3). Individual treatment with DDMN alone provided only minimal inhibition of growth (4%-12%) at low concentrations (3 μ M-6 μ M) but was substantially more effective at inhibition of growth (35%) with the highest concentration (15 μ M). Curcumin individual treatment exhibited a dose dependent response with a much stronger inhibition (25%-56%) of growth at concentrations 6 μ M-10 μ M as compared to DDMN at similar concentrations. The combination of DDMN and curcumin at half-dose serial concentrations inhibited cell viability in a dose dependent manner with an additive effect at lower concentrations of 3 μ M-5 μ M + 1 μ M-2 μ M respectively that appeared to not greatly differ from the individual treatment effects. Combination of DDMN and curcumin at higher concentrations of 4.5 μ M-7.5 μ M + 3 μ M-5 μ M respectively exhibited an antagonistic effect of less inhibition of growth than the individual treatments, as determined by the combination index previously described (Figure 4.4). Additive was defined as combination interaction index CI=1. Antagonism was defined as combination interaction index CI > 1.

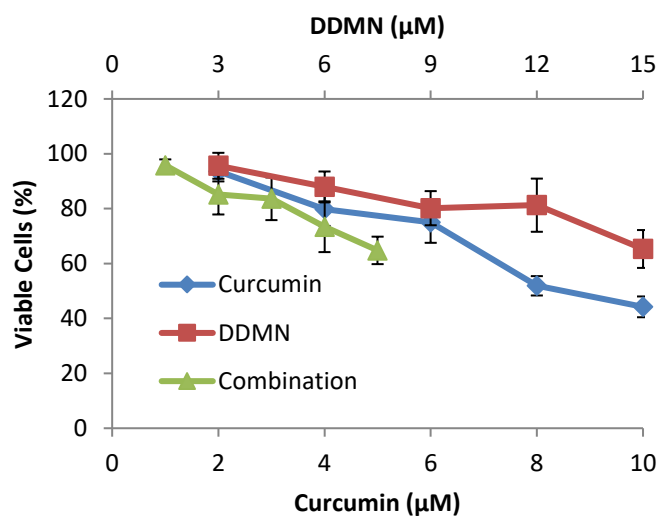


Figure 4.3 Growth inhibitory effects of curcumin and DDMN individually and in combination following serial concentrations of compound treatments in HT29 p53 mutant human colon cancer cells. Cell viability was measured by MTT assay as previously described in Section 2.3.

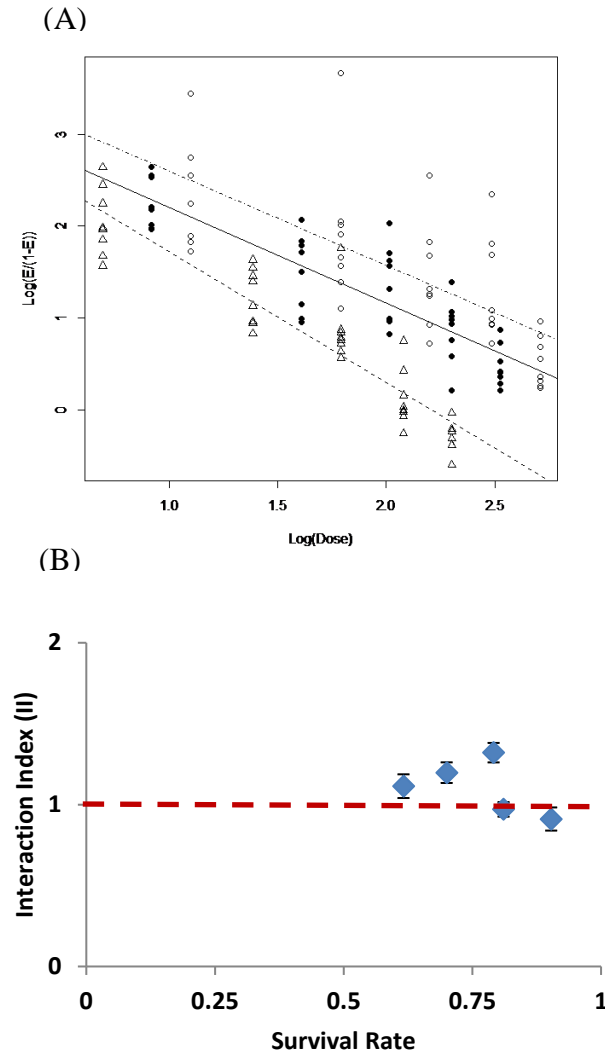


Figure 4.4 Combined treatment with curcumin and DDMN exhibited additive and antagonistic growth inhibitory effects in HT29 p53 mutant human colon cancer cells after 48 hr of compound exposure. (A) Median effect plot of curcumin, DDMN and their combination on growth inhibition of HT29 p53 mutant cells. (B) Interaction index plot for the combination effects of curcumin and DDMN on growth inhibitory effects of HT29 p53 mutant colon cancer cells. Antagonism was defined as CI interaction index > 1 . Median effect plot and interaction index plot constructed as described in Materials and Methods.

4.6.3 Discussion

Many factors point to the importance of addressing chronic intestinal inflammation both preventively and early in treatment, as progression to colon cancer is more likely to occur in cases with an earlier onset of disease, prolonged duration and severity of inflammation; each greatly increasing the risk of developing colon cancer (Terzic, Grivennikov et al. 2010; Jawad, Direkze et al. 2011). As of this date, a cure remains elusive and pharmaceutical drugs have been limited in their capability to alter the disease process (Lakatos and Lakatos 2008). The inflammatory progression of IBD often leads to unwanted surgery, complications and serious side effects associated with the drugs used to treat advanced stages of IBD. An accumulating body of research suggests dietary components exhibit anti-inflammatory properties within the same deregulated signaling pathways as chronic intestinal inflammation yet without the unwanted side effects; offering a viable alternative for the prevention and treatment of intestinal inflammation (Benavente-Garcia and Castillo 2008; Pan, Lai et al. 2009). Moreover, using dietary components in combination offers the potential for a synergistic interaction, thereby eliciting a more powerful anti-inflammatory response with low toxicity.

Nobiletin and curcumin each have shown significant anti-inflammatory properties individually in cellular and animal models (Murakami, Nakamura et al. 2000; Tanaka, Sato et al. 2004; Harada, Tominari et al. 2011). Recently we demonstrated a primary metabolite of nobiletin, 3',4' – didemethylnobiletin (DDMN) and curcumin were synergistic in the inhibition of cell growth in HCT116 colon cancer cells. In this study our findings of decreased nitric oxide (NO) production in LPS stimulated RAW 264.7 cells by individual DDMN or curcumin treatment are consistent with research conducted

by others however the anti-inflammatory effects of DDMN and curcumin in combination to our knowledge have not yet been examined (Li, Sang et al. 2007; Kim, Pae et al. 2008; Cheung, Khor et al. 2009). Therefore, we report for the first time on the synergistic anti-inflammatory inhibition of NO production by the combination of DDMN and curcumin in LPS stimulated RAW 264.7 macrophage cells. NO inhibition is an important target for the prevention of chronic intestinal inflammation and IBD-related colon cancer as IBD is characterized by elevated levels of inducible NO synthase (iNOS) resulting in high levels of nitric oxide (NO). Indeed, inducible NO synthase (iNOS) is elevated in both inflamed colonic mucosa and in colonic tumors (Hussain, Amstad et al. 2000). Peroxynitrate a product of the reaction of superoxide and nitric oxide (NO) is correlated with colonic inflammation and IBD type pathogenesis *in vivo* (Zhu and Li 2012).

This study for the first time has shown the combination of DDMN and curcumin in half-dose serial concentrations results in synergistic inhibition of NO production in LPS stimulated RAW 264.7 macrophage cells. Surprisingly, the lower half-dose concentrations of DDMN (2.5 μ M-5 μ M) + curcumin (1.5 μ M-2.5 μ M) in combination exhibited the greatest synergism; therefore a saturation or ceiling appears to potentially exist at which higher concentrations of the compounds are not as potent or efficacious in their anti-inflammatory effects when combined. Excitingly, the enhanced anti-inflammatory effect of the lower dose combinations provides an opportunity for further investigation as an effective small dose for a prolonged period of time with low toxicity would be of great benefit for those with inflammation related diseases for the inhibition of NO production.

The relationship between NO and p53 appears to be pivotal in determining the outcome of inflammation and tumorigenesis. Researchers suggest NO can be protective at early stages of chronic inflammation from IBD-related colon cancer before alteration of the p53 protein is involved (Goodman, Hofseth et al. 2004). As such, p53 under low stress conditions has been found to be protective from NO induced DNA damage when activation is triggered by iNOS and the production of NO in the colons of UC patients facilitates a negative feedback loop with p53 (Hofseth, Saito et al. 2003). Nitric oxide appears to have opposing roles, acting either as pro-tumorigenic or anti-tumorigenic depending on the status of p53. The negative feedback loop of p53 and NO is comprised with NO initiating the accumulation of p53 which then leads to the suppression of iNOS thereby limiting NO production (Forrester, Ambs et al. 1996; Ambs, Ogunfusika et al. 1998). The increased p53 activity leads to apoptosis, cell cycle arrest and the elimination of damaged cells promoting anti-tumorigenesis. In cells lacking a wild-type p53, NO production leads to cell proliferation as a result of cells no longer taking part in apoptosis and cell cycle arrest. In mice without p53 when iNOS is deleted sarcomas and lymphoma are reduced suggesting the negative feedback loop of p53 and NO is involved in the regulation of tumorigenesis (Hussain, Trivers et al. 2004; Tatemichi, Tazawa et al. 2004).

P53 also plays the role of antagonist in the inflammatory process. The nuclear factor-kappa B (NF- κ B) pathway is the primary regulator of both acute and chronic inflammation (Ben-Neriah and Karin 2011). Gudkov *et al* identifies p53 as an antagonist of NF- κ B resulting in the inhibition of inflammation (Gudkov and Komarova 2016). The relationship between p53 and NF- κ B has been described as generally antagonistic in most cell systems with both participating in a cross talk regulated by multiple mechanisms that

are still not fully defined (Schneider and Kramer 2011). NF- κ B is activated and present in biopsies of inflamed mucosa of IBD patients and can trigger iNOS to produce NO with its pro-inflammatory and potential carcinogenic effects (Rogler, Brand et al. 1998; Yamamoto and Gaynor 2001). Inhibition of NF- κ B activity in inflammation associated colon cancer mouse models halts tumor growth and suppresses tumors in xenograft studies (Gurova, Hill et al. 2005; Gasparian, Burkhart et al. 2011; Sunami and Wirth 2011; Vlantis, Wullaert et al. 2011). Interestingly, nobiletin was shown to inhibit the DNA binding of the pro-inflammatory transcription factor NF κ B in LPS stimulated RAW 264.7 macrophage cells and prevent the nuclear NF κ B translocation from the cytoplasm and its expression into the nucleus in BV-2 microglia cells, a neuroinflammation model. (Choi, Hwang et al. 2007; Cui, Wu et al. 2010). It is the deregulation of NF- κ B at any point in the NF- κ B pathway that is attributed with the constant activation of NF- κ B leading to chronic inflammation and the potential transformation to cancer (Balkwill and Coussens 2004; Lin and Karin 2007; Natarajan, Komarov et al. 2014). P53 has proven to have the capacity to suppress the activity of NF- κ B resulting in the attenuation of inflammation and its effects; likewise deregulation of NF- κ B as observed in chronic inflammation can suppress p53 and its' role as a tumor suppressor (Gudkov and Komarova 2016).

As evidence suggests p53 plays a key role in the regulation of chronic intestinal inflammation and tumorigenesis, a p53 mutation can have broad negative effects on the IBD pathogenesis and colon cancer development. Mutation of the p53 protein results in the loss of its tumor suppressor role and a gain of new functions that include increased cell proliferation and the promotion of oncogenic transformation and invasiveness.

Cooks *et al* demonstrates an extended and magnified activation of the inflammatory response by mutated p53 in addition to the absence of anti-inflammatory properties frequently observed in the non-mutated wild-type p53 (Cooks, Pateras et al. 2013).

Free radicals are generated in the inflammatory process potentially targeting genes and proteins important for homeostasis of colonocytes. Upon excessive ROS and free radical accumulation in cells a protective mechanism activates the transcription of a number of detoxifying enzymes that includes heme-oxygenase-1 (HO-1) which in turn inactivates the ROS halting any further damaging effects (Kalo, Kogan-Sakin et al. 2012). Heme-oxygenase-1 an inducible phase II enzyme, has been characterized as an endogenous inflammatory regulator, as such HO-1 knockout mice develop chronic inflammatory disease (Paine, Eiz-Vesper et al. 2010). Lipopolysaccharide (LPS) stimulated macrophages induce (HO-1) which decreases inflammatory indicators of NO, iNOS and COX-2. Interestingly, HO-1 mRNA and protein expression have been identified in intestinal cell lines and human IBD tissue samples, suggestive of an innate protective response that exists to limit tissue damage through the release of HO-1 (Paine, Eiz-Vesper et al. 2010).

The p53 protein can be targeted by these free radicals and ROS accumulation if left unchecked resulting in p53 mutations early in the pathogenesis of IBD; p53 mutations occur at a higher rate in the inflamed mucosa of IBD patients shifting the progression of inflammation to one of dysplasia and increased risk of colon cancer development (Matkowskyj, Chen et al. 2013). This sequence can be described as the “inflammation-dysplasia-carcinoma” sequence characteristic in IBD. The degree of IBD related dysplasia to carcinoma appears to correlate with the level of p53 mutations

(Gerrits, Chen et al. 2011). The changes in p53 early in the pathogenesis of IBD suggest it is an effective marker for screening of chronic inflammation of the colon and subsequent risk of colon cancer development (Matkowskyj, Chen et al. 2013).

The role of p53 in chronic inflammation and carcinogenesis is complex and multifaceted. This study is the first to report on the non-synergistic anti-proliferative effects of DDMN in combination with curcumin in the p53 mutant cell line HT29; findings indicate no interaction (additive) occurred at low combinatory concentrations of DDMN at 1.5 μ M-3 μ M + curcumin at 1 μ M-2 μ M whereas an antagonistic combination response with a reduction in anti-proliferative effects appears to exist at higher concentrations of DDMN (4.5 μ M-7.5 μ M) curcumin (3 μ M-5 μ M). These findings are in contrast to our results in the p53 wild type cell line HCT116 in which the combination demonstrated synergistic anti-proliferative effects. Our findings suggest two different mechanisms are at work in HT29 p53 mutated cells, one an additive interaction at low concentrations of DDMN + curcumin and the other an antagonistic interaction at higher concentrations of the combination. Others have reported differing mechanisms involved in curcumin's anti-cancer effects that are concentration based, with low concentrations of curcumin exhibiting an antioxidant effect and higher concentrations of curcumin demonstrating a pro-apoptotic effect (Park and Contreas 2010).

Research examining apoptosis following curcumin treatment and the role of p53 has had mixed findings (Jee, Shen et al. 1998; Choudhuri, Pal et al. 2002; Radhakrishna Pillai, Srivastava et al. 2004; Watson, Hill et al. 2008; Watson, Hill et al. 2010). Watson *et al* suggests curcumin utilizes a p-53 independent mechanism to promote apoptosis in HCT116 colon cancer cells. Other researchers compared curcumin sensitivity in p53

wild type HCT116 colon cancer cells with a p53 knockout HCT116 cell line to conclude that wild type p53 is required for curcumin sensitivity (Howells, Mitra et al. 2007).

Interestingly, clinical findings following curcumin treatment in colorectal cancer patients suggest a correlation exists between an increased expression of p53 in tumor tissue with an increase in apoptotic tumor cells and increased body weight (He, Shi et al. 2011).

Our prior study demonstrated extensive induction of apoptosis and a significant increase in p53 and HO-1 expression was associated with the synergistic inhibition of cell growth by the combination of DDMN (7.5 μ M) + curcumin (5 μ M) in HCT116 p53 wild type colon cancer cells. Indeed, the full dose combination treatment of DDMN (7.5 μ M) + curcumin (5 μ M) exhibited a substantial 110% increase in p53 expression as compared to control, resulting in a much stronger response than individual curcumin (70%) or DDMN (10%) treatments. Likewise, heme oxygenase-1 (HO-1), a phase 2 enzyme induced in response to oxidative stress was found to dramatically increase following our treatment of HCT116 p53 wild type colon cancer cells with DDMN (7.5 μ M), curcumin (5 μ M) or full dose combination treatment increasing HO-1 expression by 490%, 10% and 770% respectively. Researchers have explored the mechanism involved in the apoptotic and anti-cancer effects of HO-1 in HCT116 p53wt and p53^{-/-} colon cancer cells. Results indicate the induction of apoptosis is dependent upon a fully functioning p53 protein in order for the full expression of HO-1 and its' apoptotic effects to occur. Other research appears to support the integral role of a functioning p53. Kalo *et al* have found that HO-1 displays an anti-apoptotic effect in Caco-2 p53 mutant colon cancer cells and in other p53 mutant variants (Busserolles, Megias et al. 2006; Kalo, Kogan-Sakin et al. 2012). Mutated p53 has also been associated with a reduction in phase II enzymes such as HO-

land a resistance to cellular death. Taken together these findings suggest that HO-1 and p53 play an important role in the synergism of DDMN and curcumin in combination in HCT116 p53 wild type colon cancer cells and potentially can contribute in part to explain the differing antagonistic combination response in HT29 p53 mutant colon cancer cells.

4.7 Conclusions

In conclusion, this study for the first time has shown that the combination of DDMN and curcumin synergistically inhibits LPS-induced inflammation in RAW 264.7 macrophage cells as demonstrated by the inhibition of nitric oxide (NO) production. In addition, for the first time we have identified a non-synergistic interaction occurs in the combination of DDMN and curcumin in HT29 p53 mutant colon cancer cells resulting in diminished anti-proliferative effects that are less than individual treatments. Our results provide new insights into the characterization of the synergistic combination of DDMN and curcumin in HCT116 p53 wild-type colon cancer cells. The synergistic anti-cancer activity of this combination could be in part due to its mode of action in its ability to induce p53 in HCT116 colon cancer cells perhaps resulting in the inhibition of inflammatory pathways and the promotion of apoptosis. Further investigation is warranted for the development of new dietary combination strategies that will disrupt the transformation of chronic inflammation to potentially prevent and reduce the occurrence of IBD-related colon cancer.

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CHAPTER 5

CONCLUSIONS

Research has shown diet to be a powerful manipulator in the prevention and promotion of cancer and inflammation. Excitingly, many studies suggest dietary components exhibit anti-cancer and anti-inflammatory properties with low or no toxicity, yet little research exists examining these dietary components in combination. As we do not eat these compounds in isolation it is important to examine the interaction of dietary components when combined; examination of the biological activities and interactions of their metabolites with other dietary components can aid in eliciting a better understanding of the possible mechanisms involved *in vivo*.

Although numerous studies show that complementary mechanisms of individual dietary components can result in enhanced anti-cancer and anti-inflammatory effects when used in combination, caution is warranted as certain dietary component combinations can be antagonistic with diminished anti-cancer or anti-inflammatory effects and in some instances can even act as a promoter of carcinogenesis. In these cases, it is critical to understand how these components interact within the particular combinations so that recommendations can be made to avoid potential adverse effects. These findings underscore the need to further investigate and characterize new dietary component combination strategies that have the potential to elicit a more potent synergistic anti-cancer and anti-inflammatory response for cancer chemoprevention and inflammation.

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